UNIVERSITY OF CALIFORNIA, SAN DIEGO

Genetic mechanisms of dietary restriction-mediated protection against proteotoxicity in *C. elegans*.

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

in

Biology

by

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2012

The Dissertation of Ian Arthur Nicastro is approved, and it is acceptable in quality and format for publication on microfilm and electronically:

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DEDICATION

I dedicate this thesis to my parents who have always been unconditionally supportive of my educational endeavors and graciously provided me with the means to be able to pursue my scientific ambitions. I would also like to dedicate this thesis to my late friend Louis Nguyen who always tried his best at everything he put his hand to.

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ACKNOWLEDGEMENTS

I would like to thank numerous past and present members of the Dillin Lab for their intellectual and emotional support throughout my PhD journey. Will Mair, Suzy Wolff, Siler Panowski and Ehud Cohen were very instrumental in teaching me many of the fundamental assays that I employed during my thesis work. Will Mair was also particularly helpful in providing valuable critique and suggestions concerning the experimental design of my project. Hyun-eui Kim, Derek Joyce, Peter Douglas, and especially Nessa Morantte provided training and greatly appreciated assistance for various biochemical assays. Additionally, Derek Joyce collaborated with me on the proteasome activity assays described in Chapter 4.

Nate Baird and Carlos-Daniel De Magalhaes Filho provided technological expertise on QPCR techniques, and Carlos-Daniel De Magalhaes Filho also ran some assays with *skn-1* animals to help confirm the reproducibility of some of my findings. Rebecca Taylor, Patti Follett, Celine Riera, Kristen Berendzen, Erik Kapernick, David Vilchez, Marcela Raices, Carsten Merkwirth, Milos Simic, Ye Tian, Zheng Liu, Deepti Wilkinson, Kristan Steffen, Vidhya Ramachandran, Thomas Heimbucher, and Virginia Butel assisted with everything from offering experimental ideas to ordering supplies and reagent preparation. Over the course of my time in the Dillin Lab I became quite close to many of the outstanding scientists I mentioned above, and I'm proud to be able to call them both my colleagues and my good friends.

Additionally I'd like to thank Dr. Malene Hanson from the Sanford-Burnham Medical Research Institute for her input on the design of the autophagy assays used in my thesis as well as kindly providing strains from her lab that were used in the results presented in Chapter 4. I want to thank Andrea Carrano from the Hunter Lab at the Salk Institute for Biological Studies for her suggestions relating to the *wwp-1* component of my project in Chapter 3. I also want to thank all of the members of the Proteostasis PPG who provided input on my project.

I want to kindly thank Dr. Edward Koo for being able to participate in the UCSD School of Medicine's Ruth L. Kirschstein National Research Service Award Training Grant on the Neuroplasticity of Aging from 2006-2007, and again from 2007-2008.

I would like to thank all of the members of my thesis committee, Dr. Randy Hampton, Dr. Edward Koo, Dr. Reuben Shaw, and Dr. Gentry Patrick, for their valuable suggestions on experimental design and overall support for my project.

I would like to especially thank my advisor, Dr. Andrew Dillin, for the amazing opportunity to be a part of his lab. Andy has a real knack for recruiting some of the best minds in the field into his lab which provides a cutting-edge environment in which to work and get project support. I greatly appreciated the high degree of independence which Andy permitted, allowing me to formulate my own research goals and then providing me with valuable

suggestions and feedback. Andy has a real passion for science as well as the ability to present research findings with the utmost enthusiasm and confidence, which can't help but rub off on those who work in his lab.

I want to thank a handful of close friends, some in the field of science and some not, who at one point or another during my time in graduate school played important and supportive personal roles: Marika Orlov, Kevin Ford, Paula Patterson, Andrea Cann, Billie Laws, Kelly Marlett, and Rochelle Lorkovic.

I would like to kindly thank my family, Frederick, Diana and Graham Nicastro for their continued personal support. And lastly I would like to thank my pet bearded dragon, Baxty, who for the past four years listened to every one of my practice talks.

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ABSTRACT OF THE DISSERTATION

Genetic mechanisms of dietary restriction-mediated-protection against proteotoxicity in *C. elegans*.

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Professor Andrew Dillin, Chair

We sought to determine if the genes shown to be required for DR to increase lifespan are also critical for DR mediated protection against proteotoxicity. Here we report the novel finding that some of these genes, *hsf-1* and *skn-1*, are necessary for both DR mediated lifespan extension and protection from proteotoxicity, while other genes, *pha-4* and *wwp-1* play only a critical role in DR induced longevity and are dispensable for the DR protective response. Additionally, we identify potential downstream targets of this DR

protective response, including components of the autophagy degradation pathway.

CHAPTER ONE:

An Introduction to Aging and Dietary Restriction in *C. elegans*

Introduction:

Aging is defined as the continuous accumulation of changes in an organism over time, arising intrinsically as well as through interaction with the environment. Some of these changes are harmful to the maintenance of homeostasis and render an individual more susceptible to diseases and disorders. Eventually, accumulation of enough of these changes negatively impacts an organism's ability to respond to stresses and sets in motion a cascade of events that eventually ends in death. Aging is a universal condition that affects all life; however, different organisms age at remarkably different rates. This observation suggests that aging is not simply a stochastic response but instead has a powerful element of genetic mechanism. In seeking to understand why we age, there is potential for discovering strategies that might delay this inevitable process. There remains a multitude of theories as to why we age; below I will briefly explore a few of the more influential theories of aging.

Influential theories of aging

Evolutionary theories of aging

Important evolutionary theories of aging include Mutation Accumulation, Antagonistic Pleiotropy, and Disposable Soma. Peter Medawar's theory of Mutation Accumulation states that mutations with harmful effects that manifest

later in life, which subsequently lead to dysfunction and death, are allowed to accrue randomly because most of the population has already succumbed to extrinsic hazards. George C. Williams refined Medwar's theory by removing the element of randomness, arguing instead for the role of pleiotropic genes; those which have more than one phenotypic effect. Williams postulated that any mutation which increases in organism's reproductive fitness would be selected for even if this conferred negative effects later in life. Thomas Kirkwood's Disposable Soma theory is based on the fact that calories are a finite resource and organisms must divide that energy between reproductive output and somatic maintenance. Kirkwood argued that natural selection favors genes which result in an energy expenditure profile weighted more heavily in favor of reproduction, and over time the slight neglect of somatic maintenance leads to aging and death. Taken together these theories yield a plausible explanation for why aging exists, the unfortunate evolutionary byproduct of increased reproductive fitness.

Metabolic theories of aging

Another influential theory concerning why we age is Denham Harman's Free Radical theory which states that aging is the result of an accumulation of DNA damage from unpaired electron species known as free radicals. This has come to be known as the Oxidative Stress theory of aging because cellular metabolic processes generate reactive oxygen species (ROS), which cause

oxidative damage to proteins, lipids and DNA over time (Cui 2012). ROS include such molecules as superoxide anion, hydroxyl radical and hydrogen peroxide; the first two of which are free radicals whereas hydrogen peroxide is a potent oxidizer. ROS are primarily produced as a byproduct of mitochondrial respiration as electrons leak from the electron transport chain and react with oxygen.

Mitochondria are believed to be the main site of oxidative damage by ROS, and this observation gave rise to an additional metabolic theory of aging known as the Mitochondrial theory of aging (Harman 1972). The mitochondrial theory of aging postulates that damage to the mitochondria and specifically to mitochondrial DNA leads to mutations that render the mitochondria more prone to ROS production, initiating a downward spiral that ends in mitochondrial failure and subsequent cellular death (Miquel 1980). Cells have multiple strategies for protecting themselves against oxidative damage, including multiple DNA repair mechanisms as well as antioxidants which scavenge ROS and neutralize them. These defense strategies become weakened as an organism ages and eventually are overwhelmed.

There has been a great deal of evidence supporting these metabolic theories of aging, such as data showing reduced free radical production with increased longevity as well as observations of shortened lifespan in animals lacking antioxidant genes (Finkel 2000; Elchuri et al., 2005). Additionally, reduced expression of genes that encode for components of the electron

transport chain have been shown to extend lifespan in invertebrate model systems (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003). There has also been considerable evidence to the contrary, including results showing that overexpression of antioxidant genes or treating with exogenous antioxidant compounds does not increase lifespan in mammals (Huang et al., 2000; Sohal et al., 2006). The mixed outcomes of the research aimed at testing these theories suggest that oxidative damage and mitochondrial function play a role in aging but also demonstrate that there is more at hand that remains to be discovered.

Studying aging in *Caenonhabditis elegans*

The soil nematode *Caenonhabditis elegans* is an especially attractive model system for studying the molecular mechanisms of aging due to the organism's short lifespan and the ease by which genetic manipulations can be performed (Brenner, 1974). The somatic cells of adult worms are non-mitotic and the lifespan of *C. elegans* averages around 20 days, permitting for aging assays to be conducted quickly. Adult *C. elegans* are comprised of slightly less than 1000 cells, the identities and developmental lineages of which are known because the zygote divides in a stereotypical and reproducible fashion (Riddle et al., 1997). Adult animals are approximately 1 mm in length and can be raised on an agar filled dish with bacteria as a food source. The *C. elegans* genome has been well sequenced and feeding these nematodes bacteria

expressing dsRNA for a gene of interest will effectively knockdown expression of that gene product by a mechanism of RNA interference (Riddle et al., 1997). RNA interference (RNAi) is especially useful for genetic screening as it permits for rapid experimentation of the roles of various genes. Transgenic *C. elegans* can be created through microinjection into the germ line to produce offspring possessing heritable extra-chromosomal arrays of a transgene. These arrays can subsequently be integrated into a chromosome through additional techniques. The transparency of this nematode permits for effective use of transgenic fluorescent reporter constructs as well as visualization of various physiological processes. Of the two genders, male and hermaphrodite, the latter possesses the ability to self fertilize; eliminating the need for complicated breeding schemes. This impressive array of useful attributes has placed *C. elegans* at the forefront of aging research. This simple nematode has helped identify components of several genetic pathways that regulate aging, including mitochondrial respiration, insulin signaling and dietary restriction.

Aging and insulin/IGF-1 signaling

Research conducted in both invertebrate and mammalian model systems has shown that perturbations in the insulin/insulin-like growth factor signaling (IIS) pathway result in profound extension of longevity (Bartke et al., 2008). The genetic components of the IIS pathway are highly conserved between mammals and invertebrates, which has led to *C. elegans* being

extensively used to explore this pathway. In *C. elegans* the IIS pathway has one insulin/IGF-1 receptor, DAF-2 (Fig. 1), which when bound by insulin-like ligands, activates the phosphatidylinositol 3-kinase AGE-1 to produce PI3 (Morris et al., 1996). The phosphatase DAF-18, homologous to mammalian PTEN, functions as an 'off switch' to oppose the activity of AGE-1 (Ogg and Ruvkun, 1998). PI3 produced by AGE-1 activates PDK-1 kinase, which in turn phosphorylates the kinases AKT-1, AKT-2 and SGK-1 (Paradis and Ruvkun, 1998, Paradis et al., 1999, Hertweck et al., 2004). This kinase trio phosphorylates the forkhead transcription factor DAF-16 (orthologous to FOXO1, FOXO3a, and FOXO4 in mammals) causing it to associate with the 14-3-3 protein FTT-2 in the cytosol that keep it excluded from gene targets in the nucleus (Cahill et al., 2001, Li et al., 2007).

Upon a reduction in insulin signaling, un-phosphorylated DAF-16 enters the nucleus where it associates with co-activators like SMK-1 and binds to an evolutionarily conserved consensus sequence (Wolff et al., 2006). Additional factors function to antagonize DAF-16 within the nucleus, such as host cell factor 1 (HCF-1) which can bind DAF-16 and prevent it from interacting with target genes (Li et al., 2008). DAF-16 activates a broad range of genes that lead to increased longevity as well as amplified resistance against heat and oxidative damage (Lin et al., 1997; Ogg et al., 1997). This includes genes that neutralize ROS like the superoxide dismutase *sod-3 and the* catalases *ctl-1* and *ctl-2,* as well as genes involved in metabolism and the heat shock

response (McElwee et al., 2003; Murphy et al., 2003). Knocking down any single transcriptional target of DAF-16 fails to fully prevent IIS mediated longevity, revealing just how complicated the lifespan response is (McElwee et al., 2003; Murphy et al., 2003).

DAF-16 is not the only factor regulated by DAF-2 and the IIS pathway, as two other transcription factors serve roles in IIS mediated longevity. Heat shock factor HSF-1, which is known for orchestrating the cellular heat shock response, has been shown to be essential for IIS pathway induced lifespan extension by DAF-16 (Hsu et al., 2003; Morley and Morimoto 2004). Despite this observation, the exact signaling mechanism by which DAF-2 regulates HSF-1 still remains to be elucidated. The additional transcription factor regulated by IIS is SKN-1, which serves a previously characterized role as the master regulator of the oxidative stress response, and has been shown to be directly inhibited by the kinase cascade downstream of DAF-2 (Tullet et al., 2008). SKN-1 is not required for IIS induced longevity, but does appear necessary to obtain the maximal lifespan extension possible from modulating IIS. Neither HSF-1 nor SKN-1 is considered a canonical member of the IIS pathway due to their previously characterized roles in regulating other important pathways. The regulation of HSF-1 and SKN-1 by DAF-2 demonstrates that DAF-16 is not solely responsible for IIS mediated longevity and it is possible that other factors remain to be discovered. Beyond

mitochondrial function and IIS there are additional pathways capable of profoundly extending longevity.

Dietary restriction and aging

Overview

A significant reduction in dietary caloric intake, known as dietary restriction (DR), dramatically increases the life span of a wide assortment of eukaryotic organisms (Weindruch and Walford 1988). DR is generally defined as a 30 to 60% decrease in calorie intake compared to *ad libitum* feeding, without leading to malnutrition of the organism. It is important to point out that DR is not simply a process which removes harmful overfeeding conditions, but rather an actual significant reduction below an organism's baseline calorie intake. DR produces a graded parabolic response (Fig. 2), where the progressive reduction of calories results in increasing longevity until a level of calorie reduction is achieved that yields the greatest increase in lifespan (Mair and Dillin, 2008). Reducing calories beyond that point will result in progressively less optimal lifespan extension as malnutrition begins to set in. At levels of extreme caloric reduction malnutrition is so significant that little or no lifespan extension is observed. Protocols for applying DR vary widely, with some focusing on decreasing levels of a specific dietary macronutrient like protein, while others represent a cutback in all dietary macronutrients (Houthoofd and Vanfleteren, 2006). The end result of these varied protocols is

still the same, a total reduction in overall caloric intake without malnutrition which leads to increased longevity.

Organisms that respond to DR

Remarkably, DR appears to be able to increase longevity in almost every eukaryotic species so far tested, suggesting that it is a public mechanism of lifespan extension. DR has been shown to extend the lifespan of the classical model organisms the yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster,* the mouse *Mus musculus*, and the rat *Rattus norvegicus* (Bishop and Guarente 2007a). Longevity extension has also been observed for less commonly studied animals including insects, fish and dogs placed on DR regimes (Mair and Dillin, 2008). Several long-term studies were initiated to examine the effects of DR on primates; however, these experiments are still ongoing. The initial observations from these primate studies have demonstrated that physiological changes associated with aging are in fact delayed in the DR groups (Rezzi et al., 2009). Additionally, the preliminary lifespan data from the few monkeys that have died so far is suggestive that DR may also extend lifespan in primates (Colman et al., 2009). Large-scale experimentation of humans adhering to DR diet regimes has only recently been undertaken, and so the effects of DR on human longevity remain to be seen (Holloszy et al., 2007).

Disease reduction by DR

Beyond simply making an organism live longer, DR also appears to prolong a youthful state by delaying the onset of many diseases associated with old age. The vast majority of research on the topic of DR has been conducted in rodents, and most of these studies focused on documenting the physiological benefits of DR regimes. These observations include decreased blood glucose and insulin concentrations, improved insulin sensitivity, lowering of body temperature, and reduced LDL levels (Sohal and Weindruch 1996). These same beneficial changes in physiological parameters have subsequently been shown in primates on DR regimes (Rezzi et al., 2009). In mammals, DR has been shown to lower the occurrence of such devastating age related disorders such as diabetes, cancer, neurodegenerative conditions and cardiovascular disease (Masoro 2002). These impressive health benefits do come at a price; humans adhering to DR regimes experience reduced physical stamina, diminished immunity, lowered libido and psychological stress (Jolly 2007). DR has become an appealing target for the research community to better understand in hopes of someday developing pharmaceuticals that might be able to yield some of these same benefits in humans without the unwanted side effects.

DR as an evolutionary adaptation

Despite over 75 years of research on the topic of DR, very little is known about the mechanism by which the positive effects of DR on health and longevity arise. During DR conditions, the limited caloric resources are directed to somatic maintenance at the expense of reducing growth and reproductive output (Mair and Dillin, 2008). This has led to the hypothesis that the DR response is an evolutionary adaptation that arose during periods of famine (Holliday, 1989). Extending longevity permitted an organism to delay reproduction until a point that famine conditions have passed, affording the offspring of an organism a higher rate of survival when food is plentiful (Shanley and Kirkwood, 2000). As an evolutionary adaptation, it is possible that the genetic mechanisms behind DR may be conserved between widely diverse species. Only recently have some of the genetic pathways underlying this phenomenon begun to be understood, thanks to the use of invertebrate model systems.

Performing DR in C. elegans

C. elegans have proven to be a powerful system for examining the genetic mechanisms of DR as many recent discoveries have been made using the worm, which will be discussed in subsequent sections. In a laboratory setting, *C. elegans* are fed nonpathogenic *E. coli* strains as their primary food source. Many different protocols have been developed to study DR in *C.*

elegans, with some being more accurate than others. Any feeding protocol that is used in an attempt to induce DR in an organism must achieve certain conditions to be considered a true DR regime (Mair et al., 2009). Foremost, the DR protocol must be capable of producing a graded parabolic response in which a feeding condition that results in maximal lifespan extension can be determined. Secondly, the DR protocol must result in a reduced rate of reproduction that correlates with the observed increase in lifespan. Lastly the DR protocol must actually reduce food intake and not simply diminish any sort of overfeeding or other deleterious effects due to husbandry. These conditions can all be satisfied in *C. elegans* by employing one of two commonly used DR protocols; each of which have their own advantages and disadvantages.

The first of these *C. elegans* DR techniques is known as bacterial dietary restriction (BDR) and involves serial dilution of a bacterial food source in liquid culture media (Mair et al., 2009). A concentrated culture of bacteria at a specified optical density is used as the *ad libitum* (AL) feeding control in which adult worms are then placed. Several dilutions are made from the AL culture stock to establish a range of DR conditions, which allows for researchers to determine if a parabolic longevity extension response is present. The bacterial concentration of each dilution is kept stable by preventing bacterial reproduction through the use of nutrient-free liquid media and antibiotics. This method of DR effectively reduces food intake because

wild type worms have a maximal rate of pharyngeal pumping that cannot be increased to compensate for reduced bacterial concentrations (Mair et al., 2009). BDR is advantageous because it is relatively simple to perform and satisfies all of the conditions required for a true DR regime. The only drawback of BDR lies in the fact that RNAi knockdown through bacterial expression has proven to be incompatible with liquid culture assays (which is discussed in greater detail in Chapter 3).

The second widely accepted technique for performing DR in C elegans involves the use of mutations which reduce maximal pharyngeal pumping rates, known as EATing defective mutants (Lakowski and Hekimi 1998). The most common mutations employed for this are those that affect the *eat-2* gene locus. EAT-2 is homologous to beta subunits of the nicotinic acetylcholine receptors found in postsynaptic junctions of mammalian muscles, and is expressed in two of the posterior pharyngeal muscles of *C. elegans* (Raizen et al., 1995; www.wormbase.org). Nicotinic acetylcholine receptors are ligand gated ion channels, and so mutations in *eat-2* change the frequency by which the channels can be activated and subsequently alter the rate at which muscles contract. Plotting the pharyngeal pumping frequencies of various EAT mutants versus lifespan or reproductive output, produces scatter plots that demonstrate the expected correlations for a true DR protocol (Klass 1983; Lakowski and Hekimi 1998). The *eat-2(ad1116)* mutant allele is one of a handful of EAT mutants that yield the greatest lifespan extension and remains

one of the most commonly used strains for DR studies (Lakowski and Hekimi 1998). An advantage over BDR *is that eat-2* animals can be raised on a bacterial lawn spread over solid agar plates, which allows for use of RNAi feeding to knockdown genes of interest. It is generally accepted that wild type and *eat-2* mutants receive similar doses of RNA knockdown from RNAi feeding despite the fact that *eat-2* mutants ingest less food, as the ingested RNAi is significantly amplified once taken up into the cells (Alder et al., 2003).

DR independence from mitochondrial respiration and IIS

The exact pathway by which DR extends lifespan is still unknown, but studies have shown that DR mediated longevity functions genetically independent of other pathways known to enhance longevity, such as perturbations in the IIS signaling pathway or the mitochondrial electron transport chain (Wolff et al., 2007). The independence of these three pathways arises from the fact that each possesses different temporal requirements as well as unique genetic components. Mitochondrial respiration must be downregulated during early development to extend lifespan, while reduced IIS signaling needs to take place during early to mid adulthood to result in increased longevity (Dillin et al., 2002a; Dillin et al., 2002b). Unlike the other two pathways, DR is able to extend longevity when imposed at any point during an organism's lifespan (Mair and Dillin 2008). Each pathway has key genetic components that are dispensable for the longevity associated with

modulating other pathways; genetic components of the ETC are unique to the mitochondrial respiration pathway, *daf-16* is unique to the IIS pathway, and DR has unique transcription factors (Dillin et al., 2002b; Houthoofd et al., 2003; Panowski et al., 2007; Steinkraus et al., 2008). DR treated organisms do not show a reduction in metabolic rate, providing additional evidence for independence between the DR and mitochondrial respiration pathways (Houthoofd 2002). Lastly, modulation of more than one pathway produces a super-additive on longevity, which further suggests that each pathway extends lifespan through an independent mechanism (Dillin et al., 2002b; Lakowski and Hekimi, 1998).

