Temporal proteasome disruption has a long term and irreversible negative impact on drosophila

by

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THE CITY UNIVERSITY OF NEW YORK

Abstract

Temporal disruption of proteasome activity has a long term and irreversible negative impact on Drosophila

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The presence of unfolded, misfolded, mutant and/or oxidized proteins is a severe and never ending threat to cell survival. To prevent these abnormal proteins to form aggregates and disrupt cellular homeostasis, these proteins can be delivered to the ubiquitin proteasome system (UPS) for degradation. To investigate the proteotoxic effects induced by a dysfunctional UPS, we developed a *Drosophila* model with "tunable" proteasome impairment by modulating the expression of one of its catalytic subunits, the dβ5 subunit, through double-stranded RNA interference (RNAi). Expression of the dβ5 RNAi is controlled by an RU486 inducible Act5C promoter. Reduction of dβ5 subunit expression is caused by RU486 administration. The loss of dβ5 function causes an early onset of reduced proteasome activity. Disruption of proteasome activity has negative impacts including ubiquitinated-protein accumulation, a

shorter lifespan, low resistance to oxidative stress, and locomotor dysfunction. Many UPS-impaired models are established to address effects caused by proteasome dysfunction. However, no model investigates whether temporal proteasome disruption causes long term or irreversible effects. To address this issue, temporal proteasome disruption was induced by varying the period of RU486 administration via the conditional Gene-Switch binary system. Our results demonstrate that temporal proteasome disruption causes long term/irreversible and negative effects on lifespan, protein degradation, elimination of aggregates, and locomotor activity. Taken together, these results indicate that UPS plays critical roles in maintenance of cellular function. Proteasome disruption, even just temporally, has a long term and negative impact in drosophila.

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List of Abbreviations

αSyn α-synuclein

Aß amyloid peptide

ABAD Aβ-binding alcohol dehydrogenase

ALS amyotrophic lateral sclerosis

AD Alzheimer's disease

APP amyloid precursor protein

ATGs autophagy specific genes

ATP adenosine 5'-triphosphate

BACE1 β-site APP cleaving enzyme 1

BCA bicinchoninic acid

dsRNA double-stranded RNA

CNS central nervous system

COX cytochrome **c** oxidase

CyO curly of Oster

Ddc drosophila dopa decarboxylase

dsRNA double-stranded RNA

DTT dithiothreitol

DUB deubiquitinating enzyme

EGFP enhanced green fluorescent protein

Elav embryonic lethal abnormal vision

EtOH ethanol

Gad gracile axonal dystrophy

GS gene-switch

GSH glutathione

GFP green fluorescent protein

HD huntington disease

LBs Lewy bodies

MOMP mitochondrial outer membrane permeabilization

mRNA messenger RNA

mtDNA mitochondrial DNA

NDs neurodegenerative disorders

NFTs neurofibrillary tangles

OS oxidative stress

OXPHOS oxidative phosphorylation system

PCR polymerase chain reaction

PD Parkinson's disease

PHFs paired helical filaments

PNS peripheral nervous system

PSEN presenilin

RT-PCR real-time polymerase chain reaction

RNAi ribonucleic acid interference

ROS reactive oxygen species

RP regulatory particle

Rpn regulatory particle non-ATPase-like

Rpt regulatory particle ATPase-like

S2 Schneider 2

Sb stubble

SDH succinate dehydrogenase

SDS sodium lauryl sulfate

SN substantia nigra

SOD superoxide dismutases

TH tyrosine hydroxylase

Tb tubby

UAS upstream activation sequence

UB ubiquitin

UBP ubiquitin-specific processing protease

UCH ubiquitin carboxy-terminal hydrolases

UCHL1 ubiquitin carboxyl-terminal hydrolase isozyme L1

UPS ubiquitin proteasome system

CHAPTER I

INTRODUCTION

1. 1. THE UBIQUITIN PROTEASOME SYSTEM (UPS):

The ubiquitin proteasome system (UPS) is responsible for the turnover of the majority (~80%) of regulatory and misfolded proteins, and is essential for cell growth as well as cell viability [1]. Two major steps are involved in protein degradation through UPS ubiquitination and degradation (**Fig. 1**).

The presence of unfolded, misfolded, mutant, and/or oxidized proteins is a severe and never ending threat to cells [1]. Proteins containing aberrant conformations tend to form both soluble and insoluble aggregates. Expanding protein aggregates may confer cytotoxic effects by impairing metabolic pathways or choking the cell as the cytosolic or nuclear space is filled with insoluble aggregates, eventually [2].

1. 1. 1. Ubiquitination

Ubiquitin (Ub) is a 76 amino acid protein that is evolutionary highly conserved in all eukaryotes [3, 4]. The formation of a polyubiquitin chain containing more than 4 ubiquitins on a protein is the initial signal for targeting protein degradation. The ubiquitination of proteins is an ATP-dependent process and is carried out in three sequential steps involving three enzymes designated ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) [5-7]. In the presence of ATP, the E1 enzyme activates ubiquitin and transfers it to the E2 enzyme. Finally, the activated ubiquitin is conjugated to the substrate protein via a specific E3 ligase [6]. The linkage of ubiquitin to the target protein is through an isopeptide bond between the ubiquitin carboxyl-terminal glycine and an internal lysine on the substrate. The ubiquitin-chain elongating factor (E4) catalyzes polyubiquitination resulting in the attachment of

additional ubiquitin molecules being attached to the monoubiquitinated substrate [5]. In this way, additional ubiquitin moieties are sequentially added to each other to form a polyubiquitin chain that functions as the recognition signal for the proteasome [7].

Ubiquitins are linked to each other by an isopeptide bond between the side chain of Lys48 and the carboxyl terminus of Gly76 of successive ubiquitins. At least four ubiquitin molecules must be attached to activate proteasomal degradation. During proteasomal degradation, ubiquitin is removed and recycled, and subsequently the target protein broken down into small peptides [8].

1. 1. 2. Deubiquitination

Ubiquitin needs to be removed from tagged proteins before they enter the proteolytic core of proteasomes. Deubiquitination is catalyzed by specific proteases called deubiquitinating or DUB enzymes. There are two families of deubiquitinating enzymes: ubiquitin-specific processing proteases (UBPs) and ubiquitin carboxy-terminal hydrolases (UCH). UBP is the largest and most diverse group of DUB enzymes. These cysteine proteases contain two short but well-conserved motifs, known as the Cys and His boxes. The second ubiquitin-specific cysteine protease family is UCHs. They are generally small proteins, which were originally identified by their ability to hydrolyze small amides and esters at the C-terminus of ubiquitin [9].

1. 1. 3. The proteasome

The 26S proteasome, is responsible for the recognition, binding and proteolytic degradation of polyubiquitinated proteins. The 26S proteasome is a 2.5 MDa complex

consisting of a 20S core particle, bound at one or both ends by a 19S regulatory particle (RP). The 20S proteasome is a barrel-shaped complex. It is formed by four rings made up of two outer α-rings and two inner β-rings. The 20S proteasomes have a α7β7β7α7-ring arrangement. These α-rings and β-rings are made up of seven structurally similar α and β subunits, respectively **(Fig. 2)**.

The middle chamber of the 20S proteasome is composed of β rings and is responsible for its proteolytic activity. However, the entry of substrates into the middle chamber of the 20S proteasomes is restricted by the 19S regulatory particle (19S RP) and the α-rings. The 19S RP can be divided into two subcomplexes, known as 'base' and 'lid' [10]. The base is made up of six ATPases (Rpt1–Rpt6) and two large regulatory particle non-ATPases-like (Rpn) components, Rpn1 and Rpn2, functioning as presumptive receptors for ubiquitin-like proteins [11]. The base complex binds to the outer α-ring of the 20S proteasome and interacts with the 20S particle causing a narrow pore to open in an ATP-dependent manner, which allows access into the inner compartment of the 20S complex [12]. The base of the 19S particle exhibits chaperone-like activity *in vitro* [13]. The six ATPases of the base can supply energy for unfolding the targeted proteins before translocation into the 20S core for proteolysis. The lid contains multiple non-ATPase subunits. The role of the lid complex is less understood. Among the lid subunits, Rpn11 is known as a metalloprotease that cleaves the peptide bond between the substrate and the most proximal ubiquitin of the polyubiquitin chain [14, 15]. Rpn10 is thought to lie between the base and the lid complex and serve as one of the ubiquitin receptors [16].

The eukaryotic 20S proteasome is a tightly packed cylindrical structure, consisting of seven different α subunits and seven different ß subunits. Among the 14 different

subunits of the 20S proteasome, only three are proteolytically active in the mature 20S proteasome. These three β subunits are: β1, β2, and β5, which are responsible respectively for the caspase-like, trypsin-like and chymotrypsin-like activities of the proteasome [17-20] **(Fig. 3)**.

1. 1. 4. Mechanisms of degradation by the proteasome

The mammalian proteasome breaks down proteins in process called "biting and chewing". Multiubiquitinated substrates are recognized by the 19S particle of the proteasome. Once the polyubiquitinated tag is removed, the protein is unfolded and passes through the 19S complex and enters the 20S proteasome. Within the 20S proteasome multiple proteolytic activities with diverse specificities provide a functional advantage that allows efficient hydrolysis of targeted proteins [21]. When the protein reaches the chymotryptic site, the protein is cleaved into large peptide fragments by the β5-associated chymotrypsin-like activity. In the following steps of degradation, a caspase-like activity associated with the β1 subunit and a 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 UPS (**Fig. 4**).

1.2. NEURODEGENERATION

Neurodegenerative disorders (NDs) are characterized by progressive mental dysfunction and loss of neurons and synapses in selected areas of the nervous system. The major basic processes inducing ND are considered multifactorial caused by genetic,

environmental, and endogenous factors related to aging. However, the origin and basic molecular mechanisms of many of these diseases are not fully understood [22-25].

Alzheimer's disease (AD) [26] is the most prevalent among these disorders followed by Parkinson's disease (PD) [27]. In both AD and PD, familial cases with a genetic hereditary are in the minority, whereas sporadic cases account for the majority of AD cases [26, 27]. The percentages of people living with Alzheimer's disease among 75-, 80-, 85-, and 90-year-olds are 4.3%, 8.5%, 16.0%, and 28.5%, respectively [28]. These data demonstrate that the numbers of AD cases increase steeply with age. Huntington's disease [29] is an autosomal dominant inherited condition with a single mutation that causes the disease in almost 100% of all cases [30].

Selective loss of neurons in specific regions of the brain is the common feature of NDs. The result is often a disruption of motor, sensory or cognitive systems, resulting in severe disability of the patient. Each of the diseases involves protein misfolding and aggregation, resulting in inclusion bodies and other aggregates within cells [31]. In general, these inclusions consist of insoluble, unfolded and ubiquitinated polypeptides that fail to be targeted and degraded by the 26S proteasome [32].

1.2.1. Alzheimer disease (AD)

AD is a neurodegenerative disorder of the central nervous system (CNS). The clinical characteristics of AD patients are progressive loss of memory, deterioration of cognitive skills, gradual loss of language and mental functions, and dementia. AD is the most prevalent neurodegenerative disorder and the most common cause of dementia [33].

There are sporadic and familial AD cases. Only about 5% of all AD cases are familial cases. The familial AD cases are caused by known mutations in amyloid precursor protein (APP) or Presenilin 1 and 2 [34]. However, the cause of sporadic AD is still not clear. The pathologic hallmarks of both types of AD are the presence of plaques and neurofibrillary tangles (NFTs) as well as degeneration of synapses and neurons in the brain of AD patients. The plaques and NFTs are composed of deposits of amyloid peptide (Aß) and hyper-phosphorylated tau protein, respectively [35, 36]. Aß is derived from APP by serial cleavages involving β-secretase [also known as β-site APP cleaving enzyme 1 (BACE1)] and γ-secretase (Presenilin complex). Aß is released released into the extracellular space [37], but is also found intracellular.

1.2.2. Parkinson's disease (PD)

Parkinson's disease (PD) is a common progressive neurodegenerative disorder that is characterized by muscle tremors in stationary limbs, bradykinesia (slow movement) and difficulty in initiating and sustaining movements [38, 39]. It is caused by the degeneration of dopaminergic neurons in the substantia nigra (SN) of the midbrain and other monoaminergic neurons in the brain stem, resulting in loss of the nigrostriatal pathway and a reduction of dopamine levels in the striatum [40]. Inclusions, known as Lewy bodies, are found within neuronal cell bodies of the affected areas in most PD patients [41].

1.3. UPS IN NEURODEGENERATIVE DISORDERS

A dysfunctional UPS has been reported in multiple neuropathologies, including neurodegenerative disorders [42]. A role for the UPS in the pathology of human neurodegenerative disorders was initially suggested when polyclonal antibodies raised against ubiquitin detected ubiquitinated proteins in intracellular inclusions in a wide range of neurodegenerative diseases, such as give examples of diseases [43, 44]. Decline of proteasomal function was observed during aging [45] and in AD [46], HD [47, 48] and PD [49]. Dysregulation of the UPS appears to be both a cause and a result of NDs.

1.3.1. UPS in Alzheimer's disease

Abnormal deposition of highly insoluble protein aggregates or inclusion bodies is commonly observed AD brains [50]. In human AD brains, the inclusions bodies show ubiquitin immunoreactivity [32]. The accumulation of ubiquitin–protein conjugates in neuropathological lesions was detected in NFTs isolated from human brain [43, 51].

Alterations in the UPS have been implicated in several neurodegenerative diseases [52, 53]. Mutations in specific genes have been connected to the pathology of the disease while in other cases impairment of the UPS could be a late event in pathogenesis [54].

Recent reports with animal studies indicated that the UPS plays a crucial role in the degradation of numerous substrate proteins invoved in synaptic plasticity [52, 55]. For instance, studies in drosophila demonstrated that the UPS acutely regulates presynaptic protein turnover and synaptic efficacy [56].

The inability to change synaptic strength or synaptic plasticity results in synaptic dysfunction and leads to memory impairment in AD. In animal studies of AD, synaptic

dysfunction was observed in hippocampal slices from APP/ presenilin 1**(**PSEN1) mice, or from normal mice upon oligomeric Aβ treatment. The deterioration of the hippocampal slices was ameliorated by overexpressing ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) [57].

A role for UCHL1 in AD has been supported by other observations. For instance, in a mouse model of gracile axonal dystrophy (gad), deletion in exons 7 and 8 of the *Uchl1 gene* caused the genetic defect. The gad mice display a syndrome of ataxia. Moreover, in gad mice, accumulation of Aβ and ubiquitin- positive deposits occurs in sensory and motor neurons [58, 59].

Ubiquitinated protein aggregates appear in AD brains. The accumulation of ubiquitinated proteins in AD cases [60-62] suggests that AD might be associated with a malfunction or overload of the UPS. Neurons with dysfunctional UPS are unable to degrade intracellular proteins causing formation of protein aggregates. An ubiquitin-conjugating enzyme, E2-25K/HIP-2, is a mediator of Aβ-mediated toxicity and proteasome inhibition in an APP animal model [63]. An ubiquitin mutant, UBB^{+1} (a potent proteasome inhibitor detected in AD brains), was found to interact with E2-25K/HIP-2-mediated neurotoxicity [63].

In *in vitro* experiments, Aβ was found to bind and inhibit the proteasome, thus impairing degradation of ubiquitinated proteins [64, 65]. In addition, in an APP/PSEN1 mutant neuronal cell culture, accumulation of the Aβ peptide inhibits both the proteasome and deubiquitinating enzymes (DUBs) [66]. Moreover, paired helical filaments (PHFs) of the tau protein were also reported to impair proteasome function [67]. These data suggest that the toxicity of Aβ peptides and PHFs may be associated with impaired ubiquitin

conjugation or directly interfere with the proteasome. Other proteins implicated in AD, such as presenilins (PSENs) 1 and 2, are linked to the UPS. Both PSEN1 and PSEN2 are substrates for UPS-mediated degradation [68]. Taken together, these observations suggest that impairment of UPS components could be an important factor in AD pathology.

1.3.2. UPS in Parkinson's disease

PD is characterized by selective degeneration of dopaminergic neurons in the substantia nigra pars compacta [69]. Lewy bodies (LBs) are the morphological hallmark of PD. One of the major components of Lewy bodies is α-synuclein. α-synuclein is degraded by both the proteasome and autophagy [70, 71] indicating a link between dysfunctional UPS and development of PD. Mutations in the α-synuclein gene are associated with the development of PD [72-75]. Mutations in the α-*synuclein* gene may lead to enhanced oligomerization and fibril formation of the α-synuclein protein [76, 77]. It remains unclear how alterations in the α-*synuclein* gene cause neurodegeneration, but it has been shown that misfolded or excess levels α-*synuclein* can resist or inhibit the UPS [78]. Overexpression of α-*synuclein* in cultured cells inhibits proteasome activity leading to impairment of protein clearance and reduced dopamine release [78, 79].

In addition to mitochondrial dysfunction and oxidative stress in PD, the impairment of the ubiquitin proteasome system may also contribute to SN dopaminergic pathology. Lewy bodies contain ubiquitinated α-synuclein and parkin as the major constituents although proteasome components are also often found. Co-localization of α-synuclein and parkin (E3 ubiquitin ligase) within LBs indicates that parkin plays a role in ubiquitination and posttranslational modification of α-synuclein [80].

Mutations in a number of genes have been identified as causes of PD and many of these genes are associated with the ubiquitin/proteasome protein degradation pathway. Mutation of the ubiquitin hydrolase (UCH-L1) and parkin were found to be associated with the pathogenesis of autosomal inherited cases of PD [81, 82].

In humans, the *parkin g*ene encodes a 465 amino acid protein that functions as one of a number of E3 ubiquitin protein ligases, components of the ubiquitin-proteasome degradation pathway [83]. Promoting degradation of proteins that might damage cells is the cytoprotective effect of parkin [84]. Parkin can ubiquitinate a number of substrates including a glycosylated form of α-synuclein [85] and suppress the damaging effects of human α-synuclein [86, 87].

Mutations of UCH-L1 are associated with neuronal degeneration and progressive accumulation of ubiquitin-positive inclusions along sensory and motor neurons [88]. Analyses of AD and PD brains have shown modest decreases in the proteolytic activities of the proteasome [89, 90]. Formation of protein aggregation is associated with decreased proteasome activity.

Dysfunction of the UPS is implicated in the pathogenesis of PD [91]. In addition, the proteasome activator PA28 is scarce in the substantia nigra (SN) of PD brains, suggesting that failure in the UPS to degrade unwanted proteins leads to protein aggregation [92]. The colocalization of ubiquitinating and proteolytic enzymes and other cytoskeletal elements in LBs suggests that these inclusions could be induced to form aggresome-related structures as a means of controlling excessive levels of abnormal proteins [93].

The link between the UPS and PD is strengthened by these data. However, it is still unclear whether protein aggregation and dysfunction of the UPS is the cause or

consequence of neuronal loss in these neurodegenerative disorders.

1.3.3. Genetic and pharmacological inhibition of the UPS causes ND pathogenesis

To determine how the degradation impairment of ubiquitinated proteins by the proteasome would affect neuronal cells, the proteasomal ATPase subunit Psmc1 was conditionally knocked out in mice by using the Cre/loxP system [94]. Psmc1 was selectively knocked out in neurons of the forebrain (Psmc1fl/fl; CaMKIIa-Cre) or dopaminergic system (Psmc1fl/fl; THCre). The neuronal death caused by impairment of the proteasome results in a significant reduction in cortical thickness and expansion of ventricular cavities when Psmc1 was knocked out in the forebrain. Progressive 26S proteasome impairment not only induced neuronal degeneration but also resulted in numerous eosinophilic intraneuronal paranuclear inclusions. Those inclusions contain ubiquitin and α-synuclein. The composition of those inclusions is similar to LBs in sporadic PD. Other studies further confirm that chronic impairment of the proteasome promotes α-synuclein aggregation in cellular models [95, 96].

Many studies with animal models demonstrated that chemical proteasomal inhibition induced the PD pathology [97]. However, one major criticism of these studies was that dopaminergic neuronal cell death was determined with non-stereological assessment of tyrosine hydroxylase-positive neurons [98]. To further confirm the loss of dopaminergic neurons, a systematic stereological analysis system was utilized. The result confirmed that proteasome inhibitors indeed cause persistent nigral dopaminergeric cell loss [98]. Taken together, these studies show that the models of proteasome impairment

induced by genetic or pharmacological inhibition provide further support for the involvement of UPS-mediated processes in PD pathogenesis.

1.3.4. Oxidative stress and free radicals

Oxidative stress (OS) occurs when the production of free radicals or their products overwhelm the of antioxidant defense mechanisms [99]. Free radicals can damage biological molecules and initiate a cascade of events, including dysfunction of mitochondrial respiration, lipid peroxidation, excitotoxicity, and a fatal rise in cytosolic calcium leading to cellular dysfunction. Therefore, oxidative stress caused by free radicals is suggested to be a major factor of the pathology in many diseases including NDs [100-104].

Recent reports indicated that high levels of oxidative damage to DNA, lipids, and proteins have been found in postmortem tissues from patients with PD, AD and amyotrophic lateral sclerosis (ALS). An increase of oxidative damage may occur in the early phase of the disease progression [105]. Damage to nuclear and mitochondrial DNA (mtDNA) caused by oxidative stress was detected in the early phase of AD, PD and HD [106, 107], but also in normal aging [108].

1.3.5. Oxidative stress in AD and PD

In both human AD as well as transgenic mouse models of AD, the process of Aβ deposition produces oxidative damage resulting to OS and NDs [109-111]. OS induces apoptosis and macroautophagy for degradation of Aβ proteins [112]. Mutant APP and its derivates are involved in the generation of free radicals in mitochondria and further cause

mitochondrial oxidative damage. Aβ-induced generation of free radicals and oxidative damage are linked to AD pathology of AD [113]. Increasing evidence suggests that OS causes damage to DNA and leads to programmed cell death in AD [114].

In PD, changes of anti-oxidant systems indicate the sensitivity of cells to oxidative damage caused by generation of excess ROS or free radicals in SN [115, 116]. Glutathione (GSH) has an important role in the the antioxidative defense mechanism. GSH level declines in SN of PD. A depletion of GSH probably results in inhibition of mitochondrial complex I [117]. Superoxide dismutase (SOD) is an indicator of superoxide generation. Both isoenzymes (Cu–Zn–SOD and Mn–SOD) of SOD increase in SN of PD, in AD and ALS. High level of SOD supports an increase of superoxide generation [118]. 8-hydroxydeoxyguanosine (8-HOG) is an indicator of free radical damage to DNA. 8-HOG is involved in the degeneration of SN neurons. An increase of peripheral 8-HOG level was also detected in AD and ALS. These data suggest that oxidative damage of DNA/RNA is a common feature in these diseases [119].

In neurons, low level of OS upregulates α-synuclein [120]. OS promotes formation of α-synuclein protofibrils by translational modifications, while the aggregation of α-synuclein is inhibited by antioxidants and chaperons [121]. Rotenone is a pesticide which induces an increase in OS by inhibiting mitochondrial complex I. Chronic systemic treatment of rats with rotenone induces an increase in OS and facilitates fibrillization of α-synuclein in dopaminergic neurons [122].

In summary, oxidative damage to lipids, proteins, and nucleic acids caused by free radicals is a major contributor to the pathogenesis of many NDs.

1.3.6. Impairment in mitochondrial function

Mitochondria are the major energy (ATP) producers of cells. ATP is mostly produced via the oxidative phosphorylation system (OXPHOS) in mitochondria. Mitochondria maintain homeostasis of cellular function by producing ATP and regulating intracellular calcium levels [123, 124]. Large amounts of reactive oxidants, including ROS are produced in the process of OXPHOS. Mitochondria are both targets and sources of ROS.

Impairment in mitochondrial function has severe negative effects on cells including a reduction in ATP production, disturbance of calcium concentration, and excess generation of ROS. All of these are important factors in the pathogenesis of neurodegeneration [125, 126]. Oxidative stress disrupts mitochondrial energy metabolism. The oxidative mitochondrial damage may then promote cell death and induce degeneration [102, 124, 127].

1.3.7. Mitochondrial dysfunction in AD

In AD, there is evidence indicating an impairment of mitochondrial energy metabolism and oxidative damage [128, 129]. In AD brains, an average 50% reduction in mtRNA was detected [130].

Mutations in mitochondrial DNA (mtDNA) could cause energy depletion, increased oxidative stress and accumulation of Aβ leading to a vicious cycle with production of more mtDNA damage and oxidative stress. In AD patients and transgenic mouse models, an interaction between Aβ peptide and Aβ-binding dehydrogenase [131] in mitochondria was observed. This suggests that ABAD may be a link to Aβ peptide associated

mitochondrial toxicity [132]. Perturbations of mitochondrial function caused by phospholipase-2-mediated oligomeric Aβ lead to impairment in ATP production and an increase in oxidative stress in AD brains [133]. Caspase-cleaved tau proteins impair mitochondrial function in cortical neurons by increasing calcineurin activity in AD brains [134]. As compared to normal aging, an increasing number of COX-deficient succinate dehydrogenase (SDH)-positive hippocampal pyramidal neurons in AD brains suggests that mitochondrial enzyme activity is defective more frequently in AD.

These data support that an increase in OS leads to oxidative damage to mtDNA in AD brains causing abnormal mitochondrial dynamics and dysfunction that ultimately damage AD neurons [129, 135].

1.3.8. Mitochondrial dysfunction in PD

Mitochondrial dysfunction and OS play an important role in PD pathogenesis [136-140]. Furthermore, the function of the UPS is ATP-dependent. It is possible that dysfunctional mitochondria produce insufficient ATP thus impairing the activity of 26S proteasome mediated degradation. Excess generation of ROS during dysfunctional OXPHOS leads to the production of oxidized or misfolded proteins and these aberrant proteins depend on the UPS for degradation.

Parkin (an E3 ubiquitin ligase) maintains mitochondrial homeostasis by mediating the elimination of impaired mitochondria by autophagy, promoting DNA repair, improving mitochondrial dysfunction and preventing genotoxicity [141-145]. Mutations in parkin induce mitochondrial dysfunction and lower the resistance to OS induced by paraquat [146]. The proteasome also maintains mitochondrial homeostasis. Disruption of

proteasome activity was observed in PD. Inhibition of proteasome increases the level of ROS and alters turnover of mitochondria by lysosomal-mediated degradation [147].

High levels of mtDNA mutations were associated with respiratory chain deficiency detected in the SN neurons of PD patients, suggesting that mitochondria dysfunction is relevant to selective neuronal degeneration [148]. An interaction between α-synuclein and cytochrome ***c*** oxidase (COX) suggests that α-synuclein aggregation may be linked to mitochondrial dysfunction [149]. In addition, a dose-dependent inhibition of complex I activity by α-synuclein impairs mitochondrial function and increases OS [150, 151]. Reduced cerebral mitochondrial energy metabolism is detected in early PD [152].

All of these studies suggest that mitochondrial dysfunction is critical to the pathogenesis of NDs including AD and PD. However, if mitochondrial dysfunction is a primary cause or a part of a pathogenic process remains to be clarified [140].

1. 4. OVERALL RELEVANCE

The UPS is responsible for the regulation of biological processes including the cell cycle, transcription, signal transduction, DNA repair, antigen production, stress resistance, and the maintenance of muscle cellular architecture [4, 153]. Increasing evidence indicates that proteasome impairment causes several deleterious effects including accumulation of ubiquitinated protein conjugates, shorter lifespan, increased aggregate formation, accumulation of oxidatively modified proteins, muscle breakdown and degeneration as well as locomotor dysfunction [4, 97, 153-157].

Models of UPS impairment have been established to address the effects caused by proteasome dysfunction [94, 158-162]. However, no model has investigated whether

temporal proteasome disruption causes long term or even irreversible effects. Proteasome function may be impaired temporally under some conditions including infection by viruses and bacteria, exposure to environmental toxins, inflammation, transient ischemia/reperfusion and sports injury. These conditions may induce oxidative stress and reactive oxygen species (ROS) formation, thus disrupting the UPS directly or indirectly [163-168].

We decided to investigate the effects caused by temporal disruption of proteasome activity. To address this issue, a transgenic fly model containing drosophila β5 interfering RNA (dβ5 RNAi) was constructed. Temporal proteasome disruption was induced by using the conditional Gene-Switch system [169-171]. Our data demonstrate that RNAi directed against the 20S catalytic subunit β5, disrupted proteasome activity in flies. This model demonstrates that temporal proteasome impairment causes an accumulation of ubiquitin-conjugated proteins, an increase in protein aggregates, low resistance to oxidative stress, motor deficit, and a shorter lifespan. Particular significant, after proteasome recovery, flies were not able to overcome the accumulation and aggregation of ubiquitinated protein. Furthermore, no restoration of locomotor activity and survival was observed upon proteasome recovery.

As far as we know, our results are the first to demonstrate that a temporal reduction in proteasome activity has a long term and negative impact on lifespan, protein degradation, elimination of aggregates, and locomotor activity in drosophila.

CHAPTER 2

EXPERIMENTAL DESIGN

2.1. MODULATE THE ENDOGENOUS EXPRESSION OF THE dβ5 GENE BY USING RNA INTERFERENCE

We used RNAi to target the mRNA of the drosophila β5 gene (dβ5) to knock down the expression of the dβ5 mRNA. The efficiency of this approach was recently demonstrated to successfully target double-stranded RNA (dsRNA) of several subunits of the 26S proteasome in insect Schneider 2 (S2) cells [172]. Incubation of S2 cells with dsRNA of proteasome subunits produced significant phenotypic changes in S2 cells, including reduced growth, increased apoptosis, reduced proteasome function, and increased cellular levels of polyubiquitinated proteins, an indicator of inhibition of intracellular protein degradation via the ubiquitin-dependent pathway [173-175]. Decreased β5 expression in insect S2 cells significantly disrupted 20S proteasome assembly and decreased proteasome activity. However, exposure of insect S2 cells to β5 targeted dsRNA only knocked down temporarily the target mRNA levels. Our studies intended to address long term and short term effects caused by proteasome disruption in the whole fly, as we successfully impaired proteasome function via genetic manipulation in drosophila.

