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Deubiquitinases In Ubiquitin Homeostasis And Disease

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DEUBIQUITINASES IN UBIQUITIN HOMEOSTASIS AND DISEASE

by

GORICA RISTIC

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2017

MAJOR: PHARMACOLOGY

Approved By:

Advisor

Date

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DEDICATION

To my parents, for their unwavering love, support and encouragement.

And my grandparents, who always believed I could do anything.

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TABLE OF CONTENTS

Dedication.....	ii
Acknowledgements.....	iii
List of Tables.....	vii
List of Figures.....	viii
List of Abbreviations.....	x
Chapter 1: An Optimal Ubiquitin-Proteasome Pathway in the Nervous System; The Role of Deubiquitinating Enzymes.....	1
1.1 Introduction.....	1
1.2 UPP-Related DUBSs in the nervous system.....	6
1.2.1 Proteasome-associated DUBs.....	6
1.2.2 DUBs that function in conjunction with the UPP.....	12
1.3 The use of DUBs for therapeutic purpose.....	25
1.4 Conclusions.....	28
Chapter 2: USP5 is Dispensable for Monoubiquitin Maintenance in <i>Drosophila</i>	33
2.1 Introduction.....	33
2.2 Materials and Methods.....	35
2.3 Results.....	39
2.3.1 <i>Drosophila</i> and human USP5 disassemble unanchored ubiquitin chains similarly <i>in vitro</i>	39
2.3.2 DmUSP5, important during fly development, is dispensable in adults.....	42
2.3.3 DmUSP5 knockdown leads to increased conjugated ubiquitin species without depleting monoubiquitin.....	44
2.3.4 Accumulation of conjugated ubiquitin species during DmUSP5 knockdown is an early event.....	49

2.3.5 DmUSP5 knockdown leads to impaired proteasome activity.....	50
2.4 Discussion.....	52
Chapter 3: VCP Modulates Ataxin-3's Aggregation and Toxicity in a <i>Drosophila</i> Model of Spinocerebellar Ataxia Type-3.....	58
3.1 Introduction.....	58
3.2 Materials and Methods.....	61
3.3 Results.....	63
3.3.1 Mutating the VBM of ataxin-3 does not alter its protein or mRNA levels, or its subcellular localization.....	63
3.3.2 Mutating the VBM of ataxin-3 reduces its toxicity in <i>Drosophila</i> when expressed in all tissues and delays accumulation of SDS-resistant species.....	66
3.3.3 Ataxin-3 with a mutated VBM is less toxic in the nervous system than intact pathogenic ataxin-3.....	69
3.3.4 Mutating the VBM of ataxin-3 reduces its toxic effect on fly motility.....	72
3.3.5 Ataxin-3 with a mutated VBM is less toxic in <i>Drosophila</i> eyes.....	74
3.3.6 Knockdown of VCP improves degeneration in fly eyes.....	76
3.3.7 Ataxin-3 expression after development.....	79
3.4 Discussion.....	82
Chapter 4: Probing the interaction between ataxin-3 and VCP as a potential therapeutic Target.....	88
4.1 Introduction.....	88
4.2 Materials and Methods.....	89
4.3 Results and Discussion.....	89
Chapter 5: Conclusions.....	96
References.....	101

Abstract.....123
Autobiographical Statement.....125

LIST OF TABLES

Table 1.1 - Dubs in the nervous system.....	31
Table 2.1 - Primers used for cloning.....	57

LIST OF FIGURES

Figure 1.1 - Summary of the UPP.....	2
Figure 1.2 - Mammalian DUBs.....	4
Figure 1.3 - Roles of DUBs in Ub Homeostasis.....	5
Figure 1.4 - Summary of DUBs involved in various steps of the UPP.....	23
Figure 2.1 - Conserved catalytic activity <i>in vitro</i> between <i>Drosophila</i> and human USP5.....	41
Figure 2.2 - DmUSP5 is necessary during fly development but not in adults.....	43
Figure 2.3 - Knockdown of DmUSP5 does not deplete mono-ub.....	46
Figure 2.4 - Ub-expressing genes are not up-regulated when DmUSP5 is knocked down.....	48
Figure 2.5 - Mono-ub expression does not suppress lethality caused by knockdown of DmUSP5.....	49
Figure 2.6 - Conjugated ubiquitin accumulates early when DmUSP5 is knocked down.....	51
Figure 2.7 - Knockdown of DmUSP5 causes impairment of proteasome activity.....	52
Figure 3.1 - Ataxin-3 and VCP.....	59
Figure 3.2 - Cloning.....	64
Figure 3.3 - Mutating the VBM does not affect expression or localization of pathogenic ataxin-3.....	66
Figure 3.4 - Mutating the VBM of ataxin-3 delays its aggregation and toxicity.....	68
Figure 3.5 - A mutated VBM on pathogenic ataxin-3 decreases its toxicity in the nervous system of <i>Drosophila</i>	71
Figure 3.6 - Flies expressing pathogenic ataxin-3 with a mutated VBM perform better in motility assays.....	73
Figure 3.7 - Disrupting the interaction between ataxin-3 and VCP reduces toxicity in fly eyes.....	75

Figure 3.8 - Knockdown of VCP in fly eyes improves degeneration caused by pathogenic ataxin-3 expression.....	78
Figure 3.9 - Expression of pathogenic ataxin-3 only in adult stages does not prolong lifespan.....	80
Figure 3.10 - Model.....	87
Figure 4.1 - Ataxin-3 sequence and decoy design.....	90
Figure 4.2 - Ubiquitous expression of decoys.....	91
Figure 4.3 - Ubiquitous expression of decoys with pathogenic ataxin-3 in the fly.....	93
Figure 4.4 - Pan-neuronal expression of decoys with pathogenic ataxin-3 in the fly.....	94

LIST OF ABBREVIATIONS

BDSC - Bloomington *Drosophila* Stock Center

CHIP - C terminus of HSC70-Interacting Protein

DmUSP5 - *Drosophila melanogaster* Ubiquitin Specific Protease 5

DUB - Deubiquitinating enzyme

ESCRT - Endosomal Sorting Complex Required for Transport

JAMM - JAB1/MPN/Mov34 Metallo-enzyme Proteases

MJD - Machado-Joseph Disease

OTU - Otubain Proteases

Ub - ubiquitin

PolyQ - Polyglutamine

PQC - Protein Quality Control

qRT - quantitative Real Time

SCA3 - Spinocerebellar Ataxin Type 3

Ub - ubiquitin

UCH - Ubiquitin C-terminal Hydrolases

UPP - Ubiquitin-Proteasome Pathway

USP - Ubiquitin Specific Proteases

VCP - Valosin Containing Protein

VBM - VCP Binding Motif

VDRC - Vienna *Drosophila* RNAi Center

CHAPTER 1: AN OPTIMAL UBIQUITIN PROTEASOME PATHWAY IN THE NERVOUS SYSTEM: THE ROLE OF DEUBIQUITINATING ENZYMES

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1.1 Introduction

The Ubiquitin Proteasome Pathway (UPP) is responsible for degrading the majority of proteins in eukaryotic cells. The UPP is important to all steps and processes of the nervous system, including cell fate specification, differentiation, migration, networking and maturation, and is critical in maintaining neuronal homeostasis during aging. As post-mitotic cells, neurons cannot disperse toxic or misfolded proteins through cell division, but must instead continuously rid themselves of cellular components whose accumulation could be detrimental. The importance of the UPP to the aging nervous system is exemplified by neurodegenerative disorders such as Alzheimer's, Parkinson's and other diseases, where pathological hallmarks are the accumulation and aggregation of proteins with toxic properties.

Degradation of proteins by the UPP is a highly selective process. In order for proteins to be degraded by the proteasome, they require a tag to identify them as substrates. This "tagging" task is accomplished through the post-translational modification of substrates with the small modifier protein ubiquitin (Ub). Ubiquitination involves the covalent attachment of Ub via an isopeptide bond to a lysine residue of target proteins through the coordinated action of a Ub activating enzyme (E1; two such enzymes are known in mammals), a Ub conjugating enzyme (E2; ~50-75 in mammals) and a Ub ligase (E3; >500 in mammals) (**figure 1.1**).

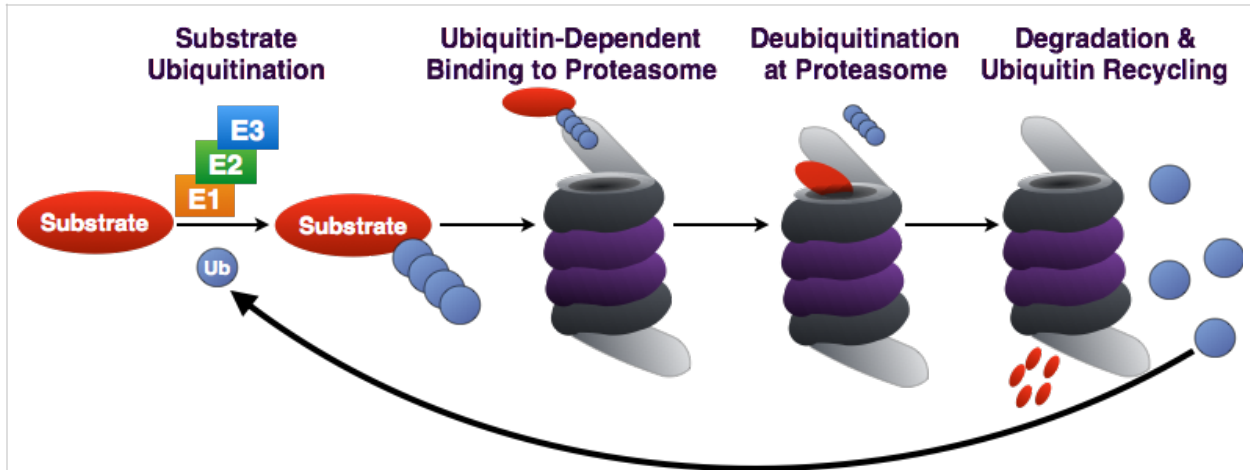


Figure 1.1: Summary of UPP.

Through the coordinated action of E1/E2/E3, Ub is conjugated to a substrate destined for proteasomal degradation. Ub-binding proteins that are components of the proteasome or that associate with it reversibly (not depicted) bind poly-Ub on the substrate, which is subsequently deubiquitinated, unfolded and degraded. Poly-Ub is then processed into mono-Ub which re-enters the cycle.

Ub can be added onto a protein as a monomer or as a poly-Ub chain. Poly-Ub can take on different topographies due to the presence of seven lysine residues on Ub itself, creating chains of different linkage types: K6, K11, K27, K29, K33, K48, and K63. Different types of chains signal different outcomes. In the case of UPP, K48-linked chains consisting of at least four Ub most commonly identify a protein as a proteasome substrate (Thrower et al., 2000; Komander and Rape, 2012; Heride et al., 2014). K11 chains have also been implicated in protein degradation during mitosis (Jin et al., 2008), although it is not clear how widely these chains are utilized by the UPP in other cellular processes.

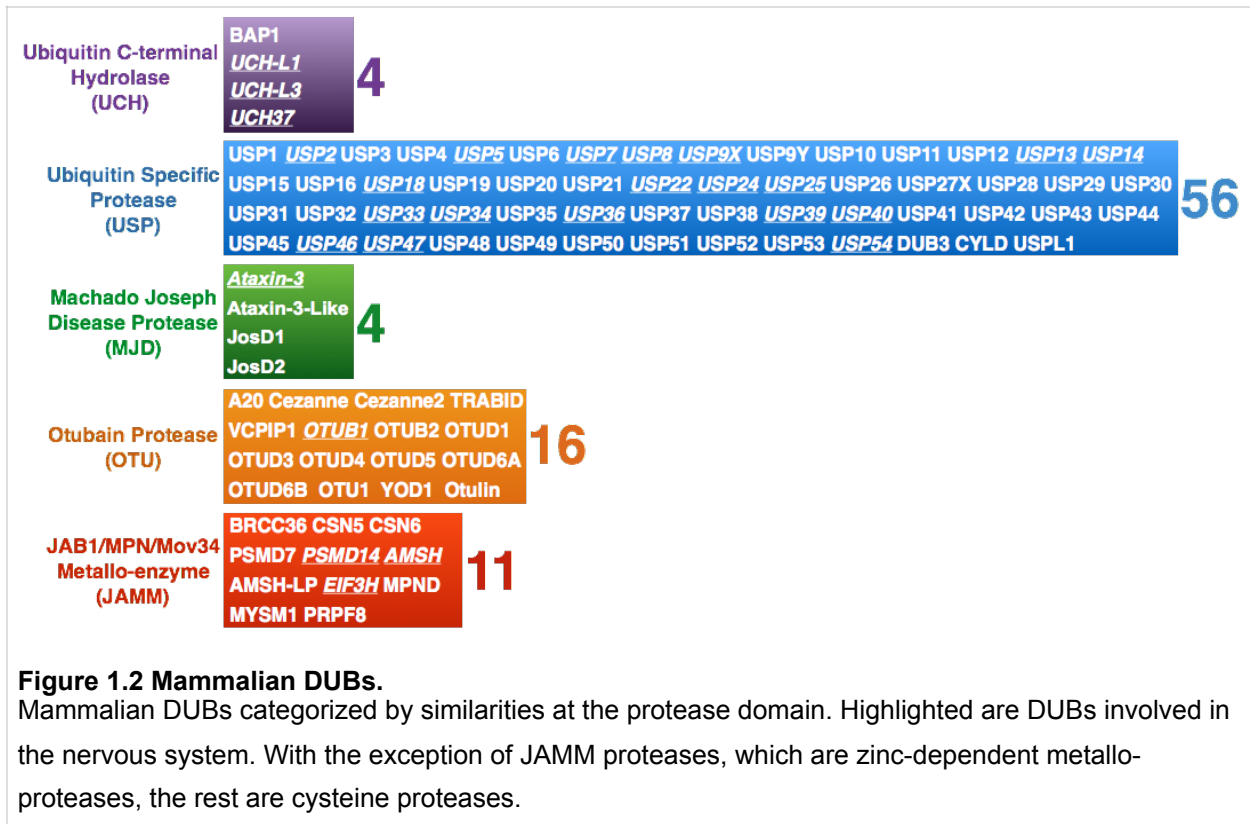
The proteasome is able to bind both K63- and K48- linked chains in reconstituted systems *in vitro* (Kim et al., 2007; Peth et al., 2010). However, in mammalian cells there appears to be selectivity for K48 poly-Ub by the proteasome (Nathan et al., 2013). K63-linked poly-Ub chains are bound by proteins involved in the Endosomal Sorting Complex Required for Transport (ESCRT) pathway, which do not function directly with the proteasome. Binding of K63-linked

poly-Ub by ESCRT proteins seemingly precludes proteasomal degradation of proteins modified with this type chain (Nathan et al., 2013). Additional specificity in poly-Ub recognition is provided by a family of proteasome-associated proteins, including the Rad23 orthologues hHR23A and hHR23B that selectively bind K48 chains (Nathan et al., 2013).

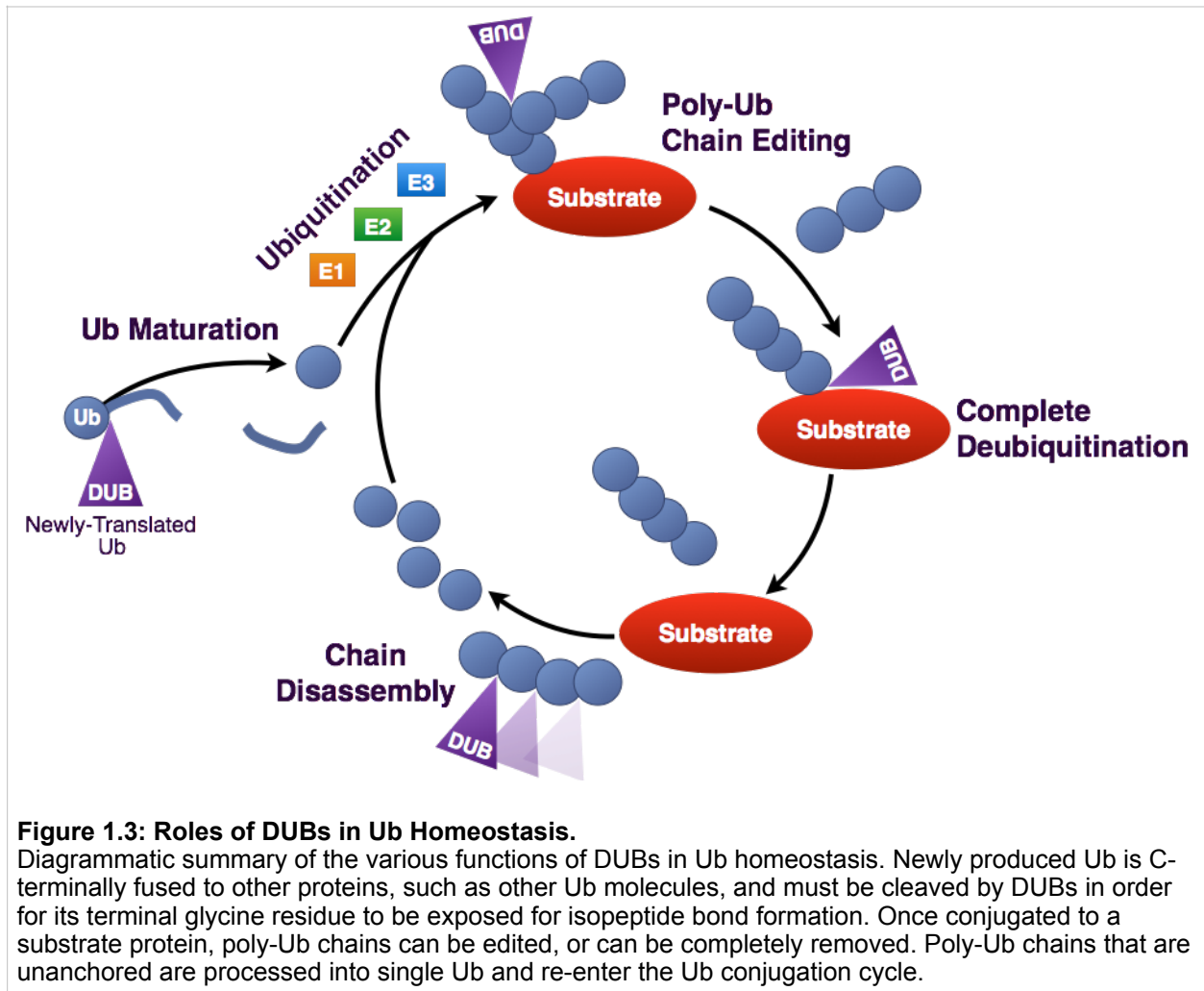
The 26S proteasome is a macromolecular structure composed of a catalytic 20S subunit and one or two 19S regulatory subunits. Ubiquitinated substrates are recognized by and bind to the 19S particle. This process is accomplished in part by the integral 19S receptors S5a and Adrm1 (Deveraux et al., 1994; Husnjak et al., 2008). Ubiquitinated proteins that are bound by the 19S proteasome are deubiquitinated and unfolded. The unfolded proteins can then pass through the hollow, cylindrical core of the 20S particle, where they are enzymatically degraded.

Erroneous ubiquitination of a protein could send it to the proteasome prematurely, or could target it for the wrong pathway (e.g. autophagy rather than the UPP), leading to unintended consequences for cells. Specificity for which proteins are ubiquitinated and the type of Ub linkage formed rests in large part with the E2/E3 pair that performs the ubiquitination process (Komander and Rape, 2012; Heride et al., 2014). An additional level of control is provided by enzymes known as deubiquitinases (DUBs), which reverse the isopeptide bond and thus help to control the status of protein ubiquitination. An increasing number of reports is being published on the role of DUBs in nearly all cellular pathways, tissues and organs, in normal homeostasis and in various diseases, including disorders of the nervous system (Todi and Paulson, 2011; Clague et al., 2013).

The nearly 95 DUBs that are encoded by the human genome are subdivided into five categories based on homology at the catalytic domain. The Ubiquitin C-terminal Hydrolases



(UCH), Ubiquitin Specific Proteases (USP), Machado-Joseph Disease Protease (MJD) and Otubain (OTU) Proteases are cysteine proteases, while JAB1/MPN/Mov34 Metallo-enzyme (JAMM) proteases are Zinc-dependent metallo-proteases (**figure 1.2**). DUBs maintain the cellular pool of mono-Ub available for conjugation by processing Ub precursors; they replenish mono-Ub by cleaving poly-Ub chains and recycling Ub; they can fully deubiquitinate substrates and reverse their outcome; or they can edit poly-Ub chains on substrates to help direct them toward a specific pathway (**figure 1.3**). Although at the most basic level all catalytically active DUBs perform the same function – disassembly of Ub-protein bonds – *in vitro* and *in vivo* studies have collected evidence that these proteases perform various non-redundant functions (Clague et al., 2013). Distinct roles stem in part from differences in the structure of the catalytic



domains of DUBs and in part from interaction domains and subcellular localization signals encoded in their amino acid sequences.

Three DUBs associate directly with the proteasome: PSMD14, USP14 and UCHL5. PSMD14 is a stoichiometric component of the 19S regulatory subunit, whereas USP14 and UCHL5 associate transiently with it during protein degradation. Several other DUBs function in conjunction with the UPP at steps that precede the proteasome (e.g. during substrate ubiquitination) or following substrate binding to the 19S (e.g. during ubiquitin chain disassembly and ubiquitin recycling; **figure 1.4** on page 23). The following sections provide details on UPP-related DUBs in the nervous system, with examples from each sub-class of this family. The

exception are OTU proteases, for which there are few mechanistic data relating them to the UPP in the nervous system. Information that has been reported so far on OTUs is included in **Table 1** at the end of this chapter, which is a comprehensive list of DUBs implicated in the nervous system, and also includes proteases that do not necessarily function through the UPP.

1.2 UPP-Related DUBs in the Nervous System

1.2.1 Proteasome-Associated DUBs

PSMD14

This metallo-protease belongs to the JAMM sub-family of DUBs. It is also known as POH1. PSMD14 is a constitutive subunit of the proteasome, a member of the 19S regulatory complex, where it neighbors the ubiquitin receptor Adrm1 (Beck et al., 2012). Its reported role is to deubiquitinate proteins at the proteasomes before they are degraded. By removing ubiquitin chains *en bloc* from substrates, PSMD14 plays an important role in facilitating protein degradation, as well as in Ub recycling. Once poly-Ub is removed from substrates by PSMD14 it is disassembled by other DUBs, for example USP5 (Clague et al., 2013; Liu and Jacobson, 2013).

PSMD14 and its role at the proteasome were uncovered through studies in yeast that examined how deubiquitination and degradation of a substrate are coupled. In yeast, PSMD14 is known as Rpn11. Through the use of an unfolded, ubiquitinated model substrate, it was shown that deubiquitination occurs after a protein is bound by the proteasome. Mutating Ub so that it can no longer be cleaved by DUBs leads to a significant reduction of substrate degradation. Other studies showed the substrate deubiquitination at the proteasome is insensitive to molecules that block cysteine proteases, whereas a zinc chelator blocks deubiquitination, suggesting a

metallo-enzyme. Mutations in Rpn11 stabilize UPP substrates and are lethal in yeast. These and other studies pointed to Rpn11 as the DUB functioning at the proteasome, bridging substrate deubiquitination and degradation (Verma et al., 2002; Yao and Cohen, 2002).

Work conducted in mammalian cell culture implicated PSMD14 in maintaining the post-mitotic status of neurons. RNAi-mediated knockdown of PSMD14 in mouse midbrain and cortical cultures leads to *de novo* synthesis of DNA and an increase in apoptosis (Staropoli and Abeliovich, 2005). The mechanism behind these observations is unclear, but the data suggest a critical role for UPP in preventing neurons from re-entering mitosis. Perhaps disturbance of the UPP (from the perspective of PSMD14) triggers a general cellular stress response that ultimately leads to neuronal death. Alternatively, the UPP could be involved in the continuous turnover of specific factors that induce mitotic re-entry. Highlighting the importance of PSMD14 to the nervous system, RNAi-mediated knockdown of its orthologue in neurons in *Drosophila* causes larval lethality (Tsou et al., 2012).

PSMD14 is one of very few DUB with a specific function, in this case, the removal of poly-Ub chains in a single swoop from substrates at the proteasome. As it will become clear through the remainder of this chapter, it is difficult to categorize most DUBs as operating in only one step, or acting on only one substrate, pathway, tissue or organ. Perhaps PSMD14 is an exception because of its continuous presence at the 19S subunit, where it is precluded from interacting with other potential partners. Most other DUBs do not appear to reside in a single constitutive molecular complex and can thus conduct different functions depending on their interactions.

UCHL5

This DUB is a member of the UCH sub-family and is also known as UCH37. Unlike PSMD14, UCHL5 is not a constitutive subunit of the proteasome but associates with it by binding to the Ub receptor Adrm1 on the 19S subunit (Lee et al., 2010a; Lee et al., 2010b). The proposed role for this DUB at the proteasome is one of poly-Ub chain trimming by hydrolyzing Ub chains at their distal end and releasing mono-Ub for re-utilization (Yao et al., 2006). UCHL5 deubiquitinating activity has been shown to increase once it is bound to the proteasome (Qiu et al., 2006; Yao et al., 2006). Based on more recent work, UCHL5 stimulates the activity of the proteasome by regulating ATP hydrolysis and 20S gate opening (Peth et al., 2009; Peth et al., 2013a; Peth et al., 2013b).

Studies in a *Uchl5*-null mouse line showed that this protease is important for brain development. *Uchl5* knockout embryos have malformations in various brain areas, including the telencephalon, mesencephalon and metencephalon (Al-Shami et al., 2010). The reasons for these specific anomalies are not known. Other proteasome-associated DUBs, such as USP14, which is discussed next and which also processes chains to yield mono-Ub, appear unable to compensate for the loss of UCHL5 in the brain, indicating a non-redundant role for this DUB in the brain.

USP14

USP14 belongs to the USP sub-family of DUBs. It reversibly associates with the 19S proteasome, where it is responsible for trimming Ub chains on substrates that are destined for degradation and thus recycling mono-Ub. The catalytic activity of USP14 is enhanced by its binding to the proteasome several hundred-fold (Lee et al., 2010a; Lee et al., 2010b), suggesting

that this DUB needs the proteasome in order to conduct functions that require its protease activity.

Depletion of USP14 alone enhances proteasome activity, whereas co-depletion with UCHL5 inhibits the proteasome (Koulich et al., 2008), indicating some cross-dependence for these DUBs during protein degradation. Critical information on the function of USP14 has come from yeast studies, where depletion of Ub leads to an upregulation of its orthologue, Ubp6 (Hanna et al., 2006), which restores Ub balance. Not all functions of USP14 at the proteasome depend on its catalytic activity: binding of USP14 to poly-Ub chains and unfolded peptides results in opening of the gates of the 20S proteasome and stimulation of the ATPase activity of the proteasome (Peth et al., 2009; Peth et al., 2013a; Peth et al., 2013b). Collectively, these findings implicated USP14 with keeping proteasomal activity under check.

A role for USP14 in the nervous system was first demonstrated by the identification of a recessive mutation in the *Usp14* gene, which causes ataxia in the *ax^J* mouse line. Homozygous *ax^J* mice suffer from progressive motor impairment including ataxia and tremor, reduced body and brain mass, paralysis and death by two months of age. The *ax^J* mutation results from the insertion of an intracisternal-A particle into intron 5 of *Usp14*, leading to ~95% lower levels of USP14 protein in the brain of homozygous mice (Wilson et al., 2002; Anderson et al., 2005). In contrast to many mouse models of neurodegenerative diseases, *ax^J* mice do not show neuronal loss or abnormal protein accumulation (Wilson et al., 2002). Instead, *ax^J* mice have reduced mono-Ub levels, particularly in synaptosomal fractions (Anderson et al., 2005; Chen et al., 2009; Chen et al., 2011). Because introduction of Ub in neurons in an *ax^J* background largely

suppresses the phenotype (Chen et al., 2011), indicating that a primary role of USP14 in the central nervous system is Ub homeostasis.

USP14 is critically important at the neuromuscular junction (NMJ). Electrophysiological Recordings in *ax^J* mice reveal defective release of the neurotransmitter acetylcholine (ACh) at the NMJ, and loss of USP14 leads to developmental anomalies at the pre- and post-synaptic terminals of the NMJ (Chen et al., 2009). The NMJs of *ax^J* mice are swollen and poorly arborized, with aberrant nerve terminals and an immature morphology of ACh receptor clusters. These changes correlate with loss of mono-Ub, and are corrected by exogenous expression of Ub in neurons (Chen et al., 2009; Chen et al., 2011).

