PROTEASOME FUNCTION DURING AGING IN *DROSOPHILA*

By

Jie Gao

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York

2012

© 2012

Jie Gao
All Rights Reserved

This Manuscript has been read and accepted by the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

Chair of Examining Committee:

____________ ____

Date **Dr. Thomas Schmidt-Glenewinkel (Hunter College, CUNY)**

Executive Officer:

____________ ____

Date **Dr. Edward J. Kennelly (Lehman College, CUNY)**

Supervisory Committee:

____________ ____

Date **Dr. Maria Figueiredo-Pereira (Hunter College, CUNY)**

____________ ____

Date **Dr. Diana Bratu (Hunter College, CUNY)**

____________ ____

Date **Dr. Christopher Cardozo (The Mount Sinai School of Medicine)**

____________ ____

Date **Dr. Marion Schmidt (Albert Einstein College of Medicine)**

THE CITY UNIVERSITY OF NEW YORK

PROTEASOME FUNCTION DURING AGING IN *DROSOPHILA*

Jie Gao

Supervisor: Thomas Schmidt Glenewinkel

Abstract

Aging is associated with dysfunction of protein homeostasis and increased protein damage caused by oxidative stress, but the detailed molecular mechanisms are unknown. The ubiquitin-proteasome pathway (UPP) is critical to the protein quality control system as it degrades a majority of intracellular proteins in eukaryotic cells. Age-related decline in proteasome function has been shown in a variety of mammalian tissues. However the role of UPP during the aging process still remains a puzzle. In this study, we compared the UPP function between two *Drosophila melanogaster* strains, the longevity mutant *methuselah (mth)* and the wild type *w1118* at different ages, which would help answer this question.

The first part of the thesis evaluated the UPP function in *mth* and *w1118* during the natual aging process and under oxidative stress condition. Mth encodes a GTP binding protein-coupled receptor (GPCR). The *mth* fly with a single Mth gene mutant has been shown to increase ~35% lifespan, but the cellular role of Mth remained elusive. We are the first to report that *mth* displays lower proteasome activity, lower proteasome levels and lower ATP steady state levels at young ages, but relatively higher levels at old ages during normal aging, when compared with control strain *w1118*. Under oxidative stress conditions, proteasome activity remained nearly unchanged

in *w1118* after 5 days of treatment with 1% H_2O_2, but it was elevated in *mth.* Moreover, while both strains exhibited a gradual increase in ubiquitinated protein conjugates and aggregates during normal aging process, *mth* produced fewer conjugates and aggregates at the comparative ages than *w1118*. Under oxidative stress conditions, the levels of ubiquitinated conjugates and aggregates were elevated in both strains, but less were observed in *mth*. Together, these data suggest that *mth* exhibits higher proteasome plasticity and maintains a more efficient protein homeostasis, contributing to longer lifespan. We propose that maintaining a steady state protein turnover rate by the ubiquitin/proteasome pathway will delay the aging process.

In the second part of the thesis, we modulated different lifespans using non-genetic approach- Dietary Restriction (DR), which is the only intervention known so far to reliably increase lifespan in a variety of organisms. Currently, it is unclear that what effects aging and DR have on proteasome-mediated protein degradation. We compared the UPP function in three *Drosophila* strains, wild type *Oregon R (OR)*, *w1118* and longevity mutant *mth* under two diets - DR and ad libitum (AL). All three fly strains responded to DR with extended lifespan. In *OR* strain, DR significantly reduced proteasome activity in male and female flies at young ages (old ages not tested) and ATP level at all age groups in male flies. These data indicate higher proteasome activity and ATP level do not always lead to longer lifespan. In addition, DR significantly reduced age-related decline in proteasome activity, and ameliorated age-related increases in ubiquitinated protein in the wild type *w1118*. However, in *mth,* DR reduced proteasome activity and displayed a slightly higher level of ubiquitinated protein conjugates under both young and old ages. Our data indicate that DR has many beneficial effects towards the function of the UPP in wild type *w1118*, and that a preservation of the UPP may be a potential mechanism by which DR extend lifespan in *w1118*. But in longevity mutant *mth*, DR can not

further ameliorate proteasome function, which suggests that other mechanisms might be responsible for the longer lifespan caused by DR. Cumulatively, these data have implications for understanding the effects of aging and DR on protein turnover and the mechanism of lifespan extension in longevity mutant *mth*.

Acknowledgements

I would like to take this opportunity to thank the people that supported me to complete this disertation.

Firstly I would like to thank my mentor Dr. Thomas Schmidt-Glenewinkel for his guidance, caring and patience through my years as a graduate student. He is always be there to answer my questions and provide encouragements and supports. His suppervision inspired me to enjoy science and allows me to develope as an independent reseacher. Secondly I would like to thank Dr. Maria Figueiredo Pereira for her unselfish and unfailing support. She is like my second mentor. She provided many insightfull advices at each stage of my reseach, which moves my project to a new level. I would also like to thank Dr. Diana Bratu for her advices for my project and showing her priceless support . She taught me how to allocate time and make a practical plan for research. I would also like to thank my committee members Dr. Marion Schmidt and Dr. Christopher Cardozo. They provided useful suggestions and invaluable assistants during progress meetings, which make this project more dialectical.

I also appreciate my past and current laboratory members, Tony, Marek, Marlon and Sireesha for their friendship and support. I also would like to thank two undergraduate students, Carmen and Sital who have been working with me over the last year. They helped sorting flies and setting up stress experiments, which help me a lot.

Last but not least I express my deepest gratitude to my beloved husband Jian, my parents Chuanxian and Wuhuang, and my brother Kun for their endless love and understanding. Without their encouragement and support I could not compelete the PhD journey.

INDEX

TITLE PAGE……………………………………………………………………………………………i

COPYRIGHT PAGE……………………………………………………………………………………ii

APPROVAL PAGE……………………………………………………………………………………iii

ABSTRACT…………………………………………………………………………………………iv

ACKNOWLEDGEMENTS……………………………………………………………………………vii

INDEX……………………………………………………………………………………………viii

LIST OF FIGURES…………………………………………………………………………………xi

LIST OF TABLES…………………………………………………………………………………xiii

LIST OF ABBREVIATIONS………………………………………………………………………xiv

CHAPTER I: INTRODUCTION……………………………………………………………………1

1.1. THE UBIQUITIN-PROTEASOME PATHWAY (UPP)……………………………………………2

1.2. AGING AND THE UPP……………………………………………………………………………5

1.3. AGING AND *DROSOPHILA MELANOGASTER*……………………………………………………7

1.3.1. Genetic approaches to understanding aging ………………………………………………8

1.3.1.1. Extended longevity mutants……………………………………………………………8

1.3.1.2. Oxidative stress, mitochondria and aging………………………………………………11

1.3.2. Nongenetic approaches to understand aging……………………………………………14

1.3.2.1. Ambient temperature…………………………………………………………………15

1.3.2.2. Physical activity………………………………………………………………………15

1.3.2.3. Gender…………………………………………………………………………………15

1.3.2.4. Reproductive status……………………………………………………………………16

1.3.2.5. Dietary restricion………………………………………………………………………16

1.4. G-PROTEIN COUPLED RECEPTOR (GPCR) AND *METHUSELAH*…………………………18

CHAPTER II: PROTEASOME FUNCTION IN THE *DROSOPHILA* LONGEVITY MUTANT *METHUSELAH*……………………………………………………………………………………………23

2.1 ABSTRACT……………………………………………………………………………………………24

2.2 INTRODUCTION……………………………………………………………………………………25

2.3. RESULTS……………………………………………………………………………………………28

2.3.1. Assessing lifespan extension in *mth*……………………………………………………28

2.3.2. Attenuated age-related decline in proteasome activity and levels in *mth*………………28

2.3.3. Attenuated age-related decline of ATP steady-state level in *mth*………………………29

2.3.4. No age-related changes in dβ5 subunit level in both *mth* and *w1118*; no age-related changes in α1 subunit level in *mth* but slight increase in w*1118*……………………………30

2.3.5. Age-related increase in protein ubiquitination is diminished in *mth*……………………31

2.3.6. Ubiquitinated protein aggregates are reduced in *mth*……………………………………31

2.3.7. *Mth* displays better locomotor ability ……………………………………………………32

2.3.8. *Mth* survives ~60% longer under oxidative stress conditions……………………………32

2.3.9. Proteasome activity and level were induced after a 5 day treatment with 1% H_2O_2 in *mth*…………33

2.3.10. Oxidative stress-induced increase in protein ubiquitination is diminished in *mth*………34

2.3.11. Oxidative stress-induced increase in ubiquitinated aggregates is diminished in *mth*……34

2.4. DISCUSSION…………………………………………………………………………………………35

2.5. MATERIALS AND METHODS………………………………………………………………………43

2.5.1. Fly stocks. ……………………………………………………………………………………43

2.5.2. Fly survival curve……………………………………………………………………………43

2.5.3. H_2O_2 administration…………………………………………………………………………43

2.5.4. In gel proteasome activity and detection……………………………………………………44

2.5.5. Western blot analysis of fly extracts…………………………………………………………45

2.5.6. ATP measurements……………………………………………………………………………46

2.5.7. Locomotor activity……………………………………………………………………………46

2.5.8. Filter trap assay………………………………………………………………………………47

2.5.9. Statistical analyses……………………………………………………………………………47

CHAPTER III: EFFECT OF AGING AND DIETARY RESTRICTION ON PROTEASOME FUNCTION IN *DROSOPHILA*……48

3.1 ABSTRACT……49

3.2 INTRODUCTION……50

3.3. RESULTS AND DISCUSSION……51

3.3.1. Assess lifespan extension via dietary restriction in *Drosophila melanogaster OR* strain……51

3.3.2. Assess the impact of DR on proteasome function and ATP level in *Drosophila OR* strain……51

3.3.3. Both *w1118* and *mth* strains exhibit extended lifespan when fed DR food……53

3.3.4. Impact of aging and DR on proteasome function in *w1118* and *mth*……54

3.3.5. Impact of aging and DR on ubiquitinated protein conjugates in *w1118* and *mth*……55

3.4. MATERIALS AND METHODS……57

3.4.1. Fly stocks. ……57

3.4.2. Dietary restriction recipe……57

3.4.3. In gel proteasome activity and detection……58

3.4.4. Western blot analysis of fly extracts……58

3.4.5. ATP measurements……58

CHAPTER IV: CONCLUSIONS AND FUTURE GOALS……59

CHAPTER V: FIGURES……65

CHAPTER VI: TABLES……97

CHAPTER VII: REFERENCES ……99

LIST OF FIGURES

Figure 1: The ubiquitin-conjugation pathway……………………………………..…….…………66

Figure 2: Proteasome structure and heterogeneity………………………..………………………..67

Figure 3: Catalytically active sites of the 20S proteasome………………………………………....68

Figure 4: Ubiquitin-proteasome pathway ……………………………………………...…...…..69

Figure 5: Life-span extension in longevity mutant *methuselah*………………………….……...…70

Figure 6: Proteasome activity and levels in *mth* mutant and *w1118* control male flies across different ages…........………………………………………………………….…..…71

Figure 7: ATP steady-state levels in *mth* and *w1118* across different ages...……...………….…… 73

Figure 8: dβ5 and α1 subunit expression in *mth* and *w1118* across different ages………………....74

Figure 9: Polyubiquitinated protein levels in *mth* and *w1118* across different ages……………………………………………………………………...75

Figure 10: Ubiquitinated protein aggregates in *mth* and *w1118* across different ages….…………...77

Figure 11: Climbing performance in *mth* and *w1118* across different ages…………………….……....78

Figure 12: H_2O_2 administration in *mth* and *w1118* in a dose-dependent manner……………………79

Figure 13: Proteasome activity and levels in *mth* and *w1118* after a 5 day treatment with 1% H_2O_2……….………………………………………..…...81

Figure 14: Polyubiquitinated protein conjugates in *mth* and *w1118* after a 5 day treatment with 1% H_2O_2…………...……………………………..……...…83

Figure 15: Polyubiquitinated protein aggregates in *mth* and *w1118* after a 5 day treatment with 1% H_2O_2…………………………….……………………..…85

Figure 16: dβ5 subunit level in *mth* and *w1118* after a 5 day treatment with 1% H_2O_2……………………………………………………......86

Figure 17: Lifespan extension by DR in *Drosophila melanogaster* *OR* strain …………………………………………………………….…………..……87

Figure 18: Impact of DR and aging on proteasome activity and level in *OR* strain……..………..…88

Figure 19: ATP steady-state levels in *OR* strain under DR across different ages…………………..…...90

Figure 20: DR extends lifespan in *Drosophila* wild type *w1118* and longevity mutant *mth*….....….91

Figure 21: Impact of DR and aging on proteasome activity and levels in *w1118* and *mth*……….…92

Figure 22: Impact of DR and aging on polyubiquitinated protein conjugate levels in *w1118* and *mth*…...……………………….…………………………………………..…..94

Figure 23: Impact of DR and aging on dβ5 subunit level in *w1118* and *mth*………..……….………96

LIST OF TABLES

Table 1: Larva and mating media ingredients……………………………..…………..……...97

Table 2: Dietary restriction treatment ingredients……………………………………..…...……98

LIST OF ABBREVIATIONS

AAA ATPase ATPases associated with diverse cellular activities

AD Alzheimer disease

AL Al Libitum

ALS Amyotrophic lateral sclerosis

Aß Amyloid peptide

ATP Adenosine triphosphate

BCA Bicinchoninic acid

CNS Central nervous system

CP 20S Core particle

DMSO Dimethylsulfoxide

DNA Deoxyribonucleic acid

DNP Dinitrophenyl hydrazone

DR Dietary restriction

DTT Dithiothreitol

ECL Enhanced chemiluminescence

EDTA Ethylenediamine tetraacetic acid

EGF Epidermal grouth factor

EGTA Ethylene glycol tetraacetic acid

ETC Electron transport chain

FLP Flippase recombination enzyme

FRT Flippase recognition target

GPCR GTP Binding Protein-Coupled Receptor

GS Gene-switch

GSH Glutathione

GSSG Glutathione-ascorbate cycle

HD Huntington disease

HNE Hydroxynonenal

HSP Heat shock protein

IFN-γ Interferon-γ

IGF-1 Insulin-like growth factor 1

Indy I am not dead yet

InR Insulin-like receptor

MDA Malondialdehyde

mRNA Messenger RNA

mSOD Manganese superoxide dismutase

mtDNA Mitochondrial DNA

Mth Methuselah

mTOR Mammalian target of rapamycin

NMJ Neuromuscular junction

Nrf2 Nuclear factor-E2-relatived factor 2

OXPHOS Oxidative phosphorylation system

PBS Phosphate-buffered saline

PD Parkinson disease

PINK1 PTEN-induced kinase 1

PLZF Promyelocytic leukemia zinc finger

RNA Ribonucleic acid

RNAi RNA interference

ROS Reactive oxygen species

RP 26S Regulatory particle

Rpn Regulatory particle, non-ATPase-like

Rpt Regulatory particle, ATPase-like

rRNA Ribosomal RNA

SDS Sodium dodecyl sulphate

SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SKP S-phase kinase-associated protein 1

SKR-5 Skp1-like protein

SOD Superoxide dismutases

TCA Trichlor acetic acid

TOR Target of rapamycin

tRNA Transfer RNA

Ub Ubiquitin

UCP Uncoupling protein

UCP5KO UCP5 knockout

UPP Ubiquitin-proteasome pathway

UPS Ubiquitin-proteasome system

CHAPTER I

INTRODUCTION

1.1. THE UBIQUITIN-PROTEASOME PATHWAY (UPP)

The ubiquitin–proteasome pathway is the major intracellular proteolytic pathway for eukaryotic intracellular non-lysosomal protein degradation. The 26S proteasome, a large proteolytic machine, degrades damaged, misfolded and short-lived regulatory proteins which are tagged with a polyubiquitin chain. Two major steps in this pathway, ubiquitination and degradation are highly ATP-dependent. The UPP thus controls a series of essential cellular processes, including the cell cycle, transcription and protein quality control (Lander et al., 2012).

Ubiquitin is a small protein of 76 amino acids in length, which can form polyubiquitin chains. These chains are formed by the successive attachment of monomers by an isopeptide bond, most frequently formed between the side chain of Lys48 in one ubiquitin and the carboxyl group of the C-terminal Gly76 of a second ubiquitin. Attachment of polyubiquitin chains to lysine residues on a protein substrate results in an over 20 fold increase in its degradation rate. Polyubiquitin chains with linkages involving lysine residues on ubiquitin other than Lys48 play distinct roles, including DNA repair, activation of NF-kB, polysome stability and endocytosis (Welchman et al., 2005).

Ubiquitination of proteins involves the following sequence of events (Figure 1): (1) Formation of a high energy thioester bond between ubiquitin and a ubiquitin-activating enzyme (E1) in a reaction that require ATP hydrolysis; (2) Formation of a thioester bond between the activated ubiquitin and ubiquitin-conjugating enzymes (E2); (3) The addition of ubiquitin to the protein substrate which is catalyzed by one of many E3s promoting the production of longer ubiquitin-chains. In some cases, ubiquitin can be transferred directly to the protein substrate by ubiquitin-conjugating enzymes (E2) (Finley, 2009).

Ubiquitin is removed from ubiquitinated proteins by de-ubiquitinating enzymes, which also disassemble polyubiquitin chains. More than 90 genes, encoding for de-ubiquitinating enzymes, have been identified, making them one of the largest family of enzymes involved in the ubiquitin pathway.

The 26S proteasome is a massive, multicatalytic protease (1.5 MDa) that degrades polyubiquitylated proteins to produce small peptides. It is composed of two subcomplexes — a 20S core particle (CP) that carries the proteolytic activities, and a 19S regulatory particle (RP) (Figure 2). The 20S CP is composed of four stacked rings, two identical outer α-rings and two identical inner β-rings. The eukaryotic α- and β-rings are each composed of seven distinct subunits. The catalytic sites are localized to three of the β-subunits. Current evidence indicates that only these three of the seven β-subunits are catalytically active, which are caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5). However, the eukaryotic proteasome cleaves a wide range of peptide bonds (Figure 3). For this reason it is capable of cleaving almost any peptide bond, having difficulty only with proline–X, glycine–X and to a lesser extent with glutamine–X bonds, where X is any of other amino acids. One or both ends of the 20S barrel can be capped by a 19S RP which is composed of ~17 distinct subunits — 9 in a "base" subcomplex, and ~8 in a "lid" subcomplex, ranging in size from 25 kDa to about 110 kDa. One important function of the 19S RP is to recognize polyubiquitinated proteins. Several ubiquitin binding subunits of the 19S RP have been identified, but their biological roles and mode of action are unclear. A second function of the 19S RP is to open an orifice in the α-ring and unfold the substrates and insert them into the 20S CP. Both the channel-opening and the substrate-unfolding functions are ATP-dependent and, indeed, the base of the 19S RP contains six AAA-ATPase subunits (RPT1–6).

Following substrate degradation, short peptides that have been derived from the substrate are released, as is reusable ubiquitin. Another regulatory particle called 11S or PA28, is a heptameric ring of PA28 subunits and is highly abundant in mammalian cells. This complex activates the 20S complex by widening the pore. The mix and match of 20S complexes, 19S complexes and 11S complexes gives rise to a variety of proteasomes (Tai et al., 2008).

Proteasome-dependent degradation of cellular proteins begins with signal recognition and ends with signal removal in typical eukaryotes (Figure 4). Substrates of the 26S proteasome are recognized following the linkage of ubiquitin to lysine residues in the substrate. Three enzymatic steps (E1, E2 and E3) are carried out in order to modify the substrate with a polyubiquitin chain. E1 activation is ATP-dependent. There are many E3 enzymes, each of which recognizes unique substrate-based signals. Therefore, E3s have the key role in substrate selection. Substrates with four or more ubiquitins attached to them have a high affinity binding to the 19S complex. After recognition by 19S, the polyubiquitinated substrates need to be de-ubiquitinated and unfolded before translocated into the proteasome core for degradation. Mechanical unfolding might occur near the site of chain attachment, but would not have to be. The rate of degradation that depends on the site of polyubiquitin-chain attachment could be improved if the substrate polypeptide chain is engaged by ATPase. Translocation through the pore of the regulatory complex is ATP dependent. Once the substrate reaches the active-site chamber inside the 20S core, it is hydrolyzed and broken into short peptides. The axial pore has to be opened by a second regulatory complex such as PA28 regulator. Therefore, the peptides exit the catalytic chamber through it. The polyubiquitin chains were removed previously from the substrate by non-ATPase on the lid of 19S such as Rpn11. This process in 26S proteasome is

strictly ATP-dependent. The polyubiquitin chain is disassembled later by non proteasomal de-ubiquitinating enzymes and will be recycled (CM et al., 2004).

1.2. AGING AND THE UPP

Aging is a nearly universal process that involves the progressive deterioration of metabolic, reproductive, cognitive and muscular functions, which ultimately affects lifespan. The underlying mechanism is still elusive. One of the more popular hypotheses about the mechanism of aging is that it is caused by an alteration in steady state protein dynamics, including a dysfunction of both protein synthesis and protein degradation (Rattan, 1996; Vellai, 2009). About 80%-90% of proteins in eukaryotic cells are degraded by the ubiquitin-proteasome pathway （UPP） with the remainder either eliminated by the lysosome or non-proteasomal proteases (Lee et al., 1998). A number of studies have shown a decline in proteasome activity with age in different animal species. (Keller et al., 2000; Gaczynska et al., 2001; Carrard et al., 2002; Martinez-Vicente et al., 2005; Vernace et al., 2007; Jana, 2012). For example, the chymotrypsin-like activity of the 20S proteasome is reported to be significantly lower in the liver, kidney, lung, heart, spinal cord and different areas of brain such as cerebral cortex and hippocampus in 24 and 28 months old rats when compared with 3 months old animals (Keller et al., 2000). Similarly in rodents, during aging the caspase-like activity of the proteasome was significantly inhibited as well (Conconi et al., 1996) . In the mouse brain, varying degrees of proteasomal inhibition were shown in different areas (Ohtsuka et al., 1995).

One of the common hallmarks of the aging related decline of UPP function is the increase in ubiquitinated proteins and aggregates (Keller et al., 2002; Vernace et al., 2007; Wang et al., 2008;

Salmon et al., 2009). In human, many age-related neurodegenerative diseases share similar morphological features, abnormal protein aggregates and inclusions in the affected areas of CNS, such as Huntingtin in Huntington disease (HD), Aβ peptide and hyperphosphorylated tau in Alzheimer disease (AD), α-Synuclein in Parkinson disease (PD) and neurofilaments in Amyotrophic lateral sclerosis (ALS) (Ross et al., 2004).

The impairment of proteasomal function during aging could have a variety of reasons including impaired assembly, damaged proteasome proteolytic sites, increased abnormal or oxidized proteins and the altered expression of proteasome subunit. (Carrard et al., 2002; Martinez-Vicente et al., 2005). For example, our previous study shows that aging related reduction of proteasome activity in *OR* wild type flies is associated with a disassembling of the 26S proteasome (Vernace et al., 2007). Mirura's group (Tonoki et al., 2009) also showed that proteasome reduction in the *w1118* wild type fly strain results from the disassembly of the 26S proteasome. They showed that the protein level of the 26S proteasome was lower in the aged fly, while 20S proteasome and 19S RP levels remained stable with age. In another study, microarray analysis of age-related variations in gene expression patterns were reported for both mitotic (human fibroblasts) and post-mitotic (rat skeletal myocytes) cells (Ly et al., 2000). A recent investigation using a reporter of global UPS has shown genetic evidences supporting that certain subunits of the 26S proteasome are differentially expressed in different tissues in *Caenorhabditis elegans*. These differences make the tissue-specific protein degradation and differential impairment of UPS during aging necessary (Hamer et al., 2010; Segref et al., 2011). However, altered subunit expression of the proteasome is not always correlated with decreased proteasome activity. Some reports have shown a decrease in proteasome activity without alteration of proteasomal subunits during aging (Keller et al., 2000; Keller et al., 2002) .

Oxidative damage has also been recognized as a major factor which contributes to aging (Harman, 1956; Harman, 2009). The oxidation of proteasome subunits (oxidation, glycation and conjugation with peroxidized lipid products) increases with age and may result in changes in UPS regulation (Carrard et al., 2002). Moreover, oxidized proteins and crosslinked proteins and lipids (all abundant in lipofuscin) can directly inhibit the proteasome (Terman et al., 2004).

What is the role that the UPP play in the aging process? The answer is still unknown. Several studies showed a positive connection between aging and proteasome function. For example, overexpressing proteasome dβ5 subunit increases the amount of assembled proteasome and confers an ameliorated response to oxidative stress and higher survival rates in WI38/T and HL60 cells (Chondrogianni et al., 2005). Knockdown of the proteasome subunit Rpn11 in *Drosophila* leads to a decrease in the 26S proteasome activity, which causes shorter lifespan (Tonoki et al., 2009). In *Saccharomyces cerevisiae*, elevating proteasome capacity by manipulating levels of the UPS-related transcription factor Rpn4, extends replicative lifespan (Kruegel et al., 2011). A recent mechanistic study about lifespan extension by EGF signaling in worms showed that increasing UPS function is involved. EGF signaling in adulthood stimulates UPS function and extends longevity by increasing the expression of various UPS components and switches the mechanism of protein homeostasis from a chaperone-based approach to protein degradation via UPS (Liu et al., 2011). Altogether, these studies reveal that the highly sophisticated molecular machinery modulates the UPS to regulate proteostasis during aging.

1.3. AGING AND *DROSOPHILA MELANOGASTER*

The fruit fly *Drosophila melanogaster*, a tiny insect about 3 mm long, has been used extensively as an animal model in biology throughout the last century. It has a relatively short

lifespan, a small genome size and is easy to culture. There exist environmental and genetic manipulations that alter lifespan. But it nevertheless contains a complex organ and endocrine system that allows studying the conserved signal transduction pathways with genetic tools. Aging research using *Drosophila* and related insects can be traced back to at least 1916. Loeb & Northrop showed that *Drosophila*'s lifespan followed the normal rules of biological processes like other animal models (Loeb et al., 1916; Loeb et al., 1917). By changing the environment temperature at which flies were cultured, they showed the inverse relationship between the temperature and lifespan. Over the last more than 90 years, *Drosophila* research has been essential for investigating the heritable and plastic nature of lifespan and mechanism in the development of many more important hypotheses of the aging process, including the rate-of-living and oxidative stress hypotheses. Critical mechanisms involved in the *Drosophila* aging process are likely to be regulated similarly in humans. The studies with *Drosophila* can help us understand and hopefully delay the aging process in humans.

There are a number of interventions known to affect *Drosophila* lifespan. They can be simply classified into the following two groups: Genetic approaches and Non-genetic approaches.

1.3.1. Genetic approaches to understand aging

1.3.1.1. Extended longevity mutants

In *Drosophila*, insertional mutagenesis with the P-element transposon has been widely used. Through simple crosses between flies with an P-element insertion and those with a transposase, single P-element insertion lines can be easily generated (Cooley et al., 1988). The newly inserted locus can be identified based on the flanking sequence of inserted transposon

(Spradling et al., 1995; Spradling et al., 1999). Several mutations in *Drosophila* created via P-element insertion have been shown to extend longevity: Methuselah (Mth) (Lin et al., 1998), I am not dead yet (Indy) (Rogina et al., 2000), Chico (Clancy et al., 2001); Insulin-like receptor (InR) (Tatar et al., 2001). In addition, there are multiways to induce controlled overexpression of a gene in *Drosophila* (Tower, 2000). Using transgenic flies with a genomic fragment containing promoter region together with the coding region, genes can be overexpressed under endogenous promoter. Or genes can be overexpressed independent of its endogenous expression pattern in particular tissues through fusing the coding region to a heterologous promoter. The following genes have been shown to extend the longevity when overexpressed: Cu/Zn-superoxide dismutase (SOD) (Sun et al., 1999), Hsp70 (Tatar et al., 1997).

The *Methuselah* (*mth*) mutant exhibited a 35% increase in average longevity and was resistant to numerous stressors including high temperature, starvation, and oxidants. The Mth gene product belongs to the family of GTP binding protein-coupled receptors (GPCR). This family of seven transmembrane spanning receptors modulates a host of signaling pathways. The function of the Mth protein is still unknown but it may be involved in regulating the stress response pathway (Lin et al., 1998).

The *Indy* mutant displays a 50% increase in lifespan. The Indy gene product resembles a protein in mammals, which is a sodium dicarboxylate cotransporter responsible for the uptake or reuptake of di- and tri-carboxylic acid Krebs cycle intermediates, such as succinate, citrate, and alpha-keto-glutarate. Indy is expressed in the fat body, gut, and oocytes, suggesting that the gene may play a role in both the absorption of metabolites and in intermediary metabolism (Rogina et al., 2000).

Chico encodes an insulin receptor substrate. The mutation of Chico extends fruit fly

median lifespan by up to 48% in homozygotes and 36% in heterozygotes. Homozygous males are slightly short-lived but heterozygous individuals also live longer. *Chico* mutants show resistance to starvation and stress, and have a higher level of SOD activity (Clancy et al., 2001).

A heteroallelic and hypomorphic genotype of the mutant *InR* yields dwarf females with up to an 85% extension of adult longevity and dwarf males with reduced late age-specific mortality. The endocrine system of *InR* dwarf female flies appears to be very much affected. Juvenile hormone synthesis was significantly reduced in the mutant females. The mutant flies are approximately half the weight of their heterozygous siblings, and they are sterile (Tatar et al., 2001).

Sun, et al. demonstrated the effects of Cu/Zn SOD overexpression on longevity using the technique called “FLP OUT” system, which overexpress a gene at desired stages of life cycle with a controlled genetic background (Basler et al., 1994). Overexpression of SOD was induced by heat-pulses, activating an *hsp70* promoter driven FLP recombinase, which excises an FRT cassette inserted between a constitutive promoter (*actin5c*) and SOD coding sequence. FRT is a marker gene with direct repeat of FRT at both ends. A genetically identical population carrying the same set of transgenes were either treated with heat-pulses to induce SOD overexpression, or kept at 25 ^{o}C throughout the experiment served as a control. Cu/ZnSOD overexpression extended the mean lifespan of flies up to 48%. Since SOD is an enzyme that scavenges superoxide anion radicals, and thus protecting cells from oxidative damage, the data suggest that oxidative damage is one of the rate-limiting factors for the life span of adult *Drosophila* (Sun et al., 1999).