2.2. DESIGN CONSIDERATIONS FOR GENOMIC-cDNA FUSION RNAi CONSTRUCTS FOR dβ5 GENE SUPRESSION

In drosophila, injection of double-stranded RNA directly into embryos effectively targets homologous gene expression in the adult [176, 177]. However, suppression of adult gene expression by double-stranded RNA is inconsistent [176]. Recent publications

showed that expression of transgenic “snap-back” inverted repeats (predicted to form dsRNA hairpins) homologous to target genes can reduce gene expression in transgenic flies [178-180]. However, the suppression is incomplete and generally produces weak phenotypes. The suppression of the target gene in transgenic flies caused only a 50% reduction in protein. This level of suppression is insufficient to produce mutant phenotypes for most genes [180].

RNAi constructs composed of inverted repeats as well as introns efficiently reduce target gene expression in transgenic adult flies [181]. The level of suppression is significantly greater than the transgenic RNAi constructs composed of simple inverted repeats or inverted repeats separated with a spacer. Such genomic-cDNA fusion constructs consisting of an intron containing genomic DNA and the corresponding inverted cDNA are transcribed and spliced normally. Following splicing, genomic-cDNA fusions of the RNAi construct will form double stranded RNA which suppresses expression of the target gene efficiently by RNA interference. One possibility is that splicing may be important to produce RNAi efficiently. For example, in the nucleus, splicing factors coupled with messenger RNA (mRNA) are exported to the cytoplasm [182]. Another possibility is that spliced RNAi transcripts are more efficiently processed and therefore reach higher levels in the cytoplasm. Transcripts encoding inverted repeats tend to form hairpins during transcription. So, transcripts including introns may stabilize pre-mRNA via reduction of hairpin formation in pre-mRNA. Furthermore, an artificial intron between inverted repeats induces greater suppression of mRNA [183]. Because RNAi constructs containing an intron efficiently reduce target gene expression, it allowed us to construct a genomic-cDNA fusion to supress *d*β5 gene expression in drosophila.

Double–stranded RNA interference effectively blocks gene function. However, microinjection of double–stranded RNA fails to persist through several days of development and to be effective in promoting gene interference for another 3-4 weeks. To circumvent this problem, we produced dsRNA endogeneously. The advantage is that once transcribed, inverted repeat sequences fold and form the double-stranded RNA molecules to trigger RNA interference to degrade target mRNA levels.

2.3. THE CONDITIONAL BINARY SYSTEM: GENE-SWITCH SYSTEM

In drosophila, the method of choice for spatially controlling transgene expression involves the GAL4-UAS (upstream activation sequence) binary system. However, we established that ubiquitious expression of UAS-dβ5 RNAi driven by an actin-GAL4 driver was embryonic lethal. To circumvent the problem of lethality, we used an inducible GAL4-progesterone receptor fusion (Gene-Switch)/UAS system.

The GAL4-progesterone receptor fusion (Gene-Switch)/UAS system is the most widely used approach for spatial and temporal control of transgene expression in drosophila. The GeneSwitch GAL4 protein was shown to induce UAS reporters *in vivo* in drosophila [171, 184]. These conditional GAL4 proteins, expressed by using tissue-specific promoters and activated by systematic application of the ligand, RU486 (Mifepristone), allow the expression of UAS transgenes in both a temporal and spatial restricted fashion. Reporter protein expression can be adjusted by the dosage of the Gene-Switch activator, RU486, which on its own at the concentrations used, has no overt effects on viability or fertility of flies [169].

We used a flexible approach to reduce dβ5 expression by varying the length of the double-stranded hairpin constructs which might change its effectiveness. In addition, we can adjust the RU486 concentration which affects expression of the transcript over a wide range. For RNAi formation we constructed inverted repeats of 501, 1062 and 1694 bp in length of the drosophila dβ5 gene in the vector pTOPO2.1 (Invitrogen, K4510-20). These inverted repeats were separated by at least 40 bp of vector sequence to facilitate hairpin formation of the transcript. Each sequence contained the dβ5 gene sequence in forward and reverse orientation separated by a hairpin loop. The RNAi construct was ligated into the P-element vector (pUAST vector) under control of the UAS promoter. Transcription of the long inverted sequence of the RNAi construct will allow formation of the dsRNA and then will trigger the RNA interference to degrade the mRNA of dβ5 gene in drosophila **(Fig. 5)**.

2.4. DESIGN OF THE FLY DRIVER LINES TO DIRECT dβ5 RNAi EXPRESSION

Since we knew that complete elimination of the dβ5 activity had a lethal effect on drosophila [185, 186], we initially tested the efficiency of dβ5 RNAi by using an inducible promoter, Act5C. The Act5C driver is an ubiquitous promoter. We activated the Gene-Switch system with various concentrations of RU486 (0 µM to 600 µM). These RU486 concentrations have been shown to be non-toxic to fruit flies [187, 188]. Under these conditions, recent reports indicate that RU486 alone does not have a significant beneficial or negative effect on the lifespan of drosophila [189, 190].

2.5. ANALYSIS OF THE TRANSFORMANT LINES

To knock down dβ5 expression, we utilized the Gene-Switch Act5C (Act5C-GS) promoter to control dβ5 RNAi expression. For this purpose the UAS-dβ5 RNAi transgenic line was crossed to the Act5C-GS driver line. The first progeny containing both UAS-dβ5 RNAi and Act5C-GS inserts were collected for analysis. In the first progeny, flies containing both UAS-dβ5 RNAi and Act5C-GS inserts were screened and separated by sex for subsequent experiments. Upon RU486 feeding, Act5C-GS binds to the UAS and activates transcription of dβ5 RNAi. Feeding flies with RU486 triggers the induction of dβ5 RNAi and then knocks down dβ5 subunit expression. Downregulation of the dβ5 subunit can thus be manipulated by feeding flies with the ligand of the Gene-Switch binary system, RU486. To assess the effect of the genetic manipulation on the catalytic properties of the proteasome, flies with the dβ5 deficiency were analyzed by several assays such as survival curve, Western blot, in-gel assay, climbing performance, and filter trap assay.

CHAPTER 3

RESULTS

3.1. ESTABLISHMENT AND CHARACTERIZATION OF A FLY MODEL WITH A TUNABLE PROTEASOME

3.1.1. Genomic and cDNA clones of the drosophila dβ5 gene

The polymerase chain reaction (PCR), applied to a BAC clone Bacr13j10 (drosophila melanogaster, chromosome 2R, region 47B-47C), was performed to generate three drosophila dβ5 genomic DNA fragments: genomic DNA A, genomic DNA B, and genomic DNA C. Each genomic DNA fragment contains one intron (61 bp). The length of the genomic DNA A, genomic DNA B, and genomic DNA C fragments is 278 bp, 559 bp, and 878 bp, respectively. RT-PCR of dβ5 mRNA was performed to generate three cDNA fragments: cDNA A, cDNA B, and cDNA C (**Fig. 6A).** The length of dβ5 cDNA A, cDNA B, and cDNA C fragments is 213 bp, 494 bp and 811 bp, respectively. To prevent rearrangement of the inserted sequences, these six constructs were cloned into the vector pTOPO2.1 (Invitrogen, K4510-20) by using *E. coli* supercompetent cells (SURE cells, Stratagene, 200238). To further confirm the length of the dβ5 genomic DNA and cDNA constructs, the fragments were digested from the pTOPO2.1 vectors and loaded on an 8% DNA agarose gel. As shown in **Fig. 6B and 6C,** the data indicated that the dβ5 genomic DNA and cDNA fragments exhibit the correct length.

3.1.2. Assembly of genomic-cDNA RNAi constructs of the drosophila dβ5 gene

A SacII-EcoRI fragment of the dβ5 genomic DNA was isolated from the pTOPO2.1 vector and cloned in to the SacII-EcoRI site of the pBluescript II SK (+) plasmid. The

resulting construct was named pBlueGen **(Fig. 7A)**. The dβ5 cDNA fragment was isolated by EcoRI-XhoI digestion of the pTOPO2.1 vector and cloned into the EcoRI-XhoI site of PblueGen **(Fig. 7B)**. The drosophila dβ5 cDNA fragment and genomic DNA fragment were then ligated using the EcoRI site in the pBluescript II SK (+) plasmid to create the RNA interference (RNAi) construct. The dβ5 RNAi construct contains the drosophila dβ5 sequence in forward and reverse orientation separated by a spacer. Three dβ5 RNAi constructs with different length were made and inserted into pBluescript II SK (+) vectors. The length of dβ5 RNAi construct A, B, and C is 486 bp, 1040 bp and 1679 bp, respectively. We constructed dβ5 RNAi with different lengths because RNAi constructs with greater lengths have higher efficiency. The pBluescript II SK (+) vectors containing dβ5 RNAi constructs were expanded and purified by using the Qiagen MaxiPrep Kit (Qiagen, 12163). To further confirm that the dβ5 RNAi constructs were inserted into the pBluescript II SK (+) vectors at the correct position, pBluescript II SK (+) vectors containing dβ5 RNAi constructs were sequential digested with SacII-XhoI and run on 8% DNA agarose gels in parallel with the non-digested construct (**Fig. 7C and 7D**). The result confirmed that dβ5 RNAi constructs were inserted correctly into the SacII-XhoI site of the pBluescript II SK (+) vectors. To transpose dβ5 RNAi constructs into drosophila chromosomes, the drosophila transformation vector, pUAST, was used for microinjection. The DNA fragment obtained from the digestion of the pBluescript II SK (+) vector containing dβ5 RNAi construct with SacII-KpnI, i.e. the dβ5 RNAi construct, was ligated into SacII and KpnI sites of the pUAST vector (**Fig. 8A**). To test whether the dβ5 RNAi constructs were inserted into the pUAST vectors correctly, undigested and pUAST vectors containing dβ5 RNAi constructs digested sequential with

SacII-KpnI were run on 8% DNA agarose gels (**Fig. 8B and 8C**). To further confirm that the pUAST vectors contained dβ5 RNAi with the inverted sequence of dβ5, the pUAST vectors containing the dβ5 RNAi constructs were purified with the Qiagen MaxiPrep Kit (Qiagen, 12163) and sequenced. After sequencing, the dβ5 RNAi constructs in pUAST vectors were sent to BestGene Inc for drosophila embryo injection to generate transgenic lines.

3.1.3. Constitutive expression of dβ5 RNAi is embryonic lethal in drosophila

Following microjection, three isogenic transgenic lines of UAS-dβ5 RNAi flies were generated. In order to disrupt the proteasome function of the flies, we used RNAi to target the dβ5 catalytic subunit of the proteasome. The drosophila genome contains two isoforms of the dβ5 mRNA: Prosbeta5 mRNA-A and Prosbeta5 mRNA-B. Translation of the two isoforms results in the same dβ5 protein with a molecular mass of 23.5 kDa. Sequence alignments indicate that our dβ5 RNAi constructs target both isoforms of Prosbeta5 mRNA. To test the efficiency of the dβ5 RNAi, UAS-dβ5 RNAi flies were crossed with the actin-GAL4 driver. However, ubiquitious expression of UAS-dβ5 RNAi driven by the actin-GAL4 driver was embryonic lethal. The lethality occurred in embryonic and larval stages. This finding indicates that dβ5 is an essential gene for drosophila.

3.1.4. Design of an inducible driver line to conditionally direct expression of the dβ5 RNAi

To circumvent the problem of lethality, we used an inducible Gene-Switch/UAS binary system. The dβ5 RNAi was thus driven by an inducible and ubiquitious Gene-Switch driver line, 9431 obtained from Bloomington stock center. The genotype of this driver line is P{hsFLP}; P{UAS-GFP}; P{Act5C-GAL4.Switch/Tb}. This inducible driver contains two homozygous inserts, UAS-GFP (green fluorescent protein) and Act5C-GAL4.Switch (Act5C-GS) on the second and third chromosome, respectively (**Fig. 9A**). Act5C-GS is a conditional GAL4-progesterone-receptor fusion protein controlled by the ubiquitious promoter Act5C. In the presence of RU486, Act5C-GS binds to UAS and activates transcription of GFP. The Act5C-GAL4.Switch driver line was starved for 6 hr and then fed with RU486. As shown in **Fig. 9B,** green fluorescence due to GFP appeared 24 hr after 200 µM RU486 administration. The intensity of the green fluorescence increased over time.

To drive expression of dβ5 RNAi conditionally, the Act5C-GS driver line has to be crossed with UAS-dβ5 RNAi flies. The progeny containing three inserts, Act5C-GAL4.Switch, UAS-GFP, and UAS-dβ5 RNAi, will be experimentally confirmed. RU486 administration to progeny not only activates dβ5 RNAi expression but also induces green fluorescence appearance. Therefore, to avoid interference of green fluorescence in immunostaining assays, we decided to remove the UAS-GFP insert from the GS-Act5C driver line.

3.1.5. Removal of the UAS-GFP insert from the GS-Act5C driver line

To remove the UAS-GFP insert, the Act5C-GS driver line was serially crossed to other transgenic flies. As shown in **Fig. 10**, after a serial segregation, both hsFLP and UAS-GFP inserts were removed from the Act5C-GS driver line. The modified GS-Act5C driver was obtained from a serial crossing. To confirm the absence of the UAS-GFP insert in the modified Act5C-GS driver, the modified Act5C-GS driver line was starved for 6 hr and then fed with 200 μM RU486. As expected, upon RU486 feeding the modified Act5C-GS driver line did not express green fluorescence in both males and females (**Fig. 11**).

As shown in **Fig. 11,** the UAS-GFP insert was removed from the modified Act5C-GS driver line. To confirm that the Act5C-GAL4.Switch insert was still present on the third chromosome after a serial segregation, the modified Act5C-GS driver line was crossed to a UAS-GFP reporter line (**Fig. 12A**). The first progeny of this cross contains both inserts, Act5C-GAL4.Switch, and UAS-GFP. A significant green fluorescence was indeed present in the first progeny fed with RU486, in both males and females. Upon 200 μM RU486 administration, the green fluorescence intensity increased in a time-dependent manner (**Fig. 12B)**. This result further confirmed the presence of the Act5C-GAL4.Switch insert in the modified GS-Act5C driver line on the third chromosome.

3.1.6. Construction of a transgenic line containing both UAS-dβ5 RNAi and Act5C-GS inserts located and balanced on individual chromosomes

To disrupt the expression of the dβ5 subunit, the dβ5 RNAi was driven by the inducible Act5C-GS driver. The UAS-dβ5 RNAi line was thus crossed with the modified

Act5C-GS driver line. The progeny contained both the Act5C-GS driver and the UAS-dβ5 RNAi inserts. The deficiency of dβ5 subunit was induced by feeding the progeny with RU486. Upon RU486 administration, Act5C-GS binds to the UAS and activates transcription of dβ5 RNAi.

We reasoned that collecting virgins, setting up crosses, and screening the first progeny would take too much time. Therefore, we tried to construct another transgenic line containing the UAS-dβ5 RNAi and Act5C-GS inserts located and balanced on the second and third chromosome, respectively. Once this transgenic line would be established, disruption of the dβ5 subunit could be induced by feeding with RU486 directly without any crosses. As shown in **Fig. 13**, following a serial crossing, a new transgenic fly line containing both the UAS-dβ5 RNAi and Act5C-GS driver was generated. Unfortunately, this transgenic line was unhealthy and vulnerable. This line did not survive well even without RU486 feeding. Although it is still unclear the vulnerability corresponds most likely to the presence of two balancers. Thus, we decided not to use this transgenic line for our project.

3.1.7. Systematic expression of the dβ5 RNAi directed by the modified Act5C-GS driver

After the UAS-dβ5 RNAi transgenic line and modified Act5C-GS driver line were generated, we next crossed these two lines and collected the first progeny containing both the UAS-dβ5 RNAi and Act5C-GS inserts. As previously discussed, three UAS-dβ5 RNAi lines containing RNAi constructs of different lengths were established. In order to have the highest efficiency of dβ5 RNAi expression, the UAS-dβ5 RNAi transgenic line

containing the longest inverted sequence (RNAi construct C, 1694 bp) was used in the crosses. To obtain high percentage of first progeny containing both inserts, homozygous UAS-dβ5 RNAi virgins were collected and crossed with males expressing the modified Act5C-GS. From the first progeny, flies containing both the UAS-dβ5 RNAi and Act5C-GS inserts were screened and separated for subsequent experiments according to their sex (**Fig. 14**). Upon RU486 feeding, Act5C-GS binds to the UAS and activates transcription of dβ5 RNAi. Therefore, feeding flies with RU486 triggers induction of dβ5 RNAi and then knocks down dβ5 subunit expression. Knock down of the dβ5 subunit can thus be manipulated by feeding flies with RU486, the ligand for the Gene-Switch binary system.

3.2. THE DISRUPTION OF PROTEASOME EXPRESSION CAUSED BY dβ5 RNAi HAS A NEGATIVE IMPACT ON DROSOPHILA

3.2.1. Administration of RU486 shortened lifespan of flies in a dose-dependent manner

UAS-dβ5 RNAi flies were generated to establish a transgenic fly model with a deficit on proteasomal activity. The dβ5 RNAi was ubiquitiously expressed in adult flies by feeding UAS-dβ5 RNAi/+; Act5C-GS /+ flies with RU486. In the presence of RU486, Act5C-GS binds to UAS and activates transcription of the dβ5 RNAi. To determine the appropriate concentration of RU486 for activation of the Genes-Switch system, we assessed the lifespan of flies upon administration of various concentrations of RU486. Male UAS-dβ5 RNAi/+; Act5C-GS /+ flies were fed different concentrations of RU486 ranging from 0 µM to 600 µM. A dose-dependent decrease in lifespan was observed with

increasing RU486 concentrations (**Fig. 15 and Table 1**). The decline in lifespan was significantly different at RU486 concentrations above 5 µM, when compared to transgenic flies that received no RU486. The mean lifespan of male transgenic flies fed 100 µM RU486 was severely reduced (12.29 ± 0.02 days) compared to controls (48.01 ± 4.35 days) (**Fig. 15 and Table 1**). Flies given 200 µM RU486 displayed a 79% decrease in lifespan. The mean lifespan of flies fed 200 µM or 600 µM RU486 did not differ, being 9.93 ± 0.12 days and 9.45 ± 0.63 days, respectively. These data suggest that the Genes-Switch system is saturated at 200 µM RU486. Based on these data, we chose to feed flies with 200 µM RU486 in subsequent experiments.

According to the decline in lifespan observed with RU486 feeding, we assumed that the dβ5 RNAi successfully disrupted proteasome activity. We reasoned that the higher RU486 concentrations tested (≥ 200 µM) induced dβ5 RNAi to reach levels that efficiently disrupted dβ5 expression leading to proteasome dysfunction.

3.2.2. Induction of dβ5 RNAi by RU486 administration decreased dβ5 expression in a time-dependent manner

To confirm that the dβ5 RNAi down-regulated the expression of endogenous dβ5, its protein levels were assessed by Western blotting. To establish the time-dependency of the RU486 effect on dβ5 knock down, separate 1 to 2 day-old adult male and female transgenic flies with the genotype UAS-dβ5 RNAi/+; Act5C-GS /+ were collected and starved for 12h. Following starvation, flies were transferred for various periods of time to new vials with fly food containing 4% ethanol (vehicle, control) or 200 µM RU486. Male and female flies were fed 200 µM RU486 for 1, 3, 5, 7, 9, and 11 days. Female flies

were additionally fed 200 µM RU486 for 13 and 15 days, as males did not survive this long. Control male and female transgenic flies were fed 4% ethanol for 11 or 15 days, respectively. Protein extracts from whole flies were subjected to SDS-PAGE (90µg protein/lane), followed by western blot analysis as described under Materials and Methods. **Fig. 16** shows that induction of dβ5 RNAi by RU486 feeding caused a time-dependent and progressive decline in endogenous dβ5 levels. A significant dβ5 decline was detected in males after 5 days (28% decline) and 9 days (52% decline) of RU486 administration. A similar pattern was observed in females: 30% decline of dβ5 level after 9 days and 70% decline after 11 days of RU486 administration.

These data demonstrate a time-dependent decrease in dβ5 protein levels in both male and female RU486 fed flies. Furthermore, feeding flies 200 µM RU486 was sufficient to induce the Gene-switch/UAS binary system and knock down the endogenous dβ5 protein as shown on the Western blots.

3.2.3. Knocking down the dβ5 subunit disrupts proteasome levels and activity

Down-regulation of the dβ5 subunit causes a disruption of proteasome activity [191]. To confirm proteasome dysfunction upon induction of dβ5 RNAi, proteasome activity was assessed by native gel electrophoresis (In-gel assay) in control and RU486 fed UAS-d*β*5 RNAi/+; Act5C-GS /+ flies. As described under Materials and Methods, the native gel was incubated with the fluoregenic substrate Suc-LLVY-AMC, which is specific for the proteasome chymotrypsin-like activity. Proteasome levels were assessed by immunoblotting with the anti-dβ5 antibody, which detects 26S (two-capped and one-capped) and 20S proteasomes. Male 1 to 2 day-old adult flies were collected and starved

for 12h. Following starvation, flies were transferred for various periods of time to new vials with food containing 4% ethanol (11 days) or 200 µM RU486 (1, 5, 9, and 11 days). Protein extracts from whole flies were subjected to native PAGE (90µg protein/lane) and immunobloting with the anti-dβ5 antibody to assess proteasome activity and levels, respectively, as described under Materials and Methods.

As shown in **Fig. 17A** and **17B**, compared to controls RU486 fed males exhibited a significant decline in proteasome chymotrypsin-like including 26S and 20S proteasomes. The greatest reduction in proteasome activity [55% in the 20S; 44% in the 26S (one capped)] was observed after 11 days of treatment. A parallel decrease in proteasome levels was detected by immunoblot quantification **(Fig. 17C** and **17D)**. At day 9, decreases of 64% (20S) and 46% (26S one capped) in proteasome levels were observed. Notably, RU486 fed male flies exhibited higher 26S proteasome (two capped) activity and levels at day 5 than controls. At day 5, the increase in 26S (two capped) proteasome activity and levels was 84% and 101%, respectively. After day 5, the activity and levels of 26S (two capped) proteasomes declined progressively in RU486 fed male flies. This effect can be explained by a compensatory mechanism to upregulate 26S (two capped) proteasome activity and level temporarily, following the knocking down of dβ5. Non-targeted subunits of the proteasome are upregulated while the dβ5 subunit is knocked down (**Fig. 20C**). The excess of non-targeted subunits of 26 proteasome may result in a temporal increase of 26S preoteasome (two capped) activity and level.

These data confirm that d*β*5 RNAi disrupted proteasome activity and levels in flies. Feeding RU486 to UAS-d*β*5 RNAi/+; Act5C-GS/+ flies did indeed induce a decline in

26S/20S proteasome activity and levels confirming the effectiveness of the Gene-Switch System.

3.2.4. Additional proteasome complex with chymotrypsin-like activity induced in the dβ5 knock down of female flies

We also assessed proteasome activity and level in female UAS-dβ5 RNAi/+; Act5C-GS/+ flies. Female 1 to 2 day-old adult UAS-dβ5 RNAi/+; Act5C-GS /+ flies were collected and starved for 12h. Following starvation, flies were transferred to new vials with fly food containing 4% ethanol (15 days) or 200 µM RU486 for 1, 7, 13, and 15 days. Protein extracts from whole flies were subjected to native PAGE (90µg protein/lane) and immunobloting with the anti-dβ5 antibody to assess proteasome activity and levels, respectively, as described under Materials and Methods.The in-gel assay and immunoblotting revealed that, just like in males, RU486 fed female flies exhibited a time-dependent decrease in 20S and 26S (one capped) proteasome activity and levels. **Fig. 18A and 18B** show that in female RU486 female flies there was a significant decline in the chymotrypsin-like activity of 20S and 26S (one capped) proteasomes compared to ethanol (vehicle) fed controls. At day 13, decreases of 39% in 20S proteasome and 34% in 26S (one capped) of proteasome activities, as well as 40% in 20S proteasome and 46% in 26S (one capped) proteasome levels were observed (**Fig. 18C and 18D)**. Like in male RU486 fed flies, an increase in activity and levels of the 26S (two capped) proteasome was detected in female RU486 fed flies. In conclusion, 26S proteasome activity and levels are impaired by dβ5 knock down in both male and female UAS-dβ5 RNAi/+; Act5C-GS /+ transgenic flies upon RU486 administration.

Surprisingly, RU486 administration to transgenic female flies induced the formation of a new complex detected below the 20S proteasome. The chymotrypsin-like activity **(Fig. 18A** and **18B)** and level (**Fig. 18C and 18D)** of the new complex were amplified in a parallel and time-dependent manner by RU486 administration. Upon day 7, 13, and 15 of RU486 administration the chymotrypsin-like activity of the new complex was raised by 111%, 304%, and 302%, and its level by 202%, 376%, and 448%, respectively.

We tested if this new proteasome complex was present in the wild type drosophila strains, yw (flies without dβ5 RNAi inserts). Flies from the yw strain were analyzed by the in-gel assay and Western blotting. Female 1 to 2 day-old adult yw flies were collected and starved for 12h. Following starvation, flies were fed for 1, 7, 13, and 15 days with fly food containing 200 μM RU486 or 4% EtOH for 15 days. Protein extracts from whole flies were subjected to native PAGE (90μg protein/lane) and immunobloting with the anti-dβ5 antibody to assess proteasome activity and levels, respectively, as described under Materials and Methods. As shown in **Fig. 19**, RU486 administration to female yw flies had no effect on proteasome activity and levels as assessed by the in-gel assay and immunoblotting, respectively. Furthermore, these assays did not detect any RU486-induced new proteasome complex in female yw flies. These results demonstrate that RU486 administration induced the formation of a new proteasome complex only in female flies with the dβ5 RNAi transgene but not in female yw flies.

3.2.5. Knock down of the dβ5 subunit upregulated the expression of other proteasome subunits

We further characterized the subunit composition of the new proteasome complex induced by administration of RU486 to female UAS-dβ5 RNAi/+; Act5C-GS /+ flies. Total fly extracts obtained from control UAS-dβ5 RNAi/+; Act5C-GS /+ female flies as well as RU486-fed transformants, were subjected to the in-gel assay and immunoblotting as described under Materials and Methods. We first detected chymotrypsin-like activity in two parallel native gels. Immunoblotting followed by probing each of the membranes with the anti-β5 or the anti-α1 antibodies. As expected, knock down of dβ5 by RNAi disrupted proteasome activity (**Fig. 20A)** and level (**Fig. 20B)** in the RU486-fed female flies. However, the signal detected with the anti-α1 antibody appeared as a smear (**Fig. 20B)**.

The smear detected with the anti-α1 antibody could correspond to "abnormal" inactive proteasome complexes formed by an excess of non-RNAi targeted proteasome subunits. To address this possibility, the same fly extracts were subjected to SDS-PAGE (90μg protein/lane) followed by western blot analyses. As shown in **Fig. 20C**, induction of dβ5 RNAi reduced dβ5 levels, but significantly increased protein levels of other non-RNAi targeted proteasome subunits, such as α1 and Rpn3. These results confirm that knock down of a single proteasome subunit by RNAi has a compensatory effect resulting in enhanced expression of non-RNAi targeted subunits. This compensatory effect was reported previously by others [172, 192].

These data suggest that the smear detected with the anti-α1 antibody most likely represents "abnormal" and inactive forms of the proteasome assembled with the excess

non-RNAi targeted subunits without dβ5, as the expression of the latter was prevented by RNAi.

The composition of the new form of the proteasome formed in female transformants upon RU486 administration requires full purification of the new complex. These experiments will be carried out in the future.

3.2.6. Disruption of proteasome activity by dβ5 RNAi promoted an age-related accumulation of ubiquitinated proteins

The proteasome maintains cellular homeostasis by degrading intracellular proteins. Most substrates of the 26S proteasome are cellular proteins modified with a polyubiquitin chain [193]. Knock down of dβ5 subunit expression impaired proteasome activity and level (**Fig. 17 and 18**). To determine whether the decline in proteasome activity induced by dβ5 RNAi results in the accumulation of polyubiquitinated proteins, separate groups of male or female UAS-dβ5 RNAi/+; GS-Act5C/+ flies were analyzed. After being starved for 12 h, flies were fed with 200 μM RU486 or 4% EtOH (vehicle, control) for varying periods of time. Male flies were fed with 200 μM RU486 for 1, 3, 5, 7, 9, and 11 days or 4% EtOH (vehicle, control) for 11 days. Female flies were fed with 200 μM RU486 for 1, 3, 5, 7, 9, 11, 13, and 15 days or 4% EtOH (vehicle, control) for 15 days. Protein extracts from whole flies were subjected to SDS-PAGE (50 μg protein/lane) and immunobloting with an anti-ubiquitin antibody as described under Materials and Methods [192].

Immunoblots from RU486 treated male and female flies displayed a high molecular-weight smear that gradually increased in intensity with time of RU486 exposure when

compared to untreated controls. In males, polyubiquitinated protein accumulation was first detected 3 days after RU486 feeding (**Fig. 21**). RU486 feeding caused an increase in polyubiquitinated protein accumulation in a time-dependent manner. At day 5, 7, 9, and 11 the increase was 298%, 409%, 514%, and 547% of control, respectively. Similar patterns were found in female flies. Polyubiquitinated protein accumulation was first detected 5 days after RU486 feeding. Time-dependent increase in polyubiquitinated proteins was also observed: at day 7, 9, 11, 13, and 15 the elevation in polyubiquinated proteins was 661%, 958%, 960%, 1107%, and 1243% of control, respectively. As we expected, the abnormal accumulation of polyubiquitinated proteins paralleled the reduction of dβ5 subunit level and proteasome dysfunction. The highest level of polyubiquitinated protein accumulation coincided with the greatest reduction in proteasome activity.