The ataxia phenotype in the *ax^J* mice suggests that the cerebellum is negatively affected by nearly absent USP14. A potential role for this DUB in regulating the activity of cerebellar Purkinje Cells has been reported. In *ax^J* mice, Purkinje Cells have increased cell surface expression of GABA_AReceptors (GABA_AR) in extrasynaptic regions, with a concomitant increase in inhibitory GABAergic currents that effectively reduces cerebellar output (Lappe-Siefke et al., 2009). GABA_AR is ubiquitinated in cells and USP14, which interacts directly with this receptor, may deubiquitinate it (Lappe-Siefke et al., 2009). Generally speaking, mono-ubiquitination controls recycling of various receptors to and from the cell membrane, including GABA_AR (Saliba et al., 2007). Consequently, USP14 deficiency may cause ataxia in the *ax^J* mice in part by perturbing the turnover and/or cell surface distribution of GABA_AR. Still, since it is not clear that USP14 directly deubiquitinates GABA_AR, it is also worth considering that this DUB may regulate the receptor indirectly. After all, USP14 is a sluggish protease outside of the

proteasomal context, leading one to wonder whether it can deubiquitinate GABA_AR in the absence of factors that enhances its catalytic activity.

Because of the early lethality phenotype in the *ax^J* mice, it has been difficult to investigate whether USP14 is important in adults. Recently, another mutation in *Usp14* was identified (Marshall et al., 2013). This mutation, *nmf375*, leads to ~95% reduction in USP14 protein levels when homozygous, similar to the reduction observed in *ax^J* mice. However, unlike *ax^J* homozygous mice, ones carrying two copies of *nmf375* do not present with phenotypes early in life. Deterioration of motor performance is observed around 12 months of age (*ax^J* mice die by 2 months) and is associated with mono-Ub depletion (Marshall et al., 2013). These data indicate that USP14 plays an important role not only for NMJ development, but also for its maintenance. They also highlight the importance of genetic modifiers; mutations that lead to similar reduction in USP14 protein levels in different genetic backgrounds have markedly different phenotypic onset and progression. Placing the *nmf375* into the same genetic background as the *ax^J* leads to a dramatically earlier phenotype, even more severe than *ax^J* (Marshall et al., 2013). Perhaps future genetic analyses will identify factors that modulate the phenotype caused by this UPP-related DUB. Could these modifiers be other DUBs related to the UPP, or are they E3 ligases that counteract USP14 function?

Studies from yeast, mammalian cell culture and mice together indicate that USP14 plays an important role in recycling mono-Ub by functioning at the proteasome. Other studies have presented the possibility that this DUB has specific substrates, potentially outside of the UPP. As described later, there is even evidence from cell culture that USP14 prevents the degradation of some ubiquitinated substrates at the proteasome, adding further complexity to the functions of

this protease (see the section on “The use of DUBs for therapeutic purposes”). Future work may find distinct binding partners of USP14 that depend on cell type, the state of neuronal activity, and on sub-cellular localization. In turn, these partners may dictate the precise function of USP14 as a DUB, or even as a scaffolding protein.

1.2.2 DUBs that function in conjunction with the UPP

UCHL1

The first reported DUB with a neuronal function, UCHL1 is a member of the UCH sub-family of DUBs. The catalytic area of UCHL1 has a loop positioned over the active site that limits the size of Ub adducts that can be processed by it to small peptides (Johnston et al., 1999; Das et al., 2006). Based on *in vitro* biochemical reactions, structural data and observations from animal studies, UCHL1 is proposed to function largely by maintaining a stable pool of mono-Ub for use in ubiquitination reactions (Clague et al., 2013). Newly translated Ub contains amino acids following the terminal glycine residue that is used for isopeptide bond formation. UCHL1 can cleave off these additional amino acids in order to expose the final glycine of Ub for conjugation. UCHL1 can also help maintain mono-Ub by reversing accidental modifications that can form during Ub activation (Larsen et al., 1998).

UCHL1 was first described in *Aplysia*, where its orthologue is known as Ap-UCH (Hegde et al., 1997). This protease was found to have a role in synaptic plasticity because it was one of the genes that was markedly upregulated in sensory neurons following LTF (long-term facilitation) and LTD (long-term depression). Inhibition of Ap-UCH induction or blockage of its function inhibits synaptic plasticity (Chain et al., 1995; Hegde et al., 1997; Fioravante et al., 2008).

UCHL1 is among the most abundant proteins in the brain, by some estimates reaching 1-2% (Jackson and Thompson, 1981; Doran et al., 1983; Wilkinson et al., 1992). Similar to Ap-UCH in *Aplysia*, mammalian UCHL1 is linked to synaptic function. Studies of mouse knockouts of the *Uchl1* gene indicate that this DUB is important for the structure and function of the NMJ. *Uchl1* knockout mice develop normally, but die prematurely after a period of spasticity and paralysis. At the level of the NMJ, *Uchl1* knockouts show a significant decrease in the release of ACh from the synaptic terminal (Chen et al., 2010), which could be due to perturbed Ub-dependent pathways as a result of decreased Ub recycling. This reduction in content release is accompanied by hindered synaptic plasticity, nerve terminal retraction and axonal degeneration (Chen et al., 2010). Supportive evidence for a role for UCHL1 at the synapse also comes from the *gad* (gracile axonal dystrophy) mouse line, which has an intragenic *Uchl1* deletion (Saigoh et al., 1999). Similar to the *Uchl1* knockout mice, ones homozygous for *gad* present with a dying-back type of axonal degeneration. Lastly, studies in rat hippocampal slices also collected evidence that UCHL1 is important for synaptic plasticity by maintaining mono-Ub. Increased UCHL1 activity leads to higher levels of mono-Ub, whereas pharmacological inhibition of this DUB had the opposite effect and is associated with anomalous synaptic spine structure (Cartier et al., 2009). Importantly, anomalies at the synaptic level during UCHL1 inhibition are rescued by the introduction of mono-Ub (Cartier et al., 2009). Together, these data indicate that a primary role for UCHL1 in the nervous system is to maintain mono-Ub available for utilization during inter-cellular communication. This pool of mono-Ub could be utilized by the UPP as well as other types of cellular pathways and processes, such as gene transcription, receptor internalization, autophagy, etc.

UCHL1 is important to the aging nervous system, as highlighted by the connection of this DUB to age-related diseases, including Alzheimer's (AD) and Parkinson's (PD). Based on proteomic studies, UCHL1 is a major target of oxidative damage in AD and PD post-mortem human brains (Choi et al., 2004). UCHL1 protein levels are reduced in the hippocampus of a transgenic mouse model of AD that has learning deficits and impaired LTF (Gong et al., 2006). Similarly, soluble UCHL1 levels are decreased in post-mortem AD brains, potentially due to its sequestration in neurofibrillary tangles (Setsuie and Wada, 2007). Introduction of UCHL1 in transgenic AD mice and in cultured cells alleviates cognitive defects and restores synaptic plasticity in a manner dependent on its catalytic activity (Gong et al., 2006). These and other findings support previously mentioned data that UCHL1 is important at synapses, and suggest that increased UCHL1 activity could counteract certain symptoms in AD.

Other work proposes that UCHL1 has specific substrates and may increase UPP-dependent degradation of the β -site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1). BACE1 sequentially cleaves APP into amyloid β (A β) protein, which is a major component of neuritic plaques, a hallmark of AD. Pharmacological inhibition of UCHL1 in cultured cells leads to an increase in BACE1 protein levels, whereas an increase in UCHL1 levels has the opposite effect and is associated with reduced levels of A β protein. Studies in cultured cells are supported by investigations in *gad* mice. Measuring hippocampal levels of BACE1 by western blotting shows an increase in its protein levels in *gad* mice compared to wild type counterparts (Zhang et al., 2012). Whether UCHL1, with its physically constrained catalytic site, can deubiquitinate BACE1 and how this would accelerate its degradation is presently

unclear. Another possibility is that reduced mono-Ub levels as a result of UCHL1 perturbation inhibits the UPP more generally, which in turn impacts BACE1 stability.

In relation to PD, a missense mutation in UCHL1 (I93M) was described in 1998 as the cause of dominantly-inherited PD in a family (Leroy et al., 1998). In *in vitro* reconstituted assays, UCHL1^{I93M} was found to have reduced DUB activity (Nishikawa et al., 2003), thus it was initially hypothesized that PD could be due to partial loss of UCHL1 activity. However, mice lacking UCHL1 do not develop neurodegenerative hallmarks of PD, such as loss of dopaminergic neurons, indicating that PD in the I93M family might not be due to reduced UCHL1 activity, but could result from a gain-of-function. To examine this latter possibility, transgenic mice were generated expressing *UCHL1*^{I93M}. These mice show loss of nigral dopaminergic neurons, as is characteristically seen in PD, and develop Ub- and UCHL1-positive inclusions, although not Lewy Bodies, which are the histopathological hallmark of PD (Setsuie and Wada, 2007; Setsuie et al., 2007; Tan and Skipper, 2007). According to these results, UCHL1^{I93M} could cause neurodegeneration through a gain-of-function mechanism. The molecular process through which this mutation would impact UCHL1 cellular function, localization or other properties is unclear.

Another report that linked UCHL1 to PD presented evidence that the DUB activity of a farnesylated, membrane-bound form of this protease rescues the PD protein, α -synuclein, from lysosomal degradation (Liu et al., 2009). α -synuclein is implicated in the buildup of aggregated structures in patient brains, and this buildup is believed to be one cause of death of dopaminergic neurons in PD (Kruger et al., 1998; Athanassiadou et al., 1999; Tan and Skipper, 2007). If it is confirmed *in vivo* that farnesylated UCHL1 has a significant role in the turnover of α -synuclein,

it would suggest this DUB as a potential therapeutic target for PD. Severing UCHL1 from the membrane might increase the degradation of α -synuclein, effectively reducing levels of this aggregation-prone protein and alleviating neuronal stress. As it will be discussed later, α -synuclein can be degraded by the proteasome as well as through autophagy. Each type of degradation rests on ubiquitin-dependent processes that are controlled by DUBs such as UCHL1 and USP9X (see below). Consequently, targeting the stability of this disease-linked protein for therapy will require the consideration of various regulatory processes.

Lastly, in 2013 three siblings from a Turkish family were discovered with a recessive, missense mutation in the Ub-binding domain of UCHL1 (mutation E7A), which leads to markedly reduced catalytic activity of this DUB *in vitro*. Patients from this family show early onset progressive neurodegenerative syndrome that includes cerebellar ataxia, spasticity, blindness and nystagmus (Bilguvar et al., 2013). These symptoms are different from those of PD patients, including the ones who carry the I93M mutation. Such phenotypic variability could be due to different effects of the E7A and I93M mutations on the activity and interactions of UCHL1, and might be compounded by other genetic differences between the families.

As we near the end of this section, it is important to note that mutations in two different DUBs, USP14 and UCHL1, which function at least by maintaining mono-Ub, cause problems at the NMJ and neurological defects in mice. Does this mean that they share duties at the molecular, cellular and tissue level? These DUBs are linked through mono-Ub: *Uchl1* transcription is upregulated in *ax^J* mice and, vice versa, *Usp14* transcription is increased in *Uchl1*-deficient mice (Walters et al., 2008), suggesting a molecular circuitry that controls the expression of these genes in response to depleted mono-Ub. However, this upregulation at the

transcription level does not lead to suppression of the phenotype, or restitution of mono-Ub levels. Neurological symptoms differ among *Uchl1*- and *Usp14*-deficient mice, which could be due to differences in genetic background. As discussed above, genetic background can have a profound effect on symptoms and progression of ataxia in *Usp14*-mutant mice (Marshall et al., 2013). Another reason why mutations in USP14 and UCHL1 lead to different phenotypes could stem from partially non-redundant functions. Perhaps there are specific cellular processes (or substrates), populations of neurons (or glia), or stages of development for which one DUB is more critical than the other. While USP14 and UCHL1 have similar effects on the levels of mono-Ub available in neurons, they likely also play divergent roles in other aspects of neuronal homeostasis that remain to be elucidated.

USP7

A member of the USP sub-family of DUBs, USP7 is also known as HAUSP. It has gained attention because it is viewed as a potential therapeutic target for malignancies. Based on numerous studies, USP7 functions closely with the UPP, where it opposes the proteasomal degradation of various substrates, including the E3 Ub ligase murine double mutant 2 (*mdm2*) and its substrate, p53, a tumor suppressor that causes cell cycle arrest and apoptosis (Clague et al., 2013). USP7 deubiquitinates both *mdm2* (which enhances p53 degradation) and p53 (which inhibits p53 degradation) (Li et al., 2002; Li et al., 2004). It is the interplay of p53 and USP7 that may be critical for the nervous system. Through a brain-restricted knockout strategy for *Usp7* in mice, it was shown that this DUB is essential: mice lacking USP7 in the brain die soon after birth and have anomalous brain development, attributed in part to p53 protein stabilization as a result of increased *mdm2* turnover in the absence of USP7 (Kon et al., 2011). p53-independent

mechanisms may also be involved in neonatal lethality, because inactivation of p53 through a knockout strategy fails to fully rescue lethality in the absence of USP7 (Kon et al., 2011).

Another potential role for USP7 in the nervous system is the regulation of the repressor element 1-silencing transcription factor (REST), which inhibits neural cell differentiation. Knockdown of USP7 in neuronal progenitor cells leads to a decrease in the levels of REST protein. USP7 and REST co-immunoprecipitate, and USP7 knockdown results in higher levels of poly-Ub REST, suggesting that USP7 controls REST by deubiquitinating it (Huang et al., 2011). During differentiation, REST is targeted for proteasomal degradation by multiple E3 ligases. The levels of one such ligase, β -TrCP, increase during neuronal differentiation, when levels of USP7 and REST are lower. When both USP7 and β -TrCP are knocked down, an intermediate level of ubiquitinated REST is observed compared to its levels in cells where only one protein is targeted (Huang et al., 2011). According to these results, β -TrCP and USP7 appear to counterbalance each other in regulating the stability of REST. This interaction between USP7 and β -TrCP is reminiscent of the USP7/mdm2 exchange with respect to p53. It is presently unclear whether p53-independent anomalies in brain development as a result of *Usp7* knockout are due to perturbation in REST signaling. Collectively, these studies suggest at least two major pathways that can be regulated by USP7 in the nervous system: neuronal differentiation and cell viability.

USP7 may also regulate other processes in the brain, including in some neurodegenerative diseases. USP7 interacts with the gene transcription protein ataxin-1 (Hong et al., 2002), mutations in which cause the age-related neurodegenerative disease Spinocerebellar Ataxia Type 1 (Williams and Paulson, 2008). The physiological implications of this interaction are uncertain, but suggest the possibility of USP7 roles in stages following nervous system

development. Lastly, recent studies indicated that the above-mentioned transcription factor, REST, is positively involved in normal aging, and its loss may be implicated in AD and mild cognitive impairment (Lu et al., 2014). Considering the role of USP7 in REST turnover during development, one can extrapolate that this DUB may be neuroprotective in the aging brain by suppressing REST degradation.

USP9X

Another member of the USP sub-family of DUBs implicated in the nervous system is USP9X. The *Drosophila* orthologue of USP9X, Faf, has been linked to the development of fly eyes and at the NMJ (Fischer-Vize et al., 1992; DiAntonio et al., 2001). Faf overexpression in *Drosophila* results in NMJ overgrowth, including an increase in synaptic span and the number of synaptic boutons. Faf genetically interacts with the E3 ligase Highwire (Hiw), because loss of function in Hiw results in a phenotype similar to Faf overexpression (DiAntonio et al., 2001). These findings implicate yet another E3 ligase/DUB pair balancing each other's functions, although the molecular events downstream of Faf and Hiw remain to be uncovered.

In fly eyes, Faf prevents over-neuralization during development. Fruit flies lacking this DUB or expressing a version that is catalytically inactive have supernumerary photoreceptors due to aberrant differentiation of cells that normally acquire non-neural fates (Fischer-Vize et al., 1992; Huang et al., 1995). The fate of these cells is specified through Notch-Delta signaling. Faf genetically interacts with Liquid facets (Lqf), an orthologue of mammalian epsins involved in endocytosis (Cadavid et al., 2000; Chen et al., 2002). Lqf is important for Delta internalization during Notch signaling and thus is a critical component of cell fate specification. In *Drosophila*, decreasing Lqf levels enhances the supernumerary photoreceptor phenotype of Faf mutants,

while increasing Lqf levels renders Faf unnecessary (Cadavid et al., 2000; Chen et al., 2002). Ubiquitination of Lqf is stabilized in Faf-less eyes, Faf and Lqf co-immunoprecipitate, and Lqf protein levels are lower in Faf-null mutants (Chen et al., 2002). These findings lead to the conclusion that Faf regulates the ubiquitination status and stability of Lqf: in the absence of Faf, ubiquitinated Lqf could be targeted for proteasomal degradation. It is possible that Lqf ubiquitination also modulates its activity. The ability of human epsins to interact with partners can be regulated by mono-ubiquitination (Nijman et al., 2005; Yi and Ehlers, 2007). Consequently, Faf-dependent deubiquitination of Lqf may control both its function and stability. The human version of Faf, USP9X, also interacts with the Lqf orthologue, epsin-1, and co-localizes with it at the synapse (Chen et al., 2003). Since RNAi against USP9X stabilizes ubiquitinated epsin-1 in cultured cells (Chen et al., 2003), we can infer an evolutionarily conserved role for USP9X in regulating epsin-1 through deubiquitination.

USP9X has been implicated in two neurodegenerative disorders: PD and Diffuse Lewy Body Disease (DLBD). USP9X expression is altered in a mouse model of PD (Zhang et al., 2010), and a portion of USP9X localizes to Lewy Bodies in PD and DLBD (Rott et al., 2011). Supporting the possibility of a role for USP9X in disease, studies have linked this DUB to the stability of α -synuclein, which has been implicated in the etiology of both PD and DLBD. USP9X is reported to regulate the cellular turnover of α -synuclein by deubiquitinating it. RNAi targeting USP9X leads to higher levels of mono-ubiquitinated α -synuclein in cultured cells and these two proteins co-immunoprecipitate from cultured cells and rat brain lysates. Higher levels of mono-ubiquitinated α -synuclein are not well tolerated by cells (Rott et al., 2011), indicating that USP9X activity can protect against toxicity from this protein. By deubiquitinating α -

synuclein, USP9X seems to specify the degradative pathway through which this disease protein is disposed: UPP or autophagy. Unlike other proteins, α -synuclein reportedly only requires mono-ubiquitination to be degraded by the proteasome. However, upon deubiquitination by USP9X, α -synuclein is removed through autophagy (Rott et al., 2011). Perhaps this information on USP9X and α -synuclein can be used for therapeutics for PD and DLBD. Activators of USP9X are expected to reduce levels of mono-ubiquitinated α -synuclein—which is toxic to cells—and to increase its degradation through autophagy, resulting in a neuroprotective effect.

As mentioned above, membrane-bound, farnesylated UCHL1 can also regulate α -synuclein in cell culture by protecting it from lysosomal degradation through a mechanism that remains to be elucidated (Liu et al., 2009). It is tempting to speculate that UCHL1 prevents the lysosomal degradation of α -synuclein by directly deubiquitinating it, but this model would not necessarily fit with the data summarized above on USP9X and the degradation of this disease protein. Potentially, UCHL1 regulates α -synuclein indirectly by controlling other proteins that dictate its turnover, including perhaps USP9X. Ultimately, therapeutic approaches based on the role of USP9X or UCHL1 on α -synuclein need to consider how these DUBs might affect each other's regulatory effect on this aggregation-prone protein.

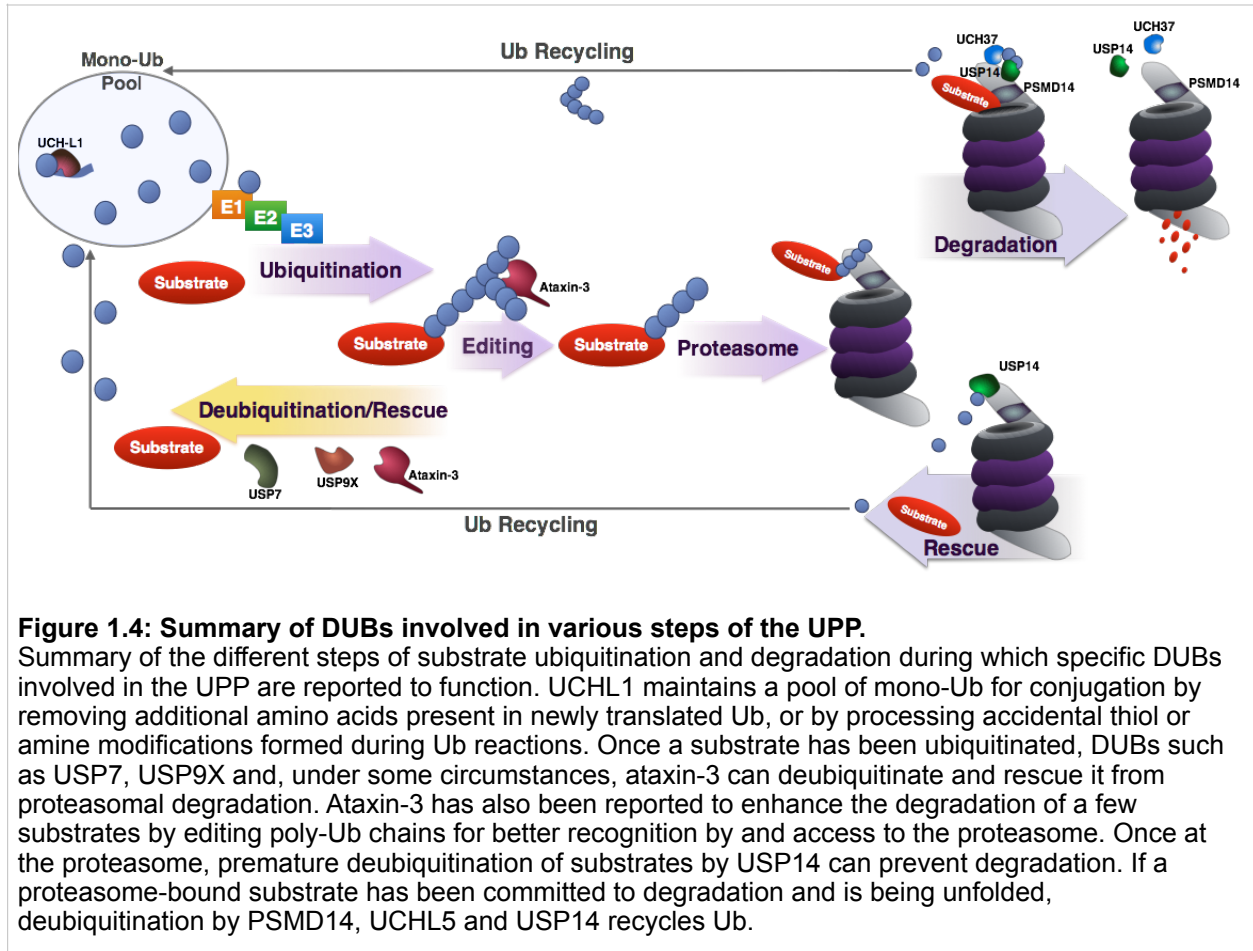
Ataxin-3

Ataxin-3 is a member of the MJD sub-family of DUBs. It first received attention because it is the disease protein in the neurodegenerative disorder Spinocerebellar Ataxia Type-3 (SCA3), also known as Machado-Joseph Disease. SCA3 is an age-related disease that belongs to the family of triplet repeat disorders, more specifically the polyglutamine repeat-related diseases that include Huntington's, Spinobulbar Muscular Atrophy, Dentatorubral-Pallidolusian Atrophy and

five more SCAs (SCA1, 2, 6, 7 and 17) (Todi et al., 2007). SCA3, which is believed to be the most common dominantly inherited ataxia in the world, is a progressive ataxia accompanied by dystonia, dysarthria, spasticity, rigidity, ophthalmoparesis, dysphagia and neuropathy. Pathology includes degeneration of cerebellar pathways and nuclei, pontine and dentate nuclei, substantia nigra, globus pallidus, cranial motor nerve nuclei and anterior horn cells. SCA3 is caused by a CAG repeat expansion in the gene *ATXN3*, which encodes the DUB ataxin-3 (Costa Mdo and Paulson, 2012).

Based on *in vitro* biochemistry, cell-based studies and *in vivo* work in mice, *C. elegans* and *Drosophila*, ataxin-3 seems to function in the UPP (Matos et al., 2011; Costa Mdo and Paulson, 2012). Under some circumstances, ataxin-3 may enhance the degradation of some proteasome substrates (e.g. in ER-Associated Degradation (Wang et al., 2006) and in relation with the ubiquitin ligase CHIP (Scaglione et al., 2011)), while under other conditions it may decelerate proteasomal degradation of other proteins (Zhong and Pittman, 2006). Since ataxin-3 interacts with several E3 ubiquitin ligases (CHIP, E4B, Hrd1, Parkin) and with the proteasome-associated proteins hHR23A, hHR23B and VCP/p97 (Costa Mdo and Paulson, 2012), this DUB may regulate the fate of numerous UPP substrates. The precise outcome of ataxin-3 function—increased or decreased stability of proteins—most likely depends on the protein partners with which it interacts.

Through a series of biochemical assays, it was shown that the E3 Ub ligase CHIP and its E2 partner Ubch5C attach long poly-Ub chains onto model substrates. Ataxin-3 cooperates with CHIP/Ubch5C to restrict or edit the length of these poly-Ub species. This collaboration serves to enhance, rather than prevent, proteasomal degradation of CHIP substrates such as iNOS



(Scaglione et al., 2011), probably because very long poly-Ub can hinder proteasomal activity (Kim et al., 2007; Kim et al., 2009). Thus, in contrast to what was described above for USP7 and mdm2, which can oppose each other's activities, ataxin-3 and CHIP appear to work together to enhance the turnover of at least some proteins. Such collaborative interactions between ligases and DUBs could be common, because a proteomic study of DUBs identified numerous E3 Ub ligases co-precipitating with these proteases (Sowa et al., 2009).

There is also evidence that ataxin-3 can suppress the degradation of some UPP substrates in cells. Work conducted on ER-Associated Degradation indicates that this DUB deubiquitinates some misfolded proteins synthesized in the ER, thus preventing their proteasomal degradation. Ataxin-3 appears to perform this function in relation with the proteasome-associated protein

VCP/p97 (Zhong and Pittman, 2006), although it has not been ruled out that ataxin-3 may also directly oppose the function of ER ligases such as Hrd1 or AMFR.

Three different knockout mouse lines for *atxn3* are viable and appear to live normal lives, indicating that *atxn3* is a non-essential gene (Schmitt et al., 2007; Reina et al., 2010; Switonski et al., 2011). However, this DUB might be required under certain physiological conditions. For example, mouse embryonic fibroblasts that lack ataxin-3 fair poorly during heat shock (Reina et al., 2010; Reina et al., 2012). Also, exogenous ataxin-3 suppresses toxicity from polyQ proteins in *Drosophila* (Warrick et al., 2005; Tsou et al., 2013). Whether ataxin-3 has a protective role *in vivo* in mice is less clear. In one study, evidence was presented that wild type ataxin-3 suppresses pathology from its disease-causing version in mouse models of SCA3 (Cemal et al., 2002). But, in a recent publication lack of ataxin-3 did not seem to enhance pathology in an HD mouse model (Zeng et al., 2013). As this latter study did not test whether higher levels of ataxin-3 had a protective effect, or whether catalytically inactive ataxin-3 worsened the HD phenotype, it remains to be clarified whether this DUB has a neuroprotective effect in mammals.

The molecular mechanisms that lead to polyglutamine-mediated neurodegeneration in SCA3 are uncertain. Based on *in vitro* studies, K48- and K63-linked poly-Ub binding and cleaving capabilities of ataxin-3 are not affected by expansions in the polyglutamine repeat (Burnett et al., 2003; Winborn et al., 2008; Todi et al., 2009), although its ability to cleave K27- and K29-linked poly-Ub is enhanced in the disease-causing version (Durcan et al., 2011). In cells, polyglutamine-expanded ataxin-3 is less efficient at reducing levels of ubiquitinated species, suggesting that some of its cellular roles are affected by expansion (Winborn et al., 2008). Supporting the notion that polyglutamine expansion alters ataxin-3 function, a disease-

causing version of this protease targets the E3 ubiquitin ligase parkin for degradation through autophagy, unlike wild type ataxin-3 (Durcan et al., 2011). Based on studies from other polyglutamine proteins such as ataxin-1 (Lam et al., 2006), polyglutamine repeat expansion may lead to both a partial loss-of- and a toxic gain-of-function for ataxin-3, although this remains to be elucidated. Since ataxin-3 appears to be non-essential in mice, perhaps one effective therapeutic route for SCA3 is to get rid of the protein, potentially without the need to discriminate between normal and pathogenic forms.