Genetic regulation of the DR longevity response

Recently there has been an extensive effort to uncover master genetic regulators of the DR longevity response. Studies using the nematode *C. elegans* have implicated four genes, *hsf-1, skn-1, pha-4,* and *wwp-1*, as being required for DR mediated life span extension (Bishop and Guarente 2007b; Carrano et al., 2009; Panowski et al., 2007; Steinkraus et al., 2008). Each of these genes has a previously characterized vital role in physiology: *hsf-1* orchestrates the cellular heat shock response, *skn-1* regulates the oxidative stress pathway, *pha-4* is required for foregut development, and *wwp-1* is an E3 ligase necessary for embryonic development (An and Blackwell et al., 2003; Carrano et al., 2009; Horner et al., 1998; Hsu et al., 2003). Three of

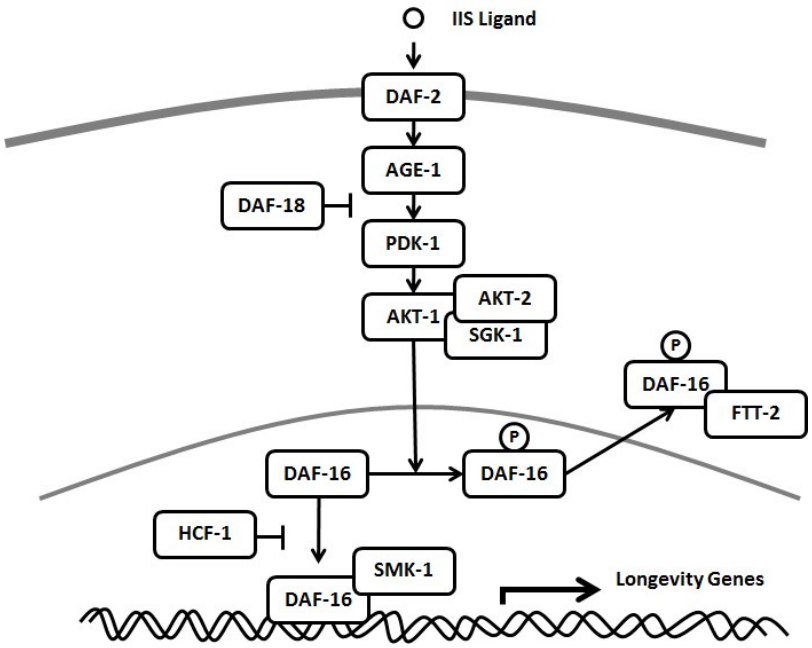
these, *hsf-1, skn-1,* and *pha-4,* are transcription factors with well characterized transcriptional targets. HSF-1 activates expression of molecular chaperones known as heat shock proteins, SKN-1 targets a broad array of detoxification and antioxidant genes, and PHA-4 induces expression of ROS scavenging superoxide dismutases (Hsu et al., 2003; Park et al., 2009; Panowski et al., 2007). Interestingly, there is some overlap between the transcriptional profiles for these factors; for example PHA-4 and SKN-1 both activate expression of *sod-1*, a cytosolic Cu/Zn superoxide dismutase (Park et al., 2009; Panowski et al., 2007). There are five sod genes in *C. elegans*, none of which have been shown to be single-handedly required for DR induced longevity. In fact, there is notable redundancy in function for many targets of all three of these transcription factors, suggesting that the longevity obtained from DR involves a complicated multi-gene downstream response.

Summary

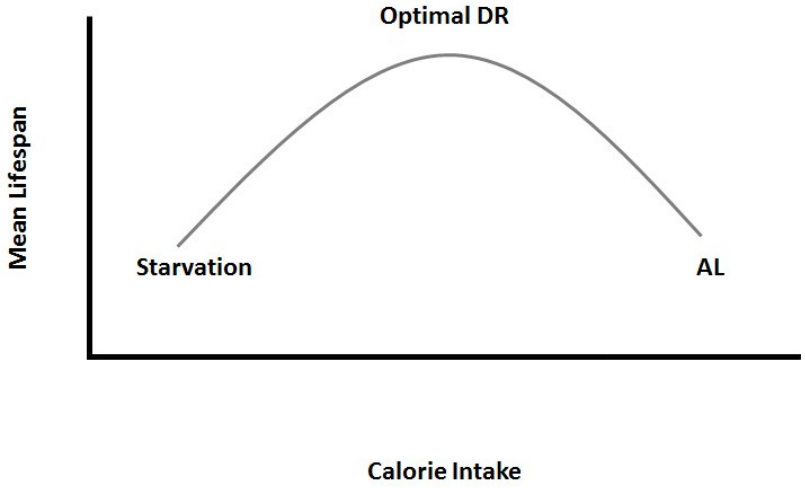
C. elegans is a very attractive model system in which to study the genetic mechanisms of how DR protects against disease. Many neurological diseases associated with aging remain without any effective treatment regimes, and so the fact that DR has been shown to be protective against neurodegenerative conditions like Alzheimer's disease make for an exciting potential source of treatment strategies. The fact that multiple genes, *hsf-1, skn-1, pha-4,* and *wwp-1,* have been shown to be required for DR to extend

lifespan begs the question as to whether these same genes might also serve a critical regulatory role in the ability of DR to protect against neurodegenerative disease. It will also be interesting to see what sort of downstream targets any transcription factors that are indicated as serving a critical role in the DR protective response are acting through.

Chapter I Figure 1: Illustration of the IIS pathway model.



Chapter I Figure 2: Illustration of a hypothetical DR longevity response.



CHAPTER TWO:

Dietary Restriction and Alzheimer's Disease

Introduction

Overview

DR has been shown to reduce the incidence and severity of aging related disorders such as diabetes, cancer, cardiovascular disease and neurodegenerative conditions (Masoro 2002). Rodents subjected to DR regimes are more resilient to neurotoxicity in models of age-related neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (Mattson et al., 2001). The neurotoxicity associated with these pathologies has been attributed to the accumulation of aggregation prone peptides, such as Aβ peptide in the brains of Alzheimer's disease patients (Stefani and Dobson 2003). Alzheimer's disease occurs in both sporadic and hereditary forms, the latter of which are earlier-onset and usually associated with mutations in Aβ precursor protein (APP) or one its processing enzymes (Tanzi and Bertram 2005). The vast majority of clinical cases belong to the sporadic category and patients generally present with symptoms after the age of 65. The disease is characterized by a progressive loss of neurons in the cortex and specific subcortical regions that lead to memory loss and the impairment of bodily functions which eventually result in death.

The exact mechanisms of Aβ induced neurotoxicity are still not entirely known, but recent work suggests that oligomeric species of Aβ might actually be significantly more toxic than the higher molecular weight aggregates of Aβ (Stefani and Dobson 2003). These oligomeric Aβ species are thought to

disrupt cellular function through interactions with membranes that lead to increased intracellular calcium levels and oxidative stress which in turn yields eventual apoptotic or necrotic cell death (Stefani and Dobson 2003). Despite the immense amount of research focusing on Alzheimer's, and other age-related neuropathies, there is still a lack of effective prevention or treatment regimes for human patients. Understanding the molecular mechanisms by which DR is able to ameliorate proteotoxicity in transgenic animal models may provide us with a means for manipulating this pathway. Therefore, we sought to better understand the molecular underpinnings that mediate DR induced protection against proteotoxicity.

Observations from mammalian models

The body of literature that addresses the topic of DR mediated protection against proteotoxicity is fairly limited. Experiments making use of murine Alzheimer's models demonstrated that DR regimes confer protection against neuronal loss, prevent behavioral deficits, and reduce the overall accumulation of Aβ (Halagappa et al., 2007; Mattson et al., 2001; Patel et al., 2005; Wang et al., 2005; Zhu et al., 1999). A study performed in nonhuman primates also revealed that DR significantly reduced levels of Aβ peptides (Qin et al., 2006). Levels of APP and APP mRNA were not altered by DR in any of these studies, suggesting that a post translational mechanism is responsible for the observed protective effect. Aβ peptide is derived from APP, which can

undergo two different proteolytic fates: alpha-secretase cleavage which yields peptide fragments associated with neurodevelopment and neuroprotection, or beta-secretase cleavage which ultimately results in the formation of toxic Aβ peptides of varying sizes (Wilquet and Strooper 2004). Some of the APP produced in the brains of normal individuals ends up processed by the beta-secretase pathway, leading to production of Aβ and the risk that Alzheimer's disease may arise in late life. Rodent models of Alzheimer's disease have shown that DR increases the expression of alpha-secretases and it has been suggested that this may be one mechanism by which DR combats Aβ toxicity, by reducing production of Aβ peptides (Wang et al., 2005). Little else is known in mammalian systems about which genes or pathways may contribute to the DR induced protective response against toxic peptides.

C. elegans models of Alzheimer's disease

C. elegans does not possess a true brain, but instead contains a nerve ring within its head region comprised of synapses between many of the 302 total neurons present in an adult animal (Riddle et al., 1997). This neuronal network allows a worm to sense environmental stimuli and execute behavioral responses by stimulating patterns of muscle contraction. Nematodes use longitudinal bands of muscle to contract their collagen-based exoskeleton to produce a slithering pattern of locomotion. Expression of aggregation prone peptides, such as Aβ, in *C. elegans* neurons has failed to yield any

quantifiable phenotypes of toxicity. In nematodes, the muscles send processes to the ventral nerve cord to form synapses, which is opposite of the way things occur in mammals, and may account for the lack of an obvious phenotype from neuronal expression of toxic peptides. Expression of human Aβ peptides in the body wall muscles of *C. elegans* produces quantifiable age-dependent muscle paralysis due to Aβ toxicity, and is widely accepted as a nematode model of Alzheimer's disease (Link 1995).

One of the most common *C. elegans* strains employed to model Alzheimer's disease is CL2006, which yields paralysis rates of 70-100% by day 12 of adulthood. Work with CL2006 has demonstrated that a reduction in IIS signaling is protective against proteotoxicity, and that this effect is mediated by HSF-1 and DAF-16 (Cohen et al., 2006). HSF-1 appears to regulate a preferred pathway in which toxic Aβ protofibrils are disaggregated and degraded, while DAF-16 regulates an additional backup pathway in which protofibrils are actively aggregated to reduce toxicity (Cohen et al., 2006). DR has been shown to extend lifespan independently of IIS pathway components such as DAF-16 and DAF-2, and therefore it is important to determine if the DR mediated protective response against proteotoxicity is also independent of IIS activity (Wolff et al., 2007). Complete bacterial deprivation (BD) of food in adult CL2006 protects against proteotoxicity, and this response was shown to be independent of DAF-16 and DAF-2 activity (Kaeberlein et al., 2006). The BD feeding protocol is not a widely accepted form of DR, as it cannot be

optimized to determine a feeding condition that produces maximal lifespan extension (Mair et al., 2009). BD also induces a starvation response and may affect physiology differently than accepted DR protocols (Mair et al., 2009). We therefore sought to employ a more widely accepted model of DR in *C. elegans* to determine if DR protects against Aβ toxicity and if this protective response is indeed independent of IIS components.

Results

DR protects against Aβ proteotoxicity in *C. elegans*

As previous findings have indicated that DR in murine models and BD in *C. elegans* models are protective against Aβ proteotoxicity, we wanted to determine if Aβ expressing *C. elegans* would demonstrate protection against proteotoxicity when subjected to widely accepted DR regimes. BDR using a broad range of bacterial concentrations, and use of *eat-2* were selected to as appropriate DR protocols. CL2006 adults were cultured in BDR using previously established bacterial concentrations, and scored for both survival and paralysis. Significant extensions in CL2006 lifespan were observed for the DR conditions versus the AL control (Figure 1A), and the DR concentrations also yielded dramatic reductions in paralysis rates compared to the AL control (Figure 1B). Plotting the mean and median lifespan values for each feeding group produced a parabolic curve as expected, with a maximal extension in lifespan at a similar concentration previously obtained for N2 wild

type animals (Figure 1C). Plotting the mean and median onset of paralysis also yielded a parabolic curve, revealing that the DR protective response can be optimized similarly to longevity by adjusting calorie consumption (Figure 1D).

In order to test the effects of the *eat-2* model of DR on Aβ toxicity, *eat-2(ad1116)* lines possessing the same human Aβ expressing construct (pCL12) used in CL2006 were created through microinjection. The resulting *eat-2;Aβ* strains were crossed to N2 wild type, to create AL controls possessing the same Aβ expressing extrachromosomal array. These matched sets of strains were then cultured on solid agar plates, and an N2;Aβ line (AGD376) with similar rates of paralysis to CL2006 was obtained. When this selected N2;Aβ strain was assayed with its corresponding *eat-2;Aβ* line (AGD342) a significant extension in lifespan was observed for the *eat-2* line as expected (Figure 2A). The AGD342 *eat-2;Aβ* line also exhibited a significant delay in both the onset and severity of paralysis compared to the AGD376 AL control (Figure 2B). These findings using the widely accepted DR protocols of BDR and *eat-2* demonstrate that a reduction in caloric intake strikingly increases resistance to proteotoxicity in *C. elegans.*

DR mediated protection is correlated with an overall reduction in Aβ levels

As mammalian models of Alzheimer's disease subjected to DR demonstrated a reduction in the levels of Aβ by ELISA, we sought to examine the effects of DR on Aβ in *C. elegans* through the use of immunoblot and ELISA (Halagappa et al., 2007; Wang et al., 2005). A scaled up BDR protocol, termed 'batch BDR,' was developed to allow for the collection of large adult *C. elegans* populations across a time course. L4 animals show some Aβ accumulation, but the bulk of Aβ protein production occurs between L4 and day 1 of adulthood (Fig. 3A). This means that most of the maximal Aβ levels observed in CL2006 are produced before the animals are subjected to DR. Following batch BDR treatment for 3 days, a significant reduction in the overall levels of Aβ was observed in the DR treated animals compared to their AL counterparts, with a more dramatic difference seen between DR and AL groups that spent 6 days in batch BDR (Fig. 3A). To compliment these Aβ ELISA results, SDS PAGE and Western blotting with 6E10 antibody was performed on batch BDR samples. The immunoblot results also demonstrated that most of the Aβ produced is present by day 1 of adulthood, and that DR treated animals have a significant reduction in Aβ levels which became more significant with increased time under DR conditions (Fig. 3B). These immunoblots as well as additional SDS PAGE and Western blotting procedures optimized to examine lower molecular weight oligomeric species of

Aβ did not reveal any striking differences between Aβ species under 40 kda. This was notable, as there has been recent mammalian work in the field of Alzheimer's disease research suggesting that oligomeric species of Aβ might actually be the most toxic species of Aβ (Stefani and Dobson 2003). Upon exposure to DR, the most dramatic decrease in Aβ that we observed was actually amongst the levels of higher molecular weight Aβ species over 80 kda (Fig 3B).

In an effort to examine the effects of *eat-2* mediated DR on Aβ levels, the Aβ expressing extrachromosomal array of the previously created AGD342 *eat-2;Aβ* strain was integrated by gamma irradiation. The resulting *eat-2;Aβ* integrated strain (AGD456) was crossed to N2 wild type to create a corresponding N2;Aβ integrated line (AGD458). SDS PAGE immunoblot with 6E10 antibody on AGD456 and AGD458 samples yielded similar results to those obtained from CL2006 on BDR, as the DR group had significantly less total Aβ with an especially notable loss in the higher molecular weight species of Aβ (Fig 3C). Considerable variability in total Aβ levels was observed between biological replicates of immunoblot for AGD456 and AGD458; however, a consistent and robust reduction in Aβ levels was maintained across the replicates for the AGD456 *eat-2;Aβ* line compared to the AGD458 AL control. Attempts at performing ELISA on AGD456 and AGD458 were unsuccessful, as a satisfactory dilution working range was unable to be obtained. This can likely be attributed to the variance in Aβ levels observed

between replicants of AGD456 and AGD458 by immunoblot. Taken together, the immunoblot and ELISA findings from CL2006, AGD456 and AGD458 point to the existence of a potent mechanism by which DR is able to reduce the levels of toxic Aβ peptides.

DR has only a minimal effect on mRNA levels of Aβ

The reduction in paralysis rates and total Aβ levels in DR versus AL treated *C. elegans*, raises the question of whether these observed benefits are due to an active degradation mechanism or an effect of DR simply lowering Aβ transgene expression. To answer this question we employed quantitative RT-PCR techniques on CL2006 samples harvested from larvae, young adults, and animals placed in batch BDR at day two of adulthood for varying durations of time. Minimal Aβ expression was detected until L4, which represents a late larval stage. When normalized to L4 expression levels, Aβ mRNA expression peaked at day 1 of adulthood and then fell off sharply in the first week of adulthood (Fig. 4A). Differences in transcript levels between groups are generally considered biologically relevant when approximately a twofold or more change is evident. AL and DR conditions 3 days after BDR was initiated showed a 45.6% difference in Aβ mRNA expression, approaching a biologically relevant change. No significant difference was observed between feeding groups after 6 days of BDR treatment. An additional set of experiments examining a longer time course reinforced these findings,

demonstrating that after 3 days in BDR there are no significant differences in Aβ expression between AL and DR conditions, and in this particular assay only a 20.1% difference in expression levels was observed between feeding groups at the day 3 time point (Fig 4B). Since maximal Aβ protein levels are reached at around day 1 of adulthood, before the animals undergo BDR treatment, it is unlikely these relatively minor differences in expression account for the reduction seen in Aβ protein levels in the DR treated animals. We also examined expression levels of *unc-54*, as the *unc-54* promoter is used to drive Aβ transgene expression in the body wall muscles, and saw a highly similar expression pattern to the Aβ transgene (Fig. 4C). This data taken together with the immunoblot and ELISA results suggest that DR is reducing Aβ levels through a process of active degradation rather than just lowering expression of Aβ transcripts.

Components of the IIS pathway function independent of the DR mediated protective response

It has been shown that DR induced lifespan extension is genetically independent of other pathways known to enhance longevity, including perturbations in the IIS pathway (Wolff et al., 2007). We therefore sought to determine if the DR mediated protection response against proteotoxicity is also independent of the IIS pathway. One of the most useful attributes of *C. elegans* is the ease by which genes can be knocked down thorough feeding

RNAi expressing bacteria on agar plates. The AGD376 N2;Aβ and AGD342 *eat-2;Aβ* lines were effectively used to examine the results of knocking down components of the IIS pathway on the DR protective response. Alternative methods were also pursued for the sake of comprehensiveness. Attempts at employing RNAi expressing bacteria in the liquid cultures of BDR to knockdown genes of interest in adult animals proved to be fruitless, as no significant gene knockdown was observed from this strategy. Multiple antibiotics are added to BDR liquid culture media in order to maintain a constant bacterial concentration, which likely severely inhibits induction and expression of RNAi. Additionally, short RNAi feeding regimes on solid plates or dsRNA soaking protocols prior to adult initiation of BDR failed to result in significant knockdown of genes of interest. Lastly, microinjection of *daf-16*(mu86) and *daf-2*(e1370) with the same pCL12 construct used in CL2006 was used to create transgenic lines expressing Aβ (AGD365-66,460 *daf-16*(mu86);Aβ and AGD367-71 *daf-2*(e1370);Aβ). We were unable to obtain any transgenic lines that demonstrated paralysis rates comparable to those observed for CL2006 cultured on solid plates for either of these IIS mutants. In the end, use of RNAi feeding on solid plates with AGD376 and AGD342 strains proved to be the most viable option to explore the function of IIS.

In order to accurately assess if IIS components play a role in the DR protective response, the AGD376 N2;Aβ and AGD342 *eat-2;Aβ* lines were raised from hatch on bacteria expressing RNAi against either *daf-16* or *daf-2.*

RNAi knockdown of *daf-16* produced a minor reduction in lifespan for both *eat-2*;Aβ and the N2;Aβ control strain but loss of daf-16 failed to block the extension in longevity of *eat-2* animals compared to controls (Fig. 5A). *daf-16* RNAi treatment produced a slight trend toward acceleration in rates of paralysis onset for *eat-2*;Aβ and N2;Aβ controls, but did not abolish the DR protective response, as *eat-2*;Aβ animals still showed a significant delay in paralysis onset compared to controls (Fig. 5B). DAF-16 has been shown previously to extend lifespan and confer protection against Aβ induced proteotoxicity, and so it's expected that down regulation of this transcription factor would produce some mild toxicity (Cohen et al., 2006; Lin et al., 1997; Ogg et al., 1997). Downregulation of DAF-2 de-represses DAF-16, and consequently RNAi knockdown of *daf-2* extends lifespan and confers protection against toxic proteins. RNAi against *daf-2* had the anticipated additive effect, further extending the lifespan of long lived AGD342 *eat-2*;Aβ animals compared to controls (Fig. 5C) and produced an additional delay in paralysis onset beyond that already observed for the AGD342 strain compared to controls (Fig. 5D). Therefore the ability of DR to combat proteotoxic stress, when assayed with the commonly accepted *eat-2* model of DR, functions independently of IIS and the *daf-16* regulated protection response.