These data show that dβ5, a catalytic subunit of the proteasome, is essential for maintaining proteasome structure and function. Knock down of endogenous dβ5 expression induced by RU486 administration caused a remarkable accumulation of non-degraded cellular protens.

These results further confirmed that our approach to specifically knock down the dβ5 subunit expression in drosophila was responsible for the disruption of proteasome activity and for the dramatic increase in the accumulation of non-degraded polyubiquitinated proteins.

3.2.7. Proteasome dysfunction caused locomotor impairment

Proteasome activity is also essential for the maintenance of muscle and neuromuscular junction architecture [153]. Moreover, administration of proteasomal inhibitors to rats and mice causes locomotor defects including bradykinesia, rigidity, tremor, and an abnormal posture [97, 155].

In our transgenic fly model, expression of the dβ5 RNAi was regulated by an inducible ubiquitious Act5C-GS driver. By feeding UAS-dβ5 RNAi/+; Act5C-GS/+ flies with RU486, dβ5 RNAi is expressed ubiquitously, including muscles and neurons. RU486 administration causes systematic disruption of proteasome activity in most tissues of the dβ5 RNAi flies. To investigate how dβ5 RNAi and proteasome impairment affect locomotion in flies, we performed a climbing assay used in previous fly studies [194, 195]. Wild type flies displayed a strong negative geotactic response. When tapped to the bottom, flies climb to the top of vials rapidly and stay there. As shown in **Fig. 22A**, transgenic flies that did not feed on RU486 displayed normal locomotor activity. In both male and female transformants, the climbing ability significantly deteriorated in a time-dependent manner upon RU486 feeding. In RU486 fed males, the significant decrease in locomotor function assessed by the climbing assay was 7%, 59%, and 88% at day 7, 9, and 11, respectively. A similar pattern was observed in RU486 fed females. The time-dependent decrease in locomotion was 9%, 42%, 66%, and 85% at day 11, 13, 15, and 17, respectively (**Fig. 22B**). Upon RU486 feeding, most flies stayed at the bottom; they tried to climb up but fell back or climbed very slowly. This rapid decline in climbing performance paralleled the decrease in dβ5 subunit level and proteasome activity. These

data confirm that proteasome activity is required for maintenance of locomotor activity as demonstrated in a by previous report [196].

3.2.8. Knock down of dβ5 shortens the lifespan of drosophila and increases sensitivity to oxidative stress

To test whether proteasome dysfunction caused by dβ5 RNAi has effects on the lifespan of flies, separate groups of males and females were analyzed. For starvation, 1 to 2 day-old UAS-dβ5 RNAi/+; Act5C-GS /+ flies were transferred to vials containing a filter paper prewetted with water. After being starved for 12 h, flies were fed 4% EtOH or 200 µM RU486 at day 0 and transferred to new vials every 5 days. The percentage of surviving flies was counted daily. This experiment was performed at 26°C.

As shown in **Fig. 23**, the lifespan of the dβ5 knockdown male and female flies was severely impaired. Compared to male untreated flies, male flies treated with 200 µM RU486 exhibited a drastic decrease (79%) in lifespan (**Fig. 23**). The mean lifespan of control males was 48.01 ± 4.35 days. The lifespan of male treated flies was shortened to 9.93 ± 0.12 days. Upon 13 days of RU486 administration only 12% of male transformants survived. A similar pattern was observed in female transformants. A dramatic decrease (79%) in the mean lifespan of female treated flies was observed. The mean lifespan for control female transformants that did not get RU486 was 65.6 ± 3.49, while those that received RU486 was 13.94 ± 0.27 days. Upon 16 days of RU486 administration only 16% of females survived. This experiment demonstrates that proteasome dysfunction induced by dβ5 RNAi, severely reduced lifespan. These data also demonstrated that proteasome activity is essential for fly survival as proteasome

impairment alone, causes a dramatic reduction in lifespan. Interestingly, we observed a sexual dimorphism in the survival assay. The average lifespan in females was longer than in males: the mean lifespan for females was 65.6 ± 3.49 days, and for males 48.01 ± 4.35 days. For unknown reasons, females exhibited higher resistance to proteolytic stress. Upon RU486 administration, their lifespan was 40% higher than in males.

The proteasome maintains protein homeostasis by degrading the majority (80%~90%) of intracellular proteins [197, 198], as well as misfolded and oxidized proteins [199-202]. We hypothesized that disruption of proteasome activity caused by dβ5 RNAi leads to an increase in vulnerability to oxidative stress. To address this hypothesis, we subjected UAS-dβ5 RNAi/+; Act5C-GS/+ flies to oxidative stress induced by 1% hydrogen peroxide (H_2O_2). 30 flies were placed in fly food containing 1% H_2O_2 or RU486 200 µM alone or in combination. As shown in **Fig. 24**, exposure to 1% H_2O_2 shortened lifespan in both male and female transformants. Compared to untreated control flies, flies with proteasome deficiency induced by RU486 showed higher sensitivity to H_2O_2, as most flies treated with RU486 and 1% H_2O_2 died after one week. This pattern was observed in both male and female flies. The mean lifespan of male flies fed with 1% H_2O_2 and 1% H_2O_2 + RU486 200 µM was 6.94 ± 0.22, and 3.71± 0.14 days, respectively. The mean lifespan of female flies fed with 1% H_2O_2 and 1% H_2O_2 + RU486 200 µM was 7.74 ± 0.19, and 3.08 ± 0.08 days, respectively. Proteasome dysfunction thus caused a decrease (47% in males; 60% in females) in the resistance to oxidative stress as assessed with the survival assay. These results suggest that proteasome activity is required for resisting oxidative stress and for protection against oxidatively damaged proteins.

3.2.9. RU486 does not decrease the lifespan and proteasome activity of wild type flies

Administration of 200 µM RU486 shortened lifespan and impaired proteasome activity in UAS-dβ5 RNAi/+; Act5C-GS/+ flies (**Fig.17 and 18**). To confirm that the effect of RU486 administration in UAS-dβ5 RNAi/+; Act5C-GS /+ flies is caused by expression of dβ5 RNAi and not by RU486 toxicity alone, wild type yw flies without the UAS-dβ5 RNAi insert were analyzed upon RU486 administration for survival and proteasome activity by the in-gel assay. Separate groups of males and females were included. 1 to 2 days-old yw flies were collected and fed with 4% EtOH or 200 µM RU486 at day 0. As shown in **Fig. 25**, no significant change in lifespan was observed in the two groups of yw flies, i.e. those that did not get RU486 (control) compared to those that did. The lifespan of control and RU486 fed male yw flies was 44.26 ± 3.83 days, and 40.77 ± 1.42 days, respectively. The lifespan of control and RU486 fed female yw flies was 59.57 ± 1.14 days, and 56.79 ± 2.3 days, respectively.

To determine if the reduction of lifespan induced by RU486 was caused by the dβ5 RNAi insert, yw and UAS-dβ5 RNAi/+; Act5C-GS/+ male and female flies were analyzed with the survival assay. RU486 administration caused a dramatic decrease in the lifespan of males (76%) and females (75%) that had the dβ5 RNAi insert (**Fig. 26**). Upon RU486 administration, the lifespan of males with or without the dβ5 RNAi insert was 9.93 ± 0.12 days, and 40.77 ± 1.42 days, respectively, and of females with or without dβ5 RNAi insert was 13.94 ± 0.27 days, and 56.79 ± 2.3 days, respectively. These data show that the effect of RU486 administration on survival of UAS-dβ5 RNAi/+; GS-Act5C/+ flies was dependent on the presence of the dβ5 RNAi insert.

To further confirm that RU486 by itself had no effect on proteasome activity and level, total extracts from yw flies, fed with RU486, were compared to those from flies that did not get RU486 by the in-gel assay. No significant difference in proteasome activity and level were observed in both groups of males (**Fig. 27**) or females (**Fig. 19**). As shown in [189, 190], our results confirm that RU486 alone has no significant effect on lifespan, proteasome activity and level.

3.3. TEMPORAL DISRUPTION OF PROTEASOME ACTIVITY HAS A LONG TERM EFFECT ON FLIES

3.3.1. Effect of temporal disruption of dβ5 subunit expression and proteasome function on the lifespan of flies

Our central hypothesis is that protein aggregation and inclusion body formation is caused by impairment of the ubiquitin proteasome pathway. We addressed whether temporal proteasome disruption has long term/irreversible effects on lifespan, protein accumulation, aggregate formation, and locomotion. We accomplish this with an inducible promoter that allows for "turning on/turning off" dβ5 gene expression in drosophila. Transgenic expression of mutant proteasome β subunits was previously used to specifically inhibit the drosophila proteasome [31, 203, 204]. In these studies a fly model with tunable proteasome activity was successfully established thus providing for a means to investigate whether temporal disruption of proteasome function has long term/or even irreversible effects on flies.

To determine whether temporal disruption of proteasome activity has an irreversible effect on lifespan of flies, UAS-dβ5 RNAi/+; Act5C-GS /+ male flies were collected and kept on fly food with 200 µM RU486 for varying periods of time (1, 3, and 5 days) and then transferred to fly food without RU486. Flies were transferred to new vials with fresh food every 5 days. We calculated the percentage of dead flies daily.

As shown in **Fig. 28**, there was no significant difference in survival of flies exposed to RU486 for 0, and 1 day as their mean lifespan was 48.01 ± 4.35 days, and 50.09 ± 2.96 days, respectively. It appears that RU486 administration for one day is not sufficient to induce dβ5 RNAi expression to impair proteasome activity and shorten the lifespan. The lifespan was reduced in flies upon 3 and 5 days exposure to RU486 by 34%, and 59%, respectively. The mean lifespan of male flies exposed to RU486 for 3, and 5 days was 31.89 ± 5.55, and 19.73 ± 2.05 days, respectively. The survival curve showed that the reduction in lifespan was parallel to the time of exposure to RU486. These data demonstrate that flies could not recover from RU486 feeding and thus temporal disruption of proteasome function has a long term effect on fly lifespan. In subsequent experiments, flies exposed to RU486 for 5 days and then transferred to fly food without RU486 were used to analyze the long term effects of temporal disruption of proteasome activity.

3.3.2. Recovery of dβ5 mRNA and protein upon temporal RU486 administration

Despite the fact that our previous data demonstrated that temporal RU486 administration caused an irreversible effect on lifespan, we could not rule out persistent activation of the Gene-Switch system. We thus investigated whether dβ5 mRNA

expression could recover after RU486 withdrawal. Two groups of male UAS-dβ5 RNAi/+; Act5C-GS/+ flies were analyzed. In the control group, flies were kept on fly food without RU486. In the "recovery" group, flies were kept on fly food with 200 μM RU486 for 5 days and then transferred to fly food without RU486. In the recovery group, flies were fed RU486 for 5 days and then kept on regular fly food (without RU486) permanently from day 6 on. The fly dβ5 mRNA level of both groups was assessed by RT-PCR. As shown in **Fig. 29**, in the recovery group, the dβ5 mRNA level declined to 70% and 53% at day 6 and 16, respectively. These data reveal a time-dependent decrease in dβ5 mRNA level from day 1 to day 16 (**Fig. 29**). Although RU486 exposure for 5 days caused a dramatic reduction in dβ5 mRNA level up to day 16, dβ5 mRNA level recovered to 80% by day 21.

We then assessed the dβ5 protein level by Western blotting. Compared to the RT-PCR results, dβ5 protein levels exhibited a similar pattern in the recovery group. In the recovery group, the dβ5 protein level at day 1, 6, and 16 was 105%, 86%, and 77%, respectively (**Fig. 30**). After day 16 dβ5 protein level recovered to control dβ5 endogenous level as it increased in day 16, 31, and 36 to 76%, 88%, and 100%, respectively.

The RT-PCR results showed that RU486 rapidly activated the Gene-Switch system to reduce dβ5 mRNA levels, but recovery after RU486 withdrawal was slow. This result was consistent with a recent report [159]. It is not clear whether the slow recovery after RU486 withdrawal is due to stability of the dβ5 RNAi, or to persistent activation of the Gene-Switch system. Although recovery of the dβ5 mRNA was slow, beginning at 21 days upon RU486 withdrawal, dβ5 protein level was restored to control endogenous

levels when mRNA level was only at 80%. These studies indicated that activation of the Gene-Switch system can be discontinued by RU486 withdrawal, although the process of recovery takes a relatively long time. However, it is clear that the irreversibility of the decline in lifespan observed upon temporal impairment of dβ5 level/proteasome activity was not caused by the persistent activation of the Gene-Switch system.

3.3.3. Proteasome activity recovered upon RU486 withdrawl

As shown in **Fig. 30**, dβ5 protein level returned to control endogenous level upon RU486 withdrawal. To examine whether proteasome activity also recovers to the control endogenous level upon RU486 withdrawal, the in gel assay was used to compare proteasome activity and level of the control and recovery groups of flies. In the control group, male UAS-dβ5 RNAi/+; Act5C-GS/+ flies were kept on fly food without RU486. In the recovery group, male UAS-dβ5 RNAi/+; Act5C-GS/+ flies were kept on fly food with 200 μM RU486 for 5 days and then transferred to fly food without RU486.

As shown in **Fig. 31**, 5 days of RU486 administration disrupted proteasome activity and level. Due to down regulation of the dβ5 subunit, proteasome activity and level declined in a time-dependent manner from day 1 to day 16. The reduction in proteasome activity and level paralleled the decline in dβ5 protein level. In the recovery group, the greatest reduction of proteasome activity and level was detected at day 16. In the recovery group, the chymotrypsin-like activity of 26S (two capped), 26S (one capped), and 20S proteasome at day 16 was 39%, 58%, and 67%, respectively (**Fig. 31A** and **31C.**) and their levels were 55%, 61%, and 67%, respectively (**Fig. 31B** and **31D**). Upon RU486 withdrawal proteasome activity and level recovered at the same pace as dβ5

expression (compare **Fig. 30** with **Fig. 31**). A progressive recovery of proteasome activity and level were observed after day 16. At day 36, the activity of 26S (two capped), 26S (one capped), and 20S proteasomes recovered to 76%, 85%, and 105%, respectively (**Fig. 31A** and **31C**) while their levels recovered to 67%, 86%, and 92%, respectively **(Fig. 31B** and **31D**). These results show that proteasome activity and level, which were impaired during RU486 administration, progressively recovered after RU486 withdrawal. Impairment and recovery of proteasome activity and level paralleled dβ5 expression, all reaching the lowest point at day 16 and recovering from then on. These studies indicate that proteasome activity and level can recover from temporal disruption via the Gene-Switch System induced by RU486 administration.

3.3.4. Proteasome recovery failed to attenuate the accumulation of ubiquitinated proteins

The ubiquitin-proteasome system represents the major pathway for regulated intracellular protein degradation in eukaryotes [160]. Polyubiquitinated substrates are recognized and degraded by the 26S proteasome. Intracellular accumulation of ubiquitinated proteins occurs in response to proteasome impairment. We investigated by immunoblot analysis, if the recovery from temporal proteasome disruption reversed the accumulation of ubiquitinated proteins.

The same control and recovery groups of male UAS-dβ5 RNAi/+; Act5C-GS/+ flies as in the previous experiment, were analyzed. In the recovery group, temporal impairment of proteasome activity by RU486 administration for 5 days, resulted in the accumulation of polyubiquitinated proteins. Surprisingly, while proteasome activity

recovered progressively with RU486 withdrawal (**Fig. 31**), the levels of polyubiquitinated proteins failed to decrease. As shown in **Fig. 32**, temporal disruption of proteasome activity upon 5 days of RU486 feeding, resulted in a gradual time-dependent accumulation of polyubiquitinated proteins detected as a high molecular-weight smear. In the recovery group, compared to controls, the increase in polyubiquitinated proteins at day 1, 6, and 16 was 8%, 74%, and 126%, respectively. The time-dependent increase in polyubiquitinated proteins paralleled the reduction in proteasome activity. In the recovery group, the decline in proteasome activity was reversed after day 16 of RU486 withdrawal (**Fig. 31 A** and **31C**). Notably, no significant reduction in polyubiquitinated proteins was displayed in the process of proteasome recovery **(Fig. 32)**.

Due to the recovery of proteasome activity, no additional increase in polyubiquitinated proteins was detected after day 16. Instead, the increase in polyubiquitinated proteins plateaued by day 16 with no change observed in the recovery group up to day 36. This study reveals that no attenuation of polyubiquitinated protein level was observed upon proteasome recovery. These data suggest that the accumulation of polyubiquitinated proteins occurring prior to proteasome recovery was stable and irreversible even when proteasome activity recovered. More importantly, these results strongly suggest that the process of intracellular accumulation of ubiquitinated proteins is irreversible. Once intracellular polyubiquitinated proteins accumulate, it is difficult to remove them. Consequently, temporal proteasome impairment could thus lead to long term and irreversible cellular damage.

3.3.5. Increase in ubiquitinated protein aggregation during proteasome recovery

Proteasome impairment is a feature of many cell models in which intracellular aggregates are formed [205]. The cytoplasmic ubiquitinated inclusions and ubiquitin aggregates are formed following proteasome inhibition [206]. Since down-regulation of the dβ5 subunit impaired proteasome activity, we investigated aggregate formation in the transgenic flies upon proteasome recovery. The same two groups of male UAS-dβ5 RNAi/+; Act5C-GS/+ flies used in previous experiments, control and recovery, were used to analyze ubiquitinated protein aggregation. In the control group, flies were kept on fly food without RU486. In the recovery group, flies were kept on fly food with 200 µM RU486 for 5 days and then transferred to fly food without RU486. In the recovery group, proteasome activity was impaired temporally by feeding flies RU486 for 5 days.

To assess the levels of SDS insoluble ubiquitinated aggregates, total extracts from both groups of flies (control and recovery) were analyzed by the filter trap assay as described in Methods and Materials. As shown in **Fig. 33**, administration of RU486 for 5 days led to a gradual increase in the levels of insoluble ubiquitinated aggregates. The time-dependent increase in ubquitinated protein aggregation at day 1, 6, and 16 was 4%, 135%, and 183%, respectively. In the recovery group, the increase in ubiquitinated protein aggregation observed from day 1 to day 16, coincided with the reduction in proteasome activity (**Fig. 31**) and the increase in ubiquitinated protein levels (**Fig. 32**). These results clearly indicate that temporal impairment of proteasome function caused by dβ5 knock down is sufficient to induce the accumulation of cellular insoluble aggregates, thus proteasome disruption alone is enough to cause aggregate formation.

Next, we investigated if the formation of insoluble aggregates in flies is a reversible or irreversible process. As demonstrated in earlier experiments (**Fig. 29, 30 and 31**), the Gene-Switch binary system is an ideal tool to assess the reversibility of aggregate formation. According to our previous experiments **(Fig. 31)**, the decline in proteasome function via RU486 feeding is reversible. In the recovery group, after day 16, the progressive restoration of proteasome activity and level from temporal disruption was observed upon RU486 withdrawal (**Fig. 31**). As shown in **Fig. 33**, temporal proteasome disruption by feeding flies RU486 for 5 days caused a progressive increase in SDS insoluble ubiquitinated aggregates. However, following recovery of proteasome activity (day 16 to day 36), instead of an attenuation of protein aggregation, we observed a gradual increase in ubiquitinated protein aggregates.

Our previous data demonstrated that upon RU486 withdrawal, the proteasome recovered from its temporal disruption, as the activity of 26S (two capped), 26S (one capped), and 20S proteasomes at day 36 was restored to 76%, 85 %, and 105%, respectively, (**Fig. 31A** and **31C**). Notably, despite proteasome recovery, flies were unable to clear the previously formed SDS insoluble, ubiquitinated aggregates. In the recovery group, the levels of aggregates at day 16, 26, and 36 was 332%, 402%, 497%, respectively, in comparison to the control group. This experiment strongly supports the view that ubiquitinated aggregate formation is caused by a deficiency in proteasome function. In addition, aggregate formation is irreversible as shown in our transgenic fly model.

3.3.6. Locomotion deterioration during temporal disruption of proteasome activity

Protein aggregation is associated with severe locomotion disability in flies [207, 208]. As shown in **Fig. 33**, proteasome impairment in our transgenic flies promoted the irreversible formation of aggregates. The climbing assay was used to determine whether the irreversible aggregate formation had an impact on locomotor activity. The same control and recovery groups of male UAS-dβ5 RNAi/+; Act5C-GS/+ flies, were analyzed for locomotor activity. As shown in **Fig. 34**, young flies in the control group displayed a strong ability to climb to the top of the cylinder after falling to the bottom upon tapping. In the control group, a time-dependent decrease in climbing ability of 7%, 30%, 36%, and 64% was detected at days 21, 26, 31, and 36, respectively.

In the recovery group, young flies were as skillful at climbing as young controls. However, over time, the climbing performance of flies in the recovery group declined rapidly. The climbing ability of the flies in the recovery group was significantly reduced after RU486 feeding for 5 days and rapidly declined after day 21. Compared to the control group, the climbing ability of flies in the recovery group decreased drastically to 73%, 74%, 88%, and 87% at days 21, 26, 31, and 36 respectively. In the recovery group, proteasome activity and level began recovering at day 21. However proteasome recovery was not sufficient to improve locomotor activity. Most likely, the rapid decline in climbing ability was associated with the parallel increase in protein aggregation. The progressive and accelerated decline in locomotor activity observed in the recovery group flies could thus be closely related to protein aggregation caused by the temporal disruption of proteasome activity.

These data support our previous finding that locomotor dysfunction is age associated [196]. Furthermore, the degree of loss in locomotor function seems to be correlated with the level of protein aggregation. In conclusion, temporal disruption of proteasome activity in flies causes a long term and irreversible deficit in locomotor activity that is closely associated with the inability to clear protein aggregates.

CHAPTER 4

DISCUSSION

To address the effects of a temporal disruption of proteasome activity in drosophila adults, we generated a transgenic fly model in which expression of the dβ5 catalytic subunit of the proteasome is knocked down by RNAi in a tunable manner. Induction of the dβ5 RNAi driven by the constitutive Act5C driver via the UAS-Gal4 system caused embryonic and larval lethality, demonstrating that the dβ5 gene is essential for embryonic and larval development in flies. To overcome this difficulty, we used an inducible binary system (Gene-Switch) in which administration of an activator (RU486) of the Gene-Switch system regulates dβ5 RNAi expression. This inducible manipulation of the dβ5 RNAi expression significantly and selectively knocked down the mRNA and protein levels of the targeted dβ5 catalytic subunit of 26S proteasome in flies. Knock down of dβ5 by RNAi had a significant impact on flies, including, proteasome impairment, ubiquitinated protein accumulation and aggregation, locomotor dysfunction and shorter lifespan.

The decline in proteasome activity and level was dependent on the reduction in dβ5 level. Since the 26S proteasome selectively degrades ubiquitinated proteins, their accumulation indicates proteasome dysfunction. Expression of dβ5 RNAi in the transgenic flies induced a substantial and rapid accumulation of ubiquitin conjugates, indicating a remarkable impact on the processing of ubiquitinated substrates. Our data also demonstrate that reduction of an individual subunit of the 26S proteasome by RNAi resulted in increased upregulation of other proteasome subunits. Knock-down of dβ5 by RNAi, upregulated the levels of other individual subunits (α1, and RPn3) of proteasome. Although the compensatory mechanism involved in the up-regulation of proteasome subunits as a consequence of RNAi is not understood, these results are in agreement with

recent reports showing that the removal of one proteasome gene results in an increase of the mRNA and protein levels of the other 20S and 19S subunits [172, 192]. Assembly of the 26S proteasome in dβ5 RNAi flies was severely impaired, as indicated by the in-gel assay. The presence of a large smear which reacted with the core particle specific antibody (anti-α1 antibody) on the native gel immunoblot, indicates that in the absence of sufficient dβ5, no well-structured 26S proteasome are assembled. The failure of the proteasome to assemble is probably in response to the deficiency of the dβ5 subunit, although other proteasome subunits are upregulated via compensatory mechanism. Surprisingly, in female dβ5 RNAi flies, knock down of dβ5 induces the formation of an extra particle that exhibits weak chymotrypsin-like activity. The mechanism involved in the generation of this extra proteasome particle as a consequence of dβ5 RNAi expression remains to be defined. Induction of the new proteasome particle only occurs in females and not in males. Sexual dimorphism was observed in the transgenic flies, as females displayed longer lifespan and greater resistance to the dβ5 deficit than males. It is not clear if this sexual dimorphism is related to the newly formed proteasome particle induced in females by dβ5 RNAi expression.

The proteasome maintains cellular homeostasis by degrading the majority of intracellular proteins [197, 198] as well as oxidized proteins [199-202]. The 26S proteasome is responsible for the degradation of the majority of intracellular proteins via an ubiquitin- and ATP-dependent pathway [20]. In contrast, oxidized proteins can be degraded in an ubiquitin and ATP-independent pathway by the 20S core proteasome [209-212]. Therefore, we reasoned that the proteasome deficient transgenic flies were more sensitivity to oxidative stress. To induce oxidative stress, 1% H_2O_2 was added to the

fly food. As expected, the transgenic flies with dysfunctional proteasome activity displayed an accelerated lethality compared to control flies. The survival curve revealed that flies with proteasome disruption were more vulnerable to H_2O_2 induced oxidative stress. During exposure to strong oxidizing toxins, the proteolytic capacity of flies is critical for the turnover of oxidized proteins. The proteasome may protect against oxidative damage by eliminating oxidative and damaged proteins. The transgenic flies with impaired proteasome activity have less capacity for the removal of oxidized proteins and therefore accumulate these damaged proteins that lead to the formation of aggregates and could contribute to lethality. Together, these results strongly support that the proteasome plays a critical role in survival under conditions of oxidative stress through elimination of oxidized, damaged cellular proteins.

A similar problem may occur in aging, or certain diseases, when proteasome activity declines below a critical threshold required to cope with oxidative stress and aging [200, 213-216]. Under such situations, oxidized proteins may not be degraded appropriately [217]. Protein oxidation induces tertiary structural changes that can cause protein aggregation. Therefore, accumulation of oxidized proteins and misfolded proteins promote aggregate formation. The resulting protein aggregates can disrupt cellular function and lead to cytotoxicity.

Proteasome function is essential for maintenance of muscle and neuromuscular junction architecture [153]. Locomotion impairment is associated with proteasome dysfunction in neurodegenerative diseases [97, 155]. Therefore, we determined if the transgenic flies with proteasome deficiency exhibited locomotor dysfunction. Our data demonstrated that proteasome disruption results in a progressive decline in locomotor

activity. This is in agreement with other studies showing similar behavioural changes after administration of a proteasomal inhibitor in rat and mouse models [155, 218].

We have characterized the molecular and behavioral features of our proteasome impaired drosophila model. This model demonstrated that proteasomal impairment causes accumulation of ubiquitinated protein conjugates, low resistance to oxidative stress, motor deficit, and a short lifespan. These results suggest that an age-related reduction of 26S proteasome activity could be a key factor in age-related features such as accumulation of misfolded or unfolded proteins, loss of locomotor activity, and higher sensitivity to oxidative stress.

Several proteasome-impaired animal models have been established, but they mostly address the effects caused by permanent proteasome dysfunction [94, 158-162]. As far as we know, there are no models that investigate whether temporal disruption of proteasome activity has long term effects. Besides aging, other factors such as infection by viruses and bacteria or exposure to environmental toxins, can also impair proteasome activity. Therefore, we decided to investigate whether the effect caused by temporal proteasome disruption is reversible or not. The transgenic fly model that we developed is optimal to address this question, because we can disrupt proteasome activity temporally and spatially via the Gene-Switch binary system. Activition and inactivation of the Gene-Switch binary system can be conditionally manipulated by RU486 administration and withdrawal, respectively. Spatial proteasome disruption can be driven to different targets by using cell-type specific promoters.

To confirm that the Gene-Switch binary system used to establish our fly model is tunable and reversible, the level of dβ5 mRNA and dβ5 protein were assessed by real-

time PCR and Western blotting, respectively. The RT-PCR data indicated that activation of the Gene-Switch system was rapid but the decay was slow after RU486 withdrawal from the fly food, which is consistent with a recent report [171]. The mechanism of slow deactivation of the Gene-switch system is not known. It is possible that the slow decay after RU486 withdrawal is due to dβ5 RNAi stability or that there is a persistent activation of Gene-Switch after RU486 withdrawal. Although the recovery of dβ5 mRNA was slow after RU486 withdrawal, western blotting revealed that the dβ5 protein reached control levels when only 80% of the mRNA was recuperated. Moreover, reduction and recovery patterns of dβ5 mRNA and protein levels coincided with that of the proteasome activity assessed by the in gel assay. These data indicate that temporal disruption of proteasome activity is accomplished by short term RU486 administration and reversed by RU486 removal.