1.3 The use of DUBs for therapeutic purposes

The UPP has attracted particular attention from a therapeutic point of view because of its critical role in protein quality control and, consequently, in regulating numerous cellular pathways. Different components of the UPP have been targeted for therapy, most commonly the proteasome itself, although not directly for neurological diseases. Therapies focusing on the proteasome have been developed and have shown promise in the clinic. Bortezomib (PS-341/Velcade®), which inhibits the activity of the 20S component, is a treatment option for multiple myeloma and mantle cell lymphoma (Clague et al., 2013). Another inhibitor, carfilzomib (PR-171/Kyprolis®), was generated to treat patients desensitized to bortezomib. Although both of these inhibitors show preference for killing cancer cells, inhibiting the proteasome itself is largely nonspecific and leads to various undesirable side effects (Colland, 2010; Mujtaba and Dou, 2011). Focusing on more specific steps and components upstream of the proteasome could provide desired therapeutic effects without perturbing general proteostasis. This is where DUBs might be particularly useful. Structural work has revealed significant differences in the catalytic areas of DUBs (Clague et al., 2013) that could be utilized to design DUB-specific inhibitors.

High-throughput screens have identified small molecule inhibitors for USP7 and UCHL1 (Colland, 2010; Todi and Das, 2012), although the efficacy and overall effect of these inhibitors in intact animals, particularly in the nervous system, is not clear.

One DUB that has been targeted therapeutically for the nervous system is USP14. As discussed earlier, this DUB associates reversibly with the proteasome and is proposed to be important in maintaining a pool of mono-Ub for utilization. USP14 may also function by rescuing certain proteins destined for degradation by deubiquitinating them at the proteasome. Cells that do not express USP14 have enhanced clearance of several disease-related proteins, including tau (related to AD), TDP-43 (AD and Amyotrophic Lateral Sclerosis) and ataxin-3 (SCA3, discussed above) (Lee et al., 2010a). This finding suggests that inhibiting the catalytic activity of USP14 may have therapeutic benefits. It is not clear what cellular conditions or substrate particularities dictate the ability of USP14 to rescue some substrates from degradation.

A screen was conducted to identify inhibitors of USP14, and one particularly promising compound was isolated, 1-[1-(4-fluorophenyl)-2,5-dimethylpropyl-3-yl]-2-pyrrolidin-1-ylethanone (abbreviated as IU1), with remarkable specificity against the catalytic activity of USP14 (Lee et al., 2010a). Treatment with IU1 enhances the clearance of disease-linked proteins in cultured cells in a manner that is proteasome dependent (Lee et al., 2010a). A series of *in vitro* and cell-based experiments indicated that trimming of poly-Ub chains by USP14 has an antagonistic effect on protein degradation by the proteasome: proteasomes incubated with USP14 show decreased degradation of a model ubiquitinated substrate compared to inactive USP14, which does not inhibit proteasomal degradation (Lee et al., 2010a). By inhibiting the deubiquitinating activity of USP14, IU1 would presumably prevent the rescue of some

ubiquitinated neurotoxic proteins at the proteasome (such as tau and ataxin-3), leading to their degradation. It remains to be determined if IU1 can prevent neurodegeneration *in vivo*, particularly in light of more recent work, which found that overall levels of ataxin-3 and tau were not different in USP14-deficient mice (Jin et al., 2012). These potential discrepancies in findings from cultured cells and intact animals could result from different handling of ataxin-3 and tau *in vitro* vs. *in vivo*. Potentially, acute inactivation of USP14 can increase the degradation of some disease proteins by the proteasome without perturbing Ub homeostasis, whereas chronic inactivation can cause an overall disturbance in mono-Ub availability that leads to compensatory mechanisms of protein turnover (such as lysosomal-dependent degradation). Additionally, inactivation or depletion of USP14 may not have beneficial effects in all neurodegenerative diseases. USP14 can reduce aggregates formed by mutant huntingtin (which causes Huntington's Disease) and can suppress cellular degeneration caused by this protein (Hyrskyluoto et al., 2014). The molecular mechanism through which USP14 has this neuroprotective effect in cells is not known, but it might involve: 1) mono-Ub availability for proper UPP function, 2) the deubiquitination of huntingtin aggregates, which are heavily ubiquitinated, for better recognition, access and degradation by the proteasome, as well as 3) changes in Endoplasmic Reticulum-Associated Degradation (Hyrskyluoto et al., 2014). Altogether, these findings stress the importance of a deep understanding of the UPP before targeting specific components for blanket therapeutics in neurodegenerative diseases.

Other studies have hinted at potential molecules that could be used to regulate DUB activities in the nervous system. A recent report that investigated the mechanism through which metal complexes inhibit proteasome function for cancer therapy found that copper pyrrithione

(CuPT) acts at two distinct steps: 1) by inhibiting the activity of the 20S component, and 2) by inhibiting USP14 and UCHL5 (Liu et al., 2014). CuPT might be a promising cancer therapeutic by inhibiting UPP and causing cellular death in malignant cells, although its utility in neurodegenerative diseases is doubtful. Inhibiting neuronal UPP would be detrimental to the nervous system and would exacerbate pathology. Still, CuPT might be an important experimental reagent to understand the function of USP14 and UCHL5 in the nervous system, especially if this complex can be modified so that its inhibitory roles on the 20S and USP14 and UCHL5 are dissociated. A molecule with such properties in fact exists: b-AP15, which inhibits the DUB activity of USP14 and UCHL5 (D'Arcy et al., 2011). To the best of our knowledge, neither compound has reportedly been utilized to investigate the nervous system.

1.4 Conclusions

Understanding the functions of enzymes important for the removal of Ub from substrates targeted for UPP-dependent degradation provides a deeper appreciation of a basic cellular process essential to all eukaryotic cells. We hope to have made a convincing case that DUBs that function in conjunction with the UPP play critical roles in nervous system development, function and disease.

The job of the UPP is to identify proteins that need to be degraded and selectively send them to be destroyed by the proteasome through the utilization of an identifier, in this case, ubiquitination. DUBs are among the various checkpoints that ensure the ubiquitination of correct substrates. Some such proteases, like ataxin-3, can function by editing the type of chain attached to a substrate in order to enhance the proteasomal degradation of a specific protein. Others, exemplified by USP7 and USP9X, can oppose the function of specific E3 Ub ligases, leading to

the stabilization of some proteasomal substrates. DUBs such as UCHL1, UCHL5 and PSMD14 seem critical for mono-Ub homeostasis; others, like USP14, control the UPP through multiple, seemingly contradictory, activities: by recycling Ub for reuse, by increasing proteasome function, and sometimes by rescuing specific substrates from degradation (**figure 1.4**).

As there are nearly 95 genes encoding DUBs in humans, one would think that there would be redundancy built into the functions of this family of proteases. However, studies in various animal models indicate that redundancy is not common among DUBs (Clague et al., 2013). Mutations or knockouts of different DUBs lead to distinct developmental anomalies or neurological disorders, supporting the idea that while some DUBs share duties, they also have roles in pathways, cells, tissues, organs and stages of development that cannot be fully compensated by other members of their family.

While we have learned a tremendous amount about the UPP and the role of DUBs in it, much remains to be uncovered, particularly in relation to the nervous system. Each time a door is opened by new research, a spiraling corridor is revealed with more possibilities to consider and opportunities to explore. Details on UPP-related DUBs in specific neuronal circuits are limited. We lack an expression and localization atlas of DUBs during development and in adults, during increased periods of activity and in resting times, in different areas of the brain and spinal cord, in different types of neuronal and glial cells and in distinct sub-cellular compartments. Such information is critical if we are to understand why the perturbation of DUBs that are supposed to have somewhat related functions has different outcomes in intact organisms. Rewards from the continued study of DUBs and UPP in the nervous system are expected to be great, both in terms

of comprehending basic mechanisms of neuronal physiology, and in understanding and treating diseases of the nervous system.

The chapters that follow summarize my thesis work and span two DUBs, USP5 and ataxin-3. In chapter two, I present data from our studies examining the physiological role of USP5 in *Drosophila*, these results have been published. In chapter three, we examine the role that the interaction between pathogenic ataxin-3 and VCP plays in Spinocerebellar Ataxia Type-3 pathogenesis in a *Drosophila* model of the disease.

Table 1.1 - DUBs in the nervous system

UCH	
UCHL1	Lack of UCHL1 causes gracile axonal dystrophy in mice. Mutations have been linked to PD and other diseases. An N-terminal truncation of this DUB may prevent PD-like damage in cultured cells, potentially by reducing oxidative stress. Primary function of this DUB appears to be mono-Ub maintenance.
UCHL3	Mutations in mice cause learning and working memory deficits. Also observed are muscular and retinal degeneration, potentially due to oxidative stress.
UCHL5/UCH37	Reversibly associates with the 19S component of the proteasome and deubiquitinates proteasome substrates. <i>Uchl5</i> knockout mice die during development due to brain malformations. In non-neural tissue, may regulate TGF- β signaling.
USP	
USP2	Involved in regulating the sensitivity to light of the circadian system in mice by deubiquitinating the transcription factor BMAL1. This DUB is upregulated in high-grade gliomas. Pan-neuronal knockdown in <i>Drosophila</i> leads to reduced locomotion and earlier adult lethality.
USP5	Co-purifies with synaptic 26S proteasome from rat cortex. Recycles mono-Ub by hydrolyzing unanchored ubiquitin chains. Pan-neuronal knockdown in <i>Drosophila</i> causes reduced motility and earlier adult death.
USP7	Largely functions by preventing the proteasomal degradation of proteins, including p53 and REST. Brain-specific knockout causes brain malformation and neonatal lethality in mice, due at least in part to p53-dependent mechanisms.
USP8	Pan-neuronal knockdown in <i>Drosophila</i> is developmentally lethal.
USP9X	Involved in neuronal fate specification and NMJ function. Evidenced to regulate the degradation of the neurodegenerative disease protein α -synuclein by deubiquitinating it. May function outside of the UPP.
USP13	Co-purifies with synaptic 26S proteasome from rat cortex.
USP14	Associates reversibly with the 19S proteasome and deubiquitinates proteasome substrates. Primary role in the nervous system appears to be maintenance of mono-Ub for reuse. May deubiquitinate specific substrates, including the neurodegenerative proteins tau and ataxin-3 to suppress their degradation, and the Wnt signaling regulator Dishevelled to regulate its interaction with partners. Mutations in mice cause ataxia and lead to abnormal NMJ structure and function, which is suppressed by reintroducing mono-Ub.
USP18	Knockouts in mice cause tremors, loss of balance, convulsions, and premature death. Anomalies appear to be related to the Ub-like protein ISG15, which USP18 can de-conjugate.
USP22	Its <i>Drosophila</i> ortholog regulates axonal projection of photoreceptor cells.
USP24	May be involved in PD susceptibility, according to gene linkage analysis.
USP25	Overexpressed in human Down syndrome brains. Evidenced to prevent the proteasomal turnover of the AD-related protein APP.
USP30	Opposes parkin-mediated mitophagy by deubiquitinating mitochondria in mammalian cells. Knockdown in <i>Drosophila</i> dopaminergic neurons suppresses PD-like degeneration.
USP33	Regulates axonal pathfinding during development by regulating the stability or surface exposure of the axonal guidance receptor Roundabout.
USP34	Pan-neuronal knockdown in <i>Drosophila</i> is developmentally lethal.

Table 1 | Continued

USP36	Pan-neuronal knockdown in <i>Drosophila</i> leads to reduced locomotion and earlier adult death.
USP39	Glial-restricted knockdown in <i>Drosophila</i> is developmentally lethal.
USP40	May be involved in PD susceptibility, according to gene linkage analysis.
USP46	Mutations in this DUB in mice cause anomalies in circadian rhythm and behavioral abnormalities during stress. In <i>C. elegans</i> , this DUB regulates lysosomal degradation of glutamate receptors.
USP47	Pan-neuronal knockdown in <i>Drosophila</i> leads to reduced locomotion and early adult death.
USP54	Pan-neuronal knockdown in <i>Drosophila</i> is developmentally lethal.
MJD	
Ataxin-3	Mutations in it cause the neurodegenerative disease SCA3. Evidenced to cooperate with several E3 Ub ligases. May assist with the degradation of various UPP substrates. Suppresses polyglutamine-dependent degeneration in <i>Drosophila</i> .
OTU	
OTUB1	Found in Lewy bodies in post-mortem PD brains.
OTUD4	Mutations in this DUB and the E3 Ub ligase RNF216 have been linked to syndromic hypogonadotropic hypogonadism, ataxia, and dementia in humans. Knockdown in zebrafish causes anomalous development of the eye, optic tectum, and cerebellum.
Otulin/Gumby	Mutations in the murine version of this DUB, which cleaves linear Ub chains, cause anomalies in angiogenesis, neuronal, and craniofacial development as a result of perturbed Wnt signaling.
JAMM	
PSMD14	Stoichiometric subunit of the 19S proteasome. Deubiquitinates substrates at the proteasome. Pan-neuronal knockdown in <i>Drosophila</i> leads to lethality during development.
EIF3H	Pan-neuronal knockdown in <i>Drosophila</i> is developmentally lethal.
AMSH	Knockout mice die young. Neuronal loss is observed in the cerebral cortex and the hippocampus.

Detailed information with references can be found in published manuscript, Ristic et. al. 2014.

CHAPTER 2: USP5 IS DISPENSIBLE FOR MONOUBIQUITIN MAINTENANCE IN *DROSOPHILA*

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2.1 Introduction

Ubiquitination is an important post-translational modification of numerous proteins in the cell, where it is involved in the regulation of various processes ranging from DNA transcription to protein degradation. Three different classes of enzymes are responsible for carrying out this modification: E1 (ubiquitin activating enzymes), E2 (ubiquitin conjugating enzymes) and E3 (ubiquitin ligases) (Pickart, 2000). Through the coordinated action of these proteins, a ubiquitin molecule is conjugated most commonly to a lysine residue of a substrate protein through an isopeptide bond. Because ubiquitin itself has seven lysine residues, which are available for isopeptide bond formation, and because ubiquitin moieties can also be connected “head-to-tail”, ubiquitin chains of different conformations are generated (Pickart and Fushman, 2004; Ristic et al., 2014). Different chains impart specific outcomes on the fate of the protein to which they are conjugated. For example, K48-linked ubiquitin targets proteins for proteasomal degradation, whereas K63-linked species have been associated with autophagy and other non-proteasomal-dependent events (Thrower et al., 2000; Komander et al., 2009; Hao et al., 2013; Nathan et al., 2013).

Ubiquitination and ubiquitin recycling are tightly controlled in order to fine-tune the process, to bring a cellular event to an end, to regulate protein fate and to recycle ubiquitin for reuse. The reversal of ubiquitination is carried out by a class of proteases known as

deubiquitinating enzymes, deubiquitinases, or DUBs. DUBs are divided into five families, based on similarity in their catalytic domains: Ubiquitin Specific Proteases, Ubiquitin C-Terminal Hydrolases, Machado-Joseph Disease Proteins, Otubain Proteases and the JAB1/MPN/Mov34 Metalloenzymes. Nearly 100 genes encoding DUBs have been identified in humans, but the functions of many of them remain to be discovered (Komander et al., 2009; Todi and Paulson, 2011; Clague et al., 2012; Clague et al., 2013; Ristic et al., 2014). Ubiquitin Specific Protease 5 (USP5, also known as Isopeptidase T) is one DUB whose structural properties are well understood. USP5 is reportedly an exopeptidase that hydrolyzes isopeptide bonds in poly-ubiquitin from the free carboxy-terminal end to produce mono-ubiquitin, which can then be re-conjugated to substrate proteins (Wilkinson et al., 1995; Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2008). Depletion of USP5 orthologues in yeast and in mammalian cells leads to accumulation of unanchored ubiquitin chains and causes proteasomal inhibition (Amerik et al., 1997). These and other findings place USP5 at the proteasome: before a protein is degraded by the proteasome, the ubiquitin chain signaling its degradation is removed *en bloc* by another DUB, RPN11/POH1, leaving unanchored poly-ubiquitin. USP5 processes this unanchored chain to yield mono-ubiquitin (Clague et al., 2013; Ristic et al., 2014). Thus, this DUB is thought of as a ubiquitin recycler, helping to maintain a mono-ubiquitin pool for reutilization (Komander et al., 2009; Grou et al., 2015; Kovacs et al., 2015). Most ubiquitin is found in conjugated form in various tissues tested, leaving only a small portion available in the unconjugated, mono-ubiquitin pool (Kaiser et al., 2011; Oh et al., 2013; Kovacs et al., 2015). As there is persistent demand for protein modification through ubiquitination, there is a constant need to generate mono-ubiquitin through recycling, or through new synthesis via ubiquitin-encoding genes.

It is not entirely clear whether a primary role for USP5 *in vivo* is mono-ubiquitin maintenance. Here, we tested this possibility in the fruit fly *Drosophila melanogaster*, whose USP5 is necessary during development (Tsou et al., 2012; Wang et al., 2014; Kovacs et al., 2015). Our biochemical and genetic experiments indicate that *Drosophila* USP5 is not necessary for maintaining a ready pool of mono-ubiquitin *in vivo*.

2.2 Materials and Methods

***Drosophila* lines and related procedures:** RNAi-1 and RNAi-2 targeting USP5 were from the Vienna *Drosophila* RNAi Center (VDRC) (Dietzl et al., 2007). These two fly lines contain the same targeting sequence inserted at different chromosomal sites. RNAi-3, the UAS-mono-ubiquitin over-expression line, and the Gal80^{ts} line were from the Bloomington *Drosophila* Stock Center (BDSC). The UAS-CL1-GFP line was a generous gift from Dr. Udai Pandey, University of Pittsburgh, the actin-Gal4 and the sqh-Gal4 lines were a generous gift from Dr. Daniel Eberl, University of Iowa, and the da-Gal4 line was generously donated by Dr. R. J. Wessells at Wayne State University. Flies were maintained at 25°C and ~40-60% humidity in regulated diurnal environments. Where noted in figures and legends, flies were maintained at 18°C and 30°C, ~40-60% humidity under diurnal cycle for Gal80 experiments.

SDS-PAGE, Western Blotting and Quantification: Larvae, pupae, or flies, as indicated in figures and legends, were homogenized in boiling SDS lysis buffer (50mM Tris pH 6.8, 2% SDS, 10% glycerol and 100 mM dithiothreitol (DTT)), sonicated, boiled for 10 minutes, centrifuged for 10 minutes at 13000 X g at room temperature, and loaded onto SDS-PAGE gels, electrophoresed at 160-170 V and transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for western blotting, as previously described (Blount et al., 2012; Tsou et al., 2012;

Blount et al., 2014; Tsou et al., 2015a; Tsou et al., 2015b). 10 larvae, 5 pupae, or 5 adults were collected per group in 15 μ L lysis buffer per larva, 20 μ L per pupa, or 30 μ L buffer per adult. For direct blue staining of PVDF membranes, 0.1% DB71 (Sigma-Aldrich) stock solution in ultra-pure water was dissolved in 40% ethanol and 10% acetic acid solvent to a final concentration of 0.01%, membrane was immersed for 5 minutes, rinsed briefly with solvent, then ultra-pure water, and air dried. Coomassie blue staining was conducted as described before (Scaglione et al., 2011; Scaglione et al., 2013). Western blots were imaged with a charge-coupled device-equipped VersaDoc 5000MP system (Bio-Rad) (Blount et al., 2012; Blount et al., 2014). Quantification of signals from sub-saturated blots was conducted with Quantity One Software (Bio-Rad) with universal background subtraction. Experimental lanes were normalized to their respective controls. Student's *t*-tests (one- or two-tailed, as appropriate) or ANOVA with Tukey's post-hoc correction were used for statistical comparisons.

Antibodies: Anti-Ubiquitin (DAKO rabbit polyclonal 1:500, catalog #Z0458), P4D1 (mouse monoclonal 1:500, Santa Cruz Biotechnology, catalog # SC2017, used only in figure 3C), anti-Tubulin (mouse monoclonal 1:5,000, Sigma-Aldrich, catalog # T5168), anti-Actin (JLA20 mouse monoclonal 1:500, Developmental Studies Hybridoma Bank), anti-HA (Y11, rabbit polyclonal, 1:1000, Santa Cruz Biotechnology, catalog # SC805), anti-GFP (mouse monoclonal, 1:1000, Roche, catalog # 11814460001), anti-CHIP (rabbit monoclonal, 1:1000, Cell Signaling Technology, catalog # 2080S), anti-HSP70 (mouse monoclonal, 1:1000, Rockland, catalog # 200-301-A27), anti-Cyclin A (A12 mouse monoclonal, 1:100, Developmental Studies Hybridoma Bank), anti-Sin3 (rabbit monoclonal, 1:2000), anti-VCP (rabbit monoclonal, 1:1000, Cell Signaling Technology catalog # 2648S) peroxidase-conjugated secondary antibodies (goat

anti-rabbit and goat anti-mouse, 1:5,000; Jackson Immunoresearch). Sin3 antibody was a generous gift from Dr. Lori Pile, Wayne State University (Pile and Wassarman, 2000). The JLA20 and A12 antibodies were procured from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. JLA20 was deposited to the DSHB by Lin, J.J.-C. A12 was deposited to the DSHB by Lehner, C.F.

Cloning and protein purification: DmUSP5 was cloned in two fragments from a *Drosophila* w¹¹¹⁸ cDNA library generated in our lab. Fragment A was PCR-amplified by using forward primer: 5'-GTT TAT GAG AAA AGA GCT GCC TAC A -3' and reverse primer: 5'- CAA TAC TGC TGT ACT TTC CCG ACT -3'. Fragment B was PCR-amplified by using forward primer: 5'- GGC AAC TCC TGC TAC ATA AAC AG -3' and reverse primer: 5'- CGA ATA ATA TTA GCT TGT GGG ACT G -3'. The fragments were ligated and inserted into pCR Blunt II TOPO vector (ThermoFisher). Full length DmUSP5 was then sub-cloned into pGEX6p1 (GE Healthcare). The human USP5 construct was purchased from Addgene, a generous gift of the Arrowsmith Lab (plasmid #25299).

DmUSP5 in pGEX6p1 and human USP5 in pET28 were transformed into BL21 *E. coli*. Individual colonies were grown at 37°C overnight in LB with ampicillin or kanamycin, as needed. 10 mL was used to inoculate 500 mL of LB and was grown for an additional 3 hours at 37°C. Protein expression was induced by 0.5mM of isopropyl-1-β-D-galactopyranoside (A.G. Scientific) for 2 hours at 30°C. DmUSP5 was purified by GST-pulldown. Bacterial cells were pelleted by centrifugation and resuspended in NETN lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 0.5% NP40), sonicated, then centrifuged for 20 minutes at 4°C. 250μL of glutathione-

sepharose beads (GE Healthcare) were washed with NETN, lysates were added to beads and tumbled at 4°C. Beads were then washed with NETN buffer thrice followed by two PBS washes. Prescission Protease (GE Healthcare) with 1% DTT was used to elute the bead-bound protein. HIS-tagged human USP5 was purified with nickel beads. Pelleted bacterial cells were resuspended in Buffer A (50mM Tris, pH 7.5, 150 mM NaCl), sonicated and centrifuged. 400µL of Nickel nitrilotriacetic acid beads (Qiagen) were washed in Buffer A and incubated with lysates. Beads were then rinsed twice each with Buffers A, B (50 mM Tris, pH 7.5, 1M NaCl, 20mM Imidazole) and C (50mM Tris, pH 7.5, 100mM NaCl, 20mM Imidazole, 0.5% TX-100). Protein was eluted with Buffer C containing 300mM imidazole and 1% DTT. Catalytically inactive DmUSP5 was created using the QuikChange mutagenesis kit (Agilent) (Blount et al., 2014). The catalytic cysteine at position 341 was mutated to an alanine. Recombinant protein concentration was determined using NanoDrop and coomassie blue staining of SDS-PAGE gels.

***In vitro* reactions:** 50 nM of recombinant DmUSP5 or human USP5 was added to kinase buffer (0.5M Tris pH 7.5, 0.5M KCl, 0.2% DTT) with 1 µM of ubiquitin chains with specific linkages for a total reaction volume of 60 µL. Reactions were incubated at 37°C for human USP5 or 25°C for fly USP5 and 15 µL were taken from each reaction at the indicated times points. Reaction was stopped by the addition of 10 µL sample loading buffer and boiled for two minutes. DUB reactions were also repeated at 37°C for *Drosophila* USP5, with similar results to those at 25°C. Ubiquitin chains were purchased from Boston Biochem.

CHIP and HSP70 ubiquitination was carried out as previously described (Scaglione et al., 2011; Scaglione et al., 2013), for 1 hour at 37°C in 100 µL kinase buffer. Ub^{mix} (2.5 mM ATP, 2.5 mM MgCl₂, 100 nM Ube1 and 250 µM ubiquitin), 1 µM E2 (Ube2w for mono-ubiquitinated

CHIP, or UbcH5c for poly-ubiquitinated CHIP and HSP70), 1 μ M CHIP, and 1 μ M HSP70. Reactions were stopped by the addition of excess EDTA, and 50 nM (final) DmUSP5 or human USP5 was added to the complex. Reaction time points were separated on 4-20% poly-acrylamide gels and analyzed by western blotting.

Quantitative, Real-Time PCR: Total RNA was extracted from larvae or pupae using TRIzol reagent (Invitrogen), followed by treatment with TURBO DNase (Ambion) to eliminate contaminating DNA. High Capacity Kit (ABI) was used to perform reverse transcription. Messenger RNA levels were quantified with PlusOne real-time quantitative system using Fast SYBR Green (ABI). rp49 was used as a control. All qRT-PCR primers are listed in table 2.1.

2.3 Results

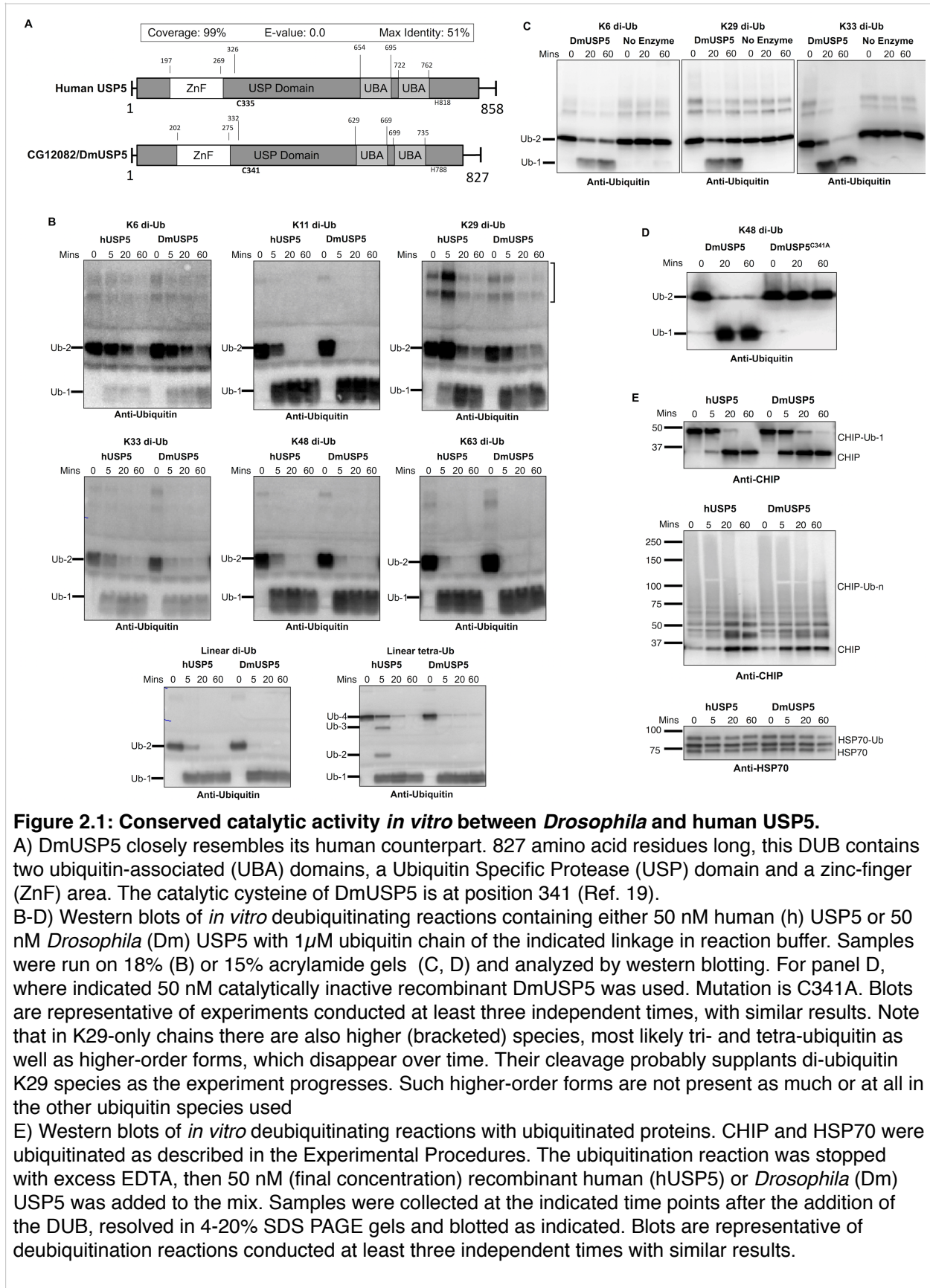
2.3.1 *Drosophila* and human USP5 disassemble unanchored ubiquitin chains similarly *in vitro*

CG12082 is the *Drosophila* gene whose product most closely aligns with mammalian USP5 (Tsou et al., 2012). Like its human counterpart, *CG12082* contains the Ubiquitin Specific Protease (USP) domain, the Ubiquitin Associated Domains (UBAs) that bind to ubiquitin moieties in poly-ubiquitin chains, and a zinc finger-like region (Falquet et al., 1995; Wilkinson et al., 1995; Raasi et al., 2005; Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2008; Avvakumov et al., 2012) (**figure 2.1A**).

