# CHARACTERIZATION OF THE 26S PROTEASOME IN *DROSOPHILA MELANOGASTER* AS A MODEL FOR AGING

*by*

Vita A.Vernace

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

2007

UMI Number: 3284379

Copyright 2007 by
Vernace, Vita A.

All rights reserved.

UMI®

UMI Microform 3284379
Copyright 2008 by ProQuest Information and Learning Company.
All rights reserved. This microform edition is protected against
unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

**© 2007**

**Vita A. Vernace**
**All Rights Reserved**

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

______________ ______________________________

**Date** **Dr. MARIA FIGUEIREDO-PEREIRA**
(Hunter College, **Chair of Examining Committee**)

______________ ______________________________

**Date** **Dr. Richard Chappell**
**Executive Officer**

______________________________

**Dr. Patricia Rockwell**
(Hunter College)

______________________________

**Dr. Schmidt-Glenwinkel**
(Hunter College)

______________________________

**Dr. German Torres**
(NYCOM/NYIT)

______________________________

**Dr. Michael Young**
(Rockefeller University)

______________________________

**Supervisory Committee**

**THE CITY UNIVERSITY OF NEW YORK**

# CHARACTERIZATION OF THE 26S PROTEASOME IN *DROSOPHILA MELANOGASTER* AS A MODEL FOR AGING

**by**

Vita A. Vernace

**Advisor: Dr. Maria E. Figueiredo-Pereira**

## Abstract

In all cells, protein degradation is a constant process that is critical for cell survival and repair. It ensures that damaged proteins are degraded and that all proteins needed by the cells for a variety of functions are available at the right time and in the right amounts.

The ubiquitin-proteasome pathway (UPP) is the major proteolytic pathway that degrades intracellular proteins. It plays critical roles in many cellular processes and diseases. Disruption of the UPP is particularly relevant to pathophysiological conditions that provoke the accumulation of aberrant proteins, such as in aging as well as in a variety of neurodegenerative disorders.

We hypothesize that one of the reasons why these neurodegenerative conditions exhibit a late onset is because proteasome activity decreases with aging. Aging-dependent impairment in proteolysis mediated by the proteasome may have profound ramifications for cell viability. It can lead to, for example, the accumulation of modified, potentially toxic proteins in cells and can cause tissue inflammation as well as premature cell death by apoptosis or necrosis.

To address this hypothesis, the major aim of this thesis was to identify mechanisms mediating the aging-dependent impairment of the proteasome in an *in vivo* setting, by using *Drosophila* as a model. Our studies revealed that:
(1) The activity of the 26S proteasome declines significantly when the flies reach an age at which overall ATP levels are highly reduced; (2) The decline in 26S proteasome activity observed in Drosophila is not a gradual process and 3) The sharp deficit in 26S proteasome activity observed in the "old" flies increases their sensitivity to proteotoxic stress.

Our results provide strong evidence for considering ATP reduction in conjunction with 26S proteasome impairment as aging hallmarks. In addition, they suggest that an "old"

age deficit in 26S proteasome activity may contribute to the late onset observed in most human neurodegenerative

The experimental approaches used in this thesis take advantage of the power of a simple *in vivo* model system, like *Drosophila,* to obtained critical information on the effects of aging on protein turnover associated with the ubiquitin-proteasome pathway.

# ACKNOWLEDGEMENTS

I would like to take this opportunity to thank the people that supported me through the last few years.

I would first like to thank my mentor, Dr. Maria Figueiredo-Pereira, who has always been there for me at any hour, anytime and even when she was traveling. She has always strived for excellence in science, and I hope that my work has made her proud. She has become like a second mother to me, and I will always cherish our time together. My leaving the laboratory is bittersweet.

I would also like to thank my committee members for reading my thesis, and offering very insightful feedback: Dr. Thomas Schmidt-Glenewinkel thank you for the collaboration over all of these years. Thank you for always stopping whatever you were doing to talk to me about science and “the outside world”. I will miss our discussions but hope to continue them via email. Thank you Dr. Patricia Rockwell for always being available to me, offering advice and support on my project, and helping me figure out my way around Grand Central. You are always welcome in our home. Thank you Dr. German Torres for changing the direction of my life 11 years ago by introducing me to neurodegenerative diseases and the human

brain. Thank you for giving me the foundation to become a successful scientist. I will always think fondly of our time in Buffalo, and always be part of the "A Team". Thank you Dr. Michael Young for allowing me to come to your laboratory and learn invaluable techniques.

I would like to say to all of my past and current laboratory members, thank you from the bottom of my heart for always being supportive, when as we all know, you had your own work to do. The time I have spent with all of you has been priceless, and I will truly miss all of you. I am not going to say good-bye because you are people I wish to know for the rest of my life. We better keep in touch!
I would like to thank each of you individually and will do so in the order that I met you. Thank you Dr. Zongmin Li for always being there for me and helping me with any aspect of my project. Thank you Marlon Jansen for always being honest with me and telling me when I am being too serious. Your helpfulness and "critiques" have penetrated and I hope to incorporate them into my life. I will truly miss working with you and only wish good things for you in the laboratory and in your personal life. Thank you Sha-Ron Pierre for all of our late nights in the lab, all of the drives home that you gave me, when I know it was out of your way, all of the lunches and dinners where we found

ourselves laughing, and for all of the memorable neuroscience meetings which you always made more fun and not just science. I will miss you terribly. Thank you Dr. Kenyon Ogburn for our Thursday nights and letting me into your world. You are a truly gifted scientist and man and I hope all good things follow you in life. Thank you Dr. Zhiyou Wang for always smiling at me across the bench and letting me not take things so seriously. You continue to be a close friend and confidant, and I hope that whatever you want in life greets you happily. Thank you Lisette Arnaud for housing me not one but twice, but on a serious note, for becoming a very good friend to me in the last few years. You have always been there for me both scientifically and personally and our friendship is invaluable to me.  Thank you for being there for me at some of the most important moments in my life (especially when my water broke). I only wish you happiness and great things in your life, and I know we will keep in touch. Thank you Natura Myeku for always being very strong and funny. I have enjoyed our discussions, and will miss our talks. Thank you Alison Larsen for always smiling and for having a heart of gold. I know we will remain friends and I cannot wait for our future “play dates”.

I would like to thank all of the students I met at Hunter College and the CUNY Graduate Center, especially those of you that started at the same time as me: Luena Papa, Evan Gomes, and Berenice Duran. You all have always supported me in my project and in my personal life. I want to say that I could not have made it through as well as I did without knowing each and every one of you. I am a better scientist and a better person for knowing each of you. Luena and Evan I will miss you both terribly and you are always welcome in my home, and we must have our children be as close as we are. Berenice, I plan to visit you in your new, beautiful home, and look forward to visiting you in Georgia. I wish only the best life has to offer to all three of you.

I would like to thank my wonderful family, who has always stood by me, I cannot list you all individually, for my acknowledgments would be longer than my thesis, but I love and thank you all for always standing beside me. I would especially like to thank my mom and dad, for without them I would not be the woman I am today-I love you.

To Sean, you let me grow and become who I am, and always supported me. You always loved me even when we were miles apart. You were always in my heart, “Together forever, even though we will be apart”, I love you always.

To my daughter Jacqueline, thank you for letting me finish my thesis before breaking “our water”. You have changed my life, and are my angel. I only foresee bright things for your future, I love you.

Last but never least, I dedicate this thesis to Luiga (Louise) Mangiaracina, my beloved Aunt Gina and godmother, and now my angel. One of the last things that you said to me was “I am always here, whatever you need, just ask”. Thank you for “listening” to me this last year and giving me the strength when I needed it most. I love you and miss you, and know I will see you again.

# Table of Contents

**TITLE PAGE** ........ **i**

**COPYRIGHT PAGE** ........ **ii**

**APPROVAL PAGE** ........ **iii**

**ABSTRACT** ........ **iv**

**ACKNOWLEDGEMENTS** ........ **vii**

**TABLE OF CONTENTS** ........ **xii**

**TABLE OF FIGURES** ........ **xiv**

**LIST OF ABBREVIATIONS** ........ **xvi**

**CHAPTER I - INTRODUCTION** ........ **1**

1.1. THE UBIQUITIN-PROTEASOME PATHWAY ........ **4**
*1.1.1. Ubiquitination* ........ **4**
*1.1.2. Deubiquitination* ........ **6**
*1.1.3. The Proteasome* ........ **7**
*1.1.3.1. Components of the Proteasome* ........ **7**
*1.1.3.2. Assembly of the Proteasome* ........ **9**
*1.1.3.3. Mechanisms of Protein Degradation by the Proteasome* ........ **11**
*1.1.3.4. Immunoproteasome* ........ **18**
1.2. AGING IN *DROSOPHILA MELANOGASTER* ........ **20**
*1.2.1. Extended Longevity Mutants* ........ **20**
*1.2.2. Oxidative Stress* ........ **23**
*1.2.3. Mitochondria* ........ **26**
*1.2.4. Dietary Restriction* ........ **28**
*1.2.5. Heat Shock Proteins* ........ **29**
*1.2.6. Gender* ........ **30**
1.3. AGING AND THE UPP ........ **31**
*1.3.1. Age-dependent decrease in proteasome activity* ........ **31**
*1.3.2. Senescence-dependent decrease in proteasome activity* ........ **32**
*1.3.3. The UPP in age-dependent disorders* ........ **36**
1.4. NEURODEGENERATION AND THE UPP ........ **38**
1.5. OVERALL RELEVANCE ........ **40**

**CHAPTER II –AGING PERTURBS THE 26S PROTEASOME ASSEMBLY IN *DROSOPHILA MELANOGASTER*** ........ **41**

2.1. ABSTRACT ........ **42**
2.2. INTRODUCTION ........ **44**

2.3. MATERIALS AND METHODS .......... 47
*2.3.1. Flies* .......... 47
*2.3.2. Peptidase activities in fly extracts* .......... 47
*2.3.3. In-gel proteasome activity and detection* .......... 48
*2.3.4. ATP measurements* .......... 50
*2.3.5. Climbing performance* .......... 51
*2.3.6. Glycerol density gradient centrifugation* .......... 52
*2.3.7. Fly treatment* .......... 53
*2.3.8. Western blot analysis of fly extracts* .......... 54
*2.3.9. Fly survival* .......... 56
*2.3.10. Statistical Analysis* .......... 56
2.4. RESULTS .......... 57
*2.4.1. The peptidase activities of the proteasome are lower in old (43–47 days) than in young (1–2 days) flies* .......... 57
*2.4.2. 26S Proteasome assembly and activity are impaired in old (43–47 days) flies* .......... 58
*2.4.3. 26S Proteasome activity declines sharply in 43 to 47 day old female and male flies* .......... 60
*2.4.4. ATP steady state levels decrease by ~50% in 43 to 47 day old female and male flies* .......... 61
*2.4.5. Climbing performance drops markedly in 43–47-day old female and male flies* .......... 62
*2.4.6. Different fractionation pattern of proteasomes from young (1–2 days) and old (43–47 days) flies* .......... 62
*2.4.7. Old (43–47 days) flies are more sensitive to the proteasome inhibitor PSI than young (1–2 days) flies* .......... 64
2.5. DISCUSSION .......... 66

**CHAPTER III – GENETIC TOOL TO MANIPULATE PROTEASOME ACTIVITY IN DROSOPHILA** .......... 73

3.1. AIM .......... 74
3.2. STRATEGY JUSTIFICATION .......... 75
3.3. METHODS AND RESULTS .......... 78
3.4. FUTURE STUDIES .......... 80
*3.4.1. Transgenic flies* .......... 80
*3.4.2. Analysis of the transformant lines* .......... 81
*3.4.3. Expected outcome* .......... 82
*3.4.4. Anticipated problems and alternatives* .......... 82
*3.4.5. Relevance* .......... 83

**CHAPTER IV – MODEL AND CONCLUSIONS** .......... 84

**CHAPTER V – FUTURE DIRECTIONS** .......... 88

**CHAPTER VI – FIGURES** .......... 93

**CHAPTER VII – REFERENCE LIST** .......... 128

# Table of Figures

Figure 1: Scheme indicating the proteolytic fate of a cellular protein subjected to stress conditions leading to misfolding 94

Figure 2: Ubiquitination and degradation of proteins by the ubiquitin-proteasome pathway (UPP) .......................... 95
Figure 3: Polyubiquitin chains target substrates for degradation by the 26S proteasome .................................. 96

Figure 4: The 26S Proteasome ............................... 97

Figure 5: Protein degradation by the 26S proteasome ......... 98

Figure 6: Predicted partial crystal structure of the *Drosophila* 20S proteasome ........................................ 99

Figure 7: Proteasome activities in young (*1-2 days*) and old (*43-47 days*) flies ........................................ 100

Figure 8: Assessing proteasome levels in flies ............. 102

Figure 9: Proteasome activity and levels in young (*Y, 1-2 days*) and old (*O, 43-47 days*) flies ............................ 104

Figure 10: Proteasome activity in female and male flies across different ages ........................................ 106

Figure 11: Results from FIVE independent experiments showing proteasome activity across different ages in separate as well as combined female and male groups of flies Figure 11: ATP steady state levels in female and male flies across different ages .............................. 108

Figure 12: ATP steady state levels in female and male flies across different age.................................110

Figure 13: Climbing performance of female and male flies across different ages ........................................ 111

Figure 14: Sedimentation velocity analysis of proteasomes from young (*1-2 days*) and old (*43-47 days*) flies ................ 112

Figure 15: Sedimentation velocity analysis of the chymotrypsin-like activity from young (*1-2 days*) and old (*43-47 days*) flies assayed in the absence and presence of the proteasome inhibitor PSI (40μM) ............................................ 114

**Figure 16: Polyubiquitinated protein and proteasome d*β*5 subunit levels in young (*1-2 days*) and old (*43-47 days*) flies** ....... **116**

**Figure 17: Survival curves for young (*1-2 days*) and old (*43-47 days*) flies** .......................................... **118**

**Figure 18: Generation of a catalytically inactive dβ5 proteasome subunit** .............................................. **119**

**Figure 19: Analysis of the genomic DNA encoding the dβ5 gene** **121**

**Figure 20: Sequencing data of the Prosβ5 gene** .............. **122**

**Figure 21: Schematic of P-element transformation** ........... **126**

**Figure 22: Establishment of isolines and localization of inserts by segregation analysis and balancing of fly stocks** ......... **127**

# List of Abbreviations

**AAA** ATPases associated with diverse cellular activities

**AD** Alzheimer's disease

**ALS** Amyotrophic lateral sclerosis

**ATP** Adenosine triphosphate

**cSOD** Copper/zinc superoxide dismutase

**CNS** Central nervous system

**COX** Cytochrome C oxidase

**CP** Core particle

**DA** Dopamine

**Ddc** Dopa decarboxylase

**DNA** Deoxyribonucleic acid

**DMSO** Dimethylsulfoxide

**DTT** Dithiothreitol

**DUBs** De-ubiquitinating enzymes

**ECL** Enhanced chemiluminescent

**EDTA** Ethylenediamine tetraacetic acid

**EGTA** Ethylene glycol tetraacetic acid

**ETS** Electron transport system

**F-MDSCs** Female muscle derived stem cells

**GFP** Green fluorescent protein

**IFN-γ** Interferon-γ

**IGF-1** Insulin-like growth factor 1

***Indy*** *I am not dead yet*

***InR*** *Insulin-like receptor*

**LBs** Lewy bodies

**L-DOPA** Levodopa

**M-MDSCs** Male muscle derived stem cells

**mSOD** Manganese superoxide dismutase

***mth*** *methuselah*

**PAEL-R** Parkin-associated endothelin receptor-like receptor

**PAN** Proteasome-activating nucleotidase

**PBS** Phosphate-buffered saline

**PCR** Polymerase chain reaction

**PD** Parkinson's disease

**PINK1** PTEN-induced kinase 1

**PIP's** Proteasome-interacting proteins

**PSI** N-benzyloxycarbonyl-Ile-Glu(O-t-butyl)-Ala-leucinal

**RNA** Ribonucleic acid

**RNAi** RNA interference

**ROS** Reactive oxygen species

**RP** Regulatory particle

**RUP** Regulated ubiquitin/proteasome-dependent processing

**SDS** sodium dodecyl sulphate

**Superoxide dismutase** SOD

**TCA** Trichloroacetic acid

**TRX** Thioredoxin

**TX** Triton X-100
**Ubiquitin** Ub

**UBPs** Ubiquitin-specific processing proteases

**UCHs** Ubiquitin carboxy-terminal hydrolases

**UCP5KO** UCP5 knockout

**UPP** Ubiquitin-proteasome pathway

**WS** Werner's syndrome

# CHAPTER I

# INTRODUCTION

**NOTE: Portions of this introduction with slight modifications were accepted for publication in the journal Aging Cell (in press):**

**"Aging and regulated protein degradation: Who has the UPPer Hand?"**

**Vita A. Vernace, Thomas Schmidt-Glenewinkel and Maria E. Figueiredo-Pereira**

Department of Biological Sciences, Hunter College of City University of New York,

New York, 10021

The ubiquitin-proteasome Pathway (UPP) is the major pathway for regulated non-lysosomal degradation of intracellular proteins in eukaryotes. Short-lived proteins such as cell cycle regulators and transcription factors, as well as abnormal proteins from the cytosol, nucleus and endoplasmic reticulum are specifically recognized and degraded through this pathway [1;2]. The UPP is of vital importance for maintaining homeostasis and normal function of eukaryotic cells [3]. In addition, the UPP degrades mutant and structurally abnormal proteins, thus preventing their accumulation and aggregation (Figure 1).

Compelling evidence supports the notion that the UPP plays a critical role in aging as well as in the pathogenesis of most neurodegenerative diseases. Accordingly, high levels of oxidized proteins detected in the aging brain are an indication of proteasome impairment, since this proteolytic complex is responsible for degrading the majority of oxidatively modified proteins. In addition, the accumulation and aggregation of ubiquitinated proteins detected in most neurodegenerative disorders is also a sign of UPP dysfunction, since this pathway degrades ubiquitinated proteins. The ubiquitinated proteins accumulate in neuronal lesions, such as neurofibrillary tangles in Alzheimer’s disease, Lewy bodies in Parkinson’s

disease, Lewy body-like inclusions in amyotrophic lateral sclerosis and nuclear inclusions in Huntington's disease.

While it is accepted that aging affects UPP function, the question is why does aging cause a decline in protein degradation by the UPP. The work described in this thesis addresses this question in *Drosophila melanogaster*. By monitoring proteasome activity as the fruit fly ages, the findings presented in this thesis characterize specific mechanisms that contribute to an age-dependent decline in protein degradation by the UPP. In addition, a tool was designed and generated to genetically manipulate proteasome activity in *Drosophila*. This tool will be used in future studies to investigate the specific effects of a decline in proteasome activity in a spatially regulated manner in transgenic flies.

Overall, these studies provide important insights into the role of aging in proteasome dysfunction.

## 1.1. THE UBIQUITIN-PROTEASOME PATHWAY (UPP)

The ubiquitin-proteasome pathway (UPP) requires most proteins to be tagged by ubiquitin to target them for degradation. Therefore, proteolysis by the UPP involves two major steps: ubiquitination and degradation. A de-ubiquitination step also plays important roles in this pathway as it edits the protein state of ubiquitination and removes the ubiquitin tag for recycling.

### 1.1.1. UBIQUITINATION

Ubiquitin (Ub) is a small protein of 76 amino acids and is crucial to the degradation of many cytosolic, nuclear and endoplasmic reticulum proteins [4]. It is so called ubiquitin because it is ubiquitous to every eukaryotic cell. There are, at least, three human ubiquitin genes, two of which, the *polyubiquitin B* and *C* genes, contain heat-shock promoters [5]. Ubiquitination of proteins is a complex process involving the following sequence of events (Figure 2): (1) formation of a high energy thioester bond between Ub and a ubiquitin-activating enzyme (E1) in a reaction that requires ATP hydrolysis; (2) formation of a thioester bond between the activated ubiquitin and

ubiquitin-conjugating enzymes (E2); (3) covalent attachment of the carboxyl terminal of Ub, usually to the ε-amino group of a lysine residue on protein substrates via an isopeptide bond; this reaction is mediated by ubiquitin ligases (E3), which confer substrate specificity to the UPP; and (4) assembly of multiubiquitin chains carried out by a novel family of ubiquitination factors (E4) which promote the production of longer Ub-chains. In some cases, ubiquitin can be transferred directly to the protein substrate by ubiquitin-conjugating enzymes (E2).

At least four molecules of ubiquitin, forming a tetra-ubiquitin chain (Figure 3), need to be attached to the substrate to ensure efficient recognition and degradation by the 26S proteasome machinery [6]. These chains are formed by the successive attachment of monomers by an isopeptide bond, most frequently formed between the side chain of Lys 48 in one Ub and the carboxyl group of the C-terminal Gly76 of a neighboring Ub. Attachment of polyubiquitin chains to lysine residues on a protein results in at least a 10-fold increase in its degradation rate [7]. Longer polyubiquitin chains exhibit increased binding affinity for the 26S proteasome, thus enhancing the degradation of the polyubiquitinated substrate [8]. Together with K48, there are seven lysine residues which

ubiquitin can utilize to link polyubiquitin chains together, which are: K6, K11, K27, K29, K33, K48 and K63. The least common of the "linkage" sites are K27 and K29. K63 linkage is used by several pathways including cell signaling, ribosomal function, DNA repair, activation of NFκB signaling complex and mitochondrial inheritance and morphogenesis. K29 has similar characteristics as K48. K6 linkage has been shown to counteract proteasomal degradation [9].

### 1.1.2. DEUBIQUITINATION

Ubiquitin is removed from ubiquitinated proteins by de-ubiquitinating enzymes (DUBs), which also disassemble polyubiquitin chains. More than 90 genes encoding de-ubiquitinating enzymes are identified, making them one of the largest families of enzymes involved in the ubiquitin pathway [10]. DUBs are cysteine proteases that hydrolyze the amide bond immediately after the COOH-terminal Gly76 [11]. There are two major classes of de-ubiquitinating enzymes: (1) Ubiquitin carboxyl-terminal hydrolases (UCHs) are smaller and remove small amides, esters, peptides and small proteins at the carboxyl terminus of ubiquitin, and (2) the larger ubiquitin-specific processing proteases (UBPs) which disassemble the polyubiquitin chains and

edit the ubiquitination state of proteins [11].

### 1.1.3. THE PROTEASOME

Covalent binding of ubiquitin to proteins marks them for subsequent degradation by the ubiquitin/ATP dependent proteinase known as the 26S proteasome (Figure 4), which is a multicomponent enzymatic complex with a native molecular mass of approximately 2000 kDa [1].

#### *1.1.3.1. COMPONENTS OF THE PROTEASOME*

The 26S proteasome complex is composed of two major particles. A cylinder-like structure, known as the 20S proteasome that is located in the center and is the proteolytic core of the enzyme, and a regulatory component, known as the 19S particle (PA700) , which may be attached to each end of the cylinder-like 20S proteasome [12].

The 19S particle is a multicomponent complex itself. It is composed of at least 17 subunits and is responsible for the ubiquitin-dependent proteolytic pathway. The 26S proteasome can be associated with one 19S particle or two, one at each end. The 19S particle can be further divided into two subcomplexes: the base and the lid. The base confers the complex its ATPase activity and consists of six AAA ATPase subunits (Rpt1-Rpt6) and two non-ATPase subunits

(Rpn 1 and Rpn2), whereas the lid is made up of eight non-ATPase subunits (Rpn3, Rpn5-9, Rpn11, and Rpn 12) which can specifically bind to polyubiquitin-tagged substrates and which may have some deubiquitination activity [12]. Together, all of the 19S components confer ubiquitin/ATP-dependency to proteolysis by the 26S proteasome.

The eukaryotic 20S proteasome consists of 28 subunits, which are arranged in four heptameric stacked rings forming a barrel-like structure, each consisting of 7 protein subunits. The α-type subunits, comprising the two outer rings of the 20S proteasome, provide binding sites for regulatory particles and form a gated channel leading to the inner proteolytic chamber. The β-type subunits contain the enzymatic-active sites of the 20S proteasome (Figure 5). The whole particle is a dimer with an $\alpha_7\beta_7\beta_7\alpha_7$ subunit arrangement [13].

The 20S proteasome degrades unfolded proteins into short peptides (of 8-9 amino acids) while the 19S proteasome guides proteins into the 20S proteasome chamber. Association between the two particles in the cell is a dynamic process and requires ATP-hydrolysis.

The 20S particle may be attached to one or two 19S particles, or it may be alternatively capped by other

complexes, such as the 11S activator (PA28). The PA28 is a cytoplasmic complex formed by equal, stoichiometric amounts of two different 28 kDa subunits, PA28α and PA28β, which are thought to form a 200 kDa heterohexamer. PA28 capped 20S proteasomes prefer substrates that are partially degraded proteins and peptides, rather than intact polyubiquitinated molecules [14].

The conserved homohexameric ring-shaped AAA ATPase known as Cdc48 in budding yeast and p97 in mammals, is also a component of the UPP. Cdc48 is a conserved chaperone-like ATPase of eukaryotic cells, which is linked to the UPP because of its ability to segregate ubiquitinated substrates from unmodified partners. Cdc48 was originally identified as a protein required for cell division. This complex is required for the degradation of artificial model substrates and is involved in the ubiquitin dependent activation of some transcription factors. Cdc48 has the ability to bind ubiquitinated proteins and unfold them prior to delivery to the proteasome.

#### ***1.1.3.2. ASSEMBLY OF THE PROTEASOME***

It is known that the 26S proteasome requires ATP hydrolysis for ATP-dependent protein degradation of ubiquitinated substrates and for the 26S proteasome

assembly. However, the roles of ATP hydrolysis in protein degradation and also in the assembly of the 26S proteasome are still unclear.

To elucidate the role of ATP in proteolysis, archaeal 20S proteasomes and the PAN (proteasome-activating nucleotidase) regulatory complex, a homolog of the eukaryotic 19S ATPases have been studied. PAN is a molecular chaperone that catalyzes the ATP-dependent unfolding of globular proteins in archaebacteria. PAN's ATPase activity can be stimulated by globular (GFP-ssrA) and unfolded (casein) substrates, and by the ssrA recognition peptide. During degradation of one molecule of globular or unfolded substrates, it was calculated that 300-400 ATP molecules were hydrolyzed. Substrate binding activates ATP hydrolysis, which promotes three processes: substrate unfolding, gate opening in the 20S particle, and protein translocation [15].

