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The Effects of Aging on Proteasome Activity,
Assembly, and Abundance in *C. elegans*

A Dissertation Submitted in Partial Satisfaction of the
Requirements for the Degree Doctor of Philosophy

in

Biology

by

Derek Christopher Joyce

Committee In Charge:

Professor Andrew Dillin, Chair
Professor Randolph Hampton
Professor Martin Hetzer
Professor Edward Koo
Professor Gentry Patrick

2012

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University of California, San Diego

2012

DEDICATION

I dedicate this thesis to wife, Christine, and daughter, Gianna. They are a constant source of joy, support, and love in every aspect of my life.

TABLE OF CONTENTS

Signature Page	iii
Dedication	iv
Table of Contents	v
List of Figures	vii
Acknowledgements	ix
Vita	x
Abstract of the Dissertation	xi

CHAPTER ONE: An Introduction to Aging and the Proteasome in

<i>C. elegans</i>	1
Introduction	2
Theories of aging	3
Mutation Accumulation	3
Antagonistic Pleiotropy	4
Disposable Soma	4
The Free Radical Theory of Aging	5
Proteostasis Collapse	6
Proteostasis	7
Protein Translation	7
Chaperones	8
Protein Trafficking/ER UPR	10
Autophagy	11
Proteasome	12
Structure and assembly	12
Targeting of proteins to proteasome by ubiquitin	15
Proteasome Regulation	16
Proteasome and aging/disease	18

CHAPTER TWO: The Effects of Aging on Proteasome Activity

in <i>C. elegans</i>	20
Introduction	21
Results	22
Proteasome activity increases with age	24
<i>daf-2</i> worms have a dampened but prolonged proteasome activity increase with age	26
Dietary restricted worms have a dampened an prolonged proteasome activity increase with age	27
Discussion	28
Experimental Procedures	30
<i>C. elegans</i> maintenance and strains	30
Preparing worm lysate	30
Plate reader activity assay	31
Native Gel activity assay	31
Native Gel Western Blots	32
Bacterial Dietary Restriction	32

CHAPTER THREE: Proteasome Activity Correlates to Proteasome Abundance with Age	33
Introduction	31
Results	36
Proteasome activity is directly correlated to proteasome abundance	36
Proteasome subunit levels correlate to activity and abundance with age	37
Knockdown of potential proteasome regulators is insufficient to alter proteasome activity increase with age	39
Ubiquitinated protein levels are stable with age	41
Discussion	42
Experimental Procedures	45
Mass Spectrometry	45
CHAPTER FOUR: Addendum	46
Introduction	47
<i>C. elegans</i> proteasomes have peculiar characteristics	47
SDS kills proteasome activity, specifically in <i>C. elegans</i>	47
Frozen worm lysate in PBS buffer shows evidence of a “trans-activator”	48
Plate reader assay results do not consistently correlate to native gels	49
GFP-ODC as an alternative full length substrate	51
Daf-2 RNAi is more protective than A β RNAi in an Alzheimer’s model worm	52
Hsf-1 is critical for L2 transition and necessary through late life in <i>C. elegans</i>	53
Summary	54
FIGURES	57
REFERENCES	76

LIST OF FIGURES

Figure 1: Chymotrypsin-like proteasome activity increases with age.	57
Figure 2: Native Gel assays of day1, day6, and day11 of adulthood.....	58
Figure 3: Native Gel proteasome assay of aged worms.	59
Figure 4: Plate reader assays show CTL activity increase is dampened in daf-2 worms.	60
Figure 3: Regulation and Localization of <i>pha-4</i> in Response to DR	61
Figure 4: Increased Dosage of <i>pha-4</i> Extends Lifespan.....	62
Figure 5: Native gel analysis of aged CF512 and daf-2 worms.	63
Figure 6: Plate reader assays show CTL activity increase is dampened in dietary restricted eat-2 worms.....	64
Figure 7: Native gel proteasome analysis of aged worms under dietary restriction in liquid culture.	65
Figure 8: Mass spec data with relative proteasome subunit abundance	66
Figure 9: Mass spec controls from Figure 8.....	67
Figure 10: aip-1 and skn-1 RNAi is insufficient to alter proteasome activity increase with age.....	68
Figure 11: RNAi of potential proteasome modulators has no effect on proteasome activity increase under normal aging	69
Figure 12: Ubiquitin levels are stable with age. Western blots of CF512 lysates from day 1 and day 9 of adulthood	70
Figure 13: Model: Proteasome activity increase compensates for age related protein damage.....	71
Supplemental Figure 1: Under simple lysis methods, old lysate is able to “activate” young lysate with mixing.....	72
Supplemental Figure 2: Much of the CTL activity may not be from proteasomes. ...	73

Supplemental Figure 3: glp-1 proteasome activity measurements differ from plate reader to native gel analysis.	.74
Supplemental Figure 4: ODC-tagged fluorescent proteins as full length proteasome substrate.	.75
Supplemental Figure 5: daf-2 RNAi is more protective from A β toxicity than A β RNAi.	.76
Supplemental Figure 6: hsf-1 expression is critical during the L2 transition and necessary throughout adulthood for maximal lifespan of daf-2 worms.	.77

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ABSTRACT OF THE DISSERTATION

The Effects of Aging on Proteasome Activity,
Assembly, and Abundance in *C. elegans*

by

Derek Christopher Joyce

Doctor of Philosophy in Biology

University of California, San Diego, 2012

Professor Andrew Dillin, Chair

Aging is associated with the accumulation of misfolded and damaged proteins. This accumulation is the result of an imbalance of proper protein folding, maintenance, and disposal. Maintaining proteostasis is critical to the health and life of an organism. While studies consistently observe that the proteostasis network diminishes with age, the effects on individual mechanisms have not been determined. Of interest is the effect of aging on protein degradation. Proteasomal degradation is a primary path of

protein degradation in all eukaryotes. Though many studies have investigated proteasome activity with age, results vary depending on the organism, tissue, and specific activity of the proteasome tested. It seems as though alterations in proteasome activity can either instigate, or potentially compensate for, a diminished proteostasis network. In order to better understand the effects of proteostasis decline on aging and disease, it is important to determine the role of the proteasome. As in most organisms, *C. elegans* show an age-related accumulation and aggregation of proteins. This suggests a failure to properly dispose of proteins, potentially by diminished proteasome activity. Surprisingly, we observe a significant increase in proteasome activity with age in the adult worm. The activity increase is apparent in the chymotrypsin-like, trypsin-like, and caspase-like activities. Lifespan extending methods have been shown to ameliorate age related toxic protein accumulation in the worm and have hallmarks of improved proteostasis. Interestingly, we observe a decrease in relative proteasome activity in long lived worms. In aging *C. elegans*, we determine that proteasome activity increases with age to compensate for the decline in general proteostasis. This increased activity is regulated by increasing proteasome abundance, likely through the regulation of limiting subunits.

CHAPTER ONE:

An Introduction to Aging and the Proteasome in *C. elegans*

Introduction

Aging can be simply described as the accumulation of changes to an organism over time. Though typically thought of in a negative sense, aging also includes the accumulation of knowledge and experience that can benefit an organism, especially humans. However, it is the deleterious changes to the physical and structural components of an organism that inevitably lead to disease and death. While aging is accepted to be universal across all known life, the rate of aging between different organisms, even those closely related, can be orders of magnitude apart. This suggests that aging is not simply a stochastic process, but has a genetic and evolutionary component. There are many theories that try to explain a universal reason how and why organisms age, I will discuss a few of the more popular evolutionary theories of aging as well as discuss specific molecular components of aging including free radical accumulation and proteostasis collapse, with an emphasis on the proteasome.

An intense amount of research has been dedicated to answering one of the most fundamental questions in biology, how and why do organisms age? Many theories have been proposed to answer the question of how and why organisms age, yet no single proposal can explain all the nuances of aging. On a mechanistic level, the primary contributor to aging may vary between the specific organism and tissue examined. Furthermore, interventions that improve one cause of aging may only have a limited benefit, as the concurrent deterioration of other pathways will also lead to death. While we may be able to target specific diseases by focusing on one aspect

of the aging paradigm, any effort to have a dramatic effect on overall aging must act more broadly. For example, the longest lived worm strains do not simply improve one aspect of aging, but rather affect a transcriptional cascade that improves many cellular processes. Because aging is associated with a concurrent malfunction of multiple biological systems, it is useful to study them as a whole, but also to focus on the individual components. My focus was directed on the effects of aging on the proteasome, using *C. elegans* as a model organism.

Theories of Aging:

Mutation Accumulation

Basic evolutionary theory predicts that any deleterious mutation, especially one causing an earlier death, will eventually be eliminated from the gene pool. Popularized by Peter Medawar in 1952, the mutation accumulation theory supposes that mutations that are deleterious at a later time point in life, when the organism would have typically died from extrinsic causes, are carried on and accumulate through generations. It is only when we remove these extrinsic causes of death, as in a laboratory setting, that we observe the damage caused by these deleterious, late acting genes. A significant problem with this theory is that any death promoting gene would likely be deleterious in early life, unless only expressed late in life. If the former, it would be selected against, if the latter, there should be no evolutionarily conserved maintenance and propagation of these genes. We would not expect to see the same genes to affect aging carried over long evolutionary time frames, but studies from yeast to humans have found conserved genes that can have deleterious effects on

maximal lifespan. One way this conflict is resolved is by supposing there are certain genes and functions that may be beneficial for increasing the number or fitness of the progeny at the expense of late life health. This is the basis behind the antagonistic pleiotropy theory of aging.

Antagonistic Pleiotropy

In 1957, George Williams proposed the theory of antagonistic pleiotropy which builds on the mutation accumulation idea, but adds in an important timing element. Additionally, it uses the fact that many genes can have multiple functions, some of which may be detrimental to organismal health. Essentially, this theory states that the evolutionary pressure is at its greatest during peak reproductive capacity, generally early in adulthood. Any gene that provides for more surviving offspring will be carried on much more efficiently, even it has fatal consequences after reproduction. This theory also predicts that many of the same genetic traits that have a negative tradeoff between lifespan and reproduction will be maintained over an evolutionary timeline. This theory fits better with the experimental data showing many conserved mechanisms, across a wide array of model organisms, which can extend lifespan at the expense of fecundity.

Disposable Soma

In 1977, Thomas Kirkwood built on the idea of reproductive tradeoff, but instead of a timing difference, focused on the tissues of the organism. Similar to

antagonistic pleiotropy, the disposable soma theory of aging supposes there is a bias towards reproduction. However, instead of looking at the time frame of reproduction, the focus is on the specific tissues. With a limited amount of resources, an organism must balance between reproduction and somatic maintenance. Evolution would only favor somatic maintenance for as long as the organism is reproductive and not succumbing to extrinsic death. However, energy spent on creating more surviving progeny would be dramatically favored, even if the gene had overall deleterious effects on the rest of the organism.

A cursory examination would lead one to think that any deleterious gene would be selected against by evolution, however, these theories show that the timing and special aspects of reproductive health, and extrinsic causes of death can, and likely do, lead to an accumulation of genetic traits that inhibit maximal lifespan. The next step in these theories of aging is to determine how these genetic traits specifically cause the aging phenomenon. I will focus on two of the most prominent theories of how organisms age, the free radical theory of aging, and proteostasis collapse.

The Free Radical Theory of Aging

Free radicals are molecules with an unpaired electron. The accumulation of free radicals can lead to aberrant chemical reactions and cause oxidative damage to a cell and the host organism. This theory was initiated by observations by Pearl in 1928 that organismal lifespan was inversely correlated to activity and metabolism levels. Additionally, in the 1950s, Harman noted that higher levels of oxygen

produced by hyperbaric chambers led to faster aging. It was postulated that the free radicals that are produced during normal respiration may be sufficient to cause accumulated damage and lead to aging and death. The mitochondrial theory of aging built on this idea, and expanded it to focus on the effect of ROS in the mitochondria. Because ROS is created in the mitochondria, the mitochondrial genome and proteome would be under increased oxidative stress and damage. This could cause a decrease in mitochondrial efficiency and a corresponding increase in ROS production, a feedback loop that eventually shuts down the mitochondria leading to the death of the organism. This theory is backed up by evidence showing older mitochondria produce more ROS and suffer more oxidative damage to their DNA and proteins, with less effective repair mechanisms (Shigenaga, Park et al. 1990; Sohal, Arnold et al. 1990). Additionally, interventions including the overexpression of antioxidant genes have a beneficial effect on lifespan. While the free radical theory of aging focusses on a cause of DNA and protein damage with age, the proteostasis collapse theory focusses on the age-related failure in protein maintenance.

Proteostasis collapse

Protein homeostasis, or proteostasis, is a concept of global protein quality control in a cell. This theory looks directly at the health of proteins as we age, especially pathways dedicated to maintenance, repair, and removal of the proteins. If protein quality control mechanisms are impaired or overwhelmed with age, entire systems in the cell could fail, causing more deterioration and the eventual death of the organism. This theory of aging has a specific focus on aging related protein

aggregation diseases. While it is not fully known why these diseases only present late in life, it is generally accepted that it is not simply a time dependent accumulation of a toxic protein, but also a breakdown in the ability to manage these proteins. The proteostasis network can be looked at in two ways, either by focusing directly on the protein maintenance mechanisms themselves, or by the regulatory control over the multiple mechanisms. To get a full picture of the network, and what may be failing in specific diseases, it is important to do both.

Proteostasis

The term proteostasis encompasses protein folding, maintenance, and degradation. While this may seem to broad a field for study, growing evidence indicates interactions between various components across the proteostasis network. Additionally, global regulatory control of proteostasis is not confined to single activity or type of activities but has effects across the proteostasis network. This is further complicated by the fact that there are multiple key regulators of proteostasis that not only induce various aspects of proteostasis machinery, but also affect each other. In order to understand the proteostasis network and the potential it has on aging, we must understand the components of the network, and how they are regulated.

Protein Translation

The first step in a proteins life cycle is translation and folding into the proper form. Even this does not occur without regulation. The very rate at which proteins

are synthesized can be modified to maintain proteostasis, and global translation rates decrease dramatically in response to protein folding stresses. Synthesis rates are modulated by the recruitment of the 40S ribosomal subunit to mRNA and the subsequent loading of the initiator tRNA (Gebauer and Hentze 2004). These are regulated by the eIF4E and eIF2a subunits respectively (Richter and Sonenberg 2005). Many interventions that have been shown to increase lifespan also reduce translation. Most notably, IIS reduction and dietary restriction (DR) have both been shown to lead to reduced translation and elongated lifespan (Wullschlegel, Loewith et al. 2006; Zid, Rogers et al. 2009). In fact, directly reducing translation through reducing TOR signaling is sufficient to extend lifespan in multiple organisms. (Vellai, Takacs-Vellai et al. 2003; Jia, Chen et al. 2004; Kapahi and Zid 2004; Kaeblerlein, Powers et al. 2005; Hansen, Taubert et al. 2007; Selman, Tullet et al. 2009). Interestingly, this extension can be additive to DR, suggesting other mechanisms, beyond translation inhibition, are important for protein quality control and organismal lifespan (Hansen, Taubert et al. 2007).

Chaperones

Once synthesized, most proteins do not spontaneously fold into their proper form, but need molecular chaperones for assistance. As nascent proteins are synthesized, chaperones attach to inhibit premature and improper folding. These chaperones not only inhibit improper folding, but actively promote the correct final structure. Some chaperones in the HSP70 family can use ATP hydrolysis to complete the final structure (Bukau, Weissman et al. 2006). One of the main issues that chaperones must combat is not just improper folding of proteins, but large protein

aggregates that can accumulate as a result and may be toxic to cells. Intuitively, there are mechanisms to remove these aggregates. Chaperones such as HSP 104 and TRiC can disaggregate these proteins, allowing them to be refolded or directing them for degradation. Interestingly, at higher protein concentrations, these same chaperones have been shown to actively aggregate these misfolded proteins to tighter, less toxic, aggregates (Shorter and Lindquist 2004; Behrends, Langer et al. 2006). One reason chaperones are so heavily studied is that they are the transcriptional targets of longevity promoting and heat shock resistance pathways. In *C. elegans*, small heat shock proteins are the target of DAF-16 and HSF-1 transcription factors, both of which play key roles in longevity assurance (Hsu, Murphy et al. 2003). Direct overexpression of some heat shock proteins has proven sufficient to increase the lifespan of both worms (Murphy, McCarroll et al. 2003; Walker and Lithgow 2003; Morley and Morimoto 2004) and flies (Morrow, Samson et al. 2004). Studies have proven that chaperones are critical to proper protein folding and maintenance, and may be a target for anti-aging therapeutics.

Once proteins are made and folded properly, environmental stresses may still induce damage, misfolding, and aggregation. Under these conditions, overall chaperone levels would need to be increased to compensate and avoid a cascade of protein misfolding and malfunction. One of the most studied protein stresses is acute heat shock. Upon thermal stress, the HSF1 transcription factor induces expression of HSPs from yeast to humans (McMillan, Xiao et al. 1998; Pirkkala, Nykanen et al. 2001; Anckar and Sistonen 2007). While the heat shock response (HSR) was defined by studying acute thermal stress, the actions of HSF1 are critical to both general organismal maintenance and essential for longevity enhancing perturbations. In *C.*

elegans, HSF-1 is most critical during development, but is still necessary in adulthood for maximum lifespan (Hsu, Murphy et al. 2003; Volovik, Maman et al. 2012). HSF-1 is so critical to lifespan, that IIS reduction or DR no longer extend lifespan in its absence (Hsu, Murphy et al. 2003; Morley and Morimoto 2004; Steinkraus, Smith et al. 2008). In normal aging, the ability to induce HSR declines with age (Ben-Zvi, Miller et al. 2009), while overexpression can increase the lifespan of both worms and flies (Lithgow, White et al. 1995; Hercus, Loeschcke et al. 2003; Hsu, Murphy et al. 2003; Morley and Morimoto 2004). The dysfunction of the chaperone network with age is a main cause of proteostasis collapse. Up-regulating these chaperones may be able to ward off age related disease and death.