Tatar, et al. showed a positive correlation between the amount of expressed Hsp70 protein and the degree of improvement in survival. The presence of Hsp70 protein increased subsequent survival at normal temperatures when expression exceeded 10–12% of basal. The flies with

extra copies of Hsp70 genes, expressed more Hsp70 proteins after being heated for 10 or 15 min at 36 ^{o}C than control and had improved survival over the two-week period after heat shock. Under these conditions, life expectancy increased by as much as 7.9% in flies with extra copies of Hsp70, which displayed the prevailing low mortality rates of young adults (Tatar et al., 1997).

1.3.1.2. Oxidative stress, mitochondria and aging

Mitochondria have been shown to be directly involved in aging in many animal models (Harman, 1981; Wallace, 2005). According to the free radical theory of aging, lifespan is determined by the ability of an organism to cope with random damages induced by reactive oxygen species (ROS) (Harman, 1956). Mitochondria consume over 90% of the cell oxygen and produce a large amount of ROS, specifically the superoxide anion radical ($O_2\bullet^-$) and its stoichiometric product, H_2O_2 (Kwong et al., 1998). The latter molecule can diffuse readily through cellular membranes and undergo scission to produce the highly reactive hydroxyl free radical, which causes a variety of macromolecular oxidative modifications. Accumulation of such oxidative damage has been widely postulated to be a primary causal factor to cause aging, and mitochondria are considered to be the most immediate targets of oxidative damage (Harman, 1972). In support of this view, mitochondrial ROS production and oxidative damage increase as a function of age (Merry, 2004). They have been hypothesized to play a major role in lifespan determination (Harman, 1972). *In vitro*, exposure of isolated mitochondria to ROS causes a variety of deleterious alterations in mitochondrial respiratory functions. On the other side, the impairment of electron transfer between oxidoreductases of the mitochondrial electron transport chain causes the upstream components to become more susceptible to autoxidation, thus decreasing respiratory activity and enhancing ROS production (Sohal et al., 1991).

Although mitochondrial involvement in the aging process is widely accepted, the

mechanism responsible for it is not fully elucidated. So far, it has been hypothesized that the aging process is associated with mitochondrial genome changes including point mutations and modifications as well as deletions (Calleja et al., 1993). mtDNA is small, highly compacted, and unprotected by histones. Consequently, mtDNA mutations in mammals accumulate at least 5-10 times faster than in a single copy nuclear DNA (Brown et al., 1979). In human, several degenerative diseases affecting muscle, nervous system, or both have been directly associated with mutations in the mtDNA (Wallace, 1992). *Drosophila melanogaster* displays an age-related decrease in mitochondrial transcripts (Calleja et al., 1993). The fruit fly mitochondrial DNA contains a 19,517 bp genome encoding 22 tRNAs, 2 rRNAs, and 13 proteins necessary for the electron transport system and phosphorylation (Schwarze et al., 1998). The amount of mtDNA recovered from whole flies or different body parts is constant during lifespan and represents approximately 1% of total DNA, but the steady-state level of multiple transcripts is significantly decreased in old flies, such as the expression of nuclear genes encoding the mitochondrial transcription factors TFAM, TFB1, TFB2, and DmTTF, which are essential for the maintenance and expression of mtDNA. Additionally, in ‘‘old’’ flies, the activities of three mitochondrial respiratory complexes (I, III, IV) sharply decline, consequently ATP synthase are reduced, and the lipid composition of the inner membrane (fatty acids and cardiolipin) is modified. However, the O_2 consumption and the subunit concentrations as measured by western blot are unaffected (Dubessay et al., 2007). The decrease in nuclear encoded mitochondrial transcripts and direct mitochondrial transcript concentrations may be one of the principal effects of aging on mitochondria, and could explain observed decreases in mitochondrial efficiency. A decrease in transcripts of genes linked to oxidative phosphorylation and to other mitochondrial functions, such as proton transport and the tricarboxylic acid cycle energy pathway, during aging has also

been unveiled by genome-wide studies using microarrays (Zou et al., 2000). The expression of these genes are elevated in long-lived *Drosophila* overexpressing a mitochondrial small heat shock protein (sHsp) (Morrow et al., 2004).

Age-related changes in carbonylation of mitochondrial proteins were determined in mitochondria from the flight muscles of *Drosophila melanogaster*. Reactivity with antibodies against (i) adducts of dinitrophenyl hydrazone (DNP), commonly assumed to react broadly with derivatized carbonyl groups, (ii) malondialdehyde (MDA), or (iii) hydroxynonenal (HNE), carbonyl-containing products of lipid peroxidation, was compared at five different ages of flies. The total amounts of immunoreactive proteins, detected by all three antibodies, were found to increase with age. These data indicate that carbonylation of mitochondrial proteins is a good marker for monitoring protein oxidative damage during aging (Toroser et al., 2007). Copeland et al. used an *in vivo* RNA interference (RNAi) strategy to inactivate electron transport chain (ETC) genes in *Drosophila melanogaster* and they reported that RNAi against five genes encoding components of mitochondrial respiratory complexes I, III, IV, and V leads to increased lifespan in flies (Copeland et al., 2009). Longevity flies with reduced ETC genes do not consistently show reduced assembly of respiratory complexes or reduced ATP levels. These data indicate that the role of mitochondrial ETC function in modulating animal aging is evolutionarily conserved and might also operate in humans.

Drosophila melanogaster has proven to be a rapid and powerful genetic platform for assessing the properties of various antioxidant supplements. Dietary supplementation of vitamin C (ascorbic acid) (Bahadorani et al., 2008), apple phenols (Peng et al., 2011) and commercially available antioxidant supplements (Vrailas-Mortimer et al., 2012) have been shown to extend adult lifespan in wild type flies. Vitamin E is protective against chemically induced lipid

peroxidation (Miquel et al., 1982). Genetic models of oxidative stress have also demonstrated that particular antioxidants can extend lifespan and reduce reactive oxygen species (ROS) in specific genetic backgrounds. As an example, loss of the detoxifying enzyme SOD1 (Cu/Zn SOD) results in a shortened lifespan,which is ameliorated by dietary supplementation with either vitamin E (α-tocopherol) or vitamin A (retinol) dietary supplementation (Bahadorani et al., 2008). Additionally, supplementation with a resveratrol complex was able to rescue the locomotor behavior defects and reduced lifespan of the alpha-synuclein induced *Drosophila* Parkinson's disease model (Long et al., 2009), but not in paraquat induced PD model (Bagatini et al., 2011).

During the aging process, the cellular redox state in *Drosophila* becomes more pro-oxidizing and is associated with the life expectancy of *Drosophila*. GSH/GSSG ratios decrease significantly with increasing age of the flies. Concentrations of Cys-Gly increased and methionine decreased with age. The amounts of protein mixed disulphides, measured as protein-cysteinyl, protein-Cys-Gly and protein-glutathionyl mixed disulphides, increased as a function of age. The glutathione-redox state and protein mixed disulphides varied according to the ambient temperature, which is inversely related to the life expectancy of the flies (Rebrin et al., 2004).

1.3.2. Nongenetic approaches to understand aging

A number of non-genetic interventions are known to affect lifespan in *Drosophila*. These include ambient temperature, gender, reproductive status, physical activity, and dietary status (Helfand-a et al., 2003; Helfand-b et al., 2003)

1.3.2.1. Ambient temperature

Decreasing ambient temperature to ~18 °C can double lifespan and change the rate of reproduction and motility in *Drosophila* (Helfand-a et al. (2003). The reasons for why lower temperature extends lifespan may be a direct effect on metabolic rates. Interestingly, more severe reductions in ambient temperature to ~11°C can cause reproductive diapause in young flies. Females arrest their egg development at temperatures of ~11 °C and both males and females can remain in low fertility stage for at least 9 to 11 weeks. After rewarming to 25 °C, they become reproductively active again and live a completely normal lifespan (Tatar et al., 2001).

1.3.2.2. Physical activity

Changes in physical activity have been reported to affect lifespan, which may also do so through an effect on metabolic rates. Sohal et al. did an interesting experiment with houseflies cultured in small containers in order to force them to reduce physical activity, not permitting them to fly. The survival data showed that it increased lifespan (Sohal et al., 1981). Neurological mutants in K^+ channels (Hyperkinetic, Shaker) increase physical activity and shorten lifespan (Trout et al., 1970).

1.3.2.3. Gender

Males and females often age at different rates resulting in longevity 'gender gaps', where one sex outlives the other. In humans, rats and flies, males have shorter lifespans than females (Barrett et al., 2011). Some studies demonstrated that male flies exhibit Parkinson-like symptoms induced by paraquat, earlier than females. The paraquat-fed flies exhibited rapid PD symptoms, which included resting tremors, bradykinesis, rotational behaviors and postural instability. Some flies even froze when undergoing the climbing assays. Paraquat treatment also caused a

concentration-dependent reduction in lifespan. Males had an average lifespan of two days after exposure, whereas females lived 12-16 hours longer. This finding in *Drosophila* reflects epidemiological studies in humans, in which incidence rates of PD in males is 1.5 to 2.5 times greater than in females (Chaudhuri et al., 2007).

1.3.2.4. Reproductive status

Reproductive status, particularly in females has a major effect on lifespan. Virgin females live about twice as long as mated cohorts (Smith, 1958). The mechanism behind might be a combination of reducing energy costs from lower egg production and mating itself. The seminal fluid transferred by males to females during mating has a direct negative effect on female lifespan (Chapman et al., 1995).

1.3.2.5. Dietary restricion

Dietary restriction prolongs the lifespan of yeast, roundworms, *Drosophila* and rodents (Piper et al., 2008). In humans, low calory intake has been shown to decrease the risk of Alzheimer's diseases (Luchsinger et al., 2002; Heilbronn et al., 2006). Studies are currently underway to test the effect of DR on lifespan in primates, with promising preliminary results (Mattison et al., 2007). More interestingly, DR has also been shown in animal models to slow and even prevent the progression of a range of age-dependent pathologies, including cardiovascular disease, multiple types of cancer, several neurodegenerative disorders and diabetes. Short term DR also reduces the risk of coronary heart disease and stroke in humans (Bishop et al., 2007). Although about 70 years of reseach has been conducted in the DR field, the mechanisms behind its effects are still elusive.

It is well established that dietary restriction increases the lifespan of *Drosophila melanogaster* (Piper et al., 2005). Commonly, the animals serving as normal controls in these studies are given unrestricted (ad libitum, AL) access to a standard laboratory diet. The ‘‘restricted’’ (DR) animals are fed daily an amount of food corresponding to some percentage of the amount of food consumed during the preceding day by AL controls of the same strain, sex, and age. In *Drosophila*, DR is usually performed by diluting the concentration of the food medium (Skorupa et al., 2008). Generally, fly lifespan can be maximized on an agar-gelled diet of sugar and lyophilized yeast, which contains all the necessary lipids, vitamins, proteins, and minerals. This diet reflects our knowledge of the ecology of *Drosophila* that feed on rotting/fermenting fruit from which they consume mainly fungus, but also some of the fruit flesh. Many studies have reported that the insulin/IGF-like signaling (IIS) and target of rapamycin (TOR) signaling pathways are involved in coupling growth to nutrition and playing a role in the control of adult lifespan in *Drosophila* (Clancy et al., 2001; Tatar et al., 2001; Giannakou et al., 2004; Hwangbo et al., 2004; Kapahi et al., 2004). Mutations that downregulate signaling through these pathways slow growth, reduce fecundity and extend lifespan. This conforms that the tradeoffs are associated with reduced nutrition. Interestingly, the long-lived IIS mutants tested possess increased lipid stores. In addition, long-lived IIS mutants are resistant to xenobiotics, and up-regulation of transcription factors that regulate xenobiotic metabolism can extend lifespan in flies (Piper et al., 2008). The interaction between DR and the lifespan-extension by the IIS mutation Chico has been reported (Clancy et al., 2002) as has the interaction between DR and reduced TOR-pathway activity. The latter results from overexpression of TSC2, which antagonizes target of rapamycin (TOR) (Kapahi et al., 2004). Both of these studies show that the maximum lifespan achieved by DR cannot be further extended by the signaling pathway

mutation at any of the food concentrations tested. Furthermore, the peak lifespan of the mutant occurs at a higher food concentration than the peak lifespan of the control flies. However, neither mutation completely abolishes lifespan variation in response to DR, which indicates that there might be several interventions responsible for mediating the effects of DR. Another interesting pathway involved in DR is sirtuins, which are NAD^+-dependent protein deacetylases. The life extending effect of DR on aging in *Drosophila* has also been reported to be dSir2 dependent (Rogina et al., 2004). But recent results showed that DR increased fly lifespan independently of dSir2 (Burnett et al., 2011).

1.4. G-PROTEIN COUPLED RECEPTOR (GPCR) AND *METHUSELAH*

Not all chemical and physical signals which bombard the surface of all cells will enter the cell directly. Some of them bind to receptors at the cell surface and initiate a flow of information that moves to the cell interior. Stimulation of these receptors activates a group of coupling proteins called guanine nucleotide–binding regulatory proteins (G proteins) that route the signals to several distinct intracellular signaling pathway. (Dohlman et al., 1991). These pathways interact with one another to form a network that regulates the biological functions of many cellular machineries, such as metabolic enzymes, ion channels, transporters and control various cellular processes, including transcription, motility, contractility and secretion (Neves et al., 2002). These receptors of G proteins are called G protein coupled receptors (GPCRs), which are among the most studied proteins in mammals. At least 39 of them are considered to be major drug targets (Lagerstrom et al., 2008). GPCRs share a seven hydrophobic α-helical domain structure. The seven hydrophobic domains spin the membrane and are linked by three extracellular loops and three intracellular loops. The extracellular NH_2-terminus is usually

glycosylated and the cytoplasmic COOH-terminus is generally phosphorylated. There exist a large diversity of GPCR genes in eukaryotic genomes. Over 1,000 GPCRs have been identified in the *Caenorhabditis elegans* genome and they just represent ~5% of its total number of genes (Brody et al., 2000). The *Drosophila* genome contains ~200 genes encoding for GPCRs, including neurotransmitter and hormone receptors, and olfactory and putative taste receptors . About 100 genes have been identified in the *Drosophila* genome that encode for putative neurotransmitter and hormone GPCRs and atypical seven-transmembrane domain (7 TM) proteins. Based on the secondary structure predictions and sequence analysis, *Drosophila* GPCRs are classified into four families: rhodopsin-like; secretin-like; metabotropic glutamate–like; and atypical 7 TM proteins.

The rhodopsin-like family is by far the largest group of GPCRs activated by a variety of ligands. It includes receptors for a large variety of stimuli, such as biogenic amine neurotransmitters, neuropeptides, peptide hormones, light, nucleotides, prostaglandins, leukotrienes, chemotactic peptides, and chemokines. This large diverse family is characterized by the presence of a protein motif (N-S-X-X-N-P-X-X-Y) in TM7 and a (DE-R-Y-F) amino acid motif at the border of TM3 and intracellular loop 2 (Schioth et al., 2005). The well-known visual rhodopsin contains structural and functional properties conserved from flies to humans. Rhodopsin in the photoreceptors in *Drosophila* responds to the presence of light. Light energy is converted to a change in the ionic permeability of the photoreceptor membrane through opening (*Drosophila*) or closing (human) of cation channels (Palczewski, 2006).

The secretin-like family includes receptors for many hormones such as secretin, calcitonin, vasoactive intestinal peptide, and parathyroid hormone and related peptides. Secretin receptors are identified by the presence of a long N-terminal domain with at least six conserved cysteine residues implicated in ligand binding (Cardoso et al., 2005). A new defined subset of the secretin family, **the Mth and mth-like family**, is a diverse and lineage specific family of receptors present mostly in *Drosophila*. Mth and mth-like family share more structural and sequence similarities to the secretin-like receptors than other families of GPCRs, which have a large (195 residues) aminoterminal extracellular domain essential for ligand binding (Heo et al., 2008).

Mth was first identified in a screen for longevity mutants in *Drosophila*. A P-element inserted within the third chromosome significantly reduces gene expression levels of Mth and increases lifespan by about 35% compared to wild type flies (Lin, 1998). These flies are resistant to all kinds of stresses including oxidative stress, starvation and high temperature. The Mth receptor is also essential for normal development since flies homozygous for the *mth* mutation displayed pre-adult lethality. In 2001, the 2.3-Å resolution crystal structure of the Mth extracellular region was revealed, which described a folding topology in which three primarily structure-containing domains meet to form a shallow interdomain groove. This groove contains a solvent-exposed tryptophan that may represent a ligand binding site (West et al., 2001). A cell based reporter system established in HEK293 cells identified “stunted” (SunA, SunB) as potential ligands for Mth (Cvejic et al., 2004). Three years later, high-affinity peptide ligands were designed by using mRNA display to bind the N-terminal ectodomain of Mth. The selected peptides are potent antagonists of Mth signaling and extend lifespan. (McGarrigle et al., 2007; William W Ja et al., 2007). The ligand, stunted is homologous to the epsilon subunit of ATP

synthase, a mitochondrial protein that regulates cellular ATP concentrations (Kidd et al., 2005). Loss of zygotic sun expression leads ultimately death. Embryos lacking maternally supplied sun (sun embryos) cause a six-fold reduction in ATP synthase activity but not completely elimination. Two additional Mth ligands were identified *in vitro* using mRNA display. This analysis revealed that effectively bound the Mth ectodomain while mutants extended lifespan (Ja et al., 2009).

Besides the investigation on the structure of Mth, very few functional studies have been conducted . Mth is required to acutely upregulate neurotransmitter exocytosis in the presynaptic motor neuron at larval *Drosophila* NMJs. Mutations in the *mth* gene reduce evoked neurotransmitter release by ~50%, and decrease synaptic area and the density of docked and clustered vesicles (Song et al., 2002). In addition, *mth* has a higher average wing-beat frequency (WBF) throughout most of its lifespan compared to parental control flies (*w1118*) and develops flight ability at a younger age, which enhances sensorimotor abilities critical to survival (Petrosyan et al., 2007). However, the connection between the mutations in *mth* and lifespan extension still remain a puzzle. Notably, part of this thesis focused on the mechanism of *mth* lifespan extension.

The metabotropic glutamate–like GPCRs are characterized by very long NH_2-terminal extracellular domains containing ~17 conserved cysteine residues. There are three ligands, calcium ions and amino acid neurotransmitters glutamate and γ-amino butyric acid (GABA). Glutamate is a major excitatory neurotransmitter for invertebrates, whereas GABA is generally released from inhibitory synaptical ternimals.

The atypical 7 TM proteins maintain the typical topology of GPCRs but do not show sequence conservation with the other GPCR family members. The Frizzled-like proteins, Starry night (Flamingo) and Bride of sevenless are decribed in this group. These receptors play important roles in tissue polarity and cell–cell signaling. The detailed signaling mechanism is not clear.

CHAPTER II

PROTEASOME FUNCTION IN THE LONGEVITY *DROSOPHILA* MUTANT *METHUSELAH*

This chapter is currently being written up as a manuscript to be submitted

2.1 Abstract

Aging is associated with dysfunction of protein homeostasis and increased protein damage caused by oxidatvie stress, but the detailed molecular mechanisms are unknown. The ubiquitin-proteasome pathway (UPP) is critical to the protein quality control system as it degrades a majority of intracellular proteins in eukaryotic cells. Here we compare proteasome function in *Drosophila* wild type *w1118* and longevity mutant *methuselah(mth),* under normal aging and oxidative stress condition. This will help to determine the role of the UPP during the aging process. We report that *mth* displays lower proteasome activity, lower proteasome levels and lower ATP stead state levels at young ages, but relatively higher levels at old ages during normal aging, when compared with control strain *w1118*. Under oxidative stress conditions, proteasome activity remained nearly unchanged in *w1118* after 5 days of treatment with 1% H_2O_2, but it was elevated in *mth.* Moreover, while both strains exhibited a gradual increase in ubiquitinated protein conjugates and aggregates with aging, *mth* produced fewer conjugates and aggregates at the respective ages as *w1118*. Under oxidative stress conditions, the levels of ubiquitinated conjugates and aggregates were elevated in both strains, but lower conjugates and aggregates were formed in *mth*. Together, these data suggest that *mth* exhibits higher proteasome plasticity and maintains a more efficient protein homeostasis contributing to longer lifespan. We propose that maintaining a steady state protein turnover rate by the ubiquitin/proteasome pathway leads to successful aging.

2.2. Introduction

The ubiquitin-proteasome pathway (UPP) degrades about 80%-90% of proteins in eukaryotic cells with the remainder being eliminated by lysosomes or non-proteasomal proteases (Lee et al., 1998). Altered UPP function can lead to a wide range of disturbances, such as abnormal signal transduction, cell cycle progression and differentiation, apoptotic pathways and survival (Hershko et al., 1998; Dahlmann, 2007; Vucic et al., 2011; Geng et al., 2012). UPP consists of two major steps: ubiquitination and degradation. The ubiquitination of substrate proteins is mediated by an enzymatic cascade that involves ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin protein ligases (E3). A chain made of at least four ubiquitin moieties targets the conjugated substrate for degradation by the 26S proteasome. The 26S proteasome is composed of a 20S core particle (CP) that carries the catalytic activities, and a 19S regulatory particle (RP). The 20S CP consists of two identical outer α-rings and two identical inner β-rings, each of which is composed of seven distinct subunits. The catalytic sites are localized to three β-subunits with caspase-like activity (β1), trypsin-like activity (β2), and chymotrypsin-like activity (β5). The ubiquitin-proteasome degradation pathway is ATP-dependent (Tai et al., 2008).

Age-related decline in proteasome function has been shown in a variety of mammalian tissues and aged flies (Chondrogianni et al., 2005; Vernace et al., 2007). Impairment of proteasome activity correlates with increased ubiquitination of proteins and intracellular deposition of aggregates (Finley, 2009). In human, many age-related neurodegenerative diseases share similar morphological features, which are abnormal protein aggregates and inclusions in the affected areas of CNS, such as huntingtin in Huntington disease (HD), Aβ peptide and hyperphosphorylated tau in Alzheimer disease (AD), α-synuclein in Parkinson disease (PD) and

neurofilaments in amyotrophic lateral sclerosis (ALS) (Ross et al., 2004). Consequently, impairment of the ubiquitin-proteasome system is commonly seen as one of the key factors in the aging process. However, the role of the UPP during aging or whether the UPP function level could be recognized as a predictor of life expectancy still remains a puzzle. Comparing UPP function between two different animals in the same species but with different life expectancies would help understand this question.

Aging research using *Drosophila* and related insects could be traced back to at least 1916 (Loeb et al., 1916; Loeb et al., 1917). *Drosophila*'s lifespan followed the normal rules of biological processes like in other animal models and has been recognized as a good model to study aging since it has a short lifespan, is easy to culture, has a relatively small genome size, and allows environmental and genetic manipulations to alter lifespan. *Methuselah* (*mth*), a *Drosophila* longevity mutant has a 35% increased longevity and enhanced resistance to various forms of stress including starvation, high temperature, and reactive oxygen species (ROS) (Lin, 1998). The Mth gene encodes for a family of GTP binding protein-coupled receptors (GPCR) that is closely related to a subgroup within the "Secretin-like receptor family" of the GPCR superfamily (Brody et al., 2000). The Mth like receptor family is considered to function as an antioxidant defense system which attenuates the damage by ROS to DNA, protein and cell structure (Harman, 2003; Barja, 2004). While there are a few studies which have demonstrated the moleculare structure, the ligands and neural mechanisms of Mth (West et al., 2001; Song et al., 2002; Cvejic et al., 2004; Wang et al., 2004), the mechanism of life extension of *mth* mutant is still unknown.

Aging is a very complex process. Aging has been associated with dyfunction of protein homeostasis and increased protein burden with oxidative modifications (Grune, 2000; Szweda et

al., 2003; Balaban et al., 2005). Oxidative stress is generally thought to be an important mechanistic contributor to the aging process (Harman, 1956). Many DNA, lipids and proteins are sensitive to oxidation, and oxidation of critical residues can negatively affect their structure and function, which might cause aging. Protein homeostasis is partly maintained by the UPP. Therefore, we hypothesized that a better maintenance of proteasome function under both normal aging and oxidative stress conditions may be important determinants of the longer lifespan of *Drosophila* longevity mutant *mth.*

In this study, we test this hypothesis by evaluating the function of ubiquitin-proteasome pathway at different ages in the longevity mutation *mth*, compared with wild type *w1118* during the normal aging process and under oxidative stress conditions. We report that the *mth* mutant fly exhibits a delay in the decline in proteasome activity and protein level caused by aging, compared with the dramatic decline observed in *w1118* flies during the normal aging process. Moreover, levels of ubiquitinated proteins and aggregates were significant lower in *mth* than in *w1118,* at all age groups tested. Upon oxidative stress treatment, the lifespan of both *mth* and *w1118* were reduced. However, proteasome function in *mth* was dramatically induced at 5 days of age. In contrast, proteasome function remained nearly unchanged in *w1118.* Furthermore, the levels of both ubiquitinated protein conjugates and aggregates were higher in *w1118* than in *mth*. Our results show that compared to *w1118,* in *mth* at comparative ages the proteasome decline is attenuated leading to less accumulation of ubiquinated proteins and fewer aggregates during normal aging and under stress conditions. Our comparative studies elucidate a key mechanistic difference that is likely to contribute to prolonged longevity and strongly implicate that maintenance of proteasome function is key to successful aging.

2.3. Results

2.3.1. Assessing lifespan extension in *mth*

The flies were raised at a constant temperature of ~25 °C and humidity ~60%, in a 12/12 hour dark/light cycle environment. Survival curves are shown in Fig. 5 with filled triangle symbols representing data from control *w1118* male and open circle symbols representing data from male *mth*. Kaplan–Meier survival analyses showed that *mth* clearly lives ~20% longer than wild type *w1118* ($P < 0.01$). The medial lifespan is 48 days for *mth* and 41 days for *w1118*. However, the mean lifespans of both lines are shorter than that previously reported, due to environmental conditions and food components known to affect fruit fly lifespan (Lin, 1998; Eric Cook-Wiensa, 2002; Timothy M. Bass, 2007).

2.3.2. Attenuated age-related decline in proteasome activity and levels in *mth*

To assess the effect of aging on the proteasome, we compared proteasome activity and levels in flies from different age groups: 1, 3, 5, 7, 9 weeks old (Fig 6). Flies from all age groups were harvested at the same date. Whole fly lysates were obtained and the cleared supernatants were used to assess proteasome activity by the in-gel assay as described in Materials and Methods. 90 µg protein of each sample was loaded onto a step-gradient native (no SDS) gel (3%, 4%, 5%), to differentiate between the two forms of the 26S proteasome (one capped and two capped) and the 20S proteasome core particle. The Suc-LLVY-AMC substrate was used to measure the chymotrypsin-like proteasome activity. We found that wild-type *w1118* flies displayed a dramatic decrease in 26S proteasome activity with aging (Fig 6A left). The proteasome activity at the old age (7 week) remarkably decreased ~ 50% in *w1118*. Both forms

of 26S-two capped and one capped, decreased with age. However, *mth* exhibited an attenuated age-related decrease in proteasome activity. At the young ages (1w, 3w and 5w) *mth* showed relatively lower proteasome activity than wild type (~20% lower at 26S two capped; ~25% lower at 26S one capped) (Fig 6A right). At the old age (7w) proteasome activity of *mth* was maintained higher than *w1118* (~5-10%). Since most of *w1118* died at 8 week, there was no sample for *w1118* at 9w. However, about 5% of *mth* survived through 9 weeks. Semi-quantification of the activity bands were detected in Fig. 6C and 6G (26S two capped; black bars and solid line for *w1118*; white bars and dashed line for *mth*) and 6E and 6I (26S one capped).

We also assessed proteasome levels with our peptide generated anti-dβ5 specific antibody (BioSynthesis) following the in-gel assay. The immunoblot data reveals a remarkable decline in 26S proteasome levels (~ 70%) (one capped) and slightly decline (~10%) in 20S level in wild type *w1118* (Fig 6B left). The *mth* proteasome levels do not exhibit the dramatic decrease with aging like in wild type. At each young age group, proteasome levels in *mth* are relatively less than in *w1118* (~25%), and are maintained at advanced age (7w and 9w) (Fig. 6B right). Semi-quantification is shown in Fig, 6D/6H and 6F/6J. Similar results were obtained in four independent experiments. These findings confirm the age dependent decline in 26S proteasome activity and levels in wild type *w1118* as we previously reported (Vernace et al., 2007). In contrast, *mth* reduced the age dependent sharply decline in both proteasome activity and levels.

2.3.3. Attenuated age-related decline of ATP steady-state level in *mth*

The function of the 26S proteasome is ATP-dependent (Liu et al., 2006), thus we compared ATP steady-state levels in the flies at each age group. ATP levels in both *mth* and *w1118* decreased about 20~40% with aging (Fig 7). At young ages (1w, 3w and 5w), *mth* had

about 10-25% less ATP than wild type. However at 7w of age, there is no significant difference of ATP levels between these two strains (the average level of ATP is ~8% higher than *w1118*). *mth* continued to survive with lower ATP levels until 9w of age. The ATP level of each age group matches their proteasome activity in both strains, which is that *mth* has lower levels at young ages (1w, 3w, 5w) but maintains activities at old ages (7w, 9w) compared to wild type.