Interestingly, while the accumulation of ubiquitinated proteins in the transgenic flies overlapped with the temporal disruption of proteasome activity, no attenuation of ubiquitinated protein levels was observed upon proteasome recovery. These results clearly indicate that once polyubiquitinated proteins accumulate due to temporal proteasome disruption they cannot be eliminated even when proteasome activity is recovered upon removal of RU486. Proteasome saturation due to high substrate levels could explain its inability to degrade the previously accumulated polyubiquitinated proteins. Upon 5 days of RU486 administration, proteasome activity declined in flies by less than 23%, however the levels of polyubiquitinated proteins increased by as much as 74%. These data suggest that when proteasome levels are at 77% of full capacity, they are not able to keep up with the proteolytic needs of the flies, thus causing accumulation

of ubiquitinated proteins. Moreover, full recovery of the proteolytic capacity of the proteasome upon RU486 removal, does not guarantee the elimination of overwhelming levels of ubiquitinated substrates that accumulated earlier during temporal disruption of proteasome activity. It is also possible that the ubiquitinated proteins that accumulate upon temporal proteasome disruption are no longer in a soluble state and are instead aggregated and thus can not be accessed by the proteasome for degradation. A recent study demonstrated that the majority of ubiquitinated proteins that accumulate upon proteasome dysfunction are not soluble and aggregate in SH-SY5Y cells [219]. Aggregates of ubiquitinated proteins have been proposed to be eliminated by lysosomes, because the aggregates are too large to have access to the proteasome catalytic chamber due of its narrow opening [220]. However, in our transgenic flies, lysosomes failed to remove the aggregates formed upon temporal proteasome disruption. The aggregates were still present even after the proteasome activity recovered due to removal of RU486. This is a very important finding, because it clearly demonstrates that short term conditions that impair proteasome activity in a reversible manner, thus mimicking the effect of the Gene-Switch binary system, exert a long-term effect, due to the irreversible nature of the protein aggregates that form upon temporal proteasome dysfunction.

The accumulation of protein aggregates occurs in response to an imbalance between generation and degradation of intracellular proteins. These aggregates exhibit strong immunoreactivity to antibodies against ubiquitin and ubiquitin conjugates [32, 43], suggesting that the proteasome plays a crucial role in aggregate formation. We confirmed that temporary proteasome impairment promotes the accumulation and aggregation of ubiquitinated proteins. Most importantly, we established that ubiquitinated protein

aggregation is an irreversible process that cannot be overcome by proteasome recovery. These data also cast doubt on the ability of lysosomes to eliminate protein aggregates formed upon transient proteasome disruption.

Temporary proteasome disruption leads to the formation of SDS insoluble ubiquitinated protein aggregates. Upon 5 days of RU486 administration, when proteasome activity declined by 29%, the level of SDS insoluble ubiquitinated aggregates increased by 68%. These data clearly indicate that dysfunction of the proteasome is responsible for formation of protein aggregates in flies. These data also reveal that ectopic expression of disease-specific proteins such as α-synuclein, Aβ, or mutant proteins, is not essential for aggregate formation. Our transgenic flies do not overexpress any of these proteins; proteasome impairment alone was enough to induce the formation of SDS insoluble ubiquitinated aggregates. This result is consistent with recent reports [94, 156, 219]. If cells have limited proteasomal capacity, misfolded proteins are not eliminated efficiently resulting in the accumulation of ubiquitinated protein conjugates leading to the formation of SDS insoluble ubiquitinated protein aggregates. As we demonstrate, a modest perturbation of proteasome activity generates aggregates in a short period of time.

In the transgenic flies upon RU486 withdrawal, the previously formed SDS insoluble ubiquitinated aggregates were not removed. Thus, the formation of SDS insoluble ubiquitinated aggregates is an irreversible process that could not be overcome even by the autophagy/lysosomal pathway. The autophagy/lysosomal pathway was postulated to play an important role in mediating the clearance of ubiquitinated protein aggregates [221]. Accumulation of ubiquitinated protein aggregates was detected in autophagy deficient

mice [222, 223]. Since in these mice, proteasome activity seems not to be affected, it suggests that autophagy is responsible for removal of ubiquitinated proteins in normal mice. Moreover, niclosamide, an autophagy-inducing compound, prevented the formation of large ubiquitin-containing aggregates generated by proteasome inhibition [219]. These data support the notion that the majority of soluble misfolded proteins are degraded by the UPS, while insoluble proteins are cleared by the autophagy/lysosomal pathway [219, 224]. It is possible that removal of protein aggregates by autophagy takes a long time. In our studies, the long term effect of temporary disruption of proteasome activity was assessed for 36 days. The autophagy/lysosomal pathway could require more time than 36 days to remove the aggregates in flies. Compared to the rate of aggregate generation, slow aggregate clearance was also observed in mice models [225]. Our data also demonstrate that the levels of ubiquitinated protein aggregates increased during the process of proteasome recovery, suggesting that autophagy induction is a slow process. If protein aggregates are not removed soon after they are formed, they could recruit other proteins such as chaperons, UPS components, centrosomal material and cytoskeletal proteins [67, 226-229]. With time, small aggregates may be converted into large insoluble aggregates. The autophagy/lysosomal pathway is not efficient in removing large insoluble aggregates in a short time. Therefore, the time-dependent increase in SDS insoluble ubiquitinated aggregates appearing during the process of proteasome recovery could very well slow down aggregate clearance by autophagy.

Whether protein aggregates are cytotoxic or cytoprotective is a controversial issue. Recent reports indicate that protein aggregates are associated with neurodegenerative diseases and are toxic to neurons. Mutations in specific proteins associated with familial

neurodegenerative diseases induce protein misfolding and aggregation [230]. For example, mutations in superoxide dismutase 1 (SOD1) are associated with familial amyotrophic lateral sclerosis; aggregates of mutant SOD1 accumulate in the spinal cord of transgenic mouse models and human patients [231]. In familial PD cases, α-synuclein mutations are related to the appearance of α-synuclein aggregates in dopaminergic neurons [76, 232, 233]. Protein aggregation is a multi-step process. Several kinds of intermediates and products, including soluble oligomers, β-sheet fibrils, and aggregates are produced in the process of aggregate formation [234]. Increasing evidence indicates that it is the soluble oligomers and fibrils that are toxic to cells [205, 206, 230]. Several reports suggest that the cytotoxic form of α-synuclein is not the amyloid fibril, but is instead the oligomer form [207, 208]. In contrast, aggregates may be inert or even cytoprotective [31, 203]. Toxic proteins could be sequestered and compartmentalized into aggregates to prevent them from spreading throughout the cell[204]. In addition, aggregate formation could facilitate elimination of toxic proteins by the autophagy/ lysosomal pathway [204, 229].

In our transgenic fly model, temporary disruption of proteasome activity causes accumulation of intracellular proteins and aggregate formation. We speculate that, initially, aggregate formation is cytoprotective as it sequesters the undegraded proteins and promotes their removal by autophagy. However, in the later stages during proteasome recovery, aggregates fail to be rapidly eliminated by autophagy. Eventually, the aggregates impair cell homeostasis resulting in cell death.

Protein aggregation is associated with severe locomotor disability [235, 236]. Our data support this view, since progressive locomotor impairment assessed by the climbing

assay in flies, was induced by temporary proteasome impairment. No recovery in fly climbing ability was observed in the process of proteasome recovery. These data imply that the long term damage to locomotor activity caused by temporary proteasome impairment could be related to the slow and inefficient clearance of protein aggregates. The irreversible aggregate formation could thus play a major role in the impairment of locomotor activity. In conclusion, our data clearly demonstrate that temporary proteasome disruption in flies, has severe, lasting consequences, including accumulation and aggregation of ubiquitinated proteins, and loss of locomotor activity. Interestingly, we previously established that a steep decline in ATP levels and proteasome activity observed in aged flies is closely related to a drastic decrease in locomotor performance [196].

Temporary disruption of proteasome activity also has an effect on lifespan. Survival curves indicated a major decline in fly survival upon transient impairment of proteasome activity. Moreover, proteasome recovery did not improve the fly survival rate. These results strongly supports that a short term disruption of proteasome activity causes an irreversible reduction in the lifespan of flies.

In summary, we establish a fly model with an overall tunable proteasome using the Gene-Switch system. Our data demonstrate that a short term disruption of proteasome activity has severe and lasting effects on the fly. These effects include (1) accumulation and aggregation of ubiquitinated proteins, (2) increased sensitivity to oxidative stress, (3) loss of locomotor activity, and finally (4) a reduction in lifespan, Upon proteasome recovery, flies were not able to (1) remove the ubiquitinated protein conjugates and aggregates previously formed, (2) recover locomotor activity, and (3) improve the

survival rate. From a protein degradation perspective, our data show that (1) accumulation and aggregation of ubiquitinated proteins result from short term proteasome impairment and (2) autophagy could not effectively eliminate the protein aggregates. It is not clear whether the protein aggregates could be cleared if flies survived longer after proteasome recovery, and whether survival and locomotor activity would fully recover upon removal of the protein aggregates.

In conclusion, our transgenic fly model demonstrates that a short term disruption in proteasome activity has a long term and possibly irreversible negative impact on flies. This finding has important clinical implications as it suggests that temporary impairment of the proteasome in humans might also cause long term damages. Increasing evidence supports that oxidative stress not only impairs ubiquitination/deubiquitination, but also directly damages UPS components [163, 237]. In addition, several transient conditions including bacterial and viral infections [164, 238], inflammation, ischemia/reperfusion [165-168], sport injuries and exposure to environmental toxins can induce temporary proteasomal disruption which will have a long term impact on health. Preventing or reducing temporal proteasome disruption could thus be a very appealing therapeutical strategy to lessen the long term impact of protein aggregation and promote human health.

CHAPTER 5

MATERIALS AND METHODS

5.1. GENERATION OF dβ5 RNAi TRANSGENIC LINES

A transgenic line with dβ5 deficiency was obtained by using an inducible RNAi method. In order to knock down the dβ5 gene, the dβ5 RNAi constructs were induced and expressed endogenously, thus leading to the formation of hairpin-loop structured RNA to trigger the drosophila RNAi mechanism. To create the RNA interference (RNAi) construct of dβ5, a drosophila dβ5 cDNA fragment and a genomic DNA fragment were ligated together via an EcoRI site in the pBluescript II SK(+) plasmid. Each fragment contains the drosophila dβ5 sequence in forward and reverse orientation separated by a spacer. The vector was purified by using the Qiagen MaxiPrep Kit (Qiagen, Valencia, CA) and sequenced to confirm the correct RNAi construct. After digesting the SacII-KpnI sites of this construct, the resulting fragment contains the drosophila dβ5 RNAi construct. This RNAi construct was cloned into the pUAST vector, a P-element transformation vector, by a directional SacII-KpnI two-way ligation. The pUAST vector containing the RNAi construct was sent to BestGene Inc. for drosophila embryo injection, and the transgenic line with the genotype UAS-dβ5 RNAi /CyO, was generated. We used the Gene-Switch system Act5C driver (Act5C-GS) line to ubiquitiously express dβ5 RNAi in flies. The Act5C-GS line was crossed with the UAS line expressing the dβ5 RNAi. Progeny expressing both the Act5C-GS and UAS-dβ5 RNAi constructs were used for analysis. Progeny with the genotype UAS-dβ5 RNAi/+; Act5C-GS/+ were collected at 1 to 2 days of age and analyzed for all experiments. All flies were maintained on a standard medium (Bloomington recipe, number 1) at 25℃

under a ~12 h/12 h light/dark cycle and 60% relative humidity. Flies were transferred to new vials with fresh food every 5-6 days.

5.2. RU486 ADMINISTRATION

We used 200 µM RU486 (Mifepristone, Sigma, M8046) for all experiments except for establishing the RU486 dose-dependent survival curve. RU486 was dissolved in ethanol (Acros Organics, 61509-0010). To feed the flies, RU486 was mixed with fly food (Carolina, Formula 4-24) and water to get the final concentration of 200 µM. The final ethanol concentration in the fly food was 4%. Adult 1 to 2 day-old flies were starved for 12 h in empty vials with filter paper prewetted with water. Following starvation, flies were transferred to new vials with fly food containing 200 µM RU486 or vehicle (4% ethanol, control). Flies were transferred to new vials with fresh food every 5 days.

5.3. SURVIVAL CURVE

30 flies per time point were monitored for survival. The numbers of dead flies were counted daily and three trials were performed for each time point.

5.4. PROTEASOME dβ5 SUBUNIT AND UBIQUITINATED PROTEINS

30 flies were harvested in 150 µl homogenization buffer containing 20 mM Tris-HCl, pH 7.5, 137 mM sodium chloride, 1 mM EGTA, 10% glycerol, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM β-

glycerophosphate, 2.5 mM sodium pyrophosphate ($Na_4P_2O_7$), 50 mM sodium fluoride (NaF), 1% Nonidet P-40 (NP40)/ Igepal (Sigma, I3021), and protease inhibitor cocktail (Sigma, P8340). Flies were homogenized on ice with a Teflon pestle (60 up and down strokes). Following a brief sonication and centrifugation (19,000 X *g*, 15 min at 4°C), cleared supernatants were transferred to new microcentrifuge tubes and protein concentration was determined with the bicinchoninic acid (BCA) (Thermo Scientific, 23227). Prior to boiling at 95°C for 5 min, cleared supernatants (90 µg protein/sample) were mixed with an equal volume with 2X Laemmli buffer and loaded onto a 12% SDS gel. The dβ5 protein level was determined with our own anti-dβ5 antibody (1:4000, generated by Bio-Synthesis, TX). Anti-ubiquitin antibodies (1:1500, Dako, Z0458) were used to determine the level of ubiquitinated proteins. Equal protein loading per well was demonstrated by probing immunoblots with anti-actin antibodies (1:3000, Sigma, A2066). Following incubation with the secondary antibody, protein levels were detected by standard chemiluminescent horseradish peroxidase method with ECL reagent.

5.5. IN-GEL PROTEASOME ACTIVITY AND DETECTION

30 flies were harvested in 150 µl Buffer A (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 10% glycerol) with 5 mM ATP (adenosine 5'-triphosphate, Sigma, A2383), and 1 mM DTT (DL-dithioththreitol, Sigma, D0632). Flies were homogenized with 60 up and down strokes on the homogenizer (Caframo, Wiarton, Ontario, Canada) with plastic knob on the line for 125-800. Following centrifugation (19,000 X *g*, 15 min at 4°C), cleared supernatants were transferred to new microcentrifuge tubes to determine protein concentration with the Bradford assay (Bio-Rad, 5000006). Bromophenol blue was added

to the cleared supernatant (90 µg protein/sample) before loading. A three-step gradient gel with (from bottom up) 5%, 4%, and 3% polyacrylamide containing Rhinohide polyacrylamide strengthener (Invitrogen, R33400) was used to assess proteasome activity and levels [196].

The non-denaturing gels were run at 150 V for 3 h at 4°C in the cold room. To detect proteasome chymotrypsin-like activity, following electrophoresis the gel was incubated with 15 ml of 0.4 mM Suc-LLVY-AMC (Enzo, BML-P802) in Buffer A with 1 mM ATP and 1 mM DTT at room temperature. Proteasome bands were visualized upon exposure to ultraviolet light (360 nM) via a UV transilluminator (Fotodyne, I-1430) and photographed with a Nikon Cool Pix 8700 camera with a 3–4219 fluorescent green filter (Peca Products, Beloit, WI, USA). Semi-quantitative analysis of the bands corresponding to proteasome activity was performed by image analysis with the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997–2006)

Subsequently, proteins on the native gel were transferred to a PVDF membrane (Millipore, IPVH 00010) at 110 mA for 2 h in the cold-room. Proteins were identified by immunobloting probed with specific antibodies. Probing with the anti-dβ5 antibody (1:4000; BioSynthesis, College Station, TX, USA) detected 26S and 20S proteasome levels because dβ5 is a subunit of the protesome core particle. Following incubation with the secondary antibody, proteasome levels were detected by a standard chemiluminescent horseradish peroxidase method with the ECL reagent.

Equal protein loading was established with an aliquot of the same samples following 10% SDS-PAGE and actin immunoblotting with the anti-actin antibody (1:3000, Sigma, A2066).

5.6. LOCOMOTOR ACTIVITY

We determined the locomotor activity of flies by using a climbing assay (negative geotaxis assay) [196]. 30 flies per time point were anesthetized and placed in a 100 ml graduated cylinder (length, 23.5cm; diameter, 3cm) marked with a line at 12.5 cm. After a 15 min recovery from CO_2 exposure, flies were gently tapped to the bottom of the graduated cylinder. The percentage of flies that could climb above the 12.5 cm line on the graduated cylinder after 20 s of climbing were calculated. Three trials were performed for each time point.

5.7. OXIDATIVE STRESS

Up to 30 adult flies (1 to 2 day-old) were collect and then transferred to polystyrene vials (diameter 24 mm, length 93 mm) closed by a cotton plug. Oxidative stress was induced by treatment with 1% hydrogen peroxide (H_2O_2). Each vial contains 10 ml of fly food with either (1) 200 µM RU486, or (2) 1 % H_2O_2 or (3) 200 µM RU486 + 1 % H_2O_2 diluted from 30% (w/w) H_2O_2 (Sigma, H1009). The vials were changed every 3 days and dead flies were removed from these vials. Three trials were performed. Lifespan was assessed by counting the number of flies that survived.

5.8. TEMPORAL DISRUPTION OF PROTEASOME ACTIVITY

Adult 1 to 2 day-old flies were starved for 12h in empty vials with filter paper prewetted with water. For the control group, following starvation, flies were transferred to new vials with fly food containing 4% ethanol. In one experimental group, which was the recovery group, following starvation and transfer to new vials with fly food containing 200 µM RU486 for 5 days, at day 6 the flies were transferred again but this time to fly food containing 4% ethanol and no RU486.

5.9. REAL-TIME PCR

Fly total RNA was extracted with RNeasy Mini Kit (Qiagen, 74104). Total RNA (2.5 µg in a 25 µl final volume) from each sample was reverse-transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368813) using random primers. To quantify the dβ5 mRNA level, real-time PCR was run with the TaqMan Gene Expression Assay (Applied Biosystems, Dm01812920_g1) and two sequence-specific PCR primers analyzed with the 7500 Fast Real-Time PCR System (Applied Biosystems). 3 µl of total cDNA in a 25 µl final volume was used for each assay. All samples were analyzed in duplicate. Each assay was run with 26 Universal Master Mix at universal cycling conditions (10 min at 95°C; 15 s at 95°C and 1 min at 60°C, 40 cycles). The pre-designed drosophila ribosomal protein L32 (RpL32) (Applied Biosystems, Dm02151827_g1) was used with the TaqMan Gene Expression Assay as the endogenous control. The amount of dβ5 mRNA was normalized by subtracting the threshold (ΔC_T) of RpL32 mRNA. We use normalized data to quantify the relative level of dβ5 mRNA

according to cycling threshold (ΔC_T) analysis. The relative dβ5 mRNA level expression for each sample was calculated as $2^{(-\Delta CT)}$.

5.10. FILTER TRAP ASSAY

30 flies were harvested with 1% SDS, and 10 mM Tris EDTA, pH7.5. Flies were homogenized on ice with a Teflon pestle (60 up and down strokes). Following heating at 95° for 5 min, a brief sonication and centrifugation (19,000 X *g*, 15 min at 4°C), cleared supernatants were transferred to new microcentrifuge tubes and protein concentration was determined with the bicinchoninic acid (BCA) (Thermo Scientific, 23227). An aliquot of each sample was normalized to the final concentration of 0.5 µg/µl by adding 2% SDS, and 10 mM Tris EDTA, pH7.5. 100 µl of each sample was vacuum-filtered through a 96-well dot blot apparatus (Minifold, Schleicher & Schuell SRC-96/0) with a 0.2 um nitrocellulose membrane (Biorad, 162-0097) prewetted with 2% SDS and 10 mM Tris EDTA, pH7.5. Each well was washed twice with a buffer containing 0.1% SDS, and 10 mM Tris EDTA, pH7.5. To detect ubiquitinated protein aggregates, the membrane was immunoblotted with anti-ubiquitin antibodies (1:1500, Dako, Z0458).

5.11. STATISTICAL ANALYSES

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

CHAPTER 6

FUTURE STUDIES

6.1. FUTURE DIRECTIONS

The ubiquitin-proteasome sytem is responsible for the degradation of the majority of intracellular proteins [239]. However, recent reports suggest that impairment of proteasome activity can induce oxidative stress and mitochondrial damage [240, 241]. In addition, proteasome inhibitors were shown to induce ROS generation, glutathione depletion, caspase activation, mitochondrial dysfunction, and apoptosis [102, 242-247]. Moreover, recent evidence suggests that mitochondrial impairment induced by proteasome inhibition plays an important role in the degeneration of neuronal cells [131, 147, 248]. Our data indicated that temporary disruption of proteasome activity causes long term and negative effects including accumulation and aggregation of ubiquitinated proteins, impaired locomotor activity and a shorter lifespan. Moreover, the negative effects of proteasome disruption persisted even when proteasome activity was restored. The irreversible effects of a temporary disruption of proteasome activity could be related to persistent factors including not only high levels of protein aggregates, but also mitochondrial dysfunction causing a buildup of ROS, and activation of mitochondrial-dependent apoptosis. Future studies should investigate whether oxidative stress and mitochondrial impairment are associated with short term proteasome dysfunction and persist even when proteasome activity is recovered. ROS overproduction and mitochondrial impairment could thus contribute to the observed short lifespan in the flies.

We propose that upregulation of antioxidant mechanism and/or activation of autophagy may rescue the irreversible negative effects induced by the temporary disruption of proteasome activity, by preventing ROS generation and facilitating the removal of protein aggregates, respectively.

6.2. EXPERIMENTAL DESIGN

6.2.1. Determine whether temporary disruption of proteasome activity induces overproduction of ROS and impairs mitochondrial function

To determine if a short term disruption of proteasome activity elevates ROS, the fluorescent dye 2,7-dichlorofluorescein-diacetate (H2DCF-DA), (Molecular Probes) will be used to assess ROS levels in flies [249]. Alterations in mitochondrial function will be assessed by measuring changes in mitochondrial membrane potential upon isolation of mitochondria from flies. Loss of mitochondrial membrane potential will be assessed by labeling mitochondria with the fluorescent lipophilic cationic dye tetramethylrhodamine methyl ester (TMRM) (Molecular Probes) [250]. This dye enters mitochondria with an intact transmembrane potential and its release serves as an indicator of mitochondrial depolarization. Mitochondrial function can also be evaluated by measuring mitochondrial respiration rates with a Clark-type oxygen electrode [250, 251]. Since mitochondria are the major intracellular source of ATP, measuring steady-state ATP level can be an indicator of mitochondrial function as well. ATP level can be assessed with a kit that uses the sensitive luciferin/ luciferase system (Invitrogen-Molecular Probes, Carlsbad, CA, USA).

6.2.2. Establish if temporal disruption of proteasome activity induces mitochondrial dependent apoptosis

Induction of mitochondrial dependent apoptosis is accompanied by mitochondrial outer membrane permeabilization (MOMP) that causes the release of cytochrome c from

the mitochondria into the cytoplasm. Once in the cytoplasm, cytochrome c triggers apoptosis by activating caspase 9 and 3 [252, 253]. To determine if mitochondrial dependent apoptosis is activated upon proteasome impairment, translocation of cytochrome c from the mitochondria to the cytoplasm will be evaluated by determining the loss of overlapping fluorescent signals emanating from a mitochondrion-selective dye, MitoTracker® Orange CMTMRos (Molecular Probes) and from immunofluorescence analysis with an anti-cytochrome c antibody. Release of cytochrome c from the mitochondria into the cytoplasm will be further confirmed by western blot analysis after mitochondrial and cytoplasmic fractions obtained from subcellular fractionation by differential centrifugation. Caspase activition in fly lysates will be measured with the fluorescence cellbased Apo-ONE Homogeneous Caspase Assays (Promega).

6.2.3. Determine whether high level of ROS, mitochondrial dysfunction and activation of mitochondrial dependent apoptosis persist over time after proteasome activity was recovered

To establish if a temporary disruption of proteasome activity in flies has a persistent effect on mitochondria function, flies will be fed RU486 for 5 days and then at day 6 RU486 will be withdrawn from fly food, as described under Materials and Methods. We previously establish that upon this feeding schedule proteasome activity will recover (**Fig. 31**). The level of ROS, mitochondrial function, and activation of mitochondrial dependent apoptosis will be evaluated as described above in 6.2.2.

6.2.4. Investigate if antioxidants maintain mitochondrial function and promote survival by inhibiting ROS production following short term proteasome disruption

How proteasome inhibition causes a dramatic overproduction of ROS in a time-dependent manner [254], is unclear. Mitochondria are both primary targets and sources of ROS. ROS damages mitochondrial DNA as well as lipids and proteins on the mitochondrial membrane. In addition, ROS increase mitochondrial membrane permeability via oxidative modification of mitochondrial lipids and channels which regulate mitochondrial permeabilization, such as the voltage-dependent anion channel (VDAC) and the adenine nucleotide translocase (ANT) [255-257]. If during oxidative phosphorylation mitochondria is impaired, ROS production is elevated initiating a vicious cycle [258]. ROS seem to be the initial signals that trigger activation of mitochondrial dependent apoptosis [254, 259]. Moreover, glutathione depletion correlates with mitochondrial dysfunction in response to a variety of stressful stimuli including proteasomal inhibition [260-262]. Based on these studies, we will investigate whether antioxidants decrease ROS production and attenuate the long-term and negative effects induced by the transient disruption of proteasome activity. Antioxidant levels in flies will be elevated by either (1) feeding flies directly with antioxidants such as N-acetyl-cysteine and/or alpha-lipoic acid, or (2) crossing transgenic flies with other transgenic lines that overexpress antioxidant proteins such as glutathione synthase or manganese superoxide dismutase. The protective effects of antioxidant will be evaluated by determining ROS levels, mitochondrial function, activation of apoptosis, and fly lifespan.

6.2.5. Investigate whether activation of autophagy facilitates the removal of ubiquitinated protein aggregates that accumulate upon temporary disruption of proteasome activity and improves fly lifespan

To pharmacologically activate autophagy, flies will be fed rapamycin or niclosamide, which are well established autophagy activators [219, 263]. To genetically activate autophagy, flies will be crossed with other transgenic lines overexpressing autophagy-specific genes (ATGs) such as ATG5 and ATG 7 [264]. Autophagy activation will be established by detecting the presence of autophagic vacuoles by transmission electron microscopy [265]. The levels of ubiquitinated protein aggregates will be measured with the filter trap assay, and lifespan will be determined as described under Materials and Methods.

6.3. EXPECTED OUTCOME

We expect that temporary disruption of proteasome activity causes the production of high levels of ROS, impairs mitochondria, and activates mitochondrial dependent apoptosis, in a persistant manner, thus shortening fly lifespan.

We also expect that administration of antioxidants and upregulation of antioxidant genes will protect flies from the transient disruption of proteasome, by scavenging excess ROS and promoting mitochondrial recovery.

Autophagy activation should facilitie the removal of ubiquitinated protein aggregates and extend fly longevity.

6.4. RELEVANCE

Genetic and biochemical models provide evidence for a link between mitochondrial dysfunction and the neurodegenerative process in AD, PD, Amyotrophic Lateral Sclerosis and Huntington's disease [266]. Our studies will address if a temporary dysregulation in proteasome mediated proteolysis induces oxidative stress and causes mitochondrial impairment thus leading to a shortening of the fly lifespan. The drosophila studies that we propose could identify new targets to prevent mitochondrial dysfunction induced by temporal disruption of proteasome activity that could be used to stop the progression of neurodegenerative disorders.

CHAPTER 7

FIGURES

Figure 1

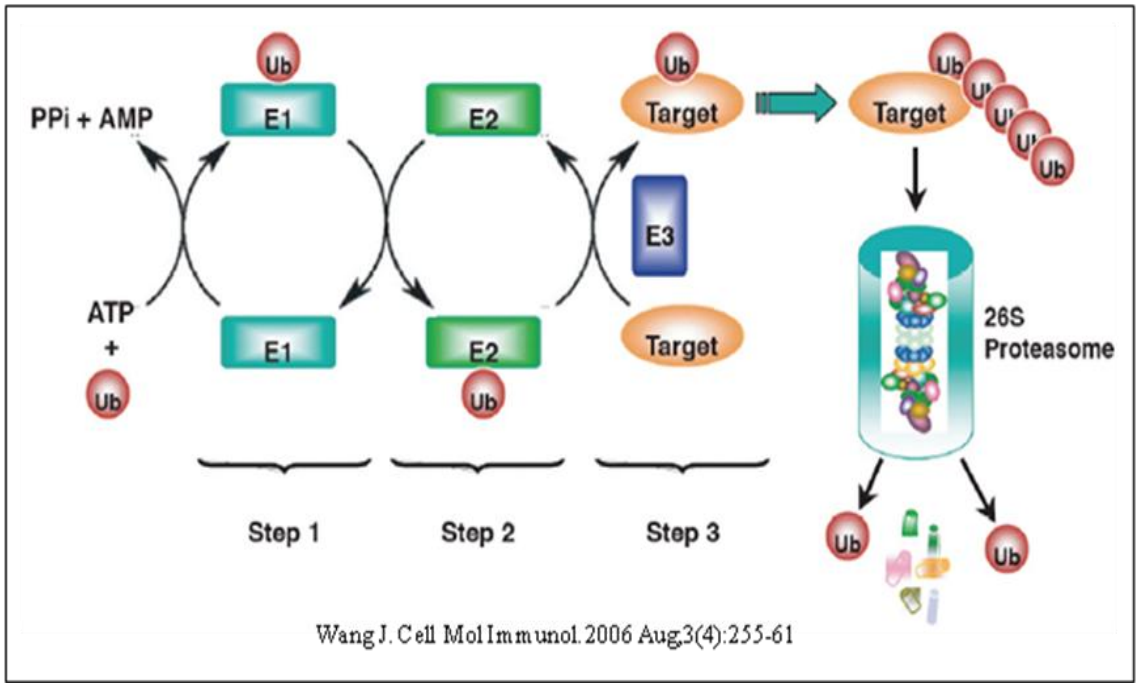

Fig. 1. Schematic of the ubiquitin-proteasome system (UPS). Ubiquitin (Ub) is added sequentially to target substrates in three sequential steps requiring enzymes E1, E2 and E3. Step 1: Ubiquination begins with the ATP-dependent activation of ubiquitin by the ubiquitin-activating enzyme E1; Step 2: Ubiquitin is then transferred to one of several forms of ubiquitin conjugating enzymes E2; Step 3: The addition of ubiquitin to the protein substrate is catalyzed by one of many E3s. Polyubiquinated proteins are recognized and degraded by the 26S proteasome. Proteasomal degradation produces small peptides and reusable free ubiquitin.