Protein trafficking/ER UPR

There are many reasons proteins need to be degraded. Whether it is due to specific pathway signaling, or removing irreparable peptides, the cellular protein degradation systems must be able to accurately and effectively remove unwanted proteins. It is not only imperative to remove unwanted proteins, but the recycling of the amino acids may be crucial to an organism on limited resources. There are two main pathways in which proteins are degraded, autophagy and the ubiquitin proteasome system (UPS). Autophagy is generally less specific and involves a membrane bound autophagosome engulfing cellular components and trafficking them to the lysosome for degradation. The UPS uses a more intricate system of ubiquitin ligases to target specific proteins which are then degraded by the proteasome. Both of these pathways are critical to all eukaryotes, and are both implicated in the process of aging.

Autophagy

Autophagy can be divided into three main categories, chaperone mediated autophagy, microautophagy, and macroautophagy. In chaperone-mediated autophagy, chaperones traffic specific proteins to the lysosome to be degraded. This is the most targeted and specific form of autophagy, more similar to the targeted degradation typically carried out by the UPS. In microautophagy, small areas of the cytosol are engulfed by the lysosome itself to be degraded. This process is poorly understood, but is differentiated from macroautophagy in that it bypasses the need for additional membrane creation. In contrast, macroautophagy involves engulfing a larger region of the cytosol, sometimes including other organelles, in a novel membrane. This autophagic vesicle then fuses with the lysosome to degrade the components within (Yorimitsu and Klionsky 2005). Autophagy is critical to the health of a cell and studies have long shown a decrease in autophagy and lysosome function with age (Terman 1995; Vittorini, Paradiso et al. 1999). Interestingly, long lived IIS depleted worms have shown an increase in autophagosomes, however this is independent of the DAF-16 transcription factor which is critical for any lifespan extension (Melendez, Tallochy et al. 2003; Hansen, Chandra et al. 2008). DR worms also possess more autophagosomes, and inhibition of macroautophagy completely negates any lifespan extension (Jia and Levine 2007; Hansen, Chandra et al. 2008). Macroautophagy is not only necessary for lifespan extension, but increasing levels in *Drosophila* is sufficient to extend lifespan (Simonsen, Cumming et al. 2008). Additionally, mTOR, a macroautophagy inducing drug, can increase the lifespan of mice, even when applied late in their lifespan (Harrison, Strong et al. 2009).

Proteasome

The ubiquitin-proteasome system (UPS) is the other main protein degrading system. The proteasome complex, its chaperones, and assembly have been well conserved from yeast through humans. In contrast to autophagy, the UPS is a much more targeted way to remove specific proteins. Generally, a protein is targeted to the proteasome by the addition of a poly-ubiquitin chain. This specificity is accomplished through ubiquitin ligases. These targeted proteins are then trafficked to the proteasome where they can be unfolded and degraded by the 26S proteasome.

Structure and assembly

The proteasome is a large, multi-subunit complex capable of rapidly unfolding and degrading proteins. The 20S core of the proteasome is formed by two beta rings and two alpha rings. These are assembled with the two catalytic beta rings sandwiched by the two regulatory alpha rings. Each of the alpha and beta rings are comprised of seven unique, but homologous subunits which must be assembled precisely. The catalytic beta rings have three protease like activities, chymotrypsin like (CTL), caspase like (CL), and trypsin like (TL) activities, with CTL shown to be the rate limiting activity (Lee and Goldberg 1998). By itself, the 20S core is generally inactive, with entrance to the catalytic beta rings blocked by the conformation of the alpha rings. The 20S core assembles independently of associated regulatory particles. Formation of the 20S starts with the assembly of the alpha subunits. However, this process requires chaperones, and the alpha subunits have been shown to be promiscuous when certain subunits are depleted (Velichutina, Connerly et al. 2004)

or produced in excess (Gerards, Enzlin et al. 1997; Yao, Toth et al. 1999). In fact, at least four 20S chaperones are critical to the final assembly. These are called proteasome assembly chaperones (PAC) 1-4. These act as heterodimers of PAC1-2 and PAC 3-4 (Hirano, Hendil et al. 2005; Le Tallec, Barrault et al. 2007), with the PAC 3-4 acting initially to attach alpha5 and recruit the proper ordering of the other alpha subunits (Kusmierczyk, Kunjappu et al. 2008; Yashiroda, Mizushima et al. 2008). Once the alpha ring is complete, the PAC3-4 disassociates to allow incorporation of the beta subunits. Unlike the PAC3-4, the PAC1-2 does not disassociate as the beta units are added (Hirano, Kaneko et al. 2008; Murata, Yashiroda et al. 2009). Another 20S chaperone is Ump1. While this has no role in alpha ring formation, it likely prevents association of the two halves of the 20S until they are fully formed. Once both halves come together to form a functional 20S, it quickly degrades both the Ump1 and the PAC1-2 chaperones (Murata, Yashiroda et al. 2009).

While the 20S core is where protein degradation takes place, it is generally thought of as inactive unless accompanied by a modulating cap. The main protein degrading proteasome in the cell is the 26S proteasome, which is formed by the addition of the 19S regulatory particle. If the 20S is capped on both ends by 19S, it can be referred to as a 30S complex. When attached to the 19S cap, the alpha ring on the 20S core is altered, allowing passage of proteins to the catalytic beta rings for degradation. In addition to opening the core, the 19S plays integral roles in targeting and unfolding proteins for degradation. The structure of the 19S cap starts with another ring of proteins, Rpt1-6. These are AAA-ATPase proteins, and together with Rpn1, Rpn2, Rpn10, and Rpn13 form the base of the 19S cap. Rpn10 and 13 act as ubiquitin receptors and are the only proteasome subunits not critical in yeast

(Finley 2009) with only Rpn13 not critical in worms (Hamer, Matilainen et al. 2010). Because of this, they are not likely critical to the formation of the functional 19S cap and final 26S proteasome. In contrast, Rpn1 and Rpn2 are thought to play a critical role as a scaffold for the 19S as they have shown protein binding to several proteasome subunits (Finley 2009). The rest of the 19S subunits create the lid complex. One subunit of note is Rpn11, which acts to deubiquitinate proteins before they get degraded (Kleijnen, Roelofs et al. 2007). Recently, Rpn6 has been implicated as important to keeping the 20S and 19S assemblies attached (Pathare, Nagy et al. 2012). At least four chaperons are needed for proper 19S assembly, Nas2, Nas6, Rpn14, and Hsm3 (Funakoshi, Tomko et al. 2009; Kaneko, Hamazaki et al. 2009; Le Tallec, Barrault et al. 2009; Park, Roelofs et al. 2009; Roelofs, Park et al. 2009; Saeki, Toh et al. 2009). These proteins have been known to be associated with, but not part of 26S proteasomes for some time (DeMartino, Proske et al. 1996; Hori, Kato et al. 1998; Verma, Chen et al. 2000), but only recently have researchers discovered their critical role as chaperones. It is thought that these chaperones act by binding specific subunits to only allow the proper final alignment, especially in the Rpt1-6 ring. With Hsm3-Rpt1, Nas6-Rpt3, Nas2-Rpt5, and Rpn14-Rpt6 pairing up to guide the final structure (Dawson, Apcher et al. 2002; Saeki, Toh et al. 2009). While 19S caps can be isolated by themselves, it is also notable that the 20S core plays an integral role, in conjunction with these chaperones, to assemble a proper 19S cap. When mutants for 20S assembly were made, there were also noted defects in 19S assembly (Kusmierczyk, Kunjappu et al. 2008). In fact, simply deleting the C-terminal domains of the Rpt proteins that interact with the 20S alpha subunits is sufficient to disturb 19S assembly (Park, Roelofs et al. 2009). These C-terminal

domains are known to strongly associate to the alpha rings and create a conformational change allowing access to the proteolytic core (Smith, Chang et al. 2007). Once attached to the 20S core, the 19S cap deubiquitinates, unfolds, and feeds the protein to the proteolytic core for degradation. Initial studies in yeast showed that the 26S particle dissociates during this cycle (Babbitt, Kiss et al. 2005), but more recent data in mammalian cells showed protein degradation without dissociation (Kriegenburg, Seeger et al. 2008). In either case, it is thought that binding and conformational changes of the cap and core complexes, in an ATP dependent fashion, are what drive the unfolding and trafficking of the proteins for degradation (Finley 2009). The 26S proteasome is complex and well conserved. The structure and function of each subunit must be correct for proper biological function.

Targeting of proteins to the proteasome by ubiquitin

Proteins are targeted to the proteasome by getting tagged with poly-ubiquitin chain by E1, E2, and E3 ubiquitin ligases. However, there are many types of ubiquitin tags, and not all lead to degradation. Ubiquitin has seven possible lysine residues to create linkages. Where lysine 48 linked proteins are targeted to the proteasome, lysine 63 tags are generally, but not always, non-proteolytic (Chen and Sun 2009; Saeki, Kudo et al. 2009). These polyubiquitin chains are recognized by Rpn10 and Rpn13, but Rpn1 and Rpt5 may have a role as well (Murata, Yashiroda et al. 2009). Because Rpn10 has been shown to dissociate freely from the 26S proteasome, it may act to shuttle tagged proteins to the 26S proteasome (Fu, Sadis et al. 1998).

Similarly, other non-proteasomal proteins may play similar functions with distinct roles including Dsk2 and Rad23 (Matiuhin, Kirkpatrick et al. 2008).

Proteasome regulation

Proteasome activity can be modulated in many fashions. The primary way specific pathways alter protein turnover by the proteasome is through modulation of the ubiquitin ligases more specific to the target protein. On a more global scale, activators such as AIRAP, that can increase the turnover rate of the proteasome upon induction of protein misfolding stress such as arsenite exposure (Sok, Calfon et al. 2001; Yun, Stanhill et al. 2008). Furthermore, post-translational modifications of specific proteasome subunits, like phosphorylation of rpt-6, have been shown to increase the local activity of the proteasome (Djakovic, Marquez-Lona et al. 2012). These proteasome and activator modifications may provide for a more rapid modulation of proteasome activity. For a more chronic or severe stress, it may be necessary to increase the number of functional proteasomes. To do this, there would need to be sufficient levels of all the subunits necessary as well as the folding chaperones to properly assemble them. While studies have shown assembly chaperones to be critical, it is the regulatory control of proteasome subunit abundance that is best understood. In yeast, the Rpn-4 transcription factor is able to simultaneously up-regulate several subunits. Rpn-4 was once thought to be a proteasome subunit since its deletion caused proteasome deficiencies. However it is now known to be a transcriptional regulator of multiple proteasome subunits. As Rpn-4 has been shown to be rapidly degraded by the proteasome in both ubiquitin

dependent and independent manners, it is only upon insufficient proteasome activity that Rpn-4 would be able to activate subunit expression. One successful method to increase proteasome abundance was employed by knocking down Ubr2, the E3 ubiquitin ligase which directs Rpn-4 for degradation (Kruegel, Robison et al. 2011). The mammalian counterpart to Rpn-4 is not a homolog, but the NRF-1/NRF-2 transcription factors (Kraft, Deocaris et al. 2006). NRF-2 has a wide range of targets, and a splice isoform, TCF11 may be the specific method in which proteasome activity is regulated in a feedback loop (Steffen, Seeger et al. 2010). Similar to RPN-4 in yeast, the NRF1-2/TCF11 transcription factor is rapidly degraded by the proteasome, and thus, its activity is inversely proportional to cellular proteasome activity. These proteasome regulatory feedbacks have been discovered and analyzed under acute proteasome stress such as addition of proteasome inhibitors or through causing general protein stress through means such as oxidation and heat stress. Interestingly, mutations in these regulatory genes do not appear to effect proteasome levels and activity under normal conditions. Additionally, the relationship to activity under aging is unknown. It is possible that there are redundant and overlapping mechanisms, including undiscovered alterations of the proteasome itself, its assembly, or proteasome activators.

Proteasome and aging/disease

The influence of the aging process of proteasome activity has been studied across many model systems. While many assume proteasome function simply decreases with age, studies have reported both increases and decreases of activity with age (Gaczynska, Osmulski et al. 2001; Gray, Tsirigotis et al. 2003; Zeng, Medhurst et al. 2005; Vernace, Arnaud et al. 2007; Yun, Stanhill et al. 2008). Additionally, even within the same organism, there can be variations in the temporal change of activity depending on the specific proteolytic activity and distinct tissue studied (Zeng, Medhurst et al. 2005). Furthermore, fly work has shown deleterious effects of aging on both the assembly and activity of proteasomes, but only in late life (Vernace, Arnaud et al. 2007). Despite the seemingly contradictory findings across different organisms or tissues, each new system tested can provide valuable insight in the role of the proteasome in protein homeostasis of aging and disease.

Studies using *C. elegans* proteasome activity have proven fruitful in determining the roles of proteasomes in aging and longevity. Specifically, the gene *aip-1*, a homologue of mammalian AIRAP (arsenic-inducible proteasomal 19S regulatory particle-associated protein), has been shown to be critical in the ability of an organism to respond to proteotoxic stress likely through activation of the UPS (Yun, Stanhill et al. 2008). Additionally, *skn-1*, the homolog of NRF1/2 may have a homologous role in regulating proteasome abundance and activity (Ferguson, Springer et al. 2010; Li, Matilainen et al. 2011). Using the worm model, it allows examination of population level changes of proteasome activity from a large synchronized population. Additionally, their transparency is ideal for *in vivo*

fluorescent proteasome sensors that have been used in other organisms. Moreover, extensive genetic tools are available in worms including bacterial RNAi libraries, the availability of many mutant strains, and the ability to create transgenic worm strains. Also, well established methods of lifespan extension by IIS reduction (*daf-2*) or dietary restriction can more than double the lifespan of *C. elegans*. This allows us to study the effects of altering lifespan on the proteasome. Using *C. elegans*, I will attempt to elucidate the connection between aging and proteasome activity and regulation.

CHAPTER TWO:

The Effects of Aging on Proteasome Activity in *C. elegans*

Introduction

As one of the critical systems in eukaryotic life, the proteasome is a target of much research. Specifically, many studies have looked at the correlation of different diseases with the proteasome, specifically those associated with misfolded proteins (Ciechanover and Brundin 2003). The influence of the aging process on proteasome activity has been studied across many model systems. While the general assumption is proteasome function simply decreases with age, studies have reported both increases and decreases of activity with age (Gaczynska, Osmulski et al. 2001; Gray, Tsirigotis et al. 2003; Zeng, Medhurst et al. 2005; Vernace, Arnaud et al. 2007; Yun, Stanhill et al. 2008). Additionally, even within the same organism, there can be variations in the temporal change of activity depending on the specific proteolytic activity and distinct tissue studied (Zeng, Medhurst et al. 2005). Furthermore, fly work has shown deleterious effects of aging on both the assembly and activity of proteasomes, but only in late life (Vernace, Arnaud et al. 2007). Interestingly, most of the defects seen in proteasome activity occur only at late life, if at all. Models in mice that use a ubiquitin tagged fluorescent protein do not show any accumulation with normal aging (Cook, Gass et al. 2009). In fact, human studies looking at healthy centenarians find that their proteasomes are still active and resemble youthful patients (Chondrogianni, Petropoulos et al. 2000; Hwang, Hwang et al. 2007). Despite the seemingly contradictory findings across different organisms or tissues, each new system tested can provide valuable insight in the role of the proteasome in protein homeostasis during aging and disease.

Studies using *C. elegans* have proven fruitful in determining the roles of proteasomes in aging and longevity. Specifically, the gene *aip-1*, a homologue of mammalian AIRAP (arsenic-inducible proteasomal 19S regulatory particle-associated protein), has been shown to be critical in the ability of an organism to respond to proteotoxic stress through the further activation of the UPS (Yun, Stanhill et al. 2008). Using the worm model, it allows examination of population level changes of proteasome activity. Additionally, their transparency is ideal for *in vivo* fluorescent proteasome sensors that have been used in other organisms. Moreover, we have the genetic tools available to worms including knockdown of the gene of interest by bacterial feeding RNAi, the availability of many mutant strains, and the ability to create transgenic worm strains.

While methods of extending lifespan like IIS reduction and DR have improved proteostasis networks, it is unclear what the effect is on the proteasome specifically. Using *C. elegans* as a model, our goal is to examine how aging alters the proteasome in the worm, and how lifespan extending interventions affect proteasome activity with age.

Results

Proteasome activity increases with age in *C. elegans*.

Multiple experiments in *C. elegans* have shown that the proteostasis capability decreases early in adulthood. Aging induces a decreased ability to properly fold proteins, as well as sufficiently degrade them. This decrease precedes and directly leads to age related declines in motility and death (Ben-Zvi, Miller et al. 2009). While

it is clear that general proteostasis declines early in worm lifespan, it is unclear what effect aging has on proteasome activity specifically. Our first goal was to determine the correlation between aging and proteasome activity in *C. elegans*. In order to measure proteasome activity in the worm, a substrate is added to worm lysate that releases a fluorescent AMC molecule when cleaved by the proteasome. The accumulation of AMC is measured by a plate reader, and the relative rate of accumulation relates to the amount of proteasome in a linear fashion (Figure 1C). CF512 (*fem-1*, *fer-15*) temperature sensitive sterile worms were used to eliminate progeny. Synchronized populations were staggered so that all lysates are collected and tested at the same time, without any freezing, to preserve the state of the proteasome. Because general proteostasis declines early in adulthood, we expected to see a similar decrease in proteasome activity. Surprisingly, we measure a dramatic increase in activity with age (Figure 1A). The activity occurs gradually over the first five days of adulthood and is maintained at levels about 2.5 fold higher than day 1 (Figure 1B). Upon further testing with different substrates, we consistently observe an increase in CTL activity, but the TL and CL activities are much more variable and do not show a consistent increase (Figure 1D). While the plate reader assay is convenient for rapid measurement of activity in many samples, it does not give any information about relative proteasome abundance or differences between 20S, 26S, and 30S assemblies.