2.3.4. No age-related changes in dβ5 subunit level in both *mth* and *w1118*; No age-related changes in α1 subunit level in *mth* but slight increase in *w1118*

The proteolytically active 20S proteasome is composed of α and β subunits. Next, we tested whether the reduction of proteasome activity and levels as age are due to the changes of individual subunit expressions. We measured the protein levels of proteasome subunits α1 and dβ5 in both strains. All age groups of whole flies were homogenized at the same date. Each supernatant was subjected to immunoblotting with anti-α1 and anti-dβ5 antibodies. The dβ5 subunit accounts for the chymotrypsin-like activity, which carries out the rate-limiting step in protein degradation by the proteasome (Groll et al., 1999). The expression of dβ5 subunits remain essential stable with age in both *w1118* and *mth* (Fig. 8). Moreover, α1 subunit in wild type was induced ~15% during the aging process; however there is no change in *mth*. These data indicate that the age-related reduction of proteasome activity and protein level is not due to a decline in α and β subunit expression. Furthermore, comparing α and β subunit levels between *mth* and *w1118*, it is apparent that the levels of these subunits were slightly lower (~15%) in *mth* than *w1118* which could account for overall lower proteasome levels (except β subunit at 3w). Furthermore, these data suggest that the longer lifespan in *mth* does not result from higher expression of 20S subunits.

2.3.5. Age-related increase in protein ubiquitination is diminished in *mth*

Proteasomes maintain intracellular protein homeostasis, thus downregulation of proteasomes usually is accompanied by increased ubiquitinated proteins. Next we measured the accumulation of ubiquitinated proteins. Fly lysates made from whole flies of both strains were immunobloted with an anti-polyubiquitin antibody. Polyubiquitinated proteins show a high molecular weight smear (Fig. 9). Both *w1118* and *mth* display a gradual accumulation of polyubiquitinated proteins with aging. At each age group, *mth* showed ~15% fewer polyubiquitinated proteins than wild type ($P<0.05$) except at 1w when there was no significant difference between the two strains. These results indicate that the age-related accumulation of polyubiquitinated proteins is observed in both strains. Furthermore, the fewer polyubiquitinated proteins in *mth* across ages suggest that proteins in *mth* might be less damaged and less tagged by ubiquitination for proteasome degradation.

2.3.6. Ubiquitinated protein aggregates are reduced in *mth*

Impairment of the ubiquintin-proteasome pathway seems to be a feature of many cell models in which intracellular aggregates are detected (Kayed et al., 2004). Most aggregates contain ubiquitinated proteins. Since *mth* showed less ubiquinated proteins, we tested if *mth* had fewer ubiquitin aggregates. In order to assess the amount of SDS-insoluble ubiquitin aggregates, proteins were extracted from whole flies in both strains. 50 µg protein of each sample was applied to a filter trap (as described in Materials and Methods) followed by western blotting. As shown in Fig. 10, both strains had few insoluble ubiquitinated aggregates during normal aging and the levels of aggregates increased as age. However, *mth* showed about 20-30% less SDS insoluble ubiquitinated aggregates than wild type across different ages. These results further

confirm that the longevity mutant *mth* resists protein damaging and misfolding, thus exhibits less aggregate formation.

2.3.7. *Mth* displays better locomotor ability

To investigate *mth*'s locomotor ability at different ages, we performed a climbing assay which has been used for studying motor behavior in transgenic fly models (Liu et al., 2008; Mohammad et al., 2009). Normal flies display a strong negative geotactic response. When tapped to the bottom of a vial, flies climb to the top of vials rapidly and stay there. As shown in Fig. 11, the climbing ability of both *mth* and *w1118* flies significantly deteriorated with age, in a time-dependent manner. In *w1118* males, a significant decrease in locomotor function with age was 15%, 70%, and 99% at 3w, 5w and 7w, respectively. In *mth* males, the time-dependent decrease of locomotor function was 8%, 42%, 60%, and 99% at 3w, 5w, 7w and 9w. At advanced age (*w1118* at 7w; *mth* at 9w), most flies stayed at the bottom, tried to climb up but fell back or climbed very slowly. Furthermore, *mth* flies were significantly better climbers than wild type at all ages except at 1w when there is no difference. At 3w, 5w and 7w, *mth* display 8%, 20% and 40% better locomotor ability than w1118 ($p<0.001$). Taken together, these results indicate that both strains display an age-dependent decline in locomotor ability and start with the same climbing ability at 1w, but *mth* maintains better locomotor ability than *w1118* at the following ages.

2.3.8. *mth* survives longer under oxidative stress conditions

The data above suggest that *mth* maintains its protein homeostasis more efficiently, and thereby optimally functional status during normal aging process. Next, we examined the ability

of *mth* flies to resist oxidative stress. 30 flies per trial were subjected to oxidative stress caused by different concentration (0%, 0.1%, 1% and 2.5%) of hydrogen peroxide (H_2O_2) and we counted fly death every day. Each trial was repeated three times. As shown in Fig. 12, increasing doses of H_2O_2 reduced lifespan of both strains in a time-dependent manner. However, *mth* survived longer than wild type at all treatment concentrations except 2.5%, at which both strains exhibited the same short lifespan. The median lifespan of *w1118* flies treated with 0%, 0.1%, 1% and 2.5% H_2O_2 was 44 days, 29 days, 4 days and 3 days respectively; in contrast, *mth* survived for 53 days, 41 days, 6.5 days and 3days respectively under the same conditions. After 5 day treatment with 1% H_2O_2, the survival percentages of *w1118* and *mth* are ~10% and ~60%, respectively. As compared to wild type, *mth* showed apparently higher resistance to H_2O_2 damages.

2.3.9. Proteasome activity and level were induced after a 5 day treatment treatment with 1% H_2O_2 in *mth*

The proteasome maintains cellular homeostasis by degrading the majority of intracellular proteins (Goldberg et al., 1997; Tanaka, 1998), as well as misfolded and oxidized proteins (Grune et al., 1997; Grune et al., 1997; Liu et al., 2003; Chen et al., 2006). Next, we compared proteasome function under 1% H_2O_2 treatment between *mth* and wild type. After 5 days of oxidative stress treatment, four samples of *mth* and *w1118* treated at 0% and 1% H_2O_2 were harvested at the same time followed by the in-gel assay for proteasome activity and western blotting for proteasome levels (Fig. 13). The Suc-LLVY-AMC substrate was used to measure the chymotrypsin-like activity of the proteasome. There is no significant difference in proteasome activity in *w1118* between the 1% H_2O_2 stress treatment and 0% H_2O_2 control treatment

(Fig. 13a). However, the proteasome activity was induced ~3 fold in the longevity mutant *mth* under 1% H_2O_2 treatment. The western blot following the in-gel assay shows that the 26S proteasome content increased ~2 folds and 20S increased ~20% in *mth* under H_2O_2 treatment (Fig.13b). In contrast, no significant changes were detected in proteasome level in wild type under stress treatment. These data indicate that *mth* exhibits an increased proteolytical capacity by increasing their proteasome activities, which allows them to cope better with oxidants. There are no changes in dβ5 subunits in both of strains (Fig. 14).

2.3.10. Oxidative stress-induced increase in protein ubiquitination is diminished in *mth*.

Proteasome usually degrades ubiquitinated proteins. Next we examined the polyubiquitinated proteins under oxidative stress in *mth* and *w1118.* Flies of both strains were harvested after 5 days of treatment with 0% and 1% H_2O_2 followed by immunoblotting with an anti-polyubiquitin antibody. As shown in Fig. 15, polyubiquitinated proteins were elevated ~60% in both strains after H_2O_2 treatment. However, *mth* displayed ~30% lower polyubiquitinated proteins than *w1118* under both 0% and 1% H_2O_2 treatment. The increase in polyubiquitinated proteins is not surprising since more proteins would be oxidized and damaged by H_2O_2 treatment. Some studies have shown mild oxidative stress would increase ubiquitin-conjugates level in lens cell (Shang et al., 2001; Zhang et al., 2008).

2.3.11. Oxidative stress -induced increase in ubiquitinated aggregates is diminished in *mth*

Consistent with previous ubiquitinated protein data, under 0% H_2O_2 treatment, *w1118* has two times more ubiqitinated aggregates than the longevity mutant *mth* (Fig. 15). Under stress conditions, *w1118* generated significantly higher levels of ubiquitinated aggregates (~3.5 fold)

than under no stress; however *mth* increased only about two-fold. The ubiquitin aggregates in *mth* under stress are about half the amount compared to *w1118* under stress. These data indicate that *mth* must have more efficient protein turnover resulting in less insoluble aggregates, which could be due to its higher proteasome activity and preserved ubiquitination ability. It further confirms that *mth* has a better resistant ability to oxidative stress than wild type flies.

2.4. Discussion

In this study, for the first time we evaluate the function of the UPP in the *Drosophila* longevity mutant *mth* during natural aging process and under oxidative stress conditions when compared with wild type *w1118*. Our data show that *mth* protein structure and integrity are better maintained during aging than that of *w1118* and this could be due to the increase proteasome capacity which enhances the turnover of negative regulators of lifespan in *mth*.

The age-related reduction of proteasome activity in *w1118* at different age groups (Fig. 6) is consistent with previous studies on humans, other mammals and flies. (Keller et al., 2002; Chondrogianni et al., 2005; Vernace et al., 2007). Thus, the age-related reduction of proteasome activity is evolutionarily conserved. Importantly, the reduction in age-related proteasome activity might result from disassembly of the 26S and 20S proteasome complexes besides dissociation of 20S and 19S. Immunodetection of proteasomes with a proteasome dβ5 subunit antibody following the native gel assay shows the decrease in both of 26S (one capped) (~75%) and 20S (~10%) proteasome protein levels at 7w compared with 1w and 3w respectively. However the protein levels of individual α1 and dβ5 subunits in *w1118* have no significant change as aging except α1 subunit slightly increase with age. These data suggest that age-related reduction of proteasome activity may result from the disassembling of 26S and 20S proteasomes into other

non-proteolytical forms or individual subunits. Moreover, since 20S proteasome level does not decrease as much as 26S does, it is possible that 26S is disassembled into 19S and 20 S. This is consistent with other studies (Vernace et al., 2007; Tonoki et al., 2009). In contrast, the longevity mutant *mth* exhibits lower activity than *w1118* at young ages (1w and 3w), but proteasome activity is maintained relatively stable at advanced age (7w and 9w). This seems surprising since several studies have shown that elevated proteasome activity will extend lifespan and that down-regulation of proteasome function will shorten lifespan in several animal models, such as *Saccharomycel cerevisiae* and *Drosophila* (Tonoki et al., 2009; Kruegel et al., 2011). However, longer lifespan in animals is not always accompanied by higher proteasome function. Salmon et al. reported that in two bat species with long lifespan proteasome activity was lower compared with short lifespan mice (Salmon et al., 2009). In addition, another protease calpain has been shown to exhibit 5 to 7-fold lower activity in brains of *T. brasiliensis* and the pallid bat (Antrozous pallidus) compared to calpain activity in mice (Baudry et al., 1986). Caplains are calcium-dependent cysteine proteases that degrade proteins associated with the cell cytoskeleton and have been implicated in degenerative processes in muscles and neurons (Storr et al., 2011). Since proteasome activity does not exhibit a higher level in *mth* at young ages, we hypothesize that it might be less needed. Proteasomes degrade misfolded and damaged proteins which are usually ubiquitinated. We measured the accumulation of soluble polyubiquitinated proteins in flies of both strains. We found that at each age group, *mth* showed apparent lower levels of ubiquitinated proteins than *w1118*. This suggests that proteins in *mth* flies might be less damaged and less marked by ubiquitination for proteasome degradation across ages. These data strongly support the notion that *mth* needs less proteasome activity for ubiquitin-linked protein removal. This is also consistent with the findings from the Salmo's group, that long lifespan bats show

lower ubiquitinated proteins than mice (Perez et al., 2009). Together, these findings suggest that protein degradation in general may be relatively low in *mth*, possibly because *mth* are able to maintain their protein structure and function better than shorter lived *w1118*, thereby reducing demand for removal of damaged and misfolded proteins.

The accumulation of protein aggregates occurs in response to an imbalance between generation and degradation of intracellular proteins. These aggregates are usually ubiquitinated (Taylor et al., 1987; Davies, 2001). The large majority of ubiquitinated aggregates are insoluble, and can not be degraded by proteasomes since they are too big and can not enter the small channel of proteasomes (Korolchuk et al., 2010). Both *mth* and *w1118* do not have many aggregates during normal aging, but *mth* showed ~20-30% less aggregates than *w1118* at each age group. This further suggests that proteins in *mth* are less damaged and therefore proteasomes are less needed. This hypothesis will be further addressed below.

UPS function is ATP-dependent, as ATP is required for ubiquitination, substrate unfolding and proteasome assembling (Eytan et al., 1989; Benaroud et al., 2003; Liu et al., 2006). ATP steady state levels will tell us whether there is an energy difference which would affect proteasome function. ATP levels decrease by about 20-40% in both fly strains as they age. This data is consistent with another ATP study in *Drosophila* (Schwarze et al., 1998). The interpretation of these data is not straightforward, especially in an entire organism where several pathways modulate ATP synthesis, its use and its steady state levels. However, the reduction in ATP levels could explain the reduction in energy input to the proteasome and the decrease in the levels of functional proteasome. Or conversely, as less proteasome is needed less ATP is produced. ATP levels in *mth* as we report decrease by ~20% at 7w of age and by ~40% at 9w. Following the proteasome activity trend, ATP level is lower at young ages (1w, 3w, 5w) and

maintained at advanced ages (7w, 9w). This confirms that proteasome function is ATP-dependent. Furthermore, western blot analysis following the in-gel assay showed that proteasome protein level in *mth* follow the same trend, lower proteasome levels at young ages and relatively higher at old ages than in *w1118*. It suggests that in *mth* the level of assembled proteasome is lower at young ages and relatively higher at advanced ages when it is most needed. The combination of all the ATP and proteasome activity and level data with ubiquitined proteins and aggregates, lead us to hypothesize that the UPP function is not fully induced in *mth* at young ages, since *mth* are able to maintain their protein structure and function more efficiently than *w1118*. At old ages, *mth* mantain higher proteasome function in order to degrade more misfolded and damaged proteins associated with aging. Protein homeostasis is maintained by ribosomes, chaparones and two proteolytic systems, the UPP and the autophagosomal/lysosomal pathway (Powers et al., 2009). Therefore, *mth* might maintain higher chaparone function and lower degradation at young ages, then later switch to higher degradation model at old ages. A recent study showed that EGF signalling regulates lifespan by switching the mechanism for maintaining protein homoeostasis from a chaperone based approach to an approach involving augmented protein degradation via UPS, as animals enter fertile adulthood (Liu et al., 2011). As animals enter adulthood, EGF signalling upregulates the expression of genes involved in the UPP, such as the Skp1- like protein SKR-5, while downregulating the expression of HSP16-type chaperones. We are not sure if EGF sigaling is involved in the lifespan extension of *mth*, but based on our data *mth* exhibit higher proteasome plasticity and induced UPP function at advanced age, which could be one option to delay aging and cause lifespan extension.

Since *mth* exhibit higher proteasome capacity at old ages during the normal aging process, whether *mth* maintain proteasome function under stress conditions is interesting to know.

Oxidative stress is generally thought to be an important mechanistic contributor to the aging process. This theory proposes that aging and age-related diseases at least partially result from the accumulation of oxidative damage to biological macromolecules (Bokov et al., 2004). *Mth* longevity mutant has been shown to have higher resistance to oxidative stress (Lin, 1998). Our oxidative stress experiments confirmed these data. After treatment with 1% H_2O_2 for 5 days, significantly fewer *w1118* (~10%) survived than *mth* (~60%). The highly induced proteasome activity in *mth* suggests that proteasome in *mth* has higher plasticity which could be induced under stress conditions. The protein homogenates from *mth* seem to exhibit less damage due to oxidative stress than *w1118.* Salmon's group also showed similar results that protein carbonyl levels following oxidative stress, which indicate oxidized protein levels are relatively lower in samples from long live bat species than those from short live mice. Oxidized proteins are reported to be degraded by the proteasome (Davies, 2001; Shringarpure et al., 2001; Chondrogianni et al., 2005). Thus, the induction of proteasomes in *mth* would contribute to longer lifespan. These data are consistent with the idea that *mth* has enhanced mechanisms for protein homeostasis. The mechanisms by which *mth* proteins are better maintained in their folded states still need to be determined. It is possible that *mth* exhibits higher expression of protein chaperones that may serve a buffer against potentially damaging stresses. It might be argued that enhanced maintenance of proteins, rather than removal of damaged proteins, may be one of the potential underlying mechanisms for maintenance of normal physiology of *mth*.

The level of ubiquitinated proteins depends on the following factors: the availability of substrates, the activity of ubiquitination enzymes and deubiquitinating enzymes, ATP levels and the activity of the 26S proteasome. When proteins are oxidized under oxidative stress condition, they become preferred substrates for ubiquitination (Dudek et al., 2005; Shang et al., 2011). Thus

it is not surprising that the levels of polyubiquitinated protein conjugates were elevated in both *w1118* and *mth*. It has been reported that mild oxidative stress increased the levels of ubiquitinated protein in some cells (Shang et al., 1997; Zhang et al., 2008). Whereas the 26S proteasome degrades the ubiquitinated substrates, induced proteasome activity in *mth* under stress could degrade more ubiquitinated proteins. Indeed, the lower levels of ubiquitinated protein in *mth* than *w1118* under 1% H_2O_2 were observed. Moreover, the higher levels of insoluble ubiquitinated aggregates in *w1118* under stress condition indicate lower efficiency to turnover proteins by proteasomes. Fewer aggregates detected in *mth* suggest that most damaged/misfolded proteins were ubiquitinated for degradation by proteasome so less would form insoluble aggregates. Together, these results further suggest that *mth* maintain better protein homeostasis, which leads to longer lifespan.

Proteasome function is essential for maintenance of muscle and neuromuscular junction architecture (Haas et al., 2007). Locomotion impairment is associated with proteasome dysfunction in neurodegenerative diseases (Bukhatwa et al.; McNaught et al., 2004). Therefore, we compared the locomotor function of flies. Both strains showed a gradual decline in climbing ability as they age. This is consistent with other studies (Toma et al., 2002; Vernace et al., 2007). However, we demonstrate that *mth* exhibits better locomotor ability across ages. These data suggest that *mth* delays the onset of age-related locomotor impairment.

Dietary restriction (DR) is the only intervention that prolongs the lifespan of all animal models such as yeast, roundworms, *Drosophila* and rodents (Piper et al., 2008). DR has been shown to ameliorate proteasome function during the aging process and decrease the damaged/misfolded proteins represented by ubiquitinated proteins and oxidized proteins (Li et al., 2008; da Cunha et al., 2011). This further confirms our hypothesis that maintaining

better protein homeostasis by UPP will contribute to lifespan extension. In addition, decreases in metabolic rate, like decreases in fertility, frequently coincide with lifespan extension. (Sohal et al., 2002). DR has been shown to share this mechanism as well (Phelan et al., 2006; Mair et al., 2008; Piper et al., 2008). These trade-off effects are characteristic of animals that have evolved to survive for long periods in inhospitable environments. In fact, it has been shown that *mth* females have significantly lower early life fecundity and significantly higher late life fecundity compared with *w1118* (Baldal et al., 2006). This trend is very similar to our findings that lower levels of ATP, proteasome activity and level changes at young ages and relatively higher levels at old ages. Early life fecundity is often found to be associated with reduced longevity and late life fecundity with increased longevity (Phelan et al., 2003). Another trade-off effect is that mutations in the *mth* gene reduce neurotransmitter release by ~50%, and decrease the synaptic area and the density of docked and clustered vesicles (Song et al., 2002). There is no direct evidence whether the reduction of synaptic activity in larval motor neurons has bearing on extending lifespan in the adult fly. However, the trade-off effects would be expected to increase lifetime resource utilization, rather than triggering a re-allocation of resources from metabolic, protein assembling or reproductive activities in order to prolong survival.

Since Mth encodes the secretin-family like receptor of GPCR, the ligand activating Mth could mediate a long-range signaling. Additional components of this pathway must be identified before we can fully understand how this mechanism regulates lifespan and stress resistance. In addition, the autophagosomal/lysosomal pathway is also very important to maintain protein homeostasis. Intriguingly, the autophagic and proteasomal system also communicate with each other (Korolchuk et al., 2010). Inhibition of the proteasome induces autophagy (Zhou et al., 2011). Therefore, the investigation on both of these two pathways will help determine the

detailed mechanisms by which pathways modulate longevity and what role they play during mammalian aging.

Collectively, we are the first to demonstrate how the ubiquitin-proteasome pathway functions in the *Drosophila* longevity mutant *methuselah* under both normal aging and oxidative stress conditions. *mth* exhibits lower ATP level, proteasome activity and level at young ages and relatively higher at old ages than *w1118* during normal aging process. Proteasome activity was induced in *mth* but remained unchanged in *w1118* under oxidative stress conditions. Ubiquitinated conjugates and aggregates are apparently fewer in *mth* during normal aging and under oxidative stress condition. Our study strongly suggests that one of mechanisms involved in lifespan extension may be through modulation of the processes responsible for maintain proper protein structure and function.

2.5. Materials and methods

2.5.1. Fly stocks.

The *mth* mutant and *w1118* parental control stocks were from Bloomington drosophila stock center (http://fly.bio.indiana.edu/). Flies were fed with a standard *Drosophila* medium as described on Bloomington database (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm). Flies were passed to fresh bottles twice a week and maintained in a humidified temperature-controlled environmental room at ~25 ^{o}C and ~60% relative humidity throughout the experiments. Flies were aged by the following steps: collect young flies at 2 days after emptying fly bottles; young flies were mated for 72 hours and male flies were transferred to new bottles; repeat this process every 2 weeks. 9 age groups of male flies: *w1118*-1 week old (1w), *w1118*-3w, *w1118*-5w, *w1118*-7w, *mth*-1w, *mth*-3w, *mth*-5w, *mth*-7w, *mth*-9w were harvested at the same date for experiments.

2.5.2. Fly survival curve

30 flies per vial and 150 flies per trial were monitored for survival. The numbers of dead flies were counted daily and three trials were performed.

2.5.3. H_2O_2 administration

Adult 1 day-old adult flies were collected, mated for 1day and then starved for 12 hours in empty vials (diameter 24 mm, length 93 mm) with filter paper pre-wetted with water. Following starvation, flies were transferred to new vials with fly food containing 0%, 0.1%, 1%, 2.5% hydrogen peroxide (H_2O_2) (Sigma H1009). The vials were changed twice a week. Dead flies

were counted for survival. To assess UPP function, flies treated with 1% H_2O_2 and 0% H_2O_2 were harvested at 5 days after stress treatment.

2.5.4. In gel proteasome activity and detection

Proteasome activity is assessed in lysates of whole fly extracts by using a synthetic peptide substrate linked to a fluorometric reporter, which is cleaved by the chymotrypsin-like activity of 20S proteasome dβ5 subunit (Glickman et al., 1998; Vernace et al., 2007). Whole fly extracts (30 flies per group) were homogenized on ice in Buffer A [50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM ATP, 1 mM DTT and 10% glycerol], which preserves 26S proteasome assembly, with a teflon pestle for microcentrifuge tubes (80 up and down strokes), followed by a 15-min centrifugation at 19,000 × g at 4°C. The supernatants were used for the determination of protein concentration and enzymatic activity. The supernatants were normalized for protein concentration determined with the Bradford assay (Bio-Rad, 5000006). Cleared supernatants were resolved on a non-denaturing three-step gradient gel (from bottom to top: 5%, 4% and 3%). 90 μg protein per sample was loaded onto the three-step gradient gel. The non-denaturing mini gels were run at 125V for 3 hours at 4 ^{o}C, followed by incubation for 5 to 30 mins at room temperature with 15 ml of 0.4 mM Suc-LLVY-AMC in buffer A (buffer A modified to contain 1mM ATP and 1mM DTT) to detect proteasome activity. Proteasome bands were visualized by exposure to UV light (360 nm) and were photographed with a NIKON Cool pix 8700 camera with a 3-4219 fluorescent green filter (Peca Producs, Inc.). Semiquantitative analysis of the bands corresponding to proteasome activity was performed by Image-J analysis.

For western blot analysis, proteins on the native gels were transferred at 110 mA for 2 hours onto PVDF membranes (Millipore, IPVH 00010). Our anti-dβ5 affinity purified antibody (1:4,000; BioSynthesis, College Station, TX, USA) was used to detect the 26S and 20S

proteasomes. Antigens were visualized by a chemiluminescent horseradish peroxidase standard method with the ECL reagent after incubation with the secondary antibody. Equal protein loading was established with an aliquot of the same samples following 10% SDS-PAGE and immunoblotting with the anti-actin antibody (1:3000, Sigma, A2066).

2.5.5. Western blot analysis of fly extracts

30 flies were harvested in 150 µl homogenization buffer [20 mM Tris-HCl, pH 7.5, 137 mM sodium chloride, 1 mM EGTA, 10% glycerol, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate ($Na_4P_2O_7$), 50 mM sodium fluoride (NaF), 1% Nonidet P-40 (NP40)/ Igepal (Sigma, I3021), and protease inhibitor cocktail (Sigma, P8340)]. Flies were homogenized on ice with a Teflon pestle (80 up and down strokes). Following a brief sonication and centrifugation (19,000 × g, 15 mins at 4°C), cleared supernatants were transferred to new microcentrifuge tubes and protein concentration was determined with the bicinchoninic acid (BCA) (Thermo Scientific, 23227). Following boiling at 95°C for 5 mins, 90 µg protein/sample were mixed with an equal volume with 2 × Laemmli buffer and loaded onto a 12% SDS gel. The dβ5 protein level was determined with our own anti-dβ5 antibody (1:4000, generated by Bio-Synthesis, TX). Anti-ubiquitinated protein antibody (1:1500, Dako, Z0458) was used to determine the level of ubiquitinated proteins. Equal protein loading per well was demonstrated by probing immunoblots with anti-actin antibodies (1:3000, Sigma, A2066). Following incubation with the secondary antibody, protein levels were detected by standard chemiluminescent horseradish peroxidase method with ECL reagent.

2.5.6. ATP measurements

Steady state ATP content in flies was assessed with an ATP determination kit using the sensitive luciferin/luciferase system (Invitrogen-Molecular Probes, Carlsbad, CA). This assay is based on the fact that luciferase requires ATP for light production using luciferin as a substrate. The nine age groups of flies (15 per group) were harvested at the same date on ice with a teflon pestle for microcentrifuge tubes (80 up and down strokes) in 75 ul of 4% TCA for denaturizing endogenous ATPases, followed by centrifugation (19,000 × g, 15 mins at 4 °C). ATP steady state levels were determined in cleared supernatants upon diluting the samples with 10M Tris-HCl, pH 8.0. Samples were then mixed with luciferin and assayed using a Luminoskan Ascent Microplate Luminometer (Thermo Electron Corporation, Waltham, MA). Protein concentration was determined with the bicinchoninic acid assay kit (Pierce, Rockford., IL) after neutralizing total fly lysates with 1M Tris, pH 10.3. Similar protocols were previously used to measure ATP levels in *Drosophila* (Schwarze et al., 1998).

2.5.7. Locomotor activity

The locomotor activity of flies was determined by using a climbing assay (negative geotaxis assay) (Vernace et al., 2007). 30 flies per age group were anesthetized and placed in a 100 ml graduated cylinder (length, 23.5cm; diameter, 3cm) marked with a line at 12.5 cm. After a 15 min recovery from CO_2 exposure, flies were gently tapped to the bottom of the graduated cylinder. The numbers of flies that could climb above the 12.5 cm line on the graduated cylinder after 20 seconds of climbing were calculated. Three trials were performed for each age group.

2.5.8. Filter trap assay

30 flies of each group were homogenized on the same date on ice in harvesting buffer [10 mM Tris EDTA, pH7.5, 1% SDS]. Following boiling at 95° for 5 min, the fly lysates were sonicated brifely and centrifuged (19,000 × g, 15 min at 4°C). Clear supernatants were collected. Protein concentration was determined in duplicates with the BCA Protein Assay (Thermo Scientific, 23227). An aliquot of each sample was normalized to final concentration (0.5 μg/μl) by adding normalize buffer [10 mM Tris EDTA, pH7.5, 2% SDS]. 100 μl of each sample was vacuum-filtered through a 96-well dot blot apparatus (Minifold, Schleicher & Schuell SRC-96/0) containing a 0.2 μm nitrocellulose membrane (Biorad, 162-0097) pre-wetted with normalizing buffer. Each well was washed two times with buffer containing [10mM Tris EDTA, pH7.5, 0.1% SDS]. The resultant membrane was blocked in 5% nonfat dry milk dissolved in blocking buffer (10 mM Tris-HCl, pH 7.3, 100 mM NaCl, and 0.1% Tween 20) for 30 min at 37°C , and then incubated overnight with an antibody for detection of ubiquitinated proteins (1:1500 dilution, Dako, Z0458). Following incubation with the secondary antibody, antigens were detected with the standard chemiluminescent horseradish peroxidase method with ECL reagent.

2.5.9. Statistical analyses

Data are expressed as the mean ± sem of at least three experiments. Statistical significance was assessed by one-way analysis of variance (ANOVA) with Graphpad software. * p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001.

CHAPTER III

EFFECT OF AGING AND DIETARY RESTRICTION ON PROTEASOME FUNCTION IN *DROSOPHILA*

3.1. Abstract

Dietary restriction (DR) is the only intervention known to reliably increase longevity in a variety of organisms including mammals and *Drosophila*. Currently it is not clear what effects aging and DR have on proteasome-mediated protein degradation. We are the first time to address the effects of DR on the ubiquitin-proteasome pathway (UPP) in *Drosophila melanogaster*. We report that all three fly strains, wild type *Oregon R* (*OR*), *w1118* and longevity mutant *methuselah* (*mth)* responded to DR with extended lifespan. In the *OR* strain, DR significantly reduced proteasome activity in male and female flies at young ages and ATP level at all age groups in male flies. These data indicate higher proteasome activity and ATP level do not always lead to longer lifespan. In addition, DR significantly reduced aging-related impairment of proteasome-mediated protein degradation, and ameliorated aging-related increases in ubiquitinated protein in the wild type *w1118* flies. However, DR reduced proteasome activity in *mth* and caused a slight increase in ubiquitinated protein conjugates at young and old ages. Furthermore, there was no change in the expression of proteasome subunit dβ5 in both *w1118* and *mth* under DR. Our data indicate that DR has many beneficial effects towards the UPP in wild type *w1118*, and that preservation of the UPP may be a potential mechanism by which DR extends lifespan in *w1118*. But in the longevity mutant *mth*, DR failed to ameliorate proteasome function, suggesting that other mechanisms might be responsible for the longer lifespan caused by DR. Cumulatively, these data have implications for understanding the effects of aging and DR on protein turnover and the mechanism of lifespan extension in longevity mutant *mth*.