ATP hydrolysis is also necessary for the rapid dissociation of the 26S proteasome into the 19S and 20S particles. Recent studies dispute previous data suggesting that ATP plays no role in proteasomal stability during the catalytic cycle. The researchers observed the dissociation of the 19S and 20S particles when PIP’s (PIP=proteasome-interacting proteins) were released from the proteasome

[15]. The formation of product peptides seems to trigger a conformational change in the adjacent ATPases by inducing an allosteric change within the 20S proteasome. Allosteric transitions are known to play a role in the function of the proteasome.

Previous studies with yeast proteasomes demonstrated that each ATPase plays a functional role in the binding and hydrolysis of ATP. For example, inactivation of the Rpt2 ATPase alone inhibits the opening of the gating channel on the 20S proteasome. The Rpt5 ATPase interacts with substrate-attached polyubiquitin chains, suggesting a role in substrate recruitment. The Rpt1 and Rpt6 ATPases were shown to bind Ubr1 and Ufd4, which are E3 ligases, implying that these ATPases play a role in the recruitment of the ubiquitination machinery [16].

### ***1.1.3.3. MECHANISMS OF PROTEIN DEGRADATION BY THE PROTEASOME***

#### ***Processive Proteolysis and Endoproteolysis***

Substrate proteolysis by the proteasome involves sequential catalytic steps suggesting a "bite-chew" processive model. An alternative non-processive model of degradation was proposed in studies with higher organisms

such as in mammals. The non-processive mechanism comprises multiple, independent cleavages with dissociation of the degradation intermediates [17].

The proteasome degrades proteins into small peptides, but in a few cases, the degradation results in limited protein processing which yields proteins of different biological activity. Recognition of ubiquitin conjugates by the 26S proteasome does not always lead to complete degradation. In some cases the proteasome only degrades specific protein segments and leaves other parts of the substrate intact. This process, termed "regulated ubiquitin/proteasome-dependent processing" or RUP is essential for the function of certain transcription factors and crucial for their regulation.

Proteolysis by the proteasome is a highly processive event indicating that RUP must invoke specific mechanisms that restrict activity of the proteasome. Studies by two different groups suggest that proteolysis by the proteasome does not always start at the N- or C-terminal ends of the substrate, but can also occur at internal polypeptide loops [18] [19]. It was demonstrated that the flexible domains within a substrate can promote hairpin formation and that the hairpins make the first contact with the active sites

within the 20S proteasome. To integrate the two aspects of protein processing into a coherent model, it was suggested that protein processing by the proteasome is initiated by the translocation of flexible domains into the proteasome. These flexible domains could be either the N- or C-terminal ends of the polypeptide chain or internal protein loops. Degradation then proceeds towards both ends of the polypeptide chain but will come to a halt when the proteasome reaches tightly folded protein domains. Segments that are not restricted by tightly folded barriers are completely degraded, whereas the folded domains (and sequences beyond these domains) are spared from degradation [18].

Similar results were obtained with the NFκB relatives Spt23 and Mga2 [20]. The tightly folded domain at the C-terminus of Mga2 does not prevent the formation of the N-terminal p90 fragment. This excludes the possibility that the processing reaction starts from the molecule’s C-terminus. In fact, the processing reaction produced an N-terminal p90 fragment and a C-terminal fragment. This finding suggests a clear precursor-product relationship. Furthermore, it strongly supports internal initiation of processing followed by bidirectional degradation toward both ends of the polypeptide, until full degradation is

challenged by tightly folded domains [20]. Thus, the proteasome possesses endoproteolytic activity. This proteolytic activity refers to the ability of proteasomes to cleave substrates in an internal region even when the ends are unable to enter the proteolytic chamber [19].

***Access to the Proteolytic Chamber***

The conventional view of how substrates enter the proteasome is that it is regulated by activators and thought to proceed sequentially, starting from one end of the substrate polypeptide. The catalytic active sites of the proteasome can hydrolyze most sequences, and it is essential that the inappropriate substrates be protected from indiscriminate proteolysis. This is ensured by the architecture of the 20S proteasome, which has the active sites in the central chamber of the 20S particle, protected by the compartments of the α-subunits in the ante-chambers. To enter the 20S proteasome, the substrate must pass the α-subunits, which exclude folded proteins. The N-terminal residues of the α-subunits form a gate. Substrates are delivered to the interior of the 20S proteasome by the 19S particle. Substrates are recognized by the 19S particle through their posttranslational ligation to ubiquitin. The

19S proteasome binds the polyubiquitin chain, cleaves the bond connecting polyubiquitin and substrate, unfolds the substrate, opens the gate to the 20S particle and translocates the substrate into the catalytic chamber.

*In vitro* studies by Liu et al. (2003) indicate that some substrates enter the 20S particle without the assistance of an activator. These substrates enter the proteasome in a hairpin conformation rather than starting from a free polypeptide terminus. Liu's group generated chimeras of natively unfolded proteins consisting of p21 or α-synuclein with a stably folded GFP. In all of their experiments, the p21 and α-synuclein portions of the substrates were degraded, whereas the GFP domain was not. This is consistent with the inability of the folded GFP to pass the α-annulus. Only the flexible parts of the chimeric substrates were degraded when the GFP was either at the N- or C- terminus, as the unfolded chains could enter the 20S proteasome. Previous studies demonstrated that the 26S proteasome unravels and degrades substrate from whichever end is tagged (e.g. with ubiquitin). The new data obtained by Liu *et al.* further demonstrate that some untagged substrates can pass the α-annulus in either direction. Interestingly, the unfolded domains of p21 and α-synuclein were proteolysed even when they were generated as

covalently closed circular constructs with no free termini. These observations strongly argue that flexible regions of proteasome substrates can pass through the α-annulus in a hairpin conformation [19].

### ***The Three Peptidase Activities of the 20S Proteasome***

Polyubiquitinated proteins are degraded by the 26S proteasome. Its proteolytic core is the 20S proteasome. Among the 14 different subunits (7 α and 7 β) of the 20S proteasome, only three were found to have active sites for peptide bond hydrolysis, namely β1 (caspase-like), β2 (trypsin-like), and β5 (chymotrypsin-like). The chymotrypsin-like activity cleaves after amino acids with large or hydrophobic side chains, the trypsin-like activity cleaves after basic residues, and the caspase-like activity is a post-glutamyl activity that recognizes acidic amino acid residues.

The β5-associated chymotrypsin-like activity seems to be the initial and the rate-limiting step in protein degradation by the 20S proteasome [21]. During this initial step, protein substrates are cleaved into large peptide fragments. In the following steps of degradation, the caspase-like activity associated with the β1 subunit and the

trypsin-like activity associated with the β2 subunit further break down the fragments generated in the initial step. These sequential catalytic steps suggest a "bite-chew" processive model for protein breakdown through the UPP although non-processive models have also been proposed (see above *"Processive Proteolysis and Endoproteolysis").* Notably, in any of the models currently proposed, the chymotrypsin-like activity associated with the β5 subunit is essential for protein degradation by the proteasome. Studies with yeast [3] and *Drosophila* [22] demonstrated that a functional β5 subunit is essential for survival.

The 20S proteasome is a threonine protease in which the nucleophilic attack is mediated by the N-terminal Thr of processed, catalytically active β subunits [23]. The three β subunits (β1, β2 and β5) bearing the active sites are first synthesized as precursor proteins each containing a propeptide at the N-terminus, which must be cleaved off for the subunits to become catalytically active [24]. In higher eukaryotes, maturation of catalytic β subunits is a two-step autocatalysis where an intact Gly-1/Thr+1 consensus motif coupled to Lys33 is essential for correct processing. The cleavage is carried out by Thr1, as the nucleophile, and Lys33, as the proton donor/acceptor [24]. Processing of the

three catalytically active subunits into mature forms occurs only after their incorporation into the 20S proteasomes.

### ***1.1.3.4 IMMUNOPROTEASOME***

Specialized proteasomes called immunoproteasomes have an 11S regulator (PA28) and are induced by interferon-γ (IFN-γ). IFN-γ is a cytokine that is released in early stages of viral infection and inhibits cell growth. IFN-γ alters proteasome activity qualitatively by incorporation of three immunosubunits LMP2 (β1i), LMP7 (β5i) and MECL-1 (β2i) to replace the constitutive β1, β2 and β5 subunits in the 20S core proteasome. The IFN-γ inducible β-subunits of the 20S proteasome enhance its capacity to generate antigenic epitopes and modify the turnover rate of specific proteins [25]. Thus, two types of proteasomes exist, the 'constitutive proteasomes', which are expressed in all somatic cells, and the 'immunoproteasomes', which are expressed under the influence of cytokines. IFN-γ also regulates the expression of two other proteins, PA28α and PA28β, which form the heptameric proteasome activator complex PA28. This heteromultimer is able to bind to the α-rings of the 20S core proteasome, thereby enhancing proteolytic activity. Interestingly, aging induces several

alterations related to proteasome activity, proteasome content, and structural replacement of proteasomal subunits (see below under ”Aging and the UPP“). However, immunoproteasomes seem to not be influenced by aging [25].

## 1.2. AGING IN *DROSOPHILA MELANOGASTER*

The fruit fly is an extremely attractive *in vivo* model to study aging, owing to a combination of a short life span (~60 days), easy genetic manipulation, rapid screen for mutations, relatively low cost and a biological complexity that is, in many ways, comparable to that of a mammal. Critical mechanisms that participate in the *Drosophila* aging process are likely to be regulated similarly in humans. The knowledge obtained from studies with *Drosophila* can thus be applied toward the understanding and prevention of the deleterious effects of human aging.

### 1.2.1. EXTENDED LONGEVITY MUTANTS

In *Drosophila melanogaster*, several mutations have been shown to extend longevity: *Methuselah,* encoding a putative G-protein coupled receptor, *Indy,* encoding a sodium dicarboxylate cotransporter, *chico,* encoding an insulin receptor substrate, and *InR,* encoding an insulin-like receptor.

In 1998, Lin et al., identified the *Methuselah (mth*) mutant whose longevity is 35% longer than wild-type flies [26]. These flies are also resistant to various stressors

including high temperature, starvation, and being fed paraquat. The *mth* gene encodes a protein similar to G-protein coupled receptors. The function of the Methuselah protein is unknown but it may be involved in regulating the stress response pathway.

In 2000, Rogina et al., identified *Indy* (*I am not dead yet)*, which is a long-lived mutant exhibiting a doubling of mean lifespan and a 50% increase in maximal lifespan [27]. *Indy* encodes a protein similar to mammalian sodium dicarboxylate cotransporters responsible for the uptake or reuptake of di- and tricarboxylic acid Krebs cycle intermediates, such as succinate, citrate, and alpha-keto-glutarate. *Indy* is expressed in the fat body, gut, and oenocytes, suggesting that it may play a role in both the absorption of metabolites and in intermediary metabolism. The reproductive period is also extended in the *Indy* mutant. *Indy* heterozygous long-lived females continue to produce viable offspring 40% longer than wild type flies.

In 2001, Clancy et al., described the *chico* mutant, encoding an insulin receptor substrate. Females exhibit increases in median lifespan (48%) and maximum lifespan (41%). 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 [28].

In 2001, Tatar et al., examined the effects of *Insulin-like receptor (InR)* mutations on *Drosophila* lifespan. They used heteroallelic combinations of *InR* alleles to produce viable and dwarf adults with substantially low level of INR kinase activity. Among the transgenic flies that were generated, only $InR^{p5545}/InR^{E19}$ females showed an extended longevity phenotype. Survivorship of $InR^{p5545}/InR^{E19}$ males was compromised as they exhibited high mortality as early adults. This high mortality could be due to potential developmental defects carried over to adults [29]. The endocrine system of *InR* dwarf female flies is anomalous. Their developmental time is extended from 10 to 20 days, but their body size is severely but proportionally reduced. The mutant flies are approximately half the weight of their heterozygous siblings, and they are sterile [2].

In conclusion, identification of intracellular pathways affected by these mutations is crucial to the dissection of the molecular mechanisms associated with aging and longevity determination. A great number of the genes that are consistently up-regulated in the extended longevity mutants are those encoding stress resistance proteins that

protect against oxidative stress and thermal damage. Since these conditions are associated with damaged proteins and the UPP is one of the major pathways that degrades intracellular damaged proteins, we reasoned that it is likely that proteasome activity is altered with aging. The studies described in this thesis address this hypothesis.

### 1.2.2. OXIDATIVE STRESS

Extended longevity phenotypes were also observed in transgenic flies overexpressing antioxidant enzymes, such as *Cu/Zn superoxide dismutase (SOD)* and *catalase* or *Cu/Zn SOD* only [30].

*Drosophila melanogaster* display an age related increase in oxidative damage. The cellular oxidative defense system in flies consists of the enzymes superoxide dismutase and catalase. Superoxide, the initial reactive oxygen species (ROS) derived from the electron transport system (ETS), is converted to $H_2O_2$ by SOD, and catalase reduces $H_2O_2$ to water and molecular oxygen. If $H_2O_2$ cannot be eliminated, hydroxyl radicals, considered to be the main species inflicting oxidative damage, are formed. *Drosophila* express two SOD genes that encode a *manganese* (mSOD) and a copper/zinc enzyme (cSOD), one of which is found located within the

mitochondrial matrix, while the other is found in the cytoplasm.

Flies with null mutations in the *sod* genes are hypersensitive to paraquat and exhibit reduced longevity. Hypersensitivity to hyperoxia, glutahione depletion, and ionizing radiation was rescued by expression of the wild-type *Cu/Zn SOD* transgene [31] in these flies. Furthermore, overexpressing human SOD1 specifically in adult motorneurons dramatically extends the longevity (up to 40%) of the transgenic flies and rescued other defects of the null mutants.

Catalase activity does not seem to limit lifespan, since mutant flies with 14% of catalase activity had normal longevity [31]. However, Schwarze et al., 1998 demonstrated that catalase null mutants exhibit a reduced life span, male sterility, and are hypersensitive to $H_2O_2$. They also demonstrated that flies overexpressing catalase and cSOD exhibit increased longevity and less oxidative damage. Fly lines with low catalase expression have a mean life span similar to that of Oregon R flies (wild-type), but when exposed to $H_2O_2$ the mortality in the mutants increases [32]. These data suggest that the effects of catalase on longevity are dependent on the targeted cell types and the level of its expression.

Parkin-associated endothelin receptor-like receptor (Pael-R), which has been implicated in the pathogenesis of Parkinson's disease (PD), is a substrate for the E3 ubiquitin ligase Parkin. Overexpression of *human Pael-R* in *Drosophila* has been shown to induce selective loss of dopaminergic (DA) neurons, which is one of the symptoms of PD. Thioredoxin (TRX) is an evolutionarily conserved antioxidant and molecular chaperone. The *Drosophila* genome contains three TRX-encoding genes, namely *TrxT*, *Trx-2* and *dhd*. When each of the TRX genes was overexpressed together with *Pael-R* in all neurons, the number of DA neurons and level of locomotor activity were significantly increased compared to control flies. Furthermore, in the absence of *Pael-R*, overexpression of TRX in all neurons increased the level of locomotor activity in aged flies and extended their mean longevity by 15% [30].

In conclusion, there is no doubt that oxidative stress is associated with aging. Signs of oxidative stress, such as lipid peroxidation, increased protein carbonyls and a decline in reduced glutathione, are considered hallmarks of aging. Oxidative stress, especially the production of free radicals and ensuing lipid peroxidation, promotes partial unfolding of cellular proteins resulting in the exposure of previously buried hydrophobic domains to ubiquitin-

conjugating as well as to proteolytic enzymes, such as the proteasome. It is clear that the proteasome plays a role in the removal of oxidatively-modified proteins.

### 1.2.3. MITOCHONDRIA

The aging process is known to affect mitochondrial function and to be associated with mitochondrial genome changes including point mutations and modifications as well as deletions [33].

*Drosophila melanogaster* display an age related decrease in mitochondrial transcripts. 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 [32]. Aged flies showed a reduction in steady-state mtRNA levels, exhibiting significant decreases (80–92%) in the mitochondrial-encoded 16s ribosomal RNA, NADH dehydrogenase subunit I and cytochrome b transcripts. Cytochrome c oxidase activity (COX) declined (48%) progressively during aging, from 1.85 μmol/min/mg at 2 days to 0.96 μmol/min/mg at 53 days. No statistically significant changes were found with aging in the ETS complexes 1 or 2. Citrate synthase activity tended to decline with age but this decrease was not statistically significant [32].

Other mitochondrial mechanisms are associated with aging. UCP5 is a novel functional mitochondrial uncoupler in *Drosophila*. UCP5 knockout (*UCP5KO*) flies are highly sensitive to starvation stress and this phenotype can be reversed by ectopically expressing UCP5 in neurons. *UCP5KO* flies live longer than controls on low-calorie diets, have a decreased level of fertility, and gain less weight than controls on high-calorie diets. However, isolated mitochondria from *UCP5KO* flies display the same respiration patterns as controls. Furthermore, total ATP levels in both *UCP5KO* and control flies are similar. However, *UCP5KO* flies have a lower body composition of sugars, and during starvation stress their triglyceride reserves are depleted more rapidly than controls. It was suggested that the loss of UCP5 activity in flies causes an unusual hormonal response leading to lower sugar levels and altered metabolic homeostasis [34]. Interestingly, targeted overexpression of another uncoupling protein (human UCP2) in the mitochondria of adult fly motorneurons led to a decrease in the generation of reactive oxygen species (ROS), a decrease in oxidative damage and a lifespan extension [35].

The PTEN-induced kinase 1 (PINK1) protein is localized to the mitochondria and is associated with sporadic forms

of PD. In *Drosophila*, PINK1 knockdown leads to mitochondrial fragmentation and increased sensitivity to multiple stresses. If PINK1 knockdown flies are treated with antioxidants, this protects them from developing PD-like neurodegeneration [36].

Overall, these studies support the notion that mitochondrial dysfunction plays an important role not only in the aging process but also in neurodegeneration.

### 1.2.4. DIETARY RESTRICTION

It is well established that dietary restriction increases the lifespan of *Drosophila melanogaster* as well as of other organisms. Recent studies involving genome expression data analysis revealed that genes encoding odorant-binding proteins were strongly affected by both age and nutrient availability. Canton S wild type flies and *Or83b* mutants were tested. Of the 62 putative odorant receptors in *Drosophila, Or83b* is atypical in that it is broadly expressed throughout olfactory tissues, interacts with conventional odorant receptors and is required for their localization to neuronal dendrites [37]. The lifespan of *Or83b*$^{2}$ mutants were measured in the presence and absence of odorants from live yeast. Fully fed female *Or83b*$^{2}$ mutant flies exhibited a 56% increase in median lifespan when

compared to wild-type animals. Mutant males were also significantly long-lived, but the magnitude of the extension was generally smaller. Heterozygous flies exhibited intermediate longevity in both sexes [38]. This interesting finding provides evidence that odorants are sufficient to modulate lifespan.

### 1.2.5. HEAT SHOCK PROTEINS

Transgenic flies overexpressing the heat shock protein (molecular chaperone) Hsp70 exhibit extended longevity phenotypes [30].

Heat shock proteins are up-regulated in response to aging. In 1997, Tatar et al., investigated the effect of the Hsp70 protein on survival during aging at normal temperatures using transgenic flies carrying a total of 12 additional copies of the *hsp70* gene. Heat shock for 10 or 15 min at 36°C improved survival over the following 2-week period at 24°C (the normal temperature). There was a positive correlation between Hsp70 levels and the degree of survival improvement, suggesting that Hsp70 plays a protective role from damages caused by aging [39].

**1.2.6. GENDER**

Recent studies demonstrated that male flies exhibit Parkinson-like symptoms induced by paraquat, earlier than females. Separate male and female flies were fed paraquat (10-20mM) starting at 1-2 days of age. 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. To try to rescue the mobility deficits by feeding the flies with paraquat, flies were co-treated with paraquat and levodopa (L-DOPA)/and or DA for 24 hours. Both L-DOPA and DA rescued the mobility deficit to 85-90% of control levels.

Notably, males exhibited the symptoms described above, 12 hours earlier than females. Males exhibited near complete loss of mobility at 24 hours and by 36 hours were totally immobile. Females were affected, but still retained some capacity for movement even at 36 hours. 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 of PD in humans, in which incidence rates of PD in males is 1.5 to 2.5 times greater than in females [40].

## 1.3. AGING AND THE UPP

### 1.3.1. Age-dependent decrease in proteasome activity

One of the most accepted theories of aging is the loss of quality control in protein turnover with the concomitant build-up of oxidatively modified proteins. Since proteasomes selectively degrade oxidatively damaged as well as ubiquitinated proteins it is postulated that proteasome activity declines with aging.

A loss of proteasome activity with aging is supported by decreased subunit expression, alterations and/or replacement of proteasome subunits and formation of inhibitory cross-linked proteins [41]; [42]. For example, in 2000, Keller et al., reported decreased chymotrypsin-like activity in the hearts, lungs, kidneys, livers, spinal cords, hippocampi, and cerebral cortexes of 12, 24 and 28 month old Fisher 344 rats compared to 3 week and/or 3 month old animals [43]. Furthermore, microarray analysis of age-related variations in gene expression patterns were reported for both mitotic (human fibroblasts) and post-mitotic (rat skeletal myocytes) cells [44]. Less than 2% of the 6347 genes monitored were affected by age under either condition. Several genes encoding either 20S proteasome or 26S proteasome subunits were found to decline with age in

both situations. Caloric restriction was found to maintain or increase proteasomal subunits and activator (*PA28*) gene expression in the rat skeletal muscle cells. Recent studies with human epidermal cells and rat myocardiac cells, also demonstrated that the accumulation of oxidatively modified proteins is associated with decreased proteasome activity and content, implying that proteasome expression is down-regulated with age [41]. Other investigators have determined that proteasome activity and expression changes with age but there is no consensus because the ages and cell types tested vary from study to study [42]. Clear mechanisms explaining the observed changes are still lacking. Food restriction, which is currently the only experimental paradigm that halts the aging process, was shown to prevent the age-dependent changes in proteasome function and structure in mice and rats, further supporting the notion that the proteasome plays a role in the aging process [45].

### 1.3.2. Senescence-dependent decrease in proteasome activity

Human fibroblasts in culture have a limited proliferative capacity and after several passages enter a state of senescence. Senescent cells are viable and functional, but have altered genetic and biochemical characteristics when

compared to young cells. Fibroblast senescence is a good model for studying aging at the cellular level and has been used in many studies to address the role of the proteasome in aging.

Human senescent fibroblasts exhibit a reduction in the levels of all three proteasome activities, proteasome content and proteasome subunit expression levels when compared to young passage cells [46]. All three proteasome activities were found to be significantly decreased by 2 to 4 fold compared to young passage cells. While the protein expression levels of the three proteolytically active subunits (β1, β2, and β5) were reduced in the late passage/senescent cells, there was no significant change in the expression of β7, α4, α6, and α7 subunits. The subunits of the 19S particle were also analyzed: S4, S5a, S6a, S6b, S8, and S14 were all down regulated in the late passage cells. In addition, senescent fibroblasts contained increased levels of oxidized as well as ubiquitinated proteins.

Early and late passage fibroblasts respond differently to treatment with IFN-γ. While young cells exhibited an increase in chymotrypsin-like and caspase-like activities following treatment with IFN-γ, senescent cells were not affected. The inability to respond to IFN-γ (by increasing

the chymotrypsin-like activity) was observed only when cells became irreversibly growth arrested. Moreover, IFN-γ treatment induced an increase in the levels of the immunosubunits in early passage cells, while there was no significant change in the constitutive subunits. In senescent cells, the induction of the immunosubunits did not occur [47].

To establish the effect of proteasome inhibition on cell proliferation and lifespan, fibroblasts were treated with proteasome inhibitors, such as epoxomicin and MG132. Epoxomicin is an irreversible inhibitor that mainly blocks the chymotrypsin-like and trypsin-like activities of the proteasome, whereas MG132 is a reversible inhibitor that acts mostly on the chymotrypsin-like activity of the proteasome. Treatment with these proteasome inhibitors reduced the chymotrypin-like activity right after treatment and low levels were still recorded one week later. The caspase-like activity was not immediately affected but neither inhibitor specifically blocks this activity [48]. Fibroblast treatment with proteasome inhibitors led to shortened lifespan, induction of a senescent-like phenotype and a dose-dependent impairment of proliferative potential [49].

In an attempt to overcome the decrease in proteasome activity observed in the senescent fibroblasts, β1 or β5 subunits were stably transfect into fibroblasts. Overexpression of the β5 subunit resulted in the overexpression of the β1 subunit and vice versa. In addition, these clones exhibited an increase in the chymotrypsin-like and caspase-like activities of the proteasome. Compared to the vector transfected cell lines, all clones overexpressing the β subunits exhibited higher survival rates when treated with the proteasome inhibitors epoxomicin or MG132 or with various oxidants and stressors [50].

Another strategy used to prevent the decrease in proteasome activity associated with senescence involved overexpressing the proteasome chaperone POMP. UMP1 and POMP proteins (yeast and human, respectively) are key molecules in the process of proteasome assembly. UMP1 is required for autocatalytic active site maturation and assembly. POMP protein interacts with the 20S proteasome and is the human homologue of UMP1. POMP overexpression in fibroblasts led to increased levels of assembled and functional proteasomes and these clones were also able to more efficiently cope with oxidants and other stressors [50].

Together these studies strengthen the prospect of

genetically manipulating the proteasomal system as a promising therapeutical approach to preventing some of the deleterious effects of aging.

### 1.3.3. The UPP in age-dependent disorders

The UPP plays a role in age-related disorders such as sarcopenia and progeroid syndromes. Sarcopenia is an age related form of muscle wasting. It is coupled to a decrease in IGF-1 signaling and to higher levels of free ubiquitin in human and rat skeletal muscle of aged individuals compared to young ones. Interestingly, injecting free ubiquitin into young healthy rats induces muscle degeneration and mimics the muscle wasting of the old rats, showing that increases in free ubiquitin contribute to muscle wasting [51].