Proteasome activity and assembly increase with age in *C. elegans*.

Our plate reader analysis showed that proteasome activity increases with age in the worm. However, this data does not show the specific activity changes in the different proteasome assemblies. Also, raw activity levels cannot give any information on how the activity is increased, by activation or increase in proteasome abundance. In order to determine the assembly states and abundance of the proteasome in the worm, lysates were run on native gels, thus maintaining the proteasome structure and allowing for measurements of both activity and protein levels of fully formed proteasomes. By soaking the native gel in the same substrates as the plate reader assay, it is possible to compare the relative activity of the various proteasome assemblies. Similar to the plate reader assay, 26S/30S proteasome activity increases with age from day 1 to day 6 of adulthood (Figure 2A). Interestingly, the proteasome activity increase is seen in the CTL as well as the TL and CL activities. Similar age related proteasome increase is seen at different time points, genetic backgrounds, and growing conditions (Figures 3A, 6A, 7A). The proteasome assemblies were confirmed by transferring the same gel for Western blot analysis (Figure 2B). As expected, the 20S specific antibody (α 1-7) is detected in the 20S as well as the 26S and 30S positions while the 19S specific Rpt-5 antibody is only detected in the 26S and 30S positions (Figure 2B). Our initial experiments showed that proteasome activity increases during the same time frame of general proteostasis decline. However, we wanted to see if there were any changes in older worm populations that have not yet started to die, but show telltale signs of aging including age onset paralysis. In contrast to earlier time points, day 11 worms no longer show maintain high proteasome activity levels (Figure 2A). Interestingly, a non-active band

(**), visible by western blot using the α 1-7 antibody indicates incomplete 20S assembly that increases with age (Figure 2B). It has long been observed that aged populations of worms contain some that move more and live longer than others (Huang, Xiong et al. 2004). In order to determine if proteasome activity decline precedes paralysis, I wanted to separate out a synchronous aged population into two groups: healthy motile worms, and ailing paralyzed worms. To do this, I utilized an observation that paralyzed worms do not easily release from bacterial plates into the M9 liquid we use to collect them. To test if motile versus immotile worms have varying proteasome activity, day 11 worms were collected using two washes. The first wash was gentle, only collecting the more motile worms. The second wash removed the remaining, less motile ones. When comparing the proteasome activity of these different sub-populations by native gel, we see that the more “youthful” worms maintained a higher proteasome activity than the immotile worms (Figure 3B). While other proteostasis declines have been measured before this late stage paralysis (Ben-Zvi, Miller et al. 2009), proteasome activity decline is concurrent, suggesting its dysfunction is caused by, not the cause of, late life deterioration of the worm shortly before death. We conclude that proteasome activity increases with age in *C. elegans*. This increase is maintained as long as the worms appear “youthful” but is rapidly reduced concurrently with age-related paralysis.

***daf-2* worms have a dampened but prolonged proteasome activity increase with age**

Our previous observations show that proteasome activity increases in an inverse relationship to proteostasis decline with age in the worm. In order to test if this relationship is direct or coincidental, we wanted to test the effects of improved proteostasis on proteasome activity. Mutations in the insulin like receptor, *daf-2*, have been shown to both increase the lifespan and proteostasis capabilities of *C. elegans*. CF512 worms, with and without the *daf-2* mutation, were grown to different ages and analyzed. Plate reader analysis shows that proteasome activity still increases with age in *daf-2* worms (Figure 4A). However, this increase is significantly lower than that observed in the CF512 background (Figure 4B). To investigate further, we looked at native gel analysis in the long lived worms. While we expect the improvement in proteostasis in the *daf-2* worms to show a dampened proteasome activity increase, the increased healthspan of these worms could ameliorate the late-life dysfunction we measured at day 11. Because *daf-2* worms have a longer and more varied development, comparing worms on day 1 is difficult, but our results indicate proteasome profiles are not significantly different. However, it is clear that by day 6 of adulthood, the proteasome activity of *daf-2* worms has increased, though at a lesser extent than the CF512 control (Figure 5A). Interestingly, at day 11, the *daf-2* worms maintain their activity while the CF512 worms show a dramatic decrease. The decrease in activity is most notable in the 30S assembly. Additionally, the CF512 worms show bands (**) in the Western blot consistent with incomplete proteasome assemblies at day 6 and 11. These “other” bands are reduced in the *daf-2* counterpart indicating the proteasome population is healthier (Figure 5B). The (**)

band is not always present in the native gel assays, and may be due to slightly different handling of lysates and dissociation of formed proteasome or biological variability of the samples. Nonetheless, all lysates within an experiment are treated equally, so the accumulation of incomplete proteasomes with age still indicates a reduced capacity to maintain fully assembled proteasomes. Similar to the differential washing experiment (Figure 3B), we do not observe a decrease in proteasome activity by day11 with *daf-2* worms since the entire population is still youthful and motile at this age. We conclude that *daf-2* mediated lifespan extension causes a moderation of the proteasome activity increase with age, but maintains these levels longer in conjunction with a lengthened healthspan.

Dietary Restricted worms have a dampened and prolonged proteasome activity increase.

To see if the effects of lifespan extension and proteasome activity are specific to *daf-2*, or a more general response to improved proteostasis and lifespan, we also measured the effects of dietary restriction (DR) induced lifespan extension on proteasome activity. Initial studies were carried out using *eat-2* mutants in a CF512 background. These worms have a slower feeding rate that induces dietary restriction. Similar to reduced insulin signaling, DR is known to improve proteostasis. If proteasome increase with age compensates for decreased proteostasis, we would expect to see a dampened response with age. Similar to *daf-2* worms, *eat-2* worms show a dampened increase in CTL activity with age by plate reader analysis (Figures 6A, 6B). Another method of dietary restricting worms is using varying bacterial

concentrations in liquid culture. Bacterial Dietary Restriction (BDR) was used at *ad libitum* (AL) OD 1.5 and maximal DR OD 0.3 on wild type N2 worms grown at 20°C. It is important to note that these worms in liquid culture live longer than those grown on plates, thus the varying time frames from previous experiments. Worms grown at *ad libitum* concentrations show a significant increase in proteasome activity by day 10 of adulthood, followed by a decrease by day 14 (Figure 7A). Dietary restricted worms still show an increase by day 10, though less than *ad libitum*. However, this activity is maintained, and by day 14, DR proteasome activity is equal to, if not higher than the AL control. These experiments show that proteasome activity increase with age is consistent across multiple genetic backgrounds, and growth conditions. Also, DR induced lifespan extension leads to a dampened but maintained increase in proteasome activity with age, similar to *daf-2* lifespan extension

Discussion

While most biological systems decline in function with age, proteasome activity does not always follow this pattern. Previous studies have shown that age can have varying effects on proteasome activity between organisms, tissues, and the specific proteolytic activity tested (Keller, Gee et al. 2002). It is well established that proper proteasome maintenance is critical for cellular function and is integral in a myriad of pathways. While perturbations to the proteasome cause rapid cellular dysfunction and organismal sickness, the reverse correlation is not typical. In fact, it seems that proteasome activity may compensate for, not be the primary cause of, age related proteostasis decline. Previous studies show a decrease in proteostasis

before dramatic changes in phenotype, such as paralysis (Ben-Zvi, Miller et al. 2009). Furthermore, mechanisms that extend organismal lifespan such as reduced insulin signaling or dietary restriction increase general proteostasis. These lifespan extending methods have been shown to increase folding capacity as well as protein clearance, specifically by autophagy (Jia and Levine 2007; Hansen, Chandra et al. 2008). Remarkably, proteasome activity does not follow this pattern. Our observations indicate that proteasome activity actually increases with age in *C. elegans*, and is maintained much later into adulthood than other proteostasis mechanisms. Furthermore, lifespan extension by either IIS reduction or dietary restriction actually dampens proteasome activity with age. It is possible that the improved folding and autophagic capabilities of both the reduced IIS worms and the DR worms indirectly reduces proteasome activity by reducing overall ubiquitinated substrate levels. Because the UPS is used to control protein levels in many pathways, simply increasing its activity may not be beneficial for an organism. Instead, maintaining proper proteasome/substrate homeostasis may be more important.

Our data shows that proteasome activity is regulated and maintained with an inverse relationship to proteostasis robustness. This indicates that there is some type of feedback mechanism that modulates relative proteasome activity to maintain ubiquitin substrate homeostasis. This activity increase might be implemented by modifications of proteasome subunits or protein activators that increase the specific activity of proteasome pools. Additionally, alterations in subunit abundance or assembly chaperones can alter total 26S and 30S abundance and overall activity. Further investigation is required to determine how proteasome activity is regulated,

and what effects the increased proteasome activity with age has on UPS homeostasis.

Experimental Procedures

***C. elegans* maintenance and strains**

All *C. elegans* strains were grown on standard growth media NGM plates. Stocks were maintained at 15°C and maintained as in (Brenner 1974). CF512: (fer-15(b26) II; fem-1(hc17ts) IV); CF596: daf-2(daf-2(mu150)III; fer-15(b26); fem-1(hc17ts)), AGD151: eat2 (eat-2(ad1116); fer-15(b26); fem-1(hc17ts); and wild type (N2) strains were obtained from the Caenorhabditis Genetics Center.

Preparing worm lysate

Stocks grown at 15°C were bleached and ~1500 eggs were plated onto ten 100mm NG plates seeded with OP50. Plates were transferred to 15°C for 8-16h for ideal hatching rates and moved to 25°C for the remainder of the lifespan. To collect different aged populations, bleaches were carried out on different days to all be collected at once. To collect the worms, M9 is used to wash the worms of the plate and collect them in 15ml falcon tubes. The worms are rinsed 2-3x to remove bacteria. The worms are then washed in 4°C proteasome buffer (50mM Tris-HCL pH7.6, 5mM MgCl₂, 1mM EDTA, 1mM DTT, 5mM ATP, 10% Glycerol). Worms are kept on ice or at 4°C for the remainder. Equal worm volumes were collected with a 1:3 worm:buffer ratio. Worms were homogenized with glass and ceramic beads using

a Precellys 24 at 3x15s @ 6500 rpm. The lysate is then centrifuged on for 10min @10,000g. BCA protein determination is used to determine protein concentration and proteasome buffer is added to normalize all samples within an experiment.

Plate reader activity assay

For each experiment, 25 µg of total protein lysate was transferred to a 96-well microtiter plate (BD Falcon) then fluorogenic substrate was added. For measuring the chymotrypsin-like activity of the proteasome either Z-Gly-Gly-Leu-AMC (Enzo) or Suc-Leu-Leu-Val-Tyr-AMC (Enzo) was used. Z-Leu-Leu-Glu-AMC (Enzo) was used to measure the caspase-like activity of the proteasome and Ac-Arg-Leu-Arg-AMC for the proteasome trypsin-like activity. Fluorescence (380-nm excitation, 460-nm emission) was monitored on a microplate fluorometer (Infinite M1000, Tecan) every minute for up to 2h at 25°C.

Native Gel Activity Assay

Worm lysates were run on 3.5% native gels prepared in resolving buffer (90mM Tris base, 90mM boric acid, 5mM MgCl₂, 0.5mM EDTA, 1mM ATP) with 5mMATP, 1mM dithiothreitol, and 3.5% acrylamide from a 40% stock solution of acrylamide and bisacrylamide in a 37.5:1 ratio (Bio-Rad, 161-0148). These were run at 110V for 3hr at 4°C. Activity assays were performed by incubating the gels in activity assay buffer (50mM Tris-HCL pH7.6, 5mM MgCl₂, 5mM ATP) plus substrate for 20minutes at 25°C and developed using a BioRad Gel Doc with UV illumination.

Native Gel Western Blots

Prior to transfer, the gels were incubated in transfer buffer (25mM Tris base, 192 mM glycine) with 1% SDS for 10 minutes followed by a 10 minute incubation in transfer buffer. The protein was transferred to PVDF at 5V for 16h to PVDF in transfer buffer using an Idea Scientific Genie Blotter. Western blot analysis was performed with anti-20S alpha 1-7 (Abcam) and anti-Rpt5 (Abcam) and analyzed using the Odyssey system (LI-COR Biosciences, Lincoln, Nebr).

Bacterial Dietary Restriction

Batch BDR was initiated with synchronized populations of eggs hatched and raised at 20°C on NG agar plates containing OP50 E. coli until young adulthood. At the onset of young adulthood FUDR at 100ug/ml was applied to the plates, and 24 hours later approximately 7000 worms were transferred into 60 ml of liquid culture in 125 ml flasks with vented tops for sterile air exchange. The liquid batch cultures were treated with FUDR at 100ug/ml and underwent moderate rocking at 20°C. Worms were pelleted by gravity, and liquid cultures changed every 3-4 days with no additional FUDR added after the first week of Batch BDR.

CHAPTER THREE:

Proteasome Activity Correlates to Increased Assembly and Subunit Levels with Age

Introduction

Proteasome activity can be modulated in many fashions. The primary way specific pathways alter protein turnover by the proteasome is through modulation of the ubiquitin ligases more specific to the target protein. On a more global scale, there are protein activators of proteasome activity, such as AIRAP, that can increase the turnover rate of the proteasome upon induction of protein misfolding stress such as arsenic exposure (Stanhill, Haynes et al. 2006). Furthermore, post-translational modifications of specific proteasome subunits, like phosphorylation of rpt-6, have been shown to increase the activity and localization of the proteasome (Djakovic, Marquez-Lona et al. 2012). These activators and proteasome modifications may provide for a more rapid modulation of proteasome activity. For a more chronic stress, it may be necessary to increase the number of functional proteasomes. To do this, there would need to be sufficient levels of all the subunits necessary as well as the folding chaperones to properly assemble them. While studies have shown assembly chaperones to be critical, the regulatory control of proteasome subunit abundance is better understood. In yeast, the RPN-4 transcription factor is able to simultaneously up-regulate several subunits (Mannhaupt, Schnall et al. 1999). As RPN-4 has been shown to be rapidly degraded by the proteasome in both ubiquitin dependent and independent manners, it is only upon insufficient proteasome activity that RPN-4 would be able to initiate subunit expression (Dohmen, Willers et al. 2007; Ha, Ju et al. 2012). The mammalian counterpart to RPN-4 is not a homolog, but the NRF-1 and NRF-2 transcription factors (Kraft, Deocaris et al. 2006; Radhakrishnan, Lee et al. 2010). NRF-2 has a wide range of targets, and a splice isoform, TCF1,1 may be the specific method in which proteasome activity is regulated in a feedback loop (Steffen,

Seeger et al. 2010). Similar to RPN-4 in yeast, the Nrf-2/Tcf-11 transcription factor is rapidly degraded by the proteasome and thus its activity is inversely proportional to cellular proteasome activity. These proteasome regulatory feedbacks have been discovered and analyzed under acute proteasome stresses like proteasome inhibitors or through causing general protein stress through means such as oxidation and heat stress. Interestingly, mutations in these regulatory genes do not appear to effect proteasome levels and activity under normal conditions. Additionally, the relationship to activity with aging is unknown. It is possible that there are redundant and overlapping mechanisms, including undiscovered alterations of the proteasome, its assembly, or proteasome activators that regulate proteasome activity with age.

C. elegans has only recently been used to study the proteasome. Initial studies found *aip-1*, a homolog to AIRAP is critical for both normal lifespan and proteotoxic stress resistance (Yun, Stanhill et al. 2008). Furthermore, overexpression of *aip-1* in *C. elegans* was sufficient to protect against acute overexpression of A β toxic protein early in adulthood (Hassan, Merin et al. 2009). Interestingly *skn-1*, the homolog of NRF1/2 in *C. elegans* can regulate expression of *aip-1* (Ferguson, Springer et al. 2010). Additionally, *skn-1* has been shown to have direct transcriptional regulation of proteasome subunit genes (Li, Matilainen et al. 2011). However, it is not known if either *skn-1* or *aip-1* have an effect on proteasome levels during normal aging. In order to determine how proteasome activity is regulated with age in *C. elegans*, we first examined how proteasome and subunit abundance changes with age. By comparing this to the activity, we should be able to determine if activity is primarily regulated through an activator, or through increased proteasome abundance. Also, we looked at the effects of knocking down known regulators *skn-1*,

and *aip-1*. Furthermore, we studied the knock down effects of potential homologs for regulators found in yeast and humans: PSMD9, HSM3, and UBR2. Finally, we investigated the effects of increased proteasome activity with age on the UPS system by measuring levels of ubiquitinated proteins.

Results

Proteasome activity is directly correlated to proteasome abundance

Our previous research shows that proteasome activity increases with age in the worm. However, this increase in activity could be due to either increased proteasome abundance, or an increase in specific activity by some modification or activator. In addition to allowing us to measure relative activity of the different proteasome assemblies, native gels also enable us to compare relative abundance of the proteasomes by transferring the same gel used in the activity assay for Western Blot analysis. By comparing both relative activity changes with the relative abundance changes, we will be able to determine if proteasome activity is increased due to an increase in abundance of assembled proteasomes or through increased specific activity of a consistent population. By comparing native gel activity assays to their corresponding native western blots, we saw a direct correlation between activity and abundance (Figures 2, 3, 4, 7, 9). Unfortunately, quantitative measurement of native gel activity is difficult. Furthermore, altering either loading volume or lysate concentration can change the migration of the proteasome in the gel, limiting the ability to determine a standard curve. Because of this, all observations regarding activity and abundance in the native gels are based on qualitative, not quantitative

observations. Analysis of all native gel proteasome profiles show a direct correlation between activity and abundance, indicating the primary method in which proteasome activity is regulated with age is through altering the number of fully formed proteasomes.