3.2. Introduction

Aging is associated with dysfunction of protein turnover (Martinez-Vicente et al., 2005). The ubiquitin-proteasome pathway (UPP) is a proteolytic system responsible for the majority of intracellular protein degradation (Finley, 2009). Compromises in the proteasome proteolytic pathway are known to occur during the aging of many animal species (Jana, 2012). Dietary restriction (DR) is currently the only intervention known to consistently and reliably slow the aging process in a variety of organisms, including mammals, flies, and nematodes (Flatt, 2009). Some studies have shown that DR is known to oppose the effects of aging on a variety of organ systems including hepatic, cardiovascular, immune, and central nervous system, leading to longer lifespan (Yu, 1996; Pahlavani, 2004; Mattson et al., 2005; Maalouf et al., 2009). However, the cellular and biochemical basis for each of these beneficial effects of DR is still not clear. In *Drosophila*, dilution of their normal diet consisting of yeast, sugar or other components leads to extension of lifespan. Insulin signaling, TOR, AMPK and Sirtuins all remain intriguing candidates for conserved regulators of DR longevity in metazoans including *Drosophila*. In this study, we examined the effects of both aging and DR on the function of UPP in three different *Drosophila* strains: two wild type strains, *Oregon R* and *w1118*, and one longevity mutant *methuselah (mth)*, which encodes one of secretin-like G-protein coupled receptors. These data demonstrate that aging and DR modulate the levels of multiple components of the ubiquitin-proteasome system including the levels of proteasome activity, proteasome amounts, subunits, and ubiquitinated proteins. Our studies contribute to the clarification of the beneficial effects of DR in protein degradation during the aging process and the role of UPP in the lifespan.

3.3. Results and Discussion

3.3.1. Assess lifespan extension via dietary restriction in *Drosophila melanogaster OR* strain

There are several protocols to modulate DR (Bass et al., 2007; Skorupa et al., 2008; Grandison et al., 2009; Wang et al., 2009). The protocol from Skorupa's group manipulates the composition of the food by varying the levels of sucrose and yeast independently in a 5×5 factorial design (Skorupa et al., 2008). The lifespan, triglyceride (fat) levels, protein levels, and feeding rates of flies maintained in each of 25 distinct nutritional regimes were fully accessed in Skorupa's study. In our study, we chose Skorupa's food recipe to modulate fly lifespan. To avoid developmental effects we reared all experimental larvae in a cornmeal-sugar-yeast media (Table 2) that was optimized for larval mobility. Following development, once-mated, adult wild type flies *OR* were transferred to one of the two adult-specific food regimes: unrestricted food (ad libitum, AL) and dietary restriction food (DR) (Table 3). The number of flies that died was counted every other day (Fig. 17). The longest lifespan of flies under AL food was ~ 36 days and under the DR was ~ 58 days. The lifespan was extended ~61%. This result confirmed that DR extends lifespan in *Drosophila* OR strain.

3.3.2. Assess the impact of DR on proteasome function and ATP level in *Drosophila OR* strain

Different age of *OR* flies maintained in both food diets, 1 week (w), 3 w, 5 w and 7 w (DR only) were harvested at the same date for analysis. The chymotrypsin-like proteasome activity was measured by the in-gel assay and ATP level with an ATP determination kit. Fig. 18 compares the proteasome activity of flies under AL and DR food. Since 95% of 5w old flies under AL died, it was hard to harvest enough flies for the assays. Thus the 5w-AL sample is

missing. Fig. 18A shows that in 3w male flies under DR food, the activities of both two caps and one cap 26 S proteasome was ~ 10-15% lower than under AL; in 1w male flies there was no significant difference between the two food diets. Under DR the proteasome activity showed an aging-dependent decline (~20%). At 7w, the two caps and one cap proteasome activity decrease by ~ 20%. Fig. 18b compares the proteasome activity 1w and 3w females maintained under the two food diets. In flies under DR, the activity of the 26S proteasome activity (including two cap and one cap) was about 50-60% lower than in flies under AL. These data showed that DR reduces the proteasome activity in 3w flies compared to AL in both male and female. Since we do not have the data for the proteasome activity at 7w under AL, we do not know if the proteasome activity under AL declined or not at 7w, and if DR attenuates the age relative changes in proteasome activity at advanced age. However, these data suggested that lower proteasome activity does not always lead to shorter lifespan.

Since proteasome function is ATP-dependent, the ATP stead state level was measured. The ATP level in flies under DR displayed an aging-relative decline (Fig.19). At 7w, the ATP level under DR was reduced by ~ 50%. Surprisingly, the average ATP level under AL increased with aging. Moreover, the average ATP level under DR was lower than AL at each age group (~15% at 1w, ~ 20% at 3w and ~ 40% at 5w). These data indicate that flies reared under richer food (AL) exhibited higher level of ATP than under diluted food (DR). Other studies showed similar results that DR reduces ATP levels in cells. Forty percent DR caused a remarkable decrease in ATP levels in erythrocytes in a mouse study (Hishinuma et al., 1990). Due to a 1.5 × enriched diet, increased ATP levels within the motor neurons could cause the activation of ATP-sensitive potassium channels leading to a reduction in excitability of the motor neuron (Ma et al., 2007). How does lower ATP level lead to longer lifespan? A recent study showed that overexpressing

mitochondria uncoupling protein-2 (ucp2) in *C. elegans* extended the median lifespan of worms by about 40% and also lowered ATP level (Sagi et al., 2012). Furthermore, DR alone extended median lifespan of the worms by 18%, *ucp2* alone extended lifespan by 40%, and dietary restriction of *ucp2* worms extended lifespan 40% compared to controls. Thus, DR compared to normal diet did not further extend the lifespan of *ucp2* worms. Based on these data, we hypothesize that the lower ATP levels in *ucp2* transgenic worms might extend lifespan via mechanisms shared by dietary restriction. Ucp2 encodes one of the mitochondrial uncoupling proteins. Its function is mitochondrial uncoupling, which allows protons to leak into mitochondria without producing ATP. According to the "uncoupling to survive" hypothesis, mitochondrial proton leakage may be beneficial because reduction of the proton motive force should reduce the production of reactive oxygen species and thereby reduce macromolecular damage during aging (Brand, 2000). Therefore, lower ATP level in DR flies might benefit from less damage from ROS leading to longer lifespan.

3.3.3. Both *w1118* and *mth* strains exhibit extended lifespan when fed DR food.

Next we compared the impact of DR and aging on wild type *w1118* and longevity mutant *mth*. After eclosion in larvae media and mating in mating media for 2 days, wild type *w1118* and longevity mutant *mth* male flies were maintained in DR and AL food. Dead flies were counted every other day. The median lifespan of *w1118* was 23 days under AL and 27 days under DR (Fig. 20). The median lifespan of *mth* was 32 days under AL and 36 days under DR. The lifespan was extended by about 17.4% in *w1118* and 12.5% in *mth*. *mth* survived longer than *w1118* under both AL and DR diets.

3.3.4. Impact of aging and DR on proteasome function in *w1118* and *mth.*

To assess the effect of aging and DR on proteasome function in *w1118* and *mth* flies, we compared the chymotrypsin-like proteasome activity of flies at young (Y, 1week) and old (O, 3.5 weeks) ages (Fig. 21A). Whole flies were homogenized and the cleared supernatants loaded onto the same gel for the in-gel assay described under Materials and Methods. Proteasome activity displayed aging-dependent decline in both strains and under both food diets. At old age, the proteasome activity decreased ~30% in *w1118* under AL, ~6% in *w1118* under DR, ~30% in *mth* under AL, and ~50% in *mth* under DR. These data indicate that the aging-dependent decline in proteasome activity is conserved under both AL and DR diets. When proteasome activity was compared between DR and AL, there were differences between the two strains. In wild type *w1118*, there was no significant difference in the activity between AL and DR at young age, but at old age, DR displays ~30% higher activity than AL. However in *mth*, the proteasome activity under DR was lower than AL at both young (~15%) and old age (~45%). These results suggest that DR attenuates the aging-dependent decline in proteasome activity at old age in *w1118*, compared with AL. However, no beneficial effects to prevent the decline in proteasome activity were detected in *mth* under DR at 1w and 3.5w ages. In conclusion, maintaining proteasome activity at old age might be one of mechanisms to extend lifespan in *w1118* under DR food. In addition, other mechanisms besides proteasome activity are involved in the lifespan extension caused by DR in *mth*.

Proteasome levels detected with our peptide generated anti-dβ5 specific antibody (BioSynthesis) following the in-gel assay are shown in Fig. 21B. The 20S proteasome level in *mth* is apparently less than in *w1118* under both food diets. When compared with AL, the 20S

proteasome levels were reduced under DR in *w1118* at both young and old ages, however they were increased in *mth*.

Next, we assessed the expression of proteasome subunits using the dβ5 antibody. Fig. 22 shows no significant difference in dβ5 protein level due to aging, food diet, or strain. It appears that the basis for the decline in proteasome function in the aging *w1118* and *mth* may be mediated in part by the disassembly of 26S and 20S proteasome complex but not by a decline in the amount of 20S proteasome dβ5 subunit. The ability of DR to preserve 20S proteasome peptidase activities in *w1118* appears not to be mediated by an increase of proteasome subunits either. Increase of assembled proteasome complexes and more efficient proteasome catalytic properties could be possible reasons. The proteasome maintains intracellular protein homeostasis usually by degrading damaged and misfolded proteins which usually are ubiquitinated. No elevation of proteasome activity in *mth* at both ages under DR as compared with AL questions if there are less damaged proteins for degradation. Therefore we next assessed the levels of poly-ubiquitintated protein conjugates by western blotting.

3.3.5. Impact of aging and DR on ubiquitinated protein conjugates in *w1118* and *mth*.

Fig. 22 shows that there was a significant elevation (~40-60%) in the levels of the polyubiquitinated protein conjugates at old age in both strains under both foods as compared to young age. This suggests that the age dependent increase in polyubiquitinated protein conjugates is conserved. In addition, *w1118* exhibited less ubiquitinated proteins under DR when compared with AL at both young and old ages. However, *mth* displayed slightly more ubiquitinated proteins under DR at both ages. These data suggest that DR significantly attenuates the observed aging-dependent increase in ubiquitinated-protein levels in wild type *w1118*. Similar results have

been reported in studies with liver, brain and heart of aging rats (Li et al., 2008; Dasuri et al., 2009). Elevations of ubiquitinated protein are believed to represent the accumulation of aberrant proteins, which may directly or indirectly contribute to deleterious alterations in cell homeostasis (Grune, 2000). Therefore, our data suggest that in wild type *w1118*, DR ameliorates aging-dependent alterations in the proteome in part via the preservation of proteasome-mediated protein degradation. However, DR does not attenuate aging-dependent increases of ubiquitinated proteins in the longevity mutant *mth*. The lifespan extension caused by DR in *mth* might be involved in some other mechanisms.

In summary, the data in the present study demonstrate for the first time the effects of aging and DR on proteasome-mediated protein degradation in *Drosophila melanogaster*. All three strains, wild type *OR*, *w1118* and longevity mutant *mth* respond to DR with extend lifespan. DR ameliorates aging dependent alterations in the proteome in part via the preservation of proteasome-mediated protein degradation in *w1118*, which leads to longer lifespan. However there are no such effects on *mth*. Our previous study in chapter two showed that *mth* maintains proteasome function at old ages during normal aging and oxidative stress conditions, when fed with normal white food. Taken together with the current DR data, we propose that DR could not further increase the capacity of the ubiquitin-protesome pathway in *mth*, which suggests that other mechanisms are responsible for longer lifespan caused by DR in *mth*.

3.4. Materials and methods

3.4.1. Fly stocks.

OR, *w1118* and *mth* stocks were bought from Bloomington drosophila stock center (http://fly.bio.indiana.edu/). Flies were maintained in cornmeal-sugar-yeast "larval" media (Table 1) for at least two generations prior to experimentation. Adult flies were collected within 24 hours of emergence and placed on "mating food" for 48 hours to mate. Male flies were then collected under light CO_2 anesthesia and transferred into single-sex bottles containing the experimental food (DR and AL) (Table 2). Flies were passed to fresh bottles twice a week and maintained in a humidified temperature-controlled environmental room at 25°C and 60% relative humidity throughout the experiments.

3.4.2. Dietary restriction recipe

Treatment medium

Water, agar, sucrose and brewer's yeast (MP Biomedicals, Irvine, CA, USA) were combined in a large Erlenmeyer flask and mixed well (Table 2). Ingredients were autoclaved with sterilization set for 30 mins at 121°C, cooled to the appropriated temperature, and tegosept 20% (w/v) in ethanol and propionic acid were added. Antibiotics (50 g/L kanamycin, 20 g/L tetracycline, Sigma-Aldrich) were added to the media to prevent bacterial growth. See Table 2 for ingredient quantities.

Larva and Mating medium

In a large kettle, water (1) and agar (Table 1) were combined and mixed well, and then slowly boiled for 15 mins. Water (2), yeast, sucrose, dextrose (MP Biomedicals), and cornmeal

(SYSCO Corp.) were combined in a separate container and mixed well. The yeast/sugar/cornmeal mixture was added to the agar and the mixing speed was increased. We ensured that the food boiled for 15 mins. The heat was turned off and food cooled to 65°C before adding tegosept, propionic acid and antibiotics. See Table 1 for the detailed ingredient quantities.

3.4.3. In gel proteasome activity and detection/

3.4.4. Western blot analysis of fly extracts/

3.4.5. ATP measurements

Please see “Materials and Methods” in Chapter II.

CHAPTER IV

CONCLUSION AND FUTURE GOALS

Age-dependent decline of proteasome activities has been shown in a variety of mammalian tissues. However, the role of the UPP during aging is elusive. In this thesis, we compared the UPP function across different ages between two *Drosophila* strains with different length of lifespan, the wild type strain *w1118* and longevity mutant *mth,* which was previously identified in a genetic screening for lifespan mutantation (Lin, 1998). We found that during the natural aging process, *mth* does not display higher but relative lower proteasome activity and proteasome level when compared with *w1118* at young ages although it has a longer lifespan. At old ages it displayed relatively stable proteasome activity and level, while *w1118* displayed a dramatical decline of proteasome activity and level during aging process. These data indicate higher levels of proteasome activity and level do not always lead to longer lifespan. The proteasome function is ATP-dependent. Intriguingly, the ATP steady level showed the similar trend as proteasome activity that *mth* displayed lower ATP at young ages but maintained the ATP level at old ages than *w1118.* Moreover, the levels of ubiquitinated protein conjugates and aggregates were apparent lower in *mth* than *w1118* in all age groups. These data suggest that there is lower protein turnover, and/or there are less damaged proteins in *mth* and therefore less would be marked by ubiquitination for degradation by proteasome. Less ATP would be needed for proteasome function and ubiquitination, thus less ATP would be produced. Consequently, less ROS were generated and less damaged were produced, which contribute to a better protein homeostasis. Together, these data indicate that during natural aging *mth* is able to maintain their protein structure and function better than *w1118*, thereby reducing demand for removal of damaged and misfolded proteins, consequently less proteasome were needed at young ages. At old ages, *mth* maintains the proteasome function in order to cope with the increased protein damages.

Age is also associated with the increase of excessive ROS which damages almost all cellular components including protein, DNA, lipids and carbohydrates, and causes dysfunction of cell, tissues and organisms. Our stress experiments showed that *mth* survived longer than *w1118* under all low-dose oxidative stress treatments (0%, 0.1% and 1%). Moreover, the proteasome activity was induced in *mth*, but remained nearly unchanged in *w1118* after 5 days 1% H_2O_2 treatment. The stress induced ubiquitinated protein conjugates and aggregates were diminished in *mth.* These data suggest that *mth* is more resistant to ROS than *w1118*, which could be due to that *mth* displays less protein damages and more efficient protein turnover by proteasome. Together, our data from natural aging and stress experiments strongly imply that *mth* exhibits a more dynamic reponse to the normal aging process and oxidative stress condition, which could be one option to delay aging and cause lifespan extension. This further confirmed that *mth* maintains better protein homeostasis, which leads to longer lifespan.

In addition, we modulated *Drosophila* lifespan, using a non-genetic approach by dietary restriction (DR). We also compared the UPP function in *Drosophila* strains with different life expectancy caused by DR. We tested three strains, the wild type *OR*, wild type *w1118* and the longevity mutant *mth*, which all responded to DR with extended lifespan. In the *OR* strain, DR significantly reduced proteasome activity in male and female flies at young ages (old age not tested) and ATP level at all age groups in male flies. These data support our previous statement that higher proteasome activity and ATP level do not always lead to longer lifespan. In the wild type *w1118*, DR reduced age-related impairment of proteasome-mediated protein degradation, and ameliorated age-related increases in ubiquitinated protein. However, DR reduced proteasome activity in *mth* and caused a slight increase in ubiquitinated protein conjugates at young and old ages. Furthermore, there was no change in the expression of proteasome subunit dβ5 in both

w1118 and *mth* under DR. These data indicate that DR has many beneficial effects towards the UPP in wild type *w1118*, and that preservation of the UPP may be a potential mechanism by which DR extends lifespan in *w1118*. But in the longevity mutant *mth*, DR failed to ameliorate proteasome function, suggesting that other mechanisms might be responsible for the longer lifespan caused by DR.

Cumulatively, our studies demonstrated the UPP function across different ages in an *in vivo* model *Drosphila*. We conclude that aging is associated with significant decline in proteasome activity and level, and increase in protein damages represented by ubiquitinated proteins and aggregates. However, this decline is not associated with the expression of proteasome individual α and β subunits since they appear almost unchanged, but with the disassembling of 26S and 20S proteasome complex besides dissociation of 20S and 19S.

Moreover, we are the first to evalute the protein degradation mediated by proteasome in longevity mutant *mth.* Our data strongly imply that *mth* displayed higher proteasome plasticity and less protein damages represented by ubiquitinated proteins and aggregates during natual aging process and under stress condition. We concluded that maintaining a higher proteasome plasticity but not absolutely higher proteasome level would lead to successful aging. Moreover lower ubiquitinated protein during normal aging or under mild stress condition also would be linked to longer lifespan.

Furthermore, we are the first to examine the DR impact on the UPP function in *Drosophila* model. The data imply that higher proteasome activity and ATP level under unrestricted AL food could be linked to shorter lifespan; reducing the age related decline in proteasome function and increase in ubiquitinated proteins by DR will lead to longer lifespan in

wild type *w1118*; there might be some other mechanisms responsible for the longer lifespan caused by DR in *mth*.

Despite showing that elevated proteasome plasticity in *mth* correlates with a longer lifespan, it is not clear which genetic pathway is involved in this elevation. A recent study has shown that EGF signaling activates the UPP to modulate *C. elegans* lifespan (Liu et al., 2011; Rajalingam et al., 2011). They reported that EGF signaling regulates lifespan through the Ras-MARK pathway and the PLZF transcription factors EOR-1 and EOR-2. It activates the ubiquitin–proteasome system (UPS) and represses the chaperone machinery by modulating the expression of several aging-related genes such as SKR-5, HSP16-type chaperones. It would be very interesting to compare those gene expressions and protein levels in *mth* across different ages with *w1118*. Besides the UPP, the autophagosomal/lysosomal pathway is another important degradation pathway which is generally used for the bulk degradation of long-lived proteins. It usually communicates with proteasome (Zhou et al., 2011). When proteasome function is reduced, autophagy will be induced. At young ages, the UPP in *mth* is not fully induced. It would be interesting to test if autophagy takes in charge.

Our stress data showed that *mth* displayed less protein damages caused by ROS. Organisms have developed multiple defense mechanisms to alleviate oxidative damage, such as antioxidant enzymes, molecular chaperones and proteolytic systems. Cellular antioxidant systems provide the primary defense for cells to cope with ROS through directly quenching ROS. The chaperon and UPP provide the second defense. Some studies showed that when the UPP was impaired, many antioxidant enzymes are upregulated due to the accumulation of the nuclear factor-E2-relatived factor 2 (Nrf2) (Sekhar et al., 2002). It would be interesting to examine the antioxidant enzymes levels and Nrf2 pathway in *mth.*

Mitochondria consume over 90% of the cell oxygen and produce a large amount of ROS. Does *mth* produce less ROS than *w1118*? I isolated mitochondria from *mth* and *w1118* and tested O_2 consumption using a clasic Clark electrod. However there is no difference in the O_2 consumptions between *mth* and *w1118*. Does ATP synthesis change? Sun has been identified as the ligand for the Methuselah receptor (Cvejic et al., 2004), which encodes mitochondrial ATP synthase ε-subunits in *Drosophila*. Loss of zygotic sun expression leads ultimately to death. Embryos lacking maternally supplied sun (sun embryos) cause a six-fold reduction in ATP synthase activity but not complete elimination, which indicates ε-subunit is required for maximal efficiency of ATP synthase. It would be very interesting to exam the mitochondria function such as the ATP synthesis, mitochondrial ETC complex activities and UCP expressions.

Another major question is still unanswered: where is *mth* expressed? It has been shown that Mth function is presynaptically required at NMJs in *Drosophila* (Song et al., 2002). However, Song reported that expression of Mth protein in motor neurons could not abolish the lifespan extension, which indicates Mth's function in aging may be outside the nervous system. Therefore, clarifying this expression pattern of *mth* will contribute to the mechanism of *mth* in lifespan extension and the GPCR in aging.

CHAPTER V

FIGURES

Figure 1

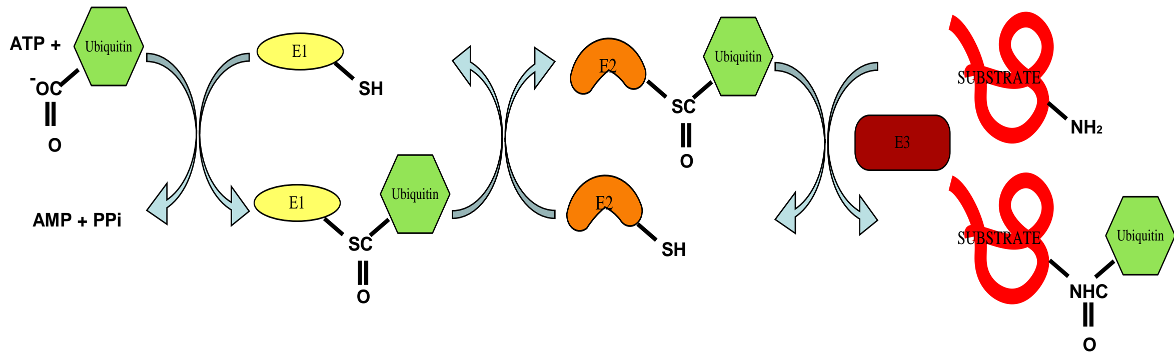


Figure 1. The ubiquitin-conjugation pathway. E1, ubiquitin activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase. The thiol ester and amide bonds involved are shown.

Figure 2

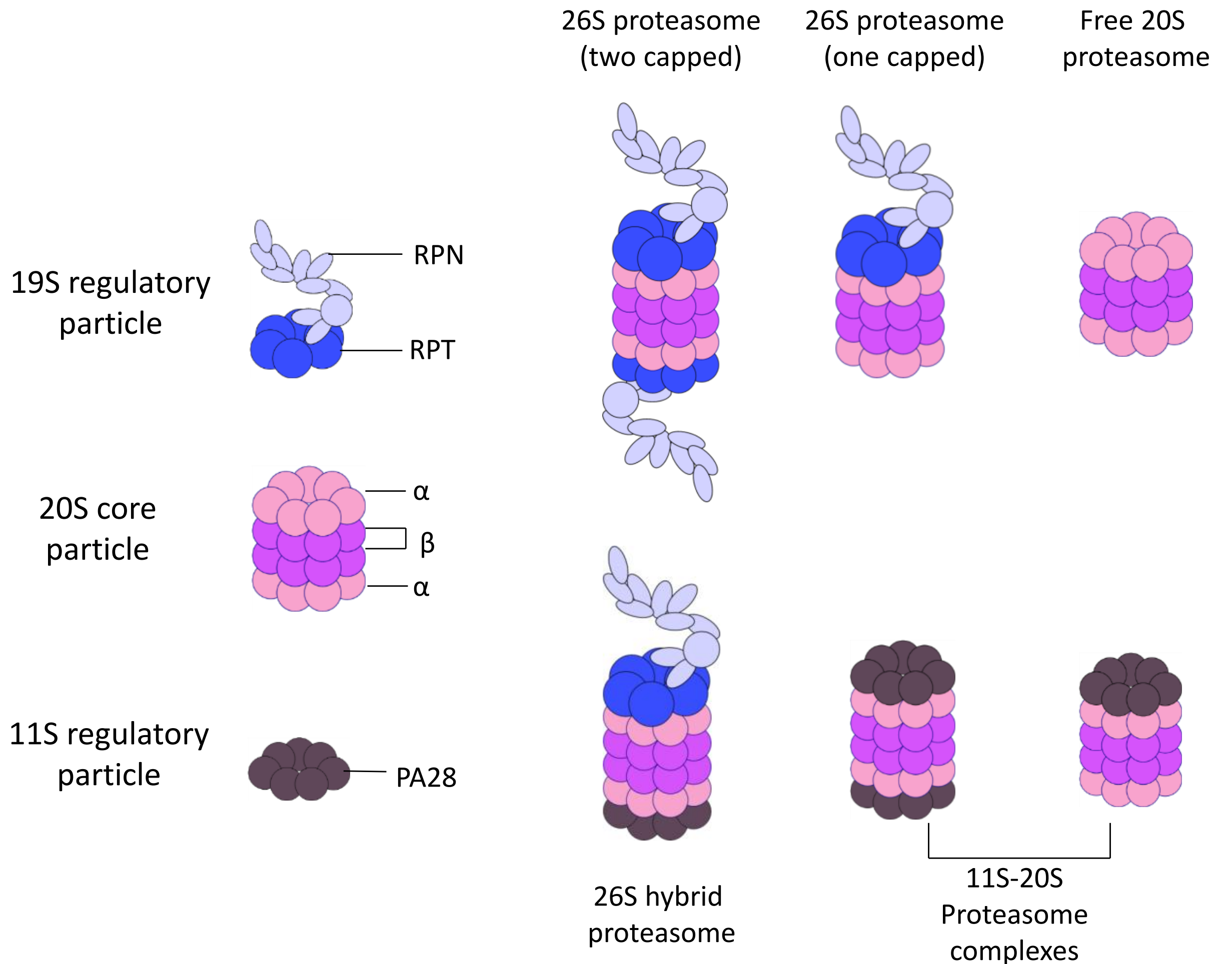


Figure 2. Proteasome structure and heterogeneity. The proteasome is a large, multisubunit protease complex including a core particle and regulatory particle(s). The 20S core complex is composed of four heptameric rings of α1–7 and β1–7 subunits. The19S regulatory complex, or PA700, contains a hexameric ring of AAA-ATPase subunits (RPT1–6) and ~12 non-ATPase (RPN) subunits. Another complex that regulates the 20S complex, called 11S, or PA28, is a heptameric ring of PA28 subunits and is highly abundant in mammalian cells. The mix and match of 20S complexes, 19S complexes and 11S complexes gives rise to a variety of proteasomes.

Figure 3

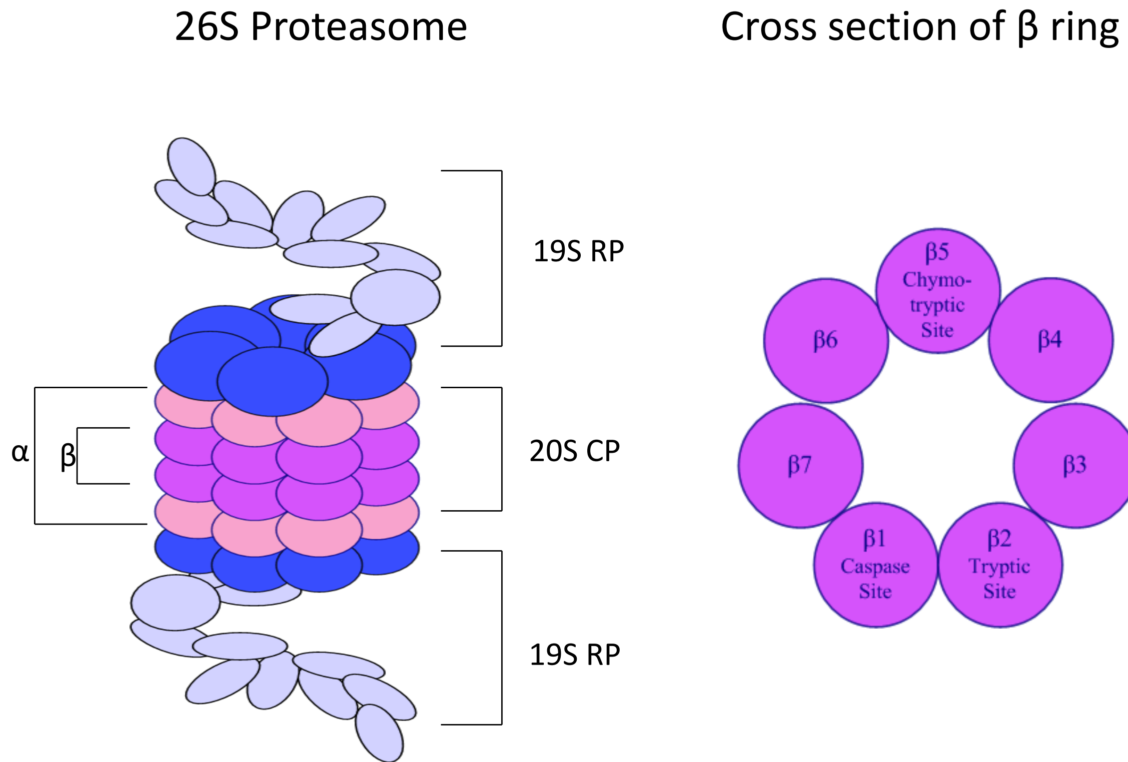


Figure 3. Catalytically active sites of the 20S proteasome. The 26S proteasome is composed of two distinct complexes: the 20S core particle (CP) and the regulatory particle (RP). The 20S CP is formed by the axial stacking of four rings made up of two outer α-rings and two inner β-rings (left). The three major proteolytic activities of 20S proteasomes are the three subunits β1 , β2 and β5 which have caspase-like, trypsin-like and chymotrypsin-like activities, respectively (right).