Figure 2

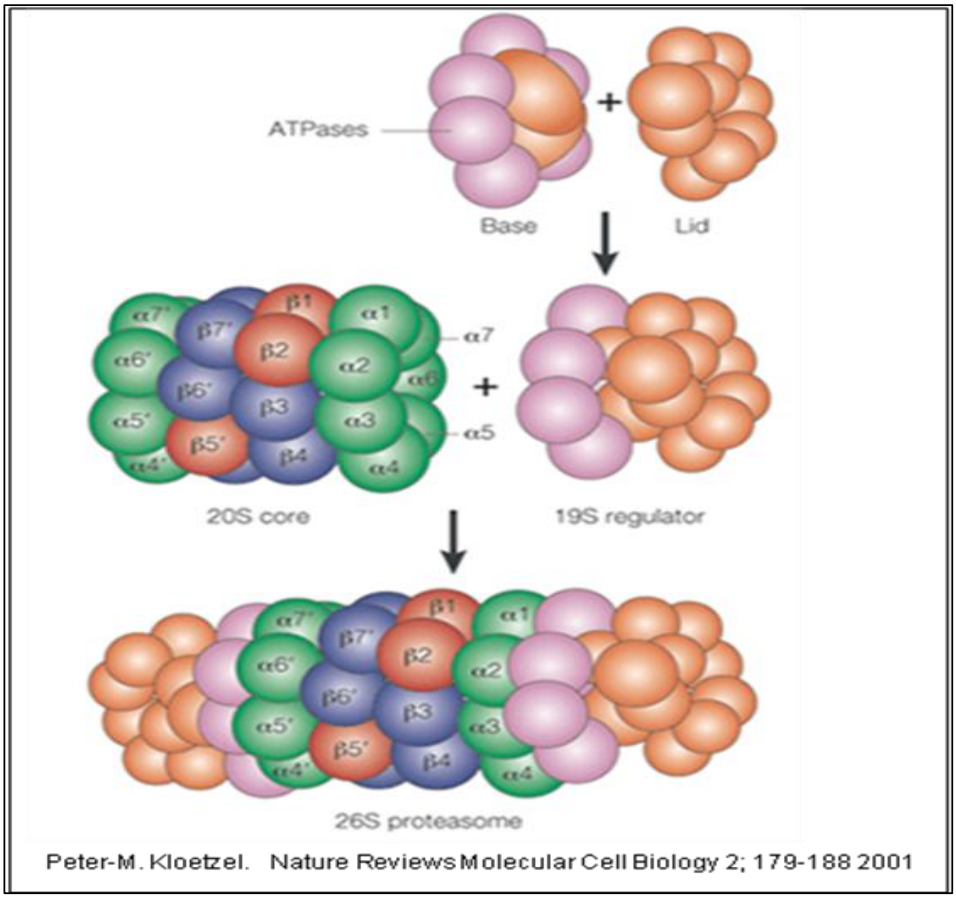

Fig. 2. The structure of the 26S proteasome. The 26S proteasome is composed of two distinct complexes: the 20S core, which constitutes the proteolytic component and the regulatory complex (RC) known as the 19S regulator. The 19S regulator contains ATPases (**base**), de-ubiquitinating enzyme and polyubiquitin-binding sites [181].

Figure 3

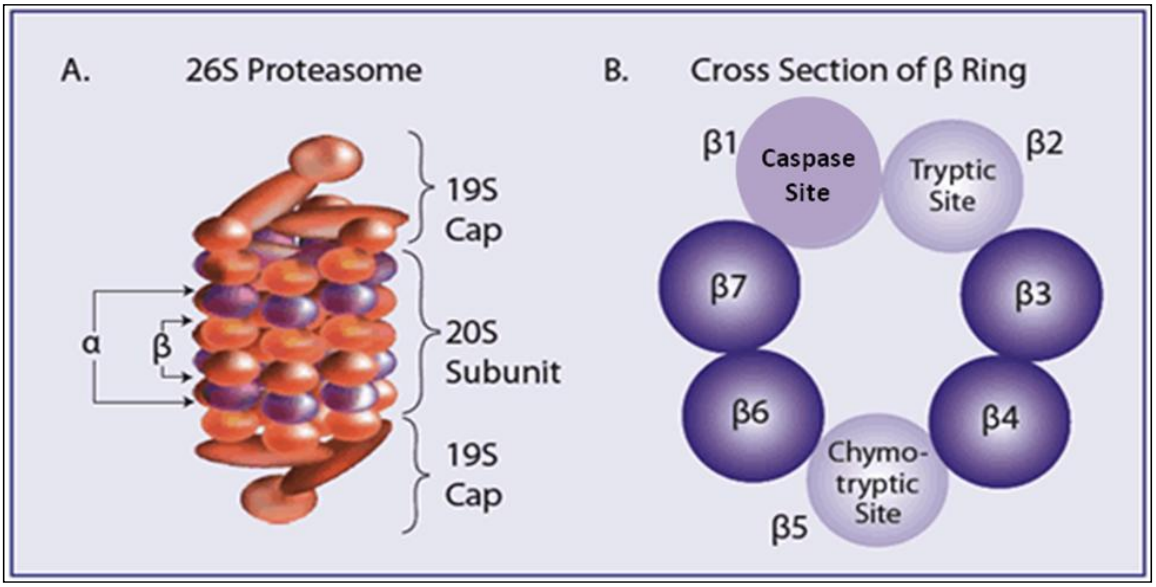

Fig. 3. The components of the 20S proteasome. The 20S proteasome is a barrel-shaped complex formed by the axial stacking of four rings made up of two outer α-rings and two inner β-rings, each consisting of seven structurally similar α- and β- subunits arranged in the following order: α7β7β7α7. The three major proteolytical activities of 20S proteasomes are the caspase-like, trypsin-like and chymotrypsin-like activities that are associated with the three subunits β1, β2 and β5, respectively.

Figure 4

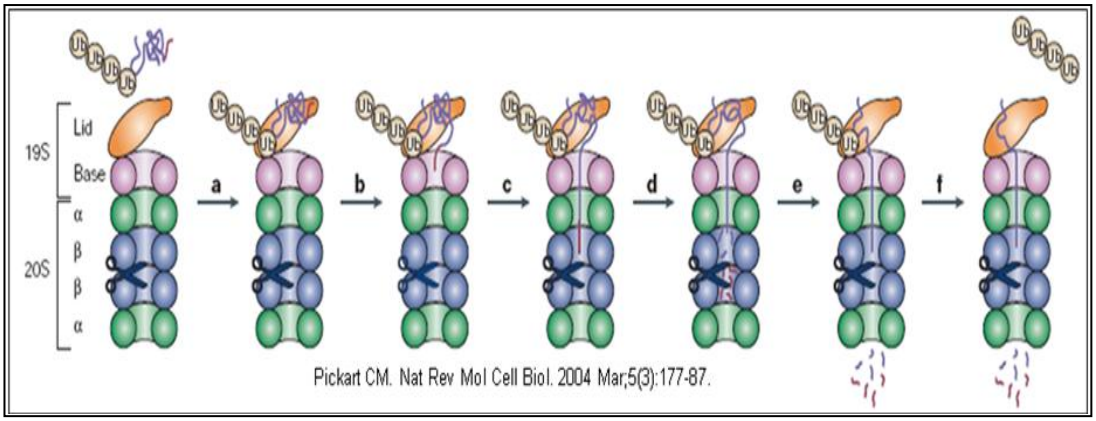

Fig.4. Steps in substrate proteolysis by 26S proteasomes. a: The substrate-linked polyubiquitin chain is recognized by 19S subunits. b: substrate translocates through a (presumptive) pore in the base subcomplex. Translocation of the substrate through the pore is driven by ATP hydrolysis. c: The substrate polypeptide transits to the juxtaposed axial pore of the 20S complex. d: The substrate is hydrolysed, which produces short peptides. e: The peptides exit the catalytic chamber through the axial pore. f: Following substrate degradation, deubiquitinating enzymes disassemble polyubiquitin chains.

Figure 5

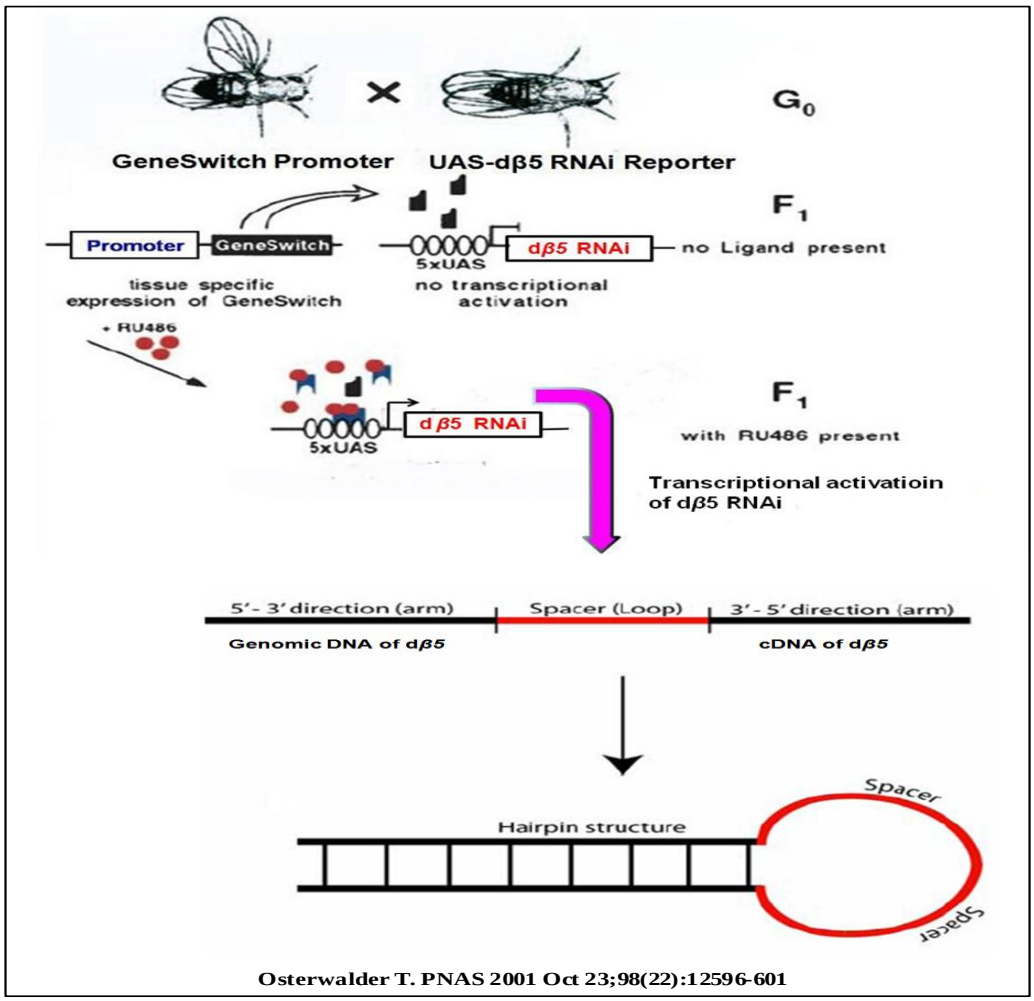

Fig. 5. The Gene-Switch/UAS expression system in drosophila. Two separate constructs were made, one carrying the Geneswitch gene under the control of the promotor, and the other carrying dβ5-RNAi under the control of the UAS. The GeneSwitch promoter lines will be crossed to UAS-dβ5 RNAi lines. Promoters drive the expression of the transcriptional activator GeneSwitch in a tissue-specific fashion. In the absence of ligand RU486, the GeneSwitch protein is expressed in target tissues but remains transcriptionally silent [152]; dβ5-RNAi is therefore not expressed. In the presence of *R*U486 (red), the GeneSwitch protein becomes transcriptionally active (blue), mediating expression of dβ5-RNAi in target tissues expressing GeneSwitch. Transcription of the dβ5-RNAi generates mRNA containing the dβ5 sequence in forward and reverse orientation separated by a spacer. The fold back of two closely positioned complementary sequences from a single-stranded mRNA molecule of dβ5 will result in a hairpin structured RNA. This hairpin structured RNA induces the RNA interference mechanism to degrade the endogenous dβ5 mRNA of flies, and therefore knock down of the dβ5 protein causing a decline in proteasome activity.

Figure 6

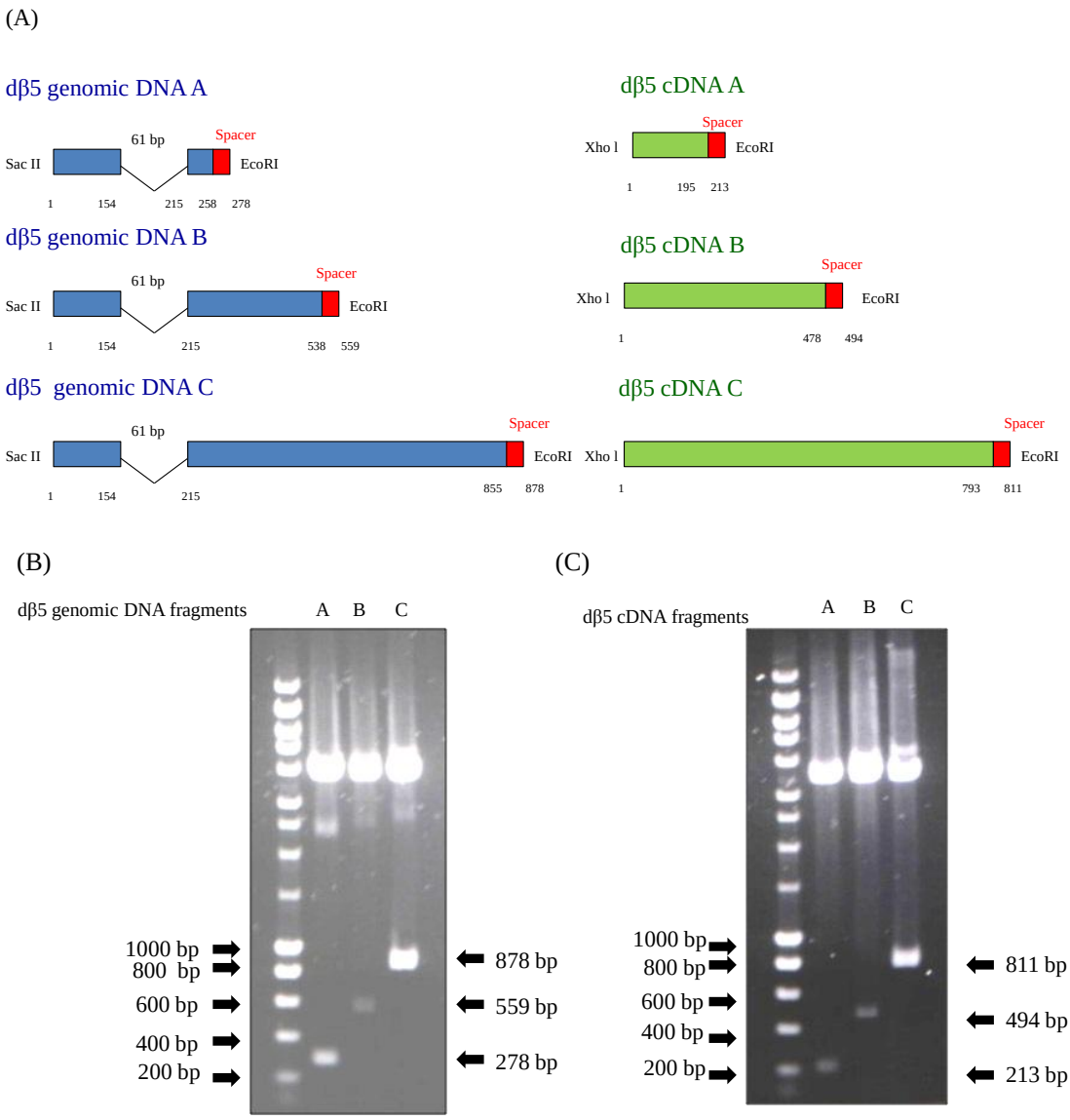

Fig. 6. Genomic DNA and cDNA fragments of the d*β*5 gene. Genomic DNA and cDNA fragments of the d*β*5 gene were generated by PCR (A). The genomic DNA and cDNA fragments were digested from the pTOPO2.1 vectors and run on 8% DNA agarose gels (B and C).

Figure 7A

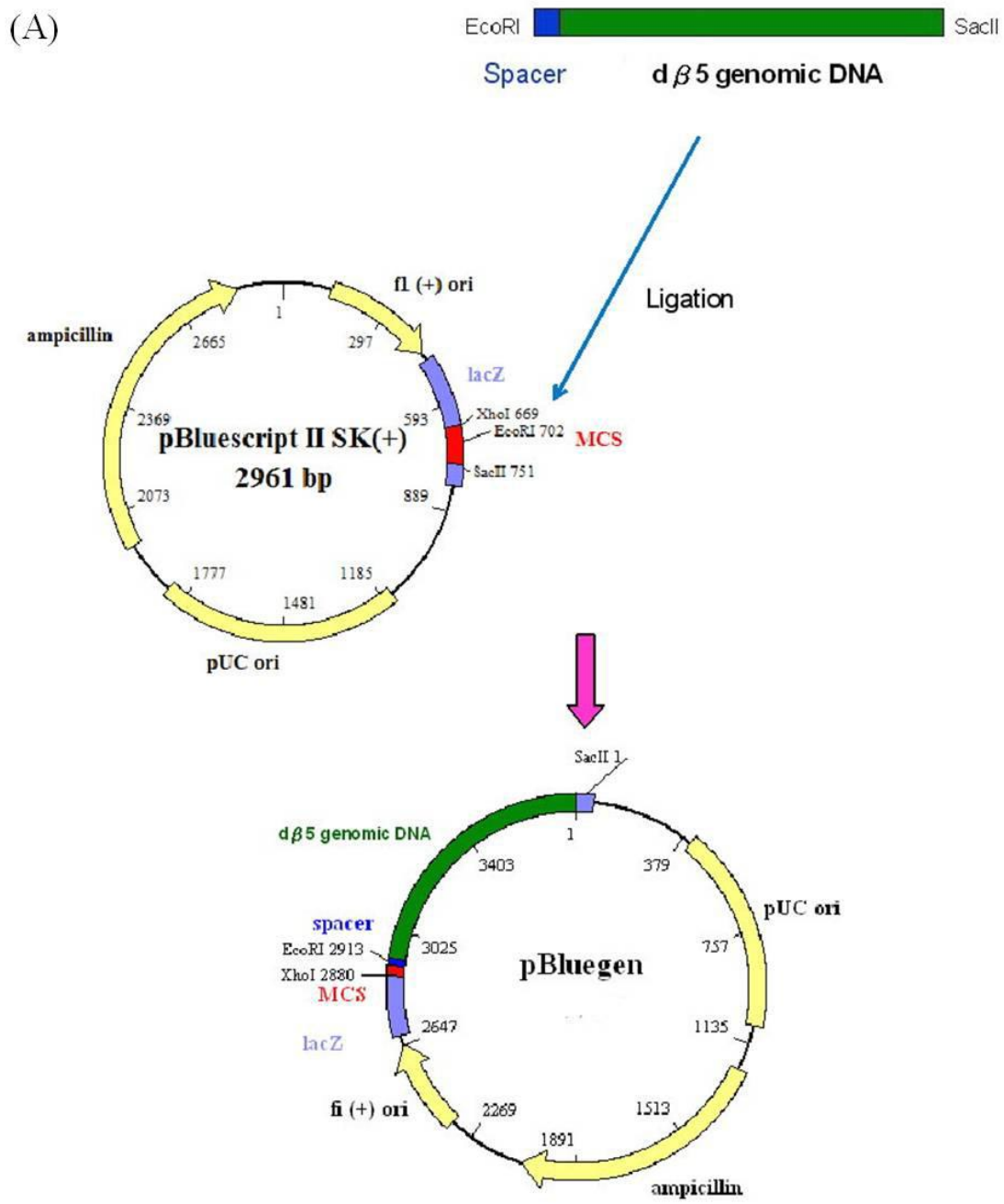
(A)
EcoRI
SacII
Spacer
dβ5 genomic DNA
Ligation
f1 (+) ori
ampicillin
lacZ
XhoI 669
EcoRI 702
MCS
SacII 751
pBluescript II SK(+)
2961 bp
pUC ori
SacII 1
dβ5 genomic DNA
spacer
EcoRI 2913
XhoI 2880
MCS
lacZ
pBluegen
pUC ori
fi (+) ori
ampicillin

Figure 7B

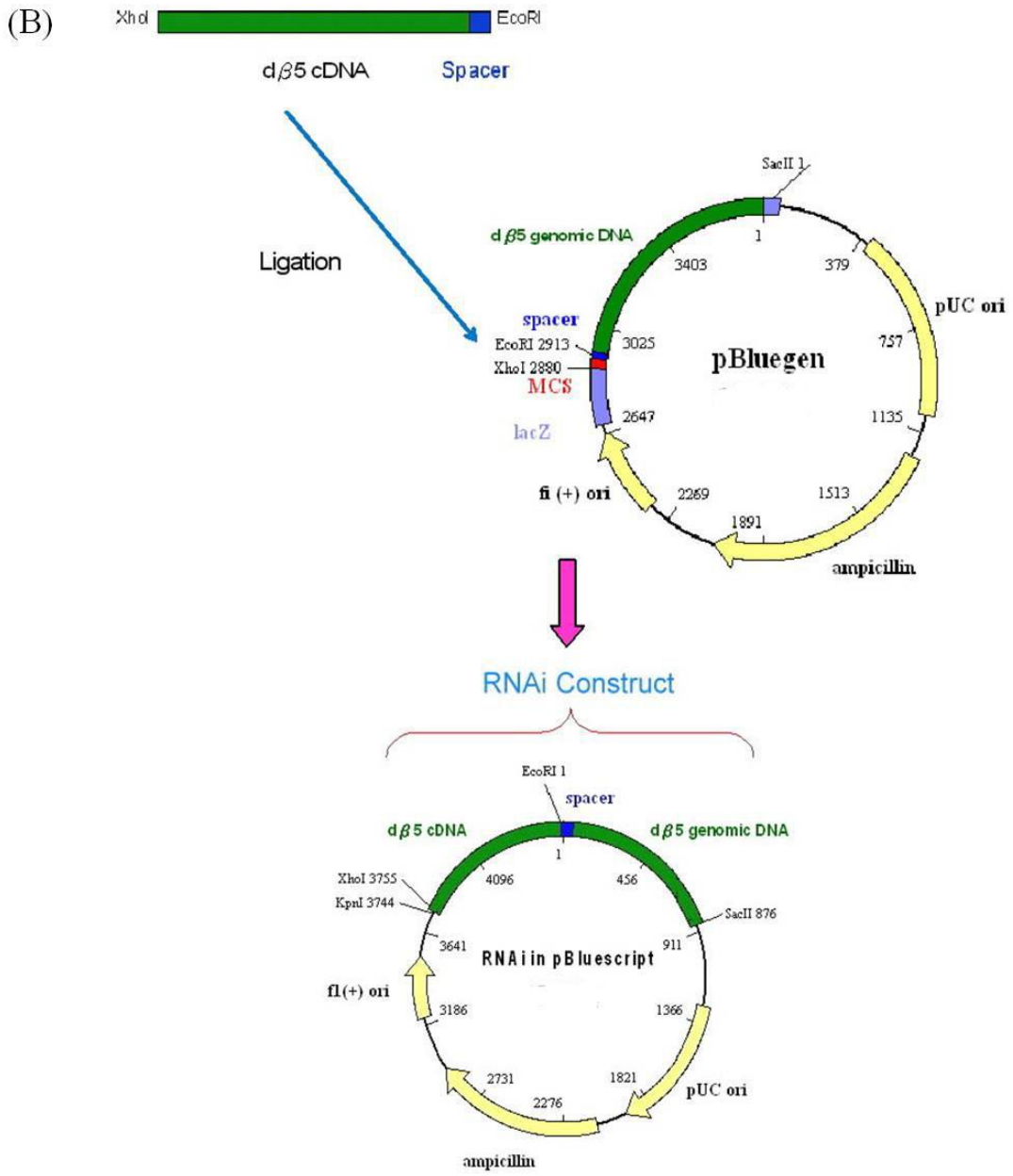
(B)
XhoI
EcoRI
dβ5 cDNA
Spacer
Ligation
SacII 1
dβ5 genomic DNA
1
3403
379
pUC ori
spacer
EcoRI 2913
XhoI 2880
MCS
3025
757
pBluegen
lacZ
2647
1135
fi (+) ori
2269
1513
1891
ampicillin
RNAi Construct
EcoRI 1
spacer
dβ5 cDNA
dβ5 genomic DNA
1
XhoI 3755
KpnI 3744
4096
456
SacII 876
3641
911
RNAi in pBluescript
fl(+) ori
3186
1366
2731
1821
2276
pUC ori
ampicillin

Figure 7C and 7D

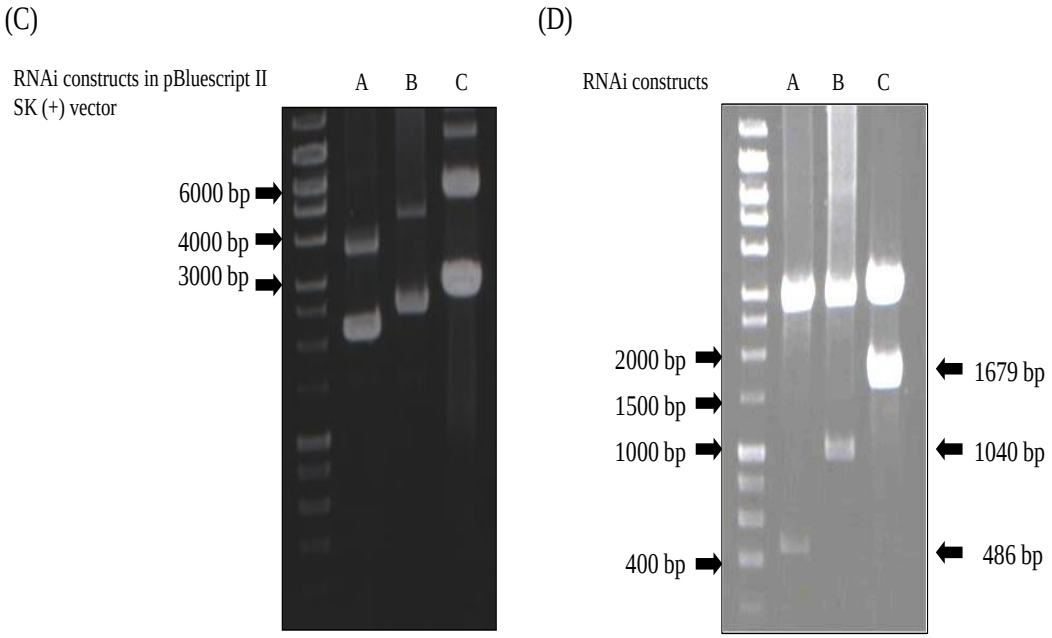

Fig. 7. Construction of the dβ5 RNAi. (A) The dβ5 genomic DNA fragment was cloned into the SacII-EcoRI site of a pBluescript II SK (+) plasmid. (B) The dβ5 cDNA fragment was isolated and cloned into the of EcoRI-XhoI site of the PblueGen vector producing a complete dβ5 RNAi construct in pBluescript II SK (+) plasmid. The pBluescript II SK (+) vectors containing the dβ5 RNAi constructs without (C**)** or with a sequential digestion (D) at SacII-XhoI were run on 8% DNA agarose gels.