Recombinant, human USP5 has been reported to hydrolyze poly-ubiquitin chains of different linkages *in vitro* (Falquet et al., 1995; Wilkinson et al., 1995; Raasi et al., 2005; Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2008; Avvakumov et al., 2012). We began our studies of *Drosophila* USP5 (DmUSP5) by comparing ubiquitin chain cleavage preferences between it and the human counterpart *in vitro*. We carried out deubiquitination reactions at 37°, optimal for

human USP5, and 25°C, optimal for *Drosophila* USP5, although the same results were also obtained at 37°C (**figure 2.1 and data not shown**). We observed that DmUSP5 and human USP5 both hydrolyze unanchored poly-ubiquitin chains of different linkages (**figure 2.1B**). The recombinant proteases have different proficiencies: K11, K48, K63 and linear (head-to-tail) species were cleaved more rapidly than K6- and K29-linked di-ubiquitin. Both DUBs were also able to cleave rapidly di- as well as tetra-ubiquitin chains (**figure 2.1B**). We noticed that as K6 and K33 di-ubiquitin disappear, mono-ubiquitin does not always seem to mirror the reduction of the substrate species (**figure 2.1B**). This is not because of indiscriminate cleaving of ubiquitin, e.g. from the presence of non-ubiquitin proteases, as the chains are stable over time in the absence of DmUSP5 (**figure 2.1C**); instead, it might result from reduced antibody affinity toward ubiquitin with only specific lysines present, which could affect epitope exposure/recognition in di- vs. mono-ubiquitin. Based on these data, we conclude that the *Drosophila* and human USP5 enzymes have similar catalytic affinity toward most ubiquitin linkages. As shown in figure 1D, the catalytic cysteine at position 341 of the *Drosophila* USP5 is necessary for its protease activity.

There is evidence that mammalian USP5 can disassemble not only unanchored chains, but that it can also deubiquitinate specific substrates (Scaglione et al., 2011; Garcia-Caballero et al., 2014). We thus examined whether DmUSP5 and human USP5 could remove ubiquitin from ubiquitinated, recombinant proteins in an *in vitro* setting. We used a previously published protocol to ubiquitinate CHIP (an E3 ubiquitin ligase) and HSP70 (the molecular chaperone (Scaglione et al., 2011)). As shown in figure 1E, both human and *Drosophila* USP5 deubiquitinate mono-ubiquitinated and poly-ubiquitinated CHIP, but do not affect ubiquitinated



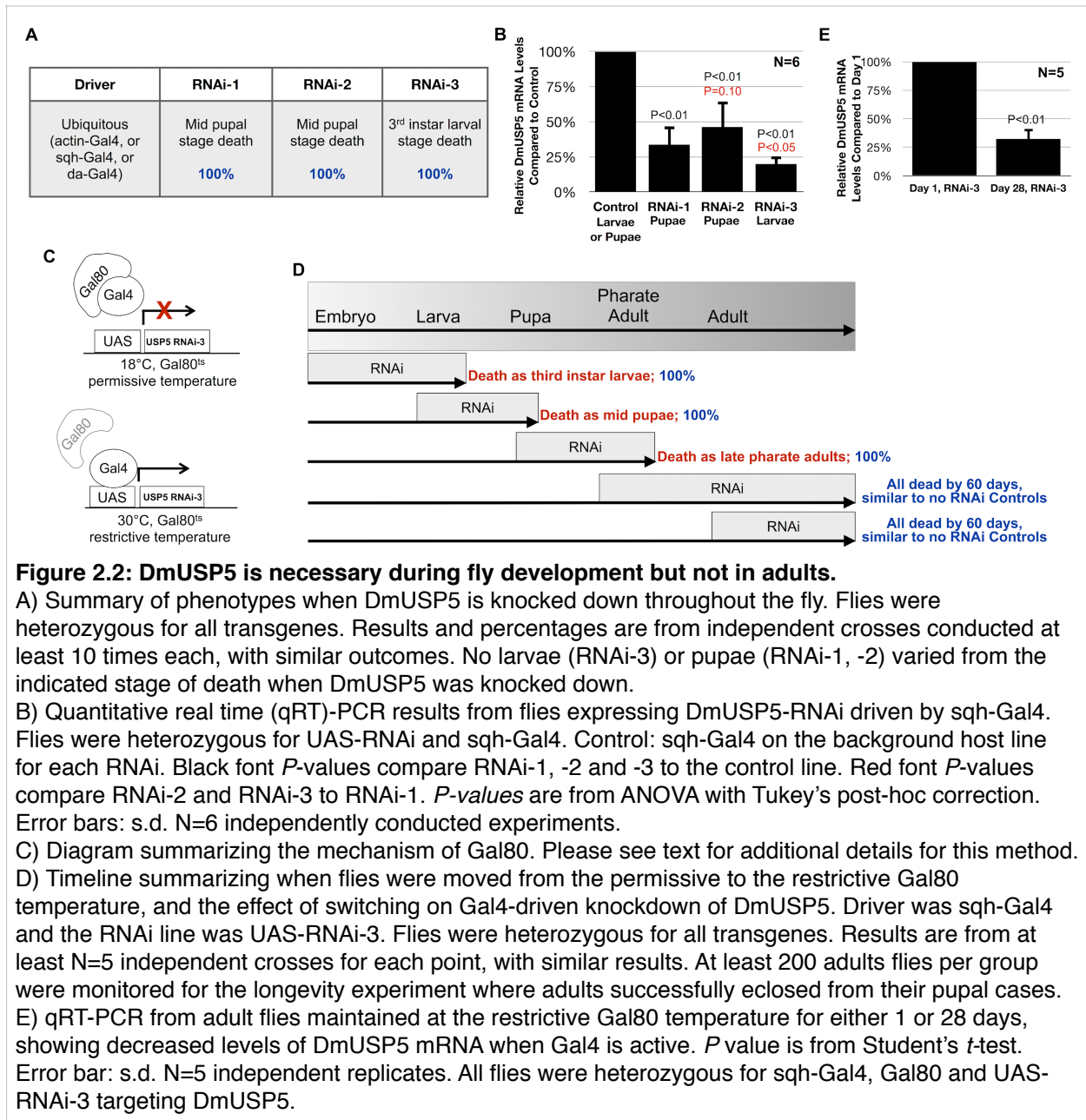
HSP70. These findings support the notion that USP5 can act on specific substrates.

2.3.2 *DmUSP5, important during fly development, is dispensable in adults*

In order to progress toward our major goal, to determine whether DmUSP5 is required for mono-ubiquitin maintenance *in vivo* in *Drosophila*, we next tested the importance of this DUB during development and in adults. Our group and others previously reported that RNA-interference (RNAi)-dependent knockdown of DmUSP5 and mutations in its gene lead to developmental lethality in the fruit fly (Tsou et al., 2012; Wang et al., 2014; Kovacs et al., 2015). To examine whether this DUB is also important in adults, we employed the power of the Gal4-UAS system (Brand and Perrimon, 1993).

We began by testing the efficacy of various RNAi lines targeting DmUSP5. DmUSP5 is expressed throughout the fly (Wang et al., 2014); therefore, we targeted this protease in the whole the organism by using the ubiquitous drivers sqh-Gal4, actin-Gal4, or da-Gal4, with similar results. We used three different RNAi lines specific for DmUSP5 that target different sites of its mRNA. RNAi-1 and -2 were generated through P-element-mediated insertions of a UAS-based transgene (Dietzl et al., 2007), whereas RNAi-3 was generated through site-specific recombination by using the phiC31 integrase (Groth et al., 2004).

Knocking down DmUSP5 in the whole fly using ubiquitous drivers causes third instar larval lethality with RNAi-3 and mid pupal lethality with RNAi-1 and -2, compared with their respective host line controls (**figure 2.2A**). Death at third instar larval stage is the same as observed with DmUSP5 null mutation (Wang et al., 2014). The level of knockdown obtained by the ubiquitous sqh-Gal4 driver was determined through quantitative real-time (qRT)-PCR and is shown in figure 2.2B. We consistently observe a more pronounced phenotype with RNAi-3



compared with the two other RNAi lines. This is most likely due to RNAi-3 reducing the levels of DmUSP5 mRNA more strongly than RNAi-1 and -2 (**figure 2.2B**).

While the importance of DmUSP5 during development is clear, it has not been reported before whether this DUB is also required in later stages, including adults. We next utilized a ubiquitously-expressed, temperature-sensitive Gal80^{ts} construct (McGuire et al., 2003) to control

when UAS-RNAi-3 targeting DmUSP5 is expressed (**figure 2.2C**). Gal80 binds to Gal4 and prevents this transcription factor from driving the expression of UAS-based transgenes, in this case, UAS-RNAi-3. When flies containing the Gal80 construct alongside RNAi-3 and the ubiquitous sqh-Gal4 driver are placed at the permissive temperature (18°C), Gal80 impedes RNAi-3 expression. When flies are moved to the Gal80 restrictive temperature (30°C), sqh-Gal4 can drive expression of UAS-RNAi-3.

We began by examining the effect of DmUSP5 knockdown when RNAi-3 targeting its mRNA is enabled on from the beginning (**figure 2.2D**); these flies die as third instar larvae, in accordance with data in figure 2.2A. Induction of USP5 knockdown beginning at larval and pupal stages results in death during pupal and pharate adult stages, respectively (**figure 2.2D**). However, when developing flies are moved to the restrictive Gal80 temperature as soon as they enter the pharate adult stage, these flies eclose normally and live a life span not different from those of their control siblings. When adults are moved to the restrictive temperature as soon as they eclose from the pupal case, they also survive and live a life span not different from controls that do not express RNAi-3 (**figure 2.2D**). Figure 2E confirms reduction of DmUSP5 mRNA when Gal80 is restricted. Based on these findings, USP5 is required during development, but is not critically important in adults.

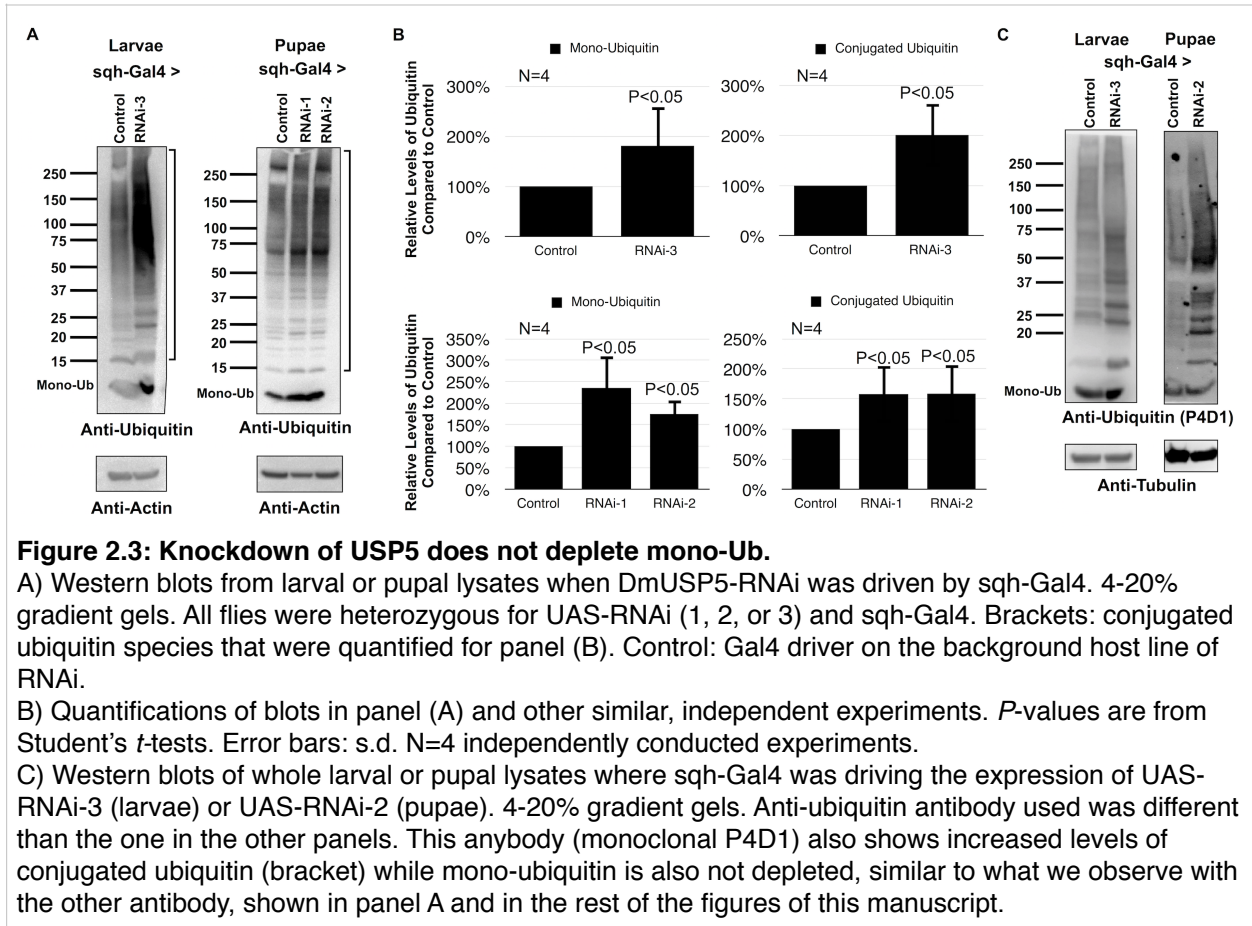
2.3.3 DmUSP5 knockdown leads to increased conjugated ubiquitin species without depleting mono-ub

USP5 is considered to be primarily a ubiquitin chain dismantler for the purpose of mono-ubiquitin recycling (Komander et al., 2009; Ristic et al., 2014; Grou et al., 2015; Kovacs et al., 2015). To examine the effect of DmUSP5 knockdown on overall ubiquitin species in developing

flies, we performed western blotting from whole larval and pupal lysates and probed for ubiquitin (**figure 2.3A**).

Lysates were prepared from developing flies harvested before death. Larvae were from crosses where UAS-RNAi-3 was driven by sqh-Gal4, while pupae were from UAS-RNAi-1 and -2, also driven by sqh-Gal4. Compared with their controls, containing sqh-Gal4 on the respective host background of the UAS-RNAi transgenes, but without DmUSP5 knockdown, we observe increased levels of conjugated ubiquitin (**brackets in figure 2.3A; quantified in 2.3B**). Interestingly, we do not observe depletion of mono-ubiquitin alongside higher levels of conjugated ubiquitin (**figure 2.3A, 2.3B**). In fact, we observe increased levels of mono-ubiquitin when DmUSP5 is knocked down. The important point, however, is that DmUSP5 knockdown in the fruit fly does not lead to decreased levels of mono-ubiquitin. We used two different anti-ubiquitin antibodies to assay conjugated and mono-ubiquitin in fly lysates, with similar results (**figure 2.3A, 2.3C**, and other supportive data not shown). Based on these outcomes, knockdown of DmUSP5 does not lead to a reduction of mono-ubiquitin in developing flies.

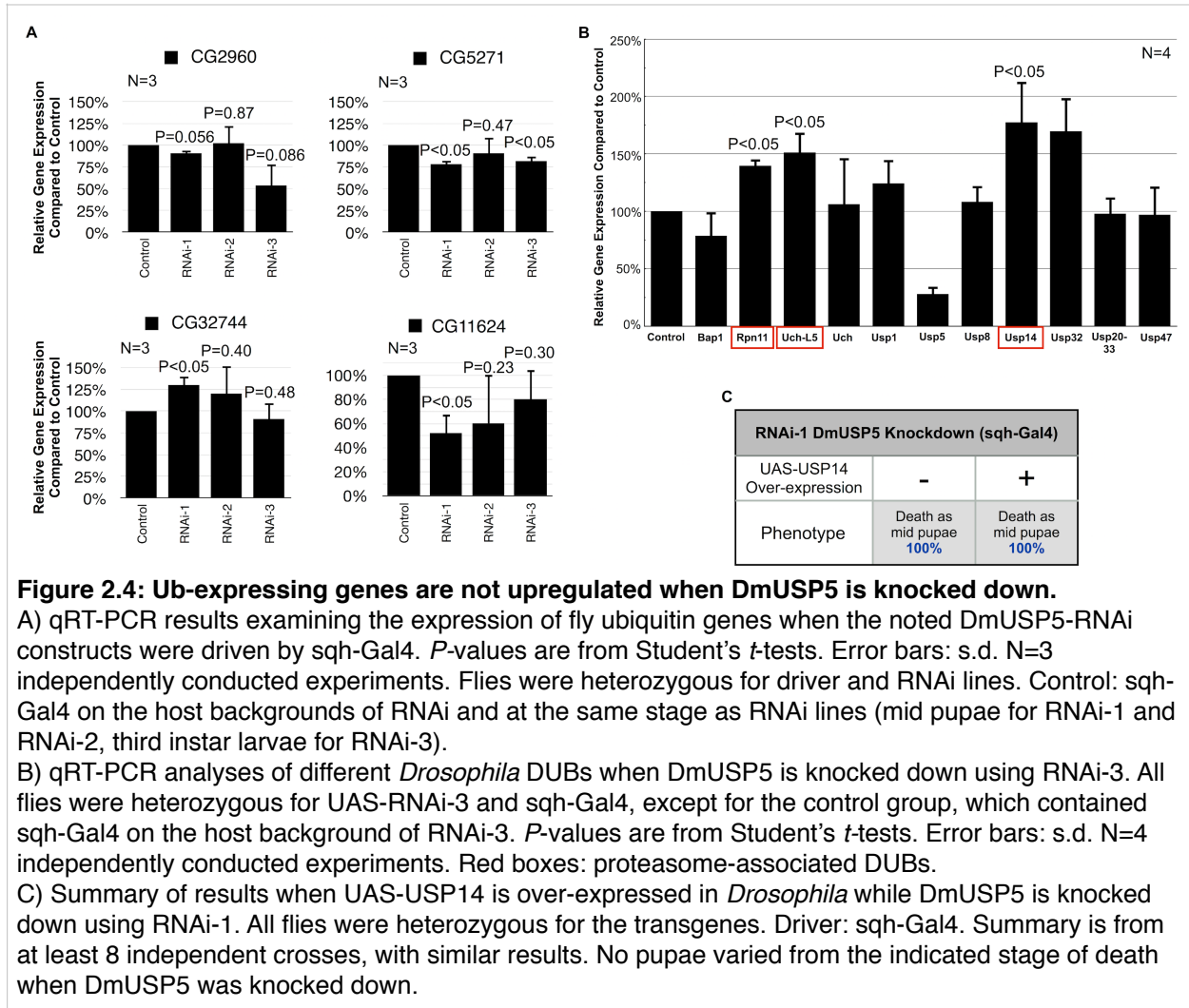
Intrigued by a lack of depletion of mono-ubiquitin when DmUSP5 is knocked down, we examined why this may be the case. Mono-ubiquitin is maintained through *de novo* synthesis from genes that encode it as a fusion protein to additional ubiquitin moieties or to other proteins, and by being cleaved from ubiquitin chains or substrates (Grou et al., 2015). We wondered whether knockdown of DmUSP5 leads to increased transcription of ubiquitin-encoding genes in *Drosophila*. We assessed by qRT-PCR the levels of different ubiquitin-encoding genes in the fly: the mono-ubiquitin encoding genes CG2960 and CG5271, which encode ribosomal fusion proteins RpL40 and RpS27A, respectively, and two poly-ubiquitin encoding genes, CG32744



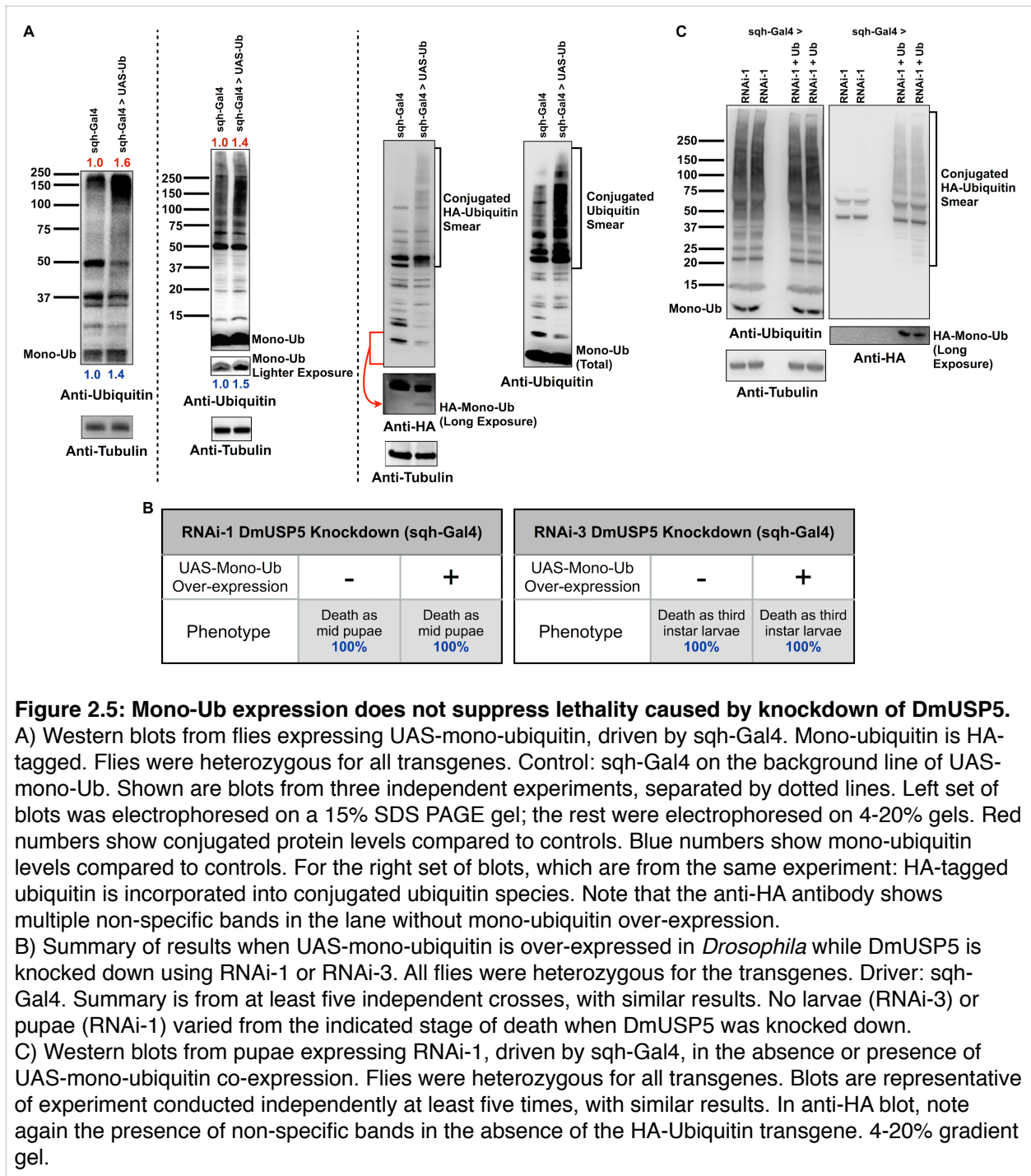
and CG11624. Another gene, CG11700, which closely aligns with CG32744 (Lu et al., 2013), was assayed through CG32744 primer sets. We identified these genes by using the FlyBase resource, through our own alignments with mammalian ubiquitin-encoding genes through BLASTp, and based on previously published work (Lu et al., 2013). Based on qRT-PCR results, there is no overall increase in ubiquitin gene expression when DmUSP5 is knocked down everywhere in the fly (**figure 2.4A**). In fact, in some instances we observe a statistically significant decrease in ubiquitin gene expression. These data suggest that lack of depletion of mono-ubiquitin when DmUSP5 is knocked down is not due to increased ubiquitin production at the transcript level.

Next, we examined the expression levels of other DUBs that have been linked to mono-ubiquitin maintenance and that function, at least in part, by associating with the proteasome, where DmUSP5 is also proposed to act: USP14, RPN11, and UCH-L5 (Tsou et al., 2012). Based on qRT-PCR, reduced DmUSP5 mRNA levels coincide with mildly, but statistically significantly, increased levels of USP14, RPN11 and UCH-L5 (**figure 2.4B**). The levels of expression of these DUBs during fly development and in adults vary from moderate to very high, similar to the expression of DmUSP5 (transcriptional data on FlyBase.org). This could suggest that some DmUSP5 functions might be complemented by increased transcription of these other DUBs, even though ultimately they fail to suppress lethality caused by the absence of DmUSP5 during development. Increased transcription of endogenous USP14, a DUB demonstrated to maintain mono-ubiquitin in mice (Anderson et al., 2005; Chen et al., 2009; Chen et al., 2011; Hallengren et al., 2013), may account for mono-ubiquitin not being depleted when DmUSP5 is knocked down in developing flies. When we tested if exogenous USP14 alleviates lethality caused by knockdown of DmUSP5, we found that this was not the case (**figure 2.4C**): USP14 over-expression alongside ubiquitous DmUSP5 knockdown results in developmental lethality at the same stage as DmUSP5 knockdown without exogenous USP14. Over-expression of USP14 by itself is not detrimental to the fly (data not shown).

Lastly, we used a UAS-based, HA-tagged mono-ubiquitin transgenic fly line to over-express wild type ubiquitin (Lee et al., 2009). Expression of ubiquitin through this line leads to an overall increase in levels of conjugated ubiquitin in intact flies (**figure 2.5A**). Importantly, as shown in the right portion of figure 2.5A, the HA-tagged mono-ubiquitin that we over-express is conjugated in flies (also see **figure 2.5C**). Mono-ubiquitin expression simultaneously with



DmUSP5 knockdown, however, does not affect the stage at which developing flies die (**figure 2.5B**). Thus, lethality caused by DmUSP5 knockdown in *Drosophila* is unlikely to be due to lack of mono-ubiquitin available for conjugation to various proteins. As shown in figure 5C, exogenous ubiquitin does not lead to an appreciable difference in ubiquitin species observed by western blotting when DmUSP5 levels are reduced. Collectively, the data in figures 3-5 lead us to conclude that DmUSP5 is not necessary for mono-ubiquitin maintenance in *Drosophila*.