Figure 4

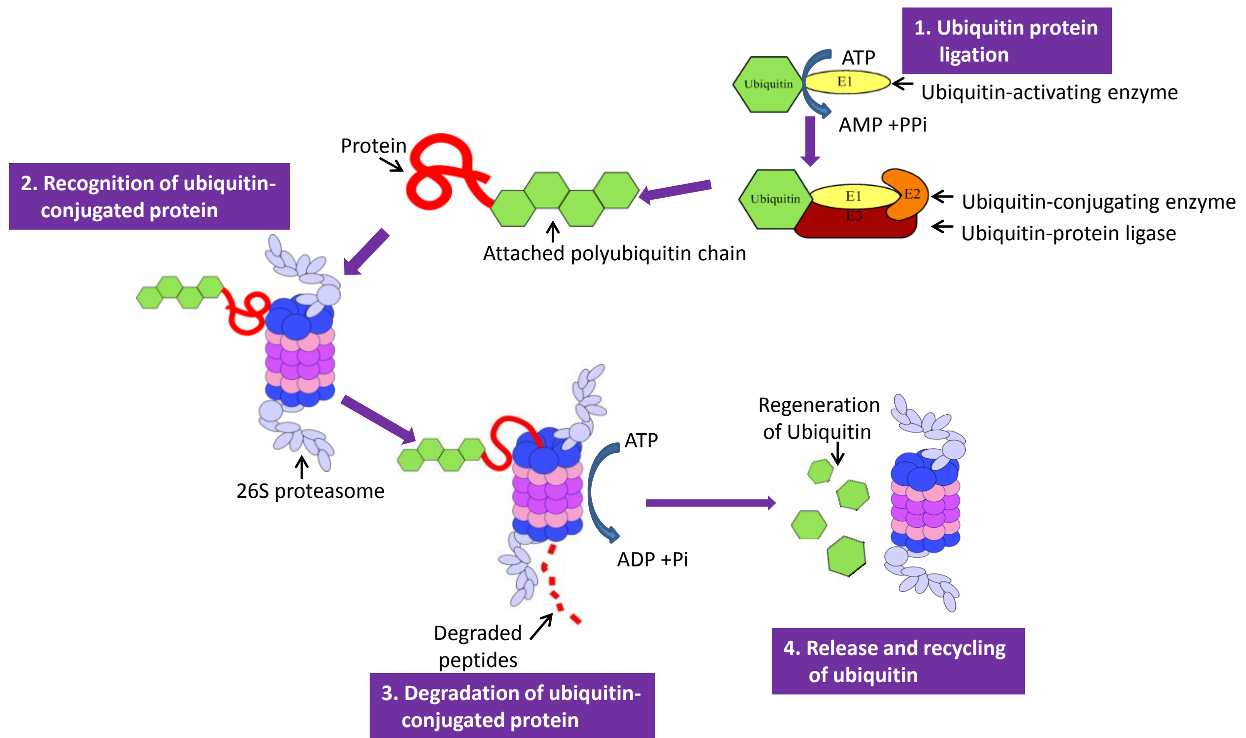


Figure 4. Ubiquitin-proteasome pathway. 1). Proteins are tagged for degradation by covalently linked ubiquitin. Ubiquitin molecules are linked by an enzymatic cascade. E1, the ubiquitin activating enzyme activates ubiquitin by a reaction that requires ATP to form a thioester bond. The ubiquitin-conjugating enzyme E2 uses the thioester bond to conjugate activated ubiquitin to the target protein. E3, a specific ubiquitin ligase helps E2 to transfer the activated ubiquitin to a lysine residue of the substrate protein. 2). A long polyubiquitin chain attached to the substrate protein is generated destined for degradation by the 26S proteasome after repeating the process several times. A protein subunit in the 19S cap of the proteasome acts as a receptor for the polyubiquitin chain. 3). The substrate translocates through a (presumptive) pore in the base subcomplex. Translocation of the substrate through the pore is driven by ATP hydrolysis. The substrate is hydrolysed, which produces short peptides. 4). Deubiquitinating enzymes disassemble polyubiquitin chains for recycle.

Figure 5

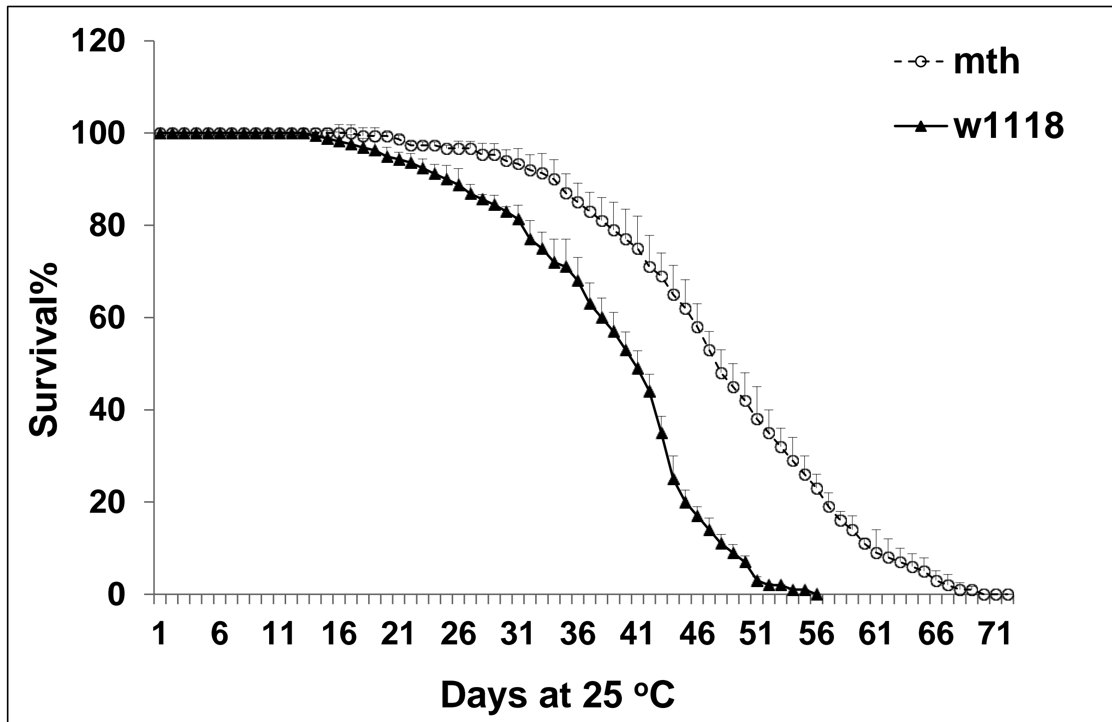
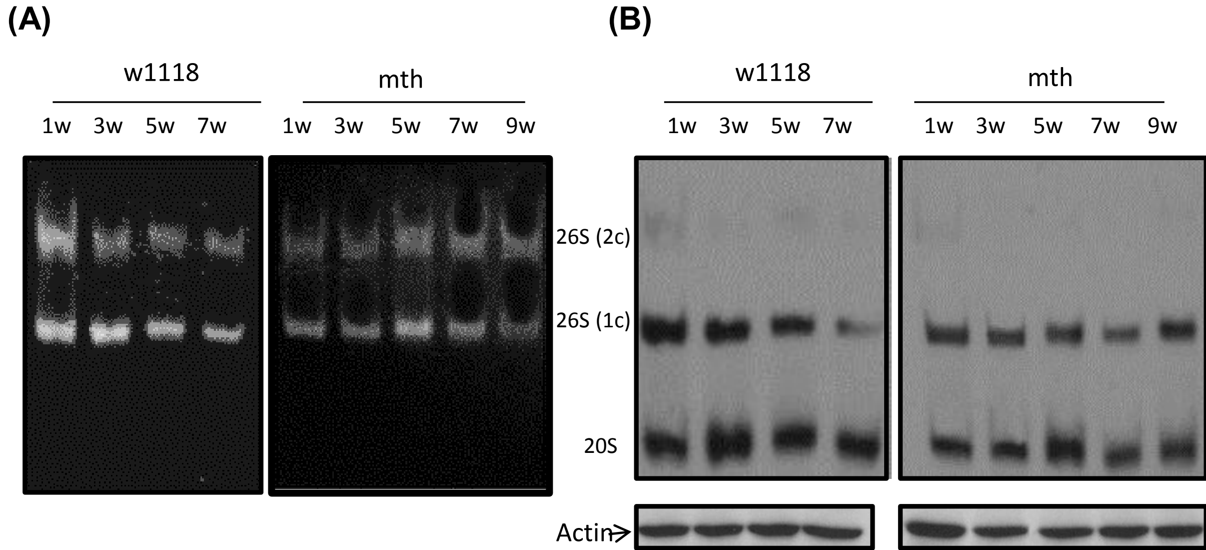
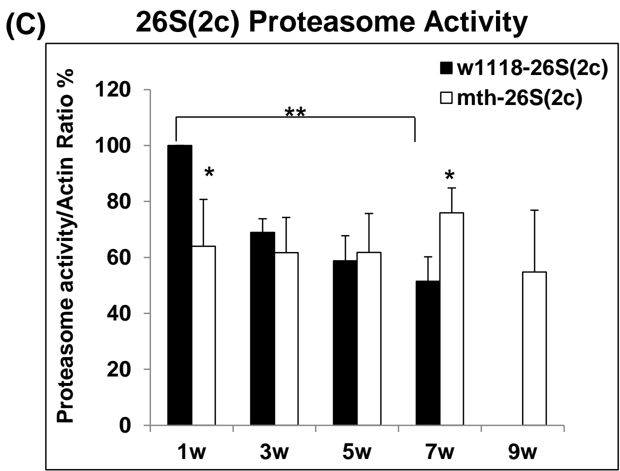
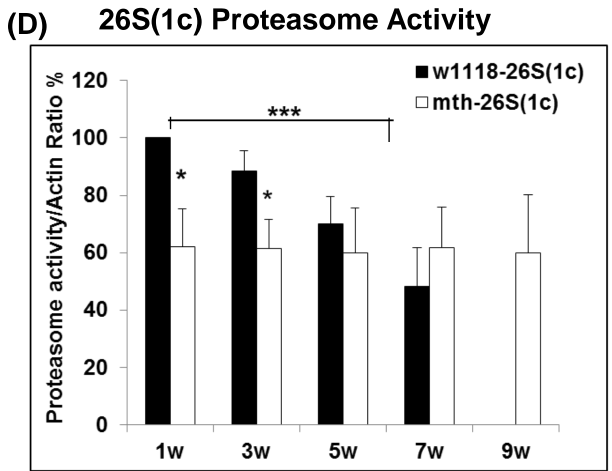


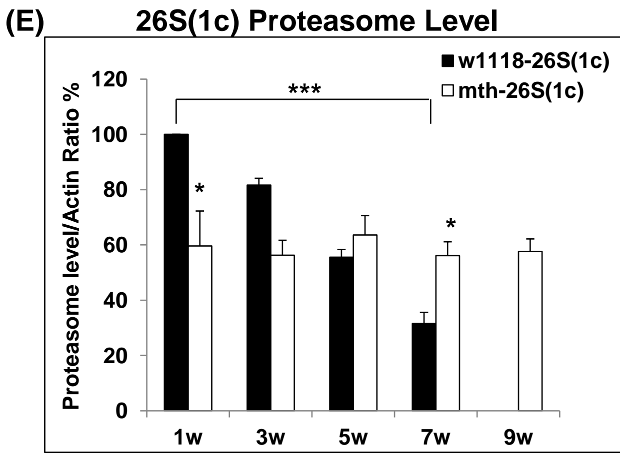
Fig 5. Life-span extension in longevity mutant *methuselah*. Male Flies of *white1118* and *methuselah* were maintained at a constant temperature (25°C), humidity (~60%), and 12/12 hour dark/light cycle environment. Flies were transferred to fresh food vials and dead flies were for survival percentage every 2 days. The average life-spans for *w1118* and *mth* were 41 and 48 days, respectively. The numbers of flies tested were 713 for *w1118* and 623 for *mth*.

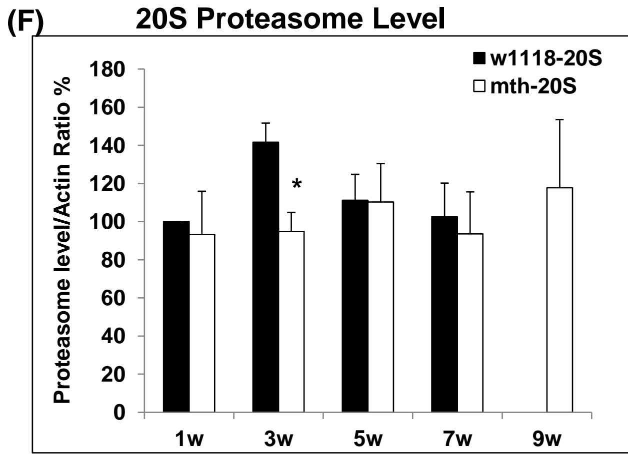
Figure 6

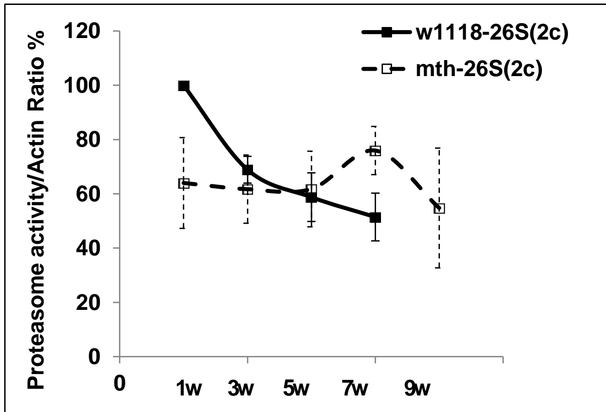


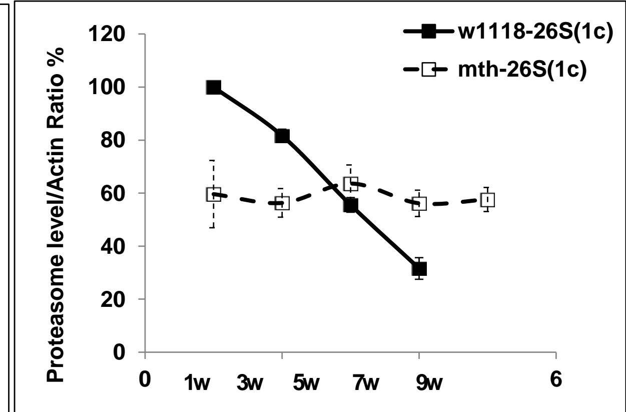


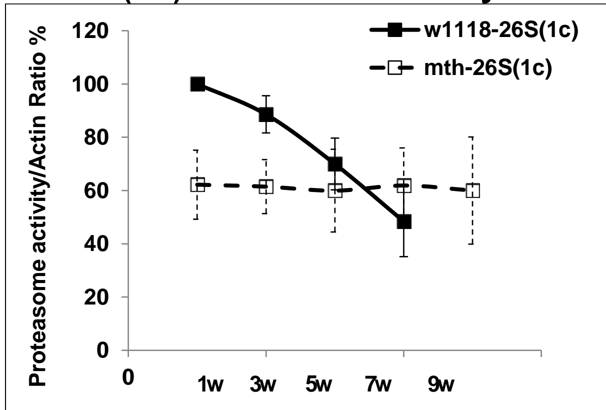












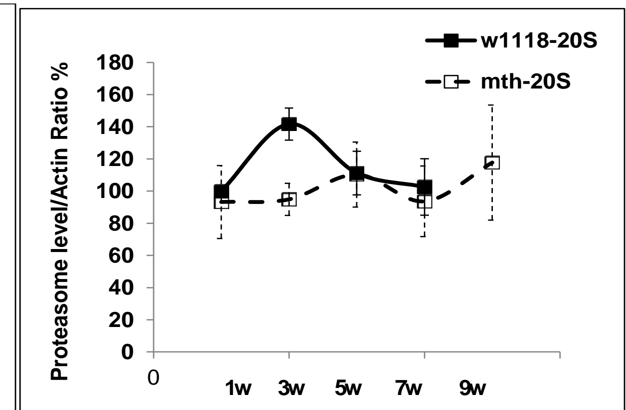


Figure 6. Proteasome activity and levels in *mth* mutant and *w1118* control male flies across different ages. (A). 26S proteasome activity in *w1118* control male flies declined with aging; 26S proteasome activity in *methuselah* mutant flies were maintained with aging. Male flies of different age (1, 3, 5, 7 and 9 weeks) were analyzed with the in-gel proteasome assay. The chymotrypsin-like activity was assessed with the substrate Suc-LLVY-AMC. 90μg of protein per lane were loaded. The 26S (single and double cap) and 20S core particle are indicated on the right. (C)/(E) and (G)/(I). Activity bands were semi-quantified by densitometry, and plotted with bar graphs and line graphs. (B). 26S proteasome level declined with aging in *w1118* control male flies, however it was maintained in *mth* mutant male flies. 26S and 20S proteasomes were detected by immunobloting with our antibody that reacts with dβ5 subunit of the core proteasome particle. As indicated in the middle, this antibody recognizes 26S (2), two caps and 26S (1), one cap, and the 20S core particle. (D)/(F) and (H)/(J). Protein level bands were semi-quantified by densitometry, and plotted with bar graphs and line graphs. Values represent mean ± sem from four trials (30 flies per each sample per each trial)

Figure 7

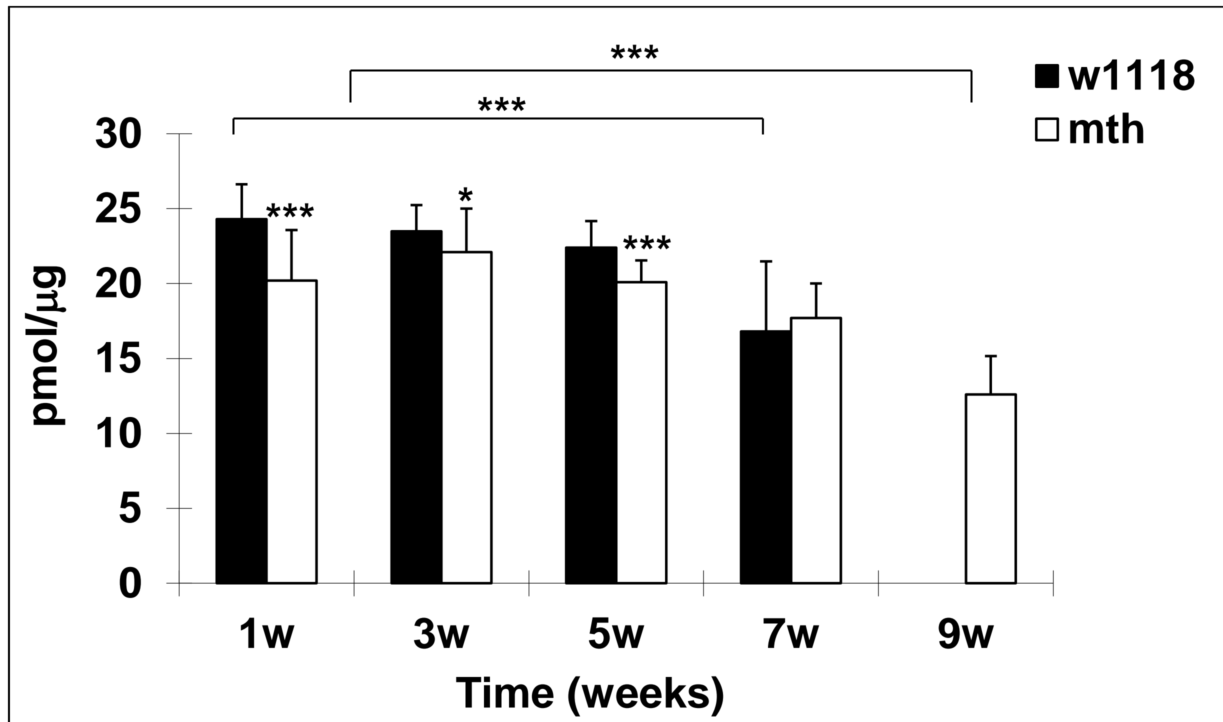


Figure 7. **ATP steady-state levels in mth and w1118 across different ages**. Both of ATP levels of *mth* and *w1118* decreased by about 20%-40% with aging. Separate groups of *mth* mutant (open bars) and *w1118* control (solid bars) of different ages (1, 3, 5, 7 and 9 weeks) were analyzed. ATP concentrations (pmoles/ug of protein) in cleared supernatants were determined with a kit using the sensitive luciferin/luciferase system. Data represent mean ± sem from four trials (15 flies per trial) per age group. (** p value ≤ 0.01)

Figure 8

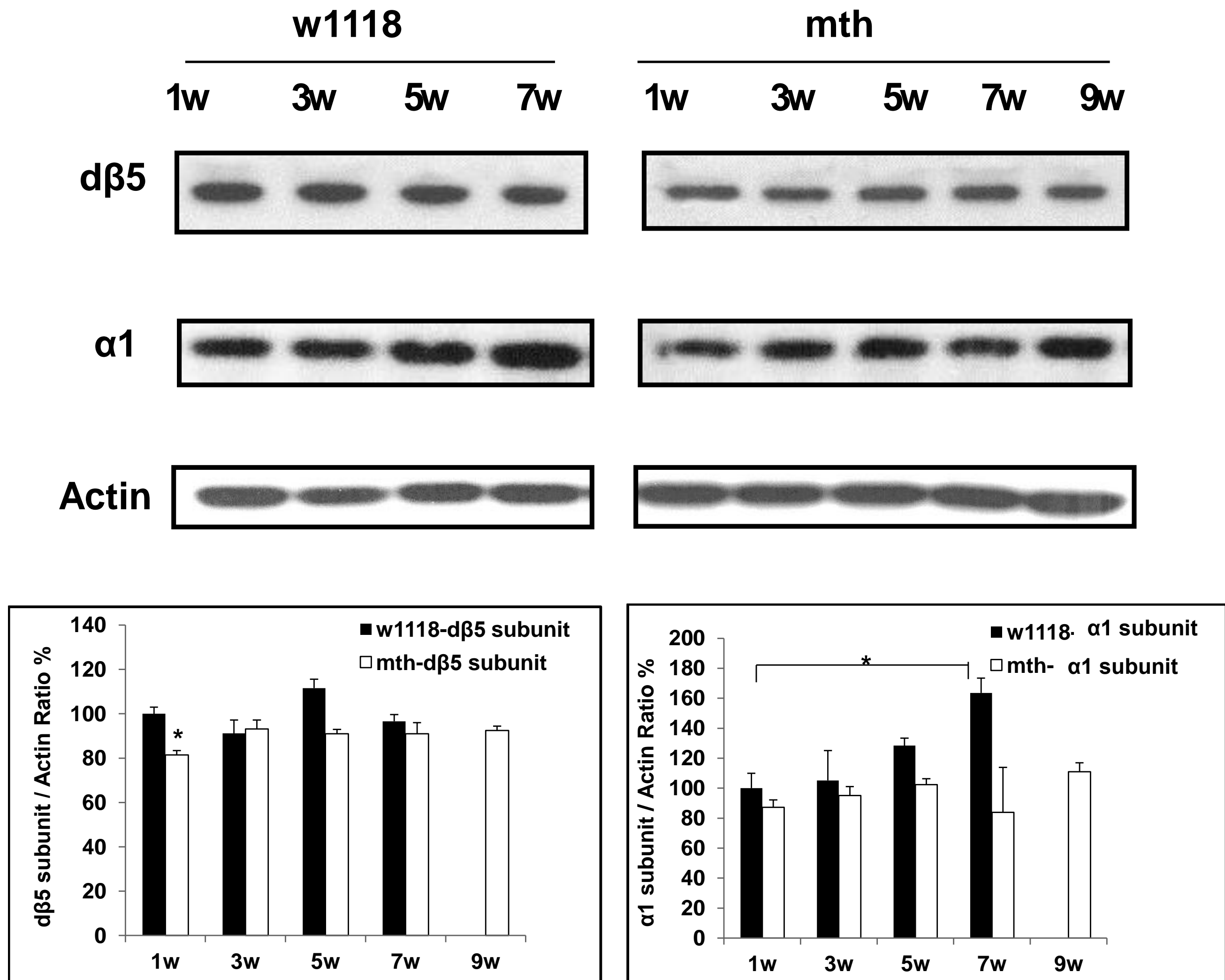
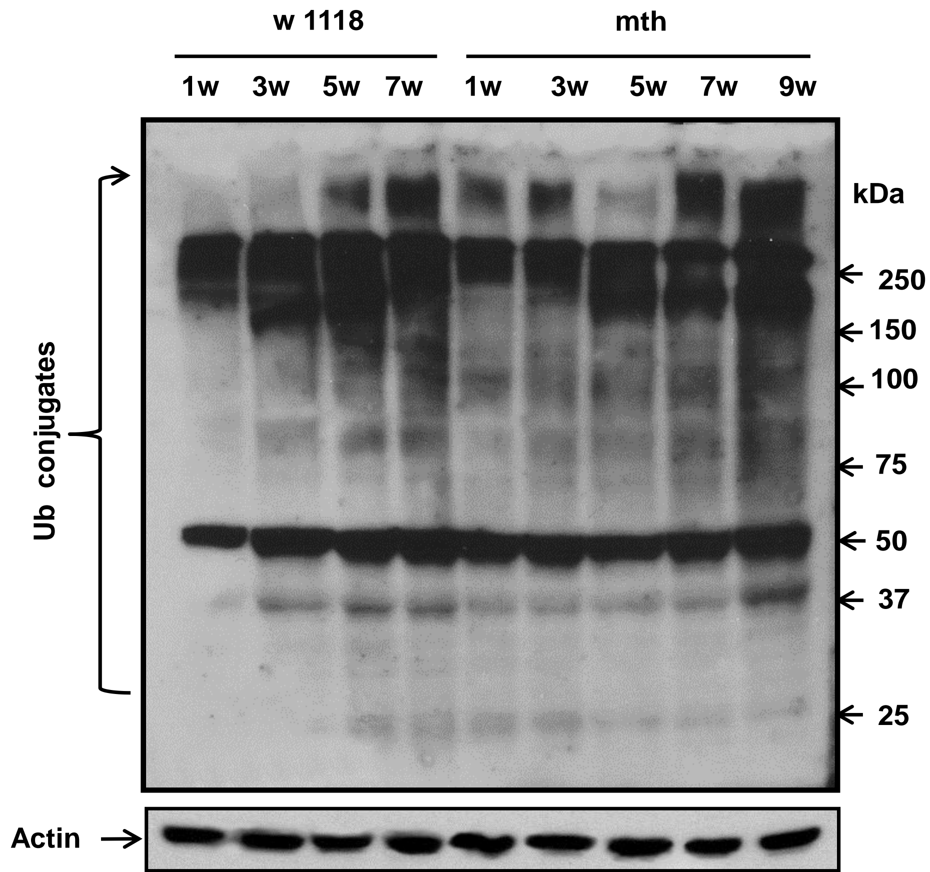


Figure 8. dβ5 and α1 subunit expression in *mth* and *w1118* across different ages. dβ5 subunit protein level did not change as a function of aging in both *mth* and w1118. α1 subunit level increase in *w1118* and was not changed in *mth*. Separate groups of *w1118* and *mth* were analyzed by western blotting (90 μg protein/sample) probed with an anti-dβ5 antibody and anti-α antibody. Equal protein loading was demonstrated by probing the immunoblots with an anti-actin antibody. The level of dβ5/actin was semi-quantified by densitometry (graph: *w1118*, solid bars; *mth*, open bars). Data represent the mean ± sem from 3 experiments). (* p value ≤ 0.05).

Figure 9



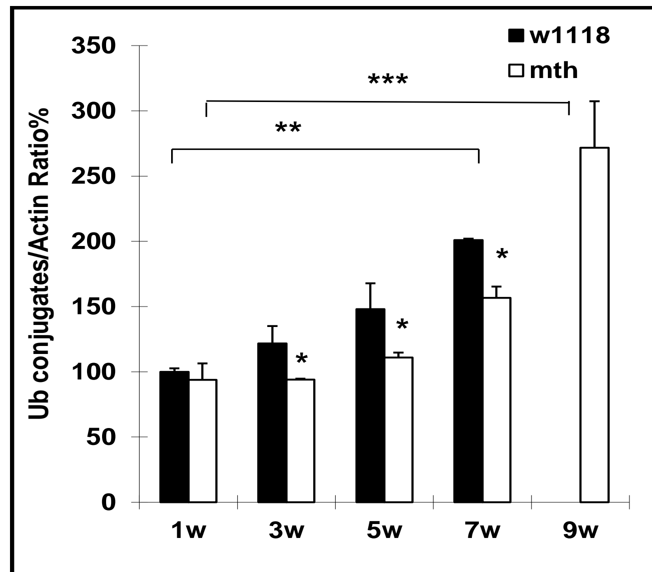


Figure 9. **Polyubiquitinated protein levels in *mth* and *w1118* across different ages.** Polyubiquitinated protein levels in both *mth* and *w1118* increase with aging. In each age group *mth* has less polyubiquitinated proteins than *w1118*. 30 males for each age group were analyzed. The levels of ubiquitinated proteins were determined by western blotting (50 μg protein/sample) probed with an anti-ubiquitin antibody. Equal protein loading was demonstrated by probing immunoblots with an anti-actin antibody. The levels of Ub-conjugates/actin were semi-quantified by densitometry (graph: *w1118*, solid bars; *mth*, open bars). Data represent the mean ± sem from 3 experiments. Protein level bands were semiquantified with ImageJ. (* p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001).