Components of the UPP were found to interact with the disease-causing mutated proteins in at least two segmental progeroid syndromes. Werner's syndrome (WS) is an autosomal recessive disease manifested by the premature onset of age-related phenotypes. Individuals with WS are characterized by a shorter than normal stature together with characteristics of normal aging, such as cataracts, osteoporosis, arteriosclerosis, hair graying, and skin aging, but they manifest these characteristics at an

earlier age [52]. The gene that is defective in WS is the *WRN* gene, which was identified in 1996 by Yu's research group [53] and is a member of the Relq family of helicases [54]. The helicase WRN associates with Cdc48/p97, an AAA ATPase implicated in the UPP [55].

RECQL4 is the dysfunctional protein in Rothmund-Thomson syndrome, another progeroid syndrome. RECQL4 interacts with UBR1 and UBR2, which are E3 ligases involved in the N-end rule pathway, although RECQL4 is not a substrate for these enzymes [56].

Together these findings clearly demonstrate that there is a relationship between the UPP and the age-related diseases described above.

## 1.4. NEURODEGENERATION AND THE UPP

There is no doubt that functional changes in the UPP are critical to the neurodegenerative process. Although selective sets of neurons are affected in different neurodegenerative disorders most of them share an intriguing morphological feature, namely the accumulation of ubiquitinated proteins (reviewed in [57]). In general, high levels of ubiquitinated proteins do not accumulate in healthy cells as they are rapidly degraded by the UPP. The inability to eliminate ubiquitinated proteins may result from a functional failure of the UPP or from structural changes in the protein substrates rendering them inaccessible to the degradation component. The UPP may, therefore, play a role in mechanisms such as oxidative stress, inflammation and apoptosis, all of which are implicated as mediators of abnormal protein deposition and cell death in neurodegeneration.

Recent findings that mutations in ubiquitin and other components of the UPP are associated with some forms of neurodegenerative disorders indicate that the ubiquitin aggregates may hold a clue to the pathological process in neurodegeneration. For example, a mutant form of ubiquitin,

known as $Ub^{+1}$, was detected only in brains of Alzheimer's disease patients and not in age matched controls [58]. $Ub^{+1}$-capped polyubiquitin chains were shown to be refractory to disassembly by de-ubiquitinating enzymes and to potently inhibit proteasome degradation of a polyubiquitinated substrate [59]. Moreover, mutations in two genes, namely *uch-l1* and *parkin*, were found to be associated with the pathogenesis of autosomal inherited cases of Parkinson's disease. UCH-L1 is a ubiquitin hydrolase and parkin is a ubiquitin ligase, thus implicating the UPP in the etiology of some familial cases of Parkinson's disease.

Collectively, these findings indicate that the UPP is deficient in neurodegenerative disorders characterized by the accumulation of ubiquitinated proteins. One of the major challenges that we are faced with is to single out the UPP as a therapeutic target for prevention of neurodegeneration. The challenge rests on developing therapeutic strategies that will enhance and maintain degradation of oxidatively-modified and toxic proteins generated by a lifetime's worth of environmental damage without compromising the normal function of the UPP.

## 1.5. OVERALL RELEVANCE

Advancements in gene cloning techniques and gene expression analyses provide compelling evidence linking the UPP with the turnover of many proteins required to maintain cellular homeostasis. These findings address new and exciting questions concerning the impact of a deregulation of proteolysis on cellular function and its causal relationship to the intracellular deposition of abnormal proteins associated with aging and neurodegeneration.

The findings described in this thesis shed light on critical mechanisms that contribute to proteasome impairment and protein aggregation. The outcome of our research is clearly relevant to the aging process as well as to the prevention and/or treatment of most neurodegenerative disorders characterized by the build-up of abnormal and ubiquitinated proteins. Furthermore, the genetic tool that we developed, which will permit the spatial manipulation of proteasome activity in *Drosophila*, will be clearly instrumental in the identification of proteasome-affected mechanisms. This knowledge will be critical to the development of potential therapeutic strategies that deal with proteasome impairment.

# CHAPTER II

# AGING PERTURBS 26S PROTEASOME ASSEMBLY IN *DROSOPHILA MELANOGASTER*

**Vita A. Vernace, Lisette Arnaud, Thomas Schmidt-Glenewinkel and Maria E. Figueiredo-Pereira**

Department of Biological Sciences, Hunter College of City University of New York,

New York, New York 10021

The FASEB Journal, Volume 21, September 2007,

Published online on April, 5, 2007

## 2.1. ABSTRACT

Aging is associated with loss of quality control in protein turnover. The ubiquitin-proteasome pathway is critical to this quality control as it degrades mutated and damaged proteins. We identified a unique aging-dependent mechanism that contributes to proteasome dysfunction in *Drosophila melanogaster*. Our studies are the first to show that the major proteasome form in old (43-47 days old) flies is the weakly active 20S core particle while in younger (1-32 days old) flies highly active 26S proteasomes are preponderant. The steep decline in 26S proteasome levels occurs in females and males after ~30 days of age attesting that it is an "old-age" event. Old flies also exhibit a decline (~50%) in ATP levels, which is relevant to 26S proteasomes as their assembly is ATP-dependent. Remarkably, the major reduction in ATP and 26S proteasome levels coincides with a marked drop in locomotor performance. Furthermore, treatment with a proteasome inhibitor increases ubiquitinated protein levels and shortens the lifespan of old but not young flies. In conclusion, our data reveal a previously unknown mechanism that perturbs proteasome activity in "old-age" female and male *Drosophila* most likely depriving them of the ability

to effectively cope with proteotoxic damages caused by environmental and/or genetic factors.

## 2.2. INTRODUCTION

In the last decade, molecular geneticists tried to identify genes that regulate longevity in yeast, worms, flies and mice (reviewed in [60]). At least four mutations were shown to extend longevity in *Drosophila melanogaster*: *methuselah*, encoding a putative G-protein coupled receptor, *Indy*, encoding a sodium dicarboxylate cotransporter, *chico*, encoding an insulin receptor substrate and *InR* encoding the insulin receptor (reviewed in [31]). The majority of genes that are consistently up-regulated in the extended longevity fly mutants are those encoding stress resistance proteins that protect against oxidative stress and thermal damage. Since these conditions are associated with damaged proteins and the ubiquitin-proteasome pathway (UPP) is the major pathway that degrades intracellular damaged proteins, it is likely that UPP impairment plays a critical role in the aging process.

A fundamental characteristic of aging [61] and age-related neurodegenerative disorders [5;62] is the accumulation and aggregation of ubiquitinated proteins in abnormal neuronal inclusions, such as neurofibrillary tangles in Alzheimer’s disease and Lewy bodies in Parkinson’s disease. The mechanisms causing the aggregation

of ubiquitinated proteins and its role in aging and age-related neurodegeneration remain elusive. No apparent changes in the levels of ubiquitin or ubiquitinating enzymes (E1, E2 and E3) with aging are reported in the literature (reviewed in [61]). However, one must keep in mind that due to the vast numbers and widely divergent substrates of E2 and E3 classes of enzymes, age-dependent changes in many of these enzymes remain to be assessed [61]. Conversely, a decline in proteasome activity with the aging process has been shown in a variety of mammalian organs and tissues (reviewed in [63]). The loss of proteasome activity with aging has been associated with decreased subunit expression, alterations and/or replacement of proteasome subunits and formation of inhibitory cross-linked proteins (reviewed in [41;42]). Food restriction, which is currently the only experimental paradigm that halts the aging process, prevents the age-dependent changes in proteasome function and structure in mice and rats, further supporting the notion that the proteasome plays a role in the aging process (reviewed in [45]). Proteasome dysfunction thus provides a link between environmental and genetic factors associated with aging and aging-related neurodegeneration.

Herein, we demonstrate a significant decline in

proteasome activity in female and male old (43-47 days of age) flies compared to younger (up to ~30 days of age) flies. Notably, we found that the major proteasome form in old flies is the 20S core particle, while in younger flies the majority of proteasomes consist of the 26S holoenzyme form. These findings establish that autoinhibited 20S proteasomes prevail in old flies whereas the fully-assembled 26S proteasome is highly active in young flies. Our results support the view that an aging-dependent disassembly of the 26S proteasome is an important risk factor in aging. Assembly of the 26S proteasome is an ATP-dependent process. We also established that ATP steady state levels decline by 50% in old versus younger flies. Other studies also demonstrated a decline in ATP levels with aging in *Drosophila* [32]. The observed ATP-depletion will perturb 26S proteasome assembly and have a negative impact on normal protein turnover and on the ability of old flies to eliminate abnormal proteins resulting from mutations and environmental damage. Remarkably, the steep reduction in ATP and 26S proteasome levels in old flies is observed when there is a major drop in their climbing performance, which might indicate that these events are milestones of the aging process.

## 2.3. MATERIALS AND METHODS

### 2.3.1. FLIES

Wild type Oregon R flies were reared on standard corn-meal agar medium [64]. Flies were passed to fresh vials every 4-6 days and maintained in humidified temperature-controlled environmental chambers at 25$^{o}$C and 60% relative humidity throughout the experiments. Flies were sorted and collected under $CO_2$. Samples containing a mixed population of females and males or separate groups of females and males for each age group were analyzed. In some experiments two main age groups of flies were compared: flies 1-2 days of age, referred to as "young" flies, and flies 43-47 days of age, referred to as "old" flies throughout the article. Where indicated, three additional age groups were analyzed: 10-12, 18-20 and 30-32 days of age.

### 2.3.2. PEPTIDASE ACTIVITIES IN FLY EXTRACTS

Mixed populations of females and males for each age group were analyzed. Whole fly extracts (30 flies per group) were prepared on ice by homogenization in 0.01MTris-EDTA, pH 7.5 buffer. The lysates were cleared by a 15-min centrifugation at 19,000xg at 4$^{o}$C. The cleared samples were

normalized for protein concentration determined by a bicinchoninic acid assay kit (Pierce, Rockf., IL). Peptidase activities were assayed colorimetrically in 20μg of protein/sample after 24h incubations at 37°C as described in [65]. The chymotrypsin-like activity was measured with the substrate Suc-LLVY-AMC, the trypsin-like activity with Z-GGR-NA and the caspase-like activity with Z-LLE-NA. All substrates were from BACHEM Bioscience Inc. (King of Prussia, PA).

### 2.3.3. IN-GEL PROTEASOME ACTIVITY AND DETECTION

Mixed or separate populations of females and males for each age group were analyzed. Flies were harvested with buffer A [50mM Tris-HCl, pH 7.4, 5mM $MgCl_2$, 5mM ATP (grade 1; Sigma, St. Louis, MO), 1mM DTT and 10% glycerol], which preserves 26S proteasome assembly [66]. Following homogenization on ice with a teflon pestle for microcentrifuge tubes (100 up and down strokes), sonication on ice (2X10s with a 5s interval) and centrifugation (19,000xg, 15-min at 4°C) the protein content of the cleared supernatants was determined with the Bradford assay (BIO RAD). The cleared supernatants were resolved by non-denaturing PAGE using a modification of the method described in [67].

To assess residual proteasome immunoreactivity in the pellet fraction, the latter was resuspended in buffer A, sonicated on ice (2X10s with a 5s interval) and resolved by non-denaturing PAGE just as the supernatant fraction. We established that the cleared supernatant fraction contains almost all of the proteasome immunoreactivity present in flies, with hardly any being detected in the residual pellet fraction.

We used a three step gradient gel with approximately similar amounts of 5%, 4% and 3% polyacrylamide containing Rhinohide™ polyacrylamide strengthener (Invitrogen-Molecular Probes, Carlsbad, CA). Bromophenol blue was added to the samples prior to loading. Non-denaturing minigels were run at 125Volts for 3 hours.

For detection of proteasome activity, the gels were incubated on a rocker for 10 to 30-min (depending on protein amount loaded) at 37°C with 15ml of 0.4mM Suc-LLVY-AMC in buffer B (buffer A modified to contain 1mM ATP). Proteasome bands were visualized upon exposure to UV light (360nm) and were photographed with a NIKON Cool Pix 8700 camera with a 3-4219 fluorescent green filter (Peca Products, Inc.). Semi-quantitative analysis of the bands corresponding to proteasome activity was performed by image analysis with the ImageJ program (Rasband, W.S., ImageJ, U.

S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2006).

Proteins on the native gels were then transferred (110mA) for 2.5h onto PVDF membranes. Western blot analysis was carried-out for detection of the 26S and 20S proteasomes with our anti-d$\beta$5 affinity purified antibody (1:4000, Bio-Synthesis, TX). The peptide $NH_2$-(GC)DSGYHWDLEDKEAQE-COOH was used to produce the anti-d$\beta$5-specific antibody and corresponds to amino acids 213-227 of the *Drosophila* d$\beta$5 subunit (Figure 6). The anti-d$\beta$5 antibody reacts with a core particle subunit (d$\beta$5) thus detecting both the 26S and 20S proteasomes. Upon incubation with the secondary antibody, antigens were visualized by a chemiluminescent horseradish peroxidase standard method with the ECL reagent.

To determine the total protein pattern of young and old flies, parallel native gels were stained with Coomassie blue after assessment of proteasome activity with Suc-LLVY-AMC.

## 2.3.4. ATP MEASUREMENTS

ATP levels were assessed in separate populations of females or males for each of the five age groups. Steady

state ATP content was measured with a 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. Flies (15 per trial) were harvested with 75 μl of 4% TCA followed by homogenization on ice with a teflon pestle for microcentrifuge tubes (100 up and down strokes) and centrifugation (11,500xg, 15-min at 4°C). ATP steady state levels were determined in cleared supernatants upon neutralizing the samples with 1M Tris-HCl, pH 8.0 (1:10 dilution). Samples were then added to the reaction buffer containing luciferin and assayed using a Luminoskan Ascent (Thermo Electron Corporation, Waltham, MA) microplate luminometer. Protein concentration was determined with the bicinchoninic acid assay kit (Pierce, Rockf., IL) upon neutralizing total fly lysates with 10mM Tris, pH 10.3. ATP levels were normalized for protein concentration. Similar protocols were previously used to measure ATP levels in *Drosophila* [32;34].

### 2.3.5. CLIMBING PERFORMANCE

We used a slight modification of a climbing assay previously established as being reliable to evaluate locomotor performance in *Drosophila* [68]. Females or males

of the five age groups (10 flies per trial) were placed in an empty graduated 100ml cylinder with a line drawn at the 66ml (2/3) mark. Flies were gently tapped to the bottom of the cylinder after a 15 min-recovery period from anesthesia. The number of flies that climbed above the 66ml mark after 20 seconds was recorded. Three trials were carried out for each group and the results were averaged.

### 2.3.6. GLYCEROL DENSITY GRADIENT CENTRIFUGATION

Mixed populations of females and males for each age group were analyzed by glycerol gradient centrifugation. Flies (~350) were harvested in 25mM Tris HCl, pH 7.5, 2mM ATP and 1mM DTT. Following homogenization and sonication the lysates were centrifuged for 10-min at 19,000xg at 4°C. The cleared supernatants (4.5 mg of protein/sample) were subjected to centrifugation at 83,000xg for 24h in a Beckman SW41 rotor in a 10-40% glycerol gradient (fractions 14 to 1) made in the same lysis buffer. Following centrifugation 14 fractions (800μl each) were collected and analyzed. Aliquots (50μl) of each fraction were assayed for chymotrypsin-like activity with the substrate Suc-LLVY-AMC colorimetrically after 24h incubations at 37°C as described in [65]. The chymotrypsin-like activity was assessed in the presence and absence of the proteasome inhibitor PSI (40μM

in 0.5% DMSO).

Proteins were precipitated with acetone from 700μl of each fraction and subjected to Western blot analysis (12% gels). The d*β*5 proteasome subunit was detected with our anti-d*β*5 antibody (1:4000, Bio-Synthesis, TX) and the S5a subunit of the 19S regulatory particle of the 26S proteasome was detected with the anti-S5a antibody (1:1,000, Abcam, Cambridge, MA). Upon incubation with the secondary antibody, antigens were visualized by a chemiluminescent horseradish peroxidase standard method with the ECL reagent.

### 2.3.7. FLY TREATMENT

Mixed populations of both females and males for each age group were analyzed. Flies were kept in narrow *Drosophila* vials which were lined with 3mm filter paper. Flies were starved for 6hrs and then fed a solution of 5% (weight/vol) sucrose with either vehicle (0.5 % DMSO, control) or with the proteasome inhibitor PSI (10μM, in DMSO) for 48hrs.

**2.3.8. WESTERN BLOT ANALYSIS OF FLY EXTRACTS**

Mixed populations of females and males for each age group were analyzed.

*Ubiquitinated proteins:* Following the indicated treatments flies were homogenized at 4°C in PBS with 1% Triton X-100 (TX) and a protease inhibitor cocktail (Sigma, St. Louis, MO). Following centrifugation (19,000xg, 15-min, 4°C) supernatants were collected (TX-soluble fraction). Pellets were resuspended in a buffer containing 50mM Tris-HCl, pH 7.5, 2% SDS and protease inhibitor cocktail, briefly sonicated and centrifuged (19,000xg, 15-min, RT), and the supernatants were collected (TX-insoluble fraction). TX-soluble and insoluble fractions were subjected to a 5-min boil at 100°C followed by a brief sonication. After determination of the protein concentration with a bicinchoninic acid assay kit (Pierce, Rockf., IL) the following was added to each sample: β-mercaptoethanol (358mM), bromophenol blue (0.005%), glycerol (20%), SDS (4%) in stacking gel buffer (0.1M Tris-Cl, pH 6.8). 50µg of protein/lane were analyzed by SDS-PAGE on polyacrylamide gels followed by Western blotting. Ubiquitinated proteins (8% gels) were detected in the TX-insoluble fractions with the anti-ubiquitin antibody (1:1,500, DAKO Corp., Carpinteria, California). No

ubiquitinated proteins were detected in the TX-soluble fraction (not shown).

*Proteasome dβ5 subunit* – Flies were harvested in a buffer containing 50mM Tris-HCl, pH 7.5, 20% glycerol, 1% Nonidet P-40, 1mM EDTA, 2mM EGTA, 274mM sodium chloride, 50mM sodium fluoride, 2.5mM sodium pyrophosphate, 1mM sodium vanadate, 1mM β-glycerophosphate, 1mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma, St. Louis, MO). Following a brief sonication, lysates were centrifuged (19,000xg, 15-min, RT). Supernatants were collected and subjected to a 5-min boil at 100°C. After determination of the protein concentration with a bicinchoninic acid assay kit (Pierce, Rockf., IL) samples were processed as described above. The dβ5 proteasome subunit (12% gels) was detected with the anti-dβ5 affinity purified antibody (1:4000, Bio-Synthesis, TX). Upon incubation with the secondary antibody, antigens were visualized by a standard chemiluminescent horseradish peroxidase method with the ECL reagent.

### 2.3.9. FLY SURVIVAL

Mixed populations of females and males for each age group were analyzed. Survival curves were generated by counting the number of dead flies at specific time intervals as indicated.

### 2.3.10. STATISTICAL ANALYSIS

Statistical comparisons were performed with the unpaired t test (for two groups) or Tukey-Kramer (for more than two groups) with Instat 2.0 Graphpad Software (San Diego, Ca).

## 2.4. RESULTS

### 2.4.1. The peptidase activities of the proteasome are lower in old (43–47 days) than in young (1–2 days) flies

We assessed the effect of aging on the peptidase activities of the proteasome in fly lysates as described in "Methods". Mixed populations of females and males from each age group were analyzed. The proteasome chymotrypsin-like activity was assayed with Suc-LLVY-AMC, the trypsin-like activity with Z-GGR-NA and the caspase-like activity with Z-LLE-NA. The three peptidase activities of the proteasome were consistently and significantly ($p \leq 0.0001$) lower ($\leq 60\%$) in old than in young flies (Fig. 7). The chromogenic substrates used to assess proteasome activity in total fly lysates may also be cleaved by other non-proteasomal proteases and thus may not specifically reflect proteasome activity in total lysates. To overcome this difficulty we also assessed proteasome activity by an in-gel assay (Fig. 9 and 10) and following glycerol density gradient centrifugation (Fig. 14 and 15).

### **2.4.2. 26S Proteasome assembly and activity are impaired in old (*43-47 days*) flies**

We postulated that the decline in proteasome activity observed in old flies could be due to impaired proteasome assembly. To test this premise we compared proteasome activity in flies of different age groups. Proteasome activity in cleared supernatants was assessed by an in-gel assay as described under "Methods". No proteasome activity could be detected in the residual pellet fraction (not shown). As a marker, an aliquot of partially purified 20S proteasomes from rabbit reticulocyte lysates (20S) was run in a parallel lane.

We also assessed proteasome levels with a peptide generated anti-d$\beta$5 specific antibody (Bio-Synthesis, TX). This antibody reacts with one band (23kDa) corresponding to the mature d$\beta$5 subunit of the proteasome core particle, when cleared supernatants were analyzed by Western blotting under denaturing conditions (Fig. 8, *top panels*). Proteasome immunoreactivity levels in the supernatant and pellet fractions obtained from total fly homogenates were assessed by Western blotting under native conditions as described in "Methods". As shown in Fig. 8 (*bottom panel*) the cleared supernatant fraction contained almost all of the proteasome immunoreactivity with very little being

detected in the residual pellet. The latter contains mostly insoluble debris and chitin.

Proteasome levels and activity (Fig. 9) from mixed populations of females and males were compared between two age groups: young (1-2 days) and old (43-47 days). In young flies, most of the proteasome activity assessed with the short substrate Suc-LLVY-AMC coincided with the 26S holoenzyme (not the 20S) form of the proteasome (Fig. 9, *left panel*). The in-gel chymotrypsin-like activity assay revealed that the proteasome activity in young flies (Y) coincided almost exclusively with the 26S holoenzyme, in its symmetrical (two capped) and asymmetrical (one capped) forms. Only extremely low levels of chymotrypsin-like activity were associated with the 20S proteasome, demonstrating that the 26S holoenzyme is the most active *in vivo* form of the proteasome in young flies. In contrast, the chymotrypsin-like activity of both the 26S and the 20S proteasomes was low in old flies (O).

Immunoblot analysis of the native gels with our anti-d$\beta$5 antibody revealed that, in young flies, proteasomes were detected as the 26S holoenzyme in its symmetric (two caps) and asymmetric (one cap) forms as well as the 20S proteasome (Fig. 9, *middle panel*). In contrast, in old flies, proteasomes were found almost exclusively as 20S

particles (Fig. 9, *middle panel*). These finding establish that low-activity 20S proteasomes prevail in old flies while the assembled 26S holoenzyme is highly active in young flies.

Following assessment of proteasome activity with Suc-LLVY-AMC, native gels were stained with Coomassie blue (Fig. 9, *right panel*). The total protein pattern of young and old flies was slightly but consistently different. This difference was not caused by post-harvesting protein degradation as similar patterns were observed when flies were harvested with or without a protease inhibitor cocktail (not shown).

### 2.4.3. 26S Proteasome activity declines sharply in 43-47 day old female and male flies

We assessed proteasome activity with the in-gel assay in separate populations of females or males across five different ages: 1-2, 10-12, 18-20, 30-32 and 43-47 days (Fig. 10A). Each lane with fly samples was loaded with an equal amount of protein (50μg). These studies clearly demonstrate that a sharp decline in 26S proteasome activity (by ≥ 89%) was observed in 43-47 days old flies compared to young 1-2 days old flies. This major decline in 26S proteasome activity was observed in both female (Fig. 10A,

*left*) and male (Fig. 10A, *right*) 43-47 days old flies. On the contrary, the activity of the 20S proteasome failed to decline with aging in both genders (Fig. 10A and *semi-quantification shown in the graph*). Total protein pattern was determined by Coomassie blue staining of the native gels following proteasome activity assessment. Changes were mainly observed in the oldest flies (43-47 days of age) of both genders (Fig. 10B).

**2.4.4. ATP steady state levels decrease by ~50% in 43-47 day old female and male flies**

The assembly of the 26S proteasome is known to be ATP-dependent [69] thus we assessed ATP steady state levels in the flies. ATP levels were analyzed in separate populations of female or male flies at five different ages: 1-2, 10-12, 18-20, 30-32 and 43-47 days. ATP levels were maintained at an average of 15.7 pmoles/μg of protein in flies of both genders up to the age of 30-32 days (Fig. 12). Remarkably, in female and male 43-47 days old flies, ATP levels decreased by more than 50% to 7.4 pmoles/μg protein.

### **2.4.5. Climbing performance drops markedly in 43-47 day old female and male flies**

Generally, as flies get older they manifest locomotor dysfunction [68]. We assessed locomotor performance in flies of different ages to compare the age at which they display locomotor dysfunction and proteasome impairment. Locomotor performance was measured with the climbing assay. *Drosophila* display a strong negative geotactic response by quickly rising to the top of a vial upon being tapped to its bottom. When tested for their climbing ability, 43-47 days old females and males exhibited a marked ($p<0.001$) decline in climbing performance compared to young (1-2 days of age) flies (Fig. 13). The oldest flies (43-47 days) made short, abortive climbs and fell back to the bottom. Flies between 18 and 32 days of age also displayed a reduction in climbing ability, albeit not as steep as the oldest 43-47 days old flies.

### **2.4.6. Different fractionation pattern of proteasomes from young (*1-2 days*) and old (*43-47 days*) flies**

To corroborate that the proteasomal chymotrypsin-like activity was lower in old than in young flies, total extracts from each age group (4.5mg/sample) were

fractionated by glycerol density gradient centrifugation. These studies focused on the two age groups that exhibited the greatest change: young (1-2 days) and old (43-47 days) flies. We also analyzed mixed populations of female and male flies, since in the above described studies no obvious difference was detected between genders.

Fractions were analyzed for chymotrypsin-like activity with Suc-LLVY-AMC. As shown in Fig. 14 (*upper panel*), the chymotrypsin-like activity of old flies (*solid squares*) was significantly reduced when compared to young flies (*open squares*), particularly in the fractions corresponding to the proteasome elution position (Fig. 14, fractions 4-10 *box*).

To establish which fractions correspond to the proteasome, aliquots from each fraction were subjected to Western blot analysis with our anti-dβ5 antibody, which reacts with a subunit of the core particle, and with the anti-S5a antibody, which reacts with a non-ATPase subunit (Rpn10) of the 19S particle (Fig. 14, *lower panels*). Immunodetection of the dβ5 subunit revealed a pronounced shift of the dβ5 peak in old flies toward lower molecular weight fractions corresponding to the 20S proteasome. Furthermore, the levels of the S5a subunit are clearly

lower in the older flies than in the young ones. From these experiments we can conclude that in old flies the 20S is the predominant proteasome form.