Figure 8A

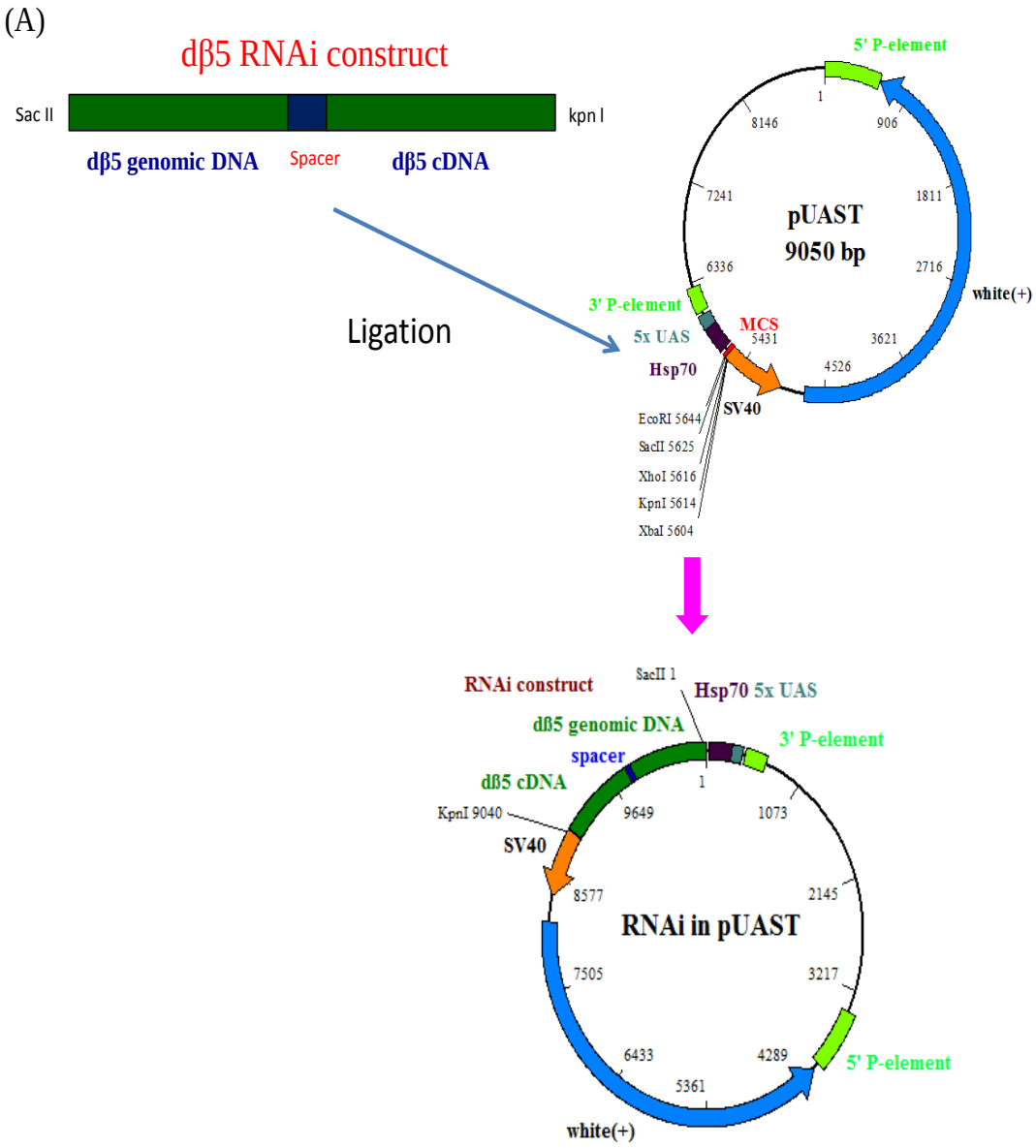
(A)
dβ5 RNAi construct
Sac II
kpn I
dβ5 genomic DNA
Spacer
dβ5 cDNA
Ligation
5' P-element
1
906
1811
2716
white(+)
3621
4526
SV40
EcoRI 5644
SacII 5625
XhoI 5616
KpnI 5614
XbaI 5604
5431
MCS
Hsp70
5x UAS
3' P-element
6336
7241
8146
pUAST
9050 bp
RNAi construct
SacII 1
Hsp70 5x UAS
dß5 genomic DNA
3' P-element
spacer
dß5 cDNA
1
1073
2145
3217
4289
5' P-element
5361
6433
white(+)
7505
8577
SV40
KpnI 9040
9649
RNAi in pUAST

Figure 8B and 8C

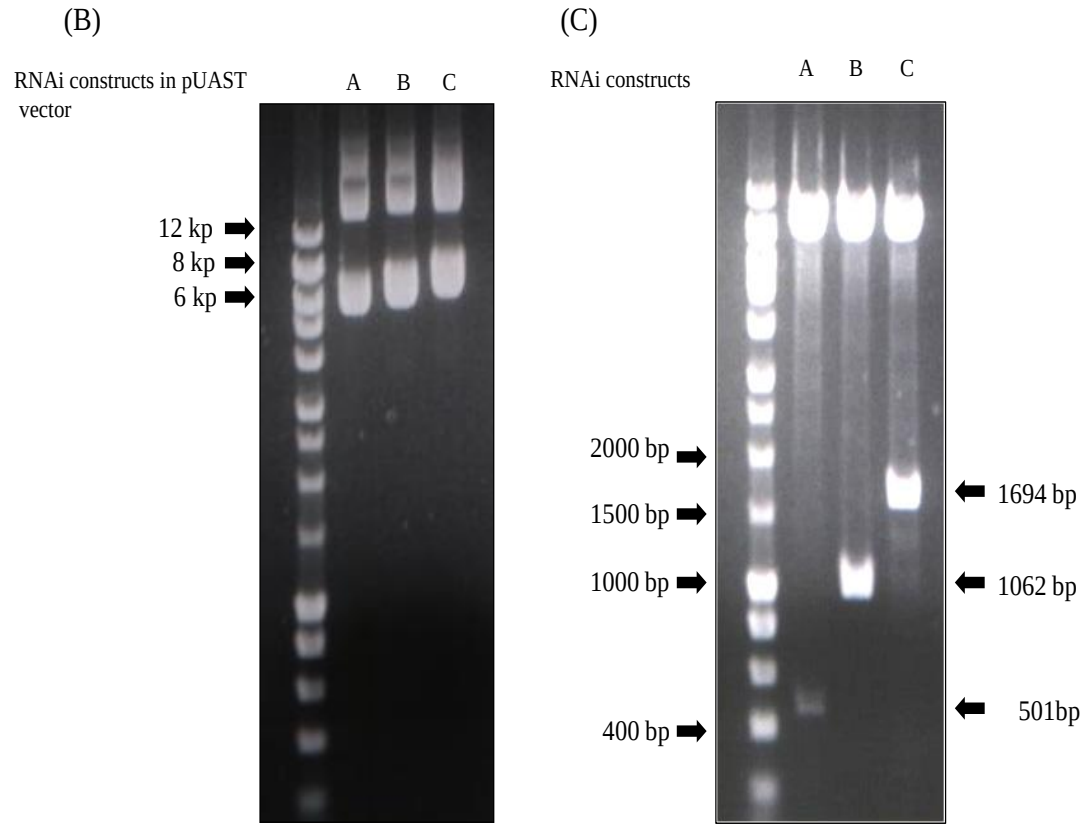

Fig. 8. The dβ5 RNAi construct was ligated into the pUAST vector. (A) The RNAi constructs were isolated from the pBluescript II SK (+) plasmid by KpnI-SacII digestion and ligated into the pUAST vector. The pUAST vectors containing the dβ5 RNAi constructs without (B) or with (D) a sequential digestion of SacII-KpnI were run on 8% DNA agarose gels

Figure 9

(A)

Genotype of the Act5C-GS driver line (Line 9431) from the Bloomington stock center:

P{hsFLP}, y w*; P{UAS-GFP}; P{Act5C-GAL4.Switch}
P{hsFLP}, y w* P{UAS-GFP} Tb

(B)

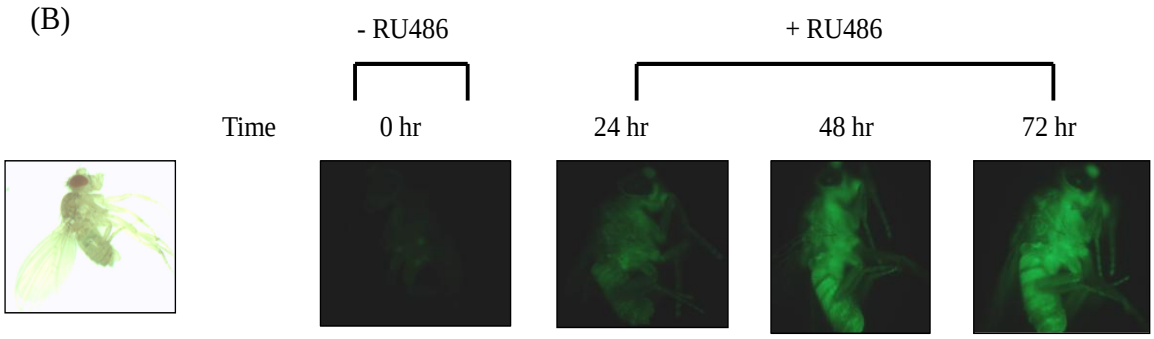

Fig. 9. GFP expression in the Act5C-GAL4.Switch driver line. Flies were starved for 6 h and then fed with 200 µM RU486**.** (A) Genotype of the Act5C-GAL4.Switch driver line. (B) The fluorescence microscopy (Nikon EFD-3) was used to visualize GFP expression in live flies anesthetized by CO_2 (image on right) and the corresponding visible light image is presented on the left. (Tb: tubby)

Figure 10

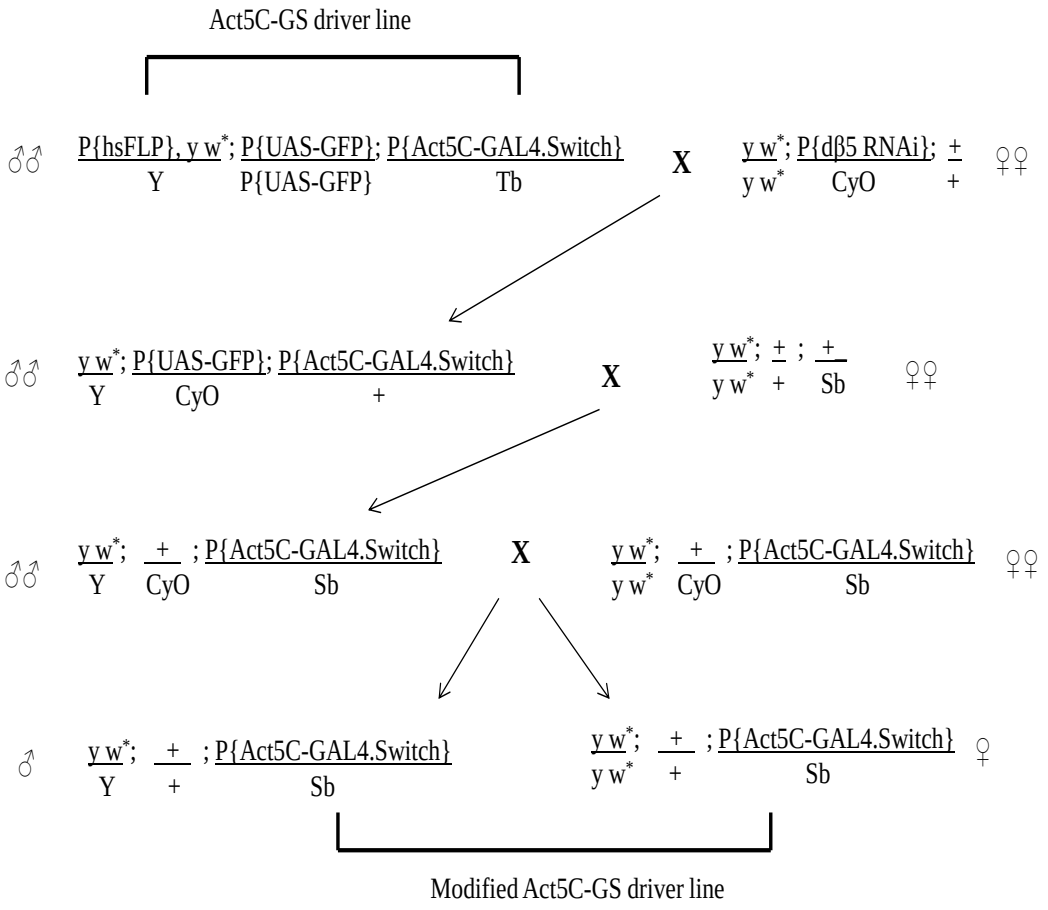

Fig. 10. Two inserts, hsFLP and UAS-GFP, were removed from the Act5C-GS driver line by a serial cross. Following a serial segregation, both hsFLP and UAS-GFP inserts were removed from the GS-Act5C driver line. (CyO: curly of Oster, Sb: stubble)

Figure 11

(A)

Genotype of the modified Act5C-GS driver line:

♂ $\frac{y\ w^{*}}{Y}$; $\frac{+}{+}$; $\frac{P\{Act5C\text{-}GAL4.Switch\}}{Sb}$

♀ $\frac{y\ w^{*}}{y\ w^{*}}$; $\frac{+}{+}$; $\frac{P\{Act5C\text{-}GAL4.Switch\}}{Sb}$

(B)

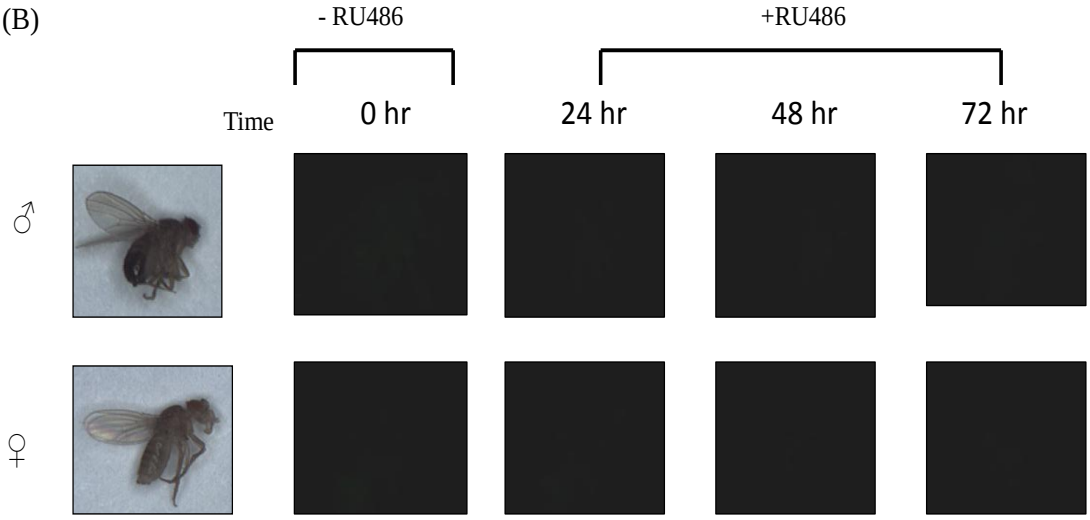

Fig. 11. No GFP expression is visible in the modified Act5C-GAL4.Switch driver line. Flies were starved for 6 h and then fed with 200 µM RU486**.** (A) Genotype of the modified Act5C-GAL4.Switch driver line. (B) The fluorescence microscopy was used to visualize GFP expression in live flies anesthetized with CO_2 image on right) and the corresponding visible light image is presented on the left. (Sb: stubble)

Figure 12

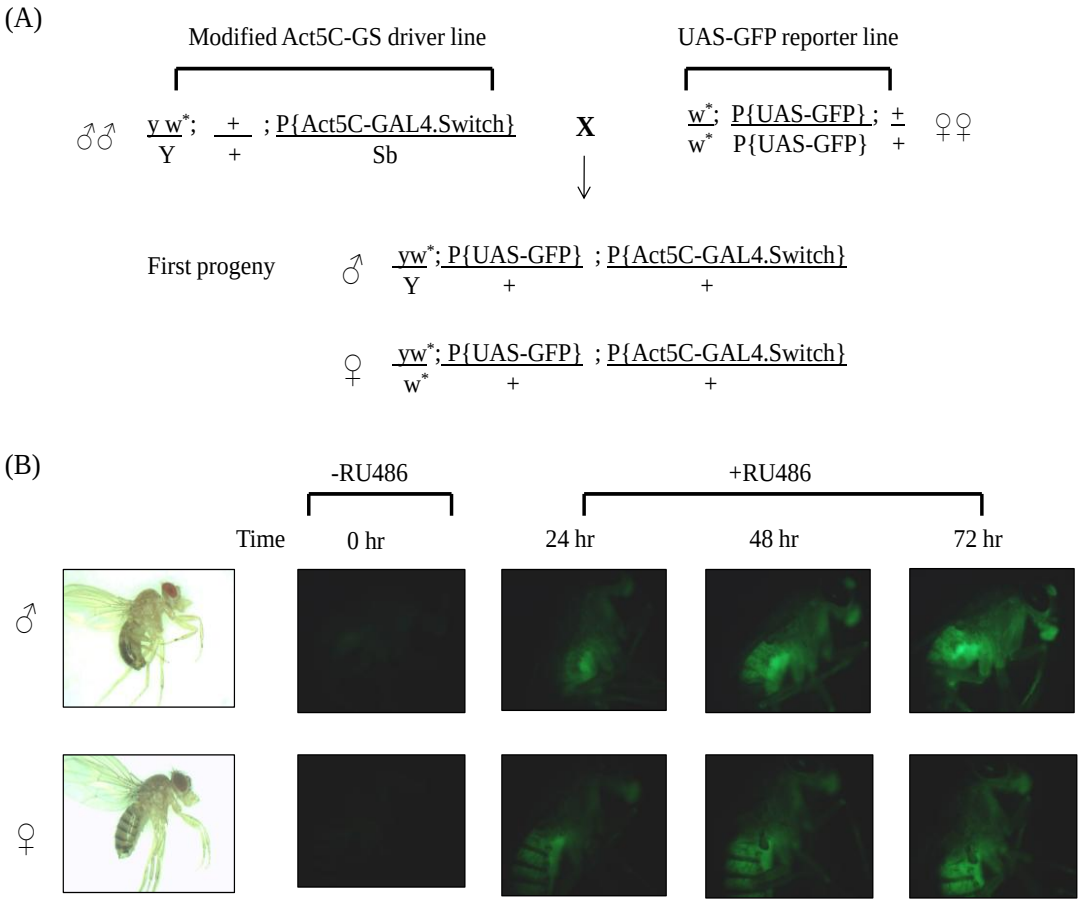

Fig. 12. The Act5C-GAL4.Switch construct is maintained in the modified Act5C-GAL4.Switch driver line. (A) The modified Act5C-GAL4.Switch driver line was crossed to the UAS-GFP reporter line. The first progeny were starved for 6 hr and then fed with 200 µM RU486**.** (B) Fluorescence microscopy was used to visualize GFP expression in the first progeny (image on right) and the corresponding visible light image is presented on the left. (Sb: stubble)

Figure 13

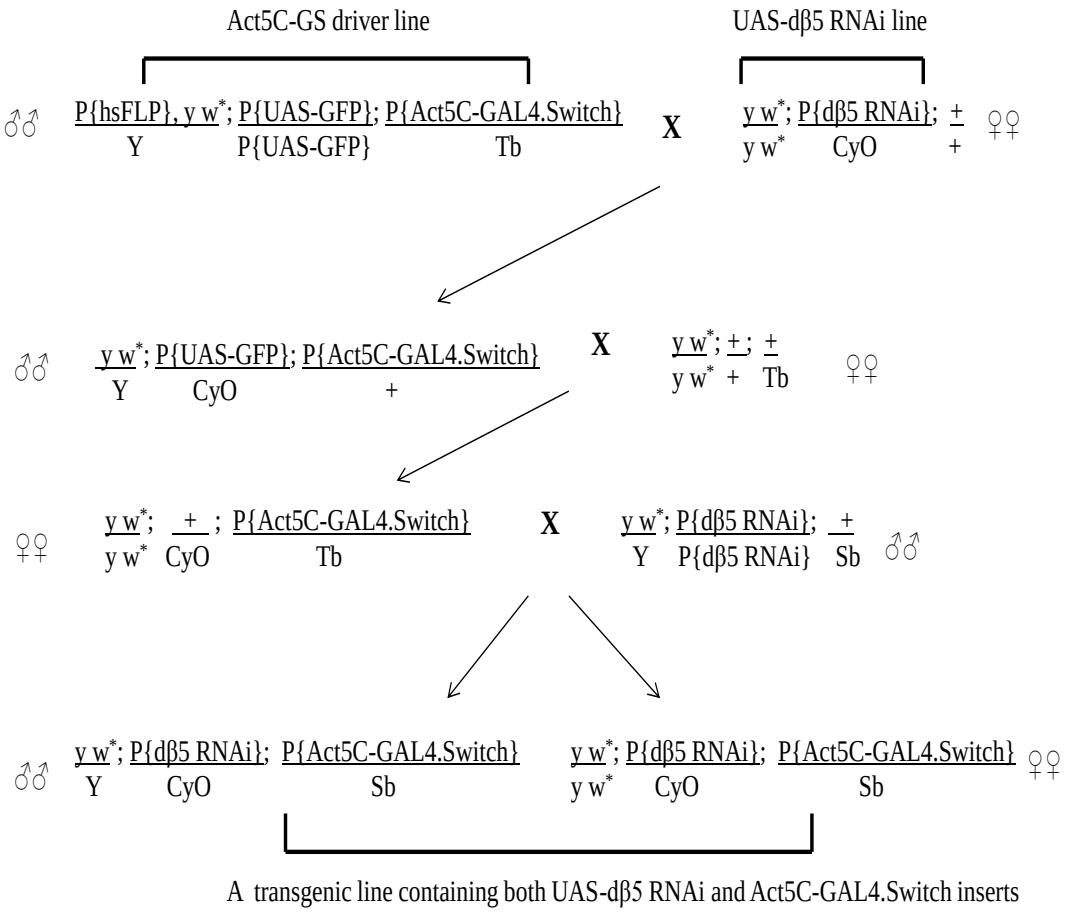

Fig. 13. Construction of a transgenic line containing both UAS-dβ5 RNAi and Act5C-GS inserts. Following a sequential cross, a new transgenic fly line containing both UAS-dβ5 RNAi and Act5C-GS driver was generated. The UAS-dβ5 RNAi and Act5C-GS inserts are located and balanced with different markers on the second and third chromosome, respectively. (CyO: curly of Oster, Sb: stubble, Tb: tubby)

Figure 14

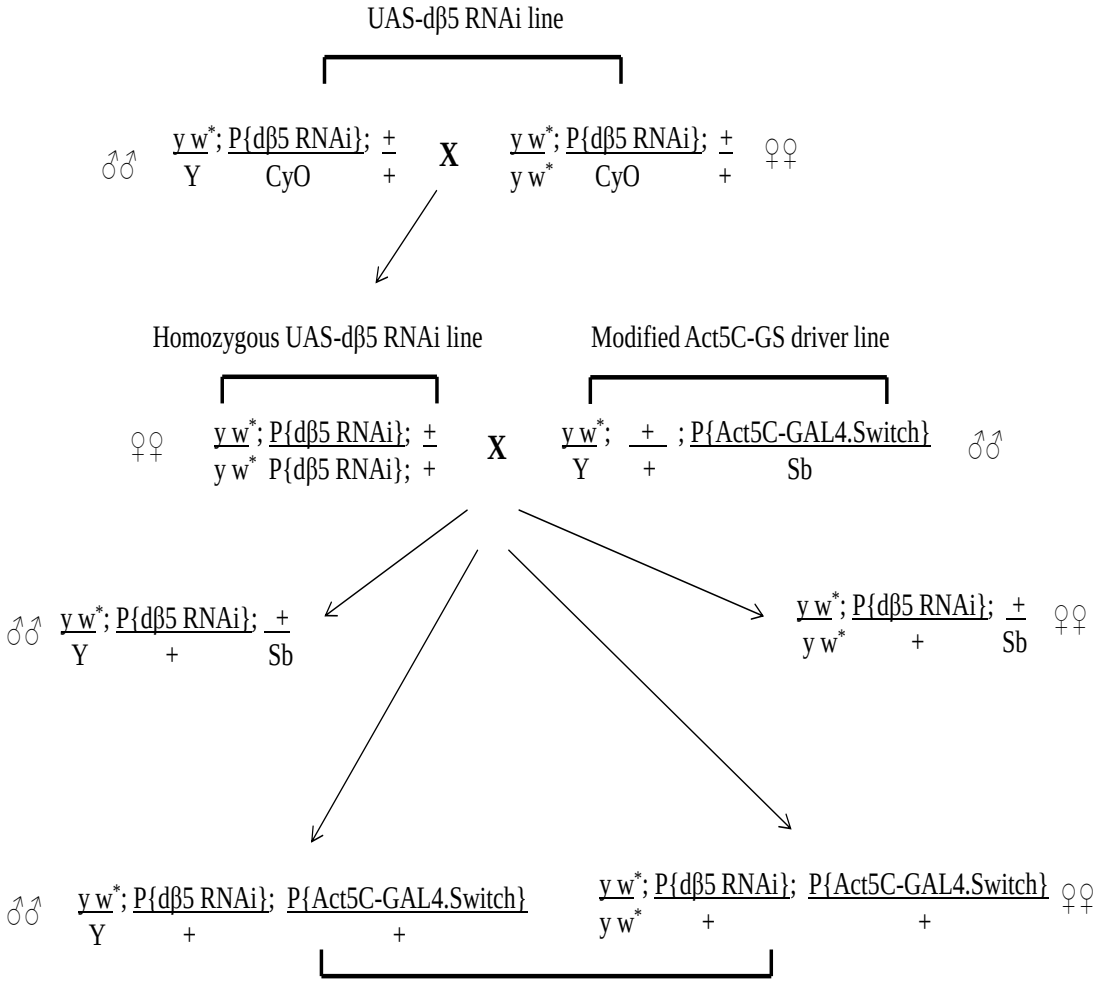

Fig. 14. UAS-dβ5 RNAi flies were crossed to modified Act5C-GS flies. Females of homozygous UAS-dβ5 RNAi flies were collected and crossed to male modified Act5C-GS flies. In the first progeny, flies containing both UAS-dβ5 RNAi and Act5C-GS inserts were screened and separated into males and females for subsequent experiments.

Figure 15

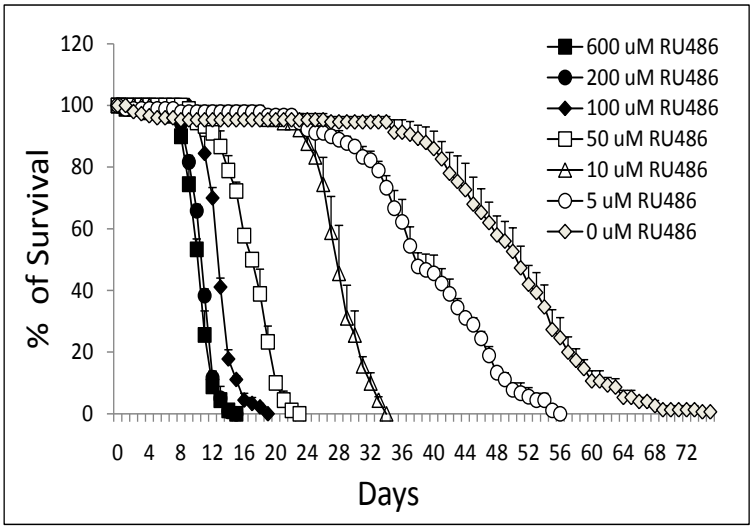

Fig. 15. RU486 administration shortened the lifespan of flies, in a dose-dependent manner. Male 1 to 2 day-old, UAS-dβ5 RNAi/+; Act5C-GS /+ flies were starved for 12 h and then fed with RU486 in varying concentrations (0, 5, 10, 50, 100, 200, and 600 µM) starting at day 0. Flies were transferred to new vials with fresh food every 6 days. The percentage of dead flies was calculated daily. Three trials were performed for each time point. Survival curves were generated. Data represent the mean ± sem of 3 experiments (n=30 flies for each treatment).

Table 1

RU486 concentration (μM)	lifespan (days)	Std
0	48.01	4.35
5	37.91	0.99
10	26.73	1.99
50	16.01	0.74
100	12.29	0.02
200	9.93	0.12
600	9.45	0.63

Table 1. The mean lifespan on flies fed with different concentrations of RU486. (Std: standard deviation)

Figure 16

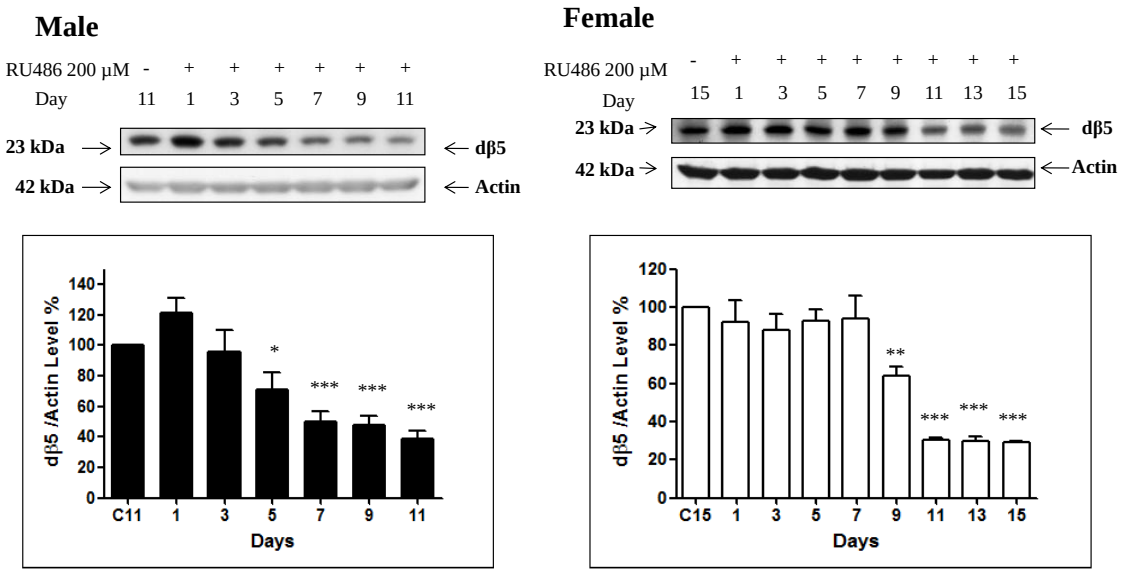

Fig. 16. Induction of d*β*5 RNAi knocked down dβ5 expression in a time-dependent manner. Separate groups of 1 to 2 day-old male and female UAS-d*β*5 RNAi/+; Act5C-GS/+ flies were starved for 12 h and then fed with 200 µM RU486 for varying periods of time. Males were fed with 200 µM RU486 for 1, 3, 5, 7, 9, and 11 days or 4% ethanol (EtOH, vehicle, control, c11) for 11 days. Females were fed with 200 µM RU486 for 1, 3, 5, 7, 9, 11, 13, and 15 days or 4% EtOH (vehicle, control, c15) for 15 days. The protein expression of dβ5 was analyzed by Western blotting (90 µg protein/sample) probed with an anti-dβ5 antibody. Equal protein loading was demonstrated by probing immunoblots with an anti-actin antibody. The level of dβ5/actin was semi-quantified by densitometry. Data represent the mean ± sem of 3 experiments. * p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001. Statistical significance was assessed by one-way analysis of variance (ANOVA).