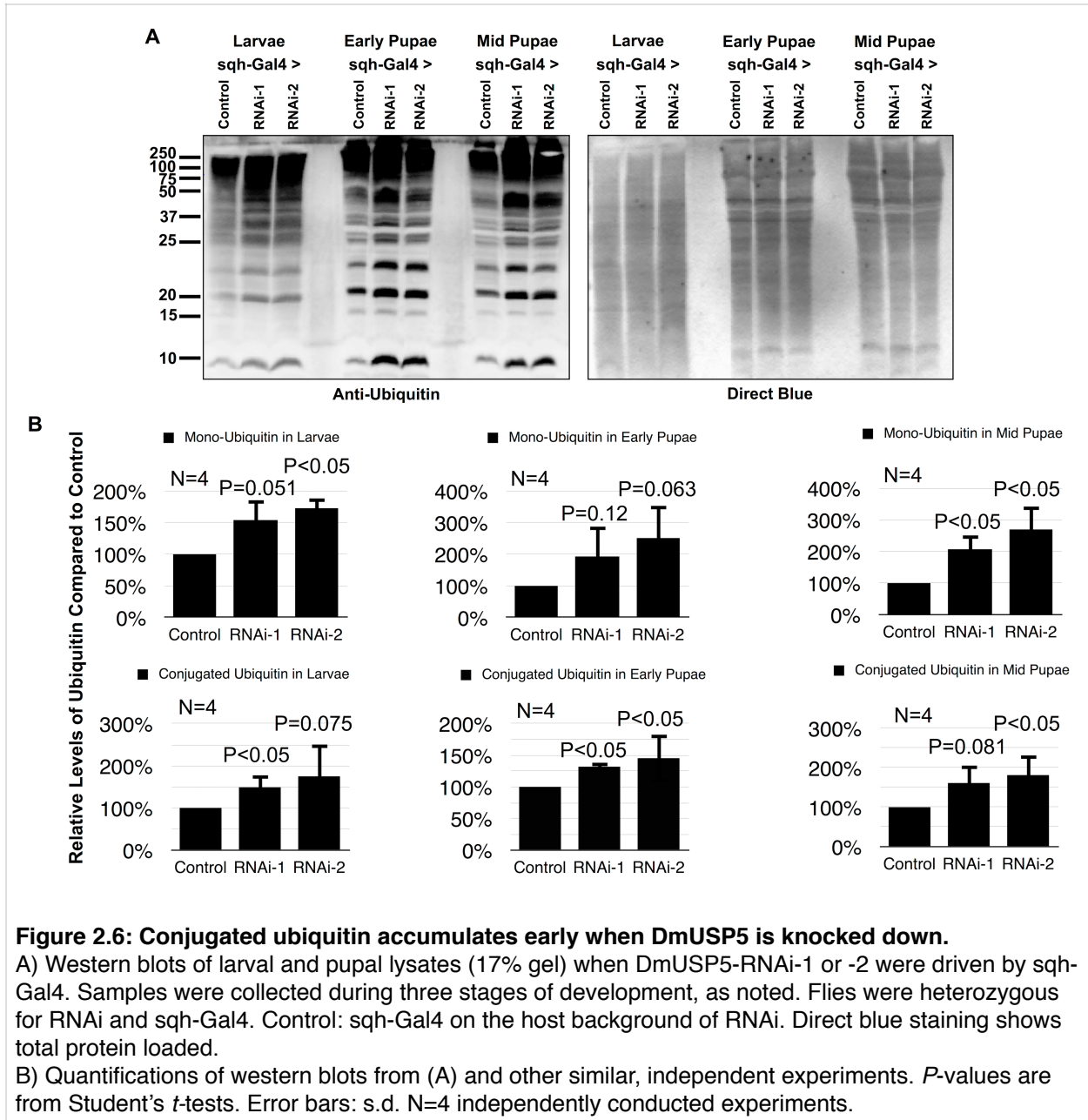
2.3.4 Accumulation of conjugated ubiquitin species during DmUSP5 knockdown is an early event

Results shown in the previous figures were from samples collected preceding larval (RNAi-3) or pupal (RNAi-1, -2) death when DmUSP5 is knocked down. In order to examine

more temporally the changes in ubiquitin species when DmUSP5 levels are reduced, we analyzed larvae and pupae of different stages when DmUSP5 is knocked down through RNA-1 or -2, which cause pupal death. As summarized in figure 2.6, when DmUSP5 is knocked down accumulation of conjugated ubiquitin species begins at least as early as third instar larvae and continues through early and mid pupal stages, when they die. This increase in conjugated species is accompanied by an increase in mono-ubiquitin that appears more robust over time (**figure 2.6**).

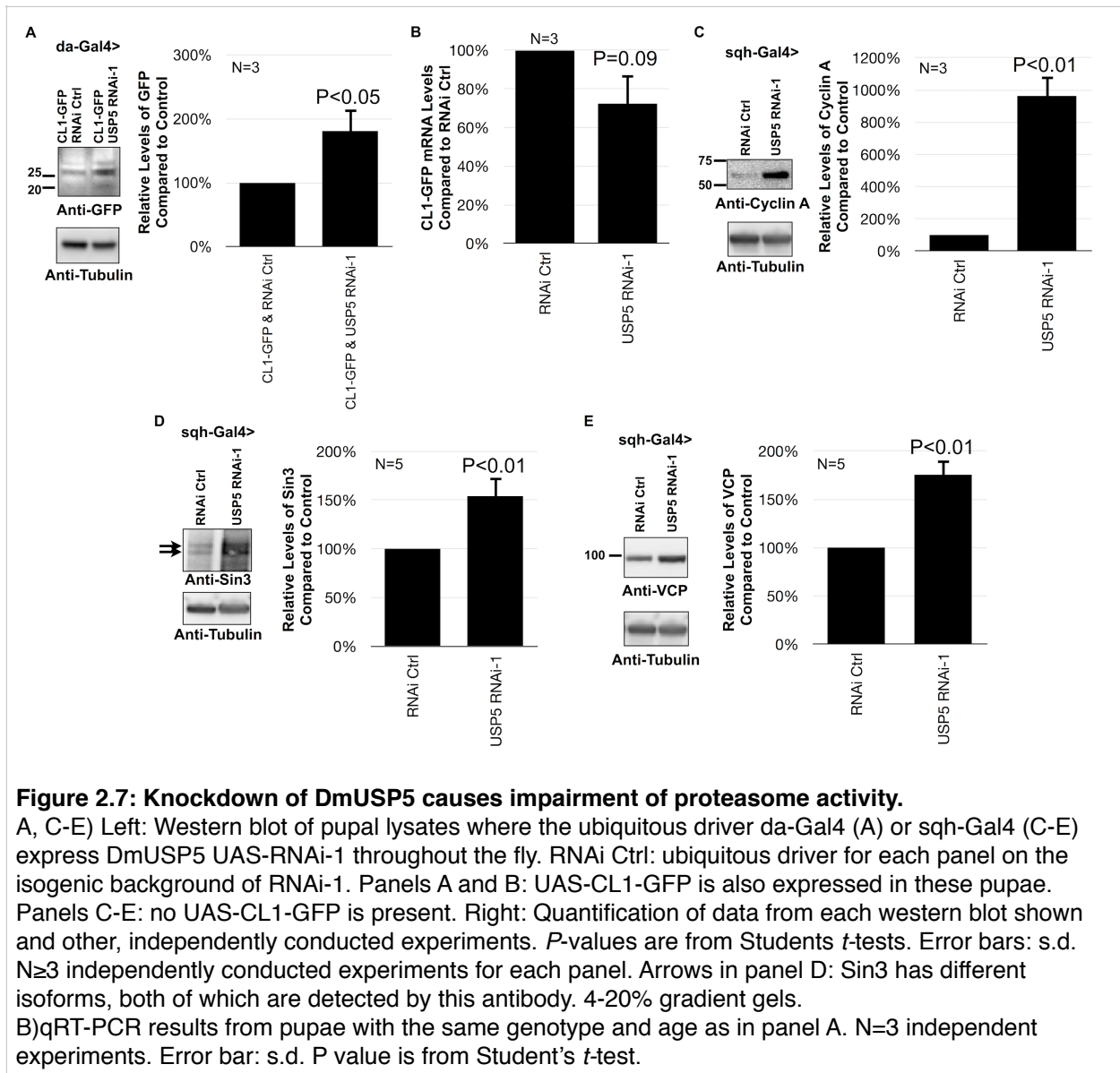
2.3.5 DmUSP5 knockdown leads to impaired proteasome activity

Excess ubiquitin chains can compete for proteasomal binding with substrates destined for degradation, thereby impairing ubiquitin-dependent proteasome activity (Beal et al., 1996; Amerik et al., 1997; Thrower et al., 2000). As shown in earlier figures, there is an accumulation of conjugated ubiquitin species that results from knocking down DmUSP5 throughout the fly. Could these species coincide with impaired proteasome function? To examine this possibility, we used a reporter of ubiquitin-dependent proteasome function, CL1-GFP. This construct consists of a GFP moiety fused to the CL1 degron, a signal which targets GFP for proteasomal degradation through ubiquitination (Bence et al., 2001; Bennett et al., 2005). We used the ubiquitous driver, da-Gal4, to express the reporter throughout the fly while simultaneously knocking down USP5 with RNAi-1. da-Gal4 also leads to death at mid pupal stages with RNAi-1 and -2, similar to what we observe with the other driver, sqh-Gal4. As the blots in figure 2.7A show, there is an increase in CL1-GFP protein level when DmUSP5 is knocked down compared with its respective, no knockdown control. Increased levels of CL1-GFP protein when DmUSP5 is



reduced are not due to higher CL1-GFP mRNA (**figure 2.7B**), indicative of impaired ubiquitin-dependent proteasomal activity.

We then examined the levels of endogenous proteins that are proteasome substrates: cyclin A (Chen et al., 2004), and Sin3 (whose orthologue in mammals is degraded by the proteasome, (Kong et al., 2010)). None of these proteins has been linked to DmUSP5 so far. We also tested the protein levels of VCP/p97, whose expression is induced when proteasome



function is reduced (Lundgren et al., 2005). As indicated by data in figure 2.7C-E, knockdown of DmUSP5 leads to statistically significantly higher levels of each of these endogenous proteins in developing flies. Based on data in figure 2.7, we conclude that ubiquitin-dependent proteasome activity is inhibited by reduced levels of DmUSP5 in *Drosophila*.

2.4 Discussion

Ubiquitination is a critical regulator of most cellular pathways. Therefore, the process of protein ubiquitination and ubiquitin availability both are carefully monitored (Pickart, 2000;

Pickart and Fushman, 2004; Shabek and Ciechanover, 2010; Ristic et al., 2014). Important regulators of protein ubiquitination are the DUBs, one of which, USP5, has been proposed to function in part by maintaining mono-ubiquitin (Komander et al., 2009; Ristic et al., 2014; Grou et al., 2015; Kovacs et al., 2015). Here, our primary question was whether the fly orthologue of this protease is important for mono-ubiquitin recycling *in vivo*. We found that this is not the case in *Drosophila*.

Prior work conducted on DmUSP5 delivered some information on its role on mono-ubiquitin levels. One study (Wang et al., 2014) found either increased levels or non-depleted mono-ubiquitin in DmUSP5 mutants in *Drosophila*. However, whether this meant that fly USP5 is dispensable for mono-ubiquitin recycling was not clarified. Another study observed increased levels of conjugated ubiquitin, while the levels of mono-ubiquitin did not appear to be consistently depleted when the gene was mutated in the fruit fly (Kovacs et al., 2015). Both of these studies arrived at a similar conclusion: USP5 is important for ubiquitin homeostasis in *Drosophila*. The issue raised in our present work, whether USP5 is needed for mono-ubiquitin recycling in *Drosophila*, required further investigation.

We presented evidence that *Drosophila* USP5, which functions similarly to its human counterpart *in vitro*, is required during fly development but seems dispensable in adults. These findings correspond well with previously published reports by others and us, which showed that this DUB is required in developing flies: ubiquitous knockdown and knockdown in select tissues (e.g. neurons and glial cells) during development has negative effects (Tsou et al., 2012; Wang et al., 2014; Kovacs et al., 2015).

In reconstituted systems, both human and fly USP5 efficiently cleave ubiquitin chains linked head-to-tail and through K11, K48 and K63. K11- and K48-linked ubiquitin have been connected to the degradation of proteins by the proteasome, while K63-linked chains have been associated with other pathways, including DNA repair, endosomal recycling and autophagy (Ikeda and Dikic, 2008; Nathan et al., 2013). Our *in vitro* data also hint at the possibility that USP5 may have specific substrates. This DUB was able to rapidly and efficiently deubiquitinate at least one recombinant, ubiquitinated protein in a reconstituted system, but not a second. There have been other reports of mammalian USP5 acting on specific substrates *in vitro* and *in vivo* (Scaglione et al., 2011; Garcia-Caballero et al., 2014).

While we found a clear importance for the presence of this DUB during larval and pupal stages, USP5 did not seem important when it was knocked down only during adult stages, even though it is well expressed in adults, based on transcriptional data aggregated on Flybase.org. If DmUSP5 is acting on specific substrates, perhaps they are not as critical in adults as in earlier stages. Alternatively, expression of other DUBs may at this point complement some of the USP5 functions that they could not in larval and pupal stages. We hope to identify specific DmUSP5 substrates in the near future through quantitative mass spectrometry.

Our collective genetic and biochemical experiments indicate that the function of DmUSP5 in flies is not to maintain mono-ubiquitin. Perhaps it is useful to compare our results with data from work conducted with another DUB, USP14, which is important for maintaining mono-ubiquitin in the nervous system of mice (Anderson et al., 2005; Crimmins et al., 2006; Chen et al., 2009; Chen et al., 2011; Hallengren et al., 2013). Mutations in USP14 lead to reduced mono-ubiquitin protein levels and increased transcription of ubiquitin-encoding genes

(Chen et al., 2011). Importantly, introduction of wild type mono-ubiquitin alleviates some of the phenotypes caused by loss of USP14 (Chen et al., 2011). In our case, depletion of DmUSP5 does not lead to lower levels of mono-ubiquitin (in fact, we see a trend of increased mono-ubiquitin at the protein level), transcription of fly ubiquitin-encoding genes is not increased, and introduction of exogenous ubiquitin does not suppress the lethality effect of DmUSP5 knockdown. Thus, our work argues that DmUSP5 is not critically needed to maintain the mono-ubiquitin pool *in vivo* in *Drosophila*.

qRT-PCR results hint that maintenance of mono-ubiquitin when DmUSP5 is knocked down may result from increased transcription of other DUBs that can yield mono-ubiquitin, such as USP14. Other investigators also described increased levels of various DUBs when DmUSP5 was knocked down or absent in the fly (Wang et al., 2014; Kovacs et al., 2015). Upregulation of these other DUBs, however, appears unable to suppress lethality caused by DmUSP5 knockdown or to normalize the levels of conjugated ubiquitin species that we observe in western blots. These findings suggest a highly regulated and flexible system for DUB expression *in vivo*, while also underscoring the non-redundant roles that these proteases play in intact organisms, as we also described before (Tsou et al., 2012).

Excess conjugated ubiquitin may hinder ubiquitin-dependent proteasomal degradation of proteins by competing for binding to the 19S proteasome. Based on our work with the reporter CL1-GFP and the endogenous proteins cyclin A, Sin3 and VCP/p97, proteasome function is indeed reduced when DmUSP5 is knocked down throughout the fly.

Our work leads us to propose that a primary function of DmUSP5 in *Drosophila* is the disassembly of conjugated ubiquitin, which helps, at least in part, to maintain proteasome

activity. When this protease is unavailable, increased transcription of other DUBs could assist with the processing of some conjugated ubiquitin to recycle mono-ubiquitin. However, these proteases appear unable to fully complement DmUSP5 activities. We suggest that as the proteasome is inhibited by species that would have been disassembled by DmUSP5, conjugated and mono-ubiquitin levels increase as ubiquitinated substrates and ubiquitin (itself a proteasome substrate, (Shabek and Ciechanover, 2010)) are degraded less rapidly. Ultimately death ensues, probably due to proteasomal inhibition.

In summary, we presented evidence that DmUSP5 is not required for mono-ubiquitin maintenance *in vivo*. Based on our additional work that DmUSP5 has wide, although not universal, ubiquitin chain linkage preferences, it will be of interest to continue to examine the various pathways and processes in which this developmentally required DUB is involved. In the next chapter, we move on to the second DUB starring in this thesis, ataxin-3.

Table 2.1 DUB Primers for qRT PCR

Target	Forward Primer 5'-3'	Reverse Primer 5'-3'
rp49	AGATCGTGAAGAAGCGCACCAAG	CACCAGGAACTTCTTGAATCCGG
bap1/ calypso	CAACAACAGTCTCAGCCACAAT	GACGAATATCTCAGCAGTTGTCTC
rpn11	CTCAATCGCCACTACTACTCGAT	CATAACCTTGTCCACCTTCTCCT
uch	GTGCCGGTAATTGTGTGTAGAA	GAATCCATTCCAAGGTGTCATC
uch-15/ uchl3	ACGGTGCTGGAAATTGGTGTCTCATTG	AAGAAGATGTTCTCCCGATCCAG
usp1	ACCTTGCGCTGCTACAGTCTAC	GCTCTTGAGCCTCTTCAATTCT
usp5	GTACGAGATCAAGGACACGTACAG	GTCAGATTAAGCCAGAGGTTGTTG
usp8	GCATTACAAGTCACCAACACCTTC	CCAGATTCTTCAGTCCAGTCAGT
usp14	ACTCCTGTCAAATTCATTGAGGAC	CAAATATAGACTTCATGGCAGACG
usp32	TTGATCTGTTCTACGGCCAGTT	AGTACTTGCAATCGGAGTTCAGAC
usp20-33	CTTGTGGAGTACATAGCAGAGCAG	CTGCTGCTGAAGTGACTGGTATT
usp47	ACGTATTCCCATCAAATTCTGTC	TCGCTGATCTGTGATAATGAAAGT
CG2960	CACACTCTCTTTTCCCTTTTTCTTT	GTGGACTCCTTCTGAATGTTGTAGT
CG5271	CGCACCTCTCTGACTATAACATT	CCGTTCTCGTCAACCTTGTAGTAT
CG32744	GGACGTCCGAGCAAGTAAAA	ATGGCTCAACCTCCAAAGTG
CG11624	CTTCGTCTCCGTGGTGGTAT	AGGGTGGACTCCTTCTGGAT
CL1-GFP	ACGTAAACGGCCACAAGTTC	AAGTCGTGCTGCTTCATGTG
Alternative DmUSP5 primers used, with similar results:		
usp5	GGCGGCCAAATACGTAAATA	CGTCCTTGTAGATGGGAGGA
usp5	CTCCGCACCTGGATAAGAAG	CGGTCAGATTAAGCCAGAGG
usp5	TCGGATGTGTTCTCCTACCC	CCATCGACTTCTCGCTCTTC

CHAPTER 3: VCP MODULATES ATAXIN-3'S AGGREGATION AND TOXICITY IN A *DROSOPHILA* MODEL OF SPINOCEREBELLAR ATAXIA TYPE-3

Chapter 2 highlighted the physiological importance of USP5 in *Drosophila*, in this chapter we switch gears to ataxin-3.

3.1 Introduction

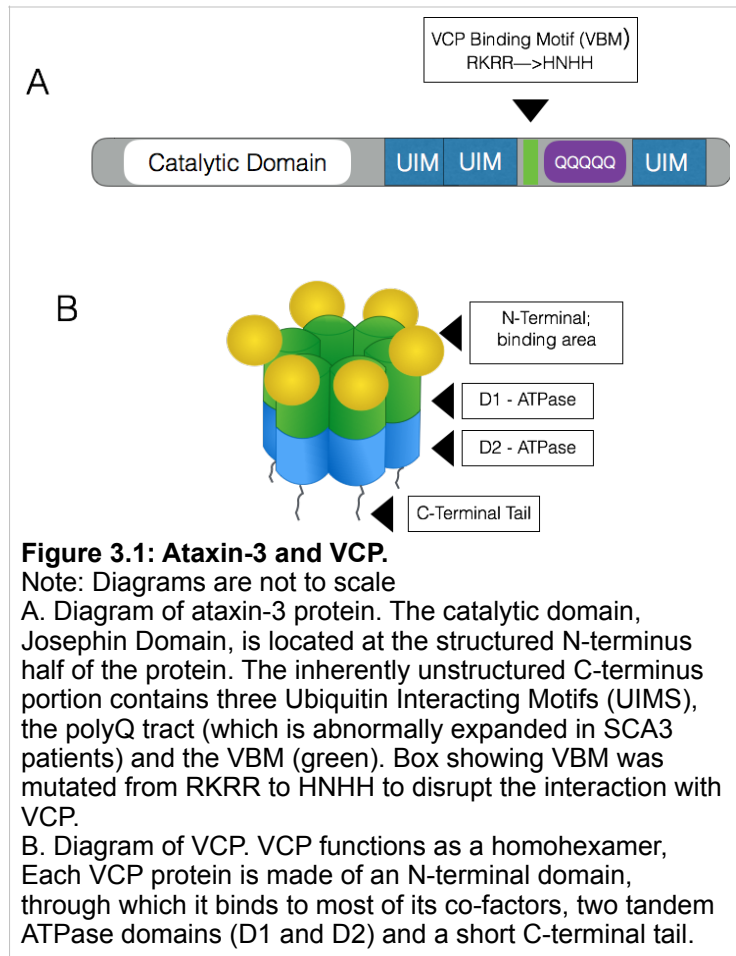
Spinocerebellar Ataxia Type-3 (SCA3, or Machado Joseph Disease) is an age-related, neurodegenerative disease that is the most common, dominantly inherited ataxia in the world. SCA3 belongs to the family of polyglutamine (polyQ) diseases along with six other SCAs (1, 2, 6, 7 and 17), Huntington's Disease, Spinal and Bulbar Muscular Atrophy and Dentatorubropallidoluysian Atrophy (Todi et al., 2007; Zoghbi and Orr, 2009; Costa Mdo and Paulson, 2012). These diseases are characterized by abnormally expanded CAG triplet repeats in the respective genes, which leads to expanded polyQ tracts in their proteins. Expanded polyQ repeats in these proteins cause their misfolding and the formation of insoluble aggregates (Todi et al., 2007; Zoghbi and Orr, 2009; Costa Mdo and Paulson, 2012). SCA3 is caused by an expansion of the CAG repeat of the *ATNX3* gene which encodes the protein ataxin-3 (**figure 3.1A**). There is no cure for SCA3; only minimally effective symptomatic therapies exist in the clinic (Costa Mdo and Paulson, 2012).

Ataxin-3 is a deubiquitinating enzyme that has been linked to protein quality control with various partners. One such partner is Valosin Containing Protein (VCP, also known as p97), a member of the AAA ATPase (ATPases associated with diverse cellular activities) family of proteins (Meyer et al., 2012). VCP contains two tandem ATPase domains, D1 and D2, an N-terminal domain which is responsible for binding to most of its co-factors and a short C-terminal tail (**figure 3.1**) (Xia et al., 2016). It functions as a homohexamer, converting chemical energy

harvested from ATP hydrolysis to mechanical energy to exert force on its substrates. It is an essential, ubiquitously expressed protein in mammalian cells. It is localized mainly in the cytoplasm of the cell but also exists in the nucleus to a lesser extent (Madeo et al., 1998; Xia et al., 2016). VCP is involved in numerous cellular processes including protein degradation (Dai et al., 1998; Buchberger et al., 2010; Meyer et al., 2012; Kim et al., 2013; Xia et al.,

2016). Ataxin-3 and VCP have been implicated in endoplasmic reticulum associated degradation (ERAD) (Zhong and Pittman, 2006; Morreale et al., 2009).

The *Drosophila* homolog of VCP, Ter49, modulates toxicity caused by polyQ expansion in generic polyQ models in the fly. Higashiyama et al. reported that higher levels of VCP exacerbates toxicity in fly eyes, whereas loss of function mutations improved degeneration caused by an isolated polyQ repeat (Higashiyama et al., 2002). Several reports demonstrated that ataxin-3 and VCP interact; one study mapped this interaction to the C-terminus of the DUB, preceding the polyQ repeat (**figure 3.1**) (Hirabayashi et al., 2001; Boeddrich et al., 2006; Fujita et al., 2013). According to biochemical and cell-based experiments, ataxin-3 binds to the N -



terminal portion of VCP through an arginine-rich area (amino acid sequence RKRR) (Boeddrich et al., 2006). Mutating the VBM (VCP-binding motif) on ataxin-3 disrupts the interaction between these two proteins (Boeddrich et al., 2006). The same group also showed that VCP stimulates fibrillogenesis of non-pathogenic, wild type ataxin-3 in a dose dependent manner in *in vitro*, reconstituted systems (Boeddrich et al., 2006). Mutating the VBM on pathogenic ataxin-3 in *Drosophila* also decreased degeneration caused by this protein in fly eyes. However, it was not clear from the earlier studies whether VCP regulates the aggregative propensity of pathogenic ataxin-3 *in vivo*; also, the relation of this direct interaction to the temporal aggregation and pathogenicity of ataxin-3 in an intact animal was not studied. Here, we set out to understand, by using *Drosophila* as a model organism, the importance of the ataxin-3-VCP interaction in the aggregation and pathogenicity of the SCA3 protein, with the hope of elucidating the targetability of this binding site for SCA3 therapeutics. Toward this end, we generated new, isogenic lines of *Drosophila melanogaster* that express in a Gal4-UAS-dependent manner (Brand and Perrimon, 1993; Brand et al., 1994; Sutton et al., 2017) full-length pathogenic ataxin-3 with a normal or mutated VBM. We found that pathogenic ataxin-3 with a mutated VBM is markedly less toxic *in vivo* in flies, even though the protein is abundantly present. Initially, flies that express the SCA3 protein with a mutated VBM are indistinguishable from controls that do not express ataxin-3. This coincides with lower levels of aggregated ataxin-3 species. With age, flies that express ataxin-3 with a mutated VBM also develop structural and physiological phenotypes, but with a timeline markedly slower than flies that express the intact, pathogenic variant of this protein. Altering the levels of endogenous VCP protein modulates the toxicity of the SCA3 protein in a manner that indicates a nucleation role for the aggregation of ataxin-3 by this AAA ATPase.

Collectively, our data highlight an important physiological role for VCP on ataxin-3-dependent toxicity, and implicate this interaction as a valuable target for SCA3 therapeutics.

3.2 Materials and Methods

***Drosophila* lines:** Husbandry was conducted at 25°C on standard cornmeal food at 40-60% humidity in regulated diurnal environments. SCA3 flies expressing pathogenic ataxin-3 with 77-80 polyQ repeats were previously generated by cloning into the pWalium10-moe vector (Sutton 2017). HNHH mutations were generated on the ataxin-3 backbone using the QuikChange mutagenesis kit (Aglient) and used to create HNHH pathogenic ataxin-3 encoding flies using this construct cloned into pWalium10-moe vector. Injections (Duke University Model System) were into y,w;+;attP2. Common fly stocks were procured from the Bloomington *Drosophila* Stock Center; isogenic host strain attP2 (#36303), VCP RNAi lines were stocks (#32869, #35608).

Western blotting: Five whole flies or 15 dissected heads per group were homogenized in hot lysis buffer (50mM Tris pH 6.8, 2% SDS, 10% glycerol, 100mM dithiothreitol), sonicated, boiled for 10 minutes, and centrifuged at top speed at room temperature for 10 minutes. Western blots were developed using charge-coupled device-equipped VersaDoc 5000MP system (BioRad). Blots were quantified using Quantity One software (BioRad). For direct blue staining PVDF membranes were submerged for 5 min in 0.1%Direct Blue 71 (Sigma-Aldrich) stock solution in ultra pure water, dissolved in 40% ethanol and 10% acetic acid solvent. Membrane was rinsed briefly in solvent, then ultra pure water, air dried and imaged.

Nuclear Cytoplasmic extraction: Experiments were conducted using the ReadyPrep Protein Extraction Kit (Cytoplasmic/Nuclear) (BioRad) to enrich cytoplasmic and nuclear proteins. 5 whole flies were lysed in cytoplasmic extraction buffer ((CPEB), BioRad). Nuclei were

resuspended in Protein Solubilization Buffer ((PSB), BioRad). Samples were run on 4-20% gradient gel (BioRad) and analyzed by western blot.

Antibodies: Anti-Ataxin-3 monoclonal (clone 1H9, mouse, 1:500-1000; Milipore), anti-tubulin (mouse monoclonal T5168, 1:10,000; Sigma-Aldrich), anti-VCP (valosin-containing protein; rabbit monoclonal, 1:1000, Cell Signalling Technology), anti-lamin (ADL84.12-5 mouse monoclonal, 1:1000, Developmental Studies Hybridoma Bank), peroxidase conjugated secondary antibodies (goat anti-mouse and goat anti-rabbit, 1:5,000; Jackson Immunoresearch)

Motility Assay (Negative Geotaxis): Motility was tested once a week for a total of four weeks. 10 flies per vial were forced to bottom of vial by light tapping on the bench. The number of flies that reached that top of the vial at 5, 15 and 30s was recorded. Flies were transferred to new vial 1h before assessment, and every 2-3 days for duration of the experiment.

Longevity Assay: Approximately 25 flies per vial were collected after eclosion from the pupal case and aged on conventional cornmeal fly media or RU-486 containing media at 25°. Flies were switched to vials with fresh food every 2-3 days. Each vial was checked for death daily, until all flies were dead.

Histology: Fly proboscises and wings were removed before fixing over night in 2% gluteraldehyde/2% paraformaldehyde in Tris-buffered saline with 0.1% Triton X-100. Fixed flies were dehydrated in series of 30, 50, 70, 100% ethanol washes and washed in propylene dioxide over night. Whole fly bodies were embedded in Poly/Bed812 (polysciences), sectioned at 5µm and stained with toluidine blue.

Quantitative PCR: Total RNA was extracted from larvae using TRIzol (Life Technologies). Extracted RNA was treated with TURBO DNase (Ambion). Reverse transcription was carried

out using the high capacity cDNA reverse transcription kit (ABI). mRNA levels were quantified using the StepOne Real-Time PCR system with Fast SYBR Green Master Mix (ABI).