Notably, we observed an additional peak of chymotrypsin-like activity that did not correspond to fractions containing proteasome subunits. This peak exhibited lower molecular weight than the proteasome-containing fractions (Fig. 14). That these fractions are not related to the proteasome is further demonstrated by their lack of sensitivity to PSI, a proteasome inhibitor (Fig. 15). This finding is not surprising, since it is well established that the substrate Suc-LLVY-AMC is cleaved not only by the proteasome [70] but also by other chymotrypsin-like proteases as well as by calpains [71]. In contrast to proteasome activity, the non-proteasomal chymotrypsin-like activity does not appear to be affected by aging (Fig. 14).

### 2.4.7. Old (*43-47 days*) flies are more sensitive to the proteasome inhibitor PSI than young (*1-2 days*) flies

Mixed populations of females and males from each age group were tested. We compared the *in vivo* sensitivity of young and old flies to the proteasome inhibitor PSI by feeding them a sucrose/PSI (10μM) diet as described under

"Methods". At this concentration, we previously showed that PSI significantly inhibits the chymotrypsin-like activity of the proteasome in mammalian cell cultures [72].

The decrease in proteasome activity did not induce the accumulation of ubiquitinated proteins in young flies, as observed by Western blot analysis with a specific antibody that detects polyubiquitinated proteins (Fig. 16, *top panel*). However, the *in vivo* administration of PSI induced the accumulation of ubiquitinated proteins in old flies (Fig. 16, *top panel*). In addition, no apparent changes in the levels of the proteasome subunit dβ5 were detected in young *versus* old flies following PSI administration (Fig. 16, *bottom panels*). The latter subunit, dβ5, accounts for the chymotrypsin-like activity, which carries-out the rate-limiting step in protein degradation by the proteasome [73].

A significant shortening (20%) of life span was observed in old flies upon the *in vivo* administration of PSI for 24h and 42h (Fig. 17). In contrast, the life span of young flies was not altered by feeding them the proteasome inhibitor.

## 2.5. DISCUSSION

In this paper we show for the first time that the aging process is associated with the disassembly of the 26S proteasome with a clear loss of its activity. The decline in 26S holoenzyme levels in old flies (43-47 days of age) coincides with increased levels of the 20S proteasome. Clearly, the activity of the 20S proteasome failed to decline. However, our studies demonstrate that the free 20S core particle is nearly inactive in all flies independently of their age group. Accordingly, the majority of the proteasome chymotrypsin-like activity coincides with the 26S proteasome, the levels of which decline sharply in 43-47 days old female and male flies. That the 20S proteasome is almost inactive is not particular to flies, as a similar phenomenon was observed in yeast [74]. It is well established that in the outer rings of the 20S core particle, the conformation of the α subunits is such that it seals the entrance into the catalytic chamber [74]. Activation of the 20S proteasome thus requires disruption of the inflexible and passive barrier provided by the outer α rings. The opening of these gates to the catalytic chamber is triggered by regulatory complexes, such as the

19S and the 11S regulatory particles (reviewed in [75]). *In vitro* studies with purified 20S proteasomes demonstrated that the closed-gate conformation is also destabilized by the binding of some hydrophobic peptides to non-catalytic sites on the core particle [76] or by certain proteins, such as p21 and α-synuclein [19]. The latter mechanism for activation of the 20S core particle has not yet been demonstrated to occur *in vivo*. However, it was proposed that because proteasomes diffuse rapidly in the cytoplasm and nucleus of mammalian cells, they may continuously collide with some of their substrates [77].

The assembly of the 26S proteasome from its 20S core and 19S regulatory particles is an ATP-dependent process [69;78]. The concentration of ATP required for half-maximal 26S proteasome assembly was estimated to be 5μM [79]. Although the mechanism of assembly of the 26S proteasome is not completely understood, it is known that the molecular chaperone Hsp90 plays a role in this process [80] and that the 20S and 19S particles undergo a structural rearrangement to produce the optimal conformation of the full complex [81]. ATP hydrolysis is also required for the actual degradation process of polyubiquitinated proteins, with an estimated Km of 12μM [79]. ATP hydrolysis is known

to promote substrate unfolding and protein translocation through the 26S holoenzyme with an apparent 300-400 ATP molecules being hydrolyzed during the degradation of one molecule of globular or unfolded substrate [15]. Recent studies suggest that ATP hydrolysis is also necessary for the rapid dissociation of the 26S proteasome into the 19S and 20S particles [16]. This dissociation coincides with release of the products of degradation. In conclusion, ATP hydrolysis is a pivotal requirement not only for the assembly-disassembly cycle of the 26S holoenzyme but also for its function, i.e. to degrade polyubiquitinated proteins.

Our current data shows that in the oldest flies tested (43-47 days of age) ATP steady state levels are reduced by more than 50%. A previous study with *Drosophila* also reported a decline (15%) in ATP levels with age, although only males were included in these experiments [32]. The decline in ATP steady state levels together with our findings described above, strongly support the notion that ATP depletion associated with the aging process may have a critical impact on 26S proteasome activity by impairing, for example, its assembly. The distribution of ATP in cells seems to be compartmentalized with different ATP pools sustaining different cell functions. For example, while ATP

produced by oxidative phosphorylation sustains contractility in smooth muscle cells, ATP generated from anaerobic glycolysis supports plasma membrane proton pumps [82;83]. Which ATP pool supports 26S proteasome activity is currently undetermined.

Changes in total cellular ATP caused by different metabolic conditions are not reflected uniformly in all intracellular compartments [84]. In cultured cells, when both glycolysis in the cytosol and oxidative phosphorylation in mitochondria were active, mitochondrial ATP was double that of other cellular compartments tested, i.e. cytosol, subplasma membrane region and nucleus. When oxidative phosphorylation was impaired, ATP levels were equilibrated in all four compartments, suggesting that the bulk of ATP was produced by glycolysis. When oxidative phosphorylation was the sole source of ATP, its levels were normal in the cytosol and subplasma membrane region, but collapsed in mitochondria and nucleus [84].

The aging process is known to affect mitochondrial function and to be associated with mitochondrial genome changes including point mutations and modifications as well as deletions (reviewed in [85]). Interestingly, aging in *Drosophila* seems to be associated with a decrease in the levels of mitochondrial RNA [33;86]. Furthermore, a decline

in skeletal muscle mitochondrial function was observed with aging in humans [87;88] and premature aging was induced in mice expressing defective mitochondrial DNA polymerase [89]. On the other hand caloric restriction, the only experimental paradigm known to extend life span in organisms ranging from yeast to mammals, was shown to promote mitochondrial biogenesis in mice [90]. In addition, calorie restriction limits the generation but not the progression of mitochondrial abnormalities in aging skeletal muscle [91]. Dietary restriction also seems to prevent a state of persistent glycolysis associated with ad libitum-fed animals (reviewed in [92]). Most glycolytic intermediates are potentially toxic because they are able to glycate proteins and other macromolecules non-enzymatically (reviewed in [92]). In conclusion, suppression of the aging process requires, among other factors, maintenance of mitochondrial function and a reduction in persistent glycolysis. Our studies uncover a potential novel factor, i.e. maintenance of 26S proteasome assembly and ensuing activity, which may be intricately related to the other two. Accordingly, 26S proteasome assembly is ATP-dependent [78] and glycated-proteins may escape proteasomal degradation [93].

Interestingly, we established that the oldest group of flies tested (43-47 days of age) exhibited a marked decline in locomotor activity assessed with a climbing assay. These data demonstrate that the steep decrease in 26S proteasome activity and ATP levels in flies is an "old-age" event that is obvious when flies exhibit a major decline in locomotor performance.

Our studies also revealed that old flies are significantly more sensitive to the peptide aldehyde PSI that is a proteasome inhibitor. The increased sensitivity was manifested by accumulation of ubiquitinated proteins and a shorter lifespan in old flies but not in young ones. Interestingly, yeast cells continue to grow when 70-80% of their proteasome activity is inhibited by peptide aldehydes or β-lactone [94]. This finding suggests that the capacity of the proteasome in yeast exceeds the required activity under homeostatic conditions. Thus, a 20-30% proteasome capacity is sufficient for yeast cell survival and growth under homeostatic conditions. It is possible that in young flies, there is also a surplus of proteasome activity. Therefore, feeding young flies sublethal concentrations of PSI has no apparent effect on survival or the levels of polyubiquitinated proteins. On the other hand, in old flies the observed disassembled state of the 26S proteasome

severely diminishes its activity, therefore rendering old flies exceptionally sensitive to proteasome inhibitors.

Our finding that the disassembly of the 26S proteasome is an “old-age” event could explain the presence of protein deposits containing ubiquitinated proteins and oxidatively modified proteins in non-pathologic aging [61] as well as in a variety of aging-related neurodegenerative disorders including Alzheimer’s and Parkinson’s diseases and amyotrophic lateral sclerosis, to name a few [95]. The reduction in assembled 26S proteasomes concurrent with ATP depletion not only will affect normal protein turnover but also most likely deprives old flies of the ability to effectively cope with proteotoxic damages caused by life long environmental and/or genetic factors.

# CHAPTER III

# GENETIC TOOL TO MANIPULATE PROTEASOME ACTIVITY IN *DROSOPHILA*

## 3.1. AIM

Our studies described in Chapter II clearly demonstrate that proteasome activity declines with age. The aim of the studies described in this chapter was to develop a genetic tool to manipulate proteasome activity in a spatially regulated manner. This tool will allow proteasome function to be genetically manipulated by expressing a dominant negative catalytically inactive dβ5 proteasome subunit in transgenic flies. Future studies will generate *Drosophila* lines expressing the dβ5 mutant proteasome subunit. These *Drosophila* lines will be used to investigate the mechanisms that are affected by proteasome impairment as well as to evaluate how different cell types and tissues in the fly respond to a decrease in proteasome activity. This knowledge will facilitate the development of potential therapeutic strategies that deal with proteasome impairment which occurs in aging and aging-associated neurodegenerative disorders.

## 3.2. STRATEGY JUSTIFICATION

Most studies addressing the outcome of proteasome inhibition in cells draw on a pharmacological approach with synthetic proteasome inhibitors, which may exert nonspecific effects as bound molecules or through interactions with other cellular proteins. In addition, in intact animals, this pharmacological approach does not allow targeting proteasome inhibition to specific cells.

An alternative approach to investigating the outcome of proteasome impairment in *Drosophila* is to genetically manipulate proteasome function. To be able to disrupt proteasome activity in *Drosophila,* we generated a construct expressing a dominant negative 20S proteasome dβ5 subunit with an active site mutation that impairs its activity, i.e. with an N-terminal threonine to alanine substitution. We focused on the constitutive dβ5 subunit because it accounts for the chymotrypsin-like activity, which carries-out the rate-limiting step in protein degradation by the proteasome [23].

Two *Drosophila* lines expressing temperature-sensitive dominant negative missense mutants of the proteasome subunits β2 and β6 were previously reported (reviewed in

[22]). When raised at permissive temperatures (22-25°C) heterozygous flies have no abnormal phenotypes but die as undifferentiated pupae at the restrictive temperature (29°C). Although these *Drosophila* lines are very useful to address the effects of proteasome disruption under certain circumstances, they can not be used in our studies since the mutant phenotypes are only effective when the flies are reared at 29°C. This temperature is known to activate the heat-shock transcription factor in *Drosophila* [96], therefore complicating the interpretation of the data.

A different approach will be used in future studies to control the expression of the wild type and mutant dβ5 proteasome subunit in *Drosophila*. The dβ5 proteasome subunit will be cloned into the pUAST vector taking advantage of a binary system [97] because a dβ5 deletion is a recessive lethal [98;99]. This binary system will permit spatial and temporal modulation of the targeted gene by the choice of promoters.

This *Drosophila* model will be ideal for identifying molecular and cellular mechanisms induced by defects in proteasomal protein degradation. In addition, the different fly lines that will be generated will determine whether the genetic manipulation of proteasome activity targeted to the

whole *Drosophila* or to its CNS in general or to the dopaminergic neurons in particular, induces premature aging and neurodegeneration with accumulation of ubiquitinated proteins.

## 3.3. METHODS AND RESULTS

To reduce proteasome activity we inserted a dβ5 gene carrying an active site T1A mutation (Figure 18) in the pBlueScript vector. We previously demonstrated that such an active site mutation (T1A) in the dβ5 gene abolished the chymotrypsin-like activity of mouse neuronal cells acting as a dominant negative mutant [100].

The N-terminal Thr of β5 was mutated to Ala because the 20S proteasome is a threonine protease in which the nucleophilic attack is mediated by the N-terminal Thr of processed, catalytically active β subunits [12]. This active site threonine is conserved in eukaryotes including *Drosophila* and, therefore, we feel certain that the mutation will lead to inactivation of the dβ5 protein.

The genomic DNA encoding dβ5 was obtained from BAC clone (BAC13J10) by PCR, using the primers 1)5′ end primer: AGGAATTCATGGCTTTACTGAAATCTGC and 2)3′ end primer: TAGGTACCGTTGGAGATGTTTA (Figure 19). Subsequently, its identity was confirmed by DNA sequencing (Figure 20). The cDNA encoding dβ5 was obtained using polyA mRNA from *Drosophila* larvae, and subsequent RT-PCR. The mutation was introduced by site-directed mutagenesis with the

oligonucleotide 5'-TTC GAT CAC GGC ***G***CC ACA ACG TTG GGC-3' (The mutated base in the oligonucleotide is indicated in bold and italic) using the QUIKChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutation generated a new *SfoI* site, which can be used to distinguish wild type and mutant forms of the *dβ5* gene.

In addition, a human c-myc tag was attached to the C-terminus of dβ5. This will allow the distinction between exogenously expressed and endogenous dβ5 subunits. The c-myc "QGTEQKLISEEDLN" epitope was used. It reacts with a commercially available antibody (Santa Cruz Biotech., Santa Cruz, CA) and was previously used successfully [101;102]. To accomplish this, an Afl III-AccI fragment centered on the carboxyl-termination codon was replaced with a new AflIII-AccI fragment synthesized from two oligonucleotides which contain the c-myc epitope in frame with the complete *dβ5* sequence. The c-myc tag was generated by my colleague Marlon T. Jansen.

```
                  AflIII
                  ~~~~~~
               TyrMetTyrGln  GluGlnLeu LysGlnGln AlaAlaLys GlnGlyThrGlu
  1     GGAGCTGCAC TACATGTACC AGGAGCAGTT GAAGCAGCAG GCCGCTAAGC AAGGCACCGA
        CCTCGACGTG ATGTACATGG TCCTCGTCAA CTTCGTCGTC CGGCGATTCG TTCCGTGGCT
                                          Stop
         GlnLysLeu IleSerGluGlu AspLeuAsn Sequence
101     ACAAAAGCTC ATCTCCGAAG AAGACCTCAA TTAGAGTTTT GATTAAGGGA ATATAAGATA
        TGTTTTCGAG TAGAGGCTTC TTCTGGAGTT AATCTCAAAA CTAATTCCCT TATATTCTAT

               AccI
              ~~~~~~~
        TTCAGTTTGT ATACGTTTGA AT
        AAGTCAAACA TATGCAAACT TA
```

Modified Afl III-Acc I fragment with the oligonucleotide sequence of the human c-myc epitope.

## 3.4. FUTURE STUDIES

### 3.4.1. – Transgenic flies

To generate transgenic flies the modified and mutagenized *dβ5* construct will be cloned into the pUAST vector taking advantage of a binary system [97]. The following GAL4 drivers will be used to express the *dβ5* gene:

**(i) Ddc** - contains the promoter of the *Drosophila* dopa decarboxylase gene and will direct expression of dβ5 to dopaminergic and serotonergic neurons.

**(ii) Elav** - the *elav* gene encodes a neuron-specific nuclear protein widely expressed in the *Drosophila* nervous system throughout all stages of development. This will allow expression of the transgene in the nervous system.

**(iii) Act5**- an ubiquitous promoter.

For P-element mediated transformation [103] the relevant

P-element vector will be co-injected with the helper element pπ25.7wc [104] to provide temporarily transposase function into $w^{1118}$ flies prior to pole cell formation (Figure 21). Embryos developing to maturity will be mated to $w^{1118}$ flies and offspring with red to orange eye colors will be selected (Figure 22). Establishment of isolines and localization of inserts by segregation analysis and balancing of stocks will be performed by a standard protocol[103].

### 3.4.2. – Analysis of the transformant lines

The effect of the genetic manipulation of proteasome activity targeted to the whole organism, to the nervous system or to dopaminergic neurons will be investigated by assessing changes in proteasome activity, accumulation of ubiquitinated proteins, accumulation of carbonylated proteins, proteasome subunit expression and longevity, using standard methods.

To establish if proteasome impairment can cause premature aging and/or neurodegeneration in the *Drosophila methuselah* mutant the dβ5 mutation will be introduced into the *methuselah* background and the respective transformants will be analyzed as described above.

**3.4.3. – Expected outcome**

The results obtained from these studies will establish if proteasome impairment is a major factor contributing to premature aging and neurodegeneration even in an extended longevity background such as in the *methuselah* mutant.

Since proteasome impairment will be targeted to the whole organism, the CNS or dopaminergic neurons, a distinction between non-neuronal and neuronal cellular responses to a decrease in proteasome activity will be determined.

Finally, as aggregate formation containing ubiquitinated proteins will be contrasted with neuronal cell death, the results obtained in these studies will give an indication of the importance of protein aggregation in the neurodegenerative process.

**3.4.4. – Anticipated problems and alternatives**

One of the concerns with the generation of the transformants harboring the dominant negative proteasome mutant is that there will be compensation in subunit expression, resulting in up-regulation of proteasome subunits to counteract the deleterious effect of proteasome impairment. Such a mechanism was observed when expression of different subunits of the 26S and 20S proteasome were modulated by RNAi in *Drosophila* S2 cells [105] and when

proteasome inhibitors were used in primary cultures of vascular smooth muscle cells [106]. To overcome this difficulty we will be able to introduce additional copies of the dominant negative dβ5 construct with the expectation that the increased gene dosage will overcome the compensatory effect.

### 3.4.5. – Relevance

These studies will address the effects of disrupting proteasome activity in the progress of aging and neurodegeneration. They will also generate optimal *Drosophila* models to test pharmacological or genetic agents that may prevent aging or neurodegeneration associated with a decline in proteasomal protein turnover.

# CHAPTER IV

# MODEL AND CONCLUSIONS

*Drosophila* is an optimal and unexplored *in vivo* system to investigate how the proteasome is altered by aging. The hypothesis tested in this thesis is that an age-dependent decline in protein turnover is the result of inappropriate regulation, expression and/or activity of the proteasomal pathway.

To address this hypothesis a major question was investigated: What are the changes in proteasome activity and structure throughout the life span of *Drosophila*? In addition, based on the outcome of our studies, a genetic tool was developed to spatially manipulate proteasome activity in *Drosophila* as a means to mimic the aging effect. This tool will be used in future studies to investigate how different tissues and/or cells mechanistically deal with a deficit in proteasome activity.

Our studies demonstrate for the first time in an *in vivo* system that aging coincides with a transition from a fully assembled 26S proteasome, which is preponderant in "young" flies, to the 20S proteasome, which prevails in "old" flies. We also established that this transition parallels a deficit in ATP levels. This may explain why 26S proteasome levels are low in old flies, as ATP is required for 26S proteasome assembly.

A decline in 26S proteasome levels will have a major

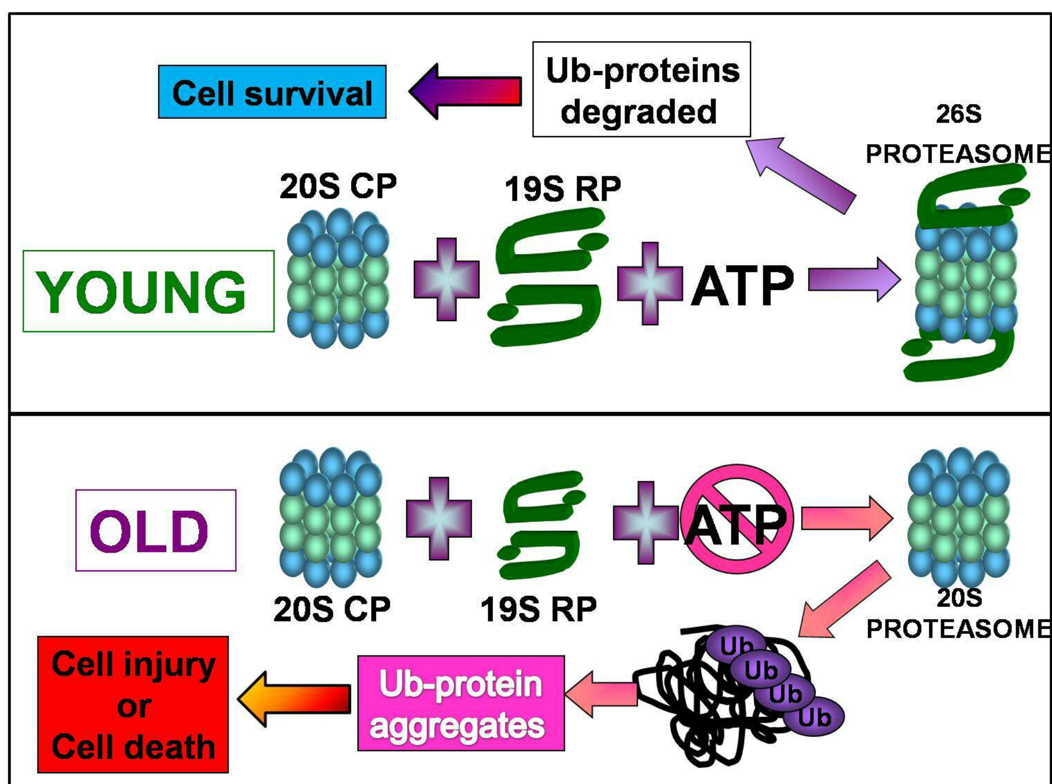
impact on overall intracellular protein turnover, as the 26S proteasome is considered to be the major system involved in protein degradation within cells. The 20S proteasome, which prevails in “old” flies, is known to be autocatalytically inhibited and is, therefore, incapable of degrading proteins without being activated in a highly regulated manner.

Our significant finding implies that intracellular protein degradation is highly impaired in “older” individuals. If this trend proves to be true in humans, it will have a major impact on the onset of many diseases. Accordingly, a decline in 26S proteasome levels will affect normal protein turnover within cells as well as their ability to effectively cope with proteotoxic damages caused by life long environmental and/or genetic factors.

Based on our findings, the following model is proposed: In “young” flies a fully assembled 26S proteasome is maintained by adequate levels of ATP. This provides them with a constant, ongoing process to degrade intracellular proteins that are no longer needed or that are defective. In “old” flies this steady state is disrupted by, for example, metabolic disturbances that reduce ATP levels. This hurdle in protein degradation will cause the abnormal build-up of intracellular protein deposits, leading to cell

injury and in many cases to cell death. In conclusion, a drop in 26S proteasome levels could be considered as a major milestone in the aging process.

The results of the studies described in this thesis provide a detailed understanding of how proteasome function is affected by aging. These results have the potential of contributing to the development of therapeutic strategies designed to overcome the major damaging effects of an age-dependent decline in protein turnover associated with the ubiquitin-proteasome pathway.




Model of the effects of aging on protein turnover by the ubiquitin-proteasome pathway. 20S CP, 20S proteasome and core particle of the 26S proteasome; 19S RP, regulatory particle of the 26S proteasome; Ub, ubiquitin. See text above for explanation.

# CHAPTER V

# FUTURE DIRECTIONS

The studies described in this thesis revealed that:

***1) The activity of the 26S proteasome declines significantly when the flies reach an age at which overall ATP levels are highly depleted.*** These results suggest that there maybe a tight correlation between ATP concentrations and 26S proteasome activity, as the latter requires ATP for its functional assembly.

***2) The decline in 26S proteasome activity observed in Drosophila is not a gradual process.*** We established that throughout most of the fly lifespan, 26S proteasome activity is maintained at a more or less constant level. However, we observed a steep decline in its activity when the flies reach an "old" age (43-47 days) that coincides with a drop in their climbing ability. These results suggest that an "old" age deficit in 26S proteasome activity may contribute to the late onset observed in most human neurodegenerative disorders.

***3) There is a gender-dependency in the aging process.*** Although this observation was not reported or addressed in this thesis, our studies revealed that significantly fewer males reached the age of 43-47 days than females. This finding suggests the existence of aging-preventive factors in females and/or aging-inducing factors in males.

Further studies should be conducted to test the hypotheses described above. Of particular interest would be to:

***1) Establish a close correlation between ATP depletion and 26S proteasome disassembly.*** The *Drosophila* temperature-sensitive *nubian* mutant displays a reduced lifespan and a 3-fold reduction in ATP levels [107]. The *nubian* mutant disrupts phosphoglycerol kinase activity, an enzyme required for the generation of ATP through the glycolytic pathway. It would be interesting to compare proteasome assembly and activity at different ages in wild type versus the *nubian* mutant flies. Consistent with decreases in ATP generation, *nubian* flies should exhibit deficits in 26S proteasome activity and assembly at an earlier age than wild type.

***2) Attempt to prolong lifespan in Drosophila by developing a transgenic fly that overexpresses the proteasome.*** It was previously demonstrated that overexpression of a proteasome subunit in cultured cells leads to an increase in overall proteasome activity [108]. A similar approach could be tested in *Drosophila* by upregulating the expression of, for example, the dβ5 proteasome subunit. This *Drosophila* model would test if an increase in proteasome activity has a delaying impact on

aging.

***3)* Cross the mutant dβ5 transgenic flies with other flies that are models for neurodegenerative diseases.** None of the currently available *Drosophila* models of neurodegeneration correlate with molecular events induced by a decline in proteasome activity. Instead, they focus specifically on the deleterious effects resulting from the expression of particular human proteins, such as α-synuclein [109] [110], tau [111], ataxin 1 [112] or ataxin 3 [113] [114] in *Drosophila*. It would be interesting to test if a deficit in proteasome activity could exacerbate and/or accelerate the neurodegenerative phenotype exhibited by the different fly lines expressing the mutant human proteins. Crossing our transgenic fly lines expressing the dβ5 subunit mutant with any of the other neurodegenerative fly models available would address this question.