Figure 10

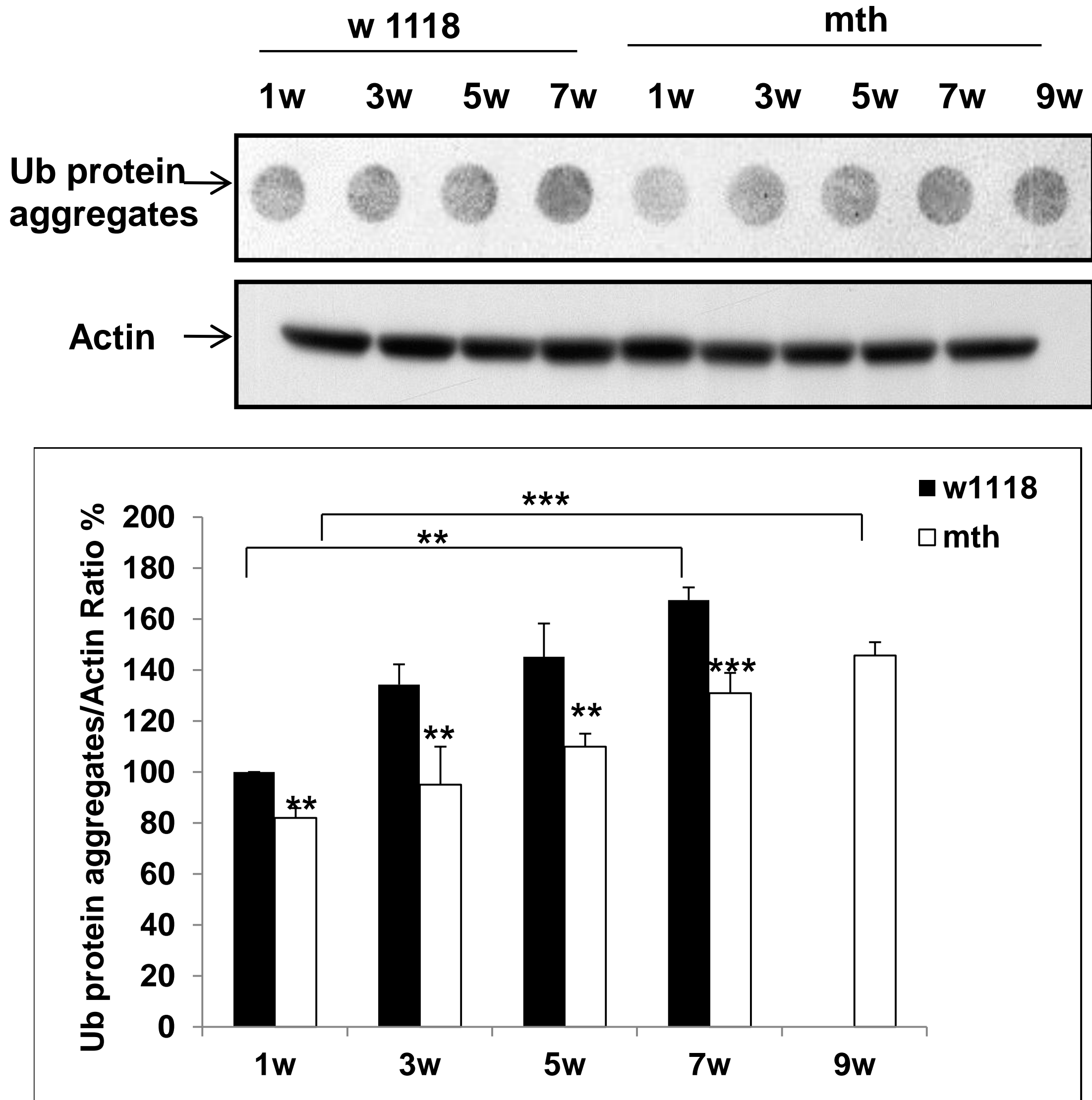


Figure 10. Ubiquitinated protein aggregates in *mth* and *w1118* across different ages. Male flies of different age of *mth* and *w1118* were analyzed for SDS insoluble protein aggregates with the filter trap assay (50 µg of protein/sample) and the anti-ubiquitin antibody. Equal protein loading was demonstrated by SDS-PAGE followed by immunoblot analysis with an anti-actin antibody. Levels of Ub-aggregates/actin were semi-quantified by densitometry (graph: *w1118*, solid bars; *mth*, open bars). Data represent the mean ± sem from 3 experiments. (** p value ≤ 0.01; *** p value ≤ 0.001)

Figure 11

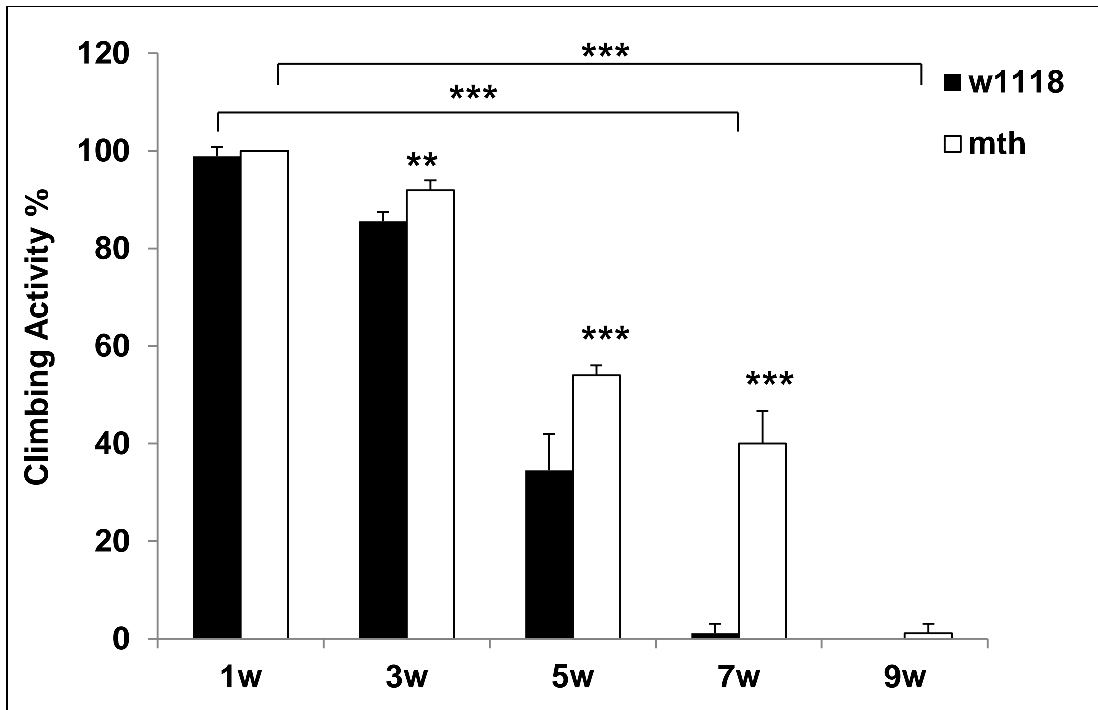
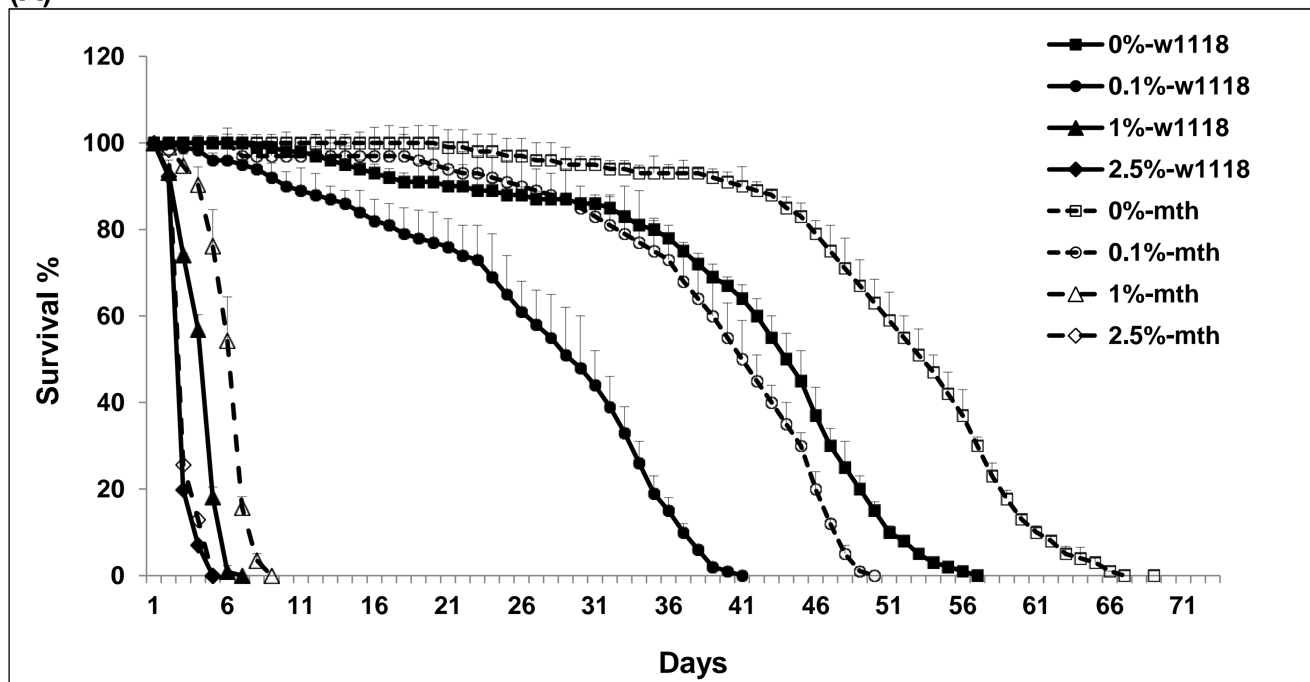


Figure 11. Climbing performance in *mth* and *w1118* across different ages. *Mth* has better climbing ability than *w1118* across different ages**.** Separate groups of *mth* (open bars) and *w1118* (solid bars) of different ages were analyzed. Locomotor performance was assessed with a climbing assay as described in "Materials and Methods". Data represent the mean ± sem from 3 experiments.

Figure 12

(A)



(B)

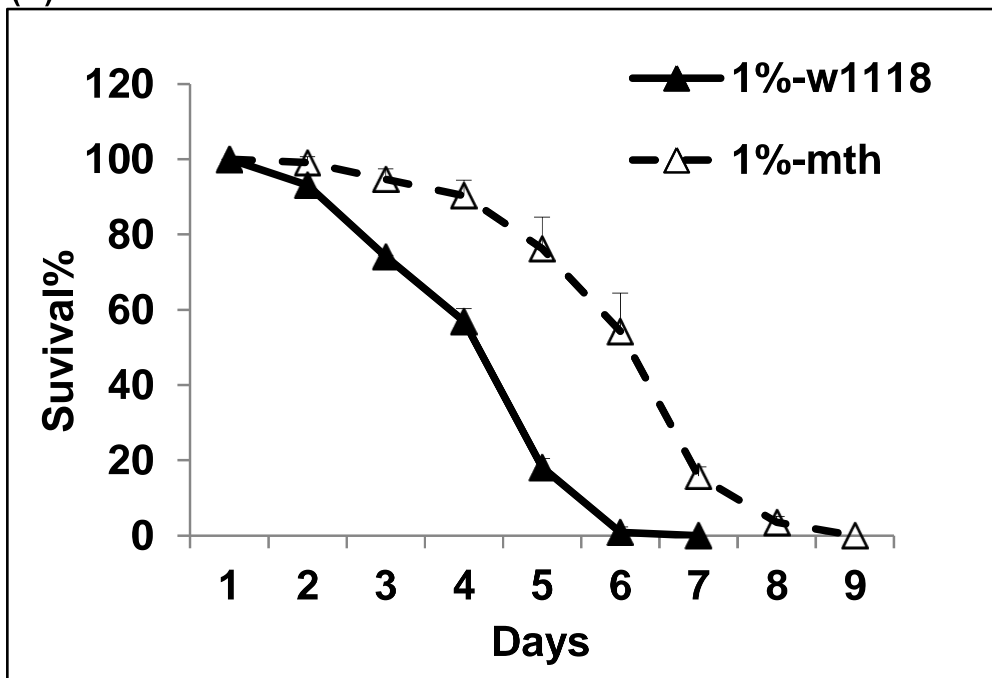
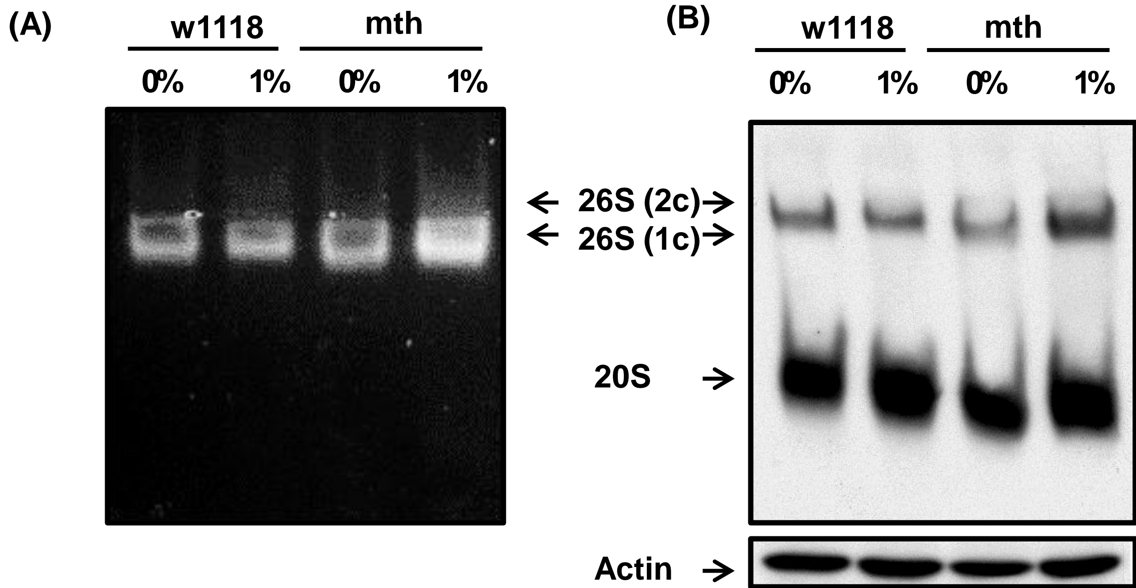
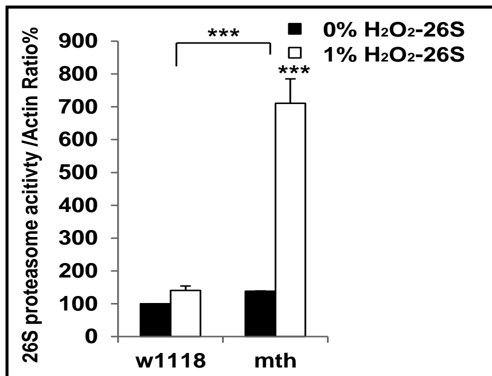
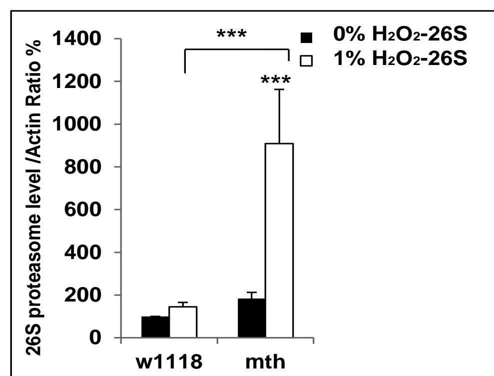


Figure 12. H_2O_2 administration in *mth* and *w1118* in a dose-dependent manner. (A). *Mth* displays enhanced resistance to oxidative stress except under 2.5%. Male 1 day-old *w1118* and *mth* were starved for 12 h and then fed H_2O_2 in varying concentrations (0, 0.1%, 1% and 2,5%) starting at day 0. Flies were transferred to new vials with fresh food twice a week. The percentage of dead flies was calculated daily. Survival curves were generated. (B) represents 1% H_2O_2 survival individually. Data represent the mean ± sem from 3 experiments (n=100 flies for each treatment).

Figure 13







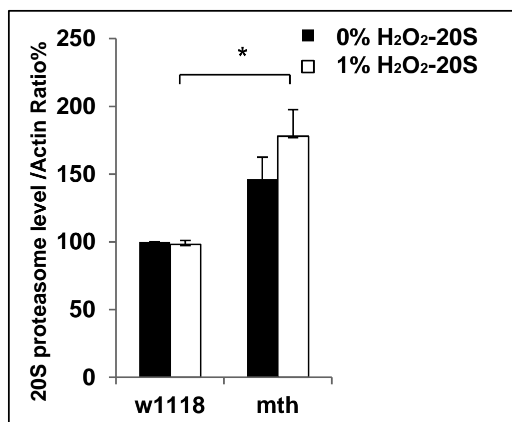
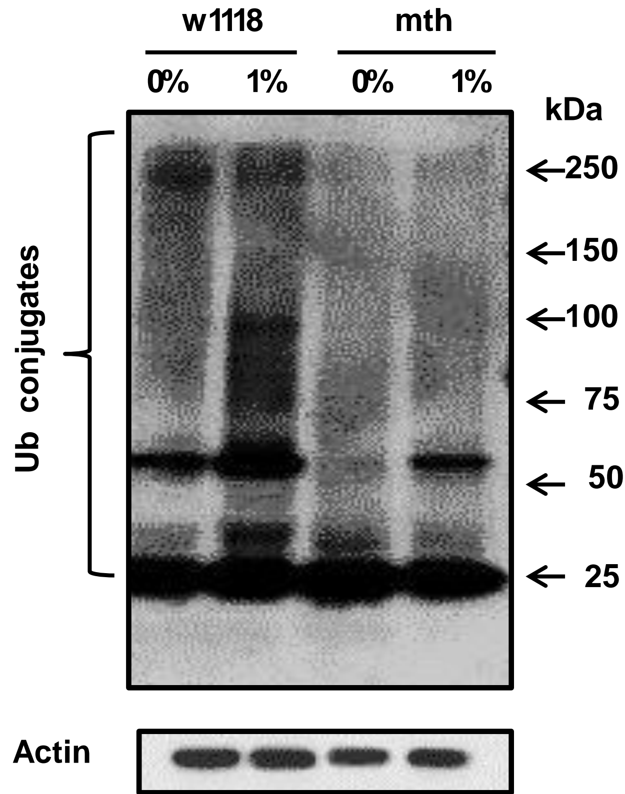


Figure 13. Proteasome activity and levels after a 5 day treatment with 1% H_2O_2. (A). Proteasome activity in *mth* is significantly induced after a 5 day treatment with 1% H_2O_2, however proteasome activity in *w1118* was maintained or reduced. Whole flies were harvested after 5 days H_2O_2 treatment and analyzed by the in-gel proteasome assay. The chymotrypsin-like activity was assessed with the substrate Suc-LLVY-AMC. The 26S (single and double cap) proteasomes and 20S core particle are indicated on the right. Open bars represent 0% H_2O_2 treatment; Solid bars represent 1% H_2O_2 treatment. Three trials were performed. (B) Proteasome levels were detected by western blotting with the anti-dβ5 antibody following the in gel assay. 26S proteasomes of *w1118* were disassembled after 1% H_2O_2 treatment; both 26S and 20S prteasome levels of *mth* were induced. (C), (D) and (E). Protein bands were semi-quantified by densitometry. Data represent the mean ± sem from 3 experiments (n=30 flies for each treatment and each age group). (* p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001).

Figure 14



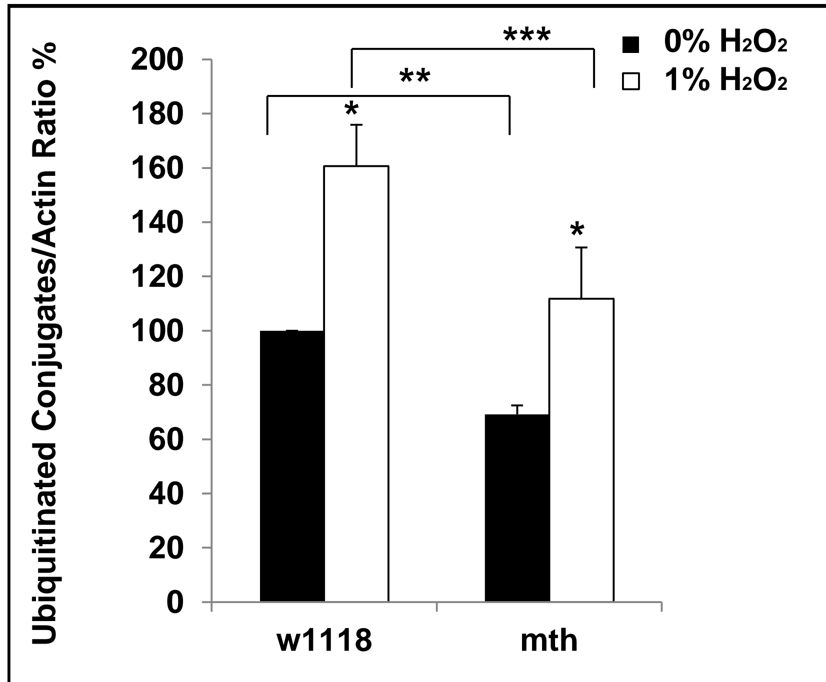


Figure 14. Polyubiquitinated protein conjugates after 1% H_2O_2 treatment. 30 male *mth* and *w1118* flies were harvested after 5 days H_2O_2 treatment and analyzed by western blotting (50 µg protein/sample) probed with anti-polyubiquitin antibody. Equal protein loading was demonstrated by probing immunoblots with an anti-actin antibody. Polyubiquitinated protein conjugates in both *w1118* and *mth* increased after treatment with 1% H_2O_2. Under both 0% and 1% H_2O_2 treatment conditions, *mth* displayed lower level of ubiquitinated-protein conjugates than *w1118*. Three trials were performed for each time point. Protein level bands were semi-quantified with ImageJ. Solid bars represent 0% H_2O_2 treatment; open bars represent 1% H_2O_2 treatment. Data represent the mean ± sem from 3 experiments (n=30 flies for each treatment). (* p value ≤ 0.05).

Figure 15

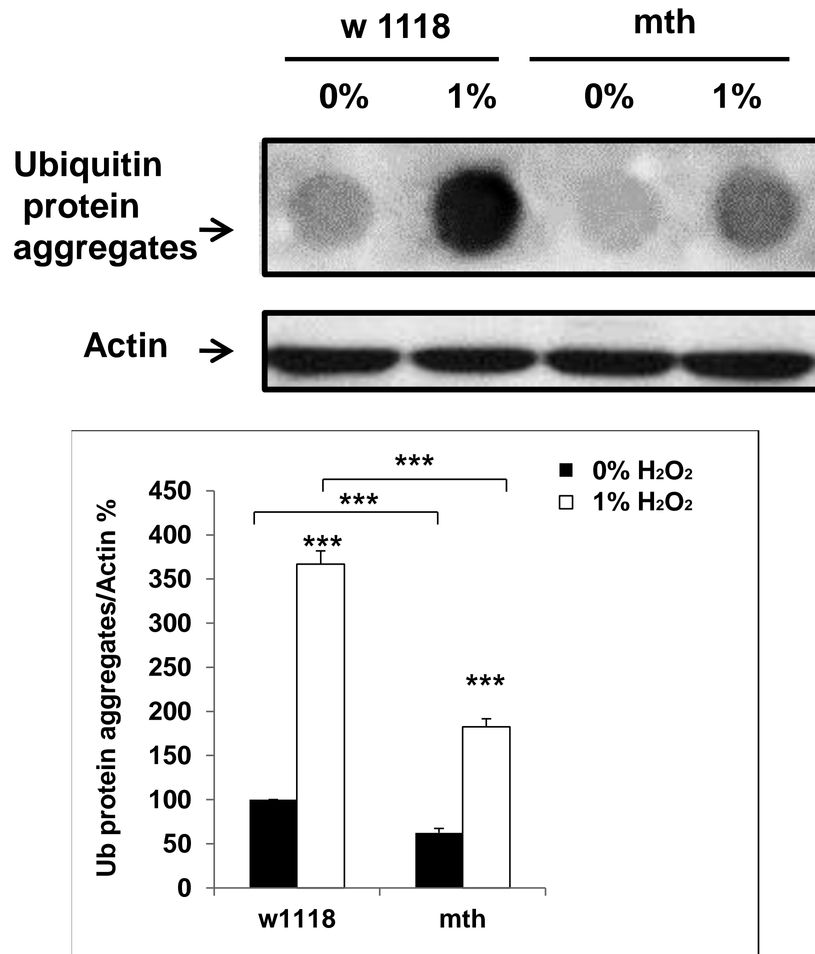


Figure 15. Polyubiquitinated protein aggregates under stress condition (A). 30 male *mth* and *w1118* flies were harvested after 5 days H_2O_2 treatment and analyzed with the filter trap assay followed by western blotting with anti-polyubiquitinated proteins antibody. Equal protein loading was demonstrated by probing the immunoblots with an anti-actin antibody. Under control conditions (0% H_2O_2), *mth* dispalyed lower basal level of ubiquitin protein aggregates than *w1118*; under stress condition (1% H_2O_2), *mth* generated much fewer aggregates than *w1118*. Three trials were performed for each time point. Protein level bands were semi-quantified with ImageJ. Solid bars represent 0% H_2O_2 treatment; open bars represent 1% H_2O_2 treatment. Data represent the mean ± sem from 3 experiments (n=30 flies for each treatment). (* p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001).

Figure 16

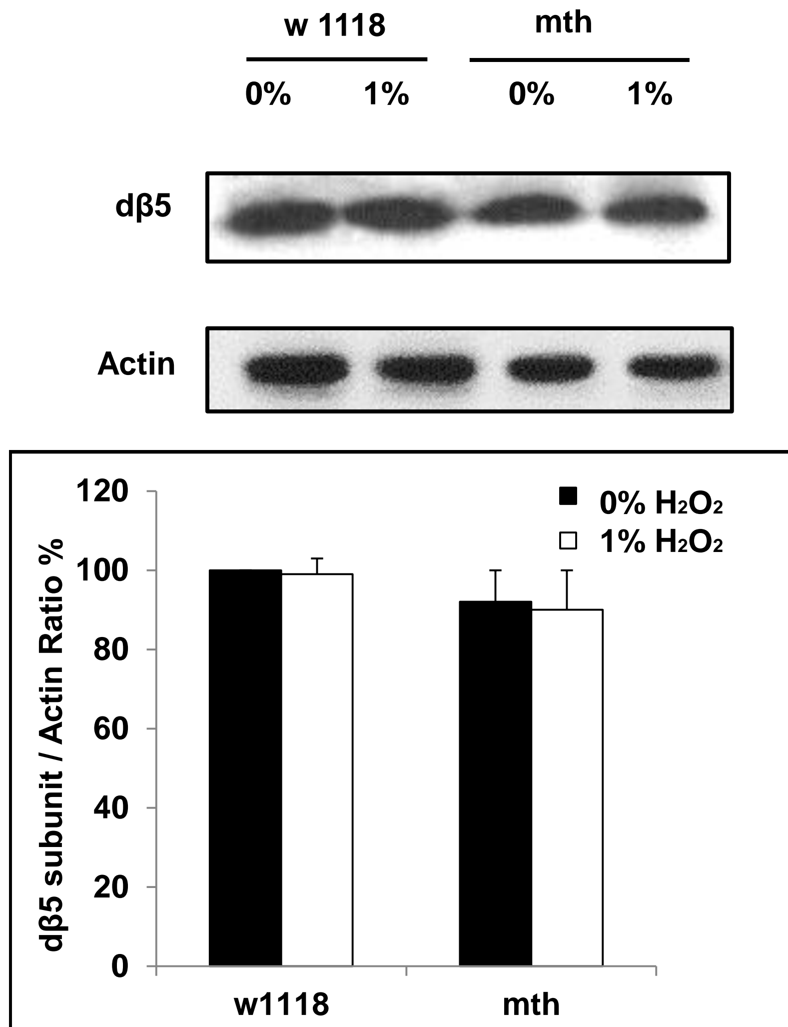


Figure 16. dβ5 subunit level in *mth* and *w1118* under stress conditions. dβ5 subunit protein levels were assessed by western blotting (90 μg protein/sample) probed with an anti-dβ5 antibody. There is no change of dβ5 subunit protein levels in both *mth* and *w1118* after 5 day treatment with % H_2O_2. The level of dβ5/actin was semi-quantified by densitometry (graph: solid bars, 0% H_2O_2; open bars, % H_2O_2). Data represent the mean ± sem from 3 experiments).