Figure 17

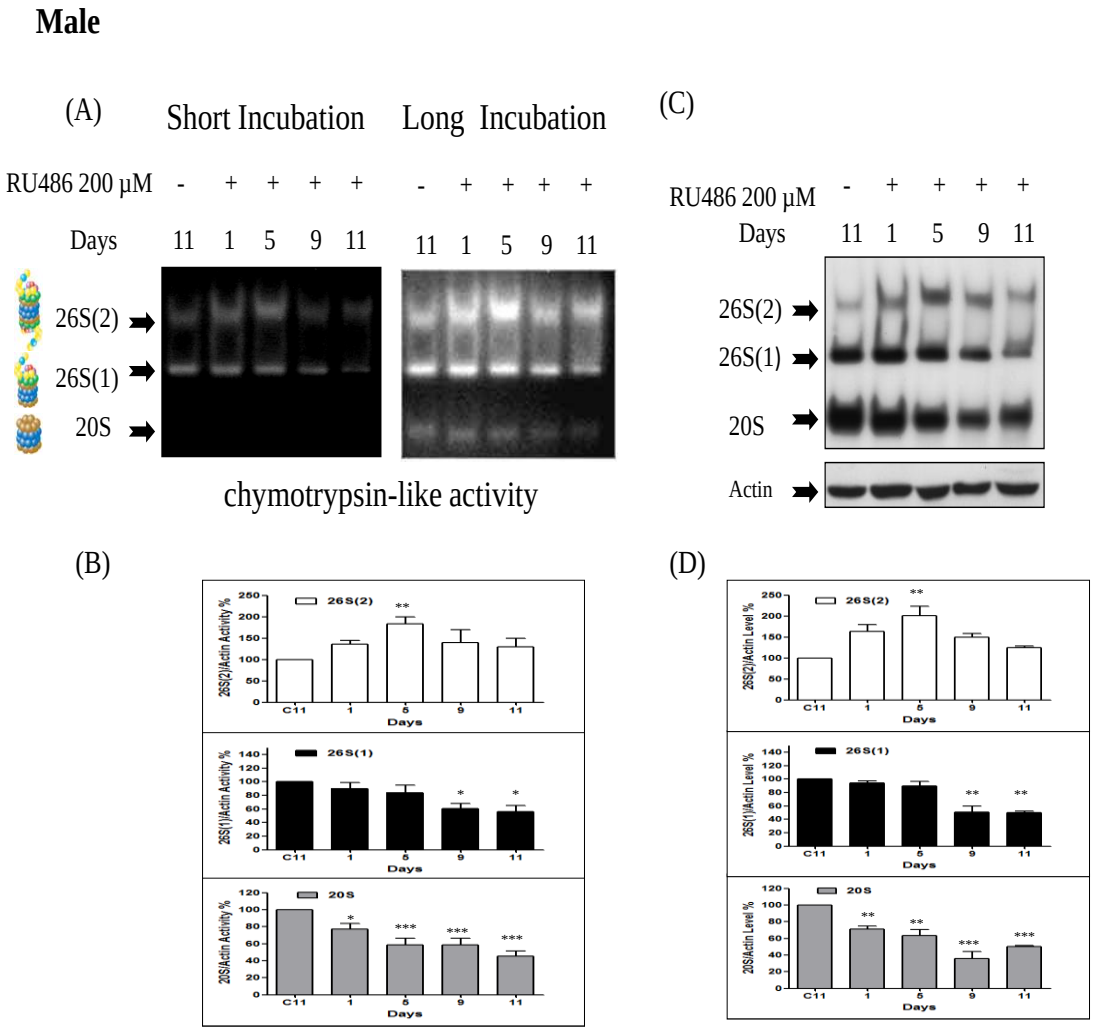

Fig. 17. Knock down of dβ5 expression in males decreases proteasome activity. Male 1 to 2 day-old UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h, and then fed with 200 µM RU486 for varying periods of time (1, 5, 9, and 11 days) or 4% EtOH (vehicle, control, c11) for 11 days. Proteasome activity and levels (90 µg protein/sample) were analyzed as described under Materials and Methods. (A) Chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the 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. (B) Activity bands were semi-quantified by densitometry. (C) Proteasome levels were assessed by immunoblotting with the anti-dβ5 antibody. Equal protein loading was demonstrated by probing with the anti-actin antibody. (D) The levels of proteasome/actin were semi-quantified by densitometry. Data represent the mean ± sem of 3 experiments (n=30 flies for each treatment). * p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001. Statistical significance was assessed by one-way analysis of variance (ANOVA).

Figure 18

Fig. 18. Knock down of dβ5 expression in females decreases proteasome activity. Female 1 to 2 day-old UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h, and then fed with 200 µM RU486 for varying periods of time (1, 7, 13, and 15 days) or 4% EtOH (vehicle, control, c15) for 15 days. Proteasome activity and levels (90 µg protein/sample) were analyzed as described under Materials and Methods. (A) Chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the 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. (B) Activity bands were semi-quantified by densitometry. (C) Proteasome levels were assessed by immunoblotting with the anti-dβ5 antibody. Equal protein loading was demonstrated by probing with the anti-actin antibody. (D) The levels of proteasome/actin were semi-quantified by densitometry. Data represent the mean ± sem of 3 experiments (n=30 flies for each treatment). * p value $\leq$ 0.05; ** p value $\leq$ 0.01; *** p value $\leq$ 0.001. Statistical significance was assessed by one-way analysis of variance (ANOVA).

Figure 18

Female

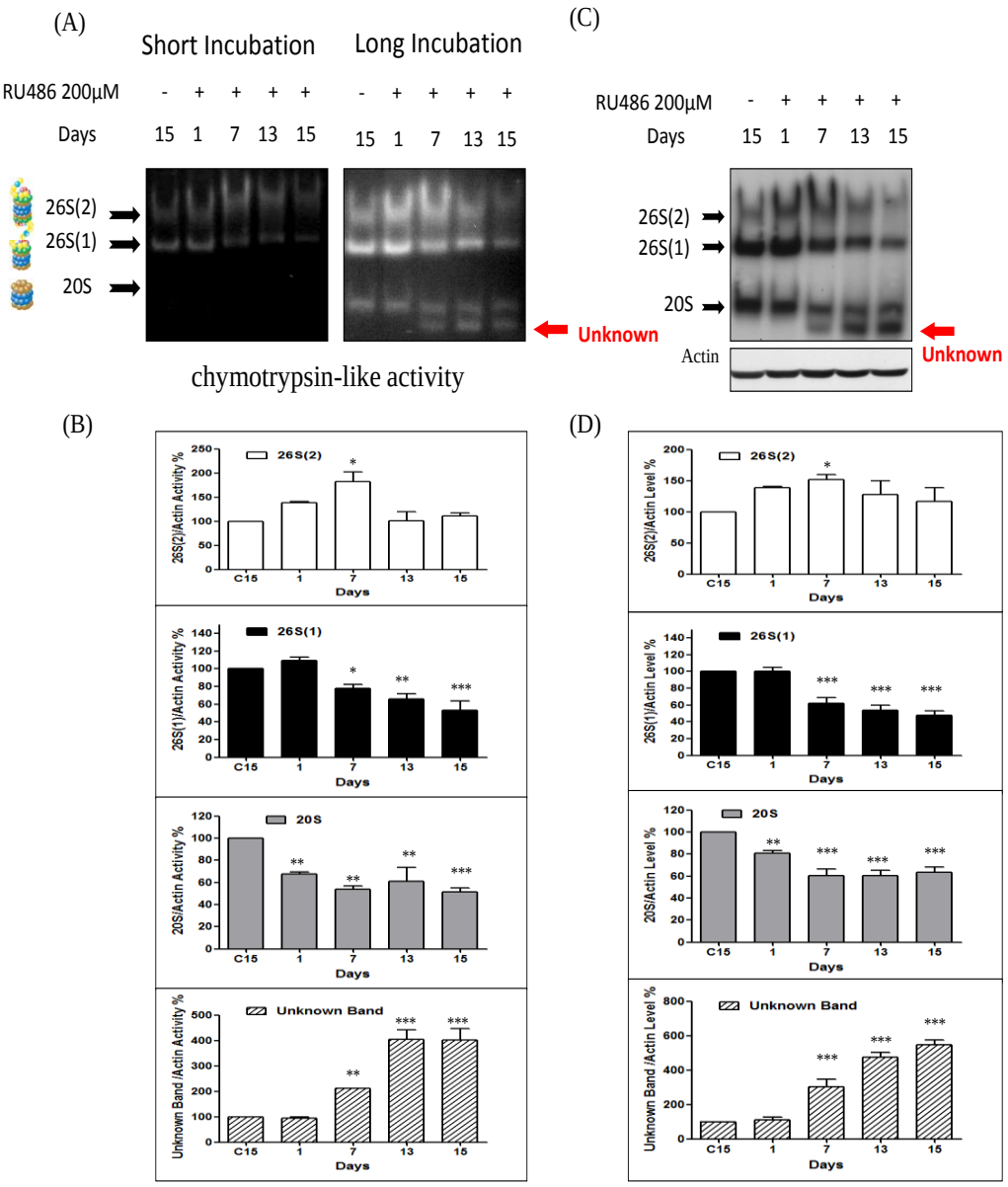

Figure 19

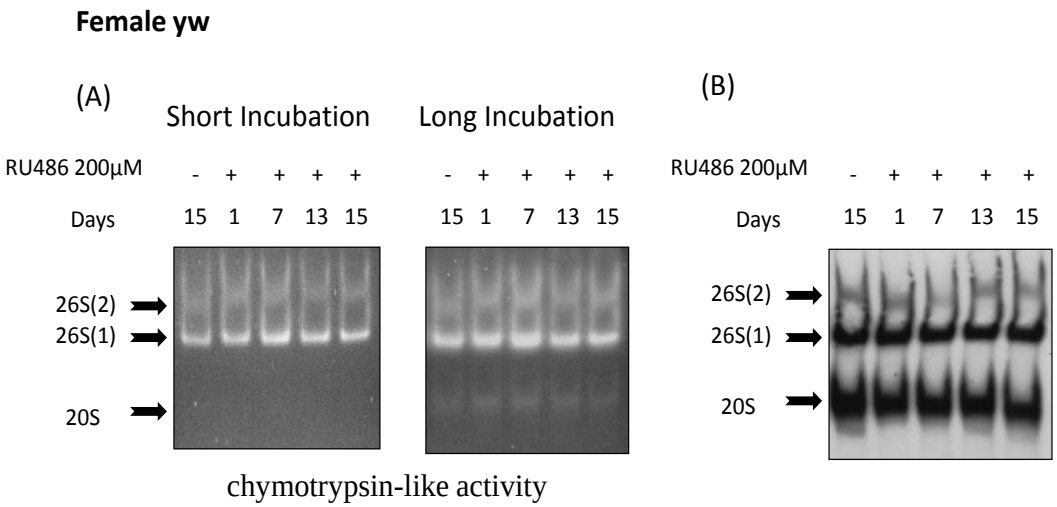

Fig. 19. The new "proteasome complex" was not induced by RU486 administration to wild type yw female flies. 1 to 2 day-old female yw flies were starved for 12 h, and then fed with 200 µM RU486 for varying periods of time (1, 7, 13, and 15 days) or 4% EtOH (vehicle, control) for 15 days. Proteasome activity and levels (90 µg protein/sample) were analyzed as described under Materials and Methods. (A) Chymotrypsin-like activity was assessed by Suc-LLVY-AMC with the 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. (B) Proteasome levels were assessed by immunoblotting with the anti-dβ5 antibody.

Figure 20

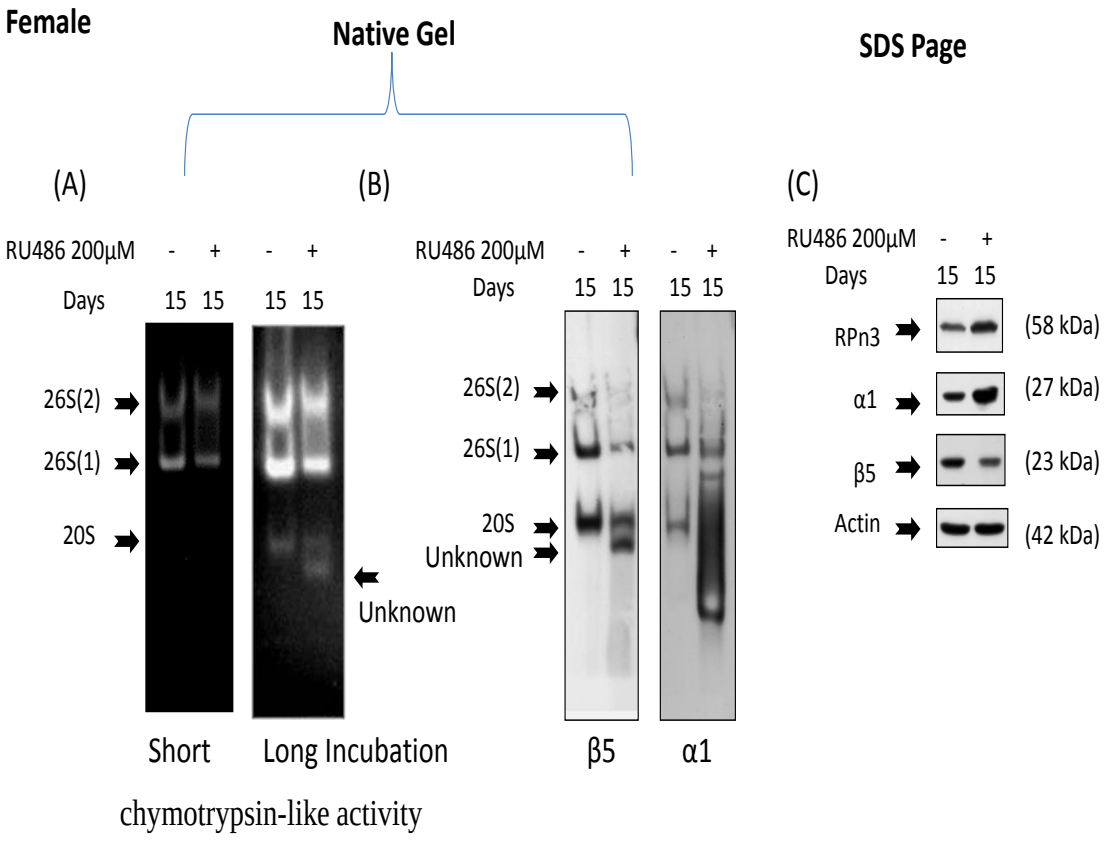

Fig . 20. Knock down of dβ5 induces upregulation of other proteasome subunits. Female 1 to 2 day-old UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h, and then fed with 200 µM RU486 or 4% EtOH (vehicle, control) for 15 days. Proteasome activity and levels (90 µg protein/sample) were analyzed as described under Materials and Methods. (A) Chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the 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. (B) Proteasome levels were assessed by immunoblotting with the following antibodies: anti-dβ5, anti-RPn3, and anti-α1 antibody. Equal protein loading was demonstrated by probing with the anti-actin antibody.

Figure 21

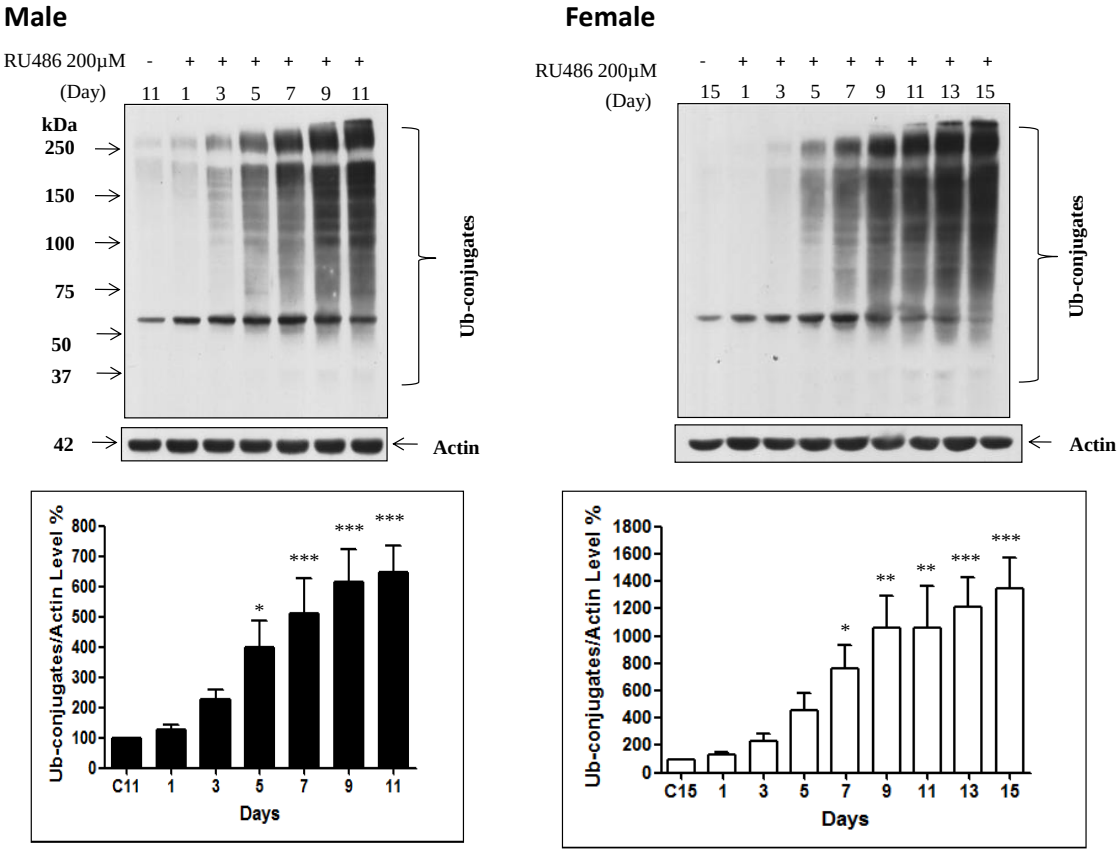

Fig. 21. Disruption of proteasome activity caused by dβ5 RNAi promotes the accumulation of ubiquitinated proteins. Separate groups of 1 to 2 day-old male and female UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h and then fed with 200 µM RU486 for varying periods of time. Males were fed with 200 µM RU486 for 1, 3, 5, 7, 9, and 11 days or 4% ethanol (EtOH, vehicle, control, c11) for 11 days. Females were fed with 200 µM RU486 for 1, 3, 5, 7, 9, 11, 13, and 15 days or 4% EtOH (vehicle, control, c15) for 15 days. The levels of ubiquitinated proteins were analyzed by western blotting (50 µg protein/sample) probed with an anti-ubiquitin antibody. Equal protein loading was demonstrated by probing immunoblots with an anti-actin antibody. The levels of Ub-conj/actin were semi-quantified by densitometry. Data represent the mean ± sem of 3 experiments. * p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001. Statistical significance was assessed by one-way analysis of variance (ANOVA).

Figure 22

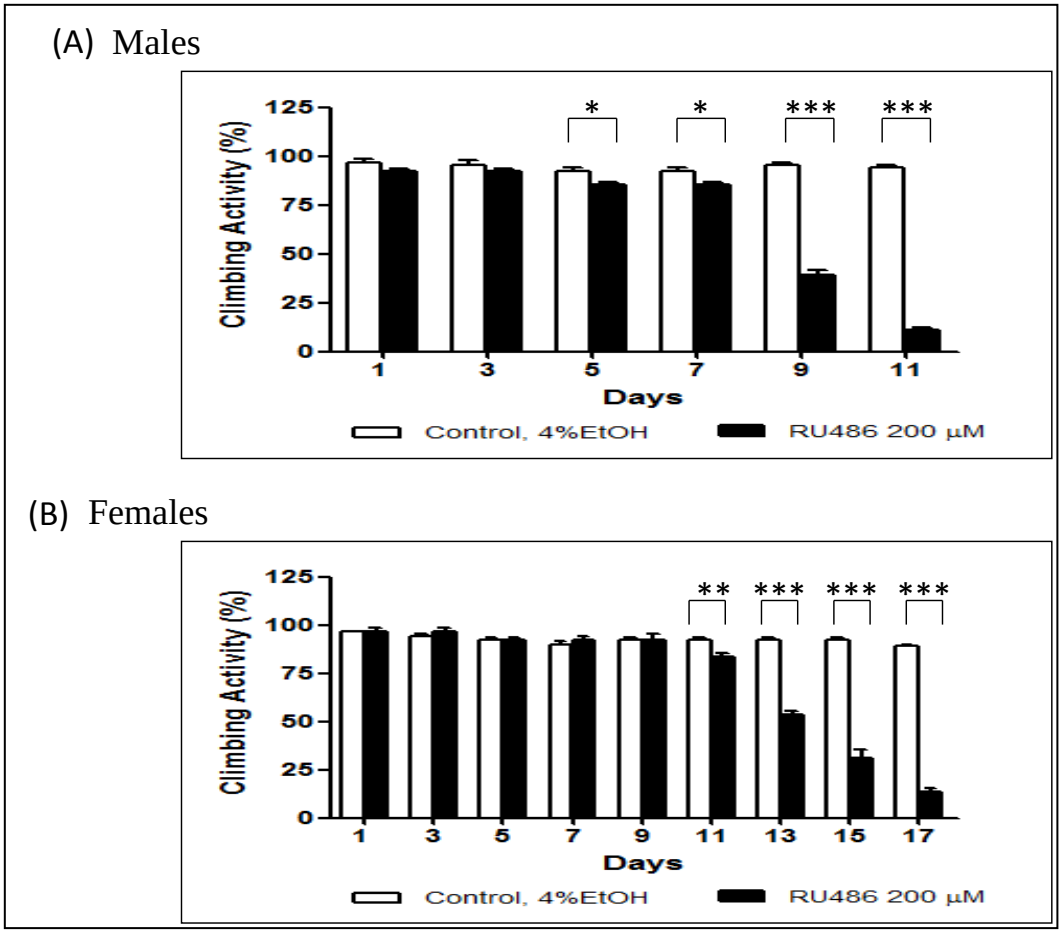

Fig. 22. Persistent proteasome disruption causes locomotor dysfunction. Separate groups of 1 to 2 day-old male and female UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h and then fed with 200 µM RU486 for varying periods of time. (A) Males were fed with 4% ethanol (EtOH, vehicle, control) or 200 µM RU486 for 1, 3, 5, 7, 9, and 11 (B) Females were fed with 4% EtOH (vehicle, control) or 200 µM RU486 for 1, 3, 5, 7, 9, 11, 13, and 15 days. The locomotor ability of flies was assessed with the climbing assay as described under Materials and Methods. Data represent the mean ± sem of 3 trials (n=30 flies for each trial). * p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001. Statistical significance was assessed by one-way analysis of variance (ANOVA).

Figure 23

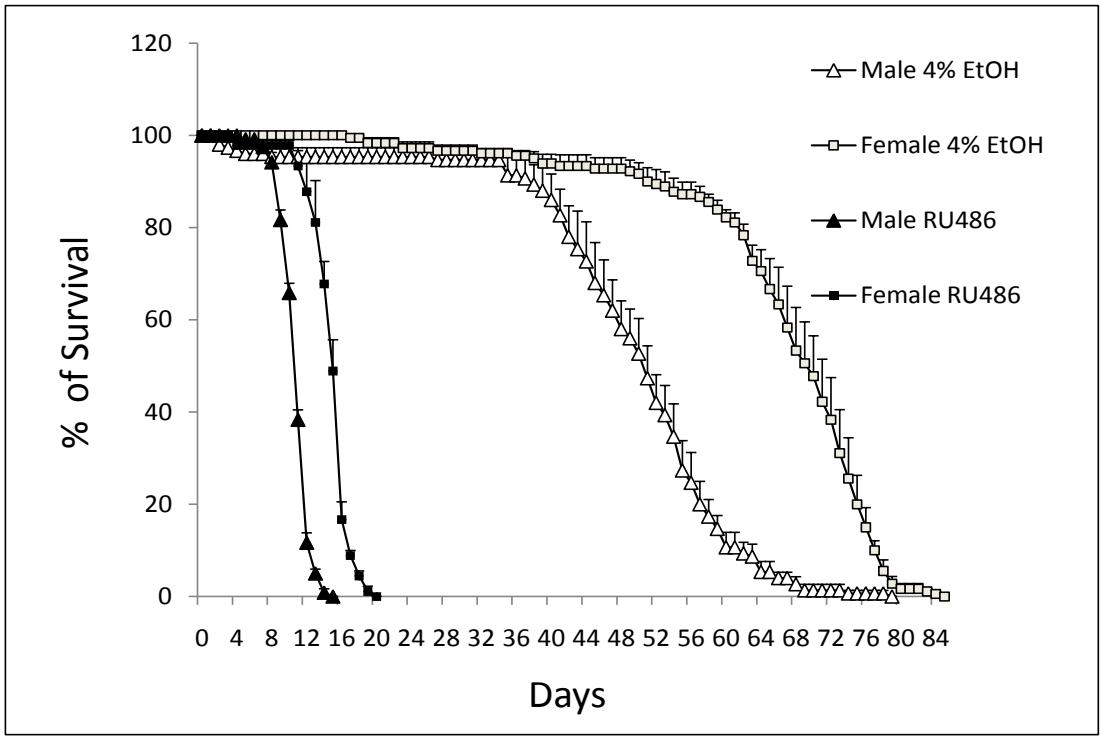

Fig. 23. Knock down of dβ5 shortens the lifespan of flies. Separate groups of 1 to 2 day-old male and female UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h and then fed with 200 µM RU486 or 4% ethanol for varying periods of time. Flies were transferred to new vials with fresh food every 6 days. The percentage of dead flies was calculated daily. Three trials were performed for each time point. Data represent the mean ± sem of 3 experiments (n=30 flies for each treatment).

Figure 24

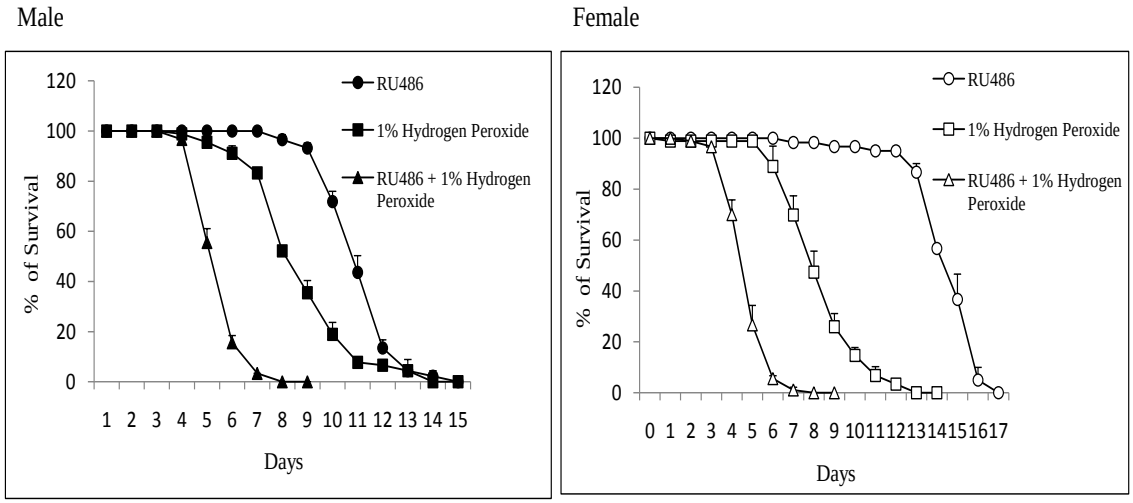

Fig. 24. Disruption of proteasome activity enhances the sensitivity to oxidative stress. Separate groups of 1 to 2 day-old male and female UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h and then fed with 200 µM RU486 or 1 % hydrogen peroxide (H_2O_2) alone or 200 µM RU486 + 1 % H_2O_2, starting at day 0. To avoid H_2O_2 inactivation, flies were transferred to new vials every 3 days. The percentage of dead flies was calculated daily. Three trials were performed for each time point. Data represent the mean ± sem of 3 experiments (n=30 flies for each treatment).

Figure 25

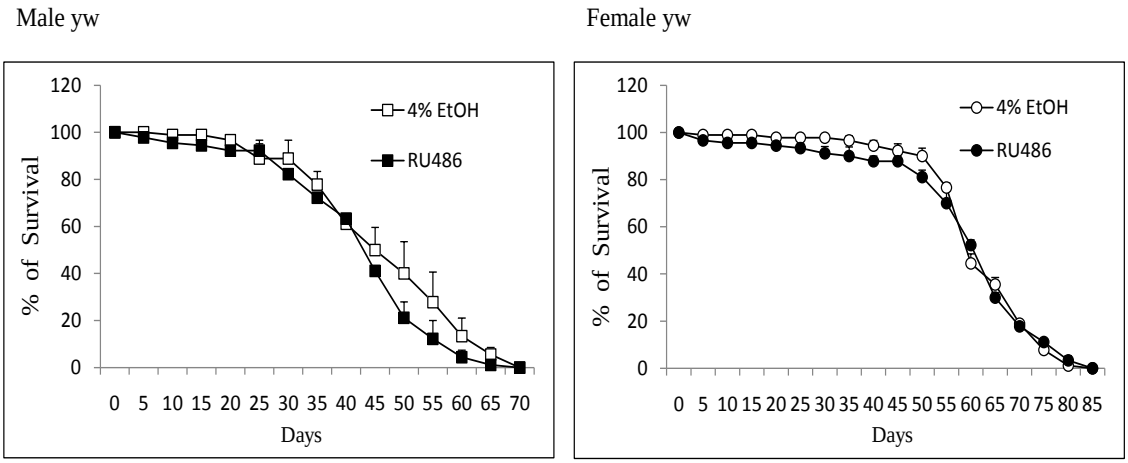

Fig. 25. Administration of 200 µM RU486 to wild type yw flies has no significant effect on their lifespan. Separate groups of 1 to 2 day-old male and female wild type yw flies were starved for 12 h and then fed with 200 µM RU486 or 4% ethanol for varying periods of time. Flies were transferred to new vials with fresh food every 6 days. The percentage of dead flies was calculated daily. Three trials were performed for each time point. Data represent the mean ± sem of 3 experiments (n=30 flies for each treatment).