**4) Identify differences in gene expression profiles between male and female flies, which may contribute to a gender-dependent susceptibility to the aging process.**

Recent studies with mice established that female muscle derived stem cells (F-MDSCs) regenerate skeletal muscle much more efficiently than male MDSCs (M-MDSCs) [115]. This higher muscle regeneration efficiency was not due to gender differences in hormonal or host immune responses.

Instead, it was caused by an innate gender variation in the number of actively expressed genes related to, for example, stress responses and apoptosis.

Interestingly, studies with *Drosophila* established that male flies are more sensitive to paraquat and exhibited PD-like symptoms earlier than females [40]. As reported above, we also observed that male flies “age” more rapidly than their female counterparts. It is possible that these gender differences are related to an innate variation in gene expression profiles between male and female flies. It would be of the utmost interest to explore by microarray analysis, age-dependent differences in gene expression profiles between male and female flies.

In conclusion, we can anticipate that the future directions proposed in this section will provide valuable information on the role of the UPP in aging as well as age-related neurodegenerative disorders.

# CHAPTER VI

# FIGURES

**Figure 1 – Scheme indicating the proteolytic fate of a cellular protein subjected to stress conditions leading to misfolding.** Once ubiquitinated, the misfolded protein should be degraded, allowing for cell recovery. However, if the modified protein is not degraded and accumulates as ubiquitin aggregates or inclusion bodies, the recovery process is hindered, leading to cell degeneration. ROS, reactive oxygen species produced by oxidative stress.

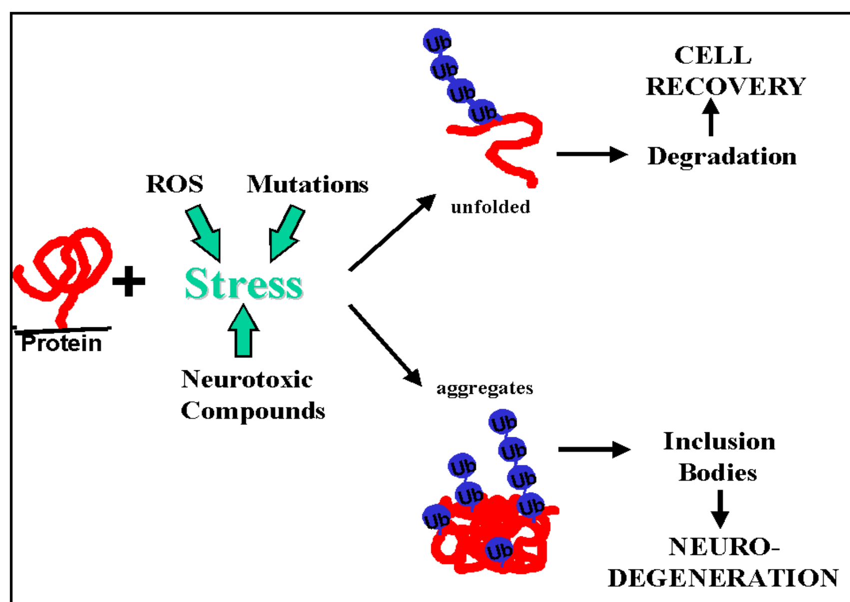



**Figure 1**

**Figure 2 – Ubiquitination and degradation of proteins by the ubiquitin-proteasome pathway (UPP).** Protein ubiquitination is a complex ATP-dependent process in which ubiquitin (Ub) is sequentially activated by ubiquitin-activating enzymes (E1), transferred to ubiquitin-conjugating enzymes (E2) and ligated to protein substrates by ubiquitin ligases (E3). Polyubiquitin chains are formed by isopeptide bonds between Gly76 and Lys48 on adjacent ubiquitin molecules. Once a protein is ubiquitinated, it is degraded rapidly by the 26S proteasome. De-ubiquitinating enzymes (DUB) remove and disassemble the polyubiquitin chain.

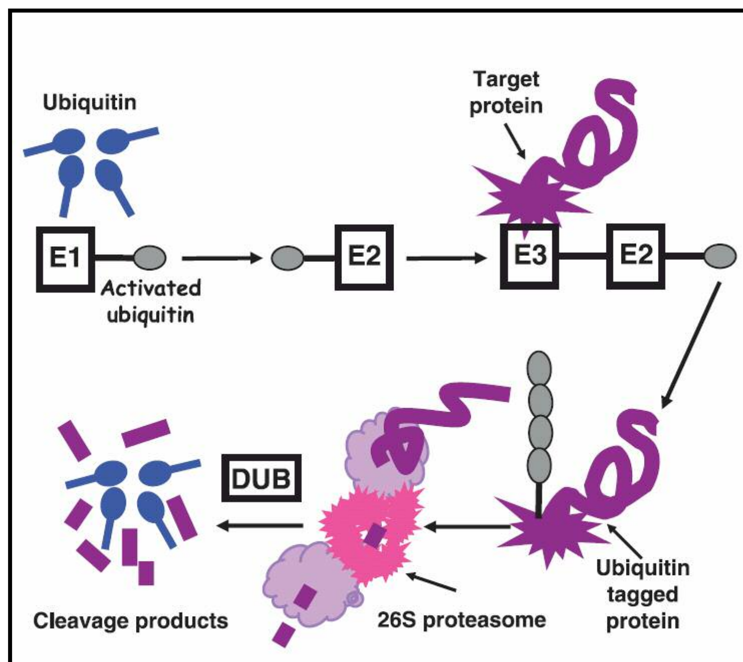
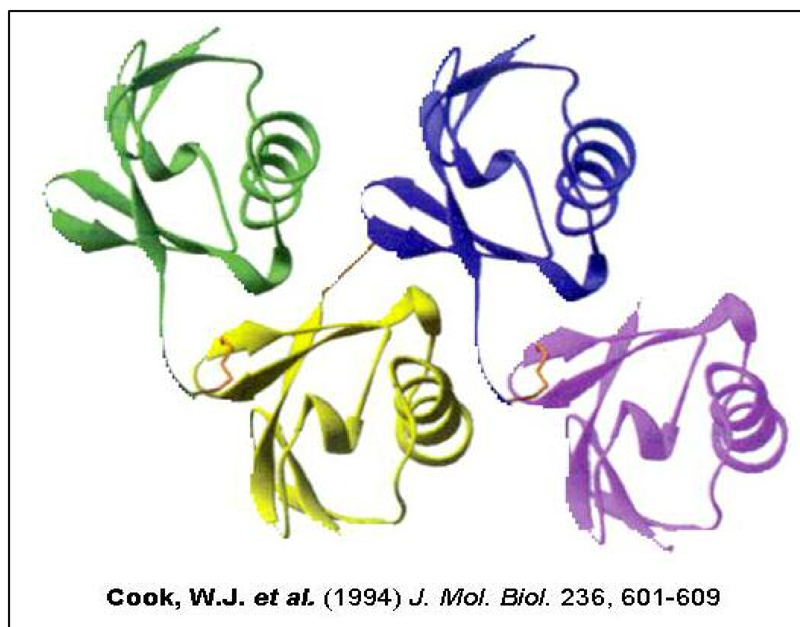



**Figure 2**

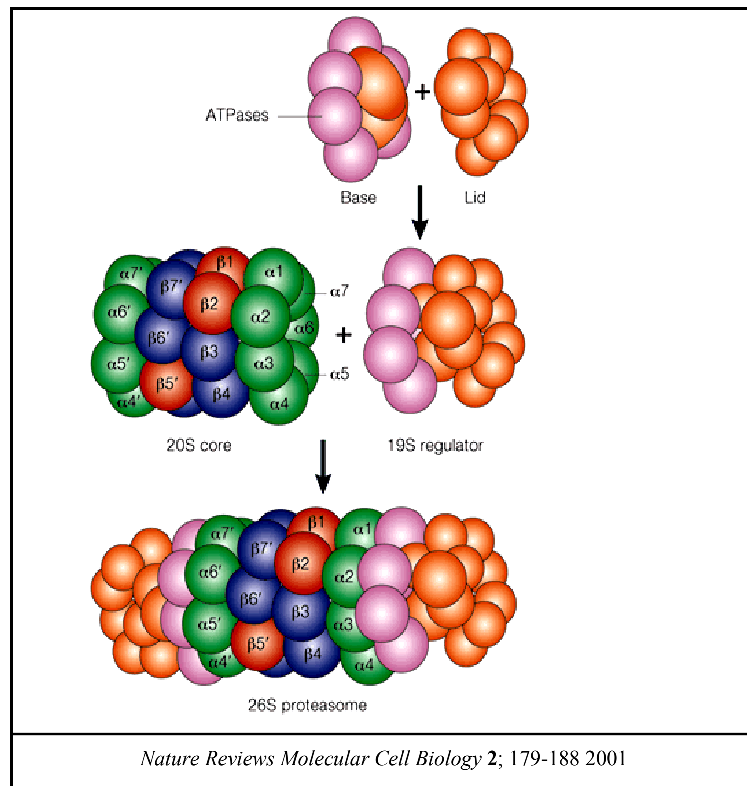
**Figure 3 – Polyubiquitin chains target substrates for degradation by the 26S proteasome.** Tetraubiquitin, as shown in this figure, is the minimum signal for efficient proteasomal targeting.



Cook, W.J. *et al.* (1994) *J. Mol. Biol.* 236, 601-609

# Figure 3

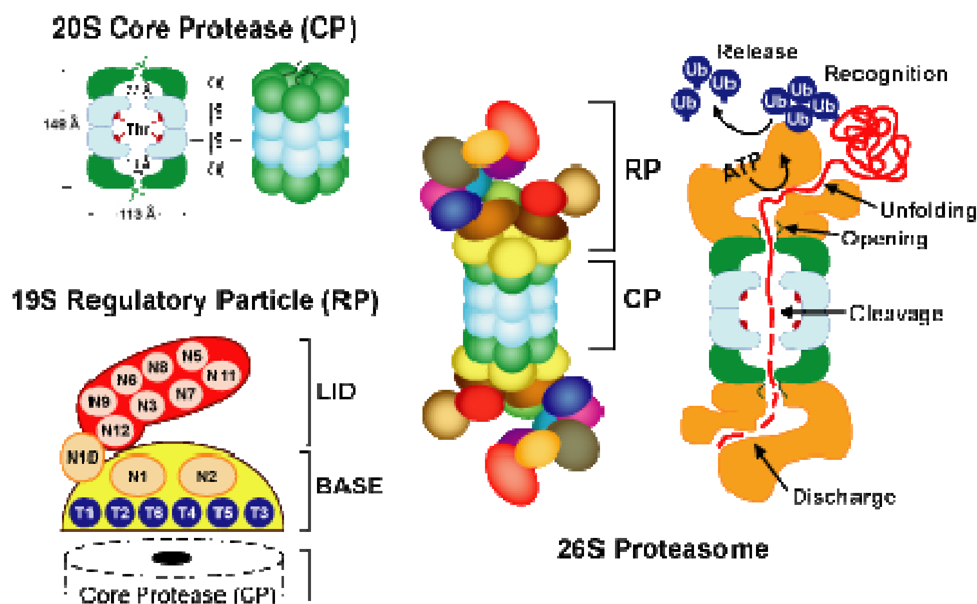
**Figure 4 – The 26S Proteasome.** When a protein becomes ubiquitinated it is degraded by the 26S proteasome, which has a regulatory moiety known as the 19S regulator and a proteolytic moiety known as the 20S core. The 19S regulator contains ATPases (base), de-ubiquitinating activity and polyubiquitin-binding sites (lid). The 20S core contains the proteolytic active sites.




*Nature Reviews Molecular Cell Biology* **2**; 179-188 2001

# Figure 4

**Figure 5 – Protein degradation by the 26S proteasome.** Polyubiquitin chains bind to receptors on the lid of the 19S particle. The lid also provides de-ubiquitinating activity to remove the polyubiquitin chain from the substrate. The ATPases in the base of the 19S particle unfold the substrate, open the gate in the 20S particle, and promote protein translocation into the proteolytic chamber of the 20S particle. Within the 20S particle, the two outer rings with the α-type subunits, act as binding sites for regulators, such as the 19S particle, and form a gated channel leading to the inner proteolytic chamber formed by β-type subunits, three of which contain the active sites for protein degradation.




http://plantsubq.genomics.purdue.edu/html/guide.html

# Figure 5

**Figure 6 – Predicted partial crystal structure of the *Drosophila* 20S proteasome.** The d$\beta$5 subunit is shown in magenta on the right (arrow). A peptide generated d$\beta$5-specific antibody was obtained from Bio-Synthesis, TX. The peptide $NH_2$ – (GC) DSGYHWDLEDKEAQE-COOH was used to produce the anti-d$\beta$5-specific antibody and corresponds to amino acids 213-227 of the *Drosophila* d$\beta$5 subunit

**Figure 6**

**Figure 7 (next page) - Proteasome activities in young (*1-2 days*) and old (*43-47 days*) flies.** Mixed populations of females and males for each age group were analyzed. Proteasome activities from young (*open bars*) and old (*solid bars*) flies were measured in cleared supernatants obtained from total fly homogenates (20μg of protein/sample). Peptidase activities were assayed colorimetrically after 24h incubations at 37°C as described under "Methods". The chymotrypsin-like activity was measured with Suc-LLVY-AMC, the caspase-like activity with Z-LLE-NA and the trypsin-like activity with Z-GGR-NA. Data represent the mean ± SEM from five experiments. The *asterisks* (*) identify the values in old flies that are significantly different ($p \leq 0.0001$) from young flies.

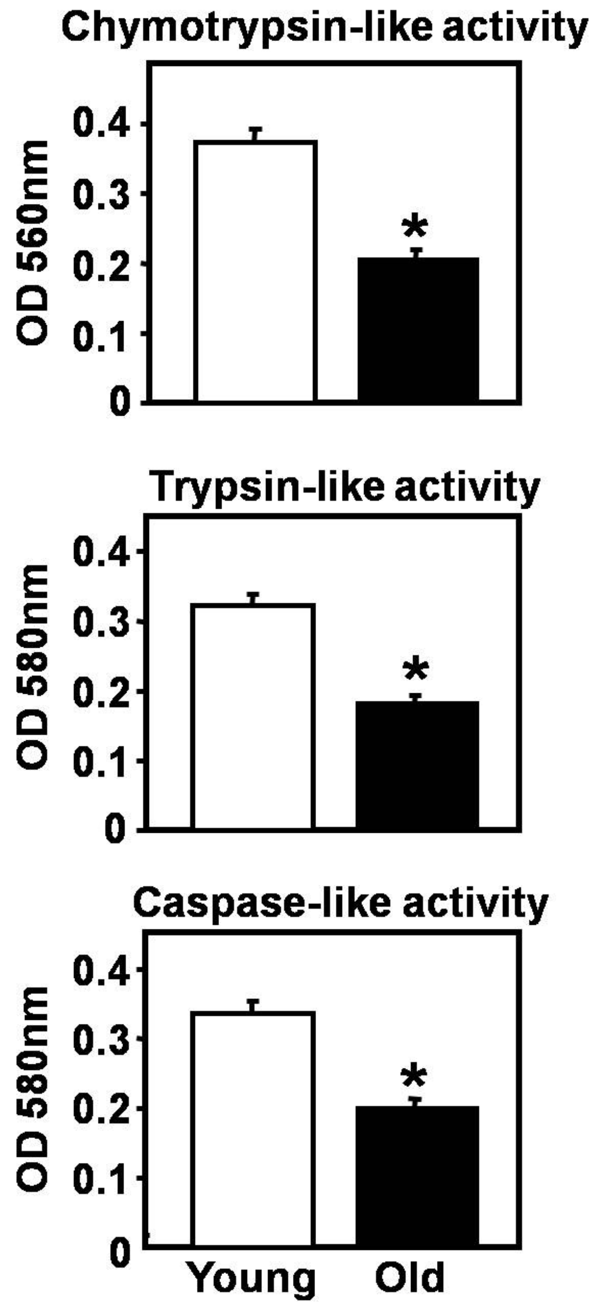
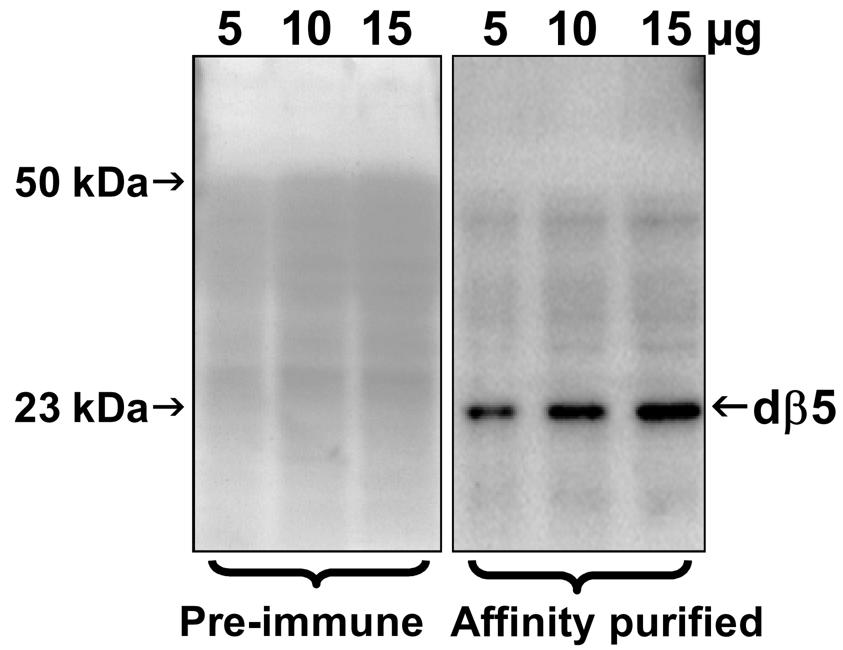



Figure 7

**Figure 8 (next page) - Assessing proteasome levels in flies.** Mixed populations of young females and males were analyzed.

*Top panel:* The specificity of our anti-d$\beta$5 antibody was assessed by Western blotting under denaturing conditions as described under "Methods". Cleared supernatants from total fly homogenates were analyzed with pre-immune serum (*left*) or an anti-d$\beta$5 (*right*) affinity purified antibody (1:4000) generated at Bio-Synthesis, TX as described in "Methods". The d$\beta$5 proteasome subunit migrates as a ~ 23kDa band.

*Bottom panel*: Proteasome levels were assessed by Western blotting under non-denaturing conditions as described under "Methods". Crude extracts were homogenized, sonicated and centrifuged. Cleared supernatant (**S**) and pellet (**P**) fractions (60μg of protein per lane) were subjected to non-denaturing gel electrophoresis and Western blotting as described under "Methods". 26S and 20S proteasomes in fly extracts were detected by immunobloting with our anti-d$\beta$5 antibody. This antibody reacts with a subunit of the core proteasome particle thus recognizing both the 20S and 26S proteasome forms. Symmetric [26S(2), two caps] and asymmetric [26S(1), one cap] 26S holoenzymes and the 20S core particle are indicated on the left.



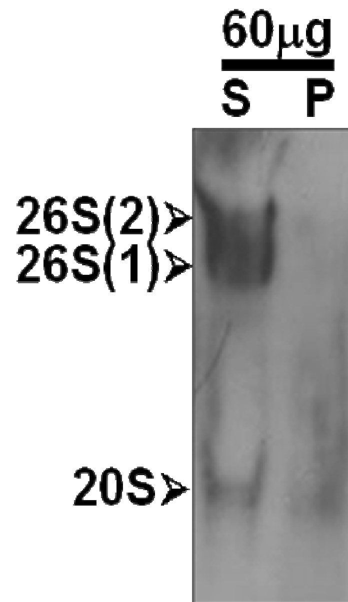




Figure 8

**Figure 9 (next page) – Proteasome activity and levels in young (*Y, 1-2 days*) and old (*O, 43-47 days*) flies.** Cleared fly lysates and 20S proteasomes partially purified from rabbit reticulocyte lysates (20S, as a marker) were subjected to non-denaturing gel electrophoresis as described under "Methods". Lysates from mixed populations of females and males of each age group were analyzed. The chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the in-gel assay (*left panel*). 26S and 20S proteasomes were detected by immunobloting with our antibody that reacts with d$\beta$5, a subunit of the core proteasome particle (*middle panel*). As indicated on the left, this antibody recognizes the symmetric [26S(2), two caps] and asymmetric [26S(1), one cap] 26S holoenzymes as well the 20S core particle. Total protein pattern was established by Coomassie blue staining of native gels (*right panel*) following assessment of proteasome activity with Suc-LLVY-AMC. Similar results were obtained in at least quadruplicate experiments.

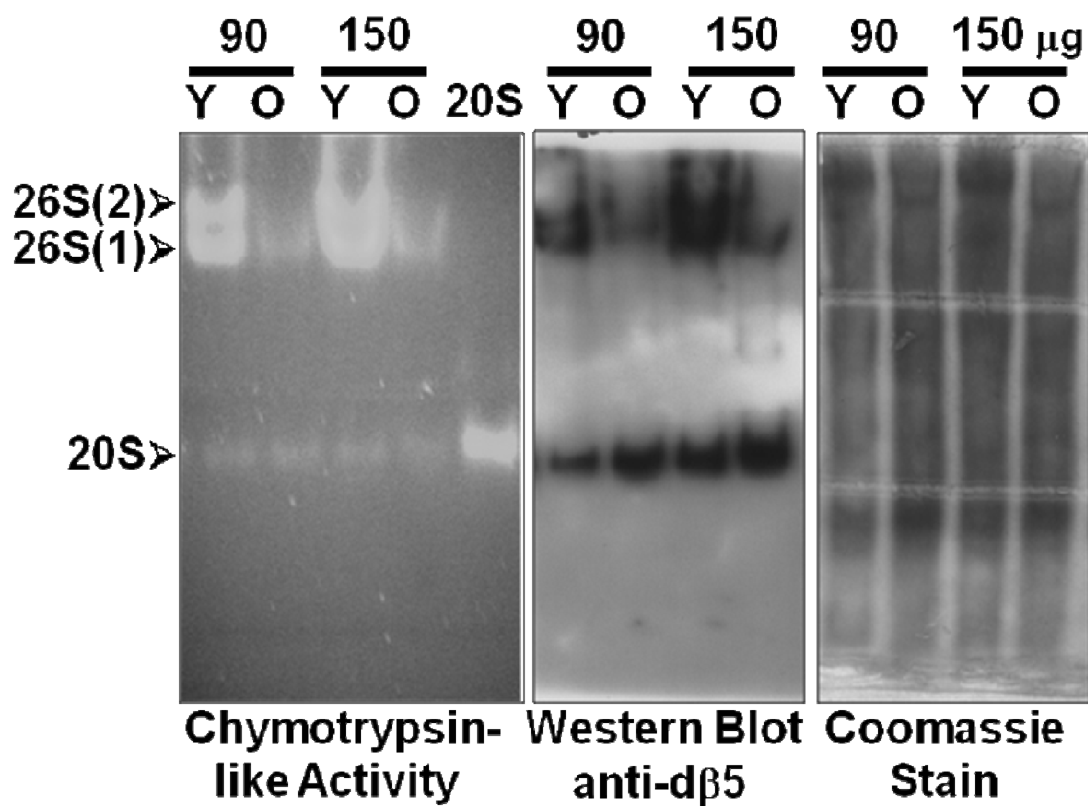
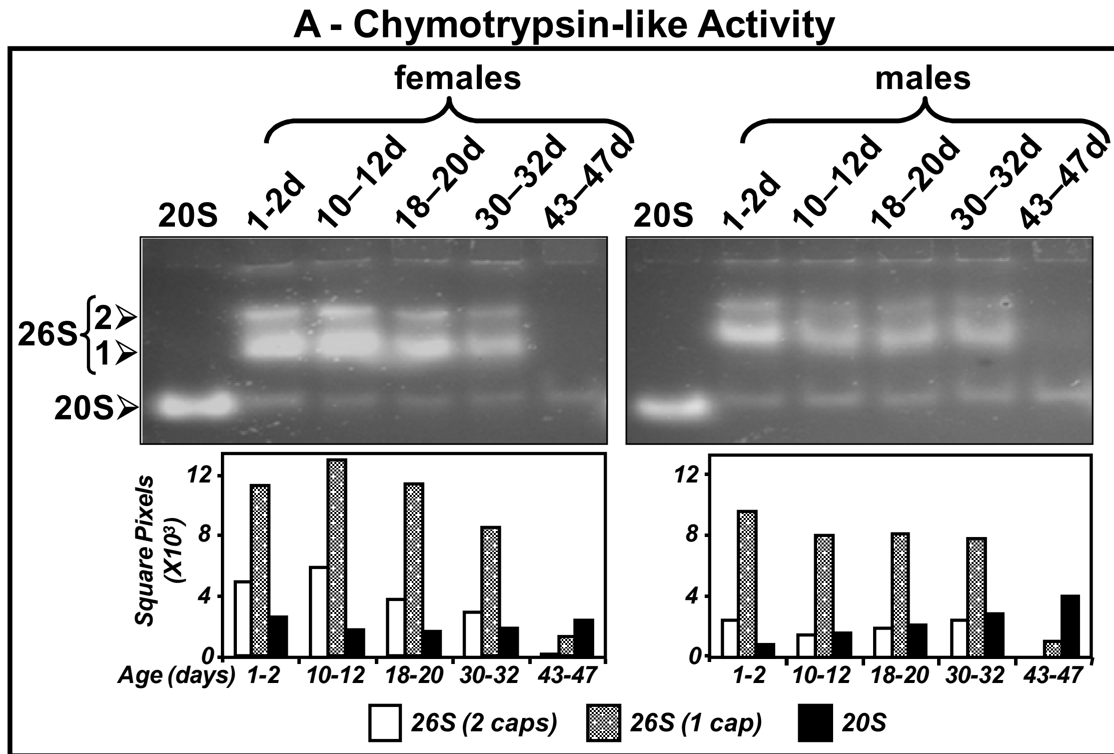



Figure 9

**Figure 10 (next page) - Proteasome activity in female and male flies across different ages.** Separate groups of females or males of different ages (1-2, 10-12, 18-20, 30-32 and 43-47 days) were analyzed. Cleared lysates as well as 20S proteasomes partially purified from rabbit reticulocyte lysates (20S, as a marker) were subjected to non-denaturing gel electrophoresis as described under "Methods". Each lane with fly samples was loaded with an equal amount of protein (50μg). *Panel A* - The chymotrypsin-like activity was assessed with Suc-LLVY-AMC by an in-gel assay. The symmetric [26S(2), two caps] and asymmetric [26S(1), one cap] 26S holoenzymes as well the 20S core particle are indicated on the left. Activity bands were semi-quantified by densitometry as described in "Methods" (*graph: open bars - 26S, two caps; stippled bars – 26S, one cap; solid bars – 20S*). *Panel B* - Total protein pattern was established by Coomassie blue staining following assessment of proteasome activity with Suc-LLVY-AMC. Only the top halves of the activity gels are shown in panel A, as no signals were detected on the bottom halves. The entire Coomasie blue stained gels are shown in panel B. Similar results were obtained in duplicate experiments.