Discussion

DR induces a potent protective response against Aβ toxicity in *C. elegans* models of Alzheimer's disease. Using two widely accepted models of DR, BDR and *eat-2*, we have shown that DR protects against proteotoxicity by delaying the onset and severity of body wall muscle paralysis arising from Aβ expression. These findings build upon the previously published results showing that complete bacterial deprivation (BD) of food in adult CL2006 protects against proteotoxicity (Kaeberlein et al., 2006). Our observations suggest that BD may actually induce a somewhat similar response to the more widely accepted DR protocols of BDR and *eat-2*, despite the fact that depriving adult animals of food also elicits a starvation response. It is important to employ proper models for DR to ensure that the observed results are entirely from the DR effect and not the result of any artifacts arising from an un-optimized model (Mair et al., 2009). The N2;Aβ and *eat-2;Aβ* strains that we have created here will permit other groups to accurately perform DR assays without having to rely on controversial models such as BD.

By employing ELISA, SDS PAGE immunoblot and QPCR we have been able to demonstrate that DR reduces overall levels of Aβ in *C. elegans* through a mechanism that that is independent of reduced Aβ mRNA production. These results are of particular interest because transgenic expression of human Aβ peptide in *C. elegans* bypasses the APP processing pathways found in mammals. Therefore, our findings suggest that DR is able

to induce a protective response against proteotoxicity that is both posttranslational and independent of Aβ production pathways, pointing to the existence of an active degradation process. These conclusions are especially important to the field, as there has been little new work in mammalian systems on the topic of how DR protects against proteotoxicity since the published findings that DR may influence APP processing to favor less Aβ production. Our observations suggest that reduced Aβ production may only be part of the picture in human patients, and that further efforts should be made in mammalian models to explore if DR conditions activate degradation mechanisms that target toxic aggregation prone peptides. This is especially enticing as a potential Alzheimer's treatment regime, as it suggests that interventions that mimic the effects of DR may be capable of reducing already accumulated Aβ levels in a subject.

We obtained novel observations by SDS PAGE and Western blotting techniques showing that the most dramatic decrease in levels of Aβ for DR treated animals, compared to AL, was actually seen amongst the pool of higher molecular weight Aβ species. Based on the growing trend in the field of Alzheimer's disease to attribute the most significant toxicity among Aβ species to oligomers of Aβ, we expected to see DR reduce the levels of lower molecular weight species of Aβ. The fact that we didn't see any drastic changes between levels of lower molecular weight species of Aβ between the DR and AL groups may be attributed to our SDS PAGE sample processing

protocol which included sonication of worm lysates with mild detergent and subsequent boiling, which could in theory have broken up significant levels of moderate molecular weight Aβ species to conceal native levels of lower molecular weight species of Aβ. It is also entirely possible that animals in AL conditions may have a more upregulated backup active aggregation mechanism then DR animals, as the degradation machinery in AL animals becomes overwhelmed by the massive amounts of Aβ being produced. Therefore, DR treated animals whose DR induced degradation machinery is able to more adequately cope with Aβ production and ensure that the overall levels of Aβ remain lower, don't induce the backup active aggregation pathway to the same degree that it is in AL animals.

Previously, DR mediated lifespan extension has been shown to function independently of IIS pathway components such as DAF-16 and DAF-2 (Wolff et al., 2007). Our results using RNAi knockdown in N2;Aβ and *eat-2;Aβ* strains demonstrated that the DR mediated protective response against proteotoxicity is also independent of IIS activity, as *daf-16* RNAi failed to abolish the DR protective response. The slight increase in paralysis rates that we observed for N2;Aβ and *eat-2;Aβ* animals treated with *daf-16* RNAi was expected, as DAF-16 has been shown to regulate a backup pathway involving active aggregation of Aβ peptides to reduce toxicity, (Cohen et al., 2006). We also demonstrated that de-repressing DAF-16 activity by knocking down DAF-2 levels with RNAi yields a delay in the onset and severity of paralysis rates for

both N2;Aβ and *eat-2;Aβ*, as would be anticipated when activating a DAF-16 active aggregation protection pathway. These findings using the widely accepted *eat-2* model of DR offered confirmation of previous results obtained with a controversial DR protocol showing that IIS components were not required for BD to extend lifespan and protect against paralysis in models of proteotoxicity (Kaeberlein et al., 2006). Our observations are interesting as they point to a mechanism independent from that used by up regulation of IIS to deal with proteotoxicity. The fact that we observed evidence of an active Aβ degradation mechanism, leads to the hypothesis that *hsf-1* may play a role in the DR protective response as this transcription factor has previously been shown to regulate a pathway that actively degrades Aβ.

Experimental Procedures

C. elegans methods and generation of transgenic lines

CF1037: *daf-16(mu86)I,* CF1041: *daf-2(e1370)III*, CL2006: N2, *I[unc-54p::hAβ1-42(pCL12), rol-6]*, DA1116: *eat-2(ad1116)*, and wild type C. elegans (N2) strains were obtained from the Caenorhabditis Genetics Center. C. elegans were raised and maintained using standard methods (Brenner, 1974). Transgenic lines were created using a mixture of plasmid DNA, containing the construct of interest, pRF4(*unc-54p::rol-6(+)*) and pIN01(*myo-2p::tdTOMATO*), which was microinjected into the gonads of adult hermaphrodite animals as per standard protocol (Mello et al., 1991). F1

progeny were selected by co-injection marker phenotype, and individual F2 worms were isolated to establish independent stable lines. AGD342: *eat-2(ad1116); uthEx410[unc-54p::hAβ1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato]* were generated by a plasmid DNA mix consisting of 10 ng/ul pIN01, 15 ng/ul pCL12, and 75 ng/ul pRF4(rol-6). *eat-2(ad1116)* were injected. AGD376: *N2; uthEx410[unc-54p::hAβ1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato]* was obtained by breeding AGD342 to N2 and selecting individual F2 worms that gave rise to offspring homozygous for N2 pharyngeal pumping rates and co-injection marker phenotypes. AGD456: *eat-2(ad1116); uthIs348[unc-54p::hAβ1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato]* was obtained through chromosomal integration by gamma irradiation of AGD342. AGD458: *N2; uthIs348[unc-54p::hAβ1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato]* was obtained by breeding AGD456 to N2 and selecting individual F2 worms that gave rise to offspring homozygous for N2 pharyngeal pumping rates and co-injection marker phenotypes. AGD365-66,460 and AGD367-71 strains sets were generated by a plasmid DNA mix consisting of 10 ng/ul pIN01, 15-25 ng/ul pCL12, and 75 ng/ul pRF4(rol-6). *daf-16*(mu86) was injected for AGD365-66,460 and *daf-2*(e1370) was injected for AGD367-71.

BDR lifespan and paralysis analysis

BDR was performed at 20ºC as described previously, with animals checked for both survival and paralysis (Panowski et al., 2007). Paralyzed animals were transferred to separate wells for each group, and assayed for survival. Batch BDR was initiated with synchronized populations of eggs hatched and raised at 20ºC 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 20ºC. 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. Liquid cultures were prepared as previously described (Panowski et al., 2007). The data presented in the accompanying figures are from one of the 2-4 biological replicates performed for every group, and are representative of the data obtained from all replicates. JMP 8 software was employed for statistical analysis to determine means and percentiles. All p values were calculated using logrank analysis.

Solid plate lifespan and paralysis analysis

Lifespan and paralysis assays were conducted simultaneously on solid plates at 20ºC as described previously, with the exception that young adults

were treated with FUDR at 100ug/ml (Cohen et al., 2006; Dillin et al., 2002a). Animals were moved to separate plates and assayed for survival after succumbing to paralysis, and paralyzed worms were reoriented every couple of days to ensure that they had continued access to food. Experimental groups had 80-100 animals, and all RNAi bacterial feeding was conducted from hatch unless otherwise noted, with RNAi obtained from the commercially available *C. elegans* ORF-RNAi Library. RNAi bacterial feeding was performed on bacterial lawns that had grown for 24 hours prior to IPTG induction. The data presented in the accompanying figures are from one of the 2-4 biological replicates performed for every group, and are representative of the data obtained from all replicates. JMP 8 software was employed for statistical analysis to determine means and percentiles. All p values were calculated using logrank analysis.

ELISA

Samples of approximately 6000 animals per group, with four biological replicates per time point, were obtained from batch BDR and thoroughly washed in M9 three times before being resuspended in 400 ul 1X PBS. Samples were then subjected to glass bead homogenization using a Precellys 24 at 4000 rpm for 90 seconds. Samples were then rested on ice for 20 minutes before undergoing tabletop centrifugation at 4ºC for 10 minutes at maximum spin to separate the resulting supernate from the cellular debris.

Protein quantification was performed by BCA, and samples were equalized for total protein content before undergoing vacuum centrifugation to concentrate the samples to approximately 4X. The concentrated samples were volume equalized to 125 ul and then subjected to formic acid extraction (250 ul of >95% formic acid added per sample) by glass bead homogenization using a Precellys 24 at 4000 rpm for 90 seconds. Samples were then placed on ice for 1hr before undergoing 135,000 x g ultracentrifugation at 4 ºC for 1 hour. 50 ul of supernatant was neutralized with 950 ul neutralization solution (1M Tris base, 0.5 M Na2HPO4, 0.05% NaN3). From this point on the standard Invitrogen human Aβ ELISA protocol was followed. The values obtained for all replicates at each time point were averaged and Student's t-test was used to determine statistical significance.

SDS PAGE and Western blotting

Samples obtained from batch BDR or from animals cultured on large solid plates were thoroughly washed and then resuspended in 1X TBS containing 0.1% NP40. Samples were then subjected to glass bead homogenization using a Precellys 24 at 4000 rpm for 90 seconds. Samples were then rested on ice for 20 minutes before undergoing tabletop centrifugation at 4ºC for 10 minutes at maximum spin to separate the resulting supernate from the cellular debris. Protein quantification was performed by BCA, and samples were equalized for total protein content. The standard

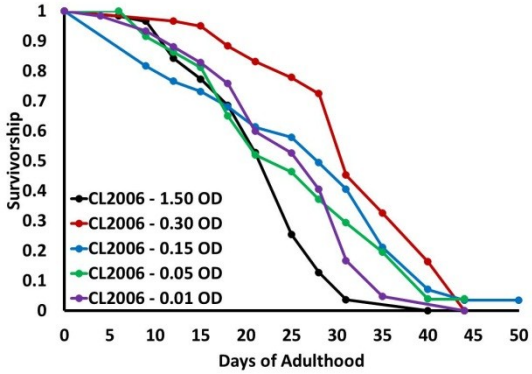
NuPAGE Bis-Tris protocol was then followed, before Western blotting with 1:1000 6E10 antibody overnight. Secondary antibody concentration was 1:10000 and membranes were visualized, then stripped and then re-probed for alpha tubulin.

RNA isolation and quantitative RT-PCR

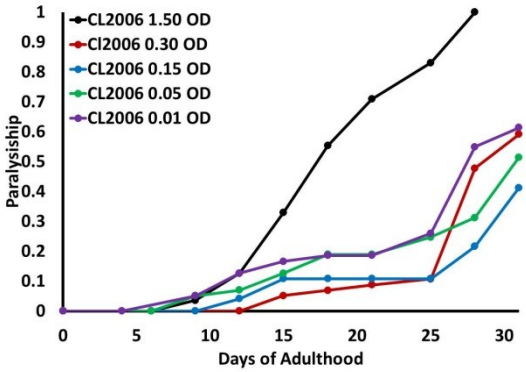
Total RNA was isolated from synchronized populations of approximately 5,000 animals for each time point, with four biological replicates per time point. Total mRNA was extracted using freeze cracking with QIAzol reagent and subsequent chloroform treatment, before undergoing ethanol and isopropanol wash steps. mRNA underwent column purification on Qiagen RNAeasy columns. cDNA was created using the Quantitec Reverse Transcriptase kit according to standard protocol (Qiagen). SybrGreen real-time QPCR experiments were performed as described in the manual using ABI Prism79000HT (Applied Biosystems) and cDNA at a 1/10 dilution. All QPCR experiments were performed in triplicate and normalized to *cdc-42* and Y45F10D.4 mRNA levels as described previously in Zhang et al., 2012. The values obtained for each time point were averaged and Student's t-test was used to determine statistical significance.

Chapter II Figure 1: DR protects against Aβ proteotoxicity in *C. elegans.* (A) Adult CL2006 animals cultured in BDR across a range of DR concentrations demonstrate lifespan extension when compared to the AL fed group (black line: 1.5OD, mean = 22.32 ± 0.95 days, median = 23 days. red line: 0.3OD, mean = 32.32 ± 1.16 days, median = 29.5 days p = <0.001. blue line: 0.15OD, mean = 26.61 ± 1.55 days, median = 26.5 days, p = 0.0003. green line: 0.05OD, mean = 25.44 ± 1.37 days, median = 23 days, p = 0.015. purple line: 0.01OD, mean = 25.39 ± 1.2 days, median = 26.5 days, p = 0.008.). (B) BDR cultured CL2006 animals in DR conditions exhibit reduced paralysis rates to the AL control group (black line: 1.5OD, mean = 19.53 ± 0.78 days, median = 16.5 days. red line: 0.3OD, mean = 30.85 ± 0.87 days, median = 29.5 days p = <0.0001. blue line: 0.15OD, mean = 32.73 ± 1.24 days, median = 33 days, p = <0.0001. green line: 0.05OD, mean = 29.20 ± 1.16 days, median = 29.5 days, p = <0.0001. purple line: 0.01OD, mean = 26.23 ± 0.98 days, median = 26.5 days, p = <0.0001.). (C) Plots of the mean and median lifespan over the bacterial food concentration range results in parabolic curves. (D) The mean and median onset of paralysis plotted over the bacterial food concentration range results yields parabolic curves.

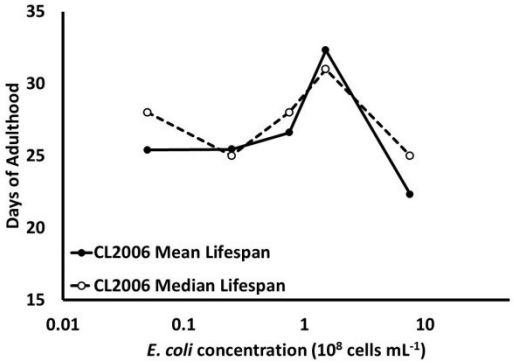
A.


Survivorship
Days of Adulthood
CL2006 - 1.50 OD
CL2006 - 0.30 OD
CL2006 - 0.15 OD
CL2006 - 0.05 OD
CL2006 - 0.01 OD

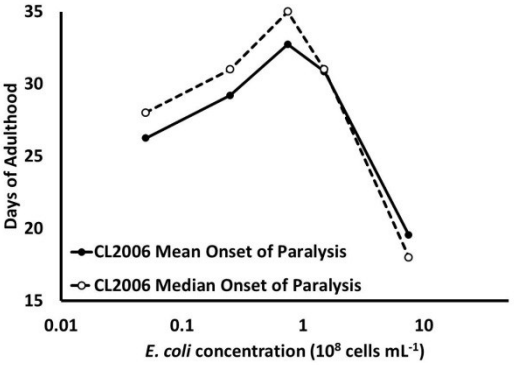
B.


Paralysiship
Days of Adulthood
CL2006 1.50 OD
Cl2006 0.30 OD
CL2006 0.15 OD
CL2006 0.05 OD
CL2006 0.01 OD

C.

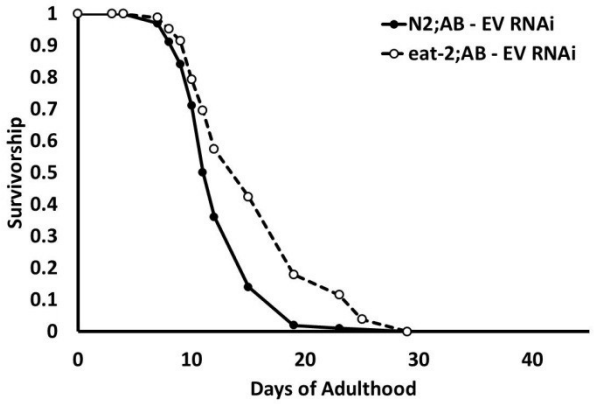

Days of Adulthood
CL2006 Mean Lifespan
CL2006 Median Lifespan
E. coli concentration (10^8 cells mL^-1)

D.

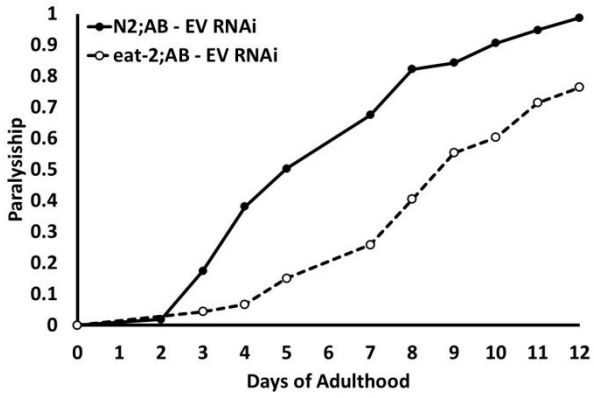

Days of Adulthood
CL2006 Mean Onset of Paralysis
CL2006 Median Onset of Paralysis
E. coli concentration (10^8 cells mL^-1)

Chapter II Figure 2: Aβ expressing *eat-2* demonstrate DR mediated protection. (A) AGD342 *eat-2;Aβ* raised on EV (dashed black line, mean = 15.87 ± 0.63 days, median = 13.5 days) were significantly longer lived than AGD376 N2;Aβ on EV (solid black line, mean = 12.72 ± 0.38 days, median = 11.5 days, p = <0.0001.). (B) *eat-2;Aβ* (dashed black line, mean = 9.06 ± 0.29 days, median = 8.5 days.) also exhibited a significant delay in both the onset and severity of paralysis compared to the N2;Aβ control (solid black line, mean = 6.22 ± 0.27 days, median = 4.5 days, p = <0.0001.).

A.

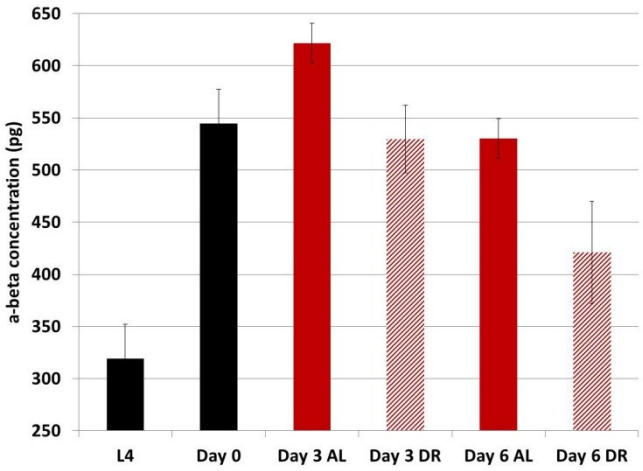

N2;AB - EV RNAi
eat-2;AB - EV RNAi
Survivorship
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
Days of Adulthood

B.

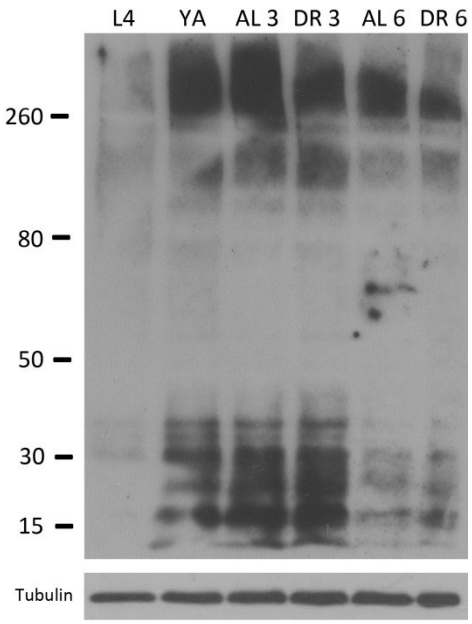

N2;AB - EV RNAi
eat-2;AB - EV RNAi
Paralysisihip
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Days of Adulthood

Chapter II Figure 3: DR mediated protection is correlated with an overall reduction in Aβ levels. (A) Four biological replicates of CL2006 grown on solid plates until adulthood and then transferred to batch BDR (BBDR) were collected at set time points and subjected to ELISA to quantify the average total Aβ levels from late larval stages through the first week of adulthood. Near-maximal Aβ production was observed at the onset of adulthood and a significant reduction in the total levels of Aβ for DR cultured animals versus AL controls was observed at both the day 3 and day 6 time points (p values: L4 to day 0 = 0.034, L4 to day 3 AL = 0.003, L4 to day 3 DR = 0.013, L4 to day 6 AL = 0.009, L4 to day 6 DR = 0.13, day 0 to day 3 AL = 0.09, day 0 to day 3 DR = 0.71, day 0 to day 6 AL = 0.70, day 0 to day 6 DR = 0.09, day 3 AL to day 3 DR = 0.009, day 3 AL to day 6 AL = 0.01, day 3 AL to day 6 DR = 0.011, day 6 AL to day 6 DR = 0.04). (B) A representative immunoblot from SDS PAGE and Western blotting performed with 6E10 antibody on batch BDR samples across a range of time points demonstrate similar changes in Aβ levels to those observed from the ELISA. (C) A representative immunoblot from SDS PAGE and Western blotting performed with 6E10 antibody on AGD456 *eat-2*;Aβ and AGD458 N2;Aβ reveals less total Aβ in the *eat-2*;Aβ group.