Proteasome subunit levels correlate to activity and abundance levels with age.

Native gel analysis shows a direct correlation between proteasome activity and abundance with age. This increase in assembled proteasomes could be regulated through increasing a pool of limiting subunits, or by increasing the chaperone mediated assembly. If the former, we would expect to see certain subunit levels to correlate to assembled proteasomes and total activity. In order to measure total subunit levels, we ran SDS-PAGE gels from the same lysate used in native gels and plate reader assays. We started by testing various antibodies for different proteasome subunits. Unfortunately, none of these were specifically made for *C. elegans*, and most had too much background to be confident about the correct band. Further complicating matters is the fact that we could not confirm a suspected band by RNAi since early RNAi causes developmental arrest, and transcriptional feedback mechanisms can counteract attempts to reduce subunits later in life. Also, many of the antibodies failed to cross-react with the confirmed proteasomes seen in native gels. Two antibodies gave unambiguous signal on both native and denature gels, an α 1-7 antibody that recognizes several of the alpha subunits, and an rpt-5 antibody that recognizes one of the ATPases that form the base of the 19S cap. In denaturing gels, there is not a clear increase in either protein with age correlated to the increase

in proteasome activity, but there is a clear decrease in abundance associated with the late life decline in activity and fully formed abundance (Supplemental Figure 3C).

In order to measure changes in subunit levels beyond the two antibodies we could confirm by Western Blot, we used quantitative MUDPIT mass spectrometry analysis to measure relative subunit levels between lysates. These are compared to an N15 labeled control to get a ratio of signal which gives reliable measurements of relative protein amount (Dong, Venable et al. 2007). We compared the relative protein levels of L4, day1, day2, day5, and day7 worms that showed the typical increase in activity in early adulthood and a decline by day7 (Figure 9B).

Normalization between lysates was performed by using the geometric mean of three standard normalization proteins: actin (act-1), tubulin (tbb-1), and GAPDH (gpd-2). Both actin levels and average relative N15 ratios are similar at all time points (Figure 9A). Using quantitative mass spec, we could simultaneously measure the majority of the subunits at most time points, only Rpn-10, Rpn-11, and Rpn-12 were insufficient to get any comparison at different ages. If certain subunits are rate limiting for the overall assembly and activity of the proteasome, we would expect to see the same trend in subunit abundance as activity, or shifted earlier, allowing time for assembly. Also, because of the stability of proteasomes, we may expect to find a decrease in the subunits preceding a decrease in activity as there would be a delay between the depletion of the active pool and the assembly or lack thereof due to subunit availability. Interestingly, the changes in subunit abundance are not dramatic, but some of them do mirror the same trends we observe in activity, though often declining already by day 5. Specifically pas-5, rpn-6, and rpn-8 have patterns that mirror what we would expect based on activity levels (Figure 8). While the extent of level

changes does not match those of the activity assays, it has been noted the mass spec analysis may underestimate the fold-change. Remarkably, *rpn-6* has been identified independently in our lab to regulate proteasome activity increase in *glp-1* sterile strains (Vilchez, unpublished). Overexpression of *rpn-6* was capable of increasing proteasome activity and vastly improving stress resistance in the worm. We conclude from the mass spec analysis that proteasome activity is likely regulated through specific subunit abundance, and late-life decrease is a consequence of a global decrease in proteasome subunits.

Knockdown of potential proteasome regulators is insufficient to alter proteasome activity increase with age.

Previous studies in *C. elegans* have shown potential roles for *aip-1* and *skn-1* in regulating proteasome activity in response to induced stress. However, neither has been tested with regards to the increase in activity we observe as worms age. To test this, we knocked down expression of potential regulators by RNAi. In order to avoid any developmental effects and focus the study on adult aging, we used RNAi to knockdown either *aip-1* or *skn-1* starting at the L4 stage. Neither *aip-1* nor *skn-1* RNAi had any observable effect on proteasome activity increase with age by either plate reader or native gel analysis (Figure 10A and 10B respectively). Unfortunately, a negative result can be hard to interpret. While *aip-1* and *skn-1* have been implicated in proteasome regulation, the aging related increase may go through a different pathway.

Additionally, we tried to knockdown other proteasome regulators that have not been fully characterized in worms. We searched for homologous *C. elegans* genes to known proteasome regulatory genes identified in other organisms. We found potential homologs to UBR2, HSM3, and PSMD9 that had RNAi available in the library. The yeast gene Rpn4 is a transcriptional regulator that acts similarly to Nrf-2 in mammals in that it is rapidly degraded by the proteasome (Mannhaupt, Schnall et al. 1999). Ubr2 is the E3 ubiquitin ligase that targets Rpn4 to the proteasome, and knocking it down can increase proteasome rates in yeast (Wang, Mao et al. 2004; Kruegel, Robison et al. 2011). Hsm3 has been shown to bind to Rpt1 in yeast to assist in 19S assembly and is required for proper 26S and 30S proteasome assembly (Roelofs, Park et al. 2009). PSMD9 is a mammalian proteasome chaperone that is required for proper assembly of the 19S base (Kaneko, Hamazaki et al. 2009). In worms, homologs to UBR2 and PSMD9 have been identified by sequence similarity and are named *ubr-1* and *psmd-9* respectively. I also found an uncharacterized gene, C31E10.5, that was the top result in a blastp search for homologs of yeast HSM3. Interestingly, all three of these genes show developmental arrest by RNAi, something we would expect from an integral proteasome regulator. RNAi knock down of these genes from L4 to day 5 of adulthood had no effect on proteasome activity as measured by plate reader or native gel assays (Figure 11). Again, the negative result is difficult to evaluate, especially because these genes are not fully characterized and homology is based solely protein similarity. Unfortunately, none of our RNAi experiments were able to determine a regulator of proteasome activity with age. While it is likely that some of these proteins are involved in proteasome regulation in

C. elegans, their activity is either not specific to the regulation due to aging, or there are redundant pathways that can compensate for the loss of one regulator.

Ubiquitinated protein levels are stable with age.

After determining that proteasome activity increases with age in *C. elegans*, we wanted to see the effect on native proteasome substrates: poly-ubiquitinated proteins. If the activity increase is not accompanied by a similar increase in target substrate, we would expect to see a change in proteasome substrate homeostasis, and a decrease in poly-ubiquitinated proteins. On the other hand, if proteasome activity is maintained with age to compensate for an increase in substrate, we would expect to see little to no change in the ubiquitin profile. Using different antibodies for total ubiquitin, mono-and poly, and poly-ubiquitinated proteins, we looked at the ubiquitin profile of worms at day1 of adult and day9 with maximal proteasome activity (figure 12). Interestingly, we do not observe a significant change in the ubiquitin profile at these different ages, despite the increased proteasome activities. Due to general proteostasis collapse early in worm adulthood, one would expect to see an increase in proteins targeted to the proteasome. However, the increase in activity seems to counteract this. Along with the negative correlation to aging and lifespan extension, the ubiquitin homeostasis points to regulated control of proteasome activity in response to increased levels of ubiquitinated substrates caused by general proteostasis collapse.

Discussion

The Ubiquitin Proteasome System is integral to cellular function and proteostasis. Mechanisms to regulate proteasome activity have been observed in almost every aspect of the UPS system. While many of the regulatory pathways have been discovered using specific targets or stresses, it remains unclear which pathways of regulation determine normal proteasome activity homeostasis with age. Additionally, while the proteasome itself is conserved in all eukaryotes, not all regulatory elements have clear homologs from yeast to mammals and *vice versa*. Determining the regulatory elements controlling proteasome activity with age is difficult in higher organisms because of varying tissues and relatively long lifespans. Conversely, aging studies in yeast are controversial and may be less relevant to multicellular aging mechanisms. Additionally, yeast may be too far removed evolutionarily to find homologs that may be important for human aging with regards to proteasome regulation. *C. elegans* has potential to be a good model to study proteasome activity with age.

Our data indicate that proteasome activity increases with age in the worm. This increase is correlated to an increase in assembled proteasomes. Likewise, proteasome subunit abundance of specific subunits, including rpn-6, correlates to assembled proteasomes, and these subunit changes may precede and predict proteasome assembly and activity. Due to the limitations of the mass spec analysis, we are unable to determine stoichiometric comparisons between the different proteasome subunits. However, we may be able to elucidate the critical limiting subunits by focusing on those that mirror or predict proteasome assembly and activity states. Known proteasome regulators, RPN4 and NRF1/2 are known to be

transcriptional regulators of proteasome subunits. I suspect that similar, possibly homologous transcriptional regulators are responsible for regulating limiting subunit levels and overall assembly and activity.

Because many controlled pathways depend on proteasome degradation, it may be more important to maintain proper substrate/proteasome homeostasis than to simply increase proteasome activity. In fact, we see that the increased proteasome activity with age does not dramatically affect the ubiquitin profile of the worms. This suggests that relative proteasome activity is regulated to keep consistent substrate removal capability. Furthermore, lifespan extending mechanisms known to increase proteostasis do not increase proteasome activity, but rather, compared to normal lived counterparts have a decrease in activity. Previous studies using a full length *in vitro* proteasome substrate show that muscle tissues are able to maintain similar degradation rates as the worm ages (Hamer, Matilainen et al. 2010). Interestingly, the muscle tissue is one of the first tissues to phenotypically age in the worm. Both morphological deterioration and phenotypic paralysis of the muscle predictably precede death. In contrast, nervous tissues do not show gross morphological aging in worms, yet *in vivo* Ub-Dendra degradation rates diminish with age. These *in vivo* substrates do not directly measure proteasome activity, but the relative rates at which substrates are degraded. Likewise, the results in the muscle mirror those of our ubiquitin western blots that despite a probable increase in the production of misfolded proteins targeted to the proteasome, the rate at which substrate are degraded remains stable. Likewise, the reduced turnover with age noted in the nervous tissue may not reflect reduced proteasome activity, but possibly an increase in substrate abundance or an alteration of the targeting of the substrate to the proteasome. It is

possible that proteasome activity decreases in neurons with age in the worm, but these changes may be masked by more abundant tissues like the muscle or intestine in our studies.

In *C. elegans*, we have determined that proteasome activity increases with age. This increase is dampened when lifespan is extended through reduced insulin signaling and dietary restriction. While the proteasome levels and activity increase significantly with age, little change occurs in its primary substrate, ubiquitinated proteins. This data suggests a feedback mechanism that increases proteasome activity in response to increased protein misfolding with age. Mass spectrometry data shows that proteasome subunits such as rpn-6 could be altered to regulate proteasome activity. Taken together, we developed a model (Figure 13) in which aging induces an increase in misfolded proteins and proteasome substrate, feedback mechanisms then increase proteasome activity through increasing 26S and 30S abundance to compensate and maintain homeostatic rates of target degradation. This increase in proteasome abundance is likely through the transcriptional regulation of select proteasome subunits.

Experimental Procedures

Quantitative MUDPIT Mass Spectrometry

Worm lysates were prepared as described earlier for plate reader and native gel analysis. An N15 standard was created by growing a mixed population of worms on N15 labeled bacteria for two generations. Mass spec analysis and quantification was performed as described previously (Dong, Venable et al. 2007).

CHAPTER FOUR:

Addendum

Introduction

During my graduate studies, along with research is to be published on the proteasome, I have worked on a number of additional projects. Here, I will briefly describe the rationale and methods of some of these projects. Additionally I will discuss some observations of the proteasome in *C. elegans* that did fit into my main thesis

***C. elegans* proteasomes have some peculiar characteristics.**

Proteasomes are less stable in C. elegans than other organisms

Much of the work studying the proteasome in *C. elegans* was troubleshooting existing protocols for yeast or mammalian cells and adapting them to the worm. For instance, with native gel activity assays, previous research has shown that in order to see activity of the 20S core, it is necessary to add some compound that opens the gating alpha rings. Typically, this is accomplished with the addition of 0.02% SDS (Elsasser, Schmidt et al. 2005). Interestingly, while we often see 20S activity without any SDS addition (Figure 2A), addition of even 0.01% SDS rapidly kills all proteasome activity. This is specific to worm samples and not purified human or rabbit proteasomes. In addition to SDS sensitivity, worm proteasomes are also much more sensitive to heat than either yeast or mammalian ones. This is not completely unexpected since worms typically grow in lower temperatures than yeast and mammals. This observation indicates that worm proteasomes differ from yeast and mammalian proteasomes in some way, and they may be less stable.

Frozen worm lysates in PBS buffer show evidence of a “trans-activator”

Initially, a great deal of focus went into examining a potential proteasome regulator that is responsible for increasing proteasome activity with age. One way to test if there is some trans-activator in older worms was to mix the lysate of young and old worms and look at the effects on proteasome activity. If there is an activator in older worms, we may expect the activity of a 1:1 mix with young worms to be higher than a simple average. On the other hand, if a repressor was present in younger worms, we could expect to see an activity of the mixture lower than an average of the two. Initial experiments not only showed that old lysate can increase the activity of young lysate, but it was very potent (Supplemental Figure 1A). Even a 4:1 mixture of young:old was able to bring the activity near the levels of 100% old lysate. This is a very promising result indicating that old worms contained some soluble proteasome activator that could rapidly and efficiently increase the activity of young lysate. However, I had concerns over the handling of the worm lysates. At this point, one synchronized population of CF512 worms was aged and different samples were collected in PBS buffer on different days and frozen at -20°C. Though all samples were frozen at least 24 hours, and lysis was performed directly before experimentation, I was concerned that the differential times of freezing, at only -20°C, with a simple PBS buffer, may not be ideal. When I tried flash freezing the samples in liquid nitrogen before storing at -80°C, I lost all “trans-activator” when mixing samples (Supplemental Figure 1B). Furthermore, upon changing protocols to use only fresh samples, in a standard proteasome buffer, I was never able to observe the “trans-activator” effect when mixing samples (Supplemental Figure 1C). While I cannot fully explain the initial mixing results, I suspect it is due to the fragile state of 26S and 30S

proteasomes. 20S and 19S complexes are notorious for dissociating unless in an ideal environment, and freezing at -20°C and using PBS likely led to increased dissociation. While the mixing results may be an artifact due to sub-optimal conditions, it is possible there is some type of “activator” that is only measurable under these conditions. It is also possible that the results seen by the plate reader assay are confounded by non-proteasome activity.

Plate reader assay results do not consistently correlate to native gels.

There are now many different ways of measuring proteasome activity. One of the original, and most quantifiable methods, is through plate reader activity assays. As the fluorogenic substrates are cleaved by the proteasome, a fluorescent AMC molecule is released and easily measured by a plate reader. One of the drawbacks of these fluorogenic substrates is that they are also cleaved by non-proteasome proteases. Typically, the amount of proteasome specific activity is confirmed by addition of proteasome specific inhibitors such as MG-132, epoxomicin, or lactacystin. In worm samples, these inhibitors knocked down 90-100% of proteasome activity by plate reader assays and abolished all activity seen on native gels. However, further analysis of these inhibitors has shown that they are not always specific to the proteasome (Meng, Mohan et al. 1999). This becomes a bigger concern when working in an organism like *C. elegans*, that has not been tested rigorously with these inhibitors. Another way to determine proteasome specificity is to separate out a lysate on a gradient or native gel, thus separating smaller proteases from the much larger multimeric proteasomes. This also allows for analysis between the 20S, 26S, and 30S assemblies. Surprisingly, in worm lysate separated by glycerol gradient

centrifugation, fractions with high CTL activity were associated with lower molecular weight fractions than those that reacted with proteasome antibodies (Supplemental Figure 2B). When mass spec analysis was performed on these two fractions, we found a large abundance of all proteasome subunits in the fractions with very little comparative CTL activity (Supplemental Figure 2A). The fractions with high CTL activity only identified four of the subunits, at less than one tenth the abundance measured in the later fraction. This, despite the fact that mass spectrometry analysis typically identifies all of the proteasome subunits in whole, unfractionated lysate. Similarly, native gels that are stopped prematurely, show a substantial smear of activity that runs faster, thus likely smaller, than the proteasomes. This activity is not seen with purified mammalian proteasomes and does not cross-react with any of the proteasome antibodies by western blot. Intriguingly, this “non-proteasome” activity is sensitive to both MG132 and epoxomicin. Also, the “non-proteasome” activity is specific to CTL activity as measured by both LLVY-AMC and Z-GGL-AMC. This may partially explain why the plate reader assays and native gels do not always directly correlate. While I saw a dramatic and consistent increase in activity in CTL activity with age, the CL and TL activities were more inconsistent (Figure 1A). However, the native gels showed similar results across all activities, which follow the hypothesis that proteasome activity is primarily regulated by relative abundance, not a change in specific activity (Figure 2A). One of the largest discrepancies between plate reader assays and native gels is seen when studying *glp-1* mutant worms. On plate reader analysis, day 6 worms have CTL activity many times higher than CF512, while RL and TL activities are up moderately and inconsistently (Supplemental Figure 3A). By native gel, the lower running “non-proteasome” activity is dramatically increased as

well as very active band that barely migrates into the gel (Supplemental Figure 3B). Additionally, short-run native gels show an increase in the “non-proteasome” activity that runs much faster than fully formed proteasomes. These activities are specific to the CTL activity and do not cross react with proteasome antibodies (Supplemental Figure 3B). In fact, native gel analysis shows that proteasome activity and abundance is actually reduced when compared to CF512 worms, matching western blot, mass spec, and QPCR data showing a reduced abundance of the majority of proteasome subunits (Supplemental Figure 3B). The discrepancy between the plate reader assays and the native gels is alarming. Because we have not identified the potential protease involved, the possibility remains that the “non-proteasome” activity seen by glycerol gradient and native gels is an artifact, and not truly an independent protease. Further analysis, potentially by mass spec of the fractions and native gel bands with the high CTL activity may be able to find this protease, if it exists.