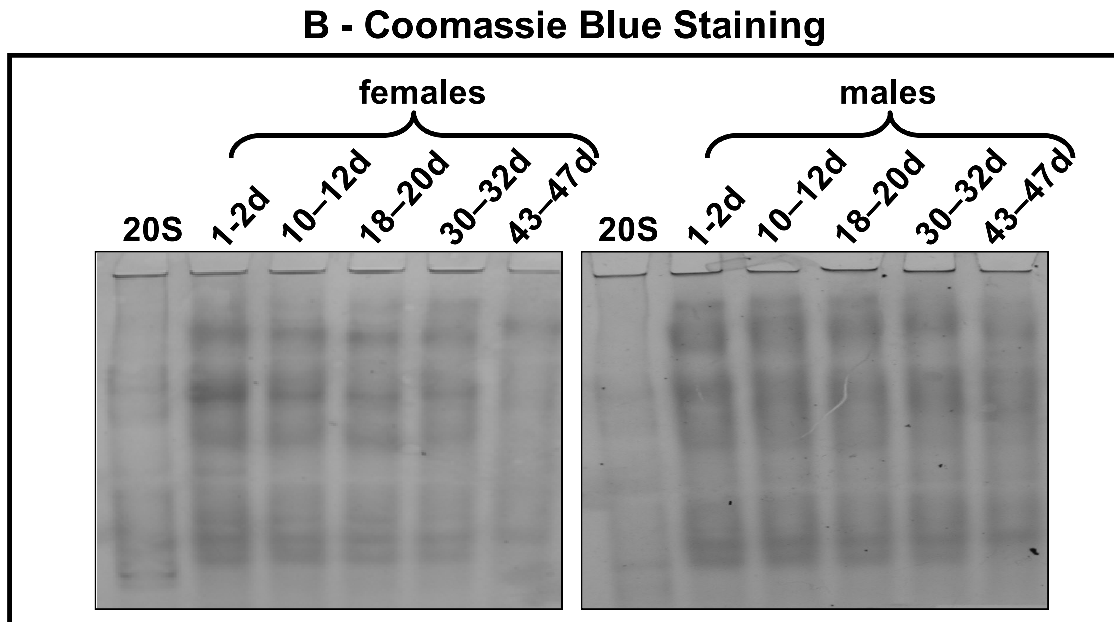
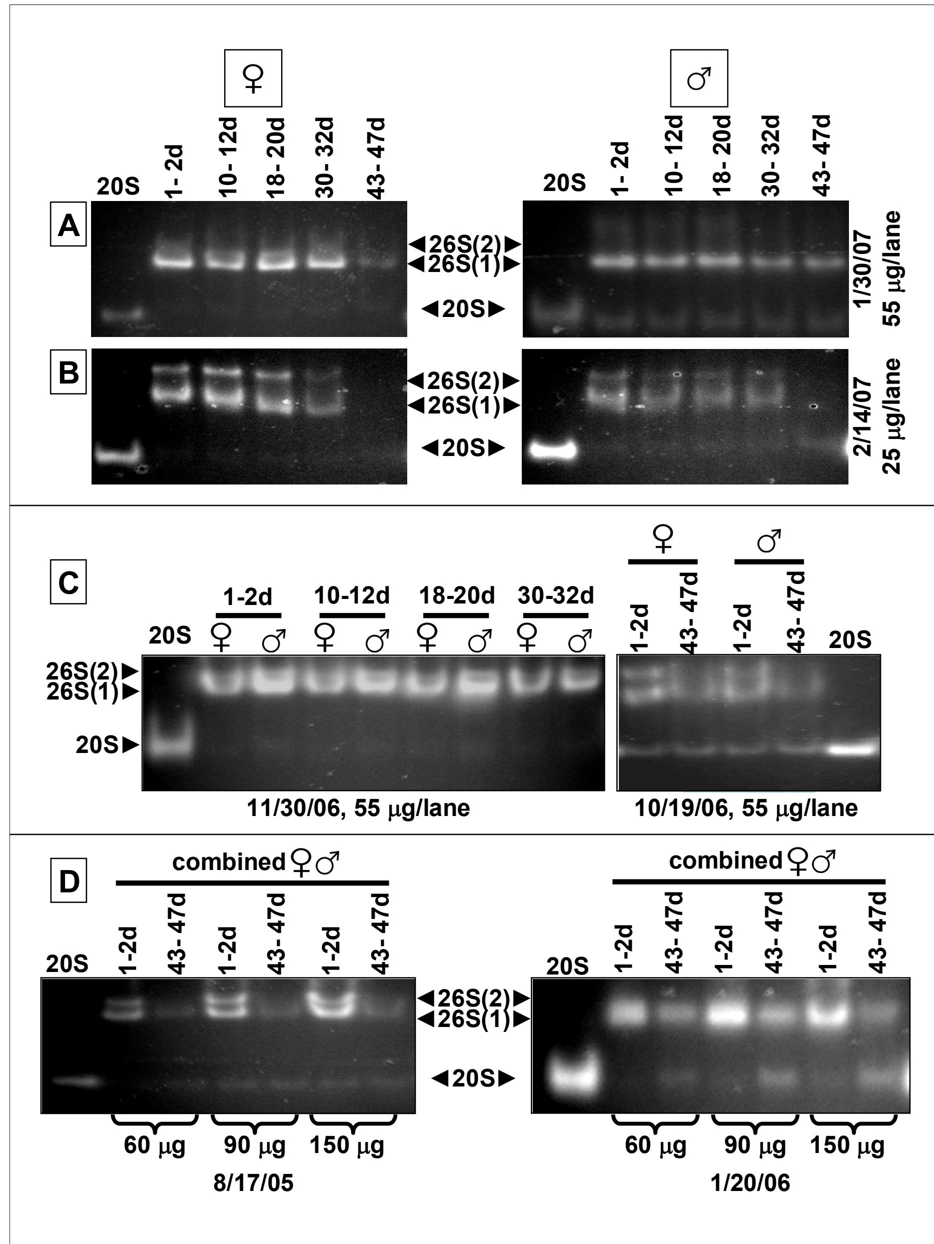




Figure 10

**Figure 11 (next page) - Results from FIVE independent experiments showing proteasome activity across different ages in separate as well as combined female and male groups of flies.**

Cleared total fly lysates and 20S proteasomes partially purified from rabbit reticulocyte lysates (20S, as a marker) were subjected to non-denaturing gel electrophoresis as described under "Methods". The chymotrypsin-like activity was assessed with Suc-LLVY-AMC by an in-gel assay. The bands corresponding to symmetric [26S(2), two caps] and asymmetric [26S(1), one cap] 26S holoenzymes as well the 20S core particle are indicated on the figure.

*A, B and C* - Separate groups of females or males across different ages (1-2, 10-12, 18-20, 30-32 and 43-47 days) were analyzed. *D* - Combined groups of females and males of two different ages (1-2 and 43-47 days) were analyzed. Each lane with fly samples was loaded with the indicated amounts of protein. The samples in panel B are the same as in figure 10, except that less protein was loaded in each lane (25μg/lane, figure 11 instead of 50μg/lane, figure 10).




# Figure 11

**Figure 12 - ATP steady state levels in female and male flies across different ages.** Separate groups of females (*open bars*) and males (*solid bars*) of different ages (1-2, 10-12, 18-20, 30-32 and 43-47 days) were analyzed. ATP concentrations (pmoles/μg of protein) in cleared supernatants were determined as described in "Methods". Data represent the mean ± SEM from four trials (15 flies per trial) per age group. The *asterisk* (*) identifies the values that are significantly different ($p<0.05$) from young (1-2 days of age) flies.

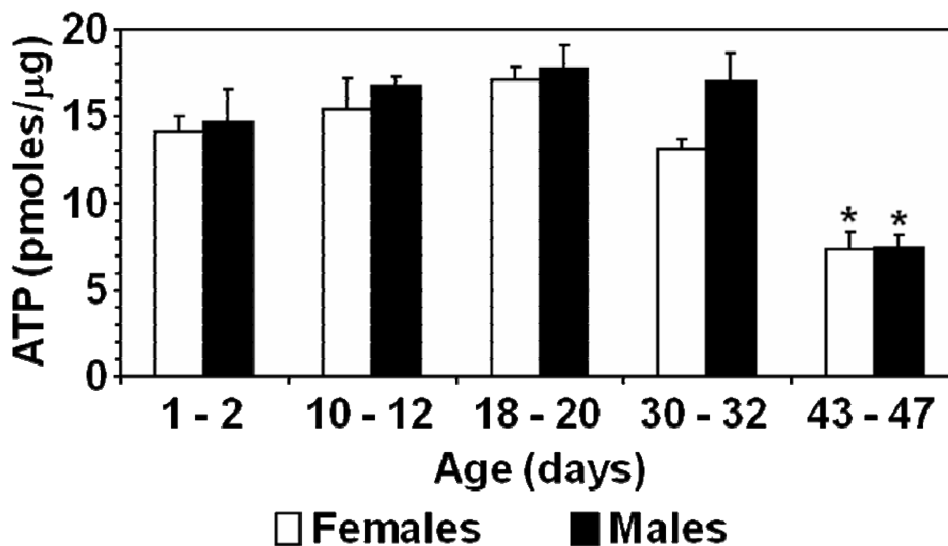



**Figure 12**

**Figure 13 - Climbing performance of female and male flies across different ages.** Separate groups of females (*open bars*) and males (*solid bars*) of different ages (1-2, 10-12, 18-20, 30-32 and 43-47 days) were analyzed. Locomotor performance was assessed with a climbing assay as described in "Methods". Data represent the mean ± SEM from three trials (10 flies per trial) per age group. *Asterisks* identify the values that are significantly different (*$p<0.05$, **$p<0.001$) from young (1-2 days of age) flies.

**CLIMBING PERFORMANCE**

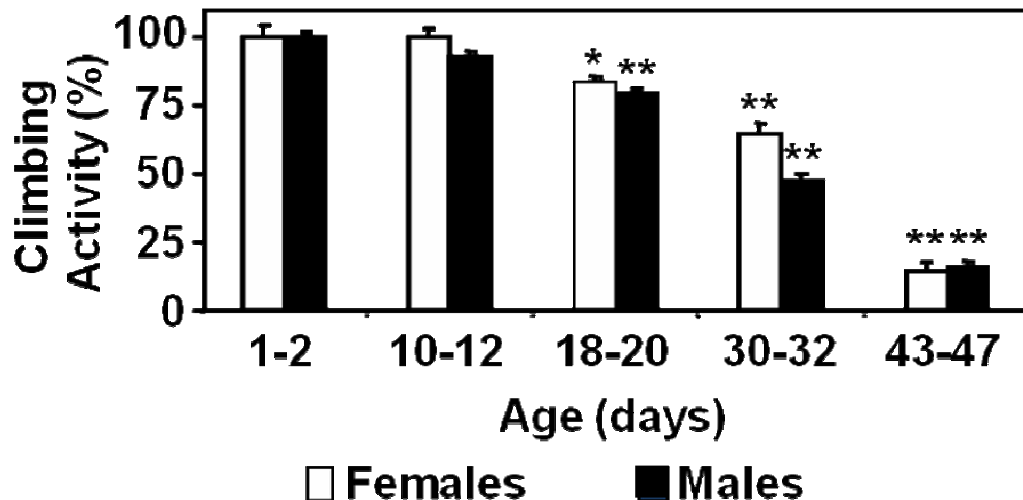



**Figure 13**

**Figure 14 (next page) - Sedimentation velocity analysis of proteasomes from young (*1-2 days*) and old (*43-47 days*) flies.** Mixed populations of females and males for each age group were analyzed. Lysates (4.5 mg protein) were fractionated by glycerol density gradient centrifugation (10-40% glycerol corresponding to fractions 14 to 1).

*Top panel* - Aliquots (50μl) of each fraction obtained from young (*open squares*) and old (*solid squares*) flies were assayed for chymotrypsin-like activity with Suc-LLVY-AMC.

*Bottom panels* - Immunoblot analysis of each fraction probed with our anti-d$\beta$5 (core particle) and the anti-S5a (19S regulatory particle, Rpn10) antibodies. Proteins were precipitated with acetone from 700μl of each fraction. The box indicates the elution of proteasome fractions. Similar results were obtained in duplicate experiments.

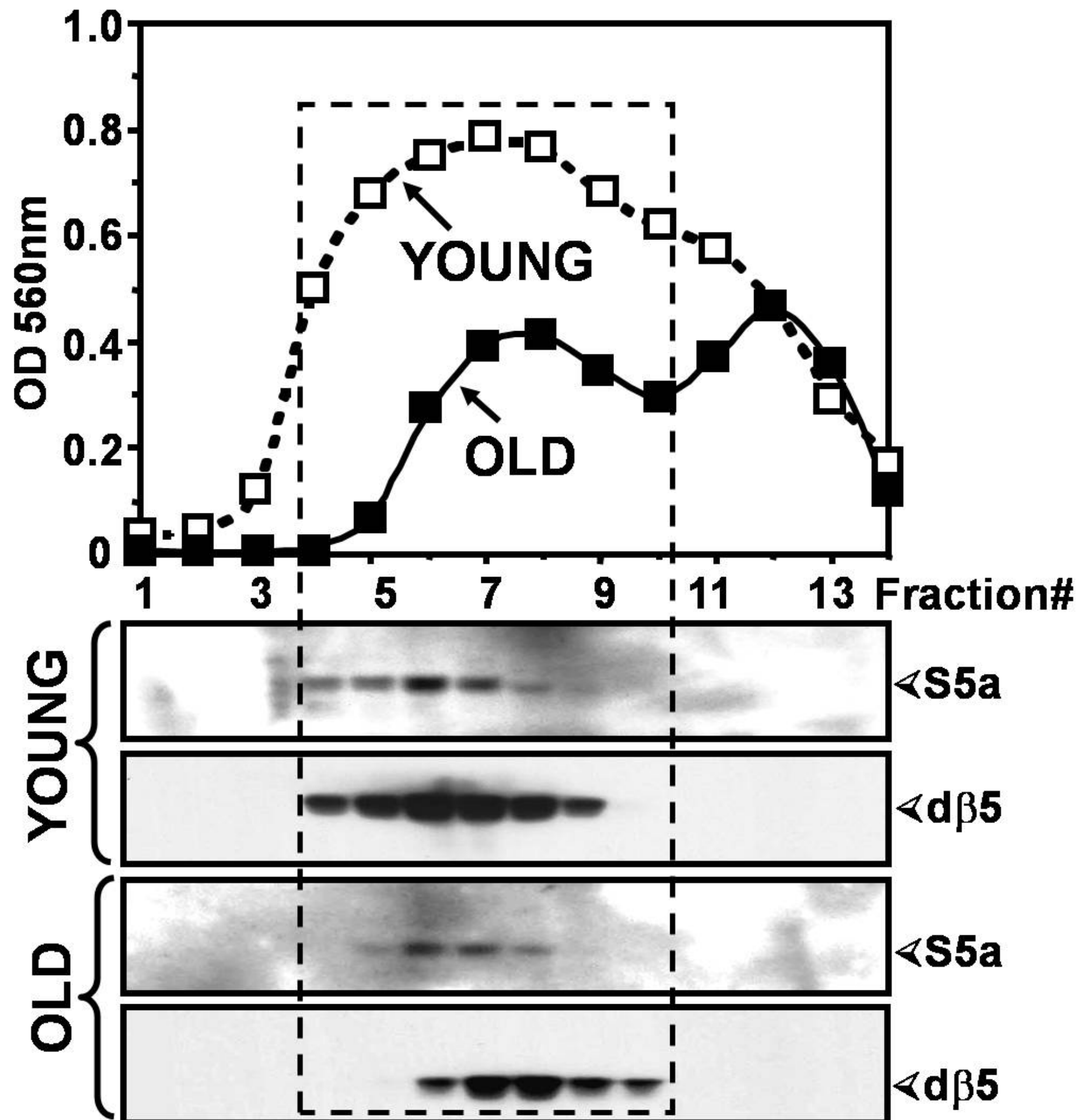



Figure 14

**Figure 15 (next page) - Sedimentation velocity analysis of the chymotrypsin-like activity from young (*1-2 days*) and old (*43-47 days*) flies assayed in the absence and presence of the proteasome inhibitor PSI (40μM).** Mixed populations of females and males for each age group were analyzed. Lysates (4.5 mg protein) were fractionated by glycerol density gradient centrifugation (10-40% glycerol, fractions 14 to 1). Aliquots (50μl) of each fraction from young (*top graph, circles*) and old (*bottom graph, squares*) flies were assayed for chymotrypsin-like activity with Suc-LLVY-AMC in the absence (*open symbols*) and presence (*solid symbols*) of the proteasome inhibitor PSI (40μM). Similar results were obtained in duplicate experiments.

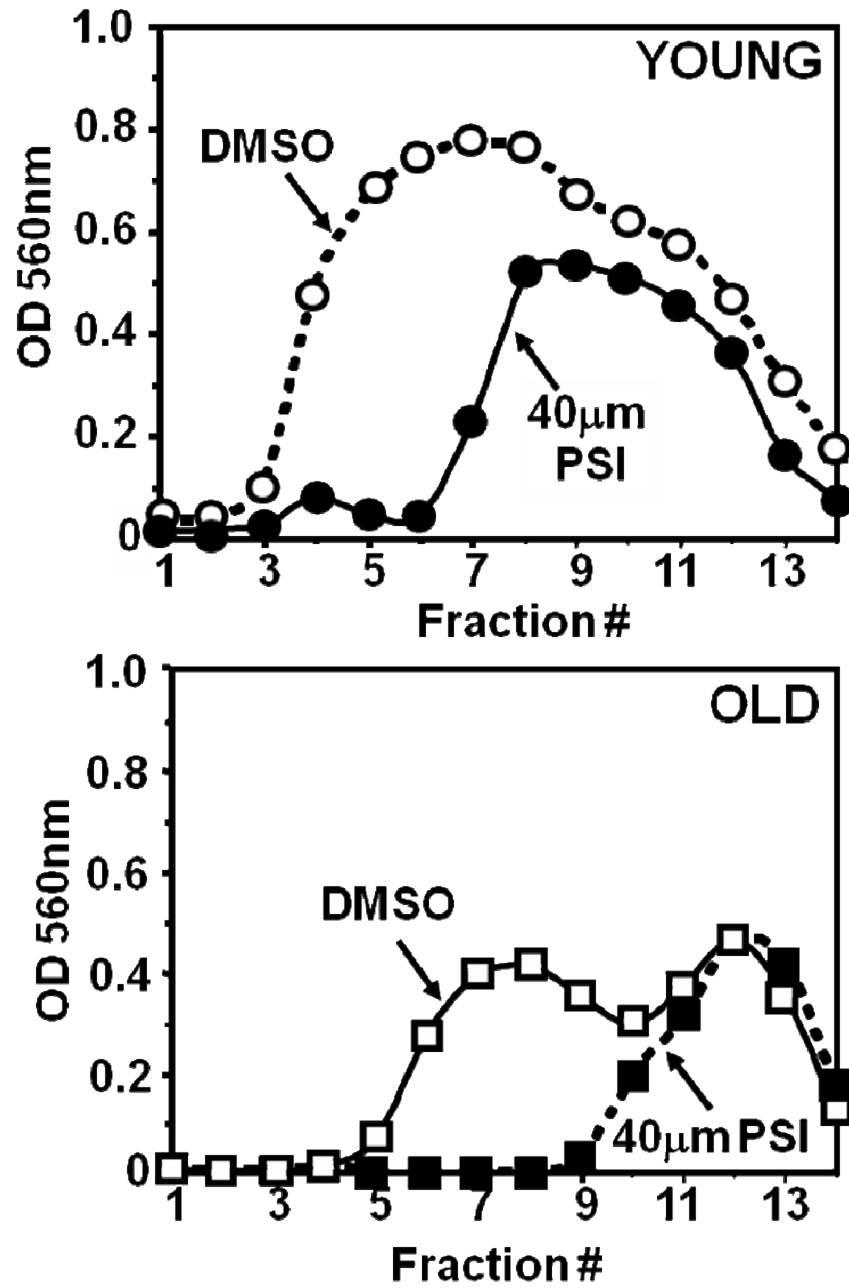



Figure 15

**Figure 16 (next page) – Polyubiquitinated protein and proteasome d$\beta$5 subunit levels in young (*1-2 days*) and old (*43-47 days*) flies.** Mixed populations of females and males for each age group were analyzed. Flies were fed 5% sucrose with DMSO (0, vehicle, control, 0.5%) or with a proteasome inhibitor (PSI, 10μM in 0.5% DMSO) for 48h after being starved for 6h. The levels of polyubiquitin-protein conjugates (Ub-conjugates) and of a proteasome subunit (d$\beta$5) were detected by Western blot analysis as described in "Methods". 50 μg of protein were loaded per lane. Similar results were obtained in duplicate experiments.

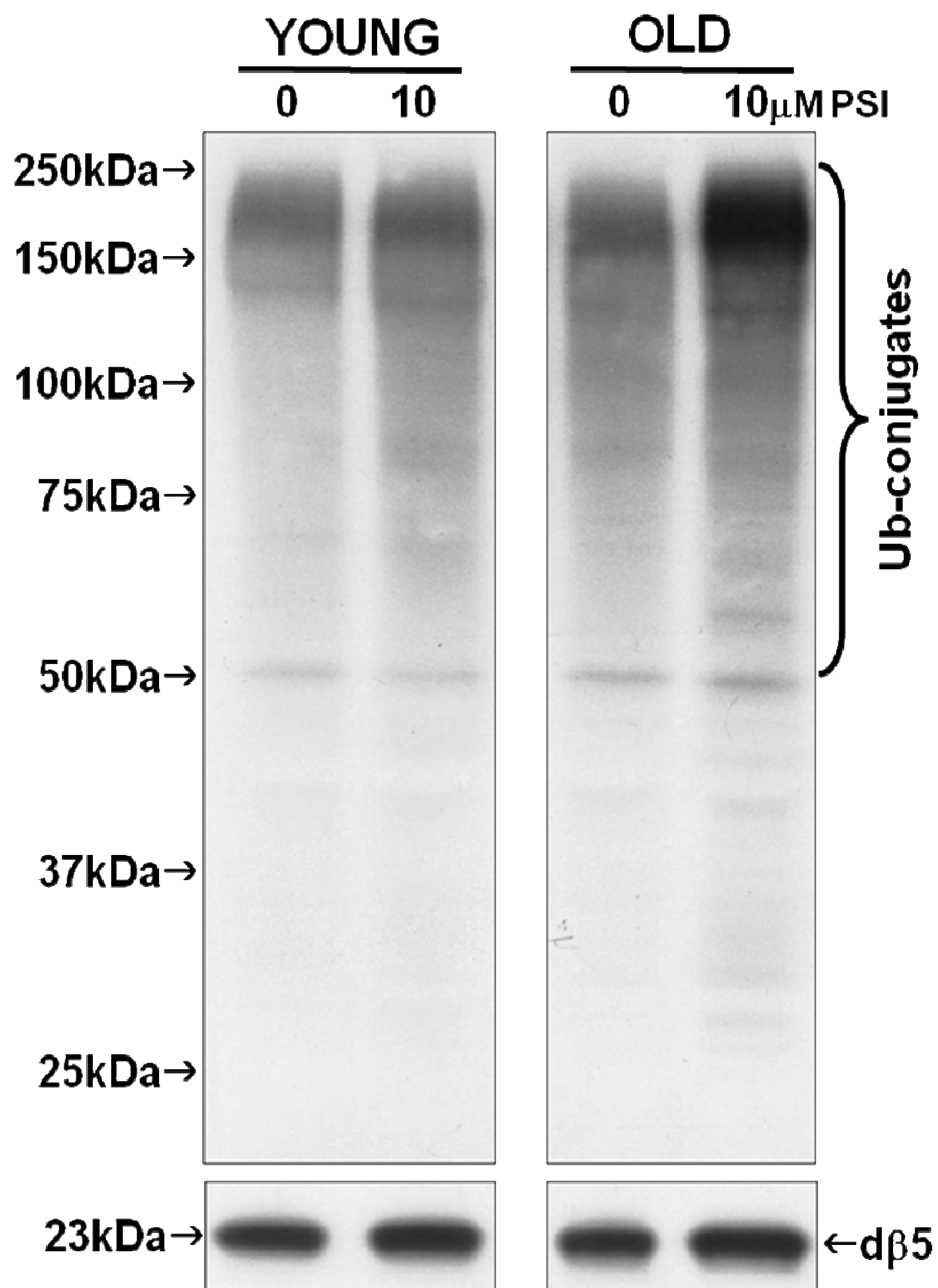



Figure 16

**Figure 17 - Survival curves for young (*1-2 days*) and old (*43-47 days*) flies.** Mixed populations of both females and males for each age group were analyzed. Flies were kept in narrow *Drosophila* vials which were lined with 3mm filter paper. Flies were starved for 6hrs and then fed a solution of 5% (weight/vol) sucrose with either vehicle (0.5 % DMSO, control) or with the proteasome inhibitor PSI (10µM, in DMSO) for 48hrs. Dead flies were counted after the time periods indicated (n=30 flies for each treatment). Data represent the mean ± SEM from three experiments. The *asterics* (*) identify the values that are significantly different ($p<0.05$) between control and PSI-treated flies for each time point within each age group.