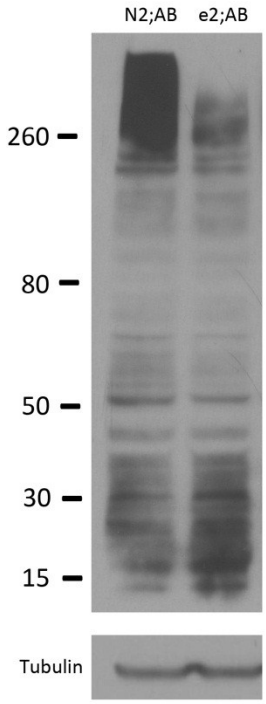
A.


650
600
550
500
450
400
350
300
250
a-beta concentration (pg)
L4
Day 0
Day 3 AL
Day 3 DR
Day 6 AL
Day 6 DR

B.

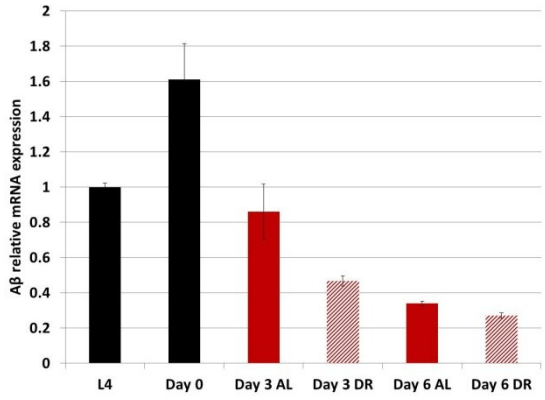

L4
YA
AL 3
DR 3
AL 6
DR 6
260
80
50
30
15
Tubulin

C.

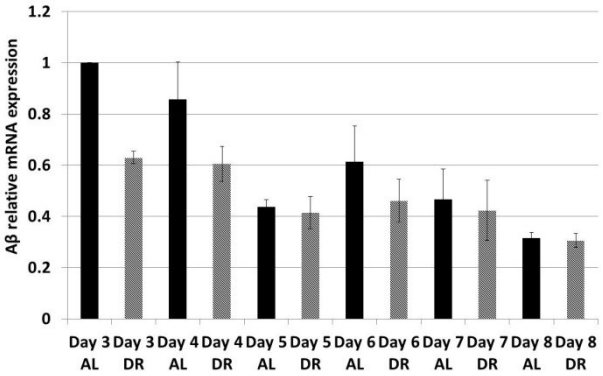

N2;AB
e2;AB
260
80
50
30
15
Tubulin

Chapter II Figure 4: DR has only a minimal effect on mRNA levels of Aβ. (A) Quantitative RT-PCR analysis of four biological replicates of CL2006 larvae, young adults, and BDR treated adults normalized to L4 expression levels shows Aβ mRNA expression peaked at young adulthood (161.17% increase in expression from L4), and then declined rapidly and significantly across the first week of adulthood. A biologically relevant difference in Aβ mRNA expression was seen between AL and DR at the 3 day time point (day 3 DR Aβ mRNA expression was 54.4% of day 3 AL expression), but no biologically relevant difference in Aβ mRNA expression was observed at the 6 day time points (day 6 DR Aβ mRNA expression was 79.7% of day 6 AL expression). (B) Additional Quantitative RT-PCR analysis performed on a longer time course of three biological replicates also shows that at time points past 3 days in BDR no biologically relevant differences in Aβ mRNA were seen between AL and DR groups (day 3 DR Aβ mRNA expression was 62.9% of day 3 AL expression, all other DR groups had over 70% the expression levels as their time matched AL counterparts). (C) Quantitative RT-PCR analysis of four biological replicates of CL2006 for mRNA levels of *unc-54* yielded a highly similar expression pattern to that observed for the Aβ transgene (day 3 DR *unc-54* mRNA expression was 36.1% of day 3 AL expression, day 6 DR *unc-54* mRNA expression was 91.4% of day 6 AL expression).

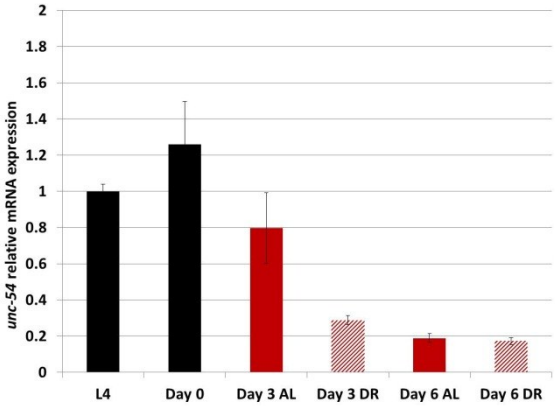
A.


Aβ relative mRNA expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
L4
Day 0
Day 3 AL
Day 3 DR
Day 6 AL
Day 6 DR

B.

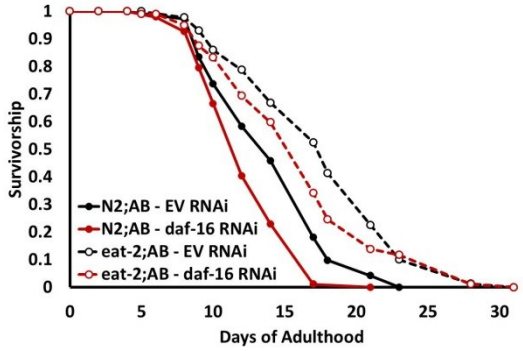

Aβ relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
Day 3 AL
Day 3 DR
Day 4 AL
Day 4 DR
Day 5 AL
Day 5 DR
Day 6 AL
Day 6 DR
Day 7 AL
Day 7 DR
Day 8 AL
Day 8 DR

C.

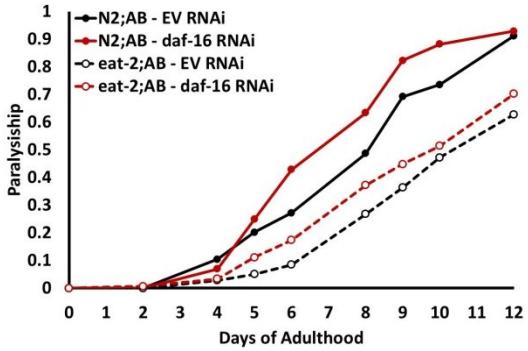

unc-54 relative mRNA expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
L4
Day 0
Day 3 AL
Day 3 DR
Day 6 AL
Day 6 DR

Chapter II Figure 5: Components of the IIS pathway function independent of the DR mediated protective response. (A) Lifespan extension was observed for EV treated AGD342 *eat-2;Aβ* (dashed black line, mean = 17.96 ± 0.61 days) compared to AGD376 N2;Aβ on EV (solid black line, mean = 14.35 ± 0.49 days, p = <0.0001). RNAi knockdown of *daf-16* from hatch reduced lifespan for N2;Aβ (solid red line, mean = 12.54 ± 0.33 days, p = 0.0004), but not *eat-2;Aβ* (dashed red line, mean = 16.62 ± 0.60 days, p = 0.13) compared to their respective EV treated controls. *daf-16* RNAi treated *eat-2*;Aβ displayed significant extension in survival compared to N2;Aβ on *daf-16* RNAi (p = <0.0001). (B) *daf-16* RNAi treatment trended toward an acceleration of paralysis onset for both N2;Aβ (solid red line, mean = 7.61 ± 0.24 days) and *eat-2;Aβ* (dashed red line, mean = 9.65 ± 0.27 days) compared to their respective EV controls, N2;Aβ (solid black line, mean = 8.5 ± 0.31 days, p = 0.06) and *eat-2;Aβ* (dashed black line, mean = 10.18 ± 0.24 days, p = 0.18) but significance was not reached. *eat-2*;Aβ on *daf-16* RNAi exhibited reduced paralysis onset rates compared to N2;Aβ on *daf-16* RNAi (p = <0.0001), similar to the reduction observed for *eat-2*;Aβ on EV RNAi compared to N2;Aβ on EV RNAi (p = <0.0001). (C) Survival rates for *eat-2;Aβ* on EV (dashed black line, mean = 15.87 ± 0.63 days) were greater than those of N2;Aβ on EV (solid black line, mean = 12.72 ± 0.38 days, p = <0.0001). RNAi knockdown of *daf-2* from hatch yielded increased longevity for both N2;Aβ (solid red line, mean = 19.17 ± 0.62 days, p = <0.0001) and *eat-2;Aβ* (dashed red line, mean = 21.26 ± 1.08 days, p = <0.0001) compared to their respective EV treated controls. *eat-2*;Aβ on *daf-2* RNAi showed a mild increase in survival compared to *N2;Aβ* on *daf-2* RNAi (p = 0.03). (D) *daf-2* RNAi treatment resulted in a reduction in paralysis onset rates for both N2;Aβ (solid red line, mean = 9.37 ± 0.27) and *eat-2;Aβ* (dashed red line, mean = 10.11 ± 0.33) versus their respective controls, EV treated N2;Aβ (solid black line, mean = 6.23 ± 0.27, p = <0.0001) and *eat-2;Aβ* (dashed black line, mean = 9.06 ± 0.29, p = 0.005). *eat-2*;Aβ on *daf-2* RNAi showed a delay in paralysis in comparison to N2;Aβ on *daf-2* RNAi (p = 0.006), similar to the reduction observed for *eat-2*;Aβ on EV RNAi compared to N2;Aβ on EV RNAi (p = <0.0001).

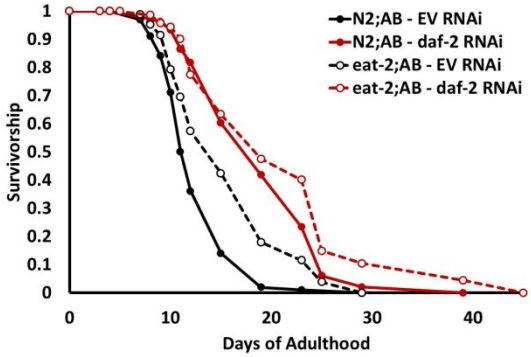
A.


Survivorship
Days of Adulthood
N2;AB - EV RNAi
N2;AB - daf-16 RNAi
eat-2;AB - EV RNAi
eat-2;AB - daf-16 RNAi

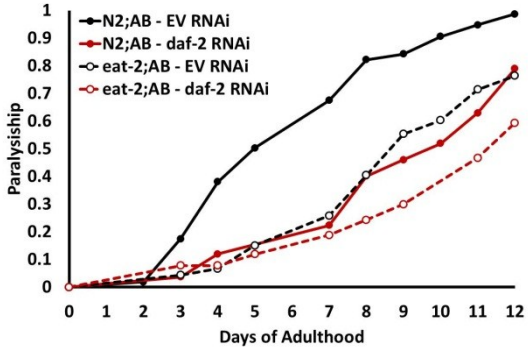
B.


Paralysishiip
Days of Adulthood
N2;AB - EV RNAi
N2;AB - daf-16 RNAi
eat-2;AB - EV RNAi
eat-2;AB - daf-16 RNAi

C.


Survivorship
Days of Adulthood
N2;AB - EV RNAi
N2;AB - daf-2 RNAi
eat-2;AB - EV RNAi
eat-2;AB - daf-2 RNAi

D.


Paralysishiip
Days of Adulthood
N2;AB - EV RNAi
N2;AB - daf-2 RNAi
eat-2;AB - EV RNAi
eat-2;AB - daf-2 RNAi

CHAPTER THREE:

Genetic Regulation of the DR Protective Response

Introduction

Overview

Multiple genetic regulators of the DR longevity response have been recently uncovered using the nematode *C. elegans*. For a gene to be considered required for DR lifespan extension, loss of that gene's function must abolish any relationship between food intake and lifespan. Whereas a wild type organism will respond to DR with a parabolic curve representing lifespan when plotted against food concentration, the loss of a critical genetic component for DR longevity will yield a flat line across the entire range of food concentrations (Mair et al., 2009). Of the genes tested so far, only four (*hsf-1, pha-4, skn-1* and *wwp-1*) have been shown to satisfy this requirement and are therefore required for DR lifespan extension (Bishop and Guarente 2007b; Carrano et al., 2009; Panowski et al., 2007; Steinkraus et al., 2008). Even though these genes have proven to be necessary for DR induced longevity, it remains to be seen how they are capable of exerting such an effect. Interestingly, all four serve additional functions besides their role in DR mediated lifespan extension, and all are critical for organismal viability. Due to their role in DR longevity, *hsf-1*, *pha-4*, *skn-1* and *wwp-1* make logical and attractive targets to test for a potential role in the DR protection against proteotoxicity response. It is important to highlight the previously characterized roles of these genes to better understand how they might potentially function in the DR protection response.

HSF-1 and the heat shock response pathway

Cellular stresses, such as exposure to heat, toxins or oxidative stress, increase the probability that proteins will undergo misfolding and be rendered unable to function properly (Lindquist and Craig 1988). Additionally, misfolded proteins that possess exposed hydrophobic regions can undergo aggregation and cause cellular toxicity by disrupting the function of other proteins as well as membranes (Stefani and Dobson 2003). It is therefore critical that protein folding is maintained during times of stress, and the heat shock response pathway is the primary mechanism by which this is achieved. Heat shock factor (HSF) is a transcription factor that rapidly induces expression of heat shock proteins (HSPs) in response to cellular stress in almost every prokaryotic and eukaryotic organism (Anckar and Sistonen 2011). Mammals have three heat shock factors (HSF1, HSF2 and HSF4) with HSF1 acting in a stress inducible fashion, while invertebrates such as *C. elegans* possess only one heat shock factor, HSF-1 (Taylor and Dillin 2011).

During non-stressed conditions HSF-1 remains as an inactive monomer in a complex with HSP90 and additional cofactors which release HSF-1 upon exposure to stress, allowing it to undergo homo-trimerization to its transcriptionally active form (Anckar and Sistonen 2011). HSF-1 induces transcription of multiple families of HSPs called chaperones that catalyze unfolding and subsequent proper re-folding of misfolded proteins (Calderwood et al., 2009). Chaperones derive their nomenclature based on their apparent

molecular weight. HSP70 and HSP90 are high molecular weight chaperones that bind unfolded peptide sequences and form complexes with cochaperones to refold target proteins in an ATP dependent manner (Lanneau et al., 2010). HSP10 and HSP60 are ATP dependent chaperones that belong to the chaperonin family which oligomerize to create barrel-shaped structures that catalyze protein refolding within (Calderwood et al., 2008). Smaller molecular weight chaperones, such as HSP27 and the *C. elegans*-specific HSP16, comprise the small heat shock protein (sHSP) family whose members undergo oligomerization to form dynamic higher molecular weight complexes that refold target proteins in an ATP-independent manner (Lanneau et al., 2010).

Evidence has shown that HSF-1 and the heat shock response play a role in both aging and age associated diseases involving aggregation-prone peptides, such as Huntington's and Alzheimer's diseases. The functionality of the heat shock response has been shown to decline significantly with age in *C. elegans* and mammals (Ben-Zvi et al., 2009; Calderwood et al., 2008). Elevated expression of specific HSPs (HSP16 and HSP90) has been observed in long lived *C. elegans*, and overexpression of HSF-1, HSP70 or HSP16 has been shown to produce lifespan extension (Hsu et al., 2003; Morley and Morimoto 2004; Walker and Lithgow 2003; Yokoyama et al., 2002). Down regulation of *hsf-1* dramatically reduces lifespan in *C. elegans* and results in an accelerated onset of polyglutamine aggregation in a transgenic *C. elegans* model of Huntington's disease (Garigan et al., 2002;

Hsu et al., 2003; Morley and Morimoto 2004). *hsf-1* knockdown in a *C. elegans* model of Alzheimer's disease dramatically accelerated the rate of observed proteotoxicity and led to increased accumulation of both small and high molecular weight Aβ aggregates (Cohen et al., 2006). *hsf-1* appears to regulate genes which act to disaggregate and degrade highly toxic Aβ aggregates, and it is believed that this pathway operates preferentially to a secondary active aggregation pathway regulated by DAF-16 (Cohen et al., 2006). Additional findings have shown that *hsf-1* needs to be expressed during larval development to orchestrate this protective response against Aβ (Cohen et al., 2009; Volovik et al, 2012).

Of the four genes that have been shown to be required for DR induced lifespan extension, only *hsf-1* has been tested to determine its role in the DR mediated protective response (Steinkraus et al., 2008). The feeding protocol employed in this assay was adult bacterial food deprivation (BD), which has been shown to extend life span (Kaeberlein et al., 2006). BD is not a widely accepted form of DR, as it is unable to produce a graded parabolic response to determine a feeding condition that produces maximal lifespan extension (Mair et al., 2009). Complete adult food deprivation in *C. elegans* also induces a starvation response and may affect physiology in a different manner than more widely accepted DR protocols (Mair et al., 2009). *hsf-1* was shown to be required for BD to extend lifespan and protect against observed proteotoxicity in *C. elegans* models of Huntington's and Alzheimer's diseases and this role of

hsf-1 was demonstrated to be independent of IIS function (Steinkraus et al., 2008). These results were interpreted to suggest that *hsf-1* is required for DR induced lifespan extension as well as DR mediated protection from proteotoxicity; however, these findings need to be repeated in a more widely accepted model of DR.

SKN-1 and the oxidative stress response pathway

Oxidative stress causes damage to proteins, lipids and DNA over time and is thought to contribute to aging (Cui 2012). Eukaryotic organisms have developed a highly conserved oxidative stress response pathway in which antioxidant and phase II detoxification enzyme genes are rapidly induced upon exposure to ROS (Sykiotis et al., 2011). *skn-1* in *C. elegans* and NRF2 in mammals are homologous members of the cap'n'collar family of transcription factors, and serve as master regulators of the oxidative stress response pathway (An and Blackwell 2003). *skn-1* also functions as a critical inducer of mesendodermal development during early embryonic stages (An and Blackwell 2003). Expression of *skn-1* in the intestinal tissue of postembryonic animals is required for normal lifespan and oxidative stress resistance (An and Blackwell 2003). Overexpression of *skn-1* yields lifespan extension, and SKN-1 signaling has been shown to decrease with age (Przybysz et al., 2009; Tullet et al., 2008).

Multiple signal transduction pathways contribute to regulation of the oxidative stress response through phosphorylation of SKN-1 at different sites. The p38 and ERK MAPK pathways, which are known for their roles in stress and growth responses, have been shown to induce SKN-1 activity through phosphorylation (Inoue et al., 2005, Okuyama et al., 2010). The IIS pathway negatively regulates SKN-1 through phosphorylation by IIS kinases AKT-1, AKT-2 and SGK-1 (Tullet et al., 2008). Glycogen synthase kinase GSK-3, which is known to play a regulatory role in many different cellular functions beyond glycogen synthesis, has also been shown to repress SKN-1 activity through phosphorylation (An et al., 2005).

In mammals, the oxidative stress response is regulated by an additional mechanism involving proteasomal degradation of SKN-1 in the absence of oxidative stress. Kelch-like ECH-associated protein (KEAP1) binds to NRF2 in the absence of ROS, to target NRF2 for proteasomal degradation through a cullin-3-dependent pathway (Nguyen et al., 2009). KEAP1 is believed to be capable of directly sensing ROS so that it can release NRF2 when oxidative stress conditions are present (Nguyen et al., 2009). Interestingly, *C. elegans* does not possess an ortholog of KEAP1, despite the fact that other invertebrates such as *Drosophila* do (Sykiotis and Bohmann 2008). The *C. elegans* WD-40 repeat containing protein WDR-23 was shown to negatively regulate SKN-1 transcriptional targets, as well as physically interact with SKN-1 and the CUL4/DDB1 ubiquitin ligase complex (Choe et al., 2009).

Knockdown of WDR-23 results in increased lifespan and oxidative stress resistance, and it has been suggested that WDR-23 may play an analogous role to KEAP1 in *C. elegans*; by targeting SKN-1 for proteasomal degradation in the absence of oxidative stress (Choe et al., 2009, Hasegawa and Miwa 2010).

Upon cellular exposure to oxidative stress, SKN-1 binds DNA as a monomer to induce target genes such as the glutathione synthesis gene *gcs-1*, multiple glutathione S-transferases (GSTs), NADH:flavin oxidoreductases, and the superoxide dismutase sod-1 (An and Blackwell 2003; Oliveira et al., 2009; Park et al., 2009). Glutathione is a tripeptide that can neutralize ROS, often through conjugation reactions catalyzed by GSTs (Hayes et al., 2005). *C. elegans* possess 52 GST-coding genes, almost half of which have been shown to be upregulated under oxidative stress (Hasegawa et al., 2008). Of the identified SKN-1 target genes, only two showed a statistically significant reduction in lifespan and resistance to oxidative stress when knocked down; the neuropeptide-like protein *nlp-7* and the ligand-gated ion channel *cup-4* (Park et al., 2010; Park et al., 2009). However, neither of these genes is considered critical to the *skn-1* induced oxidative stress response as the observed reductions in lifespan and resistance to oxidative stress were not very dramatic. It is unlikely that any single gene is critical for the oxidative stress response due to the redundant function of many *skn-1* targets.