Figure 26

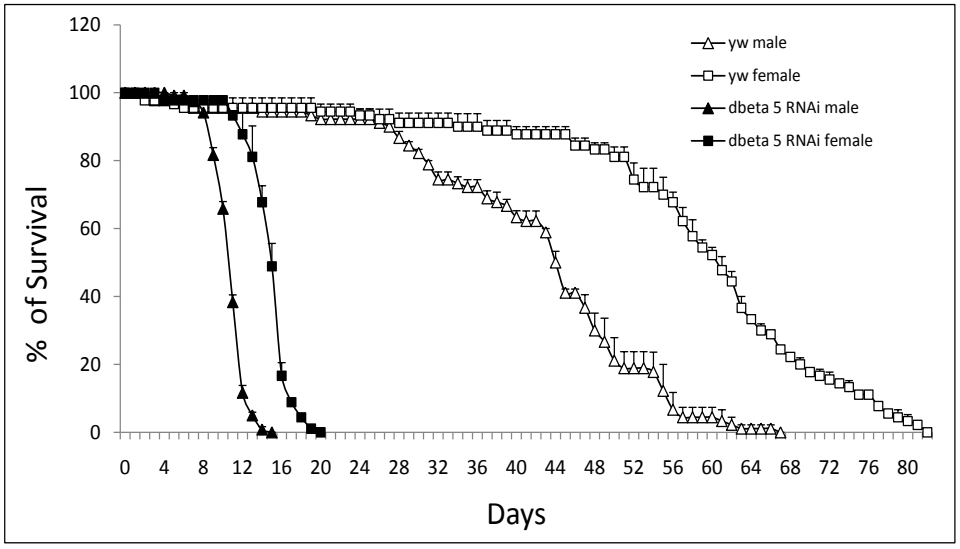

Fig. 26. Administration of 200 µM RU486 to yw flies (wild type flies containing no dβ5 RNAi insert) has no significant effect on their lifespan. Separate groups of 1 to 2 day-old male and female UAS-dβ5 RNAi/+; Act5C-GS/+ flies and wild type yw flies were starved for 12 h and then fed with 200 µM RU486 or 4% ethanol for varying periods of time. Flies were transferred to new vials with fresh food every 6 days. The percentage of dead flies was calculated daily. Three trials were performed for each time point. Data represent the mean ± sem of 3 experiments (n=30 flies for each treatment).

Figure 27

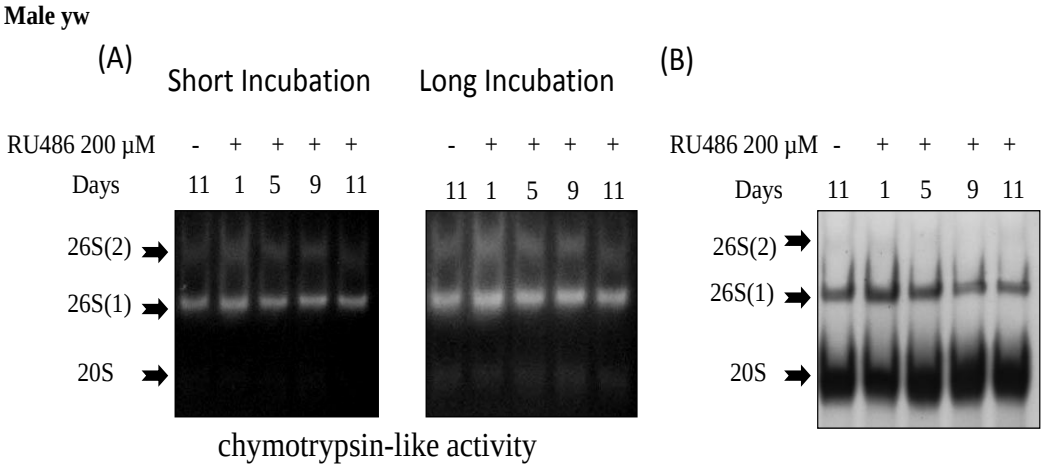

Fig. 27. Administration of 200 µM RU486 has no significant effect on proteasome activity and level on wild type yw male flies. Male 1 to 2 day-old wild type yw flies were starved for 12 h, and then fed with 200 µM RU486 for varying periods of time (1, 5, 9, and 11 days) or 4% EtOH (vehicle, control) for 11 days. Proteasome activity and levels (90 µg protein/sample) were analyzed as described under Materials and Methods. (A) Chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the 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. (B) Proteasome levels were assessed by immunoblotting with the anti-dβ5 antibody.

Figure 28

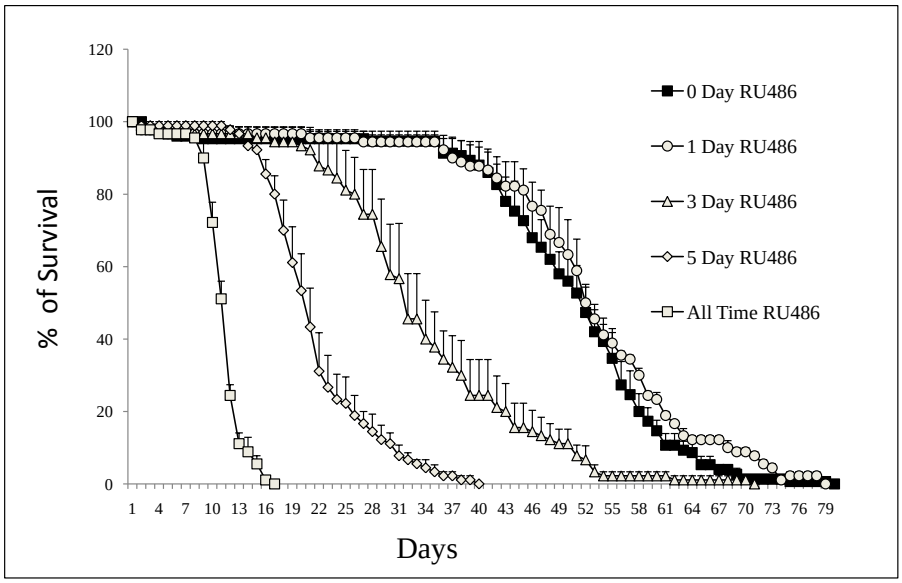

Fig. 28. Short term administration of 200 µM RU486 to transgenic flies affects their lifespan. Male 1 to 2 day-old UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h. In order to feed flies with RU486 temporally, flies were fed with 200 µM RU486 for varying periods of time (0, 1, 3, or 5 days only) at day 0 and then transferred to fly food containing only 4% EtOH (vehicle, control). To compare the effects of temporal and permanent RU486 administration, another group of flies were permanently kept in fly food containing 200 µM RU486 since day 0. Flies were transferred to new vials with fresh food every 5 days. The percentage of dead flies was calculated daily. Three trials were performed for each time point. Data represent the mean ± sem of 3 experiments (n=30 flies for each treatment).

Figure 29

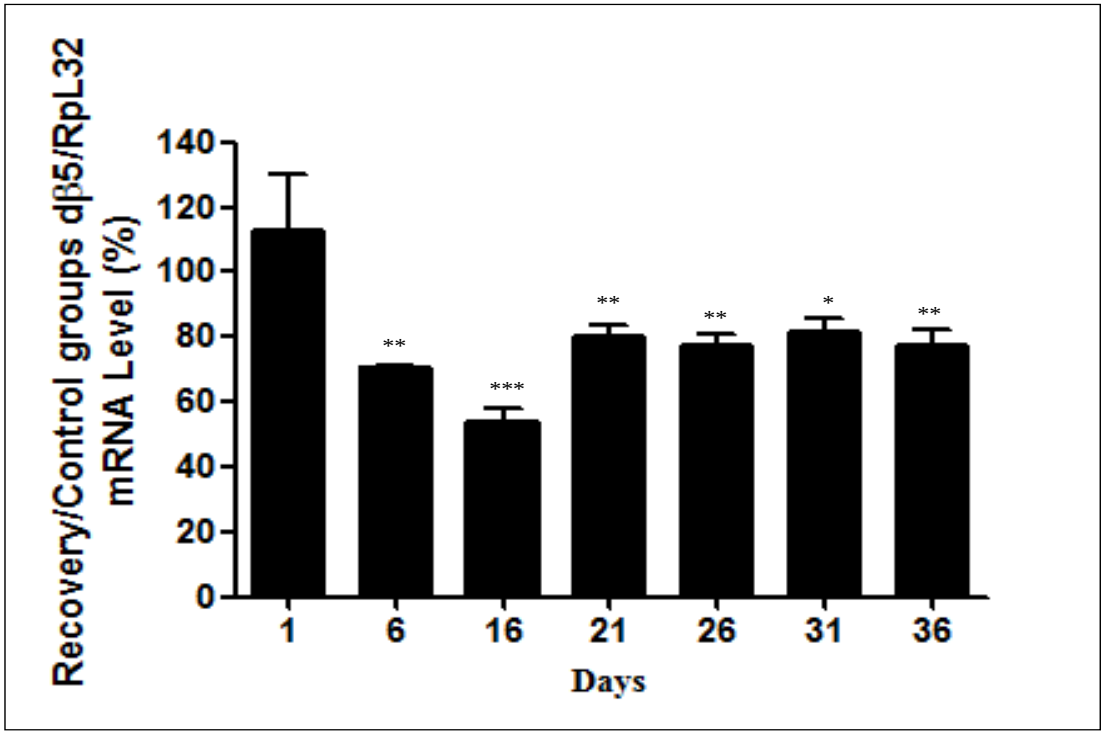

Fig. 29. The dβ5 mRNA levels recovered in dβ5 knock down transgenic flies upon RU486 withdrawal. Male 1 to 2 day-old UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h. In the control group (control, C), upon starvation flies were fed with 4% EtOH (vehicle, control) from day 0 to day 36. In the recovery group (R, flies with temporal dβ5 knock down), upon starvation flies were fed with 200 µM RU486 from day 0 to day 5, and then transferred to fly food containing 4% EtOH and not RU486, up to day 36. The fly dβ5 mRNA levels were assessed by real-time PCR as described in Materials and Methods. Data represent the mean ± sem of 3 experiments (n=30 flies for each treatment). * p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001. Statistical significance was assessed by one-way analysis of variance (ANOVA).

Figure 30

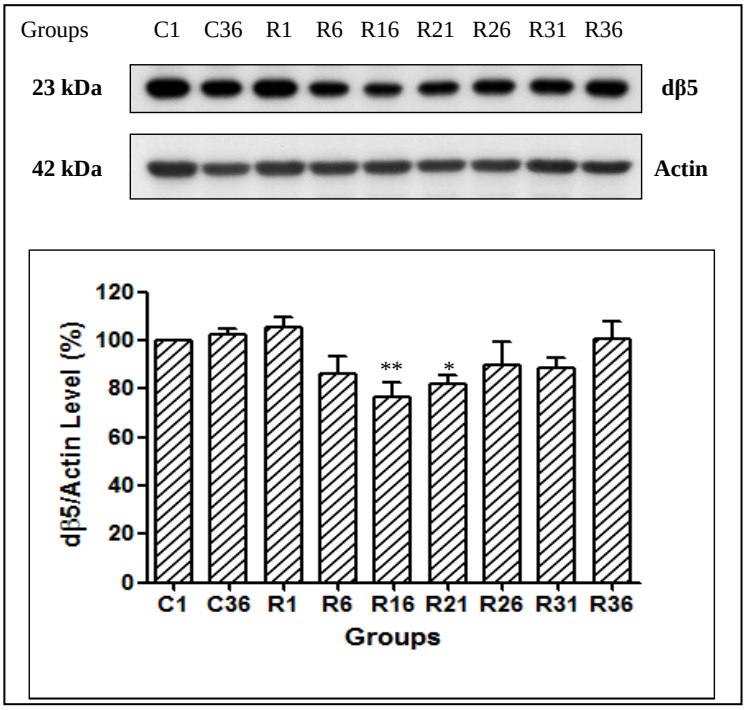

Fig. 30. The dβ5 protein levels recovered in the dβ5 knock down transgenic flies upon RU486 withdrawal. Male 1 to 2 day-old UAS-d*β*5 RNAi/+; Act5C-GS/+ flies were starved for 12 h. In the control group (control, C), upon starvation flies were fed with 4% EtOH (vehicle, control) from day 0 to day 36. In the recovery group (R, flies with temporal dβ5 knock down), upon starvation flies were fed with 200 µM RU486 from day 0 to day 5, and then transferred to fly food containing 4% EtOH and not RU486, up to day 36. The fly dβ5 protein levels were assessed by western blot analysis probed with the anti-dβ5 antibody as described in Materials and Methods. Flies were collected at different time points [day 1 (R1), day 6 (R6), day 16 (R16), day 21 (R21), day 26 (R26), day 31 (R31), and day 36 (R36)] for western blot analysis (50 µg of protein/lane). Equal protein loading was demonstrated by probing immunoblots with an anti-actin antibody. The levels of dβ5/actin were semi-quantified by densitometry. Data represent the mean ± sem of 3 experiments (n=30 flies for each treatment). * p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001. Statistical significance was assessed by one-way analysis of variance (ANOVA).

Figure 31

Fig. 31. Proteasome activity and level recovered in dβ5 knock down transgenic flies upon RU486 withdrawal. Male 1 to 2 day-old UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h. In the control group (control, C), upon starvation flies were fed with 4% EtOH (vehicle, control) from day 0 to day 36. In the recovery group (R, flies with temporal dβ5 knock down), upon starvation flies were fed with 200 μM RU486 from day 0 to day 5, and then transferred to fly food containing 4% EtOH and not RU486, up to day 36. Males were separately collected at different time points [day 1 (R1), day 6 (R6), day 16 (R16), day 21 (R21), day 26 (R26), day 31 (R31), and day 36 (R36)] for in gel and immunoblot analyses (50 μg of protein/lane) as described under Materials and Methods. (A) The proteasome chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the 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. (B) Proteasome levels were assessed by immunoblotting with the anti-dβ5 antibody and normalized with the anti-actin antibody. (C) Activity bands were semi-quantified by densitometry. (D) Protein levels of proteasome/actin were semi-quantified by densitometry. Data represent the mean ± sem of 3 experiments (n=30 flies for each treatment). * p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001. Statistical significance was assessed by one-way analysis of variance (ANOVA).

Figure 31

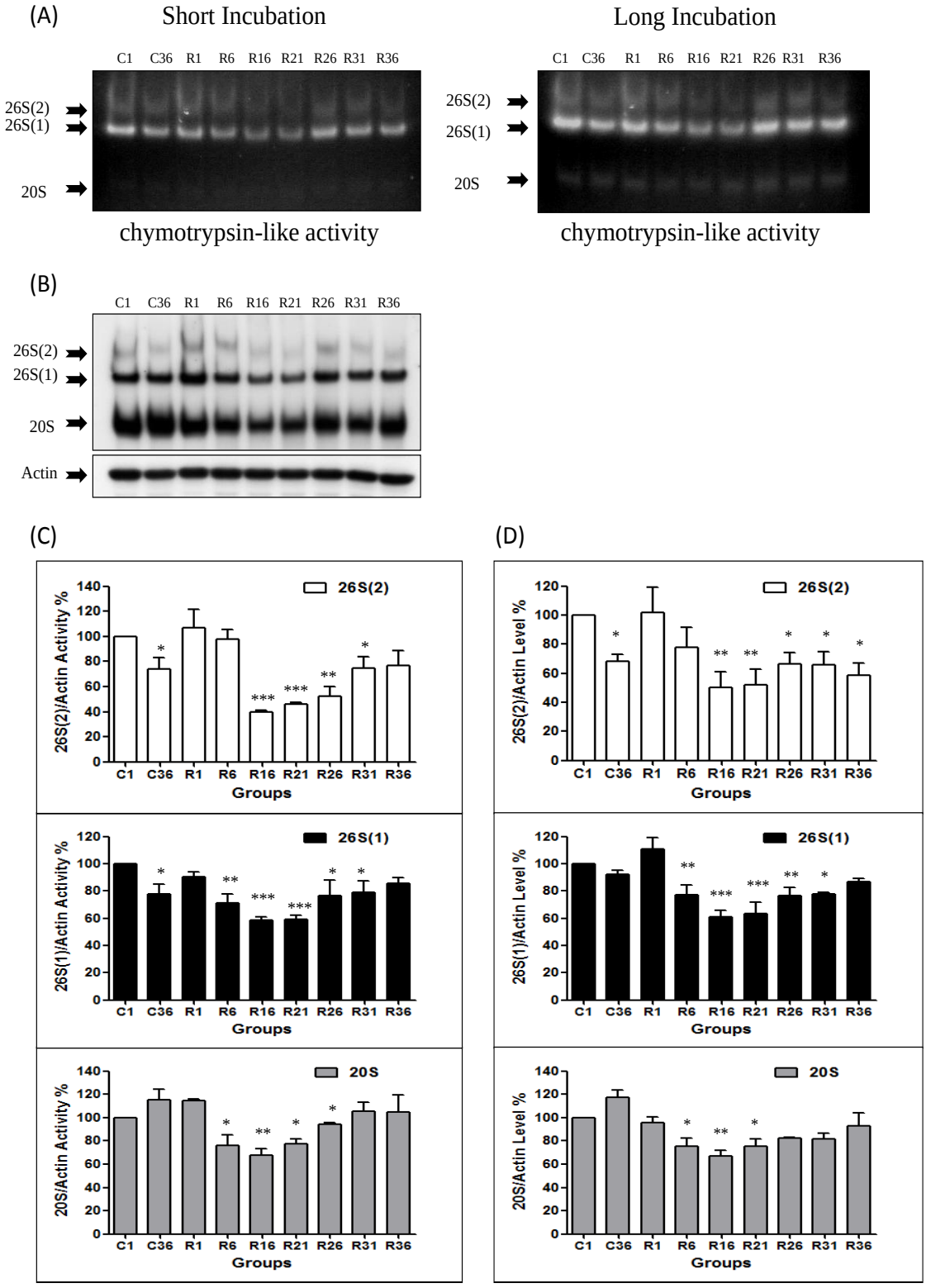
(A)
Short Incubation
Long Incubation
C1 C36 R1 R6 R16 R21 R26 R31 R36
26S(2)
26S(1)
20S
chymotrypsin-like activity
(B)
Actin
(C)
(D)
26S(2)
26S(1)
20S
26S(2)/Actin Activity %
26S(1)/Actin Activity %
20S/Actin Activity %
26S(2)/Actin Level %
26S(1)/Actin Level %
20S/Actin Level %
Groups

Figure 32

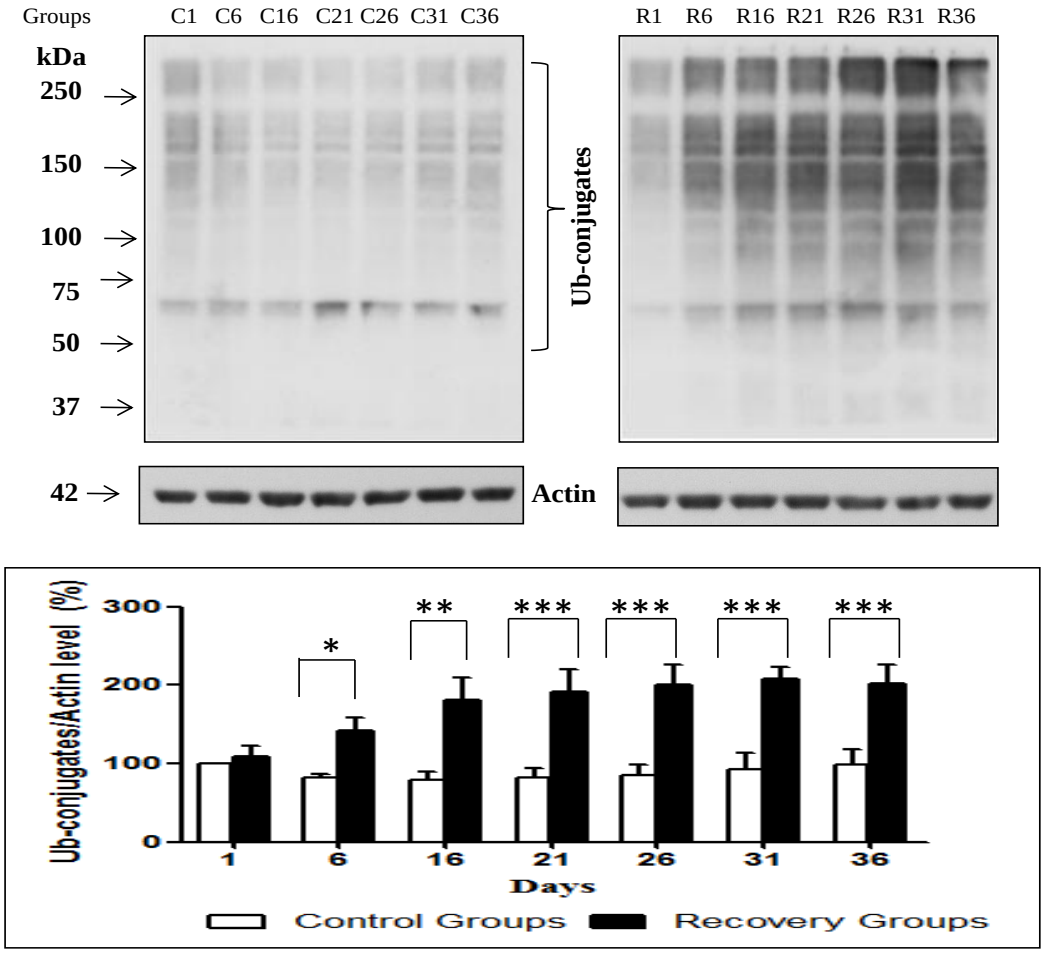

Fig. 32. High levels of ubiquitinated proteins persisted in dβ5 knock down transgenic flies even upon RU486 withdrawal. Male 1 to 2 day-old UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h. In the control group (control, C), upon starvation flies were fed with 4% EtOH (vehicle, control) from day 0 to day 36. In the recovery group (R, flies with temporal dβ5 knock down), upon starvation flies were fed with 200 µM RU486 from day 0 to day 5, and then transferred to fly food containing 4% EtOH and not RU486, up to day 36. Control (C) and recovery (R) males were separately collected at different time points (day 1, 6, 16, 21, 26, 31, and 36) for western blot analyis (50 µg of protein/lane) with the anti-ubiquitin antibody as described under Materials and Methods. Equal protein loading was demonstrated by probing the immunoblot with an anti-actin antibody. The levels of Ub-conj/actin were semi-quantified by densitometry. Data represent the mean ± sem of 3 experiments (* p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001). Statistical significance was assessed by one-way analysis of variance (ANOVA).

Figure 33

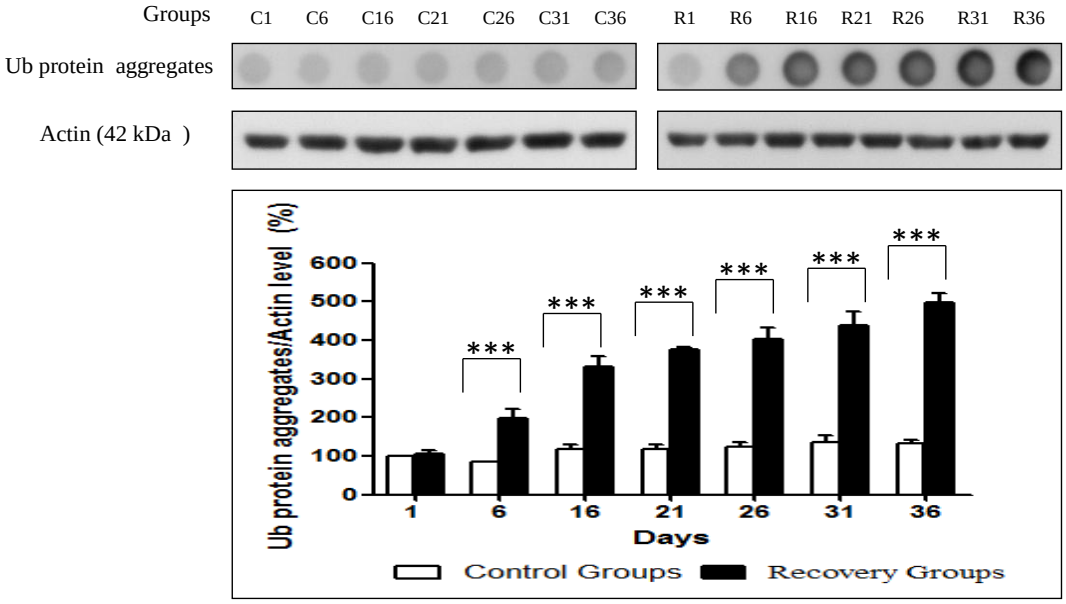

Fig. 33. Increasing levels of ubiquitinated protein aggregates persisted in dβ5 knock down transgenic flies even upon RU486 withdrawal. Male 1 to 2 day-old UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h. In the control group (control, C), upon starvation flies were fed with 4% EtOH (vehicle, control) from day 0 to day 36. In the recovery group (R, flies with temporal dβ5 knock down), upon starvation flies were fed with 200 µM RU486 from day 0 to day 5, and then transferred to fly food containing 4% EtOH and not RU486, up to day 36. Control (C) and recovery (R) males were separately collected at different time points (day 1, 6, 16, 21, 26, 31, and 36) for the filter trap assay (50 µg of protein/sample) to analyze the levels of SDS insoluble protein aggregates with the anti-ubiquitin antibody as described under Materials and Methods. Equal protein loading was demonstrated by SDS-PAGE followed by immunoblot analysis with an anti-actin antibody. Levels of Ub-aggregates/actin were semi-quantified by densitometry. Data represent the mean ± sem of 3 experiments (* p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001). Statistical significance was assessed by one-way analysis of variance (ANOVA).

Figure 34

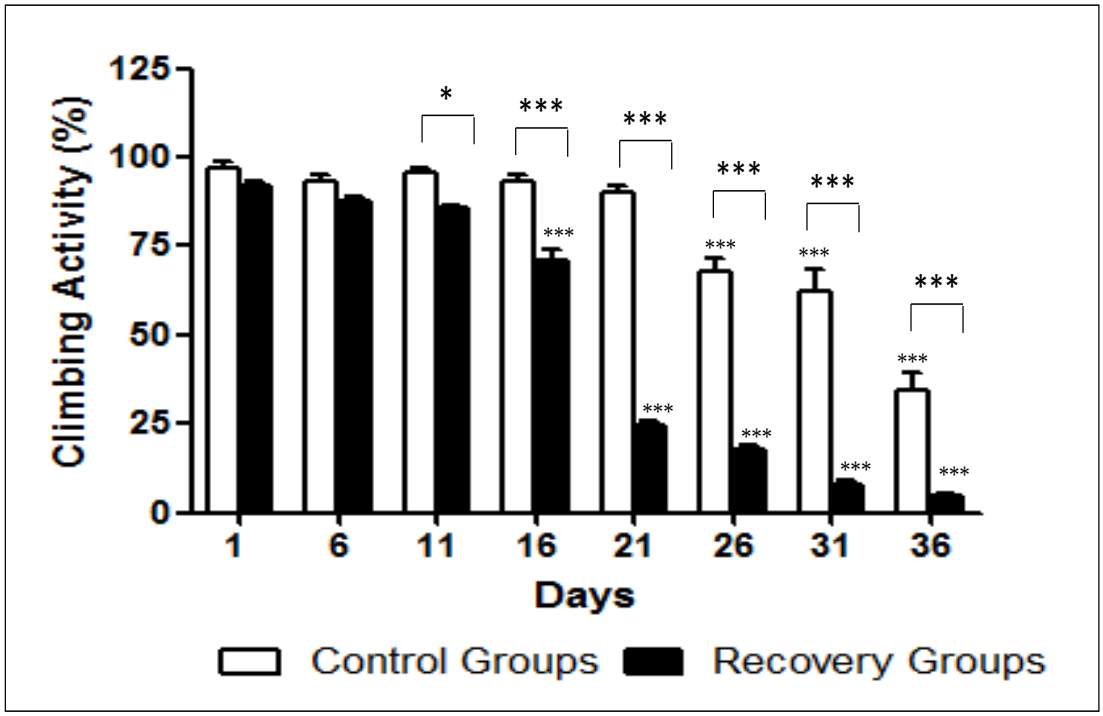

Fig. 34. Long term deficit in locomotor activity in dβ5 knock down transgenic flies even upon RU486 withdrawal. Male 1 to 2 day-old UAS-dβ5 RNAi/+; Act5C-GS/+ flies were starved for 12 h. In the control group (control, C), upon starvation flies were fed with 4% EtOH (vehicle, control) from day 0 to day 36. In the recovery group (R, flies with temporal dβ5 knock down), upon starvation flies were fed with 200 µM RU486 from day 0 to day 5, and then transferred to fly food containing 4% EtOH and not RU486, up to day 36. Control (C) and recovery (R) males were separately collected at different time points (day 1, 6, 16, 21, 26, 31, and 36) for the climbing assay recorded for 20 s, to analyze locomotor performance as described under Materials and Methods. Data represent the mean ± sem of 3 trials (n=30 flies for each trial, * p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001). Statistical significance was assessed by one-way analysis of variance (ANOVA).

CHAPTER 8

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