GFP-ODC as an alternative full length substrate

The majority of full length *in vivo* substrates use ubiquitin tagged to a fluorescent (Dantuma, Lindsten et al. 2000; Hamer, Matilainen et al. 2010). While these have been shown to be targeted and degraded by the proteasome, they are also dependent and regulated by ubiquitin ligases and deubiquitinating enzymes (DUBs). An alternative proteasome targeting method is the addition of an ODC tag which targets a protein to the proteasome independent of ubiquitination (Murakami, Matsufuji et al. 1992). By using photoconvertible GFP tagged with GFP, we should be able to measure proteasome activity independent of ubiquitin ligases and DUBs. Multiple attempts at creating a PAGFP-ODC worm strain did not provide a usable

model. While I was able to see PAGFP accumulation when larvae were treated with proteasome inhibitor, the adults did not provide an interpretable signal (Supplemental Figure 4B). This suggests that the turnover rate of PAGFP-ODC is too high for the level of expression. It is possible that other promoters may provide a useful model, also, switching the PAGFP to Dendra could be more useful since lines could be screened more easily that have high enough abundance for study. In addition to *in vivo* analysis, a GFP-ODC may be useful for *in vitro* studies. Theoretically, one could measure the degradation of a full length protein by measuring purified GFP-ODC clearance over time. Initial attempts at creating this *in-vitro* reporter were unfruitful, though I believe the idea is valid (Supplemental Figures 4C and 4D).

Daf-2 RNAi is more protective than A β RNAi in an Alzheimer's model worm.

Previous studies in our lab showed the protective effects of *daf-2* reduction on worm strains expressing a toxic A β peptide (Cohen, Bieschke et al. 2006). In order to better understand this protection, we used RNAi to knockdown *daf-2* at different time points in adulthood. Similar to previous lifespan studies (Dillin, Crawford et al. 2002), we see full protection when *daf-2* is knocked down at day 1 of adulthood (Supplemental Figure 5A)(Cohen, Du et al. 2010). Interestingly, the protective effects continue to day 9 of adulthood, later than the protective effects on lifespan. This is one of the first observations uncoupling improved proteostasis from lifespan extension. As a control to see if *daf-2* reduction was working through mediating A β levels, I used A β RNAi at the same time points. Surprisingly, A β RNAi was less effective at day 1, and had no protective effects on day 5 or later (Supplemental Figure 5B). This limited effect is most likely due to the promoter driving A β . The *unc-*

54 promoter is expressed most highly at late development and drops off dramatically after day 1. This also explains experiments that showed expression only from day 1 on was insufficient to induce any paralysis. To accomplish this, A β RNAi was applied from hatching, and then the worms were switched to *dcr-1* RNAi at different days of adulthood to initiate A β expression. This data indicates that any protocol that improves the CL2006 A β model, when applied during adulthood, is likely independent of altering A β expression. This includes recent unpublished research by Ian Nicastro in our lab showing dietary restriction dramatically reduces A β toxicity, even when applied only during adulthood.

Hsf-1 is critical for L2 transition and necessary through late life in C. elegans

HSF-1 has been shown to be critical for normal lifespan and protection against proteotoxic stress (Hsu, Murphy et al. 2003; Cohen, Bieschke et al. 2006). In order to better understand the role HSF-1 in *C. elegans*, we looked at various timing windows of *hsf-1* expression to deduce its importance at different times in the worm lifespan. When applied at hatching, *hsf-1* RNAi leads to morphologically small worms with short lifespans. Interestingly, we noticed that *daf-2* worms grown on two generations of *hsf-1* RNAi developmentally arrest at the L1/L2 stage. To pinpoint the time that *hsf-1* was critical, I shifted second generation worms to *dcr-1* RNAi to rescue expression at different time points after hatching. In order to have a very tight synchronous population, I had a plate of thousands of eggs that were harvested by bleaching, then I would wash off any L1s with M9, leaving the eggs, allow the eggs to hatch for 15minutes, and wash off any new L1s that hatched in that time. Rescue later than 18 hours after hatching led to much higher rates of arrest, indicating a

necessity for *hsf-1* expression near that time (Supplemental Figure 6A)(Volovik, Maman et al. 2012). Additionally, I performed lifespan experiments on long lived *daf-2* (e1370) worms moved to *hsf-1* RNAi at different points in adulthood (Supplemental Figure 6B). When applied during adulthood, *hsf-1* RNA led to a consistent death in an average of about 22 days (Supplemental Figure 6C). This indicates that HSF-1 is relatively stable and/or the effects of its removal take time to develop. These two experiments show that *hsf-1* plays roles in both development and adult maintenance in *C. elegans*.

Summary

The ubiquitin proteasome system is as complex as it is crucial for eukaryotic survival. While I have made a lot of observations in regards to proteasome activity, assembly, and abundance with age, there are many questions still unanswered. Complicating matters in worms is the fact that current methods for examining proteasomes in other organisms do not always translate easily to worm research. Of note, the worm proteasome seems to be more labile, and vulnerable to even very low levels of SDS or mild heat shock. Additionally, many of the assays may be confounded by “non-proteasome” protease activity, especially the CTL activity. However, it may be interesting to find out what protease(s) cause the massive amounts of “non-proteasome” activity. Interestingly, this “non-proteasome” activity is also increased with age. This may indicate some type of coordinated cellular response to increase proteolytic activity with and beyond the proteasome. While the majority of my focus was trying to focus specifically on the proteasome, there may be something interesting in this unknown CTL activity that is increased with age,

especially in *glp-1* worms. Similarly, further research into the activator seen when old and young samples are lysed in PBS could in fact be fruitful. Though I abandoned these experiments for fear that the result was an artifact, I still cannot explain why old samples mixed with young samples bring the CTL activity of the mixtures up to but not over the heightened old samples alone. Interesting findings regarding the proteasome, or possibly the “non-proteasome” activity may be possible by further investigating this phenomenon.

Beyond researching the proteasome, I designed and performed experiments looking at key regulators of lifespan and proteostasis control. Using RNAi at different time points, we were able to show that *daf-2* RNAi can be effective in protecting against proteotoxicity at time points beyond which it can extend lifespan. This was one of the first instances in which a measured improvement for a protein stress was not correlated to a longer lifespan. Also, my work looking at the expression and knockdown of A β in the worm model demonstrates that protective interventions at adulthood are likely separable from adult A β expression. Additionally, this raises further questions of how aging is affecting A β toxicity. While A β is minimally expressed after day 1 of adulthood, mean paralysis does not occur for days later. Further research in to what conformational or biological changes that occur with aging may lead to a better understanding of what exactly causes A β toxicity with age. Finally, timing experiments using *hsf-1* RNAi were able to show the critical importance of *hsf-1* at the L2 transition as well as a role in maximal lifespan. Many studies have shown *hsf-1* to be critical for normal lifespan and lifespan extension. However, there is debate whether the severe sickness caused by lack of *hsf-1* in development overstates its role with other pathways. By studying the effects of knockdown after L2,

it might clarify the pathways in which HSF-1 is more involved. Additionally, the consistent 22 day lag from *hsf-1* RNAi administration to death is intriguing. It is unclear if the delay is due to the relative stability of HSF-1, or the slow buildup of damage that is caused by its depletion. It may be possible with better antibodies to follow levels of HSF-1 as well as run experiments to look for changes of downstream targets that correlate to the observed death. These studies could find specific proteins and pathways involved with extending normal lifespan in the absence of acute stress.

FIGURES

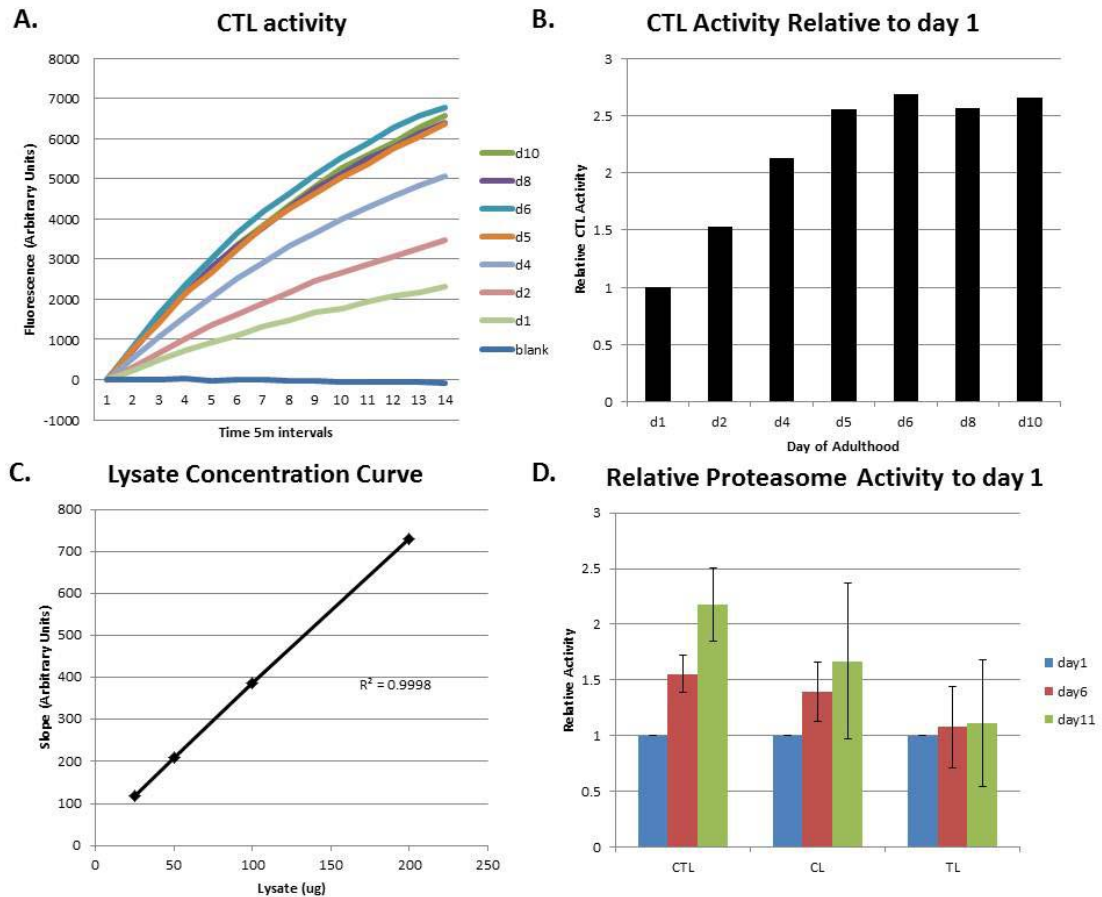


Figure 1: Chymotrypsin-like proteasome activity increases with age. (A) Plate reader activity assay shows increase in CTL activity from day 1 of adulthood to day 10 (d1-d10). Raw fluorescence readings from cleavage of CTL specific substrate (Z-GGL-AMC). (B) Quantification of activity, (slope from A) relative to day 1 (d1) of adulthood. CTL activity increases gradually to day 5 and is maintained through day 10 of adulthood. (C) Linear curve with various concentrations of lysate showing our results are within the linear range $R^2=0.9998$. (D) Relative proteasome activity of day1,6, and 11 of adulthood. CTL (LLVY-AMC) levels shows a consistent increase, while CL (LLE-AMC) and TL (RLR-AMC) do not.

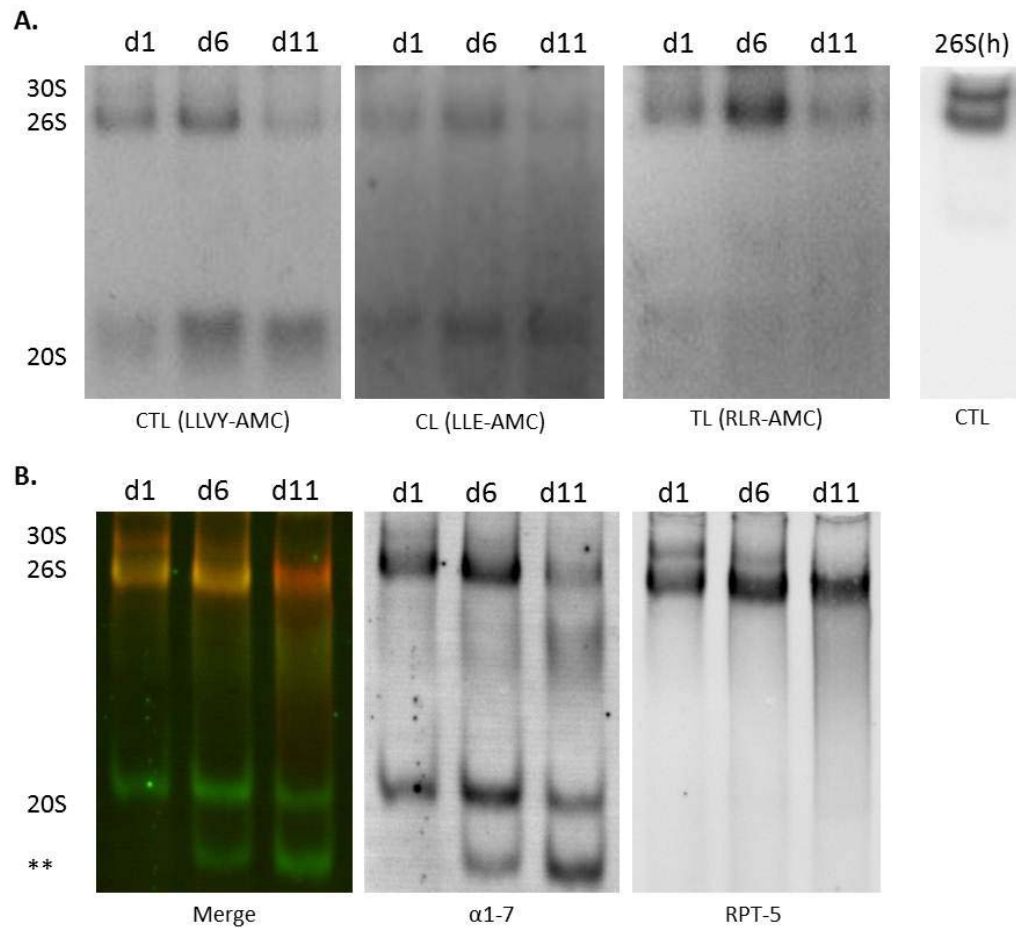


Figure 2: Native Gel assays of day1, day6, and day11 of adulthood. (A) Native Gel activity assays show an increase in activity from day 1 to day 6 in CTL, CL, and TL activities. Day 11 worms show a decrease in activity from day 6. The 20S, 26S and 30S bands were confirmed by comparing to purified human 26S proteasome (Enzo BML-PW9310) and western blot. (B) Western Blot analysis from CTL gel shown in (A). α 1-7 antibody of the 20S core cross- reacts with the corresponding 20S, 26S, and 30S proteasome bands. Another band below 20S, **, may be immature 20S. The RPT-5 antibody of the 19S cap is only present in the 26S and 30S bands.

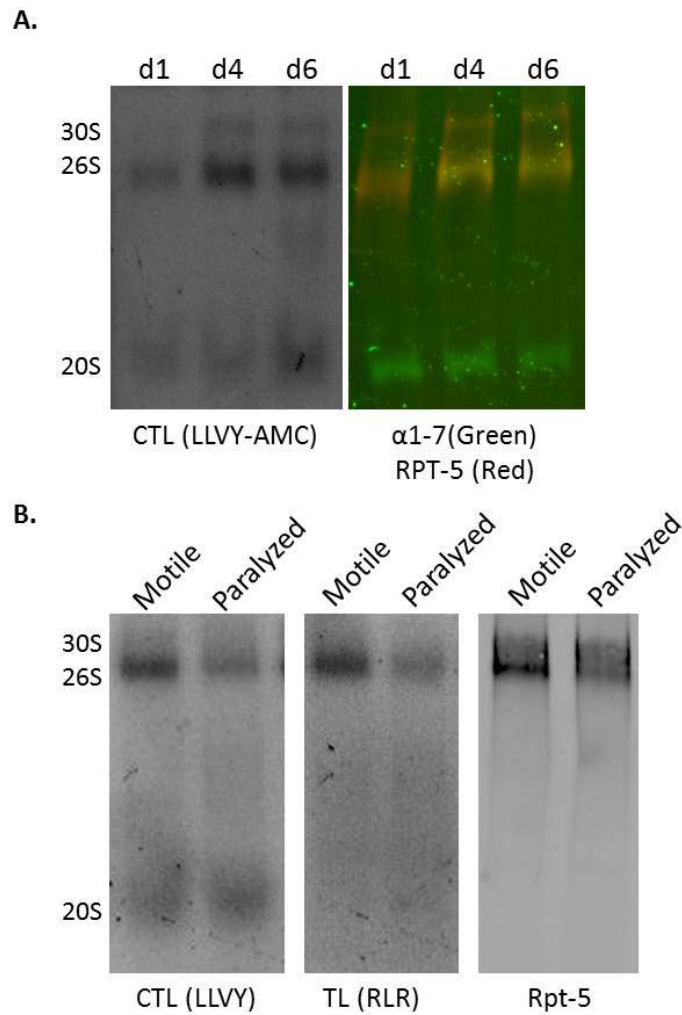


Figure 3: Native Gel proteasome assay of aged worms. (A) CTL activity assay (left) shows increase in proteasome activity by day 4 of adulthood. Western Blot analysis from same gel confirms activity is from proteasomes as α 1-7 (20S) is present in 20S, 26S, and 30S fractions while RPT-5 (19S) is only present in 26S and 30S bands. (B) Day 11 worms were separated into motile and paralyzed fractions by sequential M9 washes. The motile (first wash) worms show increases in CTL and TL proteasome activity. Western Blot with RPT-5 antibody confirms activity is proteasome specific.