Besides the role of *skn-1* in the oxidative stress response and normal longevity, SKN-1 is required for DR mediated lifespan extension (Bishop and Guarente 2007b). Of the genes that have been implicated as playing a role in lifespan extension by DR, *skn-1* is the only one of these shown to have a tissue specific expression requirement. The *skn-1* gene encodes for three protein isoforms that differ in N termini and tissue expression pattern. SKN-1C expression in the intestine is required for the oxidative stress response, whereas expression of SKN-1B in the ASI neurons is necessary for DR to extend lifespan (An and Blackwell 2003; Bishop and Guarente 2007b). DR treated *C. elegans* exhibit a significant upregulation in whole body respiration that is absent in animals lacking functional SKN-1B in the ASI neurons (Bishop and Guarente 2007b). These findings suggest that during DR conditions the ASI neurons secrete a signal to the rest of the organism that enables it to respond to DR. SKN-1 mediated lifespan extension by DR was shown to be IIS independent (Bishop and Guarente 2007b). Only one preliminary study has been conducted in mammalian systems to examine the role of NRF2 in DR mediated longevity, and this failed to show that NRF2 is required for DR mediated lifespan extension (Pearson et al., 2008).

skn-1 has been implicated in the ability of certain phytochemicals to confer beneficial effects on health and life span (Son et al., 2008). Coffee extract was demonstrated to protect against proteotoxicity in a *C. elegans* model of Alzheimer's disease, and this protective response was shown to be

dependent upon *skn-1* expression (Dostal et al., 2010). The observed role of SKN-1 in both the DR longevity response and in phytochemical induced protection against proteotoxicity, makes *skn-1* an attractive candidate to test if it also functions in the DR induced protection against proteotoxicity response.

PHA-4

pha-4 is a transcription factor in *C. elegans* that is orthologous to *forkhead* in *Drosophila*, and the three FoxA genes (*Foxa1, Foxa2 and Foxa3*) found in mammals (Horner et al., 1998). These genes represent a subfamily of proteins that contain a DNA-binding domain that gives rise to a so-called forkhead or winged helix structure in the translated protein. This DNA binding element is thought to allow interaction with chromatin before other transcription factors have gained access, permitting these genes to act as inducers of cell fate decisions during embryogenesis (Kaestner 2010). This subfamily of genes plays a highly conserved role in initiating organogenesis of components of the digestive tract in eukaryotic species. *forkhead* is necessary for gut development in *Drosophila*, and members of the FoxA family are required for development of the foregut and midgut in mammals (Costa et al., 1989; Dufort et al., 1998; Liu et al., 1991; Weigel et al., 1989). In *C. elegans, pha-4* also plays a critical role in foregut development during early embryonic stages, and is strongly expressed in the developing pharynx and intestines during development (Mango et al., 1994). Without functional *pha-4,* animals

fail to develop a pharynx and arrest as L1 larvae that are unable are to ingest bacterial food.

Regulation of *pha-4* is achieved through differences in the binding affinity of target consensus sequences, and by RNA silencing. During early embryogenesis PHA-4 levels are low and only genes with high affinity binding sites are activated. As PHA-4 levels rise during the later stages of embryogenesis, target genes with low affinity binding sites are able to be turned on (Gaudet and Mango 2002). Upon completion of larval development, the *let-7* microRNA acts to significantly reduce *pha-4* expression in the intestines (Grosshans et al., 2005). Despite the observed reduction in expression of *pha-4* during adulthood, *pha-4* and the FoxA genes have been shown to regulate aspects of metabolism in adult animals. The three FoxA genes are required for proper pancreas function, glucagon production, induction of gluconeogenesis genes, and regulation of blood glucose levels during fasting in adults mammals (Kaestner 2000). In adult nematodes, *pha-4* is critical for DR to extend lifespan and this function is independent of IIS components (Panowski et al., 2007). PHA-4 is expressed in low amounts in the intestine of adult animals, and additional expression is seen in neurons in the head and tail that is not present in larval stages. DR conditions increase PHA-4 levels, and have been shown to activate four of the five superoxide dismutase genes present in *C. elegans* (Panowski et al., 2007). There have yet to be any connections made between *pha-4* and aggregation prone

peptides associated with aging; however, the role of *pha-4* in the DR longevity response suggests that this transcription factor could play a role in DR mediated protection against proteotoxicity.

WWP-1

Ubiquitination is a process by which the small peptide ubiquitin is conjugated to proteins, often signaling them for subsequent degradation by the proteasome (Pickart 2001). Ubiquitination can also lead to regulation of protein function independent of proteasomeal degradation. Ubiquitin conjugation employs an E1 ubiquitin activating enzyme, and E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase (Scheffner et al., 1995). There are many families of E3 ubiquitin ligases, including the HECT (homologous to E6AP carboxy terminus) domain family of E3 ligases (Pickart 2001). A subfamily of HECT domain containing E3 ligases also possess modular WW domains that bind short proline rich sequences in target proteins (Kay et al., 2000). In mammals WWP1, WWP2, Itch and several other ligases comprise the HECT/WW subfamily, and these serve widely diverse functions in physiology. In *C. elegans* there is only one known ortholog of the HECT/WW subfamily, *wwp-1*, which serves a critical function during the later stages of embryogenesis (Huang et al., 2000). In adult animals *wwp-1* has been implicated in intrinsic and innate immune responses, and this role appears to be regulated by DAF-2, but independent of DAF-16 (Chen et al., 2010). *wwp-*

1 is also required for DR induced lifespan extension in adult animals, and this function is independent of IIS components (Carrano et al., 2009). Epistasis analysis has placed *wwp-1* upstream of *pha-4*, but little is known about their potential interaction other than the finding that PHA-4 is not a ubquitination target of WWP-1 (Carrano et al., 2009). *wwp-1* has not been shown to play a role in combating aggregation prone peptides associated with aging, but is still an attractive candidate for the DR mediated proteotoxicity protection response.

Results

We sought to determine if any of the four genes indicated previously as being required for DR to extend longevity (*hsf-1, skn-1, pha-4* and *wwp-1*) play a critical role in the DR protective response against proteotoxicity (Bishop and Guarente 2007b; Carrano et al., 2009; Panowski et al., 2007; Steinkraus et al., 2008). The only of these genes that had been previously tested to determine its potential function in the DR protective response was *hsf-1*, and this was accomplished using the controversial protocol of BD (Steinkraus et al., 2008). Based on our prior success using the widely accepted *eat-2* model of DR to assay the role of IIS components in the DR protective response, we also chose to employ *eat-2* to examine the function of *hsf-1, pha-4,* and *wwp-1*. AGD376 N2;Aβ and AGD342 *eat-2;Aβ* lines were fed RNAi expressing bacteria from hatch on solid plates with survival and paralysis rates quantified over time. The fact that knockdown of these genes had previously been

shown to abolish DR lifespan extension served as an effective positive control for the RNAi treatments. As RNAi knockdown in neurons is often ill-fated, extra considerations were made for assaying *skn-1* due to the known neuronal expression pattern of this particular transcription factor (An and Blackwell 2003; Bishop and Guarente 2007b). *skn-1* conditional null strains cultured in BDR conditions were employed to circumvent the neuronal limitations of RNAi feeding.

For a gene to be considered required for DR to protect against Aβ toxicity, loss of that gene's function must eliminate any relationship between food intake and proteotoxicity. In the case of the N2;Aβ and *eat-2;Aβ* lines, this means that knockdown of a gene of interest must produce nearly identical paralysis rates for both strains for that gene to be considered as being required for the DR protective response. For BDR, loss of function of a gene must result in a flat curve of mean onset of paralysis across a range of bacterial food concentrations, to qualify as serving a vital function in DR mediated protection. Here we were able to show that *hsf-1* and *skn-1* play a critical role in the DR protective response against proteotoxicity in addition to their previously characterized function in DR mediated lifespan extension. Interestingly, we also found that *pha-4* and *wwp-1* only play a necessary role in the ability of DR to extend lifespan, and are dispensable for the DR protective response against proteotoxicity.

DR requires *hsf-1* to combat Aβ proteotoxic stress

To test the function of HSF-1 in the DR protective response, AGD376 N2;Aβ and AGD342 *eat-2;Aβ* were raised on *hsf-1* RNAi expressing bacteria from hatch. We chose to initiate RNAi feeding from hatch due to the previously published observations that *hsf-1* expression during early larval development stages is required for normal lifespan (Volovik et al, 2012). Knockdown of *hsf-1* by RNAi feeding resulted in a robust and repeatable reduction in lifespan for both AGD342 and AGD376 compared to EV controls, with no significant difference in survival rates observed between these *hsf-1* RNAi treated genotypes (Fig. 1A). This observation implies that DR mediated lifespan extension requires the *hsf-1* transcription factor as *hsf-1* RNAi effectively prevented DR mediated longevity. Our findings represents the first time that *hsf-1* has been shown to be required for DR mediated lifespan extension using a widely accepted model of DR; as previous evidence of the involvement of *hsf-1* in DR longevity was obtained through a controversial BD protocol (Steinkraus et al., 2008).

Knockdown of *hsf-1* by RNAi feeding consistently and dramatically accelerated the paralysis rates for both AGD376 N2;Aβ and AGD342 *eat-2;Aβ* compared to EV controls, with both genotypes paralyzing at near simultaneous rates on *hsf-1* RNAi (Fig. 1B). Therefore, knockdown of *hsf-1* prevented the delay in paralysis onset observed for *eat-2*;Aβ on EV when compared to N2;Aβ controls. This observation reinforces the previously published result

obtained with the controversial protocol of adult BD that showed *hsf-1* being required for BD to protect against proteotoxicity, and our findings represent the first time that a more widely accepted protocol of DR has been used to show that the DR protective response requires *hsf-1* (Steinkraus et al., 2008).

To further examine these results at the protein level, we employed SDS PAGE and Western blotting techniques using 6E10 antibody against Aβ on samples of AGD458 N2;Aβ and AGD456 *eat-2;Aβ* raised on *hsf-1* RNAi expressing bacteria from L2 onward. We chose to start RNAi at the L2 stage based on the previous published findings that this is the point at which *hsf-1* is required for normal longevity (Volovik et al, 2012). Immunoblots showed clear differences between AGD456 and AGD458 raised on EV expressing bacteria, with the *eat-2;Aβ* showing less overall Aβ as expected for the control RNAi conditions (Fig. 1C). Knockdown of *hsf-1* yielded a reproducible increase in the observable levels of Aβ for both AGD456 and AGD458 raised on *hsf-1*, compared to the EV controls (Fig. 1C). These biochemical observations of Aβ levels are in accord with what would be expected based on the results of our paralysis assays, indicating that knockdown of *hsf-1* yields accelerated proteotoxicity. Interestingly, a reduction in levels of Aβ for the *hsf-1* RNAi treated DR group compared to the *hsf-1* RNAi treated AL group was still discernible in many of our biological replicates (Fig. 1C). Taken together, the results from our paralysis and biochemistry assays indicate that *hsf-1* is critical for DR to protect against proteotoxic insult.

skn-1 is critical for the DR protective response against Aβ proteotoxicity

To assay the role of *skn-1* in the DR protective response we employed *skn-1* conditional null strains. The EU35 *skn-1(zu169)* strain has a nonsense mutation in the DNA binding domain located in a C terminus of all three isoforms of *skn-1* (Bishop and Guarente 2007b). Maternal s*kn-1* is required for embryo viability and so this strain is heterozygous for the *skn-1* null allele to permit reproduction. EU35 possesses a balancer to ensure that no wild type progeny arise, which allows homozygous *skn-1(zu169)* nulls to be isolated from the offspring of these heterozygous animals (which harbor an uncoordinated phenotype marker to permit for easy differentiation). All further mention of the EU35 *skn-1(zu169)* strain will be in reference to isolated homozygous *skn-1(zu169)* nulls. We sought to repeat the previously published findings that s*kn-1* is required for DR mediated longevity by subjecting adult EU35 animals to BDR. EU35 animals subjected to BDR displayed significantly reduced lifespan compared to N2 wild type, and EU35 failed to demonstrate any lifespan extension across a range of bacterial DR concentrations when compared to the AL group (Fig. 2A). We therefore demonstrated that our BDR protocol is effective at reproducing the published findings for *skn-1*.

As expression of the SKN1B isoform in ASI neurons was previously shown to be necessary for DR to extend lifespan, and since neurons in *C. elegans* are notoriously impervious to RNAi feeding, we created Aβ-

expressing lines of the EU35 *skn-1(zu169)* conditional null strain through microinjection of the pCL12 transgene. Of the lines that resulted, one showing paralysis rates on solid plates similar to those of CL2006 and N*2*;Aβ animals was obtained, AGD393 *skn-1(zu169)*;Aβ (Fig. 2B). BDR performed with the AGD393 *skn-1*;*Aβ* line yielded similar survival rates for both AL and DR treated AGD393 animals, which were comparable to those observed for EU35 (Fig. 2C). This finding indicates that the presence of Aβ was not affecting the *skn-1* dependence of DR mediated lifespan extension. AGD458 *N2;Aβ* was assayed in both AL and DR conditions as a positive control for DR lifespan extension. AGD458 was selected as it is relatively short-lived, similar to EU35 and AGD393, yet AGD458 still displayed a statistically significant increase in longevity when cultured in DR conditions.

In BDR conditions, EU35 animals exhibit some notable age related paralysis even without an Aβ transgene (Fig. 2D). Aβ expressing AGD393 animals demonstrate significantly accelerated rates of paralysis compared to EU35 in BDR as anticipated (Fig. 2D). Excitingly, DR treated AGD393 did not show a reduction in paralysis rates compared to AL AGD393 controls, with both feeding groups paralyzing at roughly the same rate (Fig. 2D). SDS PAGE and Western blotting of AGD393 samples cultured in BBDR revealed similar levels of Aβ for the DR treated animals compared to AL controls (Fig. 2E). These novel findings implicate *skn-1* as a necessary player in the pathway by which DR combats toxic Aβ peptides.

Based on the previously reported findings that in *C. elegans* WDR-23 may play an analogous regulatory role to mammalian KEAP1, we sought to use *wdr-23* knockdown as a means of examining upregulation of *skn-1* activity on the DR protective response (Choe et al., 2009). To achieve this end we employed *wdr-23* RNAi feeding from hatch on solid plates for AGD342 and AGD376. Life span extension and increased resistance to proteotoxicity were observed for the N2;Aβ line treated with *wdr-23* RNAi compared to N2;Aβ on EV (Fig. 3A-B). Unexpectedly, *wdr-23* RNAi treated *eat;*Aβ animals displayed a significant reduction in lifespan and accelerated paralysis rates compared to EV controls (Fig. 3A-B). These observations for *eat;*Aβ failed to support the hypothesis that knockdown of *wdr-23* should result in an upregulation of *skn-1* activity, as either no change or a superadditive effect would be expected on survival and paralysis rates for *eat;*Aβ animals possessing increased *skn-1* activity. Examination of pharyngeal pumping rates showed that *wdr-23* RNAi markedly slows the pumping rates of both N2;Aβ and *eat;*Aβ, reducing N2;Aβ pumping rates to almost those of *eat;*Aβ on EV (Fig. 3C). *wdr-23* RNAi further diminished the pumping rates of *eat;*Aβ compared to EV controls, likely starving the worms and accounting for their reduced lifespan and hampered response to proteotoxicity (Fig. 3C). Therefore, use of *wdr-23* knockdown is not an effective means of studying upregulated *skn-1* activity, as the alterations in pumping rates overshadow any other effects.

pha-4 is dispensable for the DR protective response against Aβ stress

In order to test the potential requirement of *pha-4* in the DR protective response against proteotoxicity, AGD342 and AGD376 were cultured from hatch on *pha-4* RNAi expressing bacteria. Knockdown of *pha-4* by RNAi feeding did not significantly alter the lifespan of N2;Aβ animals compared to EV controls (Fig. 4A). While, *pha-4* RNAi exposure abolished DR mediated longevity in the *eat-2*;Aβ line, yielding survivorship curves similar to N2;Aβ on EV and *pha-4* RNAi (Fig. 4A). These findings are consistent with published observations for N2 and *eat-2* (without the Aβ transgene) raised on *pha-4* RNAi, and serve as an internal control assuring successful knockdown of *pha-4* in our hands (Panowski et al., 2007). RNAi knockdown of *pha-4* did not dramatically alter paralysis onset rates for both AGD342 and AGD376 compared to EV (Fig. 4B). Interestingly, *pha-4* knockdown did not suppress the delay in paralysis onset observed for *eat-2;Aβ* compared to similarly treated N2;Aβ (Fig. 4B). This observation was surprising as it indicates that PHA-4 is not a critical component of the pathway by which DR is able to confer protection against toxic Aβ peptides.

We performed microinjection on *pha-4(zu225):smg-1(cc546ts)*, a temperature inducible loss of function allele, in an attempt to create a transgenic Aβ expressing *pha-4* loss of function line. The *pha-4(zu225):smg-1(cc546ts)* line is rather difficult to work with as it exhibits significant sickness even when maintained at the permissible temperature. We observed that

individual *pha-4(zu225):smg-1(cc546ts)* animals generally only produced a few viable offspring over the course of their entire reproductive window, which was not conducive to the creation of transgenic lines expressing toxic Aβ peptides. Although not necessary, use of an Aβ expressing loss of function *pha-4* animals in BDR assays would have helped compliment the lifespan and paralysis findings obtained using *pha-4* RNAi knockdown with AGD342 and AGD376 on solid plates.

DR does not require *wwp-1* to combat Aβ proteotoxicity

AGD342 and AGD376 were raised from hatch on *wwp-1* RNAi expressing bacteria in an effort to gauge the potential role of *wwp-1* in the DR protective response. Knockdown of *wwp-1* did not significantly alter survival rates of N2;Aβ compared to EV controls (Fig. 5A). The *eat-2*;Aβ worms treated with *wwp-1* RNAi failed to show DR mediated life span extension, yielding a similar survivorship curve to the N2;Aβ animals treated with EV or *wwp-1* RNAi (Fig. 5A). These results matched what has been shown previously for non-Aβ expressing *eat-2* treated with *wwp-1* RNAi (Carrano et al., 2009). Knockdown of *wwp-1* by RNAi feeding had a negligible effect on paralysis rates for both genotypes, and did not prevent a delay in paralysis for *eat-2*;Aβ animals compared to N2;Aβ controls (Fig. 5B). Therefore, WWP-1 is not required for DR mediated protection against Aβ toxicity.

Discussion

By making use of BDR and RNAi feeding with N2;Aβ and *eat;*Aβ strains, we have determined that *hsf-1* and *skn-1* appear required for DR to combat Aβ toxicity, whereas *pha-4* and *wwp-1* do not play a critical role in the DR protective response. These findings represent the first time that *hsf-1* has been shown to be necessary for DR mediated lifespan extension and protection against proteotoxicity by using a widely accepted model of DR, as previous evidence of the requirement of *hsf-1* in DR longevity was obtained through a controversial BD protocol (Steinkraus et al., 2008). Our findings for *hsf-1* were anticipated based on the published observations that this transcription factor functions in a protective pathway against Aβ toxicity as well as its previously suggested involvement based on the BD results (Cohen et al., 2006; Steinkraus et al., 2008; Volovik et al, 2012). More excitingly, our indication that *skn-1* orchestrates the DR protective response is a completely novel finding for the field, and fits in well with the previous observations that *skn-1* plays a protective role against Aβ proteotoxicity and is required for DR mediated longevity (Bishop and Guarente 2007b; Dostal et al., 2010). It will remain to be seen what sort of interplay exists between *hsf-1* and *skn-1* in regulating pathways that degrade toxic Aβ peptides. Additionally, our results have succeeded in genetically uncoupling the DR proteotoxicity protective response from DR induced longevity through our observations that *pha-4* and *wwp-1* are dispensable for the protective response of DR. This is the first

evidence of these two DR response pathways operating genetically independently of one another, and was unanticipated. These findings suggest that when an organism is subjected to DR, its tissues detect the reduced calorie state and activate two different genetic pathways which do share some genetic overlap.

The results we obtained using biochemical techniques on the integrated AGD458 N2;Aβ and AGD456 *eat-2;Aβ* lines as well as AGD393 *skn-1(zu169)*;Aβ helped confirm the paralysis data. Knockdown of *hsf-1* dramatically accelerated paralysis rates for N2;Aβ and *eat-2;Aβ*, and we were able to show a corresponding increase in total levels of Aβ for these strains on *hsf-1* RNAi. Based on the paralysis data we would have anticipated both genotypes exhibiting almost identical total Aβ levels when treated with *hsf-1* RNAi, and so we were slightly surprised by the fact that a difference was still seen between the DR and AL groups. This may be attributed to several factors, including the fact that we initiated RNAi at L2 stage for the biochemical assays versus hatch for the paralysis assays. The L2 RNAi initiation point was done to minimize larval arrest, which is more significant when RNAi is initiated at hatch. In our paralysis studies we did not include arrested animals, but for the biochemistry assays it was unfeasible to remove individual worms from the large sample populations. An additional explanation for the fact that a DR effect at the protein level was still observed during *hsf-1* knockdown, may be due to the fact that RNAi knockdown on larger plates has been shown in our

hands to be less effective than for the smaller plates employed for lifespan and paralysis assays. As for the *skn-1(zu169)*;Aβ immunoblot results, the fact that the DR group showed slightly more Aβ than the AL controls may be explained by the observation that some of the BDR DR concentrations actually showed a more reduced lifespan then the AL group. This suggests that not only do these animals fail to respond to DR conditions with increased longevity; they are worse off under low food conditions than AL.

The results that we obtained from our *wdr-23* experiments were troubling for the field. Our findings suggest that several research groups may have published erroneous interpretations of the data they obtained using *wdr-23* knockdown, as the pharyngeal pumping effects of *wdr-23* knockdown overshadows any sort of interaction with *skn-1*. SKN-1 and WDR-23 were shown to physically interact, and therefore it is still entirely possible that that *wdr-23* may serve some sort of regulatory role over *skn-1* (Choe et al., 2009). However, the dramatic effects that knockdown of *wdr-23* has on pharyngeal pumping rates effectively forces N2 wild type animals into an *eat-2*-like DR state. *eat-2* treated with *wdr-23* are pushed so dramatically to the left of a DR parabolic response curve that they are essentially starved and lose any benefit from the DR benefits of the *eat-2* mutation alone. Although there is no published evidence showing that *wdr-23* may function to regulate pharyngeal pumping rates, unpublished data has indicated that *wdr-23* expression is associated with synaptic terminals of neurons and may be involved in

neurotransmitter release (personal communication from). Pharyngeal pumping rates are neuronally regulated and therefore *wdr-23* may actually serve some sort of important function in regulating the rates of pharyngeal pumping, but this remains to be tested.