Primers:

SCA3 F: 5'-GAATGGCAGAAGGAGGAGTTACTA- 3'

SCA3 R: 5'-GACCCGTCAAGAGAGAATTCAAGT- 3'

rp49 F: 5' -AGATCGTGAAGAAGCGCACCAAG- 3'

rp49 R: 5' -CACCAGGAACTTCTTGAATCCGG- 3'

Co-Immunoprecipitation: 30-50 fly heads per group were homogenized through mechanical disruption in PBS+PI, briefly sonicated and then supplemented with the equal volume of NETN +PI. Lysates were vortexed briefly every 5 minutes for a total of 30 minutes, centrifuged at 5,000 X g for 5 minutes and supernatant was transferred to 30 μ L slurry of anti-HA bead-bound antibody (Sigma-Aldrich). Beads with lysates were tumbled at 4°C for 4 hours, rinsed thrice with NETN+PI, and bead-bound complexes were eluted with heat and Laemmli buffer.

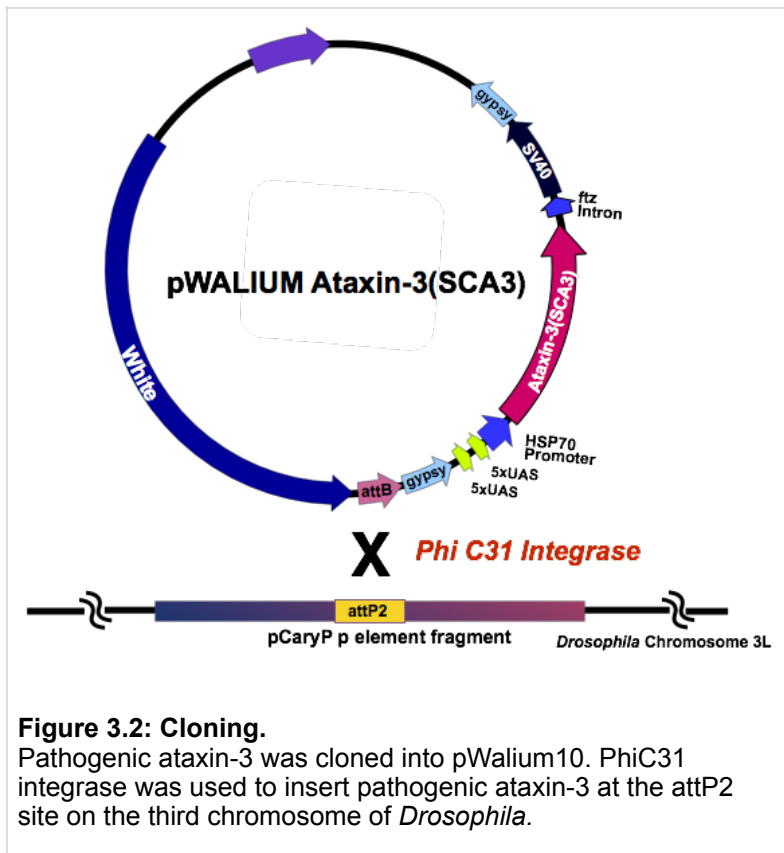
3.3 Results

3.3.1 Mutating the VBM of ataxin-3 does not alter its protein or mRNA levels, or its subcellular localization

To begin studying the role that the interaction of ataxin-3 and VCP plays in the pathogenesis of the polyQ disease Spinocerebellar Ataxia Type-3 (SCA3), we created new transgenic *Drosophila* lines expressing ataxin-3 with 80 polyQ repeats, which is within the pathogenic range of SCA3. The lines were made by cloning this ataxin-3 transgene into the pWalium10-moe vector, which was used with the phiC31 site-specific integrase system to insert the construct at the attP2 site on the third chromosome of the fly (Groth et al., 2004; Markstein et

al., 2008; Tsou et al., 2016) (**figure 3.2**). These flies are referred to as ‘Intact ataxin-3’ in our studies. Boeddrich et. al. previously showed that ataxin-3 and VCP directly bind through the VBM domain on ataxin-3 and that mutating the VBM from RKRR to HNHH disrupts the interaction between ataxin-3 and VCP (Boeddrich et al., 2006). We created another transgenic fly line which encodes the same pathogenic ataxin-3 construct inserted at the same site on chromosome 3 of the fly, but in which the VBM on ataxin-3 was mutated from RKRR to HNHH (HNHH fly line) by site-specific mutagenesis. These flies are referred to as HNHH 1 and 2, as we utilized two independently derived lines.

Before beginning our studies, we wanted to confirm that any difference in phenotype we observe among the different lines is due to the disrupted interaction between ataxin-3 and VCP and not because of a change in ataxin-3 expression levels. We examined mRNA expression

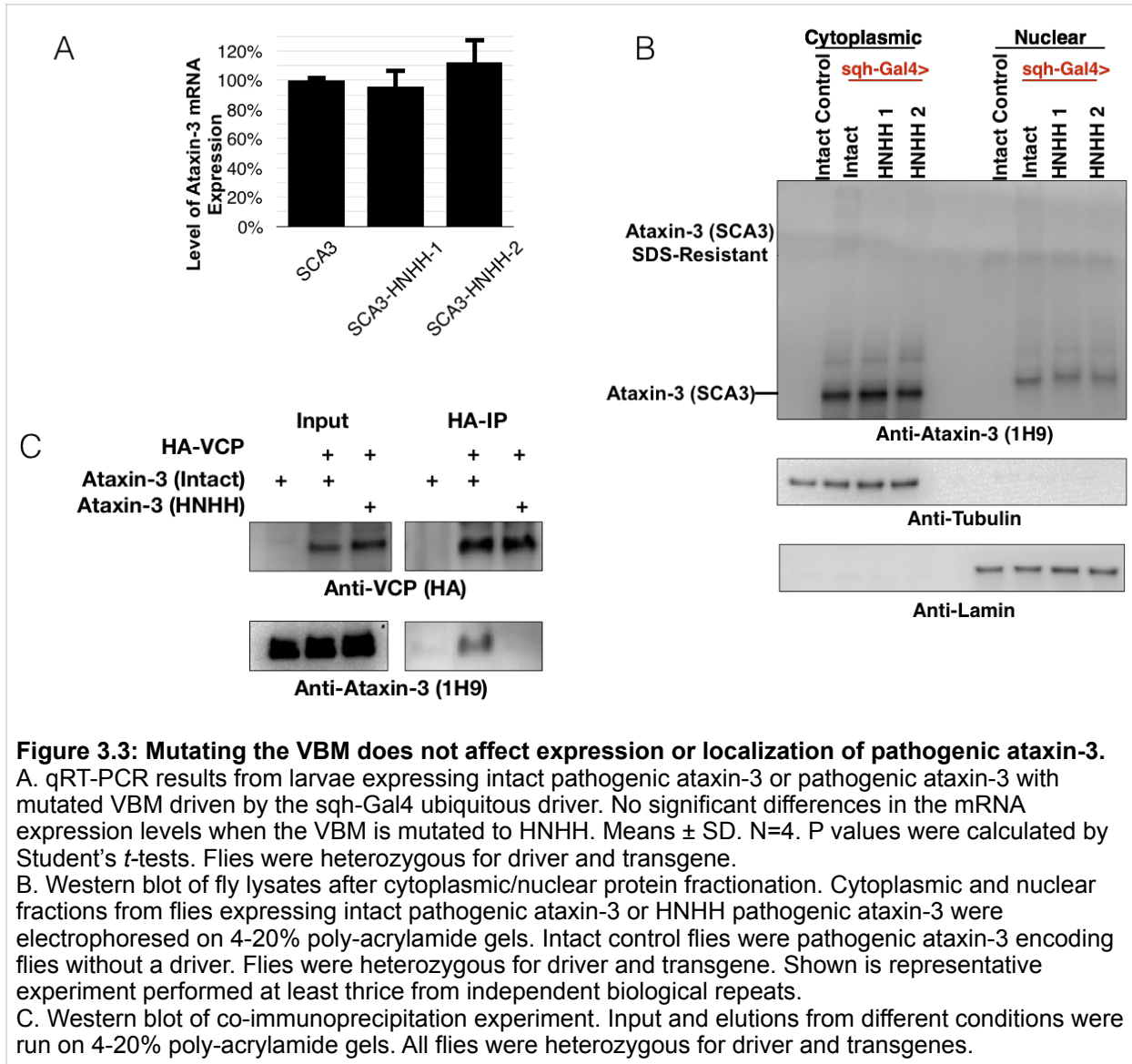


levels of ataxin-3 by qRT-PCR when the protein was expressed in the whole fly using the ubiquitous driver sqh-Gal4 (**figure 3.3A**). The HNHH-1 and -2 lines were compared to the line encoding pathogenic ataxin-3 with an intact VBM. We found that there was no statistically significant difference in the expression levels of ataxin-3 among the three fly lines.

Our lab also previously showed that ataxin-3 turnover was not affected when the VBM was mutated from RKRR to AAAA in HEK cells (Blount et al., 2014).

To make sure that mutating the VBM of ataxin-3 disrupts its interaction with VCP *in vivo* in *Drosophila* we carried out co-immunoprecipitation experiments (**figure 3.3C**). We exogenously expressed HA-tagged VCP in fly eyes along with intact pathogenic ataxin-3 or pathogenic ataxin-3 with a mutated VBM using the eye-specific GMR-Gal4 driver. Western blot analysis shows that when the VBM is mutated from RKRR to HNHH, pathogenic ataxin-3 does not co-precipitate with VCP as shown in figure 3.3C.

The VCP binding site on ataxin-3 is an arginine rich region located at the C-terminal of ataxin-3. Previously, this domain was thought to act as a nuclear localization signal (Albrecht et al., 2004). It has also been shown that ataxin-3 is able to shuttle between the cytoplasm and the nucleus, but when it contains the expanded polyQ tract it preferentially localizes in the nucleus, where it is highly toxic (Costa Mdo and Paulson, 2012). We examined whether mutating the VBM would affect the localization of pathogenic ataxin-3 in the fly by using a fractionation kit to enrich cytoplasmic and nuclear proteins from whole fly lysates when pathogenic ataxin-3 expression is driven by the ubiquitous driver sqh-Gal4. After analysis by western blotting and probing with ataxin-3 antibody, we observed no difference in protein distribution between the cytoplasm and nucleus when the VBM is mutated (**figure 3.3B**). We conclude that mutating the VBM from RKRR to HNHH does not impact the cellular localization of pathogenic ataxin-3 in the fly. These results indicate that any phenotype that we observe is not due to less or more ataxin-3 in the nucleus, and is more likely an effect of the interaction of ataxin-3 with VCP.



3.3.2 Mutating the VBM of ataxin-3 reduces its toxicity in *Drosophila* when expressed in all tissues and delays accumulation of SDS-resistant species

To physiologically assess how a VBM mutation affects the toxicity of ataxin-3 in *Drosophila*, we conducted longevity studies. As previously shown by our group (Sutton et al., 2017), pathogenic ataxin-3 is highly toxic to the fly when it is expressed ubiquitously with *sqh-Gal4* driver (Sutton et al., 2017). We observed death during late pharate adult stages and during eclosure from the pupal case, as well as shortly following eclosion. Those flies that did eclose

successfully survived for about 29 days (**figure 3.4A**). HNHH mutant flies all reached adulthood. We did not observe any death during development as we had with flies encoding ataxin-3 with the intact VBM. Interestingly, even with the reduction in toxicity in early life, in the end these flies did not live much longer. From longevity curves, we saw a clear separation in the rates of death early on: the HNHH mutants appeared healthier and more flies remained alive in the earlier days of life than their intact ataxin-3 expressing counterparts (**figure 3.4A**). We observed that about 50% of flies expressing intact pathogenic ataxin-3 die by day 11 as compared to HNHH mutant expressing flies which reached 50% death around day 21. When compared with the control flies that do not express pathogenic ataxin-3, both intact VBM containing flies as well as those with the mutated VBM domain live a lifespan that is greatly shortened (**figure 3.4A**). Control flies in this experiment contain the sqh-Gal4 driver as well as the cloning vector we used (pWalium10-moe) also inserted at the attP2 site, but without an ataxin-3 transgene in it.

We assessed by western blotting the expression levels of ataxin-3 protein with one day old whole fly lysates where pathogenic ataxin-3 with an intact VBM or pathogenic ataxin-3 with a mutated VBM were expressed in the whole fly using the ubiquitous driver, sqh-Gal4. Our results show that flies which harbor pathogenic ataxin-3 with an intact VBM have an accumulation of higher molecular species, indicative of ataxin-3 SDS-resistant aggregates as early as day one after eclosion, whereas flies with a mutated VBM do not have this high molecular smear rather: they show more soluble ataxin-3 (**figure 3.4B**).

As already noted, SCA3 is an age-related, progressive neurodegenerative disease. While aging the flies in the longevity experiment, we collected flies for western blot analysis at day one after eclosion, day 7 and day 14 to examine the state of SDS-resistant species as flies age and to

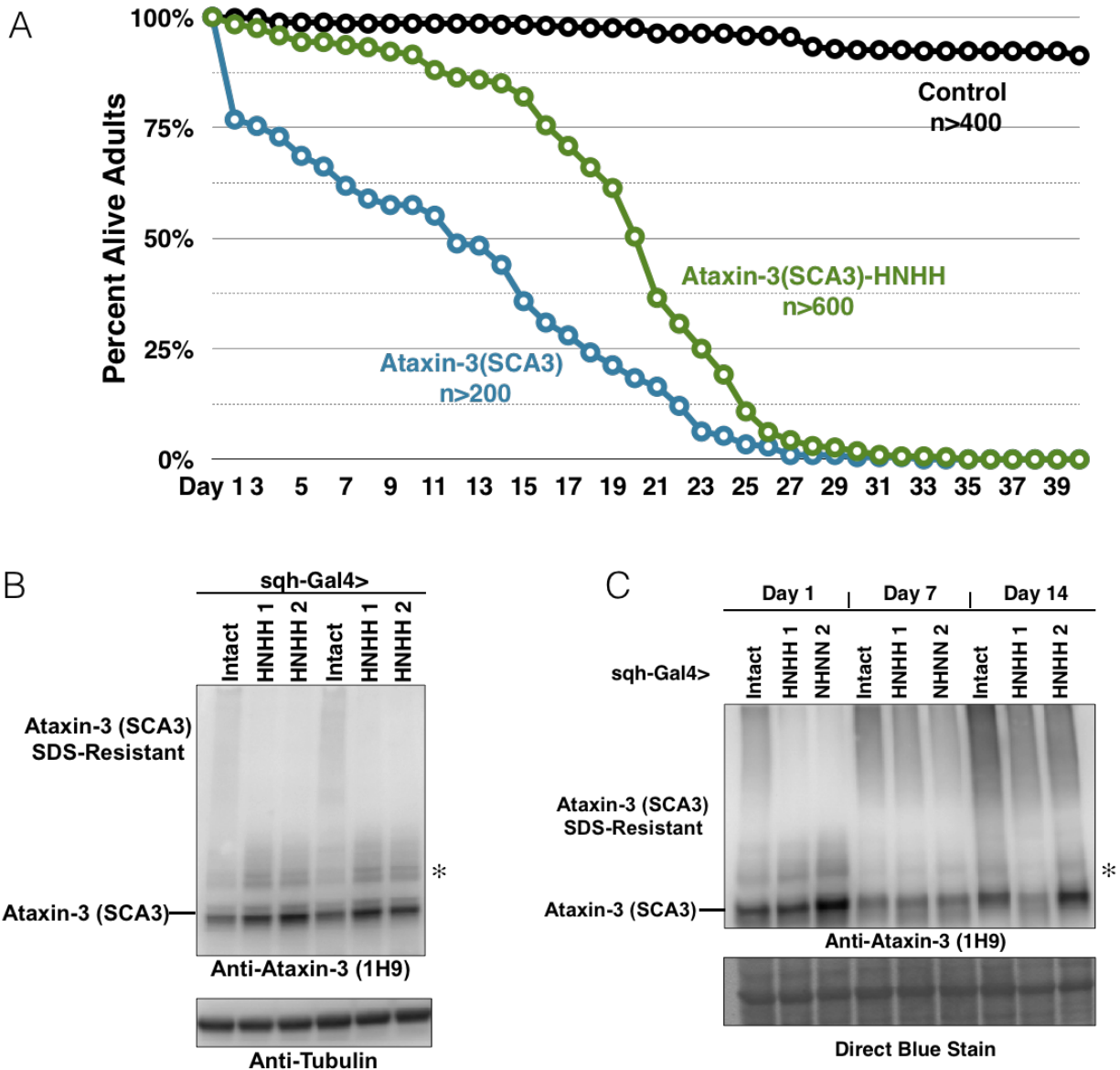


Figure 3.4: Mutating the VBM of ataxin-3 delays its aggregation and toxicity.

A. Longevity assay. Flies were collected immediately after eclosing from the pupal case and aged until death. Vials were monitored for death on a daily basis. Control flies contain the ubiquitous, *sqh-Gal4* driver and cloning vector (*pWalium10-moe*) inserted at the *attP2* site, but without an *ataxin-3* transgene. Control flies lived up to 90 days.

B. Western blots of one day old fly lysates, electrophoresed on 4-20% poly-acrylamide gels, when *ataxin-3* transgenes were driven by the ubiquitous, *sqh-Gal4* driver. Flies were heterozygous for driver and transgene. Blot shown is representative of experiment done three times from independent biological repeats.

C. Timeline of aggregate accumulation. Fly lysates collected on noted dates after eclosure. Pathogenic *ataxin-3* protein transgenes were expressed in whole fly by the ubiquitous, *sqh-Gal4* driver. Flies were heterozygous for all constructs. Representative blot of at least three independent biological repeats.

* ub-*ataxin-3*.

correlate shortened lifespan with ataxin-3 aggregates (**figure 3.4C**). We observed that at day one, SDS-resistant aggregates are already present in flies which expressed the intact pathogenic protein, whereas with HNHH mutant flies we only begin to see accumulation of insoluble protein species at day 7, and increasing accumulation with age. It is also worth noting that HNHH mutants show higher levels of SDS-soluble ataxin-3 as compared to intact counterparts. These levels are concomitantly reduced as SDS-resistant species accumulate. Interestingly, we observe that the accumulation of high molecular species occurred at a point which preceded death in HNHH flies, which suggests a temporal relationship between the accumulating protein aggregates and ensuing death in flies exists.

Collectively, the above experiments show that mutation of the VBM of ataxin-3 does not affect its mRNA or protein expression levels, or the cellular localization of the protein when the protein is expressed in the whole fly. When the VBM is mutated, there is a delay in the accumulation of SDS-resistant protein species in flies as compared to their intact pathogenic ataxin-3 expressing counterparts. Ultimately, disrupting the interaction between pathogenic ataxin-3 and VCP does not prolong the life span of flies, but initial toxicity is markedly decreased (**figure 3.4A**), as shown by the separation in longevity chart.

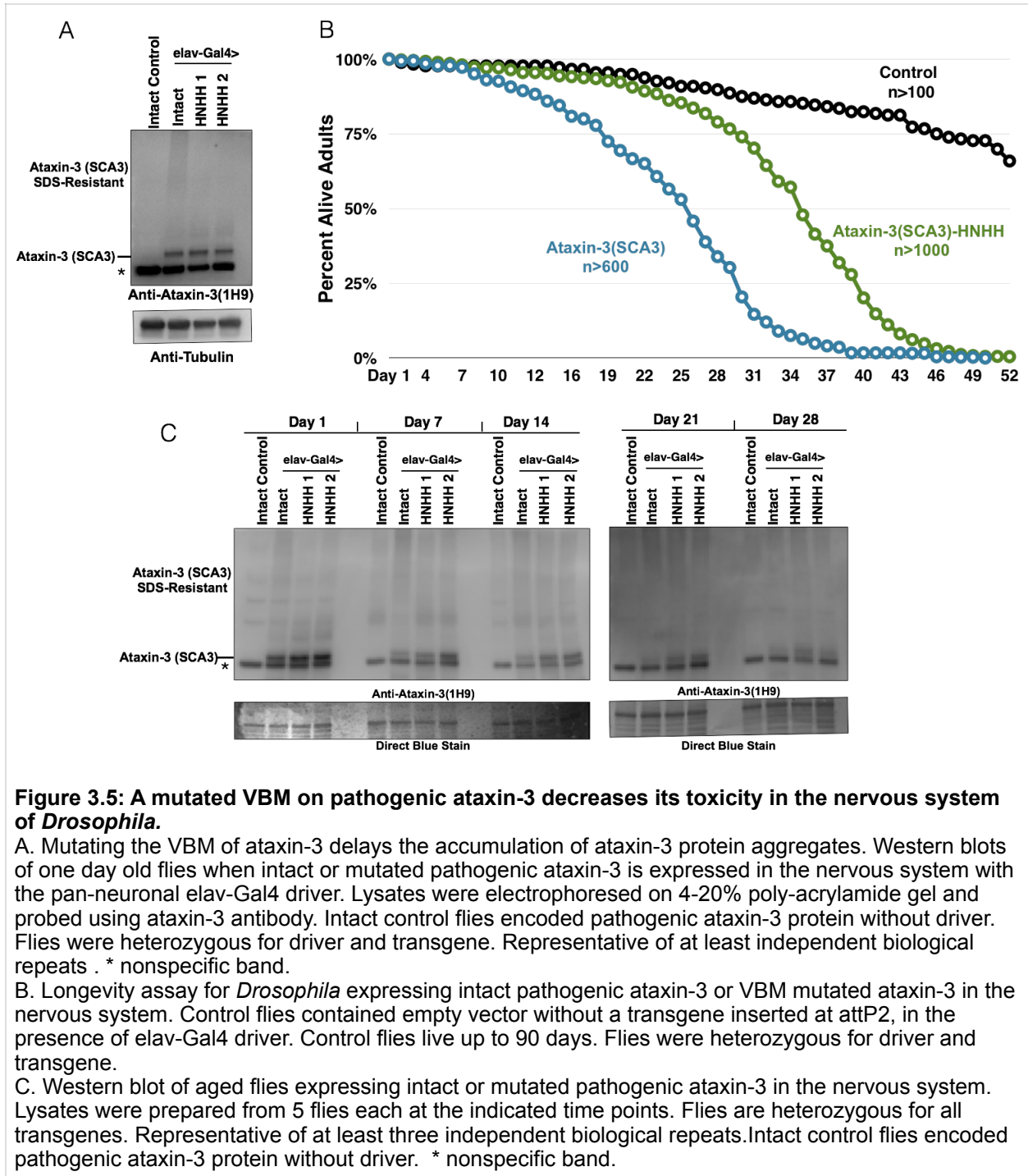
3.3.3 Ataxin-3 with a mutated VBM is less toxic in the nervous system than the intact pathogenic protein

Ataxin-3 protein is expressed throughout all tissues and cells in the human body. However, the polyQ disease SCA3 manifests as a neurodegenerative disorder with degeneration specifically in the brain, affecting the brainstem and the cerebellum (Costa Mdo and Paulson,

2012). With the power of fly genetics, we are able to restrict the expression of ataxin-3 only to the nervous system using a neuronal-specific driver, *elav-Gal4*, and examine its effects in the fly.

Western blot analysis from whole one day old fly lysates where pathogenic ataxin-3 with an intact VBM is expressed pan-neuronally already show accumulation of SDS-resistant ataxin-3 species (**figure 3.5A**). Comparing the HNHH, VBM-mutant flies to those flies expressing the intact pathogenic protein, the high molecular smear is largely absent with greater presence of lower molecular weight, SDS-soluble ataxin-3 species. The control flies used in this experiment were flies which contain an intact pathogenic ataxin-3 without the pan-neuronal driver, and thus, there was no ataxin-3 expression in these flies.

We next aged flies to investigate how ataxin-3 expression in the nervous system affects their life span. We observed that the lifespan of flies expressing either intact pathogenic ataxin-3 or the HNHH mutant counterpart was shortened as compared to control flies which encode and express the empty vector control. Control flies live up to 90 days (**figure 3.5B**). When we compare the lifespan of flies expressing pathogenic ataxin-3 with a mutated VBM to those expressing the intact pathogenic protein, a higher percentage of flies remain alive initially; that is, we observe a 50% reduction of flies expressing ataxin-3 with an intact VBM by day 25 whereas this reduction occurs around day 35 in flies expressing pathogenic ataxin-3 with a mutated VBM. However, even though in earlier time points more HNHH mutant flies remain alive, both fly lines have an average life span of 40 to 45 days (**figure 3.5B**). Flies expressing either variant of pathogenic ataxin-3 pan-neuronally live longer than those expressing the same constructs ubiquitously with the *sqh-Gal4* driver. Ataxin-3 is ubiquitously expressed in all tissues in the human body but toxicity manifests specifically in the nervous system. The difference in

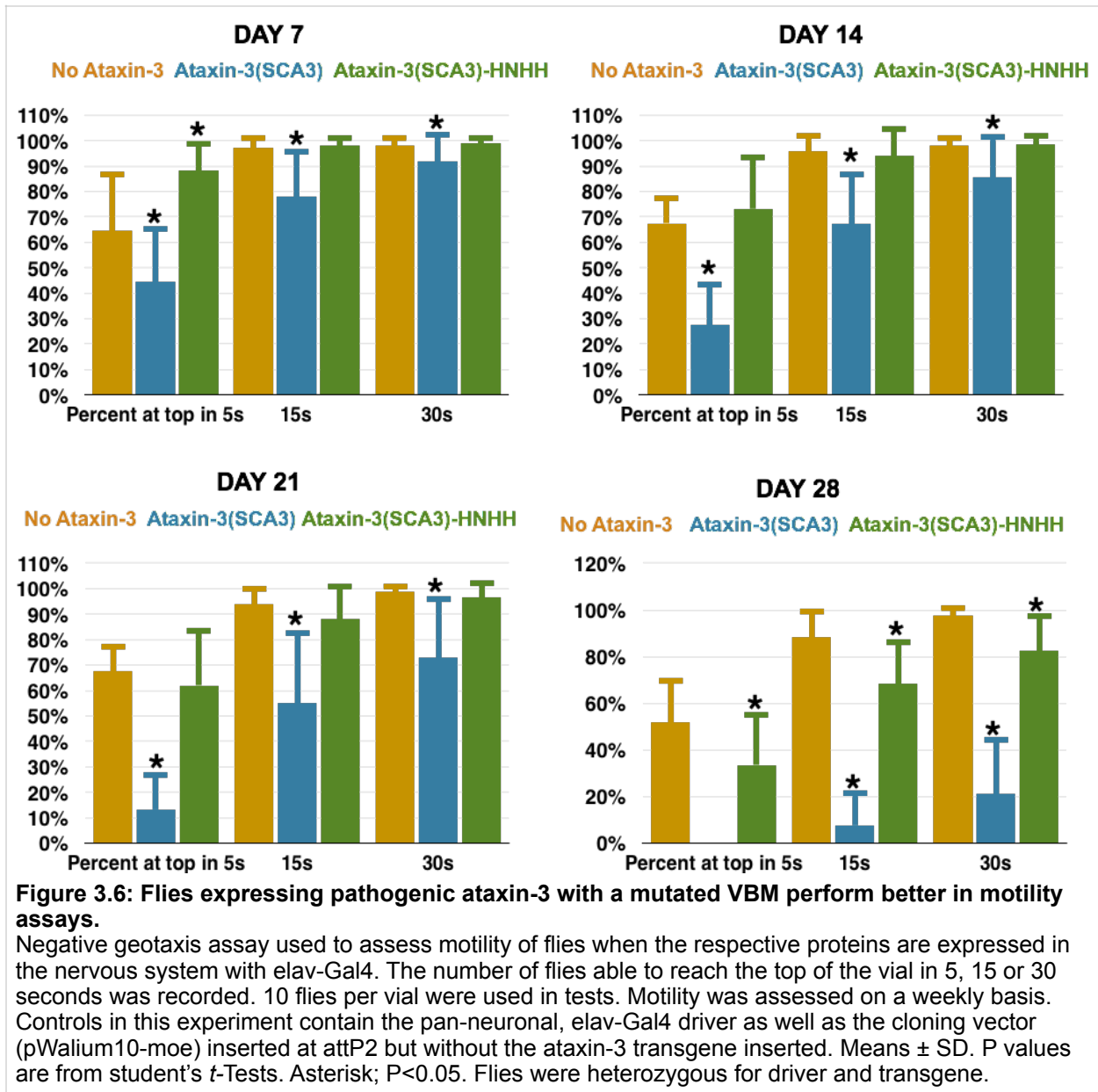


toxicity observed in this case may be due to expression in all tissues of the fly as compared to only in nervous system cells because this excludes glial cells.

To investigate the relationship between fly lifespan and the presence of SDS-resistant species in the pan-neuronal expression model, we examined by western blotting whole fly lysates at five different time points, day 1 to day twenty eight (**figure 3.5C**). As previously shown, (**figure 3.5A**), presence of SDS-resistant pathogenic ataxin-3 species is evident at day one in flies expressing the protein with an intact VBM. Conversely in those flies expressing pathogenic ataxin-3 with a mutated VBM high molecular species smear are not prominent until day 14 (**figure 3.5C**). In both cases, though, accumulation of high molecular species precedes the point at which death is observed in the longevity studies (**figure 3.5.B, figure 3.5C**).