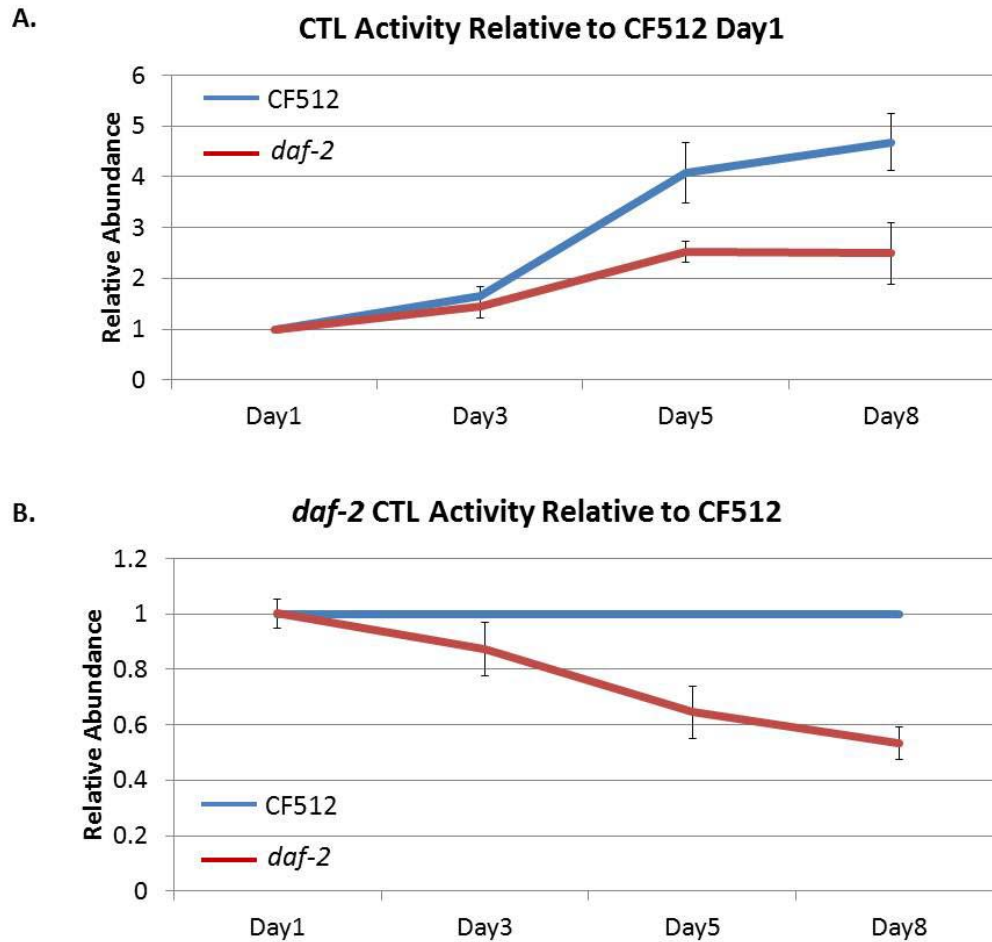


Figure 4: Plate reader assays show CTL activity increase is dampened in *daf-2* worms. (A) *daf-2(mu150)* in a CF512 background were harvested at day1,3,5 and 8 of adulthood. CTL (Z-GGL-AMC) activity measured by plate reader analysis is plotted relative to day1 of CF512. Proteasome increases with age in *daf-2* worms, but to a lesser extent than the CF512 control. (B) The same data in (A), but relative to CF512 at each time point. By day 3, *daf-2* has significantly less CTL activity than CF512 control. This discrepancy increases at both day 5 and day 8.

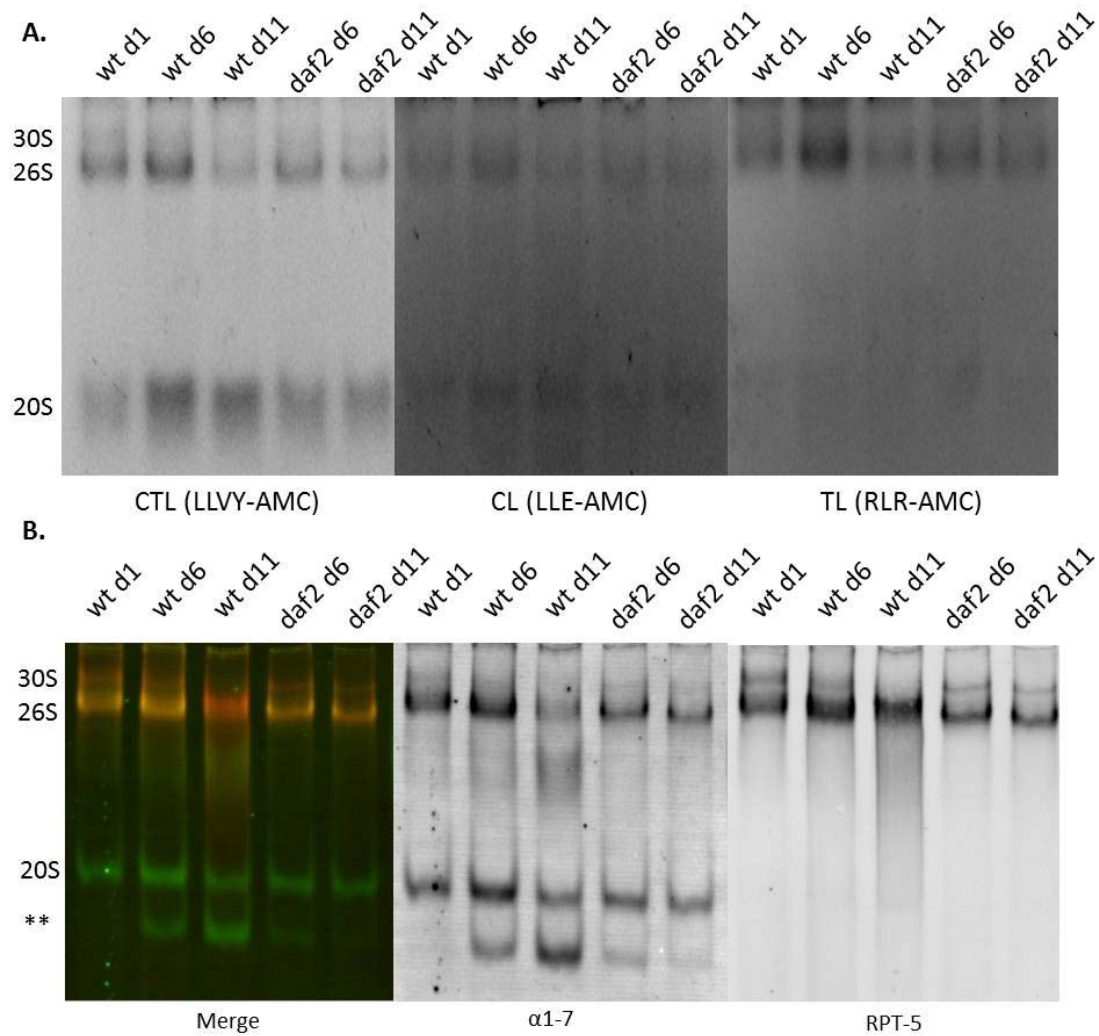


Figure 5: Native gel analysis of aged CF512 and *daf-2* worms. (A) Native Gel Activity assays were performed on wild type(wt) control worms and *daf-2*(mu150) worms in a CF512 background at day 6 and 11. *daf-2*proteasome activity (CTL,CL,TL) is less than CF512 control at day 6, but does not decrease at day 11 of adulthood. (B) Western blot analysis confirms activity is from proteasome. The ** band that increases with age in CF512 worms is much reduced in *daf-2* worms.

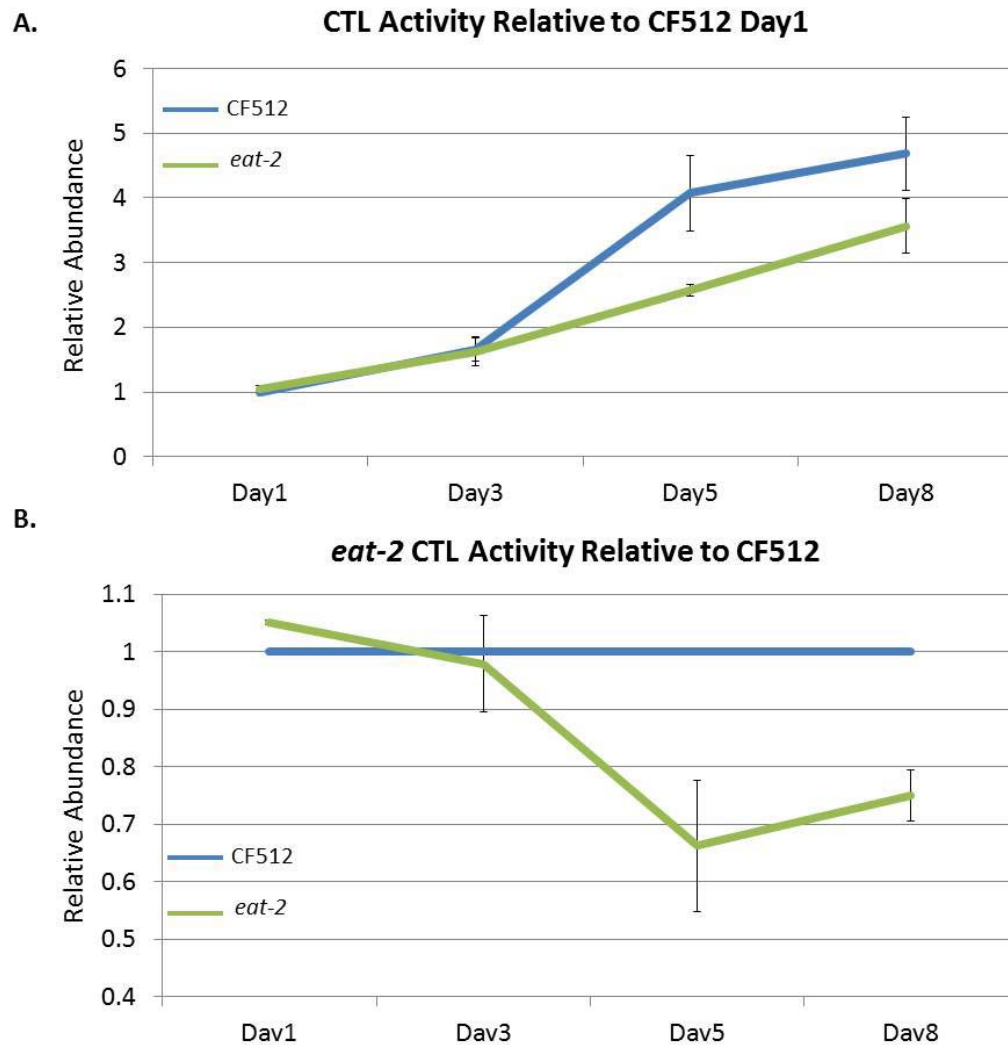


Figure 6: Plate reader assays show CTL activity increase is dampened in dietary restricted *eat-2* worms. (A) *eat-2* and wild type worms in a CF512 background were harvested at day1,3,5 and 8 of adulthood. CTL (Z-GGL-AMC) activity measured by plate reader analysis is plotted relative to day1 of CF512. Proteasome increases with age in *eat-2* worms, but to a lesser extent than the CF512 control. (B) The same data in (A), but relative to CF512 at each time point. Relative CTL activity is significantly decreased in *eat-2* worms at day 5 and day 8 of adulthood.

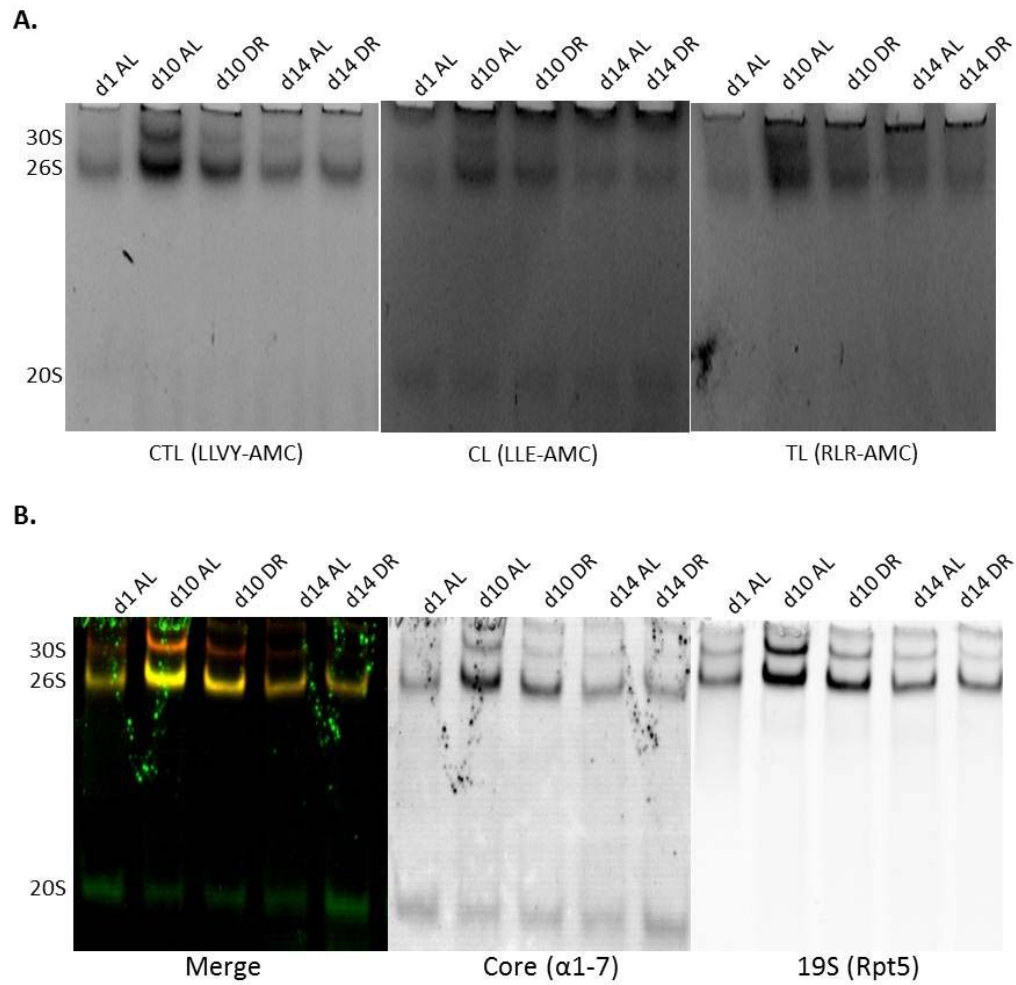


Figure 7: Native gel proteasome analysis of aged worms under dietary restriction in liquid culture. (A) Wild type N2 worms were grown in liquid culture under *ad libitum* (AL) or dietary restriction (DR) conditions from and harvested at day 1, 10, and 14 of adulthood. AL worms show an increase in all proteasome activity between day 1 and day 10. This increase is attenuated under DR conditions. (B) Western Blot analysis confirms activity is proteasome specific. Additionally, relative proteasome abundance is similar to the activities measured in (A).



Figure 8: Mass spec data with relative proteasome subunit abundance. Relative subunit abundance from L4, days 1,2,5, and 7 was performed by mass spec in comparison to common N15 labeled standard. Relative abundance is in comparison to L4. Few of the subunits increase in abundance as activity does between L4 and day2. Notably, pas-5, rpn-6, and rpn-8 levels mimic the activity (see Figure 9). All of the subunits show a decrease in abundance by day 7, more notably in the 19S (rpn's, rpt's).