Figure 17

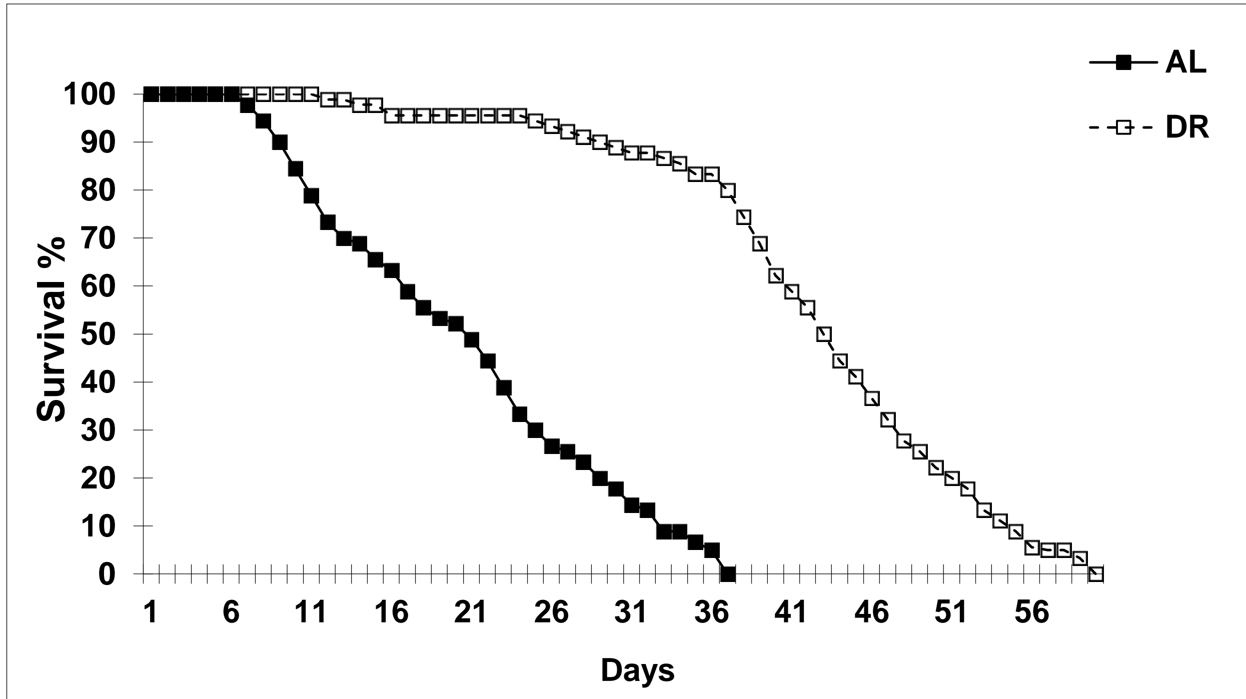
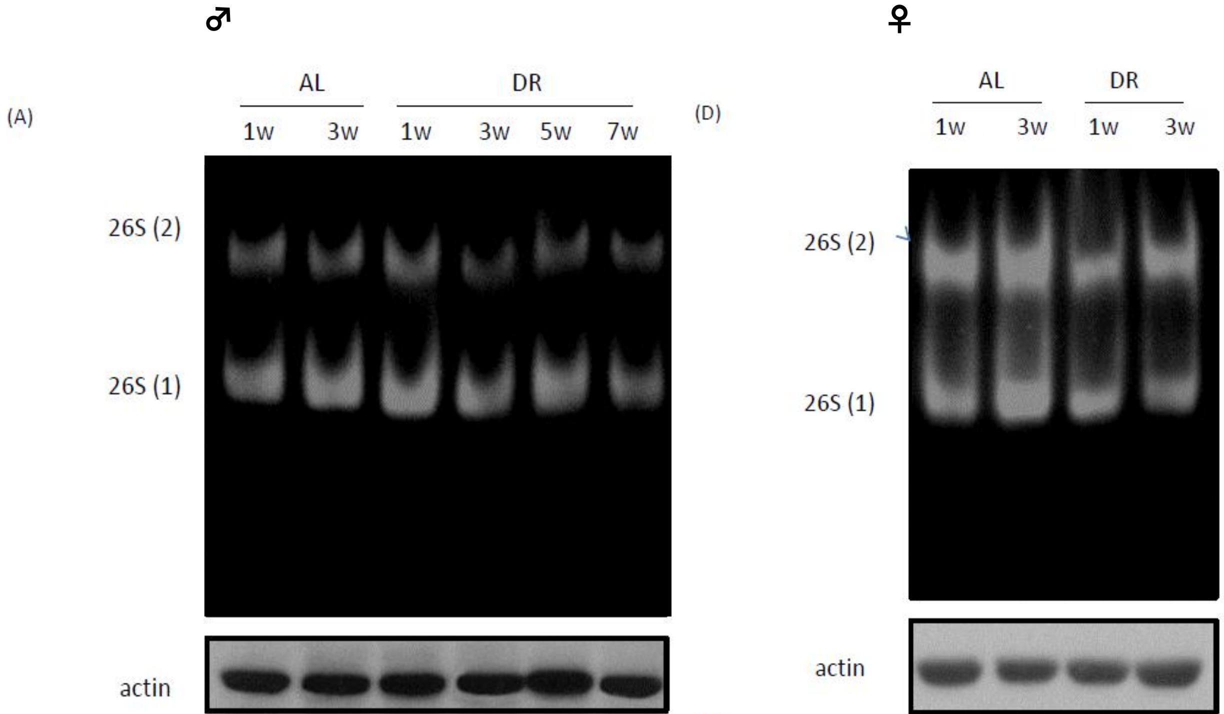
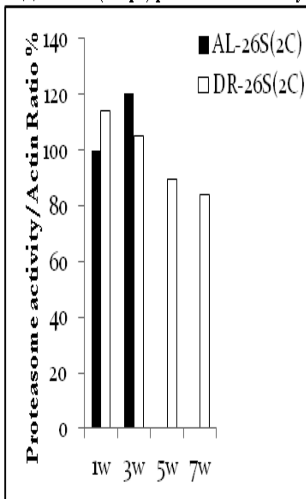


Figure 17. Lifespan extension by DR in *Drosophila melanogaster OR* strain. Flies were maintained in at a constant temperature (25 ℃), humidity (~60%), and 12/12 hour dark/light cycle environment.1-2 day old male *OR* flies were collected from larva food and then transferred to mating food. After 2 days mating, they were transferred to DR or AL food. Dead flies were counted every other day. Flies survive longer in DR food than in AL food. Data represent the mean value from 3 experiments (n=100 flies for each treatment).

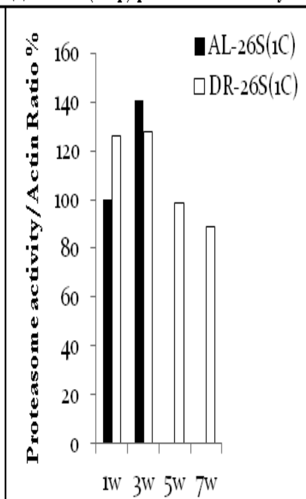
Figure 18



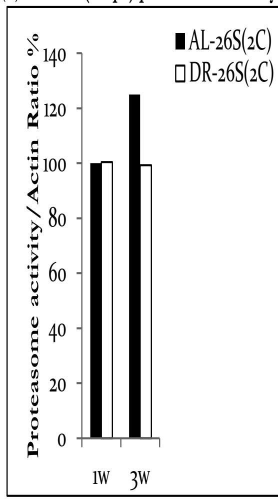
(B) ♂26S (2caps) proteasome activity



(C) ♂26S (1cap) proteasome activity



(E) ♀26S (2caps) proteasome activity



(F) ♀26S (1cap) proteasome activity

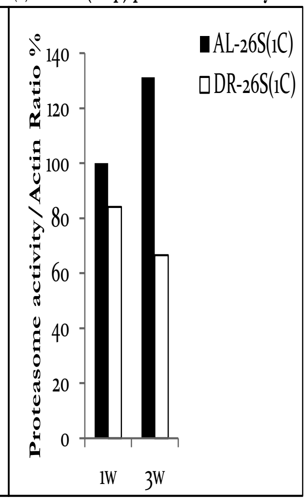


Figure 18. Impact of DR and aging on proteasome activity and level in *OR* strain. Separate gender groups of flies under *DR* food and AL food at different ages were analyzed. The proteasome activity of male and female *OR* strains were assessed with the in-gel assay. The chymotrypsin-like activity was assessed with the substrate Suc-LLVY-AMC. DR causes lower proteasome activity in both male (3w) and female flies (1w; 3w) at young ages. (A). Proteasome activity of male flies displayed an age-dependent decrease under DR food. The 5 week old male group under AL was missing due to very low survival at 5w under AL. (B)/(C). Proteasome activity in male flies was semi-quantified by densitometry. (graph : solid bars, 0% H_2O_2; open bars, 1 % H_2O_2) (D). Proteasome activity of 1w and 3w female flies (E)/ (F). Proteasome activity in female flies was semi-quantified by densitometry.

Figure 19

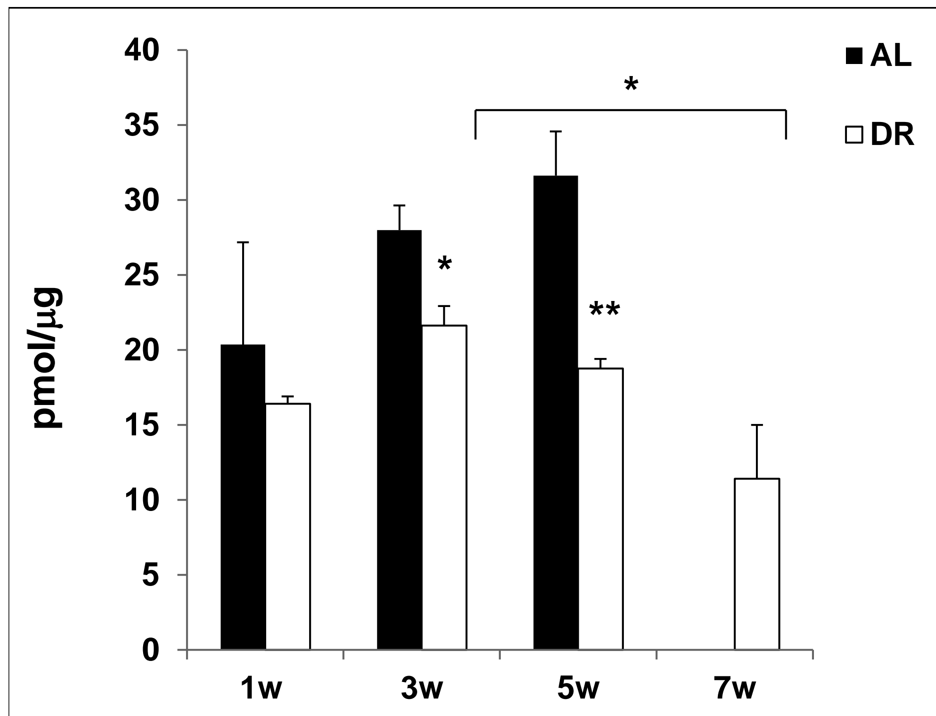


Figure 19. **ATP steady-state levels in *OR* strain under DR across different ages**. Separate groups of flies under DR (open bars) and AL (solid bars) at different ages (1, 3, 5, 7 and 9 weeks) were analyzed. ATP concentrations (pmoles/ug of protein) in cleared supernatants were determined with a kit using the sensitive luciferin/luciferase system. ATP levels decreased in an aging-dependent manner under DR food; however, they increased with aging under AL food. At each age group, ATP level is higher under AL than under DR. Data represent data from four trials (15 flies per trial) per age group. (** p value ≤ 0.01)

Figure 20

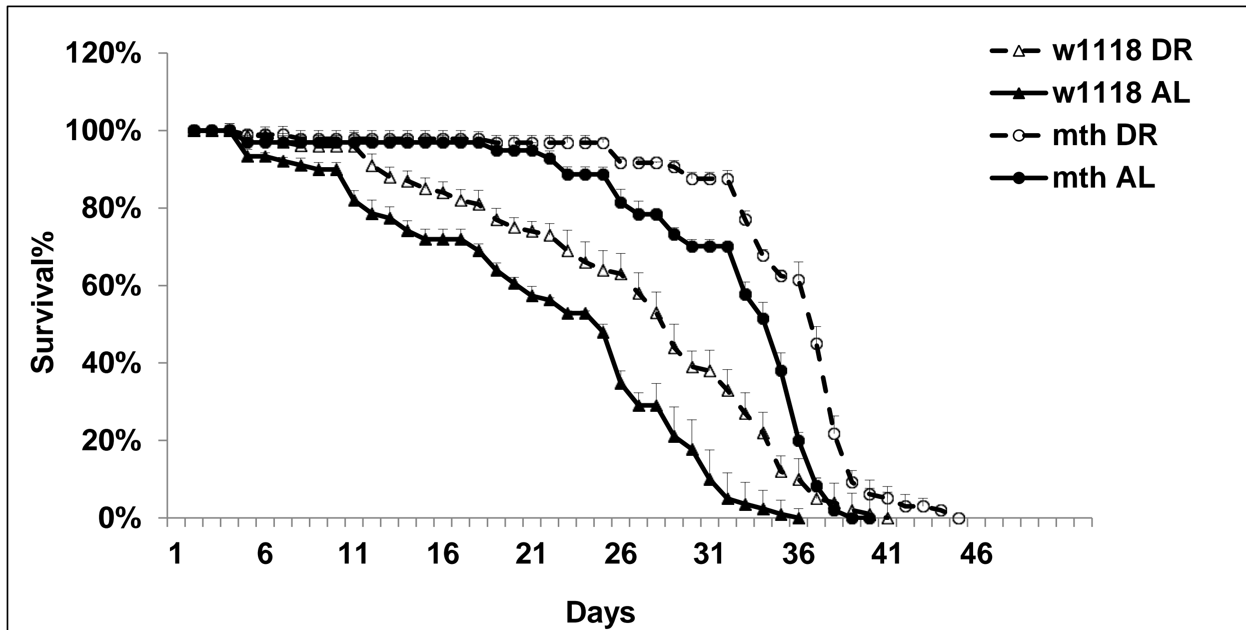
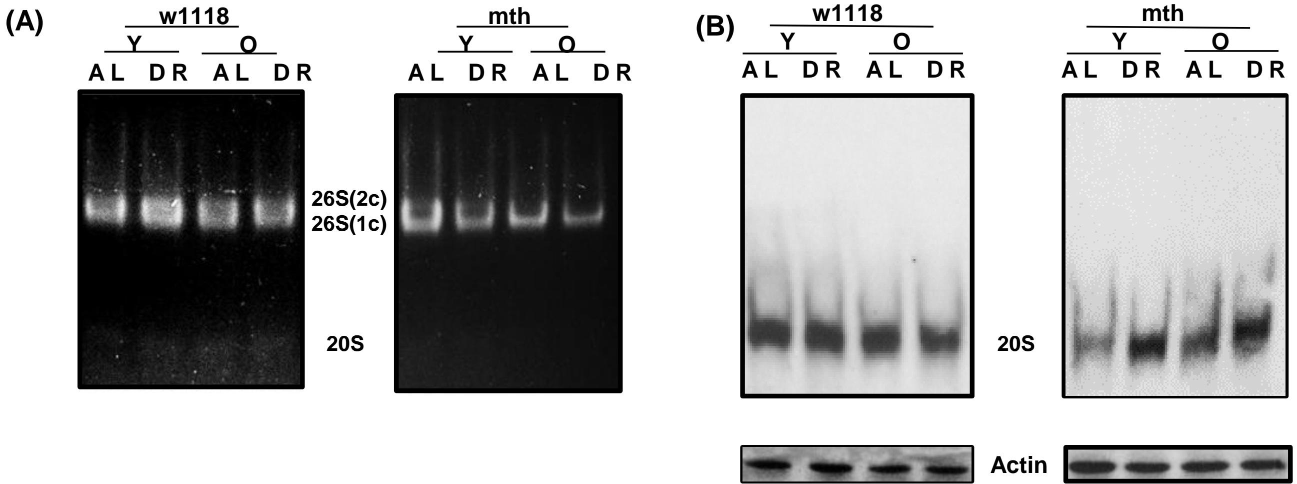


Figure 20. DR extends lifespan in *Drosophila* wild type *w1118* and longevity mutant *mth*. Male flies were maintained at a constant temperature (25°C), humidity (~60%), and 12/12 hour dark/light cycle environment. 1-2 day old male flies were collected from larva food and then transferred to mating food. After 2 days mating, they were transferred to DR and AL food. Dead flies were counted every other day. DR extends lifespan in both strains. *Mth* survives longer than *w1118* under both DR and AL food. Data represent the mean ± sem from 3 experiments (n=100 flies for each treatment).

Figure 21



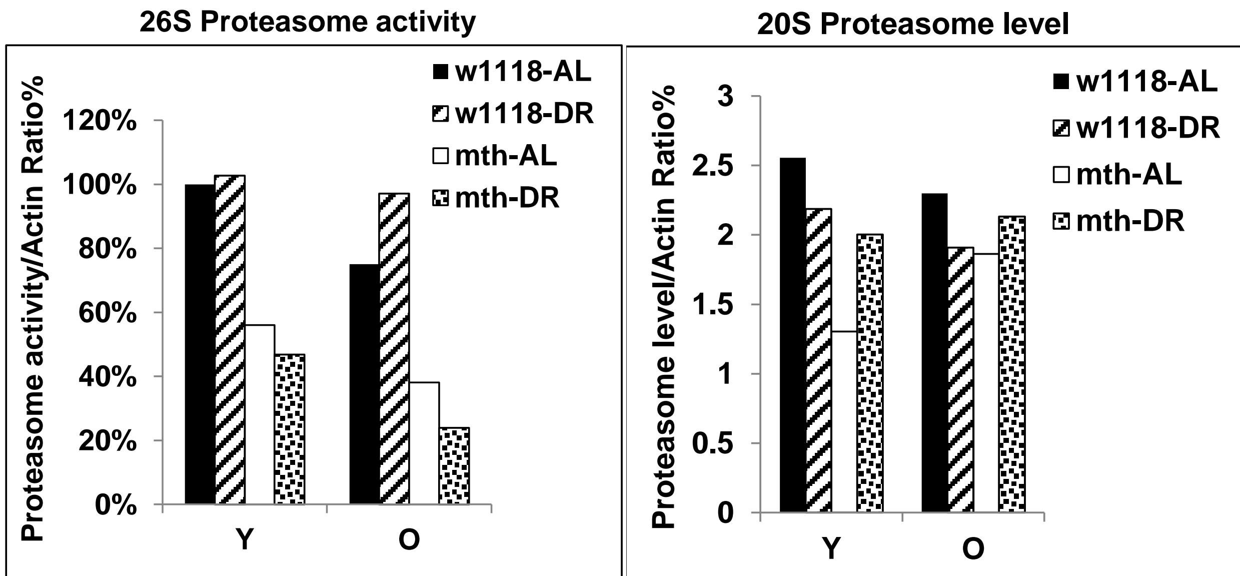
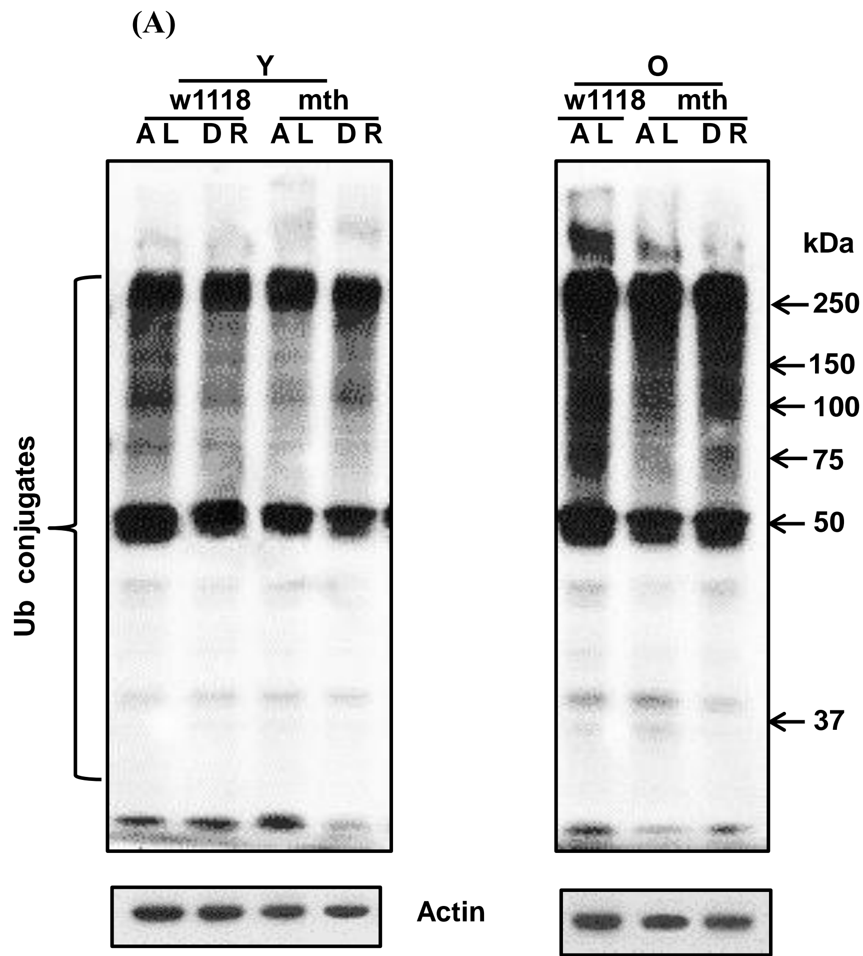
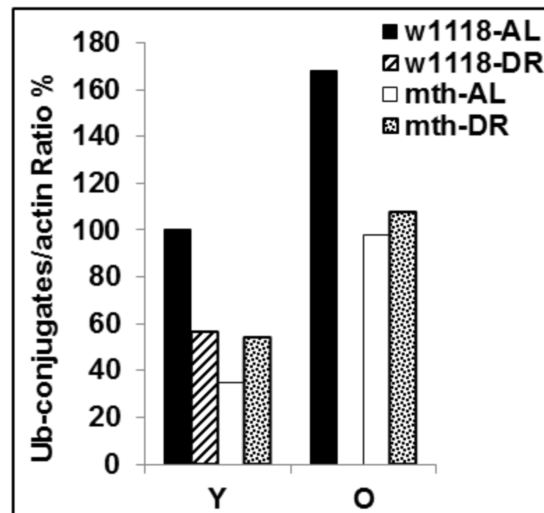


Figure 21. Impact of DR and aging on proteasome activity and levels in *w1118* and *mth*. Young (1 week old) and old (3.5 weeks old) male flies fed with DR and AL food were analyzed. The chymotrypsin-like proteasome activity was assessed with the in-gel assay with the substrate Suc-LLVY-AMC. (A) DR increases proteasome activity at both young and old ages in *w1118* (left fig.). DR decreased proteasome activity at both young and old age in *mth* (right fig.). (B). 20S proteasome levels were detected by western blotting following the in-gel assay using the proteasome dβ5 antibody. DR reduced 20S proteasome level at both young and old age in *w1118*. (left fig.). DR increased 20S proteasome level in *mth* at both young and old ages (right fig.). Proteasome activity and level were semi-quantified by densitometry. (graph: solid bars, *w1118* under AL; stripped bars, *w1118* under DR; open bars, *mth* under AL; dotted bars, *mth* under DR.)

Figure 22

(A)





(B)

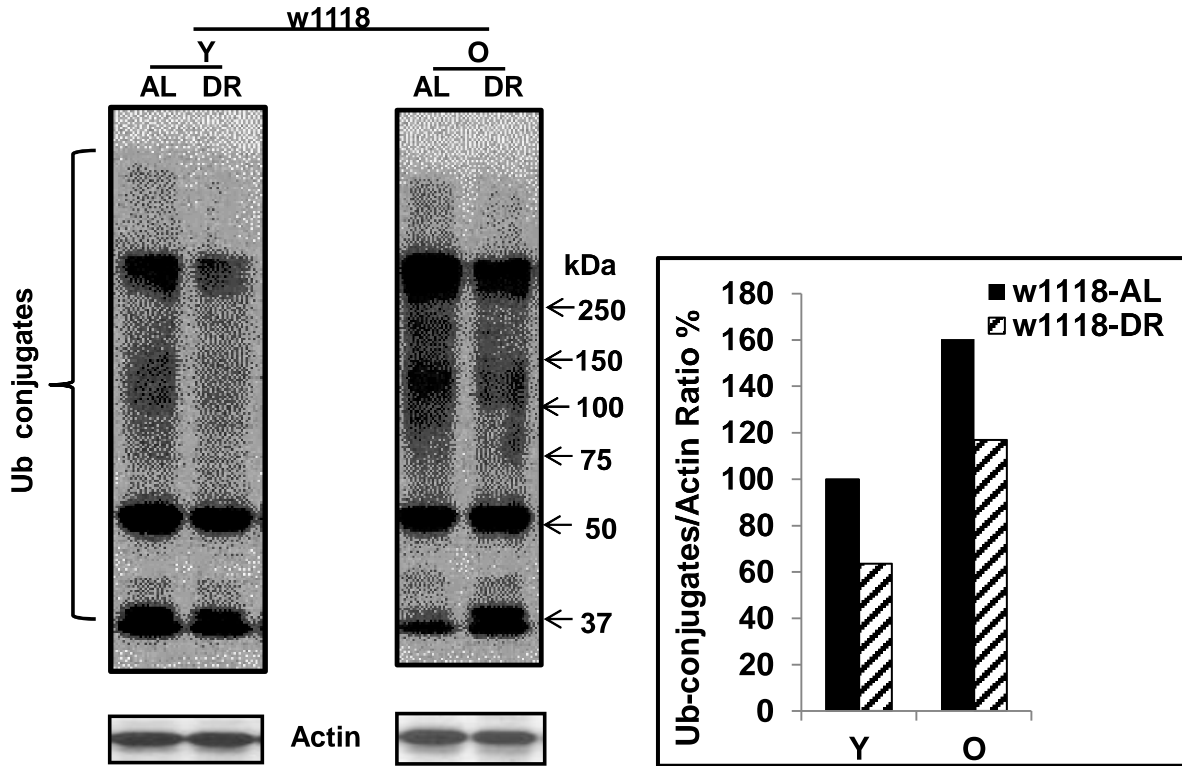
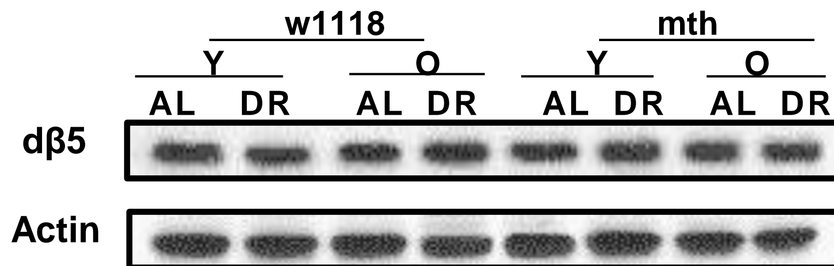


Figure 22. Impact of DR and aging on polyubiquitinated protein conjugate levels in *w1118* and *mth*. Young (1 week old) and old (3.5 weeks old) male flies under DR and AL food were analyzed by western blotting (50 µg protein/sample) probed with an anti-ubiquitin antibody. Equal protein loading was demonstrated by probing immunoblots with an anti-actin antibody. (A) *w1118* old fly samples under DR were missing due to infection. We retested this sample in Fig. (B). DR decreased polyubiquitinated protein conjugates in *w1118* at both young and old ages; however DR increased the ubiquitin conjugates levels in *mth* at both young and old ages, but the increase at old age is diminished compared with at young age. *Mth* displayed lower Ubiquitin conjugate levels than *w1118* at both young and old ages under Al and lower at young ages under DR. (B). Impact of DR and aging on polyubiquitinate protein conjugate levels in *w1118* (retested). Semi-quantification by densitometry. (Graph: solid bars, *w1118* under AL; stripped bars, *w1118* under DR; open bars, *mth* under AL; dotted bars, *mth* under DR.)

Figure 23



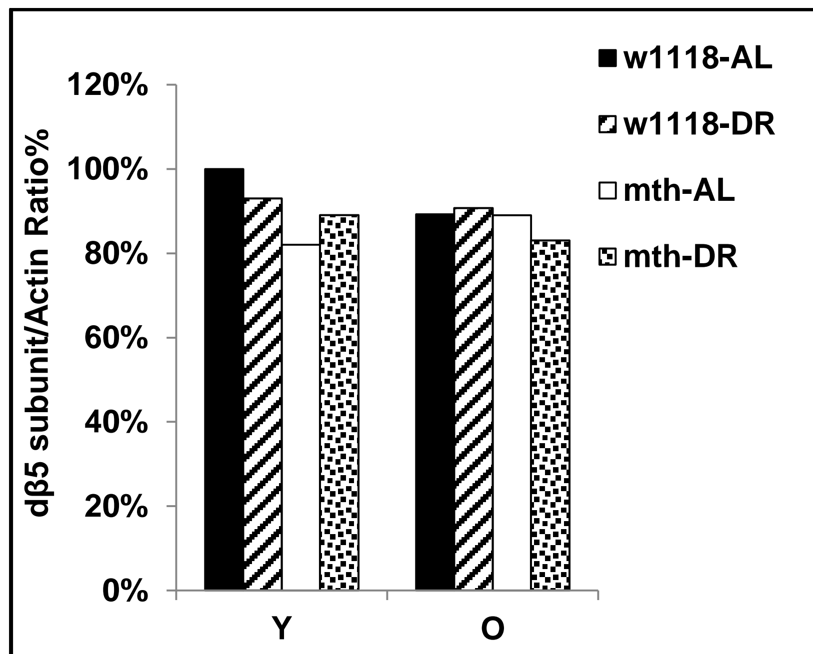


Figure 23. Impact of DR and aging on dβ5 subunit level in *w1118* and *mth*. dβ5 subunit protein levels were assessed by western blotting (90 μg protein/sample) probed with an anti-dβ5 antibody. There is no significant change in dβ5 subunit protein levels in both *mth* and *w1118* maintained under both foods. The level of dβ5/actin was semi-quantified by densitometry. (Graph: solid bars, *w1118* under AL; stripped bars, *w1118* under DR; open bars, *mth* under AL; dotted bars, *mth* under DR.)