3.3.4 Mutating the VBM of ataxin-3 reduces its toxic effect on fly motility

SCA3 is a movement disorder, marked by progressive ataxia followed by death. We wanted to test the neuronal function of flies when the pathogenic ataxin-3 proteins were expressed specifically in the nervous system. Motility is assessed by measuring how many flies reach the top of the vial in 5, 15 and 30 seconds. The flies were kept over a period of four weeks and motility was assessed weekly (**figure 3.6**). As early as day seven, flies expressing the intact pathogenic protein were slower than control flies containing the empty vector across all three time points. This difference increased markedly with age, and at four weeks of age the majority of flies did not reach the top of the vial even during the longest time interval, 30 seconds (**figure 3.6, blue bars**). Conversely, flies expressing pathogenic ataxin-3 with the mutated VBM performed as well as control counterparts for up to three weeks of age. At four weeks of age, these flies are slower than empty vector control counterparts but perform markedly better than flies expressing intact pathogenic ataxin-3 (**figure 3.6, green bars**).



In conclusion, expression of intact pathogenic ataxin-3 and HNHH mutant pathogenic ataxin-3 pan-neuronally is less toxic than ubiquitous expression in the fly (**figure 3.4**). When the VBM of ataxin-3 is mutated, aggregate accumulation in the fly is delayed as was observed with ubiquitous expression of the respective proteins. Furthermore, flies expressing pathogenic ataxin-3 with a mutated VBM perform significantly better than their intact pathogenic ataxin-3 expressing counterparts in the negative geotaxis motility assay, comparable to control flies

expressing empty vector until three weeks of age. We observed that reduced performance by the flies expressing pathogenic ataxin-3 with mutated VBM happens on the same day that we first observe accumulation of SDS-resistance species by western blotting (**figure 3.5B**). Flies expressing intact pathogenic ataxin-3 have poor performance from the first time point tested (week one). These flies already have accumulation of SDS-resistant species as early as day one after eclosion (**figure 3.5A, figure 3.5B**).

3.3.5 Ataxin-3 with mutated VBM is less toxic in Drosophila eyes

Drosophila eyes serve as a widely accepted model to study degeneration in polyQ diseases. Histological sections allow visualization of degeneration and accumulation of proteinaceous aggregates. Using the eye-specific, GMR-Gal4 driver, we expressed our transgenes in the fly eyes and conducted histological assays at different points of the fly life to characterize the degeneration caused by expression of pathogenic intact ataxin-3 and what effects mutating the VBM has on the phenotype of the eye. The ommatidium is the functional unit of the fly eye. Each eye contains approximately 800 ommatidia arranged in a structured array. The highly iterative structure of the eye makes it a good model to easily and reliably observe any perturbations arising from toxicity (Kumar, 2012).

Histological sections from one week old flies showed that there was already some disruption of the internal ommatidial array of the eye compared to the control flies which did not express any pathogenic ataxin-3 protein (**figure 3.7A**, panel II compared to panel I). The HNHH mutant eyes very closely resembled control eyes, with a well arranged ommatidial array and no signs of degeneration (**figure 3.7A**, panel III and IV). As SCA3 is a progressive disease, we expected to observe more degeneration as flies aged over the course of four weeks. This was the

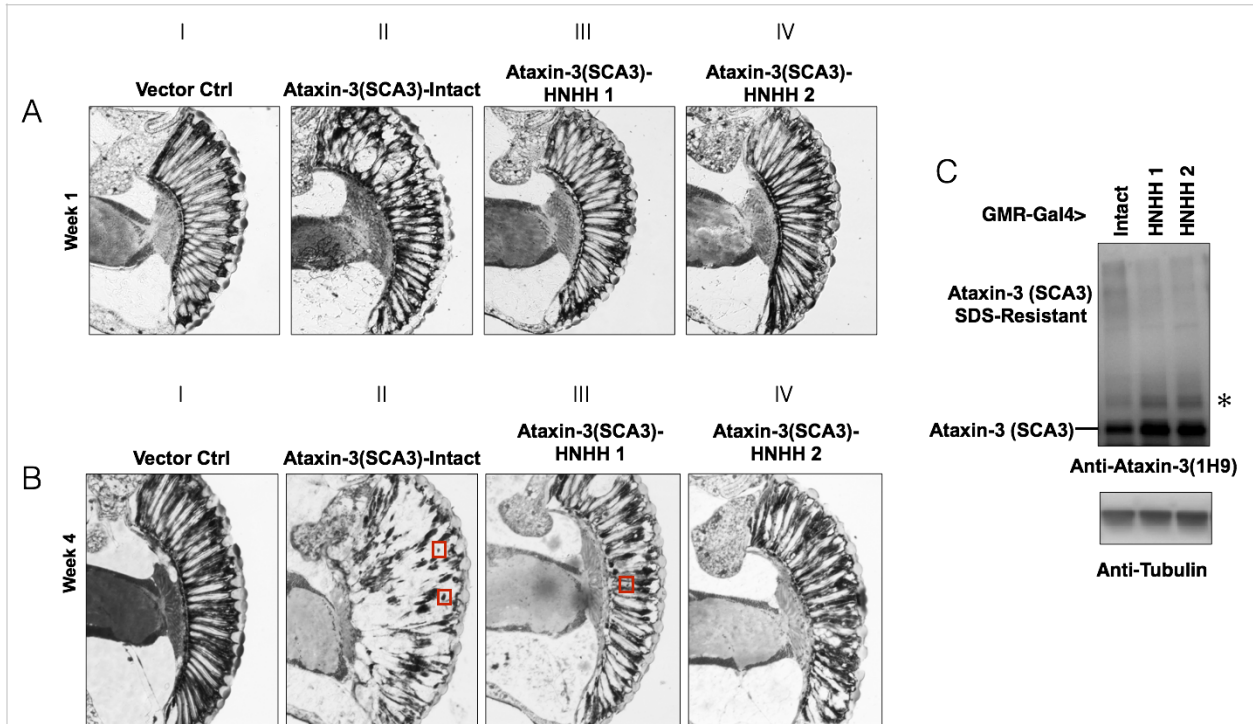


Figure 3.7: Disrupting the interaction between ataxin-3 and VCP reduces toxicity in fly eyes. (A, B). Histological sections of fly eyes at one week of age (A) or four weeks (B) when pathogenic ataxin-3, intact or HNHH, was expressed in the eyes using GMR-Gal4. Controls in this experiment contain the eye-specific, elav-Gal4 driver as well as the cloning vector (pWalium10-moe) inserted at attP2 but without the ataxin-3 transgene inserted. (C). Western blots of one day old fly head lysates, electrophoresed on 4-20% acrylamide gel. Flies are heterozygous for all constructs. Representative of at least three independent biological repeats. * Ub-ataxin-3. Red boxes: example of densely staining structure.

case for four week old fly eyes expressing the intact pathogenic ataxin-3 protein, where we saw substantial degeneration and loss of structure (**figure 3.7B**, panel II). We also observed densely staining structures, indicative of protein aggregates (Red boxes). In HNHH mutant eyes, we also began to observe degradation. However, the ommatidial array was still clearly present and remained rather intact over the course of the examinations (**figure 3.7B**, panel III and IV). In summary, from histological sections of the fly eye, we see that mutating the VBM of pathogenic ataxin-3 reduces toxicity and preserves normal eye structure.

We coupled our histological assays with western blotting from dissected fly heads. Western blots from one day old fly heads show that there is already ataxin-3 SDS-resistant

species as indicated by the high molecular smears when we probe with ataxin-3 antibody (**figure 3.7C**). We do not observe these species when the HNHH mutant pathogenic ataxin-3 is expressed in the fly eyes (**figure 3.7C**). We can draw the conclusion from these results that mutating the VBM of pathogenic ataxin-3 delays the formation of aggregates and in turn slows the degeneration of fly eyes. Expression of intact pathogenic ataxin-3 causes large degeneration of the fly eye within four weeks of age, whereas expression of pathogenic ataxin-3 with a mutated VBM showed much less toxicity at the same age.

Altogether, the studies conducted in the various tissues with the different forms of pathogenic ataxin-3 lead us to conclude that VCP influences the aggregation and toxicity of pathogenic ataxin-3, increasing the accumulation of aggregated species and expediting the toxicity of this protein *in vivo*. To further probe into this model, we next turned to endogenous VCP in the fly.

3.3.6 Knockdown of VCP improves degeneration in fly eyes

The results we obtained with our earlier experiments indicate that expression of pathogenic ataxin-3 protein is less toxic when the VBM is mutated to disrupt interaction with VCP. We wanted to confirm that the differences in phenotype we observed were due to the interaction, or lack thereof, between ataxin-3 and VCP, by targeting VCP itself.

VCP is an essential protein with numerous important functions in the cell; knockdown of VCP in the whole fly as well as in the nervous system is developmentally lethal (personal communication). However, knockdown of VCP in the fly eyes is tolerated (our lab's data). Thus, we examined whether knockdown of VCP in fly eyes expressing pathogenic ataxin-3 with an intact VBM would impact the structure of the eye.

From histological sections at four weeks of age, as previously shown (**figure 3.8A**), expression of intact pathogenic ataxin-3 in the eyes using GMR-Gal4 was toxic and caused the breakdown of the ommatidial array (**Figure 3.8A, panel 2**). We used two different RNAi lines targeting VCP, both of which were generated using phiC31 mediated site specific integration and were inserted on the third chromosome of the fly at the attP2 (BDSC). As shown in **figure 3.8B**, knockdown of VCP by both RNAi lines led to statistically significant reduction in VCP protein levels in the fly head. An important note here is that the blots underestimate the precise level of VCP reduction. This is because we target RNAi to the fly eyes, but we examined protein levels from intact fly heads.

Knockdown of VCP in fly eye led to improvement of pathology caused by ataxin-3. As shown in figure 3.8, knocking down VCP with either RNAi-1 or -2 reduced ommatidial disarray in the presence of pathogenic ataxin-3 (**figure 3.8A**). Importantly, concomitant with improved pathology we observed reduced levels of SDS-resistant species of ataxin-3 when VCP was knocked down. In figure 3.8B, and to a lesser extent in figures 3.4B and C and 3.7C, a higher proportion of SDS-soluble ataxin-3 in flies where VCP is knocked down or the VBM of pathogenic ataxin-3 is mutated seems to be ubiquitinated. It is possible that when ataxin-3 proteins bind to VCP they are brought in close proximity and deubiquitinate each other. It is also possible that when pathogenic ataxin-3 is ubiquitinated it is less aggregation-prone due to steric hindrance.

In conclusion, we observe decreased toxicity from ataxin-3 when VCP is knocked down in the eyes. Similar to the mutation of VBM on ataxin-3 to disrupt its interaction with VCP, decreasing the levels of VCP in the cell suggests that there are less VCP proteins binding

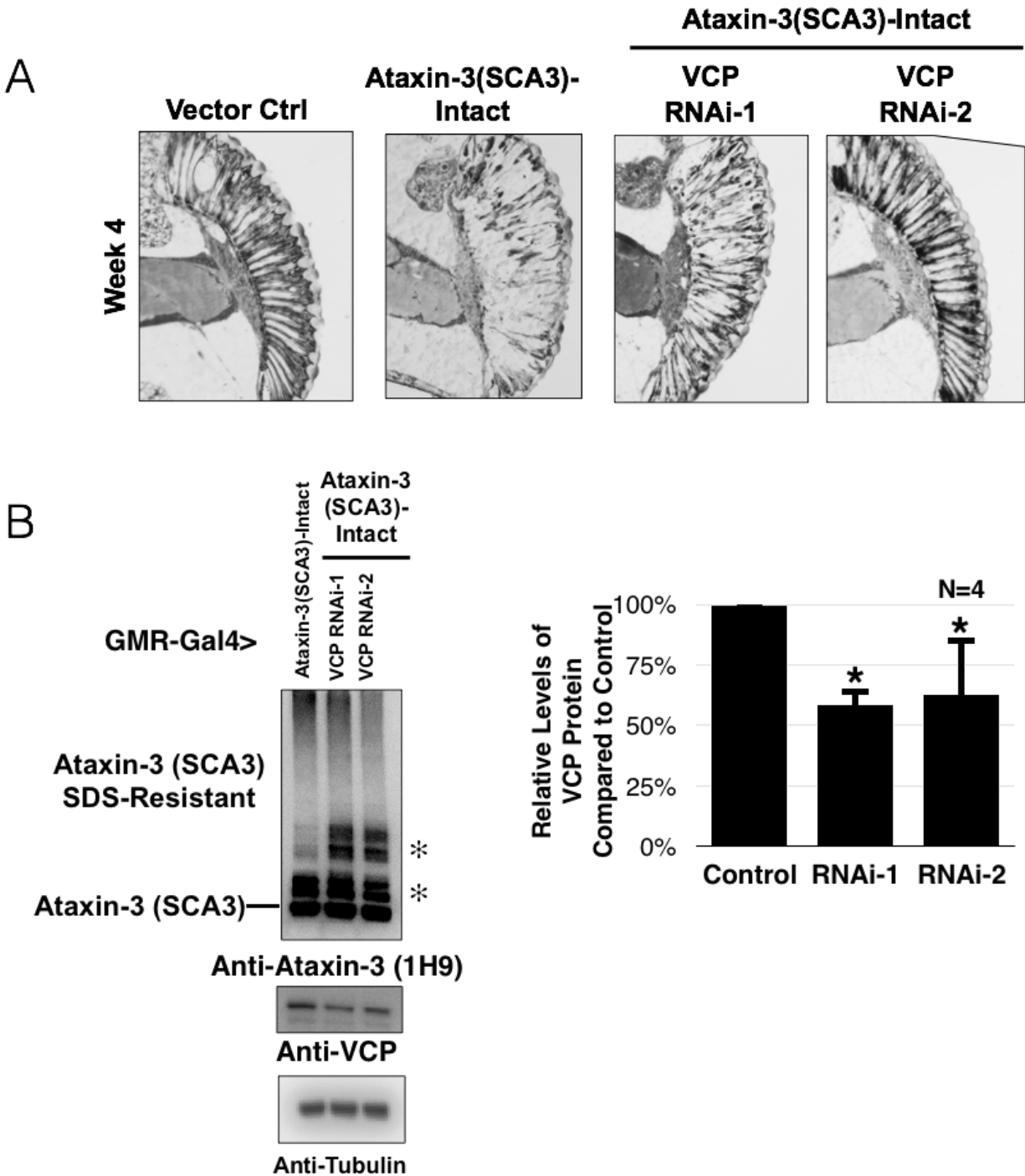


Figure 3.8: Knockdown of VCP in fly eyes improves degeneration caused by pathogenic ataxin-3 expression.

(A). Histological sections of four week old fly eyes. GMR-Gal4 was used to express pathogenic ataxin-3 with or without VCP RNAi in the eyes. Vector control contains no ataxin-3 expression.

*: ub-ataxin-3

(B). Western blot of one day old fly head lysates when pathogenic ataxin-3 is expressed alone or with VCP RNAi in fly eyes. Right; Quantification of VCP knockdown from four separate experiments. Means \pm SD. Asterisks: $P < 0.05$. P values are from students *t*-Tests. Flies are heterozygous for all constructs.

ataxin-3 and bringing them closer together to form aggregates. Collectively, these data support our overall model that VCP enhances ataxin-3 aggregation and toxicity.

3.3.7 Ataxin-3 expression after development

Intrigued by our results with the ubiquitous driver, which showed the presence of SDS-resistant ataxin-3 species before we observed lethality in flies, as early as day one after eclosing with expression of intact pathogenic ataxin-3, we wanted to further examine the temporal relationship between these SDS-resistant species and death. In our previous experiments, expression of pathogenic ataxin-3 proteins (intact and HHH mutant) was driven throughout development and in adults. In the next round of experiments, we harvested the power of fly genetics to switch on ataxin-3 expression after adults eclosed and for specific points of time to examine the possibility of a correlation between ataxin-3 death and the accumulation of SDS-resistant ataxin-3 species in neural tissues.

We used the tissue specific and inducible GeneSwitch Gal4 driver system (Osterwalder et al., 2001; Nicholson et al., 2008). Male fly and virgin female flies are crossed, one encoding an ataxin-3 construct and the other a pan-neuronal GeneSwitch, elav-GAL4 driver. The flies are crossed on normal fly media, lacking the GeneSwitch activator, RU-486. Once offspring from the cross eclosed, we switched them to fly media containing the inducer, RU-486. RU-486 activates the GeneSwitch protein, which in turn drives the expression of the respective pathogenic ataxin-3 transgenes pan-neuronally. The offspring were aged on RU-486-containing food and collected for lysates on day one, four, seven, ten, 14, 21, 28 and 35 and further examined by western blotting with ataxin-3 antibody for protein aggregation (**figure 3.9A, left panel**).

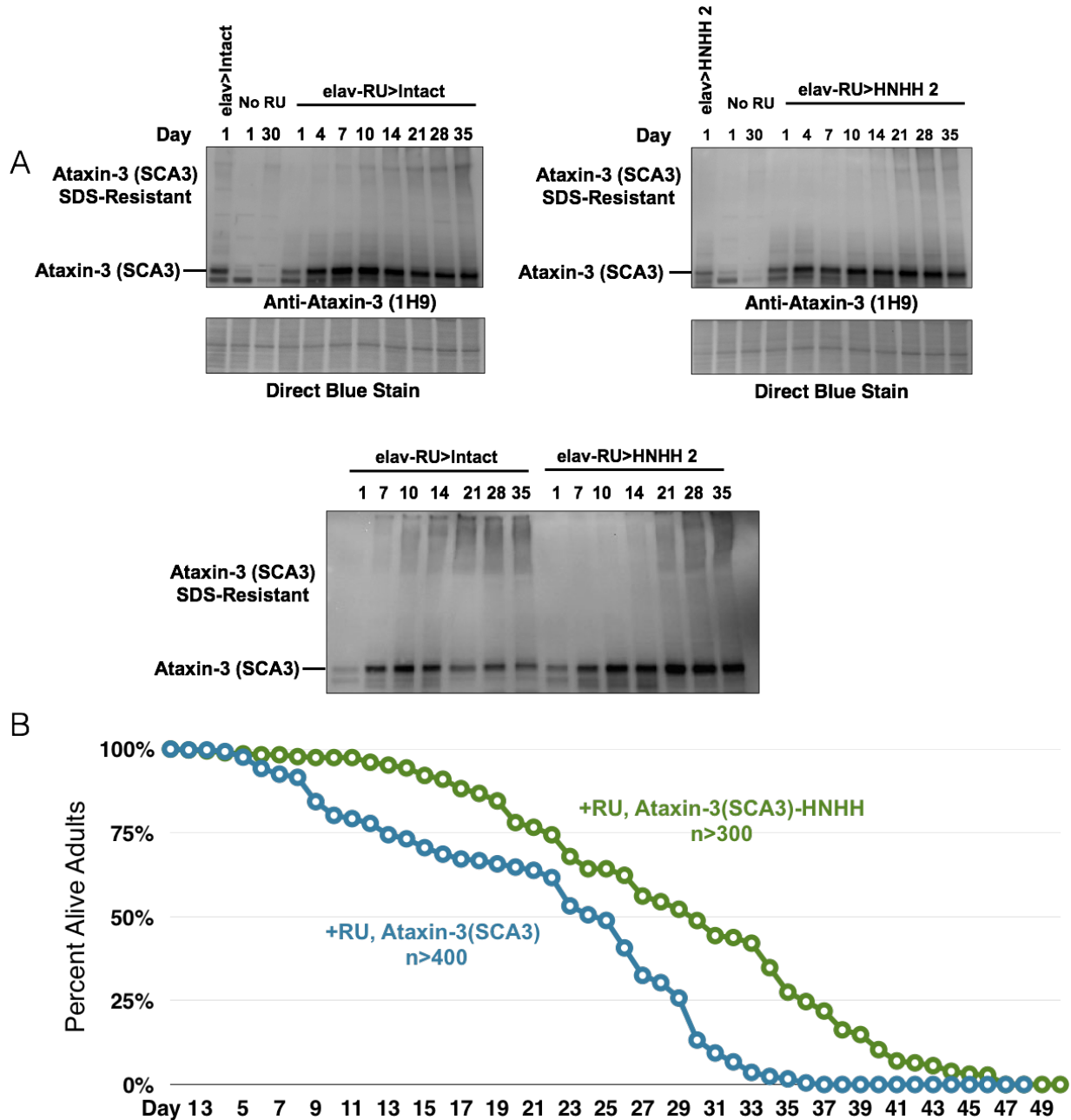


Figure 3.9: Expression of pathogenic ataxin-3 only in adult stages does not prolong lifespan.

When expression of intact or mutated pathogenic ataxin-3 expression is started after flies eclose from the pupal case, life span does not differ from that of flies in which the proteins are expressed throughout development as well (**figure 3.5**).

(A). Left panel; western blot of fly lysates at indicated ages (days) when pathogenic ataxin-3 is expressed in the nervous system by the RU-486-inducible GeneSwitch elav-Gal4 driver. Right panel; blots from HNHH mutant pathogenic ataxin-3 expressed pan-neuronally by the inducible driver. Control lysate for this experiment was from flies expressing intact pathogenic ataxin-3 or HNHH pathogenic ataxin-3 by the constitutive pan-neuronal elav-Gal4 driver at day one (driver that was used in **fig. 3.5**). Bottom blot, samples re-run on the same poly-acrylamide gel. Blots representative of three independent biological repeats. Flies are heterozygous for all transgenes.

(B). Longevity assay for flies expressing intact or mutated pathogenic ataxin-3 by RU496-inducible pan-neuronal driver. Flies are heterozygous for all constructs.

When intact pathogenic ataxin-3 is driven by the RU-486 inducible elav-Gal4 driver, we do not observe SDS-resistant species until day ten, compared to flies in which intact pathogenic protein expression is driven by the pan-neuronal driver elav-Gal4, where we observed SDS-resistant species at day one (**Figure 3.9A**). Intriguingly, we do not observe a delay in the time of death between flies that express the pathogenic, intact ataxin-3 protein throughout development and adulthood (elav-Gal4, **figure 3.5**) and those in which expression of the pathogenic protein is started in early adulthood with the GeneSwitch driver (**Figure 3.9A**). In fact, flies expressing pathogenic ataxin-3 by RU-486-inducible elav-Gal4 driver may overall die earlier than their counterparts driven by the non-inducible driver (**figure 3.9B blue line, compare to figure 3.5B blue line**).

A similar pattern is observed when pathogenic ataxin-3 with a mutated VBM is conditionally expressed in flies after they eclose (**figure 3.9, right panel**). SDS-resistant species are evident around day 21 by western blotting. As in experiments shown above (**figures 3.4 and 3.5**), mutating the VBM of pathogenic ataxin-3 delays the accumulation of SDS-resistant species in *Drosophila*. We do not observe accumulation of SDS-resistant species with HHHH mutants with the inducible or non-inducible drivers used previously at day one. However, the flies in which expression of pathogenic ataxin-3 begins during adulthood ultimately die at the same time as their counterparts in which the protein is expressed throughout development as well, around day 45 of age (**Figure 3.9B**). We do observe that flies which express pathogenic ataxin-3 with a mutated VBM driven by the inducible pan-neuronal driver show 50% death around day 27, whereas with the non-inducible elav-Gal4 driver 50% death is not observed until day 35 as noted above (**figure 3.9B green line, figure 3.5B green line**).

Collectively from these experiments, expression of intact pathogenic ataxin-3 or pathogenic ataxin-3 with a mutated VBM pan-neuronally in the fly using the RU-486-inducible Gal4 driver to express after fly eclosion does not extend lifespan of the flies as compared to those flies in which the pathogenic proteins are expressed throughout development. One explanation for the similarity in lifespan is that the inducible driver drives higher expression of ataxin-3 protein and thus is more toxic to the fly and causes death at a similar time point as in flies with ataxin-3 expression all throughout development (**figure 3.9B, 3.5B**). Similar to our other experiments, we also observed that mutating the VBM extends the time it takes for SDS-resistant species to accumulate in the fly as shown by western blots (**figure 3.9A, 3.5C**). There is about a 10 day delay in the accumulation of SDS-resistant species in flies expressing the mutated version of pathogenic ataxin-3 in comparison to flies expressing the intact pathogenic protein (**figure 3.9A**). Another explanation for the similar time of death when pathogenic ataxin-3 is expressed conditionally in adult as compared to all throughout development is that developing neurons may have better mechanisms to withstand the burden from accumulating SDS-resistant species. It could be that degradation systems are not as effective in the terminally differentiated neurons, combined with higher pathogenic ataxin-3 expression could explain the earlier 50% death, and overall death at the same age.

3.4 Discussion

SCA3 is a devastating, progressive disease with no available cure and poor symptomatic therapies. Further studies to better understand ataxin-3 functions, interacting partners and mechanisms will be instrumental in designing therapies and hopefully finding a cure for this disease. PolyQ disease associated protein aggregation has long been studied at terminal stage,

after aggregation has already occurred. We decided to take a different approach and examine the factors that are involved in the initial stages of promoting protein aggregation in the disease state. Boeddrich et. al. previously showed that ataxin-3 and VCP directly interact through a VBM domain on the C-terminal of ataxin-3, and when this domain is mutated, binding between the two proteins is disrupted *in vitro* and in mammalian cells (Boeddrich et al., 2006). Our studies showed that disrupting the interaction between ataxin-3 and VCP in a *Drosophila* model of SCA3 delays aggregation of pathogenic ataxin-3 and decreases its toxicity.

We showed that expression of pathogenic ataxin-3 with a mutated VBM did not alter mRNA expression levels as compared to its intact ataxin-3-expressing counterparts, nor did it affect the cellular localization of the protein. When the proteins were expressed ubiquitously or pan-neuronally, using the *sqh*- and *elav*-Gal4 drivers respectively, the HNHH mutant expressing flies did not show accumulation of protein aggregates by western blotting until 14 and 21 days of age. Conversely, flies expressing the intact pathogenic protein with the same drivers showed accumulation of SDS-resistant species by western blot at day one after adults eclosed from the pupal case. This delay we observed in aggregate accumulation may explain the trend that occurs in the longevity curves with both ubiquitous and pan-neuronal expression of the ataxin-3 variants. In both instances we saw that a larger number of HNHH mutant expressing flies remain alive initially, whereas those expressing intact pathogenic ataxin-3 started to decline much earlier (**figure 3.4A, figure 3.5C**). Similar to the delay we see in aggregate accumulation, we also observe a delay in death when ataxin-3 VBM is mutated. Ultimately, however, there was no difference in lifespan between these two fly lines with either driver. Even though mutating the VBM leads to a disrupted interaction between pathogenic ataxin-3 and VCP, it cannot ultimately

prevent aggregation of ataxin-3 which has an inherent ability to aggregate, whether wild type or pathogenic (Masino et al., 2004; Ellisdon et al., 2006).

Our studies address the role of VCP in the early stages of ataxin-3 aggregation, which seems to bring pathogenic proteins closer together to accelerate their aggregation. VCP may also act as a mediator in ataxin-3 aggregation providing more structure to the process, whereas without VCP ataxin-3 does not aggregate as rapidly. Accumulation of SDS-resistant species occurs faster in flies expressing intact pathogenic ataxin-3 than in flies expressing pathogenic ataxin-3 with a mutated VBM (**figure 3.4C, figure 3.5C**). Flies expressing intact pathogenic ataxin-3 reach a toxic threshold of SDS-resistant species earlier than their counterparts expressing pathogenic ataxin-3 with a mutated VBM (**figure 3.4B, C and figure 3.5A, C**). This corresponds to the division in longevity curves shown in figures 3.4A and 3.5B, where we begin to observe death in intact pathogenic ataxin-3 expressing flies much earlier than in flies expressing pathogenic ataxin-3 with a mutated VBM. Once the flies expressing HNHH ataxin-3 variant reach a threshold of SDS-resistant species, death occurs rapidly. This could also be explained by the motility assay where we see the flies that express pathogenic ataxin-3 with a mutated VBM perform very similarly to control counterparts without pathogenic ataxin-3 expression (**figure 3.6**), but their motility is greatly reduced at the point that coincides with the accumulation of SDS-resistant species (**figure 3.5B, figure 3.6**). Flies expressing intact pathogenic ataxin-3, which have SDS-resistant species present at day one after eclosion, perform consistently worse than control flies and HNHH-mutant counterparts.