Experimental Procedures

C. elegans methods and generation of transgenic lines

Wild type C. elegans (N2) strains were obtained from the Caenorhabditis Genetics Center. EU35: *skn-1(zu169) IV/nT1[aIs51] (IV;V)* (Bishop and Guarente 2007b) was kindly provided to us by Dr. Leonard Guarente, with permission from Dr. Keith Blackwell. *pha-4(zu225):smg-1(cc546ts)* (Gaudet and Mango, 2002) was kindly provided by Dr. Susan Mango. AGD342: *eat-2(ad1116); uthEx410[unc-54p::hAβ1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato],* AGD376: *N2; uthEx410[unc-54p::hAβ1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato],* AGD456: *eat-2(ad1116); uthIs348[unc-54p::hAβ1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato],* AGD458: *N2; uthIs348[unc-54p::hAβ1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato]* were obtained as described in the Chapter 2 methods. C. elegans were raised and maintained using standard methods (Brenner, 1974). AGD393: *skn-1(zu169) IV/nT1[aIs51] (IV;V)*; *uthIs445[unc-54p::hAβ1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato]* were generated by a plasmid DNA mix consisting of 10 ng/ul

pIN01, 15 ng/ul pCL12, and 75 ng/ul pRF4(rol-6) injected into *eat-2(ad1116)* EU35 *skn-1(zu169) IV/nT1[als51] (IV;V)* following the protocol previously described in the Chapter 2 methods for creation of transgenic animals. *pha-4(zu225):smg-1(cc546ts)* were injected with the same DNA mix used for generating AGD393.

Solid plate lifespan and paralysis analysis

Lifespan and paralysis experiments on solid plates were as described in the Chapter 2 methods section.

BDR lifespan and paralysis analysis

BDR and batch BDR assays were performed as described previously in the Chapter 2 methods section.

SDS PAGE and Western blotting

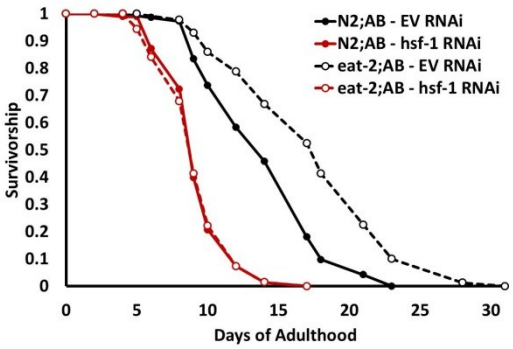
SDS PAGE and Western blotting assays were performed as described previously in the Chapter 2 methods section, with the one addition that populations grown on large size solid plates for subsequent biochemistry analysis had RNAi feeding initiated at the L2 larval stage.

Pharyngeal pumping rate assay

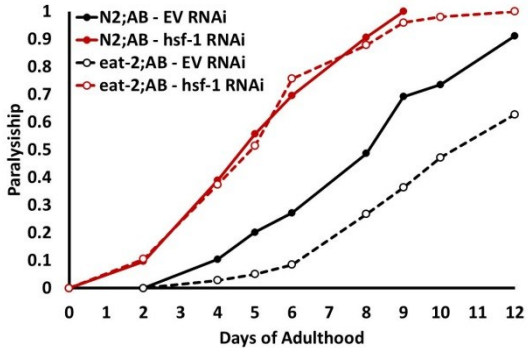
Pumping rates on various RNAi conditions were determined by counting pumps of the terminal pharyngeal bulb for one minute intervals to determine pumps/min. The pumping rates of ten worms per group across two biological replicates were averaged and Student's t-test was used to determine statistical significance. Worms were raised on RNAi expressing bacteria from hatch, and pumping rates were determined 24 hrs following detection of the first visible eggs forming in young adults.

Chapter III Figure 1: DR requires *hsf-1* to combat proteotoxic stress. (A) Survival rates for EV treated AGD342 *eat-2;Aβ* (dashed black line, mean = 17.96 ± 0.61 days) were significantly longer than those of AGD376 N2;Aβ on EV (solid black line, mean = 14.35 ± 0.49 days, p = <0.0001). RNAi knockdown of *hsf-1* from hatch produced a profound reduction in lifespan for both N2;Aβ (solid red line, mean = 9.45 ± 0.27 days, p = <0.0001) and *eat-2;Aβ* (dashed red line, mean = 9.35 ± 0.29 days, p = <0.0001) compared to their respective EV treated controls. h*sf-1* RNAi *eat-2;Aβ* failed to exhibit any lifespan extension compared to N2;Aβ on *hsf-1* (p = 0.92). (B) Both N2;Aβ (solid red line, mean = 5.56 ± 0.24 days) and *eat-2;Aβ* (dashed red line, mean = 5.59 ± 0.26 days) showed a dramatic acceleration in paralysis onset when treated with *hsf-1* RNAi versus their respective controls, EV treated N2;Aβ (solid black line, mean = 8.5 ± 0.31 days, p = <0.0001) and *eat-2;Aβ* (dashed black line, mean = 10.18 ± 0.24 days, p = <0.0001). *eat-2*;Aβ paralyzed at near simultaneous rates as N2;Aβ (p = 0.82) on *hsf-1* RNAi, in contrast to *eat-2*;Aβ on EV which demonstrated a delay in paralysis compared to N2;Aβ on EV (p = <0.0001). (C) Integrated AGD458 N2;Aβ and AGD456 *eat-2;Aβ* raised on *hsf-1* from L2 stage onward were subject to SDS-PAGE and Western blot analysis. *eat;*Aβ animals grown on EV displayed significantly reduced total levels of Aβ, with an especially notable loss of higher molecular weight species, compared to N2;Aβ controls. Both N2;Aβ and *eat-2*;Aβ raised on *hsf-1* RNAi had significantly increased levels of higher molecular weight Aβ species compared to EV controls; however *hsf-1* RNAi treated eat-2;Aβ animals still displayed a reduction in Aβ levels compared to N2;Aβ. Displayed are three representative biological replicates.

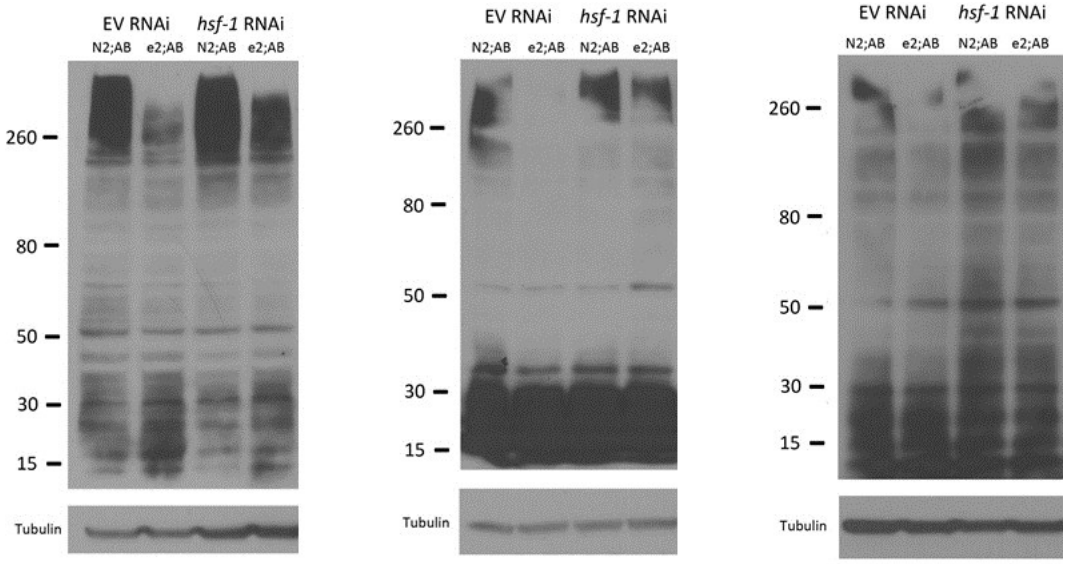
A.


Survivorship
Days of Adulthood
N2;AB - EV RNAi
N2;AB - hsf-1 RNAi
eat-2;AB - EV RNAi
eat-2;AB - hsf-1 RNAi

B.

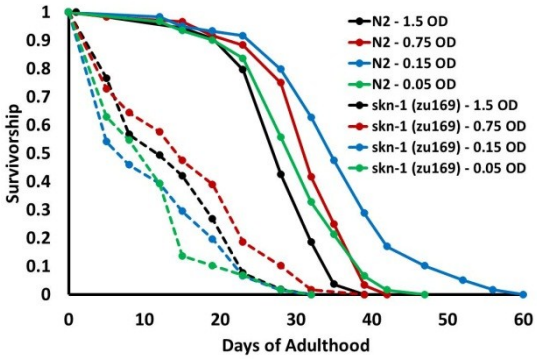

Paralysishiip
Days of Adulthood
N2;AB - EV RNAi
N2;AB - hsf-1 RNAi
eat-2;AB - EV RNAi
eat-2;AB - hsf-1 RNAi

C.

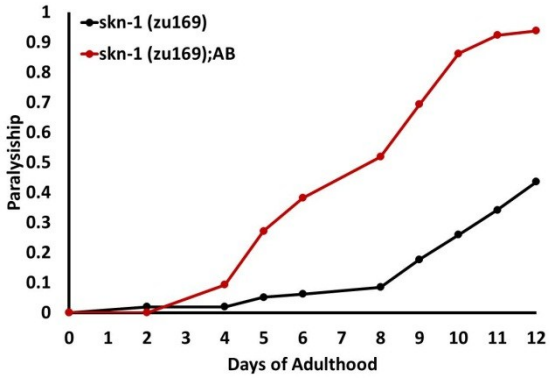

EV RNAi
hsf-1 RNAi
N2;AB
e2;AB
N2;AB
e2;AB
260
80
50
30
15
Tubulin

Chapter III Figure 2: *skn-1* is critical for the DR protective response. (A) N2 animals cultured in BDR across a range of reduced food concentrations exhibit lifespan extension compared to the AL fed group (solid black line: 1.5OD, mean = 28.8 ± 0.75 days. solid red line: 0.75OD, mean = 32.13 ± 0.88 days, p = <0.0001. solid green line: 0.15OD, mean = 36.54 ± 1.26 days, p = <0.0001. solid blue line: 0.05OD: mean = 30.77 ± 0.94 days, p = 0.015.). EU35 *skn-1(zu169)* animals subjected to BDR displayed significantly reduced lifespan compared to N2 wild type, and EU35 *skn-1(zu169)* failed to demonstrate any lifespan extension across a range of bacterial DR concentrations when compared to the AL group (dashed black line: 1.5OD, mean = 14.26 ± 1.05 days. dashed red line: 0.75OD, mean = 16.72 ± 1.28 days, p = 0.066. dashed green line: 0.15OD, mean = 12.0 ± 1.04 days, p = 0.19. dashed blue line: 0.05OD: mean = 11.62 ± 0.89 days, p = 0.065.). (B) AGD393 *skn-1(zu169)*;*Aβ* (red line, mean = 7.87 ± 0.29 days) raised on solid plates showed marked paralysis compared to EU35 controls (black line, mean = 10.9 ± 0.22 days, p = <0.0001). (C) *skn-1*;Aβ in DR conditions (dashed blue line, mean = 17.52 ± 1.38 days) demonstrated similar lifespan rates to *skn-1*;Aβ AL controls (solid blue line, mean = 18.09 ± 0.94 days, p = 0.84) as well as EU35 AL (solid red line, mean = 18.56 ± 0.91 days, p = 0.81) and EU35 DR (dashed red line, mean = 16.37 ± 0.85 days, p = 0.23) controls. As a positive control, AGD458 N2;Aβ in DR (dashed black line, mean = 14.48 ± 0.88 days) demonstrated an increase in lifespan compared to N2;Aβ in AL (solid black line, mean = 11.46 ± 0.69 days, p = 0.0041). (D) EU35 in both AL (solid red line, mean = 14.07 ± 0.61 days) and DR (dashed red line, mean = 12.36 ± 0.68 days) conditions display some age related paralysis, whereas *skn-1*;Aβ demonstrated accelerated rates of paralysis onset for both AL (solid blue line mean = 8.92 ± 0.69 days, p = <0.0001) and DR (dashed blue line, mean = 7.89 ± 0.56 days, p = <0.0001) conditions when compared to their respective EU35 feeding control. Paralysis rates for *skn-1*;Aβ were unchanged between the two feeding groups (p = 0.53). As a positive control, AGD458 N2;Aβ in DR (dashed black line, mean = 11.8 ± 0.47 days) demonstrated a delay in the onset of paralysis compared to AL conditions (solid black line, mean = 9.45 ± 0.50 days, p = 0.0003). (E) Immunoblot revealed that DR treated *skn-1*;Aβ possessed similar levels of total Aβ to their AL controls, even showing up elevated levels of higher molecular weight species in the DR group.

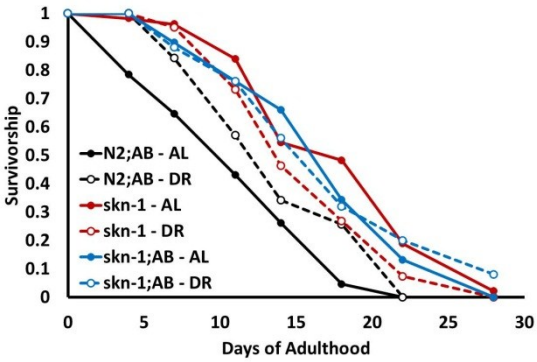
A.


Survivorship
Days of Adulthood
N2 - 1.5 OD
N2 - 0.75 OD
N2 - 0.15 OD
N2 - 0.05 OD
skn-1 (zu169) - 1.5 OD
skn-1 (zu169) - 0.75 OD
skn-1 (zu169) - 0.15 OD
skn-1 (zu169) - 0.05 OD

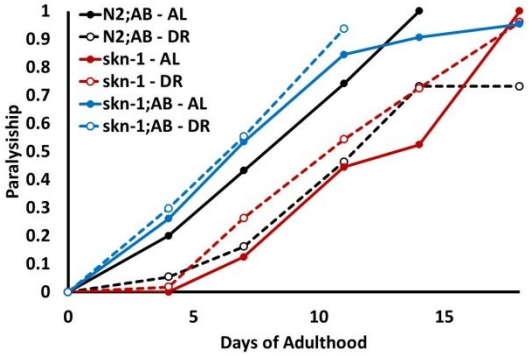
B.


Paralysisihip
Days of Adulthood
skn-1 (zu169)
skn-1 (zu169);AB

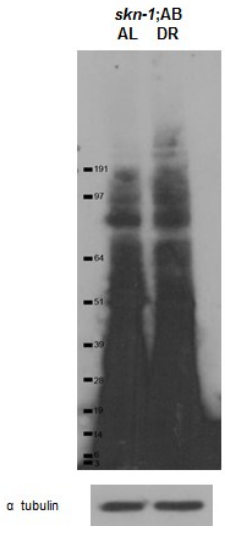
C.


Survivorship
Days of Adulthood
N2;AB - AL
N2;AB - DR
skn-1 - AL
skn-1 - DR
skn-1;AB - AL
skn-1;AB - DR

D.

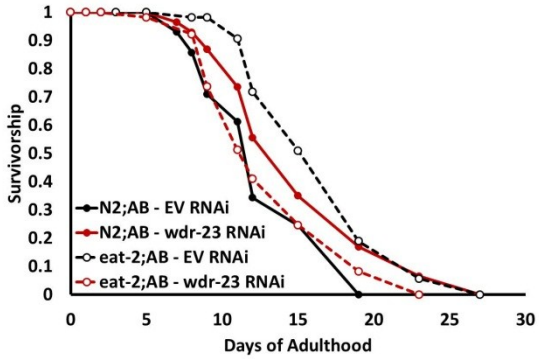

Paralysisihip
Days of Adulthood
N2;AB - AL
N2;AB - DR
skn-1 - AL
skn-1 - DR
skn-1;AB - AL
skn-1;AB - DR

E.

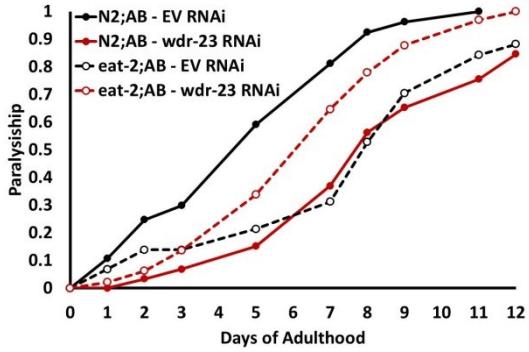

skn-1;AB
AL DR
α tubulin

Chapter III Figure 3: *wdr-23* knockdown reduces pharyngeal pumping rates, and alters lifespan accordingly. (A) Survival rates for EV treated AGD342 *eat-2;Aβ* (dashed black line, mean = 17.03 ± 0.64 days) demonstrated lifespan extension compared to AGD376 N2;Aβ on EV (solid black line, mean = 12.82 ± 0.64 days, p = <0.0001). N2;Aβ on *wdr-23* RNAi (solid red line, mean = 15.37 ± 0.59 days) from hatch demonstrated life span extension compared to N2;Aβ on EV (p = 0.008). *wdr-23* RNAi treated *eat;*Aβ (dashed red line, mean = 13.4 ± 0.66 days) animals displayed shorter survival than *eat-2;Aβ* on EV (p = 0.0002). (B) N2;Aβ on *wdr-23* RNAi (solid red line, mean = 8.54 ± 0.29 days) showed a dramatic delay in paralysis compared to N2;Aβ on EV (solid black line, mean = 5.2 ± 0.37 days, p = <0.0001). *wdr-23* RNAi treated *eat-2;Aβ* (dashed red line, mean = 6.82 ± 0.34 days) displayed accelerated paralysis versus EV treated *eat-2;Aβ* (dashed black line, mean = 8.0 ± 0.4 days, p = 0.01). (C) Pharyngeal pumping rates for *eat-2;Aβ* on EV (dashed black bar, mean) were significantly lower than those for N2;Aβ on EV (solid black bar, mean, p). w*dr-23* RNAi treated N2;Aβ (solid red bar, mean) possessed reduced pumping rates compared to N2;Aβ on EV (solid black bar, mean, p). Pumping rates for *eat-2;Aβ* (dashed red line, mean) also displayed reduced pumping rates in comparison to *eat;*Aβ on EV (dashed black bar, mean).

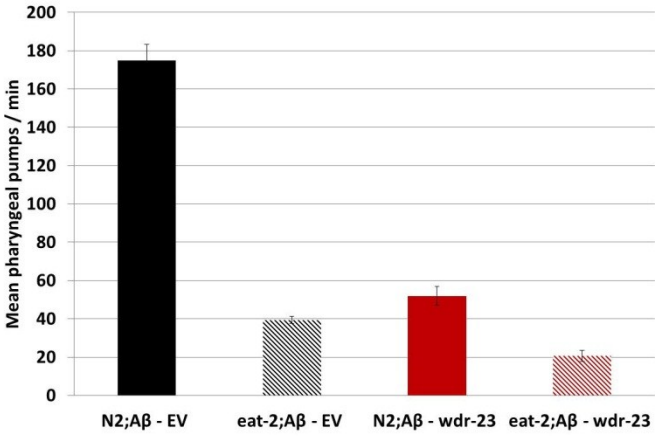
A.


Survivorship
Days of Adulthood
N2;AB - EV RNAi
N2;AB - wdr-23 RNAi
eat-2;AB - EV RNAi
eat-2;AB - wdr-23 RNAi

B.

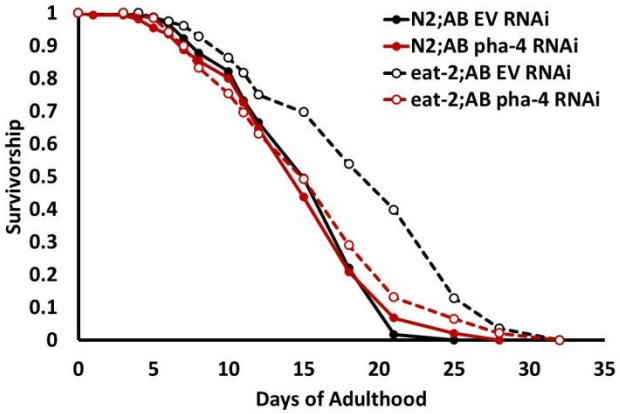

Paralysisihip
Days of Adulthood
N2;AB - EV RNAi
N2;AB - wdr-23 RNAi
eat-2;AB - EV RNAi
eat-2;AB - wdr-23 RNAi

C.