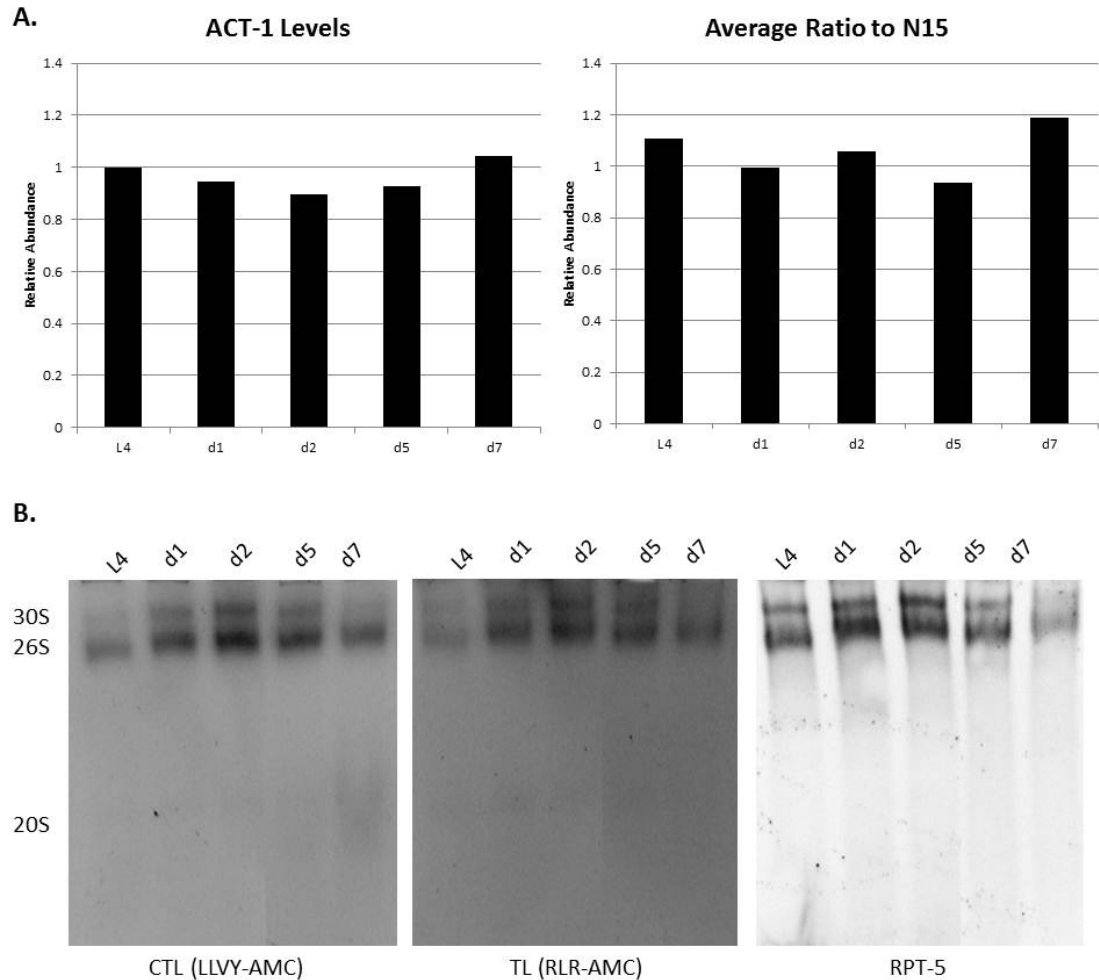


Figure 9: Mass spec controls from Figure 8. (A) Control standards confirm total protein levels consistent with age. (left) Actin (act-1) control protein levels indicate well normalized total protein between different aged worms. (right) An average ratio of all proteins in each sample compared to the n15 standard. There is little difference between different ages, confirming proper normalization. (B) Native gel analysis of samples used for mass spec. (left and middle) CTL and TL activities go up from L4 to day 2, this is maintained at day 5, dropping off at day 7. (right) Western blot analysis using the 19S specific RPT-5 antibody shows activity is proteasome specific and relative activity change over time is similar to change in relative abundance of 26S and 30S proteasomes.

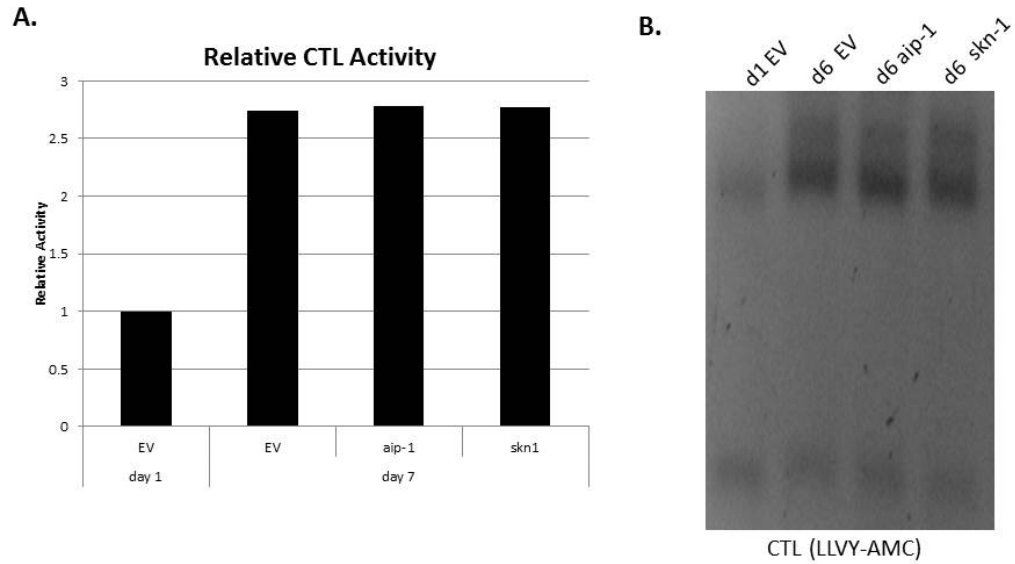


Figure 10: *aip-1* and *skn-1* RNAi is insufficient to alter proteasome activity increase with age. (A) Plate reader analysis measuring CTL activity (LLVY-AMC) shows that neither *aip-1* nor *skn-1* RNAi has an effect on the age related increase in activity with age. RNAi was started at L4 to avoid developmental defects. (B) Native gel analysis shows adult RNAi of *aip-1* or *skn-1* does not affect the activity or assembly of the proteasome with age.

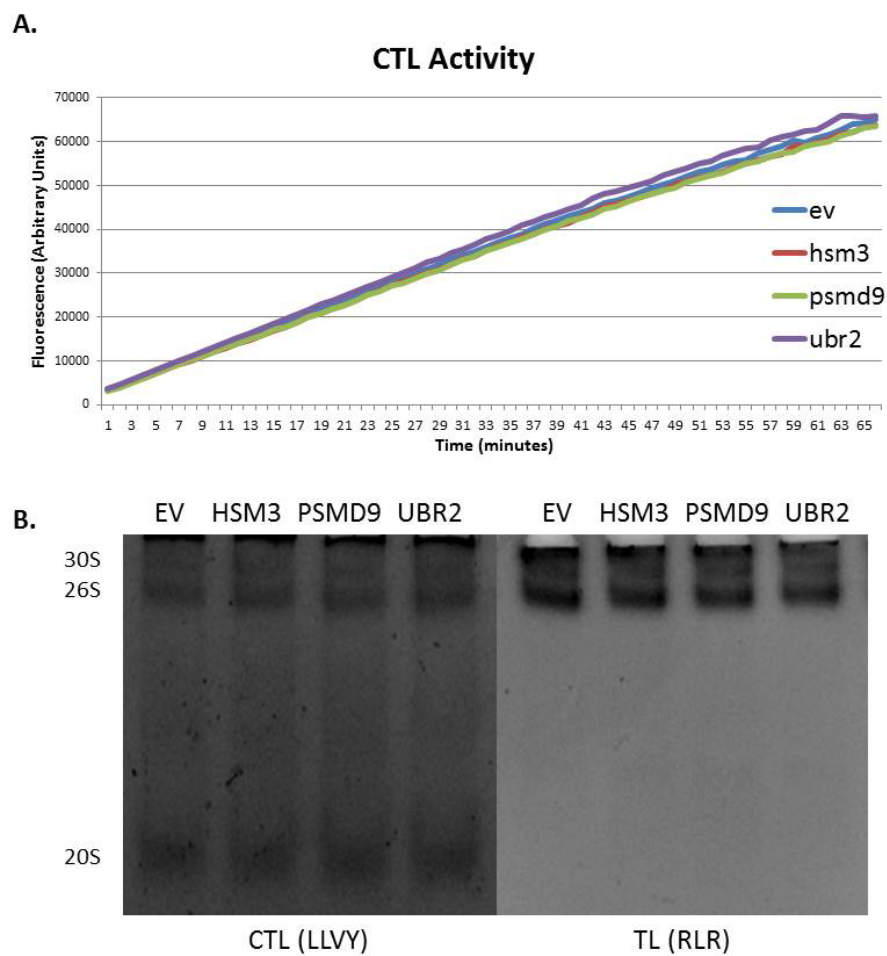


Figure 11: RNAi of potential proteasome modulators has no effect on proteasome activity increase under normal aging. (A) Plate reader activity assay shows similar levels of CTL activity for worms grown on EV or switched to RNAi for potential homologs of HSM3, PSMD9, or UBR2.

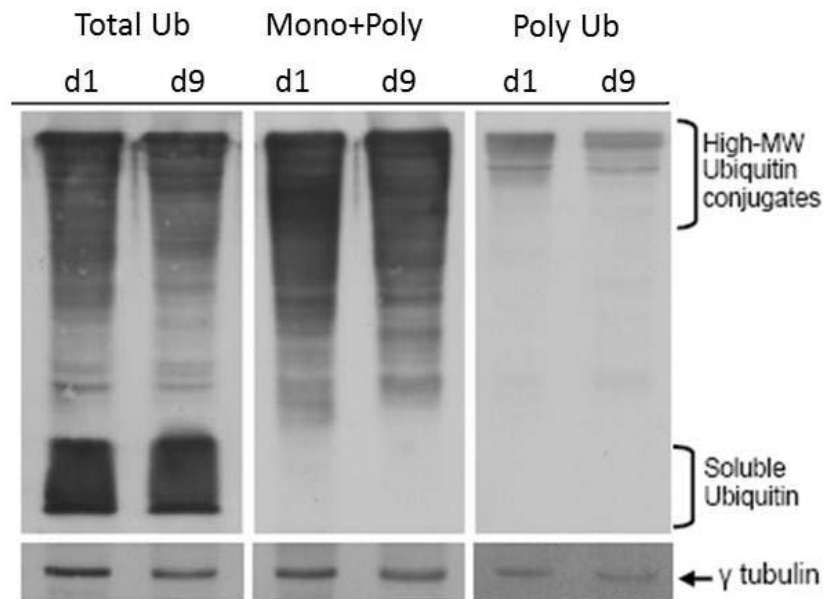


Figure 12: Ubiquitin levels are stable with age. Western blots of CF512 lysates from day 1 and day 9 of adulthood. Antibodies against total Ubiquitin (MMS258R), mono and poly-ubiquitin (FK2) or poly-ubiquitin (FK1) show similar levels between day1 and day9. Tubulin antibody was used as a loading control.

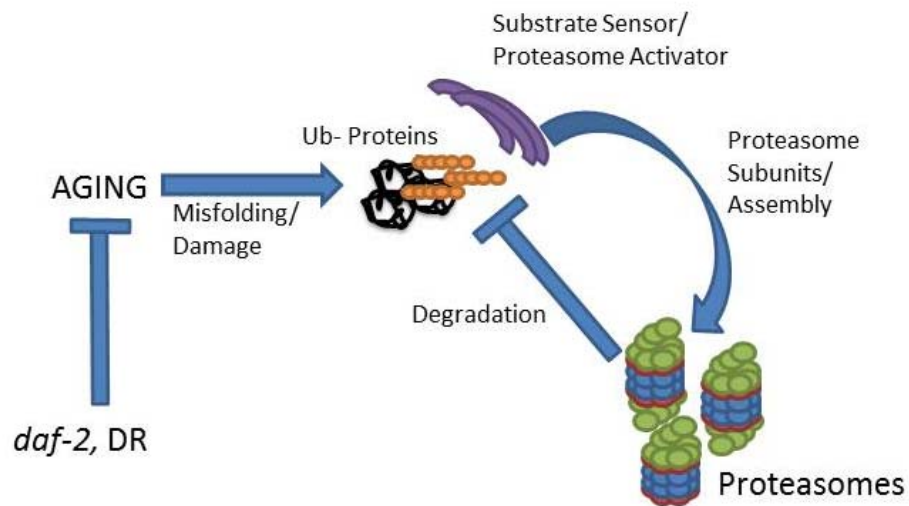
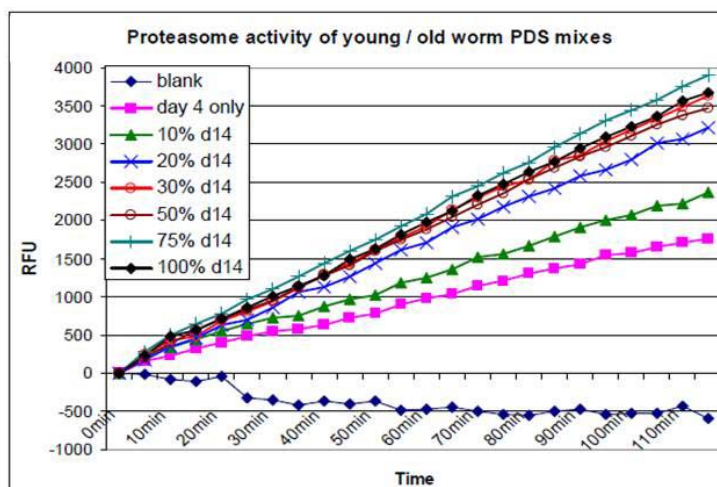
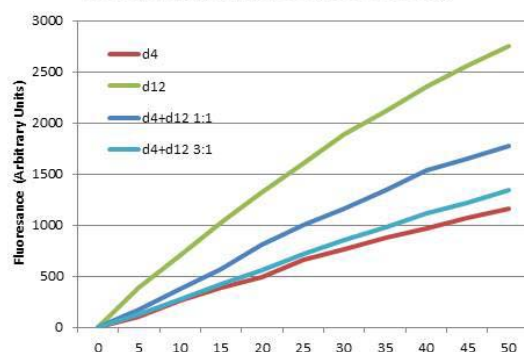


Figure 13: Model: Proteasome activity increase compensates for age related protein damage. As the worm ages, the ability to properly fold and maintain proteins, decreases. Many of these damaged proteins are targeted to the proteasome for degradation. In order to maintain proper UPS capability, proteasome abundance and activity is increased to compensate. Lifespan extending mechanisms such as reduced IIS (*daf-2*) or dietary restriction (DR) improves protein quality and requires less proteasomes for optimal UPS capability.

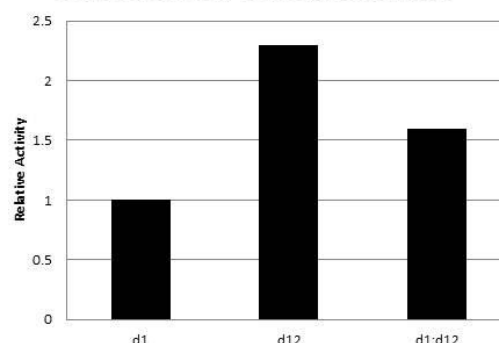
A.