Table 1

Larva and mating media ingredients

Component	Larval Media Amount	Mating Media Amount
Water (1)	800 ml	750 ml
Water (2)	200 ml	250 ml
Agar	10 g	20 g
Dextrose	55 g	-
Corn Meal	60 g	-
Sucrose	30 g	100 g
Yeast	25 g	100 g
20% Tegosept	15 ml	15 ml
Propionic Acid	3 ml	3 ml

(Skorupa et al., 2008)

Table 2

Treatment media ingredients

Dietary Restriction Food Recipe		
Components (/dL)	**DR**	**AL**
Water	**100ml**	**100ml**
Agar	**1.5g**	**1.5g**
Sugar	**10g**	**30g**
Yeast	**10g**	**30g**
Tegosept(20%)	**1.5ml**	**1.5ml**
Propionic Acid	**3ml**	**3ml**

CHAPTER VI

REFERENCES

Bahadorani, S., P. Bahadorani, et al. (2008). "The effects of vitamin supplementation on Drosophila life span under normoxia and under oxidative stress." J Gerontol A Biol Sci Med Sci **63**(1): 35-42.

Balaban, R. S., S. Nemoto, et al. (2005). "Mitochondria, oxidants, and aging." Cell **120**(4): 483-495.

Baldal, E. A., W. Baktawar, et al. (2006). "Methuselah life history in a variety of conditions, implications for the use of mutants in longevity research." Exp Gerontol **41**(11): 1126-1135.

Barja, G. (2004). "Free radicals and aging." Trends Neurosci **27**(10): 595-600.

Barrett, E. L. and D. S. Richardson (2011). "Sex differences in telomeres and lifespan." Aging Cell **10**(6): 913-921.

Basler, K. and G. Struhl (1994). "Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein." Nature **368**(6468): 208-214.

Bass, T. M., R. C. Grandison, et al. (2007). "Optimization of dietary restriction protocols in Drosophila." J Gerontol A Biol Sci Med Sci **62**(10): 1071-1081.

Baudry, M., R. DuBrin, et al. (1986). "Low levels of calpain activity in Chiroptera brain: implications for mechanisms of aging." Neurobiol Aging **7**(4): 255-258.

Benaroud, N., P. Zwickl, et al. (2003). "ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation." Mol. Cell **11**: 69.

Bishop, N. A. and L. Guarente (2007). "Genetic links between diet and lifespan: shared mechanisms from yeast to humans." Nat Rev Genet **8**(11): 835-844.

Bokov, A., A. Chaudhuri, et al. (2004). "The role of oxidative damage and stress in aging." Mech Ageing Dev **125**(10-11): 811-826.

Brand, M. D. (2000). "Uncoupling to survive? The role of mitochondrial inefficiency in ageing." Exp Gerontol **35**(6-7): 811-820.

Brody, T. and A. Cravchik (2000). "Drosophila melanogaster G protein-coupled receptors." J Cell Biol **150**(2): F83-88.

Brown, W. M., M. George, Jr., et al. (1979). "Rapid evolution of animal mitochondrial DNA." Proc Natl Acad Sci U S A **76**(4): 1967-1971.

Bukhatwa, S., B. Y. Zeng, et al. "The effects of dose and route of administration of PSI on behavioural and biochemical indices of neuronal degeneration in the rat brain." Brain Res **1354**: 236-242.

Burnett, C., S. Valentini, et al. (2011). "Absence of effects of Sir2 overexpression on lifespan in C. elegans and Drosophila." Nature **477**(7365): 482-485.

Calleja, M., P. Pena, et al. (1993). "Mitochondrial DNA remains intact during Drosophila aging, but the levels of mitochondrial transcripts are significantly reduced." J Biol Chem **268**(25): 18891-18897.

Cardoso, J. C., M. S. Clark, et al. (2005). "The secretin G-protein-coupled receptor family: teleost receptors." J Mol Endocrinol **34**(3): 753-765.

Carrard, G., A. L. Bulteau, et al. (2002). "Impairment of proteasome structure and function in aging." Int J Biochem Cell Biol **34**(11): 1461-1474.

Chapman, T., L. F. Liddle, et al. (1995). "Cost of mating in Drosophila melanogaster females is mediated by male accessory gland products." Nature **373**(6511): 241-244.

Chaudhuri, A., K. Bowling, et al. (2007). "Interaction of genetic and environmental factors in a Drosophila parkinsonism model." J Neurosci **27**(10): 2457-2467.

Chen, Q., J. Thorpe, et al. (2006). "Ump1 extends yeast lifespan and enhances viability during

oxidative stress: central role for the proteasome?" Free Radic Biol Med **40**(1): 120-126.

Chondrogianni, N. and E. S. Gonos (2005). "Proteasome dysfunction in mammalian aging: steps and factors involved." Exp Gerontol **40**(12): 931-938.

Chondrogianni, N., C. Tzavelas, et al. (2005). "Overexpression of proteasome beta5 assembled subunit increases the amount of proteasome and confers ameliorated response to oxidative stress and higher survival rates." J Biol Chem **280**(12): 11840-11850.

Clancy, D. J., D. Gems, et al. (2001). "Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein." Science **292**(5514): 104-106.

CM, P. and C. RE (2004). "Proteasomes and their kin: proteases in the machine age." Nat. Rev. Mol. Cell Biol. **5**: 177.

Conconi, M., L. I. Szweda, et al. (1996). "Age-related decline of rat liver multicatalytic proteinase activity and protection from oxidative inactivation by heat-shock protein 90." Arch Biochem Biophys **331**(2): 232-240.

Cooley, L., R. Kelley, et al. (1988). "Insertional mutagenesis of the Drosophila genome with single P elements." Science **239**(4844): 1121-1128.

Copeland, J. M., J. Cho, et al. (2009). "Extension of Drosophila life span by RNAi of the mitochondrial respiratory chain." Curr Biol **19**(19): 1591-1598.

Cvejic, S., Z. Zhu, et al. (2004). "The endogenous ligand Stunted of the GPCR Methuselah extends lifespan in Drosophila." Nat Cell Biol **6**(6): 540-546.

da Cunha, F. M., M. Demasi, et al. (2011). "Aging and calorie restriction modulate yeast redox state, oxidized protein removal, and the ubiquitin-proteasome system." Free Radic Biol Med **51**(3): 664-670.

Dahlmann, B. (2007). "Role of proteasomes in disease." BMC Biochem **8 Suppl 1**: S3.

Dasuri, K., L. Zhang, et al. (2009). "Aging and dietary restriction alter proteasome biogenesis and composition in the brain and liver." Mech Ageing Dev **130**(11-12): 777-783.

Davies, K. J. (2001). "Degradation of oxidized proteins by the 20S proteasome." Biochimie **83**(3-4): 301-310.

Dohlman, H. G., J. Thorner, et al. (1991). "Model systems for the study of seven-transmembrane-segment receptors." Annu Rev Biochem **60**: 653-688.

Dubessay, P., I. Garreau-Balandier, et al. (2007). "Aging impact on biochemical activities and gene expression of Drosophila melanogaster mitochondria." Biochimie **89**(8): 988-1001.

Dudek, E. J., F. Shang, et al. (2005). "Selectivity of the ubiquitin pathway for oxidatively modified proteins: relevance to protein precipitation diseases." FASEB J **19**(12): 1707-1709.

Eric Cook-Wiensa, M. S. G. (2002). "<Dissociation between functional senescence and oxidative stress resistance in Drosophila.pdf>." Experimental Gerontology **37**: 1345–1355.

Eytan, E., D. Ganoth, et al. (1989). "ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated to ubiquitin." Proc Natl Acad Sci U S A **86**(20): 7751-7755.

Finley, D. (2009). "Recognition and processing of ubiquitin-protein conjugates by the proteasome." Annu Rev Biochem **78**: 477-513.

Flatt, T. (2009). "Ageing: Diet and longevity in the balance." Nature **462**(7276): 989-990.

Gaczynska, M., P. A. Osmulski, et al. (2001). "Caretaker or undertaker? The role of the proteasome in aging." Mech Ageing Dev **122**(3): 235-254.

Geng, F., S. Wenzel, et al. (2012). "Ubiquitin and proteasomes in transcription." Annu Rev Biochem **81**: 177-201.

Giannakou, M. E., M. Goss, et al. (2004). "Long-lived Drosophila with overexpressed dFOXO in adult fat body." Science **305**(5682): 361.

Glickman, M. H., D. M. Rubin, et al. (1998). "The regulatory particle of the Saccharomyces cerevisiae proteasome." Mol Cell Biol **18**(6): 3149-3162.

Goldberg, A. L., T. N. Akopian, et al. (1997). "New insights into the mechanisms and importance of the proteasome in intracellular protein degradation." Biol Chem **378**(3-4): 131-140.

Grandison, R. C., R. Wong, et al. (2009). "Effect of a standardised dietary restriction protocol on multiple laboratory strains of Drosophila melanogaster." PLoS One **4**(1): e4067.

Groll, M., W. Heinemeyer, et al. (1999). "The catalytic sites of 20S proteasomes and their role in subunit maturation: a mutational and crystallographic study." Proc Natl Acad Sci U S A **96**(20): 10976-10983.

Grune, T. (2000). "Oxidative stress, aging and the proteasomal system." Biogerontology **1**(1): 31-40.

Grune, T. and K. J. Davies (1997). "Breakdown of oxidized proteins as a part of secondary antioxidant defenses in mammalian cells." Biofactors **6**(2): 165-172.

Grune, T., T. Reinheckel, et al. (1997). "Degradation of oxidized proteins in mammalian cells." FASEB J **11**(7): 526-534.

Haas, K. F., E. Woodruff, 3rd, et al. (2007). "Proteasome function is required to maintain muscle cellular architecture." Biol Cell **99**(11): 615-626.

Hamer, G., O. Matilainen, et al. (2010). "A photoconvertible reporter of the ubiquitin-proteasome system in vivo." Nat Methods **7**(6): 473-478.

Harman, D. (1956). "Aging: a theory based on free radical and radiation chemistry." J Gerontol **11**(3): 298-300.

Harman, D. (1972). "The biologic clock: the mitochondria?" J Am Geriatr Soc **20**(4): 145-147.

Harman, D. (1981). "The aging process." Proc Natl Acad Sci U S A **78**(11): 7124-7128.

Harman, D. (2003). "The free radical theory of aging." Antioxid Redox Signal **5**(5): 557-561.

Harman, D. (2009). "Origin and evolution of the free radical theory of aging: a brief personal history, 1954-2009." Biogerontology **10**(6): 773-781.

Heilbronn, L. K., L. de Jonge, et al. (2006). "Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial." Jama **295**(13): 1539-1548.

Helfand-a, S. L. and B. Rogina (2003). "Molecular genetics of aging in the fly: is this the end of the beginning?" Bioessays **25**(2): 134-141.

Helfand-b, S. L. and B. Rogina (2003). "Genetics of aging in the fruit fly, Drosophila melanogaster." Annu Rev Genet **37**: 329-348.

Heo, J., W. W. Ja, et al. (2008). "The predicted binding site and dynamics of peptide inhibitors to the Methuselah GPCR from Drosophila melanogaster." Biochemistry **47**(48): 12740-12749.

Hershko, A. and A. Ciechanover (1998). "The ubiquitin system." Annu Rev Biochem **67**: 425-479.

Hishinuma, K. and S. Kimura (1990). "Significant calcium accumulation in fragile erythrocytes by dietary restriction in mice." Int J Vitam Nutr Res **60**(4): 372-378.

Hwangbo, D. S., B. Gershman, et al. (2004). "Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body." Nature **429**(6991): 562-566.

Ja, W. W., G. B. Carvalho, et al. (2009). "The Drosophila G protein-coupled receptor, Methuselah, exhibits a promiscuous response to peptides." Protein Sci **18**(11): 2203-

2208.

Jana, N. R. (2012). "Protein homeostasis and aging: role of ubiquitin protein ligases." Neurochem Int **60**(5): 443-447.

Kapahi, P., B. M. Zid, et al. (2004). "Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway." Curr Biol **14**(10): 885-890.

Kayed, R., Y. Sokolov, et al. (2004). "Permeabilization of lipid bilayers is a common conformation-dependent activity of soluble amyloid oligomers in protein misfolding diseases." J Biol Chem **279**(45): 46363-46366.

Keller, J. N., J. Gee, et al. (2002). "The proteasome in brain aging." Ageing Res Rev **1**(2): 279-293.

Keller, J. N., K. B. Hanni, et al. (2000). "Possible involvement of proteasome inhibition in aging: implications for oxidative stress." Mech Ageing Dev **113**(1): 61-70.

Keller, J. N., F. F. Huang, et al. (2000). "Decreased levels of proteasome activity and proteasome expression in aging spinal cord." Neuroscience **98**(1): 149-156.

Kidd, T., R. Abu-Shumays, et al. (2005). "The epsilon-subunit of mitochondrial ATP synthase is required for normal spindle orientation during the Drosophila embryonic divisions." Genetics **170**(2): 697-708.

Korolchuk, V. I., F. M. Menzies, et al. (2010). "Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems." FEBS Lett **584**(7): 1393-1398.

Kruegel, U., B. Robison, et al. (2011). "Elevated proteasome capacity extends replicative lifespan in Saccharomyces cerevisiae." PLoS Genet **7**(9): e1002253.

Kwong, L. K. and R. S. Sohal (1998). "Substrate and site specificity of hydrogen peroxide generation in mouse mitochondria." Arch Biochem Biophys **350**(1): 118-126.

Lagerstrom, M. C. and H. B. Schioth (2008). "Structural diversity of G protein-coupled receptors and significance for drug discovery." Nat Rev Drug Discov **7**(4): 339-357.

Lander, G. C., E. Estrin, et al. (2012). "Complete subunit architecture of the proteasome regulatory particle." Nature **482**(7384): 186-191.

Lee, D. H. and A. L. Goldberg (1998). "Proteasome inhibitors: valuable new tools for cell biologists." Trends Cell Biol **8**(10): 397-403.

Li, F., L. Zhang, et al. (2008). "Aging and dietary restriction effects on ubiquitination, sumoylation, and the proteasome in the heart." Mech Ageing Dev **129**(9): 515-521.

Lin, Y. (1998). "Extended Life-Span and Stress Resistance in the Drosophila Mutant methuselah." Science **282**(5390): 943-946.

Lin, Y. J., L. Seroude, et al. (1998). "Extended life-span and stress resistance in the Drosophila mutant methuselah." Science **282**(5390): 943-946.

Liu, C. W., M. J. Corboy, et al. (2003). "Endoproteolytic activity of the proteasome." Science **299**(5605): 408-411.

Liu, C. W., X. Li, et al. (2006). "ATP binding and ATP hydrolysis play distinct roles in the function of 26S proteasome." Mol Cell **24**(1): 39-50.

Liu, G., J. Rogers, et al. (2011). "EGF signalling activates the ubiquitin proteasome system to modulate C. elegans lifespan." EMBO J **30**(15): 2990-3003.

Liu, Z., X. Wang, et al. (2008). "A Drosophila model for LRRK2-linked parkinsonism." Proc Natl Acad Sci U S A **105**(7): 2693-2698.

Loeb, J. and J. H. Northrop (1916). "Is There a Temperature Coefficient for the Duration of Life?" Proc Natl Acad Sci U S A **2**(8): 456-457.

Loeb, J. and J. H. Northrop (1917). "What Determines the Duration of Life in Metazoa?" Proc

Natl Acad Sci U S A **3**(5): 382-386.

Luchsinger, J. A., M. X. Tang, et al. (2002). "Caloric intake and the risk of Alzheimer disease." Arch Neurol **59**(8): 1258-1263.

Ly, D. H., D. J. Lockhart, et al. (2000). "Mitotic misregulation and human aging." Science **287**(5462): 2486-2492.

Ma, W., J. Berg, et al. (2007). "Ketogenic diet metabolites reduce firing in central neurons by opening K(ATP) channels." J Neurosci **27**(14): 3618-3625.

Maalouf, M., J. M. Rho, et al. (2009). "The neuroprotective properties of calorie restriction, the ketogenic diet, and ketone bodies." Brain Res Rev **59**(2): 293-315.

Mair, W. and A. Dillin (2008). "Aging and survival: the genetics of life span extension by dietary restriction." Annu Rev Biochem **77**: 727-754.

Martinez-Vicente, M., G. Sovak, et al. (2005). "Protein degradation and aging." Exp Gerontol **40**(8-9): 622-633.

Mattison, J. A., G. S. Roth, et al. (2007). "Dietary restriction in aging nonhuman primates." Interdiscip Top Gerontol **35**: 137-158.

Mattson, M. P. and R. Wan (2005). "Beneficial effects of intermittent fasting and caloric restriction on the cardiovascular and cerebrovascular systems." J Nutr Biochem **16**(3): 129-137.

McGarrigle, D. and X. Y. Huang (2007). "Methuselah antagonist extends life span." Nature Chemical Biology **3**(7): 371-372.

McNaught, K. S., D. P. Perl, et al. (2004). "Systemic exposure to proteasome inhibitors causes a progressive model of Parkinson's disease." Ann Neurol **56**(1): 149-162.

Merry, B. J. (2004). "Oxidative stress and mitochondrial function with aging--the effects of

calorie restriction." Aging Cell **3**(1): 7-12.

Miquel, J., J. Fleming, et al. (1982). "Antioxidants, metabolic rate and aging in Drosophila." Arch Gerontol Geriatr **1**(2): 159-165.

Mohammad, F., P. Singh, et al. (2009). "A Drosophila systems model of pentylenetetrazole induced locomotor plasticity responsive to antiepileptic drugs." BMC Syst Biol **3**: 11.

Morrow, G., M. Samson, et al. (2004). "Overexpression of the small mitochondrial Hsp22 extends Drosophila life span and increases resistance to oxidative stress." Faseb J **18**(3): 598-599.

Neves, S. R., P. T. Ram, et al. (2002). "G protein pathways." Science **296**(5573): 1636-1639.

Ohtsuka, H., R. Takahashi, et al. (1995). "Age-related accumulation of high-molecular-weight ubiquitin protein conjugates in mouse brains." J Gerontol A Biol Sci Med Sci **50**(5): B277-281.

Pahlavani, M. A. (2004). "Influence of caloric restriction on aging immune system." J Nutr Health Aging **8**(1): 38-47.

Palczewski, K. (2006). "G protein-coupled receptor rhodopsin." Annu Rev Biochem **75**: 743-767.

Peng, C., H. Y. Chan, et al. (2011). "Apple polyphenols extend the mean lifespan of Drosophila melanogaster." J Agric Food Chem **59**(5): 2097-2106.

Perez, V. I., R. Buffenstein, et al. (2009). "Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat." Proc Natl Acad Sci U S A **106**(9): 3059-3064.

Petrosyan, A., I. H. Hsieh, et al. (2007). "Age-dependent stability of sensorimotor functions in the life-extended Drosophila mutant methuselah." Behav Genet **37**(4): 585-594.

Phelan, J. P., M. A. Archer, et al. (2003). "Breakdown in correlations during laboratory evolution. I. Comparative analyses of Drosophila populations." Evolution **57**(3): 527-535.

Phelan, J. P. and M. R. Rose (2006). "Caloric restriction increases longevity substantially only when the reaction norm is steep." Biogerontology **7**(3): 161-164.

Piper, M. D. and A. Bartke (2008). "Diet and aging." Cell Metab **8**(2): 99-104.

Piper, M. D., C. Selman, et al. (2008). "Separating cause from effect: how does insulin/IGF signalling control lifespan in worms, flies and mice?" J Intern Med **263**(2): 179-191.

Piper, M. D., D. Skorupa, et al. (2005). "Diet, metabolism and lifespan in Drosophila." Exp Gerontol **40**(11): 857-862.

Powers, E. T., R. I. Morimoto, et al. (2009). "Biological and chemical approaches to diseases of proteostasis deficiency." Annu Rev Biochem **78**: 959-991.

Rajalingam, K. and I. Dikic (2011). "Healthy ageing through regulated proteostasis." EMBO J **30**(15): 2983-2985.

Rattan, S. I. (1996). "Synthesis, modifications, and turnover of proteins during aging." Exp Gerontol **31**(1-2): 33-47.

Rebrin, I., A. C. Bayne, et al. (2004). "Free aminothiols, glutathione redox state and protein mixed disulphides in aging Drosophila melanogaster." Biochem J **382**(Pt 1): 131-136.

Rogina, B. and S. L. Helfand (2004). "Sir2 mediates longevity in the fly through a pathway related to calorie restriction." Proc Natl Acad Sci U S A **101**(45): 15998-16003.

Rogina, B., R. A. Reenan, et al. (2000). "Extended life-span conferred by cotransporter gene mutations in Drosophila." Science **290**(5499): 2137-2140.

Ross, C. A. and M. A. Poirier (2004). "Protein aggregation and neurodegenerative disease." Nat Med **10 Suppl**: S10-17.

Sagi, D. and S. K. Kim (2012). "An Engineering Approach to Extending Lifespan in C. elegans." PLoS Genet **8**(6): e1002780.

Salmon, A. B., S. Leonard, et al. (2009). "The long lifespan of two bat species is correlated with resistance to protein oxidation and enhanced protein homeostasis." FASEB J **23**(7): 2317-2326.

Schioth, H. B. and R. Fredriksson (2005). "The GRAFS classification system of G-protein coupled receptors in comparative perspective." Gen Comp Endocrinol **142**(1-2): 94-101.

Schwarze, S. R., R. Weindruch, et al. (1998). "Oxidative stress and aging reduce COX I RNA and cytochrome oxidase activity in Drosophila." Free Radic Biol Med **25**(6): 740-747.

Segref, A., S. Torres, et al. (2011). "A screenable in vivo assay to study proteostasis networks in Caenorhabditis elegans." Genetics **187**(4): 1235-1240.

Sekhar, K. R., X. X. Yan, et al. (2002). "Nrf2 degradation by the ubiquitin proteasome pathway is inhibited by KIAA0132, the human homolog to INrf2." Oncogene **21**(44): 6829-6834.

Shang, F., X. Gong, et al. (1997). "Activity of ubiquitin-dependent pathway in response to oxidative stress. Ubiquitin-activating enzyme is transiently up-regulated." J Biol Chem **272**(37): 23086-23093.

Shang, F., T. R. Nowell, Jr., et al. (2001). "Removal of oxidatively damaged proteins from lens cells by the ubiquitin-proteasome pathway." Exp Eye Res **73**(2): 229-238.

Shang, F. and A. Taylor (2011). "Ubiquitin-proteasome pathway and cellular responses to oxidative stress." Free Radic Biol Med **51**(1): 5-16.

Shringarpure, R., T. Grune, et al. (2001). "Protein oxidation and 20S proteasome-dependent proteolysis in mammalian cells." Cell Mol Life Sci **58**(10): 1442-1450.

Skorupa, D. A., A. Dervisefendic, et al. (2008). "Dietary composition specifies consumption,

obesity, and lifespan in Drosophila melanogaster." Aging Cell **7**(4): 478-490.

Smith, J. M. (1958). "THE EFFECTS OF TEMPERATURE AND OF EGG-LAYING ON THE LONGEVITY OF DROSOPHILA-SUBOBSCURA." Journal of Experimental Biology **35**(4): 832-842.

Sohal, R. S. and P. B. Buchan (1981). "Relationship between physical activity and life span in the adult housefly, Musca domestica." Exp Gerontol **16**(2): 157-162.

Sohal, R. S., R. J. Mockett, et al. (2002). "Mechanisms of aging: an appraisal of the oxidative stress hypothesis." Free Radic Biol Med **33**(5): 575-586.

Sohal, R. S. and B. H. Sohal (1991). "Hydrogen peroxide release by mitochondria increases during aging." Mech Ageing Dev **57**(2): 187-202.

Song, W., R. Ranjan, et al. (2002). "Presynaptic regulation of neurotransmission in Drosophila by the g protein-coupled receptor methuselah." Neuron **36**(1): 105-119.

Song, W., R. Ranjan, et al. (2002). "Presynaptic regulation of neurotransmission in Drosophila by the G protein-coupled receptor Methuselah." Neuron **36**(1): 105-119.

Spradling, A. C., D. Stern, et al. (1999). "The Berkeley Drosophila Genome Project gene disruption project: Single P-element insertions mutating 25% of vital Drosophila genes." Genetics **153**(1): 135-177.

Spradling, A. C., D. M. Stern, et al. (1995). "Gene disruptions using P transposable elements: an integral component of the Drosophila genome project." Proc Natl Acad Sci U S A **92**(24): 10824-10830.

Storr, S. J., N. O. Carragher, et al. (2011). "The calpain system and cancer." Nat Rev Cancer **11**(5): 364-374.

Sun, J. and J. Tower (1999). "FLP recombinase-mediated induction of Cu/Zn-superoxide

dismutase transgene expression can extend the life span of adult Drosophila melanogaster flies." Mol Cell Biol **19**(1): 216-228.

Szweda, P. A., M. Camouse, et al. (2003). "Aging, lipofuscin formation, and free radical-mediated inhibition of cellular proteolytic systems." Ageing Res Rev **2**(4): 383-405.

Tai, H. C. and E. M. Schuman (2008). "Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction." Nat Rev Neurosci **9**(11): 826-838.

Tanaka, K. (1998). "Proteasomes: structure and biology." J Biochem **123**(2): 195-204.

Tatar, M., A. A. Khazaeli, et al. (1997). "Chaperoning extended life." Nature **390**(6655): 30.

Tatar, M., A. Kopelman, et al. (2001). "A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function." Science **292**(5514): 107-110.

Tatar, M. and C. Yin (2001). "Slow aging during insect reproductive diapause: why butterflies, grasshoppers and flies are like worms." Exp Gerontol **36**(4-6): 723-738.

Taylor, A. and K. J. Davies (1987). "Protein oxidation and loss of protease activity may lead to cataract formation in the aged lens." Free Radic Biol Med **3**(6): 371-377.

Terman, A. and U. T. Brunk (2004). "Lipofuscin." Int J Biochem Cell Biol **36**(8): 1400-1404.

Timothy M. Bass, R. C. G., Richard Wong, Pedro Martinez, Linda Partridge, and Matthew D. W. Piper (2007). "<Optimization of Dietary Restriction Protocols in Drosophila.pdf>." Journal of Gerontology: BIOLOGICAL SCIENCES **62A**(10): 1071-1081.

Toma, D. P., K. P. White, et al. (2002). "Identification of genes involved in Drosophila melanogaster geotaxis, a complex behavioral trait." Nat Genet **31**(4): 349-353.

Tonoki, A., E. Kuranaga, et al. (2009). "Genetic evidence linking age-dependent attenuation of the 26S proteasome with the aging process." Mol Cell Biol **29**(4): 1095-1106.

Toroser, D., W. C. Orr, et al. (2007). "Carbonylation of mitochondrial proteins in Drosophila

melanogaster during aging." Biochem Biophys Res Commun **363**(2): 418-424.

Tower, J. (2000). "Transgenic methods for increasing Drosophila life span." Mech Ageing Dev **118**(1-2): 1-14.

Trout, W. E. and W. D. Kaplan (1970). "A relation between longevity, metabolic rate, and activity in shaker mutants of Drosophila melanogaster." Exp Gerontol **5**(1): 83-92.

Vellai, T. (2009). "Autophagy genes and ageing." Cell Death Differ **16**(1): 94-102.

Vernace, V. A., L. Arnaud, et al. (2007). "Aging perturbs 26S proteasome assembly in Drosophila melanogaster." FASEB J **21**(11): 2672-2682.

Vrailas-Mortimer, A., R. Gomez, et al. (2012). "A survey of the protective effects of some commercially available antioxidant supplements in genetically and chemically induced models of oxidative stress in Drosophila melanogaster." Exp Gerontol.

Vucic, D., V. M. Dixit, et al. (2011). "Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death." Nat Rev Mol Cell Biol **12**(7): 439-452.

Wallace, D. C. (1992). "Mitochondrial genetics: a paradigm for aging and degenerative diseases?" Science **256**(5057): 628-632.

Wallace, D. C. (2005). "A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine." Annu Rev Genet **39**: 359-407.

Wang, H. D., P. Kazemi-Esfarjani, et al. (2004). "Multiple-stress analysis for isolation of Drosophila longevity genes." Proc Natl Acad Sci U S A **101**(34): 12610-12615.

Wang, J., C. E. Wang, et al. (2008). "Impaired ubiquitin-proteasome system activity in the synapses of Huntington's disease mice." J Cell Biol **180**(6): 1177-1189.

Wang, P. Y., N. Neretti, et al. (2009). "Long-lived Indy and calorie restriction interact to extend life span." Proc Natl Acad Sci U S A **106**(23): 9262-9267.

Welchman, R. L., C. Gordon, et al. (2005). "Ubiquitin and ubiquitin-like proteins as multifunctional signals." Nat Rev Mol Cell Biol **6**(8): 599-609.

West, A. P., Jr., L. L. Llamas, et al. (2001). "Crystal structure of the ectodomain of Methuselah, a Drosophila G protein-coupled receptor associated with extended lifespan." Proc Natl Acad Sci U S A **98**(7): 3744-3749.

William W Ja, A. P. W., Jr, Silvia L Delker, Pamela J Bjorkman, Seymour Benzer, and R. W. Roberts (2007). "Extension of Drosophila melanogaster life span with a GPCR peptide inhibitor." Nature Chemical Biology **3**(7): 415-419.

Yu, B. P. (1996). "Aging and oxidative stress: modulation by dietary restriction." Free Radic Biol Med **21**(5): 651-668.

Zhang, X., J. Zhou, et al. (2008). "The proteasome: a target of oxidative damage in cultured human retina pigment epithelial cells." Invest Ophthalmol Vis Sci **49**(8): 3622-3630.

Zhou, F., T. van Laar, et al. (2011). "APP and APLP1 are degraded through autophagy in response to proteasome inhibition in neuronal cells." Protein Cell **2**(5): 377-383.

Zou, S., S. Meadows, et al. (2000). "Genome-wide study of aging and oxidative stress response in Drosophila melanogaster." Proc Natl Acad Sci U S A **97**(25): 13726-13731.