Mean pharyngeal pumps / min
N2;Aβ - EV
eat-2;Aβ - EV
N2;Aβ - wdr-23
eat-2;Aβ - wdr-23

Chapter III Figure 4: *pha-4* is dispensable for the DR protective response against Aβ proteotoxicity. (A) EV treated AGD342 *eat-2;Aβ* (dashed black line, mean = 19.54 ± 0.56 days) were significantly longer lived than AGD376 N2;Aβ on EV (solid black line, mean = 15.38 ± 0.34 days, p = <0.0001). *pha-4* knockdown by RNAi feeding from hatch did not significantly alter the lifespan of N2;Aβ (solid red line, mean = 15.18 ± 0.45 days) animals compared to EV control (p = 0.97). *eat-2;Aβ* treated with *pha-4* RNAi (dashed red line, mean = 15.99 ± 0.55 days) failed to show a DR mediated longevity response when compared to *pha-4* RNAi treated N2;Aβ (p = 0.1). The *pha-4* RNAi *eat-2*;Aβ survivorship curve was also not statistically different from that of N2;Aβ on EV (p = 0.08). (B) Downregulation of *pha-4* by RNAi did not dramatically alter paralysis onset rates of both N2;Aβ (solid red line, mean = 7.07 ± 0.24 days) and *eat-2;Aβ* (dashed red line, mean = 9.2 ± 0.27 days) when compared to their respective EV controls, N2;Aβ (solid black line, mean = 7.47 ± 0.23 days, p = 0.13) and *eat-2;Aβ* (dashed black line, mean = 9.93 ± 0.23 days, p = 0.054). *pha-4* RNAi treated *eat-2*;Aβ still demonstrated a significant delay in paralysis onset compared to N2;Aβ on *pha-4* RNAi (p = <0.0001), similar to that of *eat-2*;Aβ and N2;Aβ on EV (p = <0.0001).

A.


N2;AB EV RNAi
N2;AB pha-4 RNAi
eat-2;AB EV RNAi
eat-2;AB pha-4 RNAi
Survivorship
Days of Adulthood
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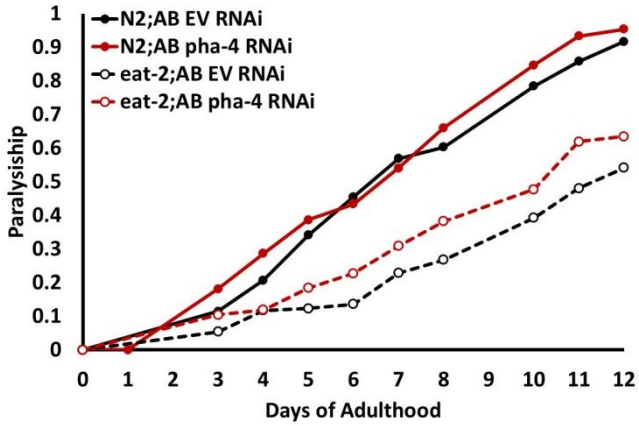
B.


N2;AB EV RNAi
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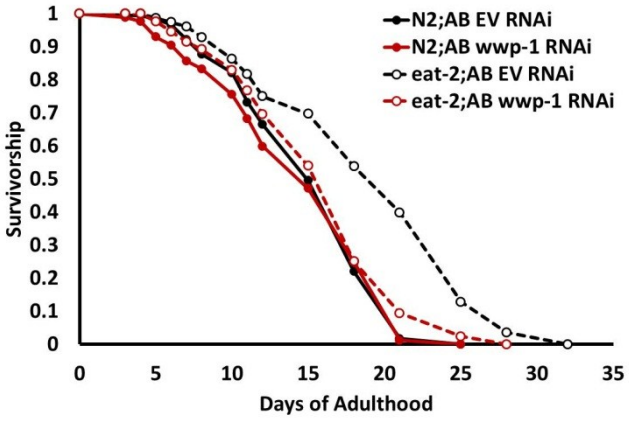
Chapter III Figure 5: DR does not require *wwp-1* to combat Aβ proteotoxic stress. (A) Lifespan extension was observed for EV treated AGD342 *eat-2;Aβ* (dashed black line, mean = 19.54 ± 0.56 days) compared to AGD376 N2;Aβ on EV (solid black line, mean = 15.38 ± 0.34 days, p = <0.0001). RNAi knockdown of *wwp-1* from hatch did not significantly alter the lifespan of N2;Aβ (solid red line, mean = 14.76 ± 0.42 days) animals compared to EV control (p = 0.71). *wwp-1* RNAi treated *eat-2;Aβ* (dashed red line, mean = 16.12 ± 0.48 days) did not demonstrate a DR induced longevity response when compared to N2;Aβ on *wwp-1* RNAi (p = 0.06). N2;Aβ on EV show a statistically similar survivorship curve to *wwp-1* RNAi treated *eat-2*;Aβ (p = 0.11). (B) RNAi knockdown of *wwp-1* led to a very minor increase in the paralysis rates of N2;Aβ (solid red line, mean = 6.82 ± 0.24 days) compared to N2;Aβ on EV (solid black line, mean = 7.47 ± 0.23 days, p = 0.005). *eat-2;Aβ* on *wwp-1* RNAi (dashed red line, mean = 10.14 ± 0.25 days) demonstrated similar paralysis rates to EV treated *eat-2;Aβ* (dashed black line, mean = 9.93 ± 0.23 days, p = 0.45). *wwp-1* RNAi treated *eat-2*;Aβ did still show a significant delay in paralysis compared to N2;Aβ on *wwp-1* RNAi (p = <0.0001), similar to *eat-2*;Aβ on EV compared to N2;Aβ on EV (p = <0.0001).

A.


N2;AB EV RNAi
N2;AB wwp-1 RNAi
eat-2;AB EV RNAi
eat-2;AB wwp-1 RNAi
Survivorship
Days of Adulthood
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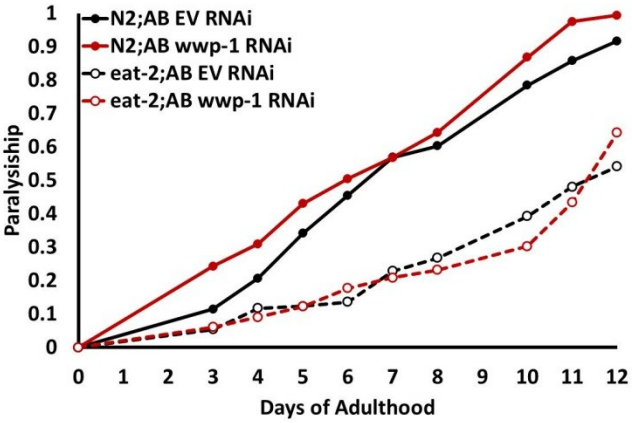
N2;AB EV RNAi
N2;AB wwp-1 RNAi
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eat-2;AB wwp-1 RNAi
Paralysiship
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CHAPTER FOUR:

Potential Degradation Mechanisms of the DR Protective Response

Introduction

Overview

With the implication that *hsf-1* and *skn-1* are critical mediators of a DR protection response which potently degrades Aβ, we sought to determine possible downstream mechanisms by which this could be achieved. Although both of these transcription factors have already been shown to regulate processes that degrade toxic Aβ, there is still no indication as to the identity of the degradation machinery (Cohen et al., 2006; Dostal et al., 2010). We hypothesized that the downstream mechanism by which DR induced degradation occurs may involve proteasome degradation and/or autophagy. Both *hsf-1* and *skn-1* have previously indicated involvement in the ubiquitin-proteasome pathway used by cells to degrade proteins. Autophagy is another attractive candidate mechanism for DR induced Aβ degradation, as heat shock proteins function in the autophagy pathway. Interestingly, *skn-1* has never been shown to function in the autophagy pathway; however, *skn-1* shares in operon with the critical autophagy gene *bec-1.*

Ubiquitin Proteasome System:

The ubiquitin proteasome system is a well conserved pathway that plays a significant role in the degradation of proteins that reside in the cytosol, nucleus and ER of eukaryotes. Proteins are signaled for degradation by the proteasome through ubiquitination, a process by which the small globular

peptide ubiquitin is conjugated to a target protein in succession to form a small ubiquitin polymer (Pickart, 2001). Ubiquitination does not always result in proteasome degradation of the target, as some ubiquitin linkages serve a signaling role or modify the targets function (Wong and Cuervo 2010). Ubiquitin conjugation employs an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase (Scheffner et al., 1995). E3 ubiquitin ligases are responsible for the selection of protein substrate, usually through recognition and binding of exposed degradation sequences found on unstable proteins. Eukaryotes possess over 1000 E3s which allows for significant specificity in targeting proteins for degradation by the proteasome (Staub and Rotin 2006).

The proteasome is a large protease complex consisting of a catalytic 20S core segment and an 11S or 19S regulatory cap that can dock at one or both sides of the core to form proteasome species of various sizes (Wong and Cuervo 2010). The 26S proteasome, which is formed by one 19S cap and a 20S core, plays the most well characterized role in protein degradation and quality control. The 20S core is comprised of two outer α rings that serve as a gateway to the two inner β rings which function as the site of proteolysis (Pickart and Cohen 2004). Ubiquitinated protein substrates that arrive at the regulatory cap of a 26S proteasome undergo deubiquitination and unfolding, which requires energy supplied by ATPase subunits present in the regulatory cap. The target peptides are then passed through the α ring gateway and

exposed to the potent caspase-like, trypsin-like, and chymotrypsin-like protease activities of the β rings (Wong and Cuervo 2010).

The transcription factors *hsf-1* and *skn-1* serve indirect regulatory roles in the ubiquitin proteasome system. Heat shock protein targets of *hsf-1* act as E3 cofactors and interact directly with assembled proteasomes. CHIP is an important E3 that interacts with members of the hsp70 and hsp90 families of heat shock pathway chaperones to ubiquitinate target proteins and flag them for destruction by the proteasome (Kumar et al., 2007; Tyedmers et al., 2010). CHIP and hsp70 have been shown to function in mechanisms that reduce Aβ levels in mammalian systems (Kumar et al., 2007). hsp90 also plays a critical role in maintaining the structure and function of fully assembled proteasome complexes (Imai et al., 2003). s*kn-1* plays an even more direct regulatory role in proteasome function, as it serves as a transcription factor for specific subunits of the proteasome (Kahn et al., 2008; Li et al., 2011). s*kn-1* also activates *aip-1* (arsenic inducible RNA-associated protein), which is known to bind to and enhance function of the proteasome as well as contribute to degradation of Aβ (Ferguson et al., 2010, Hassan et al., 2009).

Human Alzheimer's disease patients exhibit a significant decrease in proteasome activity in specific brain regions compared to healthy controls (Riederer et al., 2011). Additionally, colocalization of 26S proteasomes with protein aggregates as well as observations of delayed removal of aggregates in the presence of proteasome inhibition yields further evidence for the role of

proteasome degradation of toxic peptides (Tyedmers et al., 2010). However, the 26S proteasome has never been shown to directly degrade aggregated proteins, and protein aggregates actually reduce the activity of nearby proteasomes (Tyedmers et al., 2010). Therefore, the proteasome may likely only play a very small role in degradation of Aβ peptides.

Autophagy:

Autophagy is the recycling of organelles and cytoplasmic constituents through a process which involves sequestering and then delivering these materials to lysosomes for degradation (Yang and Klionsky 2009). Different avenues of acquisition and subsequent delivery to lysosomes exist, including macroautophagy, chaperone mediated autophagy and microautophagy. Macroautophagy is well conserved among eukaryotes and is the best characterized of the three avenues of autophagy, with the term autophagy often used to refer specifically to macroautophagy (Mizushima et al., 2010). Macroautophagy begins with the formation of an isolation membrane that elongates and eventually fuses on itself to surround a portion of cytoplasm, forming a double membraned vesicle termed an autophagosome (Rubinsztein et al., 2011). The outer membrane of the autophagosome fuses with a lysosome to form an autolysosome, which leads to degradation of the inner membrane and the cytoplasmic contents within it by lysosomal hydrolases (proteases, lipases, glycosideases, and nucleotidases). The resulting amino

acids and other molecules liberated by hydrolysis are then returned to the cytoplasm through permeases for recycling (Ravikumar et al., 2010).

Autophagy is highly regulated and it functions at relatively low levels until specific cellular stresses are detected (Rubinsztein et al., 2011). Starvation is one of the most potent inducers of the autophagy response, as breakdown of cytoplasmic macromolecules can serve as an emergency source of energy. Autophagy has shown to be induced under DR conditions and the longevity associated with DR requires specific genetic components of the autophagy pathway (Hansen et al., 2008). The highly conserved eukaryotic nutrient sensor TOR (Target of Rapamycin) plays a well characterized regulatory role by which it inhibits macroautophagy under normal nutrient conditions (Suzuki and Ohsumi 2007). Atg1 is a component of the Atg1/unc-51-like kinase (ULK) complex required for initiation of the isolation membrane, and TOR inhibits Atg1 activity when a cell's energy needs are met (Yang and Klionsky 2009). Under low nutrient conditions TOR inhibition of Atg1 is removed and ULK activity leads to formation of a protein complex that includes the class III PI3K Vps34 and Beclin1 (*bec-1* in *C. elegans)*, which both also serve a necessary role in initiation of the isolation membrane (Ravikumar et al., 2010). Approximately 35 genes have been shown to function in macroautophagy, most of which are identified by the nomenclature prefix Atg. Atg8, also known as LC3 in mammals and *lgg-1* in *C. elegans*, serves a necessary role in the elongation of autophagosome

membranes and is a commonly used marker for autophagosomes (Mizushima et al., 2010). Another important autophagy gene is Atg18, atg-18 in *C. elegans*, which possesses WD40 domains and is an effector protein for the PI3K enzymatic product PI3P (Lu et al., 2011).

Chaperone mediated autophagy involves the use of chaperones to bind and deliver specific soluble cytosolic proteins to lysosomes for degradation (Wong and Cuervo 2010). The chaperone hsc70 is a constitutively expressed hsp70 family member that recognizes specific motifs in target proteins and complexes with them (Li et al., 2011). Hsc70 and its target bind Lamp2a receptors on a lysosomal membrane, and the target protein is unfolded and then translocated into the lysosomal lumen for degradation (Li et al., 2011). The heat shock response pathway chaperones hsp90 and hsp40, as well as CHIP, act as cofactors for hsc70 and regulate its activity (Li et al., 2011). While these components of the heat shock response pathway have only been shown to play a direct role in chaperone mediated autophagy, indirect evidence indicates that *hsf-1* may also function in macroautophagy. This evidence hinges on the fact that DR and IIS, which are dependent on *hsf-1*, both activate macroautophagy (Hansen et al., 2008).

Autophagy has been indicated in the degradation of aggregation prone peptides associated with neurodegenerative disease such as Parkinson's, Huntington’s and Alzheimer's (Menzies et al., 2011). Both Aβ expressing *C. elegans* and the brains of Alzheimer’s disease patients exhibit accumulations

of autophagic vacuoles that have failed to proceed with lysosomal fusion, suggesting Aβ impairs autophagy (Boland et al., 2008; Florez-McClure et al., 2007). Human Alzheimer's disease patients exhibit reduced levels of Beclin1, and genetic knockdown of Beclin1 in the brains of Alzheimer's disease model mice accelerated neurodegeneration and Aβ accumulation (Pickford et al., 2008). Evidence has shown that ubiquitination can mark misfolded and aggregated proteins for removal by a recently discovered form of receptor mediated macroautophagy (Tyedmers et al., 2010). This acts as a link between the ubiquitin proteasome degradation system and autophagy, yielding an additional avenue besides chaperone mediated autophagy in which heat shock response proteins can direct targets for autophagic degradation. This collection of observations suggests a significant role for autophagy in the removal of aggregation prone peptides associated with neurodegenerative disease.

Results

DR animals exhibit a youthful proteasome activity profile

We have previously observed that proteasome activity of *C. elegans* increases with age, and that some long-lived strains, such as *daf-2 and eat-2*, exhibit reduced levels of proteasome activity compared to age-matched N2 controls. These assays are performed with fluorogenic proteasome substrates, and activity can be measured both in native gels and by using

sample lysates in a plate reader. Using a native gel assay with fluorogenic proteasome substrates, we observed an increase of proteasome activity with age for AL fed CL2006 during the first two weeks of adulthood (Fig. 1A). This increase in proteasome activity was evident in all three different types of proteasome protease activities, caspase-like, trypsin-like, and chymotrypsin-like (Fig. 1A). Additionally, animals cultured in DR conditions exhibit a dampened increase in proteasome activity compared to the AL group at a 10 day time point, with little change seen between the groups at 14 days, but an effect seen at the 19 day time point as well.

Native gel Western blot for CL2006 proteasome core subunits α1-7 as well as the rpt5 component of the 19S regulatory cap demonstrated an increase in expression of proteasome subunits for the AL fed 10 day time point compared to AL fed young adult animals (Fig. 1B). We observed that DR cultured worms exhibit a reduced induction of proteasome subunit expression at day 10 when compared to AL controls, which matched the activity assay results (Fig. 1B). The day 14 time point showed little difference between AL and DR conditions, however at the day 19 time point we saw a dramatic change with very little proteasome signal in the DR group. Subsequent biological replicates of the fluorogenic proteasome substrate activity assay and immunoblots for proteasome subunits yielded similar findings. Taken together, the fluorogenic proteasome substrate activity assay and proteasome

subunit expression data for animals cultured in DR conditions suggests a more youthful proteasome activity profile compared to AL matched counterparts.

Autophagy pathway genes are required for the DR protective response.

To test the potential role of autophagy in the DR protective response, AGD376 N2;Aβ and AGD342 *eat-2;Aβ* were raised from hatch on *bec-1* RNAi expressing bacteria. Knockdown of *bec-1* did not significantly alter survival rates of N2;Aβ compared to EV controls (Fig. 2A). The *bec-1* RNAi treated *eat-2*;Aβ failed to demonstrate DR mediated life span extension, instead displaying a survivorship curve similar to N2;Aβ animals treated with EV or *bec-1* RNAi (Fig. 2A). These observations matched the published data for non-Aβ expressing *eat-2* treated with *bec-1* RNAi which showed that DR mediated lifespan extension requires *bec-1* (Hansen et al., 2008). *bec-1* RNAi knockdown significantly accelerated the paralysis rates for both AGD376 N2;Aβ and AGD342 *eat-2;Aβ* compared to EV controls, with both genotypes paralyzing at near simultaneous rates on *bec-1* RNAi (Fig. 2B). Therefore, knockdown of *bec-1* abolished the DR protective response observed for *eat-2*;Aβ on EV when compared to N2;Aβ controls.

We examined AGD376 N2;Aβ and AGD342 *eat-2;Aβ* raised from hatch on RNAi against another critical autophagy gene, *atg-18*. Knockdown of *atg-18* did slightly reduce the lifespan of N2;Aβ animals compared to EV controls, and also abolished DR mediated longevity in *eat-2*;Aβ; yielding survivorship

curves for *atg-18* treated *eat-2;Aβ* that were even lower than those for N2;Aβ on EV or *atg-18* RNAi (Fig. 3A). RNAi knockdown of *atg-18* repeatedly accelerated the paralysis rates for both AGD342 and AGD376 compared to EV, and suppressed the delay in paralysis observed for *eat-2;Aβ* on EV compared to similarly treated N2;Aβ (Fig. 3B). Therefore, two critical autophagy genes have been shown in our hands to be required for both DR induced longevity as well as DR mediated protection against proteotoxicity.

We performed SDS PAGE and Western blotting with 6E10 antibody on samples of AGD458 N2;Aβ and AGD456 *eat-2;Aβ* raised on *bec-1* and *atg-18* RNAi. Control immunoblots demonstrated that *eat-2;Aβ* possess less overall Aβ compared to N2;Aβ EV controls as expected (Fig. 2C and 3C). Knockdown of *bec-1* or *atg-18* from L2 yielded a reproducible increase in the observable levels of Aβ for both AGD456 and AGD458, compared to the EV controls (Fig. 2C and 3C). This increase in Aβ levels matches well with our paralysis observations which showed that knockdown of *bec-1* or *atg-18* yields accelerated proteotoxicity. We did observe a noticeable reduction in levels of Aβ for either of the experimental RNAi treated DR groups when compared to their matching RNAi treated AL samples (Fig. 2C and 3C). These observations were similar to what we had seen for *hsf-1* treated *eat-2;Aβ* versus N2;Aβ. This biochemical evidence combined with our paralysis data, implicates autophagy as a likely downstream target of the pathway by which DR is able to combat aggregation prone Aβ peptides.

The current gold standard for gauging relative activity of the autophagy pathway in *C. elegans* involves counting LGG-1::GFP foci (Mizushima et al., 2010). Previously, *C. elegans lgg-1*::GFP reporter strains expressing Aβ have been developed, but these strains make use of a temperature inducible Aβ expression system (Florez-McClure et al., 2007). In our hands, use of temperature inducible Aβ expression systems have proven to be incompatible with DR assays, as the rates of paralysis in these strains reach approximately 80-100% within the first 24 hours after adulthood begins. This time point is normally when animals would be placed in DR, and so inducible Aβ expression strains simply paralyze too fast to be useful for DR studies.

In an attempt to circumvent the timing issue of the inducible Aβ expression strains we obtained DA2123 (*N2;lgg-1:GFP*) and MAH34 (*eat-2;lgg-1:GFP*), and attempted to cross these to AGD458 N2;Aβ and AGD456 *eat-2;Aβ*, respectively. The offspring we obtained from homozygous F2 animals from these two crosses displayed high levels of the abnormal cuticle formation phenotype known as ‘dumpy,’ and subsequent generations quickly exhibited sterility. Counting LGG-1::GFP foci in DR treated Aβ expressing animals appears to be unfeasible, and so alternative methods needed to be pursued to examine levels of autophagy.