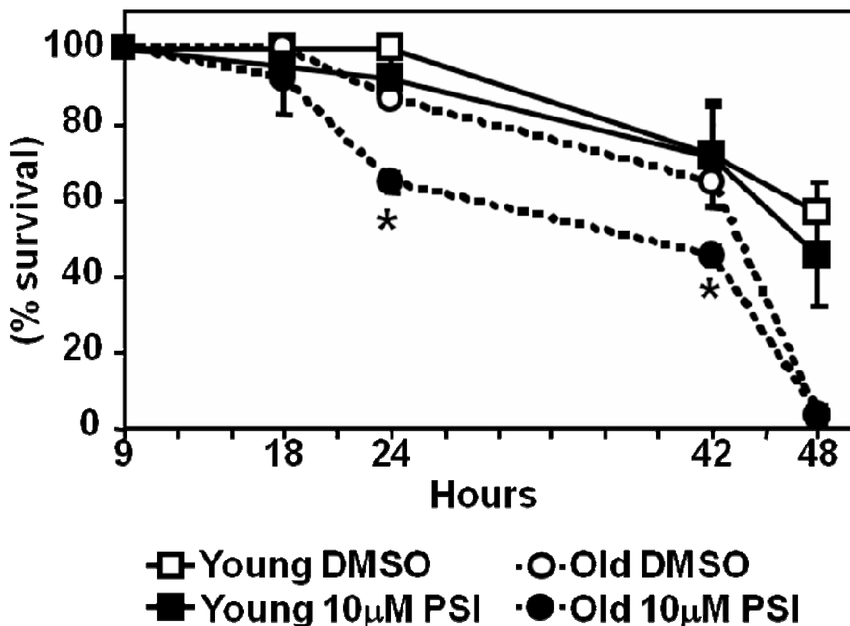
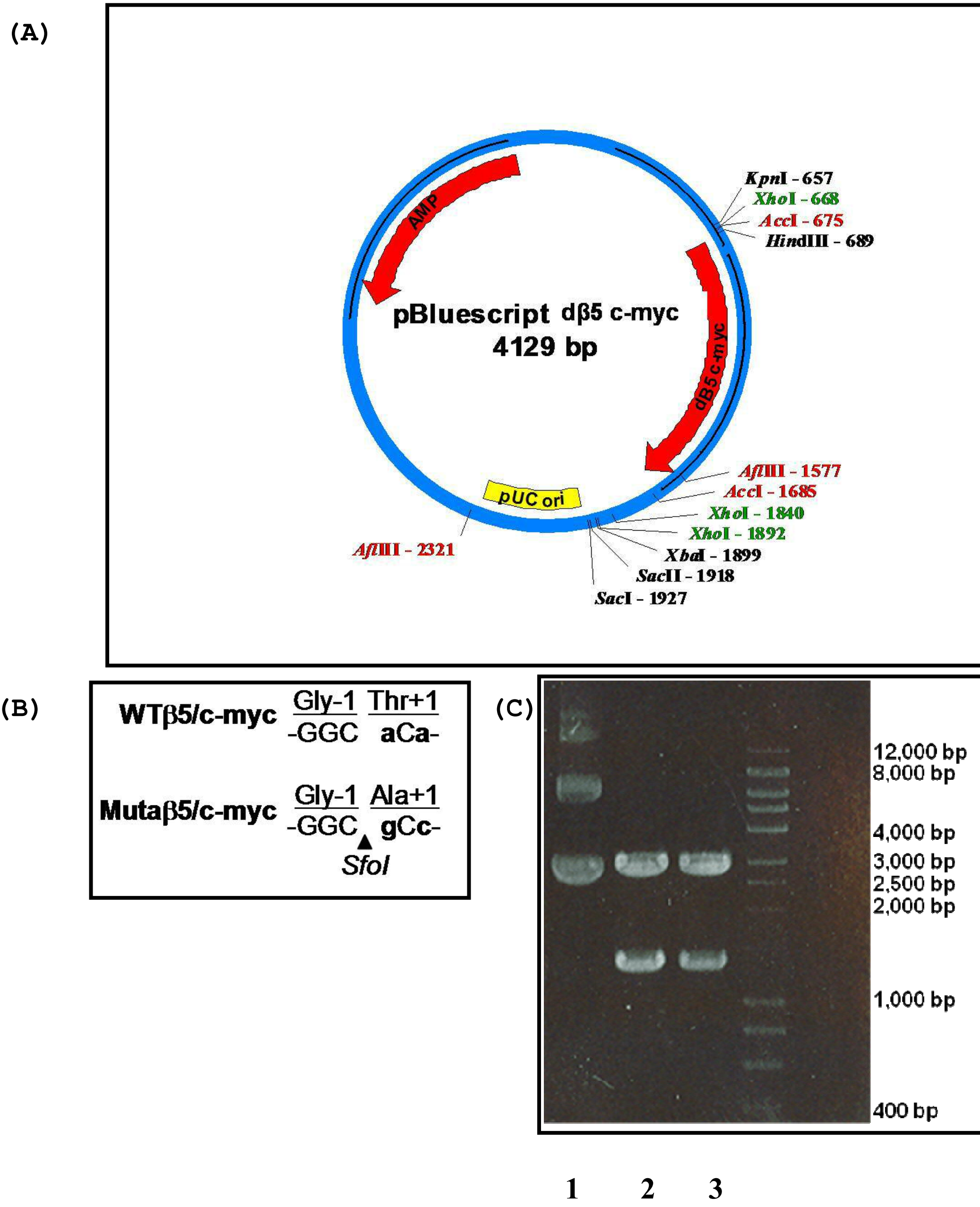



**Figure 17**

**Figure 18 - (next page) Generation of a catalytically inactive dβ5 proteasome subunit.** **(A)** To reduce proteasome activity a dβ5 gene carrying an active site T1A mutation was inserted into the pBlueScript vector. **(B)** The two mutated base pairs (*a* to *g* and *a* to *c*) established the Thr1Ala mutation and the unique Sfo1 site.

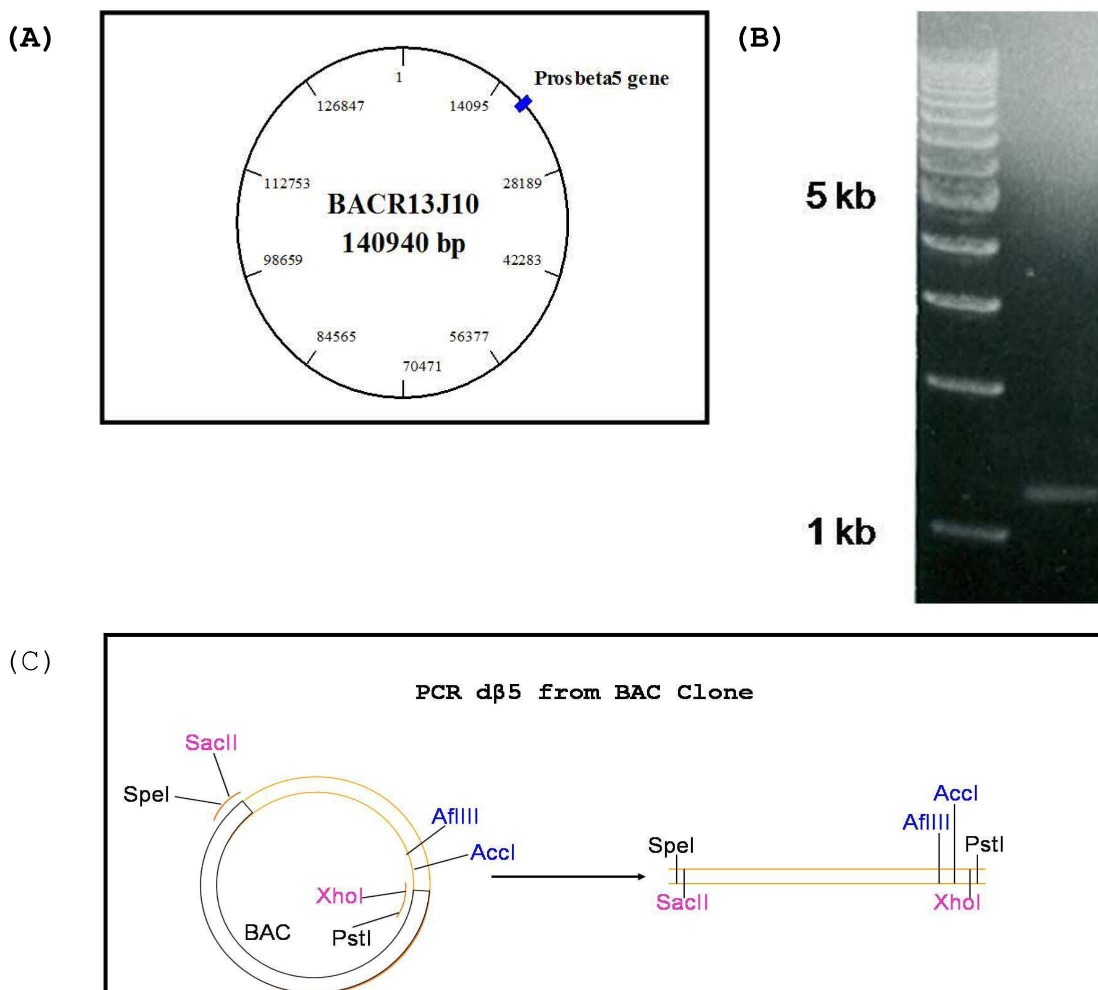
**(C)** Restriction analysis of the pBlueScript vector with the mutated dβ5-c-myc. *Lane 1*, uncut pBlueScript-mutated dβ5-c-myc; *Lanes 2 and 3*, two possible clones of pBlueScript-mutated dβ5-c-myc cut with the restriction enzyme XhoI. Both clones rendered the appropriate size fragment corresponding to the mutated dβ5 gene.




# Figure 18

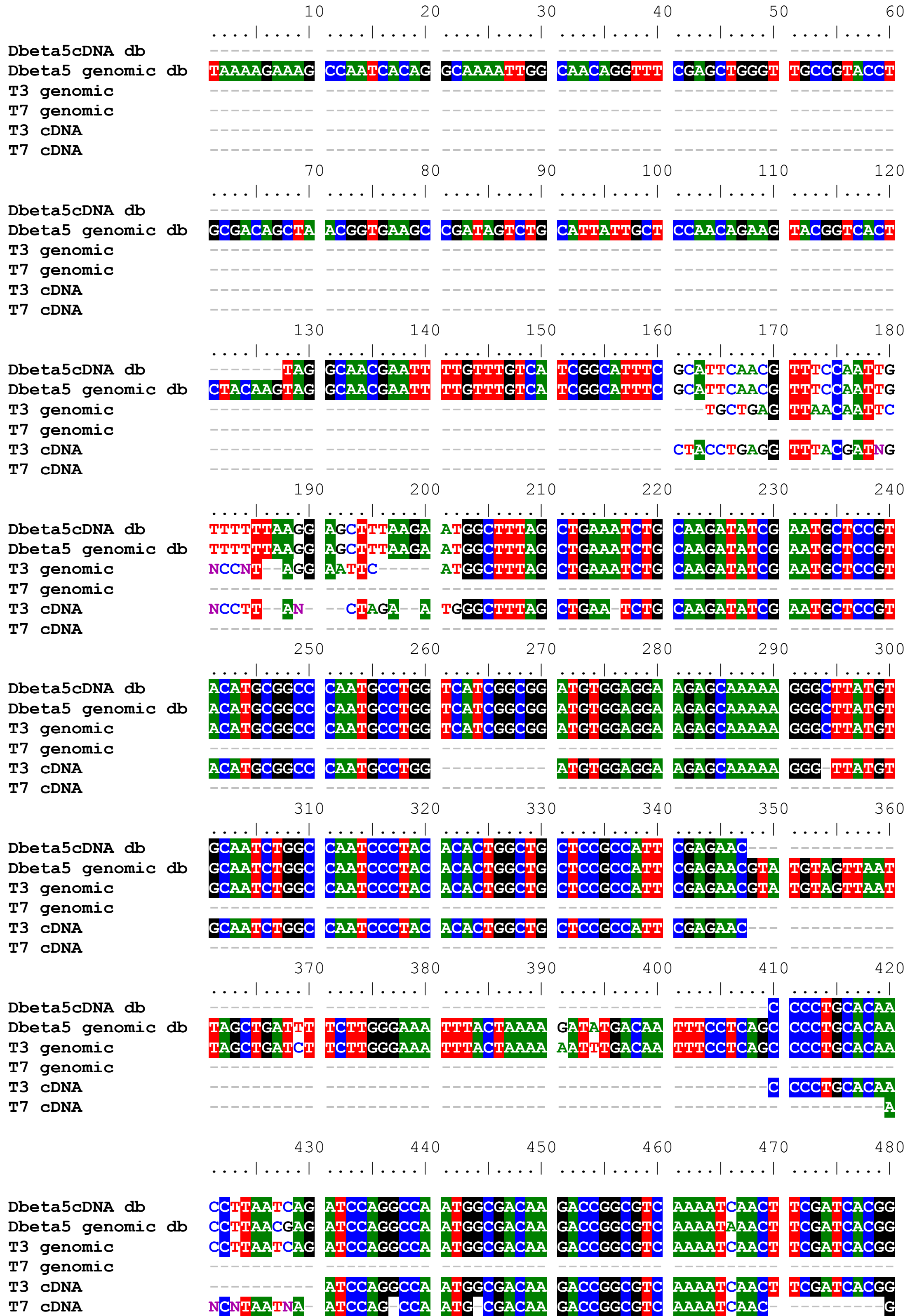
**Figure 19 - Analysis of the genomic DNA encoding the dβ5 gene. (A)** BACR13J10 clone from which the Prosβ5 gene (dβ5) was obtained by PCR. **(B)** PCR analysis of the dβ5 gene from the BAC clone.

**(C)** Schematic of the PCR analysis




# Figure 19

**Figure 20-(next page) Sequencing data of the Prosβ5 gene.** The Prosβ5 cDNA and genomic DNA sequences were obtained from Fly Base. The next four lines were the generated sequenced from the T3 and T7 directions for each. This figure displays the consensus of my sequences versus the sequences in the data base.



```
                           490        500        510        520        530        540
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Dbeta5cDNA db       CACCACAACG TTGGGCTTCA AGTTCAAGGG CGGCGTTCTC CTGGCAGTCG ATTCCCGTGC
Dbeta5 genomic db   CACCACAACG TTGGGCTTCA AGTTCAAGGG CGGCGTTCTC CTGGCAGTCG ATTCCCGTGC
T3 genomic          CACCACAACG TTGGGCTTCA AGTTCAAGGG CGGCGTTCTC CTGGCAGTCG ATTCCCGTGC
T7 genomic          ---------- ---------- ---------- ---------- ---------- ----------
T3 cDNA             CACCACAACG TTGGGCTTCA AGTTCAAGGG CGGTGTTCTC ---------- ATTCCCGTGC
T7 cDNA             CACCACAACG TTGG-CTTCA A-TTCAAGGG CGGTGTTCTC CTGGCAGTCG ATTCCCGTGC

                           550        560        570        580        590        600
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Dbeta5cDNA db       CACGGGTGGA TCGTACATTG GCTCCCAGTC GATGAAGAAG ATCGTG--GA GATCAATCAG
Dbeta5 genomic db   CACGGGTGGA TCGTACATTG GCTCCCAGTC GATGAAGAAG ATCGTG--GA GATCAATCAG
T3 genomic          CACGGGTGGA TCGTACATTG GCTCCCAGTC GATGAAGAAG ATCGTG--GA GATCAATCAG
T7 genomic          ---------- ---------- ---------- ---------- ---------- ----------
T3 cDNA             CACGGGTGGA TCGTACATTG GCTCCCAGTC GATGAAGAAG ATCGTGTGGA GATCAATCAG
T7 cDNA             CACGGGTGGA TCGTACATTG GCTCCCAGTC G--------- -TCGTG--GA GATCAATCAG

                           610        620        630        640        650        660
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Dbeta5cDNA db       TTCATGCTGG GCACCTTGGC CGGTGGCGCA GCCGATTGCG TTTACTGGGA CAGGGTCCTT
Dbeta5 genomic db   TTCATGCTGG GCACCTTGGC CGGTGGCGCA GCCGATTGCG TTTACTGGGA CAGGGTCCTT
T3 genomic          TTCATGCTGG GCACCTTGGC CGGTGGCGCA GCCGATTGCG TTTACTGGGA CAGGGTCCTT
T7 genomic          ---------- ---------- ---------- ---------- ---------- ----------
T3 cDNA             TTCATGCTGG GCACCTTGGC CG-------- --CGATTGCG TTTACTGGGA CAGGGTCCTT
T7 cDNA             TTCATGCTGG GCACCTTCGC CGGTGGCGCA GCCGATTGCG TTTACTGGGA CAGTGTCCTT

                           670        680        690        700        710        720
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Dbeta5cDNA db       TCGAA-GGAA TGCCGCCTTC ACGAGCTTCG AAACAAGGAA CGCATCTCGG TGGCCGCCGC
Dbeta5 genomic db   TCGAA-GGAA TGCCGCCTTC ACGAGCTTCG AAACAAGGAA CGCATCTCGG TGGCCGCCGC
T3 genomic          TCGAA-GGAA TGCCGCCTTC ACGAGCTTCG AAACAAGGAA CGCATCTCGG TGGCCGCCGC
T7 genomic          ---------- ---------- ---------- ---------- ---------- ----------
T3 cDNA             TCGAA-GGAA TGCCGCCTTC ACGAGCTGCG AAACAAAGAA CGCATCTCGG TGGCCGCCGC
T7 cDNA             TCGAAAGGAA TGCCGC---- ------TGCG AAACAAAGAA CGCATCTCGG TGGCCGCCGC

                           730        740        750        760        770        780
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Dbeta5cDNA db       CAGCAAGATA ATGGCCAACA TTGCCCACGA ATACAAGGGA ATGGGTCTGA GCATGGGCAT
Dbeta5 genomic db   CAGCAAGATA ATGGCCAACA TTGCCCACGA ATACAAGGGA ATGGGTCTGA GCATGGGCAT
T3 genomic          CAGCAAGATA ATGGCCAACA TTGCCCACGA ATACAAGGGA ATGGGTCTGA -CATGGGCAT
T7 genomic          ---------- ---------- ---------- ---------- ---------- ----------
T3 cDNA             CAG------- ---GCCAACA TTGCCCACGA ATACAAGGGA ATGGGTCTGA GCATGGGCAT
T7 cDNA             CAGCAAGATA ATGGCCAACA TTGCCCACGA ATACAAGGGA ATGGGTCTGA GCAT------

                           790        800        810        820        830        840
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Dbeta5cDNA db       GATGCTGGCC GGTTAC-GAT AA--GCGTGG TCC-AGGCC- TCTACTA-TG TGGACTCC-G
Dbeta5 genomic db   GATGCTGGCC GGTTAC-GAT AA--GCGTGG TCC-AGGCC- TCTACTA-TG TGGACTCC-G
T3 genomic          GATGCTGGCC GGTTAC-GAT AA--GCGTGG TCC-AGGCC- TCTACTA-TG TGGACTCC-G
T7 genomic          --------CC GGTTTCCNAN AAAGGGGGGG TCCCAGGCCC TTTNTTAATG GGGACTCCCG
T3 cDNA             GATGCTGGCC GGTTAC-NAT AA---CGTGG TCC-AGNC-- TCTACTA-TG TGGA--TC--
T7 cDNA             ----CTGGCC GGTTAC-GAT AA--GCGTGG TCCACGGCC- TCTACTA-TG TGGACTCC-G

                           850        860        870        880        890        900
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Dbeta5cDNA db       AGGGATCTCG CACGCCTGGC -AATTTGTTC TCTGTTGGTA GTGGATCGCT GTACGCCTAC
Dbeta5 genomic db   AGGGATCTCG CACGCCTGGC -AATCTGTTC TCTGTTGGTA GTGGATCGCT GTACGCCTAC
T3 genomic          AGGGATCTCG CACGCCTGGC -AATTG--TC TCTGTC---- ---------- ----------
T7 genomic          AGGGATNTCN CACCCCTGGC CAATTTGTTC TCTGTTGGTA GTGGATCGCT GTACGCTTAC
T3 cDNA             -------TCG CACGCCTGGN -AATCTGTTC TCTGTTGG-- ---------- ----------
T7 cDNA             AGGGTTCTCG CACGCCTGGC -AATCTGTTC TCTGTTGGTA GT-------- --ACGCCTAC

                           910        920        930        940        950        960
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Dbeta5cDNA db       GGTGTCCTGG ACTCTGGCTA TCATTGGGAC CTGGAGGACA AGGAGGCCCA GGAGCTGGGA
Dbeta5 genomic db   GGTGTCCTGG ACTCTGGCTA TCATTGGGAC CTGGAGGACA AGGAGGCCCA GGAGCTGGGA
T3 genomic          ---------- ---------- ---------- ---------- ---------- ----------
T7 genomic          GGTGTCCTGG ACTCTGGCTA TCATTGGGAC CTGGAGGACA AGGAGGCCCA GGAGCTGGGA
```

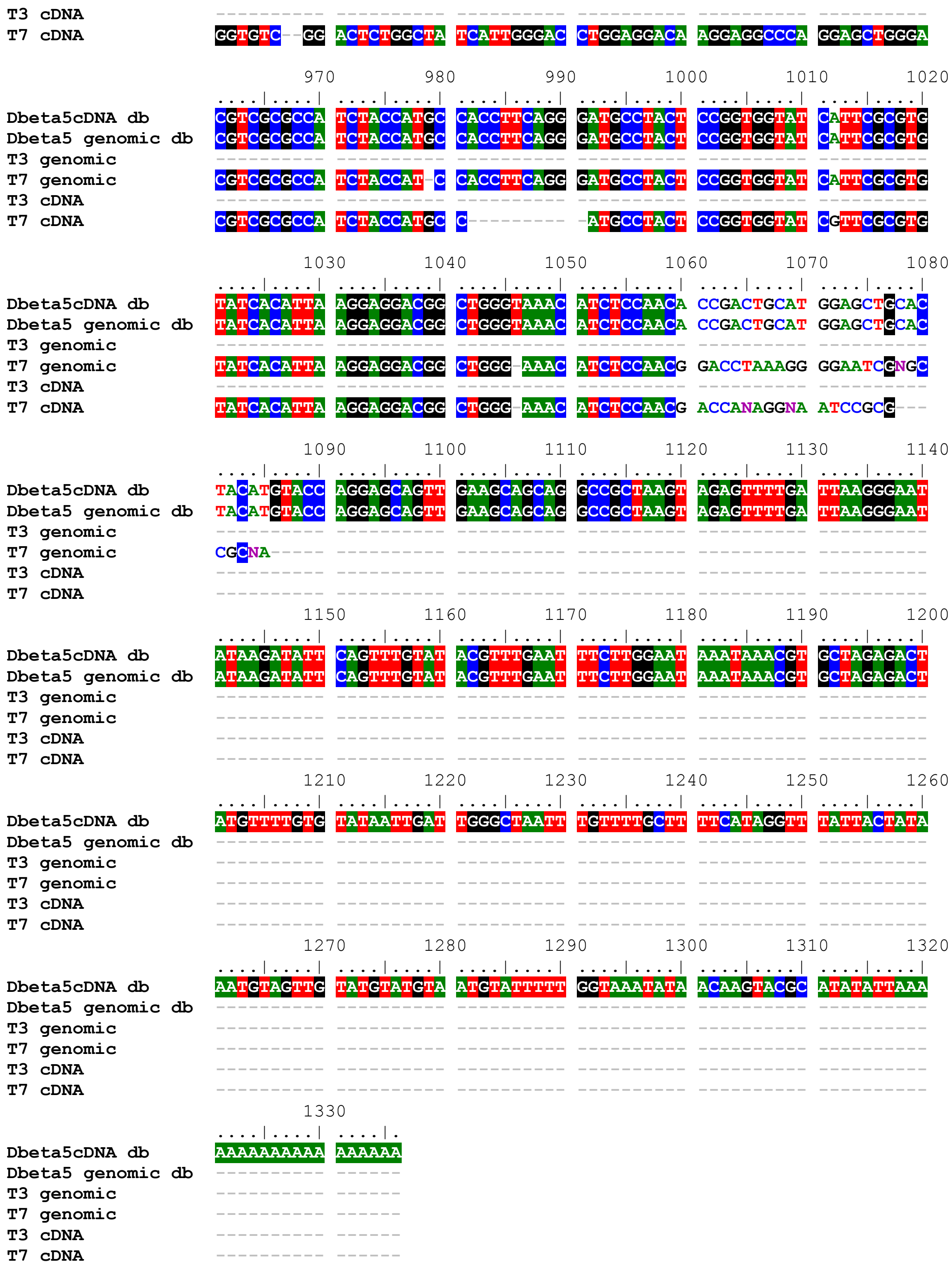


Figure 20

**Figure 21-Schematic of P-element transformation.** The P-element vector will be co-injected with the helper plasmid p∏25.7wc to provide temporary transposase function into $w^{1118}$ flies prior to pole cell formation.