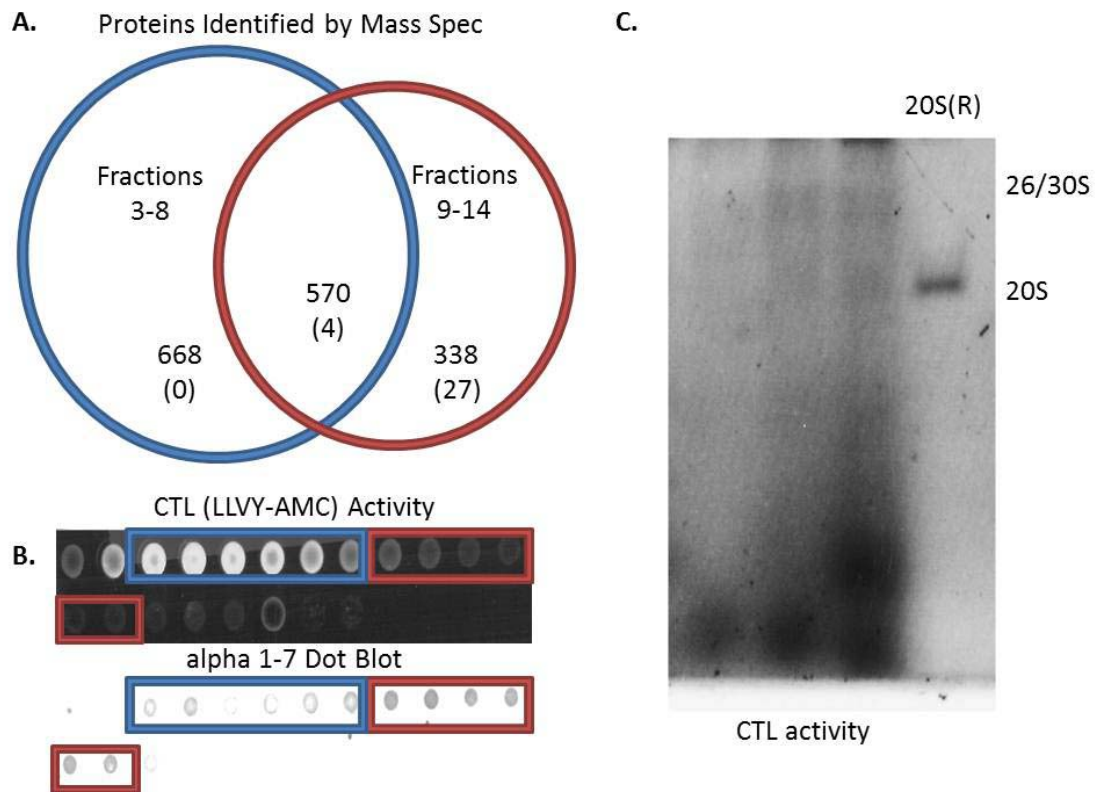
B. CTL Activity After Flash Freezing



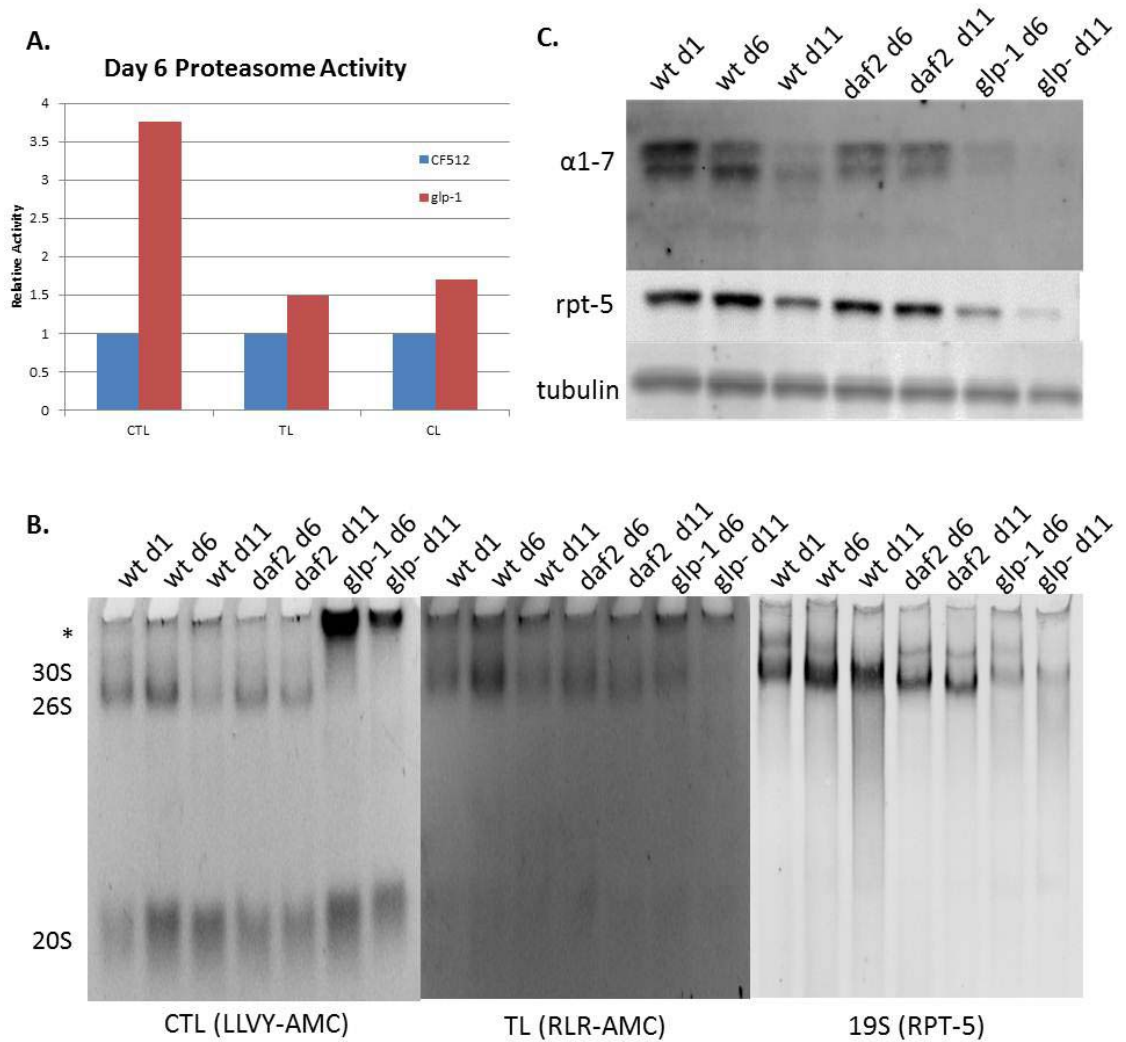
C. Fresh Samples/ Proteasome Buffer



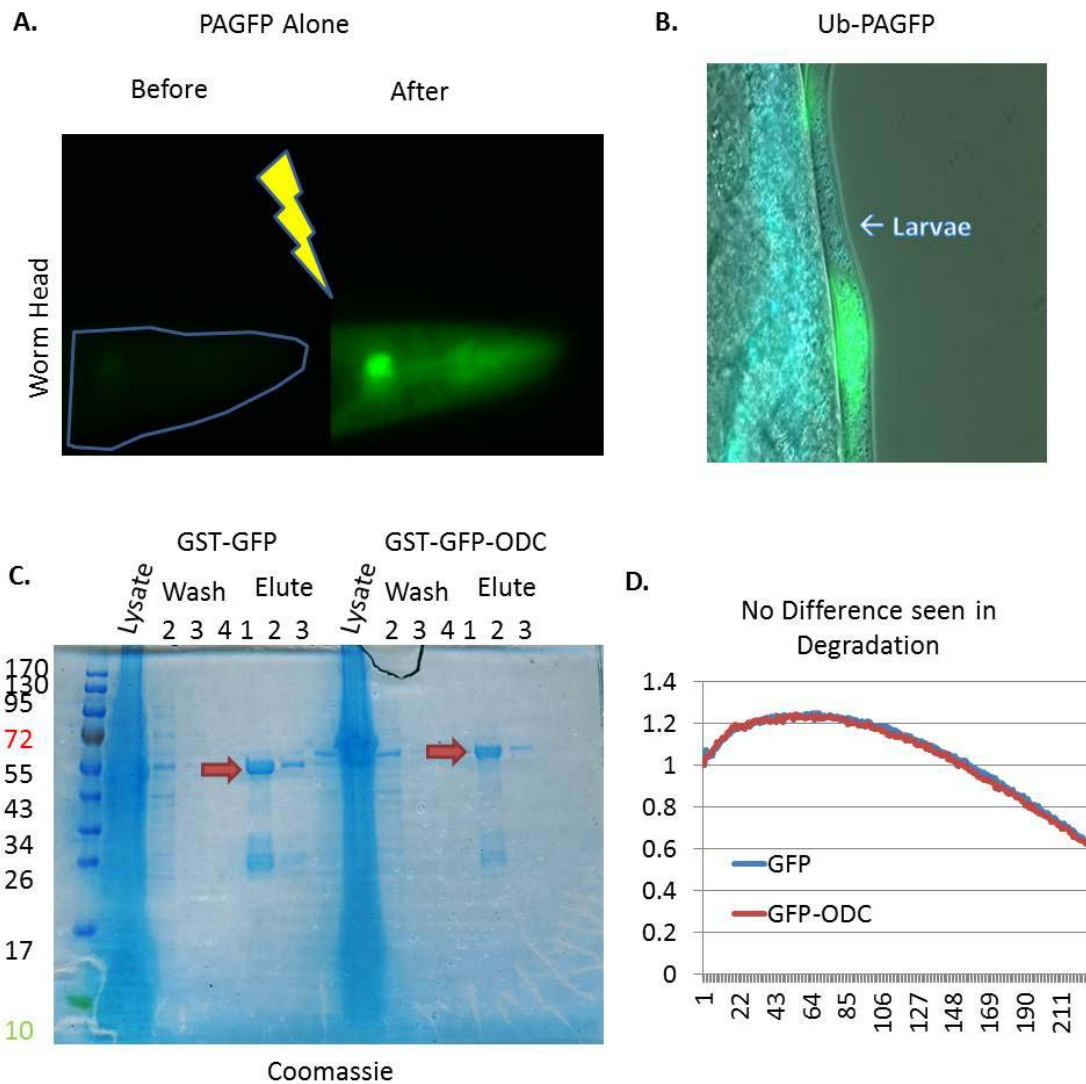
Supplemental Figure 1: Under simple lysis methods, old lysate is able to “activate” young lysate with mixing. (A) Different proportions of young (day4=day1 of adult) and old (d14=d10 adult) lysate are mixed together and tested for CTL (Z-GGL-AMC) activity. At only 20-30% old sample, the activity is equivalent to 100%. This indicates a trans-activator that can increase the activity of the young samples. Worms were from one bleaching, collected and frozen at -20°C and lysed in PBS. (B) Same as (A) except worms were flash frozen in liquid nitrogen and stored at -80°C. No activation is visible. (C) Aged worms were collected on the same day and lysed in proteasome buffer without any freezing. No activator activity is observed.



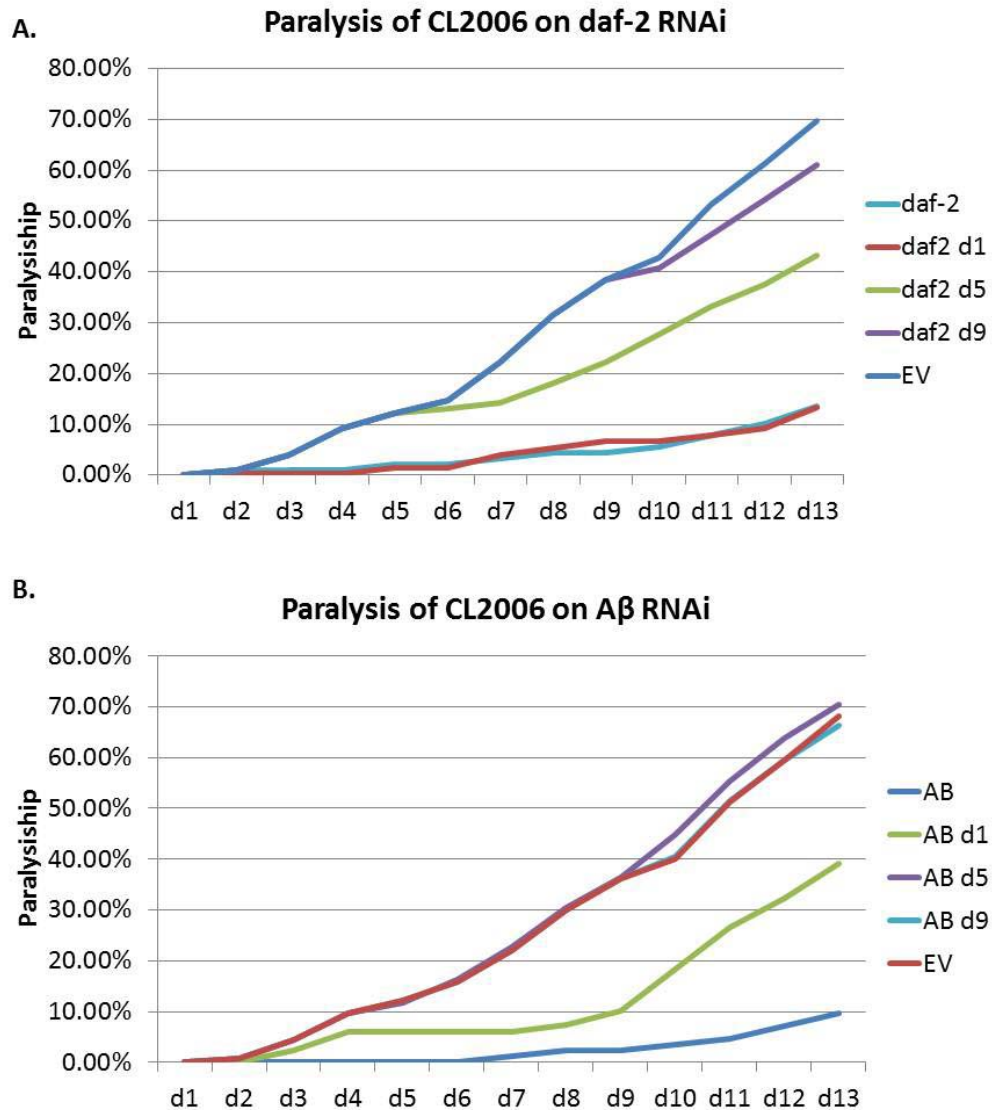
Supplemental Figure 2: Much of the CTL activity may not be from proteasomes. (A) Mass spec data from a sucrose gradient. Fractions 3-8 represents the portion of the gradient with high CTL activity and low alpha1-7 antibody cross reactivity (see B). Fractions 9-14 represent a portion of the gradient with much lower CTL activity but more alpha subunit detection. Only 4 of the 31 major proteasome subunits (pas1-7;pbs1-7;rpt1-6;rpn1-3,5-12) are detected in fractions 3-8. All 31 subunits are detected in fractions 9-14. Those identified in both have spec counts over 10-fold higher in fractions 9-14. This strongly suggests the activity in fractions 3-8 is not from fully formed proteasomes. (B) CTL plate reader assay viewed with Gel-Doc for comparison to dot blot using alpha 1-7 antibody. Fractions 3-8 (blue) have high CTL activity and low alpha subunit reactivity. Fractions 9-14 have lower CTL activity and higher alpha 1-7 signal by dot blot. (C) Native gel activity assay of CF512 worms at day1,6, or 11 of adulthood. Purified 20S rabbit antibody shown. This gel was run for only 60min vs 180min, thus retaining more (likely smaller) proteins. A large amount of CTL activity, that increases with age, is visible well below the full formed proteasomes.



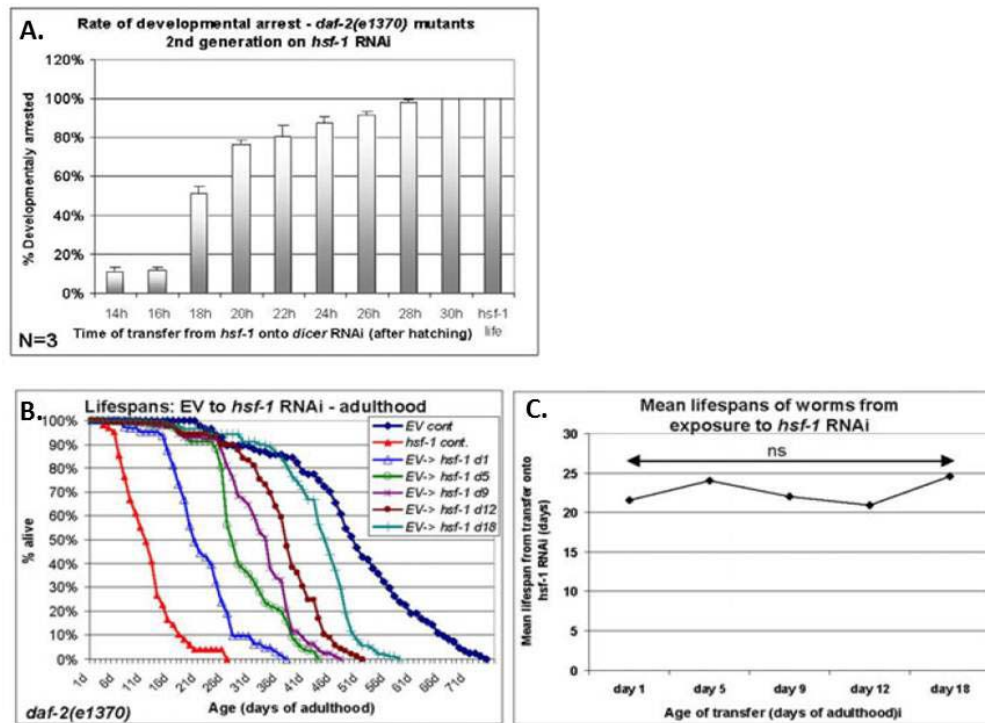
Supplemental Figure 3: *glp-1* proteasome activity measurements differ from plate reader to native gel analysis. (A) Plate reader activity assay from day 6 CF512 and *glp-1* worms. (LLVY-AMC) activity is increased much more than TL or CL activity. (B) Native gel proteasome assays show strong CTL activity retained at the very top of the gel (*). TL activity does not show this same increase. Western blot analysis with RPT-5 antibody shows little to no cross reactivity with the (*) CTL activity.



Supplemental Figure 4: ODC-tagged fluorescent proteins as full length proteasome substrate. (A) PAGFP expressed in all tissues (sur-5 promoter) shows sufficient abundance and activation by 405nm. (B) PAGFP-ODC is only visible in larvae of worms treated with proteasome inhibitor MG-132. This shows the PAGFP-ODC is folding well and likely rapidly degraded by the worm proteasome. (C) GFP-ODC expression and purification using GST tag. Red arrows indicate expected bands for GST-GFP and GST-GFP-ODC respectively. (D) The GFP and GFP-ODC were added to worm extract similar to plate reader proteasome assays. Relative fluorescence depletion is similar with and without ODC tag, indicating it is not sufficiently targeted and degraded by the proteasome.



Supplemental Figure 5: *daf-2* RNAi is more protective from A β toxicity than A β RNAi. (A) CL2006 worms expressing A β were grown on *daf-2* RNAi from hatch, or switched from EV to *daf-2* at day1,5, or 9 of adulthood. Hatch and day 1 time points have similar paralysis rates. All time points show protection from paralysis soon after switch to RNAi. (B) CL2006 worms were transferred to A β RNAi. From hatch, A β RNAi sufficiently protects worms from paralysis. By day 1, there is protection, but much less than *daf-2* RNAi (A). No protection from paralysis is seen when A β RNAi is started at day5 or day9 of adulthood.



Supplemental Figure 6: *hsf-1* expression is critical during the L2 transition and necessary throughout adulthood for maximal lifespan of *daf-2* worms. (A) *daf-2(e1370)* worms grown on two generations of *hsf-1* RNAi developmentally arrest at the L1/L2 transition. Highly synchronized *daf-2* worms were grown on a second generation of *hsf-1* RNAi then transferred to *dcr-1* to allow *hsf-1* expression at the given time points after hatching. If *dcr-1* is not started by 18h after hatching, over half the worms remain developmentally arrested. (B) Lifespan of *daf-2* worms grown on *hsf-1* RNAi from hatching or shifted from EV at the given time points. (C) Plot of mean lifespan of *daf-2* worms after transfer to *hsf-1* RNAi. There is a consistent ~22 day lifespan after transfer to *hsf-1* RNAi.

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