Examination of mRNA levels of genes in *C. elegans* that encode critical components of the autophagy pathway has been used once previously as a surrogate for assessing relative levels of autophagic activity between samples

(Lapierre et al., 2011). We obtained the QPCR primers used for this published assay in an effort to determine what effect DR is having on autophagy levels in CL2006 raised in BDR conditions. We hypothesized that adult AL fed CL2006 would exhibit high levels of *lgg-1* mRNA compared to L4 CL2006, since Aβ expression has been shown to hinder successful fusion of autophagic vacuoles with lysosomes (Florez-McClure et al., 2007). Therefore, heavy accumulation of autophagosomes would be expected as animals enter adulthood and Aβ expression peaks. Additionally, we hypothesized that adult CL2006 in DR conditions would possess *lgg-1* mRNA levels higher than those of L4 controls, and that the DR CL2006 *lgg-1* mRNA levels would be lower than the AL treated CL2006 as we would expect DR to increase successful autophagic degradation (Hansen et al., 2008). The results of a QPCR assay for levels of *lgg-1* mRNA did trend in such a way as to match our hypotheses (Fig. 4). However, significance was not obtained for any of these trends, and the changes observed for not large enough to be biologically relevant.

Discussion

The fluorogenic proteasome substrate activity assay data that we obtained matched previously unpublished work from our lab demonstrating that proteasome activity increases with age. We believe that the dramatic observations at the day 19 time point may likely be attributed to the fact that at this observation point statistically significant numbers of worms in each group

have died from age, which may have confounded the activity data. We are still confident in our observations taken at earlier time points as those samples did not display significant amounts of death. Our results also matched previous observations that DR treated animals display a dampened increase in proteasome activity with age compared to AL controls. We were able to show that Aβ expression did not alter the phenomena of increased proteasome activity with age, or the DR induced dampening effect on proteasome activity induction with age. It has previously been concluded that DR maintains proteasomes in a more youthful state, but we are unsure if this is the result of more efficient proteasome targeting or if it is merely a side effect of DR inducing other protein degradation pathways. In all, it appears that the DR protective response does not depend upon increased proteasome activity, suggesting that other degradation pathways such as autophagy are the likely mechanism by which DR degrades toxic aggregation prone peptides.

RNAi knockdown of the essential autophagy pathway genes *bec-1* and *atg-18* abolished both the DR longevity and protective responses in our hands. We were able to show this through the use of lifespan and paralysis assays with our our N2;Aβ and *eat-2;Aβ* strains, as well as by examining levels of Aβ by SDS PAGE and Western blotting techniques. The observation that autophagy is required for DR induced lifespan extension has already been published on, and so this particular result was expected and it shows that we can recapitulate the findings of others using our N2;Aβ and *eat-2;Aβ* strains

(Hansen et al., 2008). Our observation that DR requires components of the autophagy pathway to combat proteotoxic stress that results from Aβ expression is an entirely novel and exciting finding. This finding is not altogether unexpected based on previous results showing that Autophagy appears to be involved in the degradation of aggregation prone peptides associated with neurodegenerative disease; however, our results mark the first time that a connection has been made between the DR protective response and autophagy (Menzies et al., 2011).

Based on the paralysis data for *bec-1* and *atg-18,* we anticipated that the AGD458 N2;Aβ and AGD456 *eat-2;Aβ* genotypes would exhibit almost identical total Aβ levels when treated with RNAi for either component of the autophagy pathway. We were somewhat surprised that a difference in levels of Aβ was still seen between the DR and AL groups on *bec-1* and *atg-18* RNAi. Similar to what was discussed in Chapter 3 in regards to the *hsf-1* biochemistry data; we attribute this difference in levels of Aβ to the fact that we initiated RNAi at L2 stage for the biochemical assays versus hatch for the paralysis assays.

We were disappointed that we were unable to create a *C. elegans lgg-1*::GFP reporter strain expressing Aβ that we could effectively use in BDR conditions. Strains that have mutations or transgenes that cause some toxicity can be very difficult to introduce the Aβ transgene into, as expression of Aβ in *C. elegans* results in significant toxicity. It appears that when Aβ expression is

combined with the *lgg-1*::GFP reporter construct, unavoidable and profound disturbances in both cuticle formation and reproduction occur. Our attempts to bypass this issue and use mRNA levels of autophagy genes as a surrogate for studying autophagy activity was also ineffective. It is important to point out that using mRNA levels of autophagy genes as a substitute for visualizing autophagy activity through *lgg-1*::GFP reporter expression is not a widely accepted strategy in the field. It was at least encouraging to see that the trends in mRNA expression matched our hypotheses, but it appears that other means will need to be employed in order to examine autophagy activity in Aβ expressing animals in BDR. In our future directions we plan on obtaining antibodies against components of the autophagy pathway and using SDS PAGE and Western blotting techniques to directly compare relative levels of key players in autophagy between samples.

Experimental Procedures

C. elegans methods and generation of transgenic lines

Wild type C. elegans (N2) strains were obtained from the Caenorhabditis Genetics Center. DA2123: *N2; asIs2122[GFP::LGG-1, unc-54p::rol-6(+)]* and MAH34: *eat-2(ad1116) II; asIs2122[GFP::LGG-1, unc-54p::rol-6(+)]* were kindly provided by Dr. Malene Hansen. AGD342: *eat-2(ad1116); uthEx410[unc-54p::hAB1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato],* AGD376: *N2; uthEx410[unc-54p::hAB1-42(pCL12),*

pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato], AGD456: *eat-2(ad1116); uthIs348[unc-54p::hAB1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato],* AGD458: *N2; uthIs348[unc-54p::hAB1-42(pCL12), pRF4(unc-54p::rol-6(+)), myo-2p::tdTomato]* were obtained as described in the Chapter 2 methods. C. elegans were raised and maintained using standard methods (Brenner, 1974). DA2123 and MAH34 were crossed to AGD458 and AGD456, respectively, but the resulting lines were unstable and became sterile within two generations.

Proteasome Activity Assay

Populations of approximately 6000 worms were raised following the same batch BDR protocol described previously in the Chapter 2 methods section. Samples underwent glass bead homogenization with a Precellys 24 at 4000 rpm for 90 seconds. Samples then underwent tabletop centrifugation at 4ºC for 10 minutes at maximum spin to separate the lysate from the cellular debris. Protein quantification was performed by BCA, and samples were equalized for total protein content. Equalized lysates were prepared on 3.5% native gels prepared in resolving buffer (90mM Tris base, 90mM boric acid, 5mM MgCl2, 0.5mM EDTA, 1mM ATP) with 5mM ATP, 1mM dithiothreitol, and 3.5% acrylamide. These lysates were run on triplicate gels at 110V for 3hr at 4ºC. Proteasome activity assays were performed by incubating each gel in activity assay buffer with LLVY-AMC, LLE-AMC or RLR-AMC for 20 minutes at

25ºC and then visualizing using a BioRad Gel Doc with UV illumination. The protein in the gels then underwent transfer to PVDF at 5V for 16h in transfer buffer using an Idea Scientific Genie Blotter. Western blot analysis was performed with anti-20S alpha 1-7 (Abcam) or anti-Rpt5 (Abcam) and analyzed using an Odyssey system (LI-COR Biosciences, Lincon, Nebr).

Solid plate lifespan and paralysis analysis

Lifespan and paralysis experiments on solid plates were as described in the Chapter 2 methods section.

SDS PAGE and Western blotting

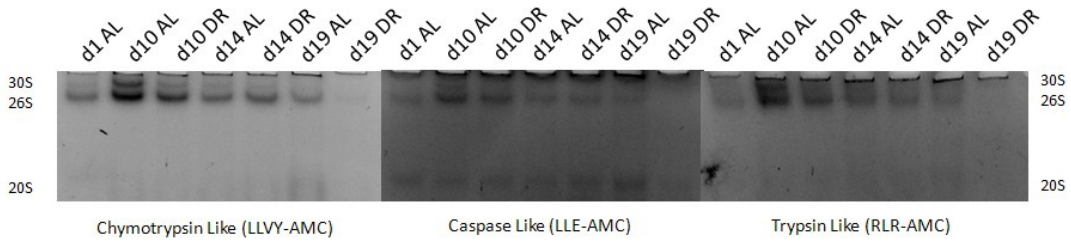
SDS PAGE and Western blotting assays were performed as described previously in the Chapter 2 methods section, with the one addition that populations grown on large size solid plates for subsequent biochemistry analysis had RNAi feeding initiated at the L2 larval stage.

RNA isolation and quantitative RT-PCR

RNA isolation and quantitative RT-PCR assays were performed as described previously in the Chapter 2 methods section.

Chapter IV Figure 1: DR restricted animals exhibit altered proteasome activity. (A) Adult CL2006 animals show an increase in proteasome activity during the first two weeks of life, compared to day one adults. Animals in batch BDR DR conditions demonstrate reduced levels of proteasome activity compared to age-matched AL fed animals at a 10 day time point, with no effect seen at day 14, and an effect observed at day 19. (B) Immunoblot for subunits of the proteasome showed that there were less fully formed proteasomes in the DR treated animals compared to AL controls at the 10 day time point, but not at the day 14 time point. We believe the dramatic observations at the day 19 time point, are due to the fact that at this time point significant numbers of animals have started to die, skewing the data.

A.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d10 DR
d14 AL
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d19 AL
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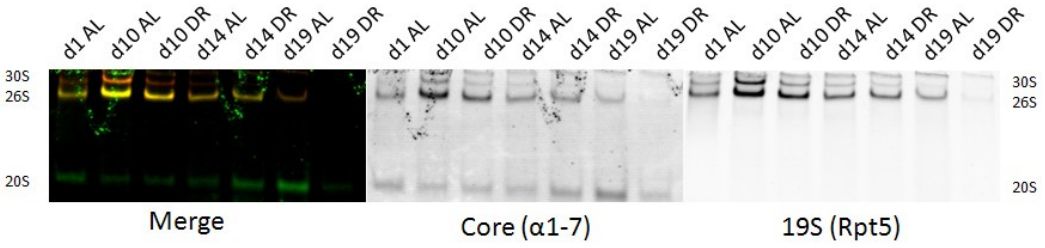
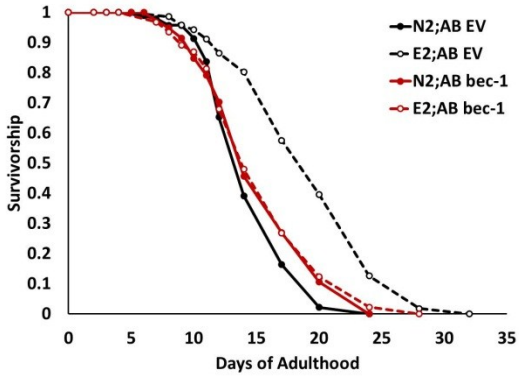
30S
26S
20S
Chymotrypsin Like (LLVY-AMC)
Caspase Like (LLE-AMC)
Trypsin Like (RLR-AMC)

B.

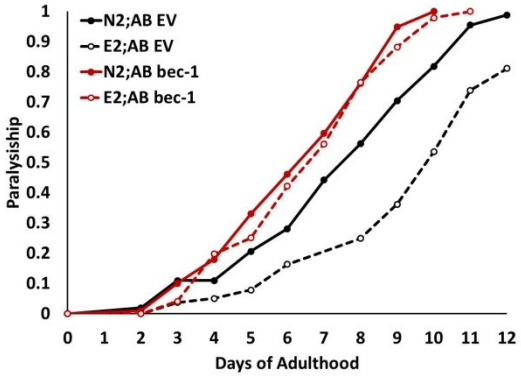

d1 AL
d10 AL
d10 DR
d14 AL
d14 DR
d19 AL
d19 DR
30S
26S
20S
Merge
Core (α1-7)
19S (Rpt5)

Chapter IV Figure 2: *bec-1* is required for the DR protective response. (A) EV treated AGD342 *eat-2;Aβ* (dashed black line, mean = 19.82 ± 0.71 days) displayed lifespan extension when compared to AGD376 *N2;Aβ* on EV (solid black line, mean = 14.69 ± 0.38 days, p = <0.0001). *bec-1* knockdown by RNAi feeding from hatch did not significantly change the lifespan of N2;Aβ (solid red line, mean = 15.51 ± 0.48 days) animals compared to EV control (p = 0.09). *eat-2;Aβ* treated with *bec-1* RNAi (dashed red line, mean = 15.66 ± 0.52 days) didn't show a DR mediated longevity response when compared to *bec-1* RNAi treated N2;Aβ (p = 0.75), and were statistically different then *eat-2;Aβ* controls (p = <0.0001). (B) Both N2;Aβ (solid red line, mean = 6.81 ± 0.18 days) and *eat-2;Aβ* (dashed red line, mean = 7.13 ± 0.25 days) demonstrated faster paralysis onset when treated with *bec-1* RNAi versus their respective controls, EV treated *N2;Aβ* (solid black line, mean = 7.89 ± 0.26 days, p = 0.0006) and *eat-2;Aβ* (dashed black line, mean = 8.91 ± 0.36 days, p = <0.0001). *eat-2*;Aβ paralyzed at near simultaneous rates as N2;Aβ (p = 0.36) on *bec-1*, in contrast to *eat-2*;Aβ on EV which demonstrated a delay in paralysis compared to *N2;Aβ* on EV (p = <0.0001). (C) Integrated AGD458 *N2;Aβ* and AGD456 *eat-2;Aβ* raised on *bec-1* from L2 stage onward were subject to SDS-PAGE and Western blot analysis. *eat;*Aβ animals grown on EV displayed less total Aβ, with a reduction in higher molecular weight species, compared to N2;Aβ controls. Both N2;Aβ and *eat-2*;Aβ raised on *bec-1* RNAi had significantly increased levels of higher molecular weight Aβ species compared to EV controls; however *bec-1* RNAi treated eat-2;Aβ animals still displayed a reduction in Aβ levels compared to N2;Aβ. Displayed are three representative biological replicates.

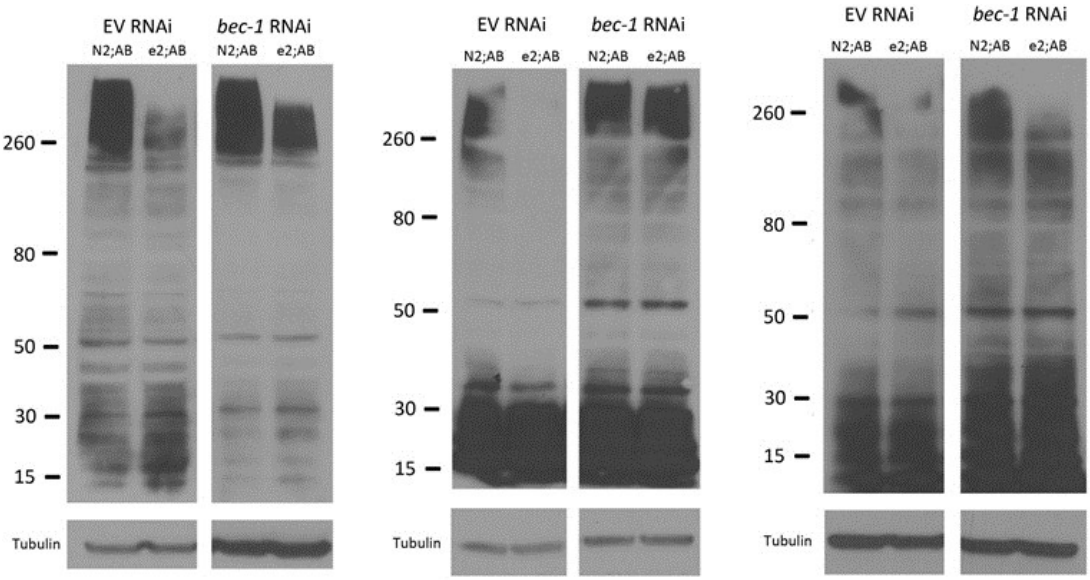
A.


Survivorship
Days of Adulthood
N2;AB EV
E2;AB EV
N2;AB bec-1
E2;AB bec-1

B.

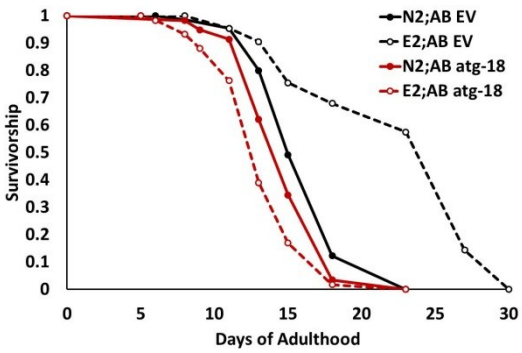

Paralysiship
Days of Adulthood
N2;AB EV
E2;AB EV
N2;AB bec-1
E2;AB bec-1

C.

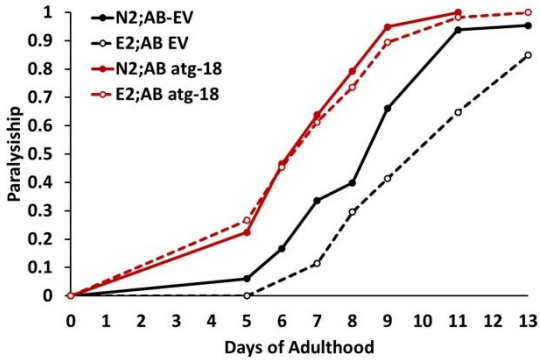

EV RNAi
bec-1 RNAi
N2;AB
e2;AB
260
80
50
30
15
Tubulin

Chapter IV Figure 3: *atg-18* is required for the DR protective response. (A) EV treated AGD342 *eat-2;Aβ* (dashed black line, mean = 23.11 ± 1.04 days) displayed lifespan extension when compared to AGD376 *N2;Aβ* on EV (solid black line, mean = 16.55 ± 0.41 days, p = <0.0001). *atg-18* knockdown by RNAi feeding from hatch did slightly reduce the lifespan of N2;Aβ (solid red line, mean = 15.16 ± 0.39 days) animals compared to EV control (p = 0.018). *eat-2;Aβ* treated with *atg-18* RNAi (dashed red line, mean = 13.56 ± 0.40 days) didn't show a DR mediated longevity response when compared to *atg-18* RNAi treated N2;Aβ, and actually showed a reduced mean lifespan compared to this AL fed RNAi group (p = 0.006). (B) Both N2;Aβ (solid red line, mean = 6.98 ± 0.22 days) and *eat-2;Aβ* (dashed red line, mean = 7.18 ± 0.26 days) demonstrated faster paralysis onset when treated with *atg-18* RNAi versus their respective controls, EV treated *N2;Aβ* (solid black line, mean = 8.93 ± 0.30 days, p = <0.0001) and *eat-2;Aβ* (dashed black line, mean = 11 ± 0.48 days, p = <0.0001). *eat-2*;Aβ paralyzed at near simultaneous rates as N2;Aβ (p = 0.48) on *atg-18*, in contrast to *eat-2*;Aβ on EV which demonstrated a delay in paralysis compared to *N2;Aβ* on EV (p = 0.0006). (C) Integrated AGD458 *N2;Aβ* and AGD456 *eat-2;Aβ* raised on *atg-18* from L2 stage onward were subject to SDS-PAGE and Western blot analysis. *eat;*Aβ animals grown on EV showed reduced levels of Aβ, with less higher molecular weight species, compared to N2;Aβ controls. Both N2;Aβ and *eat-2*;Aβ raised on *atg-18* RNAi displayed greater amounts of higher molecular weight Aβ species compared to EV controls; however *atg-18* RNAi treated eat-2;Aβ animals still showed a reduction in Aβ levels compared to N2;Aβ. Displayed is one representative biological replicate.

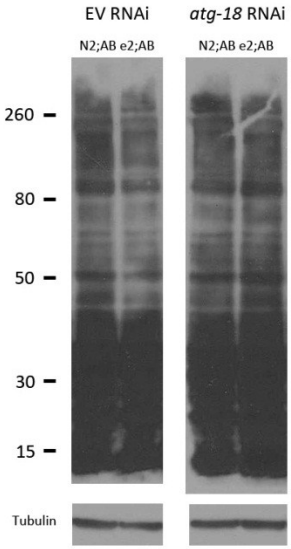
A.


N2;AB EV
E2;AB EV
N2;AB atg-18
E2;AB atg-18
Survivorship
Days of Adulthood

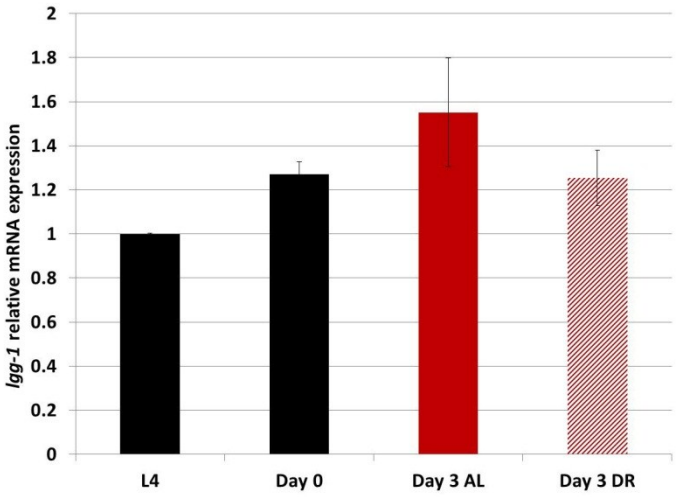
B.


N2;AB-EV
E2;AB EV
N2;AB atg-18
E2;AB atg-18
Paralysisihip
Days of Adulthood

C.


EV RNAi
atg-18 RNAi
N2;AB e2;AB
N2;AB e2;AB
260
80
50
30
15
Tubulin

Chapter IV Figure 4: *lgg-1* mRNA levels in BDR treated CL2006. Day 0 young adults showed a significant (p = 0.05) increase in *lgg-1* mRNA levels compared to L4 levels. Day 3 AL *lgg-1* mRNA levels did not show significance compared to Day 0 levels (p = 0.74). Day 3 DR *lgg-1* mRNA levels also failed to show significance compared to Day 3 AL levels (p = 0.54).



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