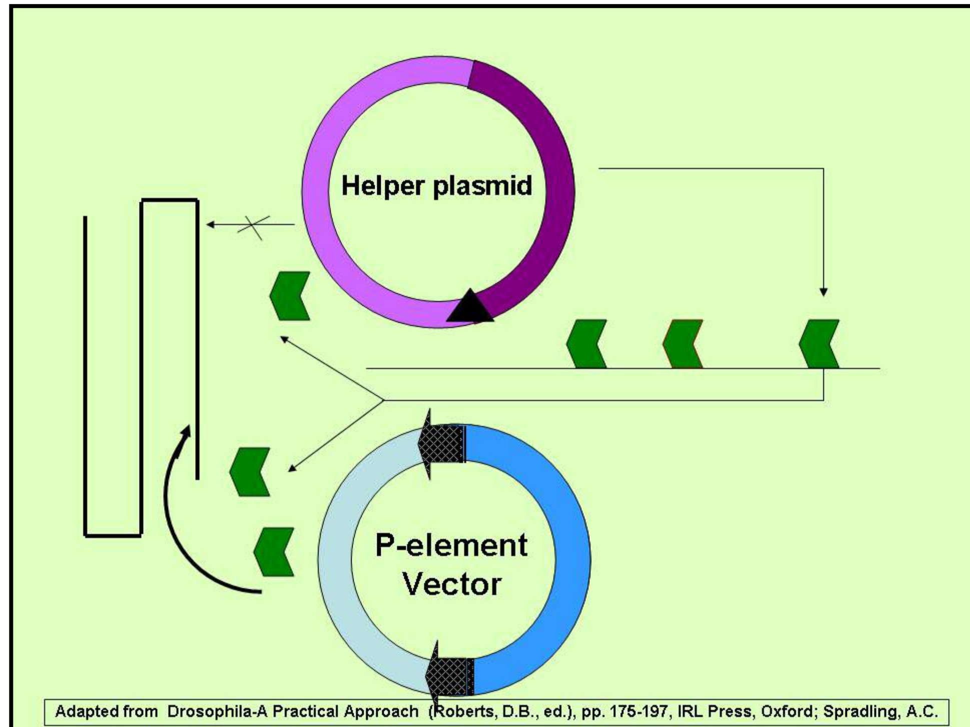



**Figure 21**

**Figure 22-Establishment of isolines and localization of inserts by segregation analysis and balancing of fly stocks.**

Generation

pUAST + pπ25.7wc
co-microinjection

$G_0$ yw ♂ or ♀ × yw or ♂♂ or ♀♀

Select yw$^+$

$G_1$ P[(w+)A] or yw+ ♂or♀ × yw ♂♂ or ♀♀

Select yw+

$G_2$ Isolines

localize insert(s)

$G_3$ Single Insert Lines

$G_4$ Homozygous Stocks

Balanced Stock

Adapted from Drosophila-A Practical Approach (Roberts, D.B., ed.), pp. 175-197, IRL Press, Oxford; Spradling, A.C.

**Figure 22**

# CHAPTER VII

# REFERENCES

## Reference List

1. Coux O, Tanaka K, Goldberg AL. Structure and Functions of the 20S and 26S Proteasomes. *Annual Review of Biochemistry* 1996; 65:801-847.

2. Chen P, Hochstrasser M. Autocatalytic Subunit Processing Couples Active Site Formation in the 20S Proteasome to Completion of Assembly. *Cell* 1996; 86:961-972.

3. Heinemeyer W, Gruhler A, Mohrle V, Mahe Y, Wolf DH. PRE2, highly homologous to the human major histocompatibility complex- linked RING10 gene, codes for a yeast proteasome subunit necessary for chrymotryptic activity and degradation of ubiquitinated proteins. *J Biol Chem* 1993; 268:5115-5120.

4. Hochstrasser M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Current Opinion in Cell Biology* 1995; 7:215-223.

5. Mayer RJ, Arnold J, Laszlo L, Landon M, Lowe J. Ubiquitin in health and disease. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* 1991; 1089:141-157.

6. Cook WJ, Jeffrey LC, Kasperek E, Pickart CM. Structure of Tetraubiquitin Shows How Multiubiquitin Chains Can Be Formed. *Journal of Molecular Biology* 1994; 236:601-609.

7. Beal R, Deveraux Q, Xia G, Rechsteiner M, Pickart C. Surface hydrophobic residues of multiubiquitin chains essential for proteolytic targeting. *PNAS* 1996; 93:861-866.

8. Pickart CM. Targeting of substrates to the 26S proteasome. *FASEB J* 1997; 11:1055-1066.

9. Shang F, Deng G, Liu Q, Guo W, Haas AL, Crosas B et al. Lys6-modified Ubiquitin Inhibits Ubiquitin-dependent Protein Degradation. *J Biol Chem* 2005; 280:20365-20374.

10. Chung CH, Baek SH. Deubiquitinating Enzymes: Their Diversity and Emerging Roles. *Biochemical and Biophysical Research Communications* 1999; 266:633-640.

11. Wilkinson KD. Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *FASEB J* 1997; 11:1245-1256.

12. DeMartino GN, Slaughter CA. The Proteasome, a Novel Protease Regulated by Multiple Mechanisms. *J Biol Chem* 1999; 274:22123-22126.

13. Chan HYE, Warrick JM, Gray-Board, Paulson HL, Bonini NM. Mechanisms of chaperone suppression of polyglutamine disease: selectivity, synergy and modulation of protein solubility in Drosophila. *Hum Mol Genet* 2000; 9:2811-2820.

14. McBride WH, Iwamoto KS, Syljuasen R, Pervan M, Pajonk F. The role of the ubiquitin/proteasome system in cellular responses to radiation. *Oncogene* 2003; 22:5755-5773.

15. Benaroudj N, Zwickl P, Seemuller E, Baumeister W, Goldberg AL. ATP Hydrolysis by the Proteasome Regulatory Complex PAN Serves Multiple Functions in Protein Degradation. *Molecular Cell* 2003; 11:69-78.

16. Babbitt SE, Kiss A, Deffenbaugh AE, Chang YH, Bailly E, Erdjument-Bromage H et al. ATP Hydrolysis-Dependent Disassembly of the 26S Proteasome Is Part of the Catalytic Cycle. *Cell* 2005; 121:553-565.

17. Wang R, Chait BT, Wolf I, Kohanski RA, Cardozo C. Lysozyme Degradation by the Bovine Multicatalytic Proteinase Complex (Proteasome): Evidence for a Nonprocessive Mode of Degradation. *Biochemistry* 1999; 38:14573-14581.

18. Rape M, Jentsch S. Productive RUPture: activation of transcription factors by proteasomal processing. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 2004; 1695:209-213.

19. Liu CW, Corboy MJ, DeMartino GN, Thomas PJ. Endoproteolytic Activity of the Proteasome. *Science* 2003; 299:408-411.

20. Piwko W, Jentsch S. Proteasome-mediated protein processing by bidirectional degradation initiated from an internal site. *Nat Struct Mol Biol* 2006; 13:691-697.

21. Kisselev AF, Akopian TN, Castillo V, Goldberg AL. Proteasome Active Sites Allosterically Regulate Each Other, Suggesting a Cyclical Bite-Chew Mechanism for Protein Breakdown. *Molecular Cell* 1999; 4:395-402.

22. Belote JM, Fortier E. Targeted expression of dominant negative proteasome mutants in Drosophila melanogaster. *Genesis* 2002; 34:80-82.

23. Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD et al. Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature* 1997; 386:463-471.

24. Schmidtke G, Kraft R, Kostka S, Henklein P, Frommel C, Lowe J et al. Analysis of mammalian 20S proteasome biogenesis: the maturation of beta-subunits is an ordered two-step mechanism involving autocatalysis. *EMBO J* 1996; 15:6887-6898.

25. Mishto M, Santoro A, Bellavista E, Bonafe M, Monti D, Franceschi C. Immunoproteasomes and immunosenescence. *Ageing Research Reviews* 2003; 2:419-432.

26. Lin YJ, Seroude L, Benzer S. Extended Life-Span and Stress Resistance in the Drosophila Mutant methuselah. *Science* 1998; 282:943-946.

27. Rogina B, Reenan RA, Nilsen SP, Helfand SL. Extended Life-Span Conferred by Cotransporter Gene Mutations in Drosophila. *Science* 2000; 290:2137-2140.

28. Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E et al. Extension of Life-Span by Loss of CHICO, a Drosophila Insulin Receptor Substrate Protein. *Science* 2001; 292:104-106.

29. Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM, Garofalo RS. A Mutant Drosophila Insulin Receptor Homolog That Extends Life-Span and Impairs Neuroendocrine Function. *Science* 2001; 292:107-110.

30. Umeda-Kameyama Y, Tsuda M, Ohkura C, Matsuo T, Namba Y, Ohuchi Y et al. Thioredoxin suppresses Pael-R-induced neurotoxicity and extends longevity in drosophila. *J Biol Chem* 2007;M700937200.

31. Aigaki T, Seong Kh, Matsuo T. Longevity determination genes in Drosophila melanogaster. *Mechanisms of Ageing and Development* 2002; 123:1531-1541.

32. Schwarze SR, Weindruch R, Aiken JM. Oxidative stress and aging reduce COX I RNA and cytochrome oxidase activity in Drosophila. *Free Radical Biology and Medicine* 1998; 25:740-747.

33. Calleja M, Pena P, Ugalde C, Ferreiro C, Marco R, Garesse R. Mitochondrial DNA remains intact during Drosophila aging, but the levels of mitochondrial transcripts are significantly reduced. *J Biol Chem* 1993; 268:18891-18897.

34. Sanchez-Blanco A, Fridell YW, Helfand SL. Involvement of Drosophila Uncoupling Protein 5 in Metabolism and Aging. *Genetics* 2006; 172:1699-1710.

35. Fridell YW, Sanchez-Blanco A, Silvia BA, Helfand SL. Targeted expression of the human uncoupling protein 2 (hUCP2) to adult neurons extends life span in the fly. *Cell Metabolism* 2005; 1:145-152.

36. Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH et al. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature* 2006; 441:1162-1166.

37. Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, Vosshall LB. Or83b Encodes a Broadly Expressed Odorant Receptor Essential for Drosophila Olfaction. *Neuron* 2004; 43:703-714.

38. Libert S, Zwiener J, Chu X, VanVoorhies W, Roman G, Pletcher SD. Regulation of Drosophila Life Span by Olfaction and Food-Derived Odors. *Science* 2007; 315:1133-1137.

39. Tatar M, Khazaeli AA, Curtsinger JW. Chaperoning extended life. *Nature* 1997; 390:30.

40. Chaudhuri A, Bowling K, Funderburk C, Lawal H, Inamdar A, Wang Z et al. Interaction of Genetic and Environmental Factors in a Drosophila Parkinsonism Model. *J Neurosci* 2007; 27:2457-2467.

41. Carrard G, Bulteau AL, Petropoulos I, Friguet B. Impairment of proteasome structure and function in aging. *The International Journal of Biochemistry & Cell Biology* 2002; 34:1461-1474.

42. Keller JN, Gee J, Ding Q. The proteasome in brain aging. *Ageing Research Reviews* 2002; 1:279-293.

43. Keller JN, Huang FF, Markesbery WR. Decreased levels of proteasome activity and proteasome expression in aging spinal cord. *Neuroscience* 2000; 98:149-156.

44. Ly DH, Lockhart DJ, Lerner RA, Schultz PG. Mitotic Misregulation and Human Aging. *Science* 2000; 287:2486-2492.

45. Gaczynska M, Osmulski PA, Ward WF. Caretaker or undertaker? The role of the proteasome in aging. *Mechanisms of Ageing and Development* 2001; 122:235-254.

46. Chondrogianni N, Stratford FLL, Trougakos IP, Friguet B, Rivett AJ, Gonos ES. Central Role of the Proteasome in Senescence and Survival of Human Fibroblasts: INDUCTION OF A SENESCENCE-LIKE PHENOTYPE UPON ITS INHIBITION AND RESISTANCE TO STRESS UPON ITS ACTIVATION. *J Biol Chem* 2003; 278:28026-28037.

47. Stratford FLL, Chondrogianni N, Trougakos IP, Gonos ES, Rivett AJ. Proteasome response to interferon-[gamma] is altered in senescent human fibroblasts. *FEBS Letters* 2006; 580:3989-3994.

48. Chondrogianni N, Gonos ES. Proteasome inhibition induces a senescence-like phenotype in primary human fibroblasts cultures. *Biogerontology* 2004; 5:55-61.

49. Torres C, Lewis L, Cristofalo VJ. Proteasome inhibitors shorten replicative life span and induce a senescent-like phenotype of human fibroblasts. *J Cell Physiol* 2006; 207:845-853.

50. Chondrogianni N, Gonos ES. Overexpression of hUMP1/POMP proteasome accessory protein enhances proteasome-mediated antioxidant defence. *Experimental Gerontology* In Press, Corrected Proof.

51. Cai D, Li M, Lee K, Lee K, Wong W, Chan K. Age-related changes of aqueous protein profiles in rat fast and slow twitch skeletal muscles. *Electrophoresis* 2000; 21:465-472.

52. Davis T, Kipling D. Werner Syndrome as an example of inflamm-aging: possible therapeutic opportunities for a progeroid syndrome? *Rejuvenation Res* 2006; 9:402-407.

53. Yu CE, Oshima J, Fu YH, Wijsman EM, Hisama F, Alisch R et al. Positional cloning of the Werner's syndrome gene. *Science* 1996; 272:258-262.

54. Balajee AS, Machwe A, May A, Gray MD, Oshima J, Martin GM et al. The Werner Syndrome Protein Is Involved in RNA Polymerase II Transcription. *Mol Biol Cell* 1999; 10:2655-2668.

55. Wojcik C, Yano M, DeMartino GN. RNA interference of valosin-containing protein (VCP/p97) reveals multiple cellular roles linked to ubiquitin/proteasome-dependent proteolysis. *J Cell Sci* 2004; 117:281-292.

56. Yin J, Kwon YT, Varshavsky A, Wang W. RECQL4, mutated in the Rothmund-Thomson and RAPADILINO syndromes, interacts with ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway. *Hum Mol Genet* 2004; 13:2421-2430.

57. Figueiredo-Pereira ME, Rockwell P. The ubiquitin/proteasome pathway in neurological disorders, Role of Proteolysis in the Pathophysiology of Neurodegenerative Diseases. A.Lajtha NLB (editor). New York, NY: Kluwer Academic/Plenum Publishers; 2001. pp. 137-153.

58. van Leeuwen FW, de Kleijn DP, van den Hurk HH, Neubauer A, Sonnemans MA, Sluijs JA et al. Frameshift Mutants of {beta}?Amyloid Precursor Protein and Ubiquitin-B in Alzheimer's and Down Patients. *Science* 1998; 279:242-247.

59. Lam YA, Pickart CM, Alban A, Landon M, Jamieson C, Ramage R et al. Inhibition of the ubiquitin-proteasome system in Alzheimer's disease. *PNAS* 2000; 97:9902-9906.

60. Longo VD. Search for methuselah genes heats up. *Sci Aging Knowledge Environ* 2004; 2004:e6.

61. Gray DA, Tsirigotis M, Woulfe J. Ubiquitin, proteasomes, and the aging brain. *Sci Aging Knowledge Environ* 2003; 2003:RE6.

62. ves-Rodrigues A, Gregori L, Figueiredo-Pereira ME. Ubiquitin, cellular inclusions and their role in neurodegeneration. *Trends in Neurosciences* 1998; 21:516-520.

63. Chondrogianni N, Gonos ES. Proteasome dysfunction in mammalian aging: Steps and factors involved. *Experimental Gerontology* 2005; 40:931-938.

64. Ashburner M. Drosophila: a laboratory handbook. Cold Spring Harbor, New York: Cold Spring Harbor Press; 1989.

65. Wilk S, Orlowski M. Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic protease complex. *J Neurochem* 1983; 40:842-849.

66. Elsasser S, Schmidt M, Finley D. Characterization of the Proteasome Using Native Gel Electrophoresis. In: Methods in Enzymology
Ubiquitin and Protein Degradation, Part A. Raymond JD (editor). Academic Press; 1907. pp. 353-363.

67. Glickman MH, Rubin DM, Fried VA, Finley D. The Regulatory Particle of the Saccharomyces cerevisiae Proteasome. *Mol Cell Biol* 1998; 18:3149-3162.

68. Orso G, Martinuzzi A, Rossetto MG, Sartori E, Feany M, Daga A. Disease-related phenotypes in a Drosophila model of hereditary spastic paraplegia are ameliorated by treatment with vinblastine. *J Clin Invest* 2005; 115:3026-3034.

69. Liu CW, Li X, Thompson D, Wooding K, Chang Tl, Tang Z et al. ATP Binding and ATP Hydrolysis Play Distinct Roles in the Function of 26S Proteasome. *Molecular Cell* 2006; 24:39-50.

70. Stein RL, Melandri F, Dick L. Kinetic characterization of the chymotryptic activity of the 20S proteasome. *Biochemistry* 1996; 35:3899-3908.

71. Sasaki T, Kikuchi T, Yumoto N, Yoshimura N, Murachi T. Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. *J Biol Chem* 1984; 259:12489-12494.

72. Figueiredo-Pereira ME, Berg KA, Wilk S. A New Inhibitor of the Chymotrypsin-Like Activity of the Multicatalytic Proteinase Complex (20S Proteasome) Induces Accumulation of Ubiquitin-Protein Conjugates in a Neuronal Cell. *Journal of Neurochemistry* 1994; 63:1578-1581.

73. Groll M, Heinemeyer W, Jager S, Ullrich T, Bochtler M, Wolf DH et al. The catalytic sites of 20S proteasomes and their role in subunit maturation: A mutational and crystallographic study. *PNAS* 1999; 96:10976-10983.

74. Groll M, Bajorek M, Kohler A, Moroder L, Rubin DM, Huber R et al. A gated channel into the proteasome core particle. *Nat Struct Mol Biol* 2000; 7:1062-1067.

75. Forster A, Hill CP. Proteasome degradation: enter the substrate. *Trends in Cell Biology* 2003; 13:550-553.

76. Kisselev AF, Kaganovich D, Goldberg AL. Binding of Hydrophobic Peptides to Several Non-catalytic Sites Promotes Peptide Hydrolysis by All Active Sites of 20 S Proteasomes. EVIDENCE FOR PEPTIDE-INDUCED CHANNEL OPENING IN THE alpha -RINGS. *J Biol Chem* 2002; 277:22260-22270.

77. Reits EA, Benham AM, Plougastel B, Neefjes J, Trowsdale J. Dynamics of proteasome distribution in living cells. *EMBO J* 1997; 16:6087-6094.

78. Eytan E, Ganoth D, Armon T, Hershko A. ATP-Dependent Incorporation of 20S Protease into the 26S Complex that Degrades Proteins Conjugated to Ubiquitin. *PNAS* 1989; 86:7751-7755.

79. Laura H, Martin R. Effects of nucleotides on assembly of the 26S proteasome and degradation of ubiquitin conjugates. *Molecular Biology Reports* 1997; V24:13-16.

80. Yang Y, Gehrke S, Haque M, Imai Y, Kosek J, Yang L et al. Inactivation of Drosophila DJ-1 leads to impairments of oxidative stress response and phosphatidylinositol 3-kinase/Akt signaling. *PNAS* 2005; 102:13670-13675.

81. Kurucz E, Ando I, Sumegi M, Holzl H, Kapelari B, Baumeister W et al. Assembly of the Drosophila 26 S proteasome is accompanied by extensive subunit rearrangements. *Biochem J* 2002; 365:527-536.

82. Saks VA, Khuchua ZA, Vasilyeva EV, Belikova OY, Kuznetsov AV. Metabolic compartmentation and substrate channelling in muscle cells. Role of coupled creatine kinases in in vivo regulation of cellular respiration--a synthesis. *Mol Cell Biochem* 1994; 133-134:155-192.

83. Ishida Y, Riesinger I, Wallimann T, Paul RJ. Compartmentation of ATP synthesis and utilization in smooth muscle: roles of aerobic glycolysis and creatine kinase. *Mol Cell Biochem* 1994; 133-134:39-50.

84. Gajewski CD, Yang L, Schon EA, Manfredi G. New Insights into the Bioenergetics of Mitochondrial Disorders Using Intracellular ATP Reporters. *Mol Biol Cell* 2003; 14:3628-3635.

85. Lee CM, Weindruch R, Aiken JM. Age-Associated Alterations of the Mitochondrial Genome. *Free Radical Biology and Medicine* 1997; 22:1259-1269.

86. Schwarze SR, Weindruch R, Aiken JM. Decreased mitochondrial RNA levels without accumulation of mitochondrial DNA deletions in aging Drosophila melanogaster. *Mutat Res* 1998; 382:99-107.

87. Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S et al. From the Cover: Decline in skeletal muscle mitochondrial function with aging in humans. *PNAS* 2005; 102:5618-5623.

88. Bua E, Johnson J, Herbst A, Delong B, McKenzie D, Salamat S et al. Mitochondrial DNA-Deletion Mutations Accumulate Intracellularly to Detrimental Levels in Aged Human Skeletal Muscle Fibers. *Am J Hum Genet* 2006; 79:469-480.

89. Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE et al. Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* 2004; 429:417-423.

90. Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L et al. Calorie Restriction Promotes Mitochondrial Biogenesis by Inducing the Expression of eNOS. *Science* 2005; 310:314-317.

91. Bua EA, McKiernan SH, Aiken JM. Calorie restriction limits the generation but not the progression of mitochondrial abnormalities in aging skeletal muscle. *FASEB J* 2004;03-0668fje.

92. Hipkiss AR. On the mechanisms of ageing suppression by dietary restriction--is persistent glycolysis the problem? *Mechanisms of Ageing and Development* 2006; 127:8-15.

93. Bulteau AL, Verbeke P, Petropoulos I, Chaffotte AF, Friguet B. Proteasome Inhibition in Glyoxal-treated Fibroblasts and Resistance of Glycated Glucose-6-phosphate Dehydrogenase to 20 S Proteasome Degradation in Vitro. *J Biol Chem* 2001; 276:45662-45668.

94. Lee DH, Goldberg AL. Selective Inhibitors of the Proteasome-dependent and Vacuolar Pathways of Protein Degradation in Saccharomyces cerevisiae. *J Biol Chem* 1996; 271:27280-27284.

95. Lowe J, Blanchard A, Morrell K, Lennox G, Reynolds L, Billett M et al. Ubiquitin is a common factor in intermediate filament inclusion bodies of diverse type in man, including those of Parkinson's disease, Pick's disease, and Alzheimer's disease, as well as Rosenthal fibers in cerebellar astrocytomas, cytoplasmic bodies in muscle, and Mallory bodies in alcoholic liver disease. *J Pathol* 1988; 155:9-15.

96. Oughtred R, Bedard N, Adegoke OAJ, Morales CR, Trasler J, Rajapurohitam V et al. Characterization of Rat100, a 300-Kilodalton Ubiquitin-Protein Ligase Induced in Germ Cells of the Rat Testis and Similar to the Drosophila Hyperplastic Discs Gene. *Endocrinology* 2002; 143:3740-3747.

97. Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 1993; 118:401-415.

98. Covi JA, Belote JM, Mykles DL. Subunit Compositions and Catalytic Properties of Proteasomes from Developmental Temperature- Sensitive Mutants of Drosophila melanogaster. *Archives of Biochemistry and Biophysics* 1999; 368:85-97.

99. Smyth KA, Belote JM. The Dominant Temperature-Sensitive Lethal DTS7 of Drosophila melanogaster Encodes an Altered 20S Proteasome ¯-Type Subunit. *Genetics* 1999; 151:211-220.

100. Li Z, Arnaud L, Rockwell P, Figueiredo-Pereira ME. A single amino acid substitution in a proteasome subunit triggers aggregation of ubiquitinated proteins in stressed neuronal cells. *J Neurochem* 2004; 90:19-28.

101. Han KA, Millar NS, Grotewiel MS, Davis RL. DAMB, a Novel Dopamine Receptor Expressed Specifically in Drosophila Mushroom Bodies. *Neuron* 1996; 16:1127-1135.

102. Evan GI, Lewis GK, Ramsay G, Bishop JM. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol* 1985; 5:3610-3616.

103. Spradling AC, Rubin GM. Transposition of cloned P elements into Drosophila germ line chromosomes. *Science* 1982; 218:341-347.

104. Karess RE, Rubin GM. Analysis of P transposable element functions in drosophila. *Cell* 1984; 38:135-146.

105. Wojcik C, DeMartino GN. Analysis of Drosophila 26 S Proteasome Using RNA Interference. *J Biol Chem* 2002; 277:6188-6197.

106. Meiners S, Heyken D, Weller A, Ludwig A, Stangl K, Kloetzel PM et al. Inhibition of Proteasome Activity Induces Concerted Expression of Proteasome Genes and de Novo Formation of Mammalian Proteasomes. *J Biol Chem* 2003; 278:21517-21525.

107. Wang P, Saraswati S, Guan Z, Watkins CJ, Wurtman RJ, Littleton JT. A Drosophila Temperature-Sensitive Seizure Mutant in Phosphoglycerate Kinase Disrupts ATP Generation and Alters Synaptic Function. *J Neurosci* 2004; 24:4518-4529.

108. Chondrogianni N, Tzavelas C, Pemberton AJ, Nezis IP, Rivett AJ, Gonos ES. Overexpression of Proteasome {beta}5 Assembled Subunit Increases the Amount of Proteasome and Confers Ameliorated Response to Oxidative Stress and Higher Survival Rates. *J Biol Chem* 2005; 280:11840-11850.

109. Feany MB, Bender WW. A Drosophila model of Parkinson's disease. *Nature* 2000; 404:394-398.

110. Auluck PK, Chan HYE, Trojanowski JQ, Lee VMY, Bonini NM. Chaperone Suppression of alpha -Synuclein Toxicity in a Drosophila Model for Parkinson's Disease. *Science* 2002; 295:865-868.

111. Wittmann CW, Wszolek MF, Shulman JM, Salvaterra PM, Lewis J, Hutton M et al. Tauopathy in Drosophila: Neurodegeneration Without Neurofibrillary Tangles. *Science* 2001; 293:711-714.

112. Fernandez-Funez P, Nino-Rosales ML, de Gouyon B, She WC, Luchak JM, Martinez P et al. Identification of genes that modify ataxin-1-induced neurodegeneration. *Nature* 2000; 408:101-106.

113. Warrick JM, Chan HYE, Gray-Board, Chai Y, Paulson HL, Bonini NM. Suppression of polyglutamine-mediated neurodegeneration in Drosophila by the molecular chaperone HSP70. *Nat Genet* 1999; 23:425-428.

114. Chan HYE, Warrick JM, Gray-Board, Paulson HL, Bonini NM. Mechanisms of chaperone suppression of polyglutamine disease: selectivity, synergy and modulation of protein solubility in Drosophila. *Hum Mol Genet* 2000; 9:2811-2820.

115. Deasy BM, Lu A, Tebbets JC, Feduska JM, Schugar RC, Pollett JB et al. A role for cell sex in stem cell-mediated skeletal muscle regeneration: female cells have higher muscle regeneration efficiency. *J Cell Biol* 2007; 177:73-86.

116. Spradling, AC. Drosophila-A Practical Approach.Roberts, D. B.1(editor) IRL, Oxford. 1986. pp. 175–197.