We showed that disrupting the interaction between these two proteins, ataxin-3 and VCP, improves motility in flies. HNHH mutant expressing flies performed as well as control flies until

about three weeks of age, but those flies expressing intact pathogenic ataxin-3 were already slower at day seven. Furthermore, we showed that mutating the VBM decreases toxicity of pathogenic ataxin-3 when expressed in fly eyes. Substantial degeneration is seen with intact pathogenic ataxin-3 expression in the eyes, complete loss of ommatidial structure with accompanying aggregates. Mutation of the VBM to disrupt the interaction between ataxin-3 and VCP retains eye structure at four weeks and is more comparable to control eyes without pathogenic ataxin-3 expression. These findings were complemented with studies where we knocked down endogenous VCP in fly eyes in the presence of pathogenic ataxin-3 and found that decreasing levels of VCP was also able to reduce some of the toxicity that was observed when pathogenic ataxin-3 was expressed in the fly eyes alone.

Collectively, our results show that mutating the VBM of pathogenic ataxin-3 delays the aggregation of ataxin-3 into SDS-resistant aggregates in a *Drosophila* model of SCA3. This delay in aggregate accumulation coincides with a delay in toxicity in the flies. We observed with all Gal4 drivers, sqh-Gal4, elav-Gal4, and GMR-Gal4, that pathogenic ataxin-3 with mutated VBM was less toxic initially in flies, but did not alter ultimate fate of the flies.

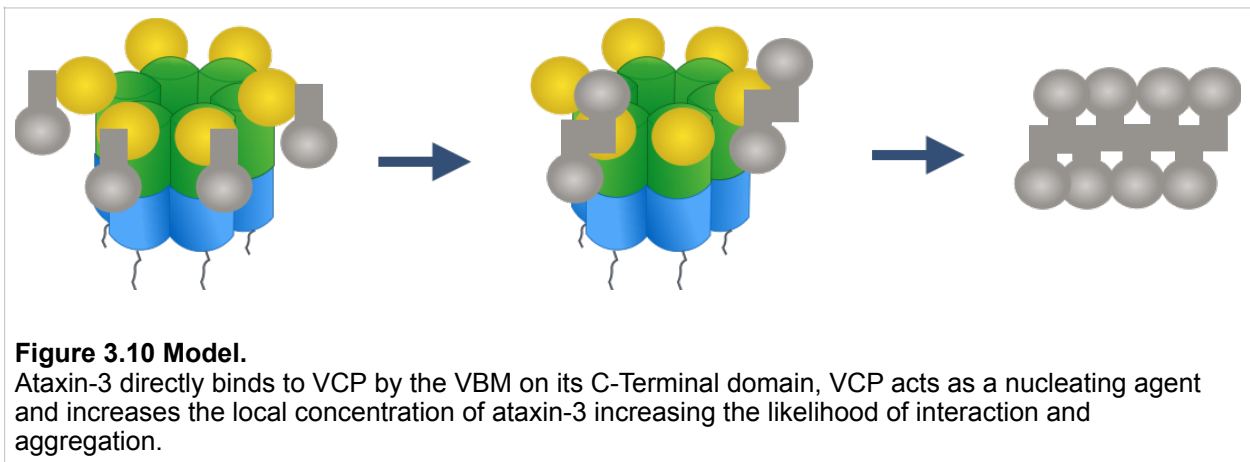
An interesting finding arose from our studies when we induced pan-neuronal expression of ataxin-3 proteins after flies eclosed from the pupal case, that is, when we restricted expression to only during adulthood. We saw that these flies died at the same age as their counterparts in which the ataxin-3 proteins were expressed throughout development and adulthood (**figure 3.5C**, **figure 3.9B**). When intact pathogenic ataxin-3 is expressed during development with the elav-Gal4 driver, flies already show accumulation of SDS-resistant ataxin-3 species at day one, whereas those flies in which pathogenic ataxin-3 expression is induced starting at day one

accumulation of ataxin-3 starts at day 10 of age (**figure 3.5A and C, figure 3.9B**). Similarly, in flies expressing HNHH mutated pathogenic ataxin-3 driven by the RU-486-inducible driver die at about the same age as those flies where protein expression is driven during development. Initially, this experiment was designed to assess when protein aggregation begins and when death ensues in SCA3 flies. The results of this experiment lead to the question of how flies are able to tolerate protein accumulation during development, since flies expressing intact pathogenic ataxin-3 throughout development and in adults show protein accumulation at day one (**figure 3.5A**). Further experiments are necessary to look at differences in expression levels by the different drivers, and whether we observe death at the same time simply due to high expression levels caused by RU-486-inducible elav-Gal4 driver as opposed to the other non inducible elav-Gal4 driver.

Non-pathogenic ataxin-3 itself has a propensity to aggregate. This is believed to happen through a two step process based on *in vitro* data with reconstituted systems using purified, recombinant proteins (Masino et al., 2004; Ellisdon et al., 2006; Masino et al., 2011). Ataxin-3 that does not contain an expanded polyQ tract is thought to first dimerize through the catalytic Josephin Domain in the structured N-terminal; in a second less understood step the C-terminal tails of the proteins come together to create a ‘bead-on-a-string’ type structure and these further assemble into SDS-soluble protein aggregates (Ellisdon et al., 2006; Masino et al., 2011). This process is also thought to occur in the same two step manner when ataxin-3 contains an expanded polyQ region, although, in that instance highly stable, SDS-insoluble aggregates are formed, again *in vitro* (Ellisdon et al., 2006; Masino et al., 2011). Based on the data we presented here, we propose that VCP acts as a nucleating agent in initial ataxin-3 binding and fibrilization.

Ataxin-3 directly binds to VCP through its VBM domain. VCP functions as a hexameric complex and could potentially bind up to six ataxin-3 proteins (Boeddrich et al., 2006; Xia et al., 2016). We propose that VCP, by binding to ataxin-3, increases the local concentration of the protein and also increases the likelihood of binding between ataxin-3 molecules and eventual formation into larger protein aggregates (**figure 3.10**).

In summary, we presented work that shows VCP involvement in protein aggregation in the polyQ disease, SCA3. This interaction may not only be limited to this protein interaction as there is work that points to VCP interacting with other polyQ disease related proteins (Fujita et al., 2013). Thus, the interaction between VCP and ataxin-3 can be applied to other polyQ diseases. One potential way to address the interaction between VCP and pathogenic ataxin-3 in the cell would be to create peptides which could compete with ataxin-3 for binding to VCP by mimicking the arginine-lysine rich RKRR sequence of the VBM of ataxin-3 (**figure 4.1**). This is what we examined next.



CHAPTER 4: PROBING THE INTERACTION BETWEEN ATAXIN-3 AND VCP AS A POTENTIAL THERAPEUTIC TARGET

4.1 Introduction

As stated earlier, there is currently no cure for SCA3. Some relief is available to patients through symptomatic therapies, unfortunately, all those affected succumb to this progressive disease (Costa Mdo and Paulson, 2012). Our goal was to study the interaction between ataxin-3 and VCP and hope that this interaction could be exploited as a potential therapeutic target. Our results above showed that disrupting this interaction reduces the toxicity observed from pathogenic ataxin-3 in *Drosophila*. We presented evidence that disrupting the interaction between ataxin-3 and VCP delays the accumulation of SDS-resistant species in the fly and delayed toxicity: flies remained healthier for a longer period of time and performed better in physiological assays. Our collective results led us to propose a model in which VCP, through direct binding to ataxin-3, acts as a nucleation site for aggregation, bringing together in closer proximity pathogenic ataxin-3 proteins and increasing the likelihood of interaction and eventually fibrilization and protein aggregate accumulation (**Figure 3.10**). To date, no studies have shown that VCP is required for ataxin-3 function, except in ERAD (Wang et al., 2006; Zhong and Pittman, 2006) and that role for ataxin-3 is highly debatable. This led us to the idea of disrupting this interaction for SCA3 therapy.

We wanted to design an approach that could disrupt the interaction between these two proteins. We began by creating peptides that would potentially disrupt the interaction between ataxin-3 and VCP, by utilizing the RKRR region of ataxin-3. We created five decoy peptides that contained the VBM sequence RKRR from ataxin-3 with increasing amino acids from the

ataxin-3 backbone on either side of it, with a HIS⁶-tag for detection (**figure 4.1**). These peptides were transgenically expressed in flies. We created five new *Drosophila* lines, each one encoding a different peptide (Note: Decoy 2 has not yet been utilized in experiments). This was done using the PhiC31 site specific integrase, to insert the decoy peptides at the attP40 landing site, on chromosome 2 of the fly (Markstein et al., 2008).

4.2 Materials and Methods

Drosophila lines: Flies encoding decoys were generated by cloning the different decoy constructs into the pWalium10-moe vector (as described in Sutton et. al. 2017). Injections (Duke University Model System) were into y,w; attP40; +.

Longevity assay: Conducted as previously described in chapter 3: flies were collected after eclosure and aged until death. Flies were switched to vials containing new food every two to three days.

4.3 Results and Discussion

We began by examining first whether these decoy peptides were toxic to the flies when expressed ubiquitously in all tissues (**Figure 4.2**). The reason for this initial approach was due to the fact that VCP binds to other proteins through their VBM including, HDR1 and E4B (Morreale et al., 2009). Thus, we wanted to make sure that the decoys were not toxic due to interrupting all VCP interactions. VCP and ataxin-3 are both ubiquitously expressed in human tissues. Expression of the decoys by themselves did not appear to be toxic, as the longevity of flies expressing the different decoys followed the longevity curve of the control flies for the most part (**figure 4.2**). The control flies in this experiment were host flies that were used to create our transgenic decoy flies (y,w; attp40; +), that also contained the ubiquitous, sqh-Gal4 driver but no

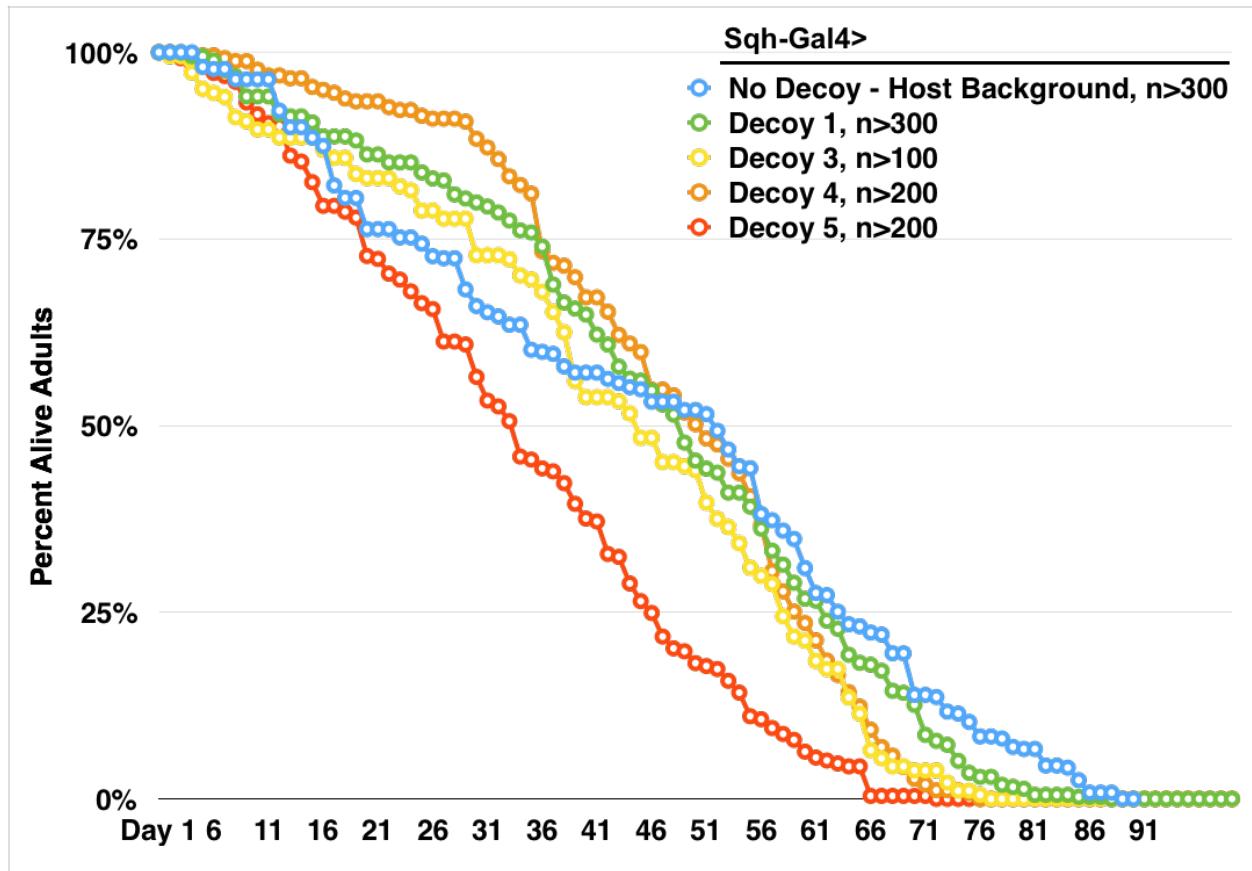


Figure 4.2; Ubiquitous expression of decoys.

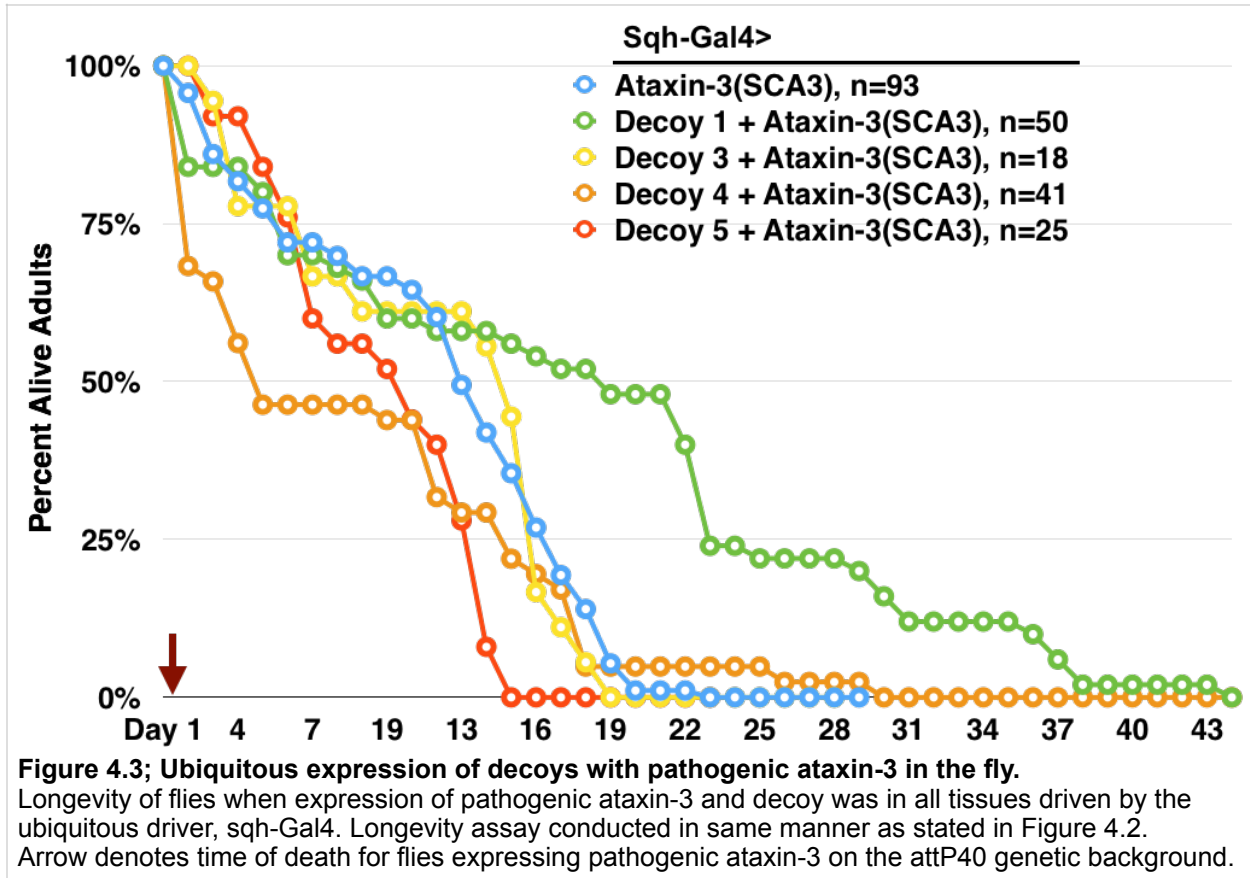
Longevity curves for flies expressing respective decoys in all tissues of the fly with the ubiquitous driver, sqh-Gal4. Approximately 25 flies per vial were collected shortly following eclosion and aged. Death was monitored on a daily basis until all flies were dead. Control flies were flies with the same isogenic background originally used to create decoy containing transgenic fly line, with the sqh-Gal4 driver. Flies were transferred to vials containing new food every two to three days.

used in experiments described in Chapter 3, which lived up to ~90 days (**figure 3.4, 3.5**). This is due to the difference in genetic backgrounds used to make the decoy flies, in which constructs were specifically inserted at a specific site, attP40, on chromosome two as compared to chromosome three site attP2 for the pathogenic ataxin-3 encoding transgenic flies. This point highlights the need to always control for the proper genetic background when examining fly longevity.

We were ultimately interested in whether these decoys could reduce or prevent the toxicity we observe from intact pathogenic ataxin-3. In other words, could these decoys disrupt

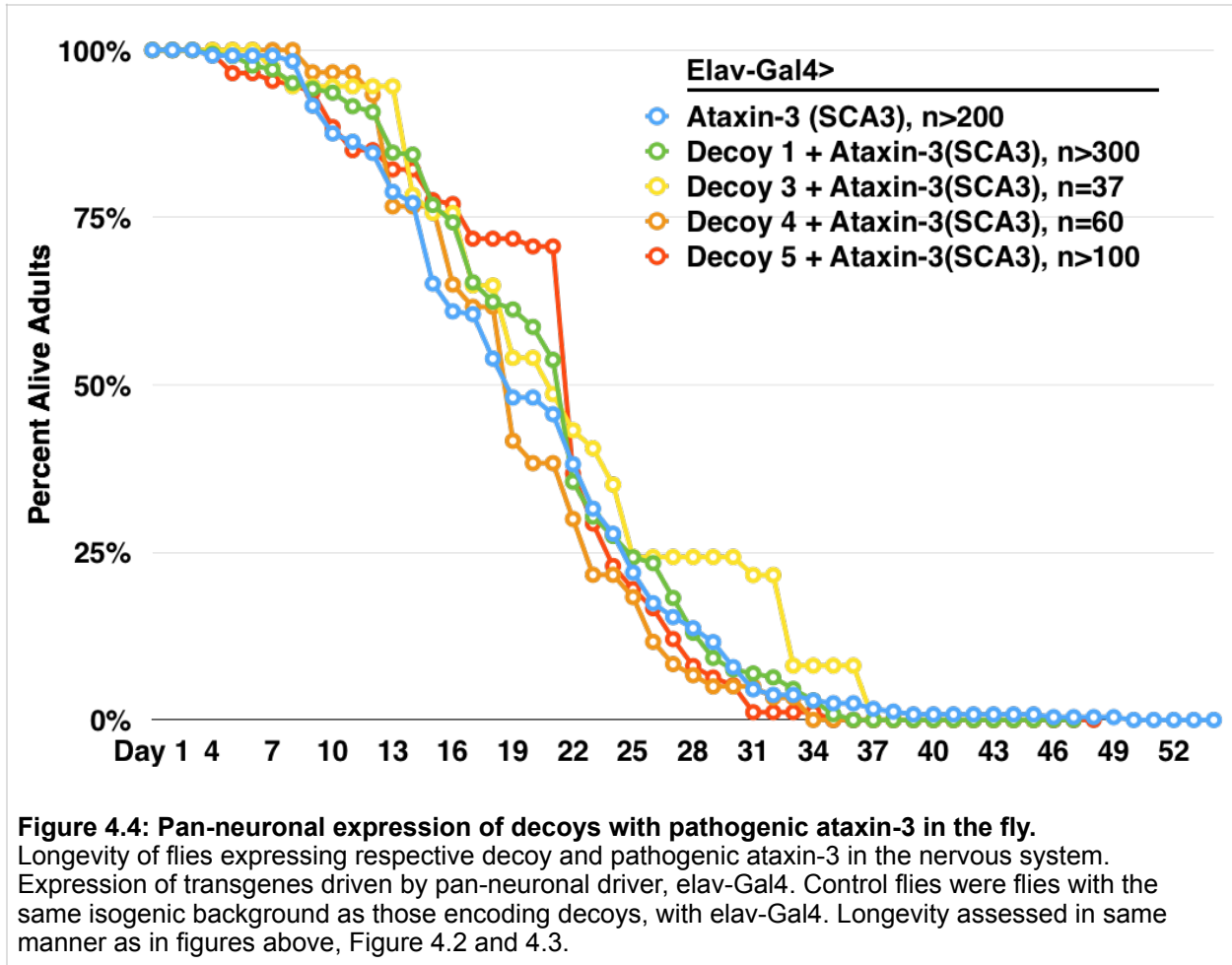
the interaction between ataxin-3 and VCP like mutating the VBM of ataxin-3 did, to delay the accumulation of aggregates in the fly? To better assess how toxicity of pathogenic ataxin-3 is affected by expression of decoy peptides in the whole fly, we first expressed pathogenic ataxin-3 on the same genetic host background as flies encoding the decoys. Using the ubiquitous, *sqh-Gal4* driver to express pathogenic ataxin-3 in the whole fly, we observed that ataxin-3 on this genetic background was extremely toxic and flies died as pharate adults: no flies eclosed from the pupal case (**figure 4.3, red arrow**). In comparison, when we expressed any of the decoys alongside ataxin-3 in the whole fly, toxicity was reduced and flies eclosed from the pupal case. We observed a similar trend as the above experiment (**Figure 4.2**): flies expressing decoy one and pathogenic ataxin-3 lived longer than flies expressing the other decoys with pathogenic ataxin-3 (**Figure 4.3, green line**), and those flies expressing decoy five exhibited the shortest lifespan (**Figure 4.3, red line**). Overall, however, these flies are still not outliving their counterparts expressing pathogenic ataxin-3 at the *attP2* site (**figure 4.3, blue line**). Future work needs to confirm that the decoys work as intended. It needs to be confirmed that they are specific for the ataxin-3-VCP interaction and are not disrupting interaction between VCP and other partners such as HRD1 and E4B.

As we showed in Chapter 3, expression of pathogenic ataxin-3 in the nervous system is less toxic to the fly than ubiquitous expression; flies expressing pathogenic ataxin-3 pan-neuronally all eclose from the pupal case and live longer than flies expressing pathogenic ataxin-3 ubiquitously (**figure 3.4A and figure 3.5B**). This gave us a longer timeline to examine decoy expression and its outcomes in flies. It is also a good model to examine the effects of pathogenic ataxin-3 and the decoys in the nervous system specifically. Thus, we expressed the



different decoys alongside pathogenic ataxin-3 with the pan-neuronal driver, elav-Gal4 (**figure 4.4**). We did not observe a difference in lifespan between flies expressing any decoy along side pathogenic ataxin-3 and flies in which pathogenic ataxin-3 was expressed alone pan-neuronally (**figure 4.4, blue line**). Leading us to the conclusion that pan-neuronal expression of decoy peptides does not reduce toxicity caused by pathogenic ataxin-3.

A shortcoming of these studies is that the line that was used to create the transgenic decoy flies is not as healthy as the fly line used to create the pathogenic ataxin-3 fly line. The decoys were inserted into the second chromosome, at the attP40 landing site, whereas the ataxin-3 transgene was inserted at the attP2 site on the third chromosome. We noticed during our studies that the fly line encoding the attP40 site was not as healthy as the other fly lines we used in the lab. This has made it difficult to easily examine whether the decoy peptides are improving the



phenotype that we see when pathogenic ataxin-3 is expressed, as the lifespan of flies is greatly shortened.

These experiments are still informative, however, and at the very least indicate that the decoys might not be as useful as originally intended to suppress toxicity from ataxin-3 in the fly. Further biochemical and physiological assays are necessary to understand their ability to engage VCP, to disrupt ataxin-3 interaction with VCP (and potentially other proteins that bind VCP through a similar region as ataxin-3's), and to reduce the aggregation of ataxin-3 *in vivo*. It will be beneficial to test these decoys in cells, by performing immuno-precipitation experiments to see how well each decoy binds to VCP, co-immunoprecipitation experiments to study how

expression of the decoys affects ataxin-3 binding to VCP and then further experiments to examine the effects of these decoys on protein aggregation in cells. In preliminary data, decoys 3 and 5 bind fly and human VCP very well, based on biochemical studies (Todi. S, Sutton. J, data not shown). The goal of transgenically expressing the decoy peptides was to obtain a general idea of whether or not we are able to disrupt the interaction between pathogenic ataxin-3 and VCP to decrease toxicity similarly to how we do by mutating the VBM. Future studies of these decoys should include orally feeding the decoys to *Drosophila*, although, based on my data so far this might not be a successful approach overall.

CHAPTER 5: CONCLUSIONS

The UPP is a vital pathway in the cell. It is necessary to rid the cell of mutant, misfolded or aberrant proteins in order to keep the cell functioning properly. Ubiquitination and deubiquitination play an important role in making sure this process happens in a controlled manner, by tagging proteins for degradation by ubiquitination, or saving them by removing their ubiquitin tag. When perturbations in this system arise, they can result in various deleterious diseases as highlighted in chapter one of this thesis. The overall goal of the research described in this thesis was to tackle two DUBs, one critical for normal physiological function (USP5), and the other directly linked to disease (ataxin-3).

I began my journey by investigating USP5. The findings in chapter two examined physiological function of USP5 *in vivo* in the fly. Although earlier reports proposed that the main function of USP5 was that of a mono-Ub recycler, our results showed that knockdown of USP5 in the fly, which was lethal during development, resulted in the accumulation of poly-Ub chains but not the depletion of mono-Ub. This led us to propose that USP5 was acting to hydrolyze ubiquitin chains to prevent their accumulation and hindrance of protein degradation in the cell. Reports published following ours also indicated the importance of USP5 handling poly-Ub chains to keep protein degradation operational. One group showed that USP5 mutant flies had abnormal neuromuscular junction morphology of the post synaptic end due to impairment of protein degradation (Wang et al., 2017). Other work highlighted the importance of USP5 as a direct DUB for specific proteins, supporting my *in vitro* results that USP5 can and does act on specific proteins (Garcia-Caballero et al., 2014).

As mentioned in chapter two, a paper by Garcia-Caballero et. al. showed that USP5 interacted with and deubiquinated the T-type calcium channel $Ca_v3.2$ in inflammatory and neuropathic pain models in rodents (Garcia-Caballero et al., 2014). The authors showed that USP5 could modulate the levels of this channel by deubiquitinating it and thus rescuing it from proteasomal degradation, increasing its overall cellular levels. We also showed that USP5 could have substrates other than unanchored poly-Ub chains. In a series of *in vitro* deubiquitinating reactions with recombinant proteins, we showed that USP5 could deubiquitinate CHIP, but it did not deubiquitinate Hsp70. These results raise the distinct possibility that USP5 has other specific substrates in the cell. This would be an interesting route to explore, since even though there are some overlapping roles between different DUBs, nothing seems able to compensate for loss of function of USP5, as its mutation or knockdown results in death during fly development.

My *in vitro* results with USP5 and CHIP uncover a potential link that could connect USP5 and ataxin-3. Studies have shown that wild type ataxin-3 can deubiquitinate CHIP; pathogenic ataxin-3 is unable to perform this function as well, and leads to decreased CHIP proteins levels in a SCA3 mouse model (Scaglione et al., 2011). CHIP has been shown over and over again to play an important role in suppressing polyQ related protein aggregation (Miller et al., 2005; Williams and Paulson, 2008). Whether CHIP is a substrate of USP5 *in vivo* remains to be studied, but it would be interesting to investigate whether USP5 deubiquitination of CHIP leads to an increase in protein levels as a protective mechanism in polyQ disease, or if it additionally decreases its levels and causes more toxicity.

Another interesting question to explore in terms of pathogenic ataxin-3 and aggregation in *Drosophila* is whether it is a reversible process, or if it is a point of no return. Is there a point

at which the cell could remove protein aggregates, when there is no additional burden by expression of pathogenic ataxin-3? Can neurons handle an amount of aggregates and survive with them, or once accumulation starts does it ever stop? I propose that stopping expression of the disease protein could reduce toxicity and prolong the lifespan of the SCA3 flies. This type of experiment would also be able to show us whether aggregates are cleared from the cell: do the smears on western blots decrease with time, or will they stop accumulating and remain at the same level? With the tools available to us, we would be able to express this protein in the whole fly and in a tissue specific manner. We would also be able to control when to start expression of ataxin-3, and as a natural extension of these tools we would also be able to utilize them to stop the expression of ataxin-3 in the fly. To study how stopping the expression of pathogenic ataxin-3 may affect toxicity in flies, I would propose to once again utilize the RU-486-inducible drivers. We would cross and raise flies on regular media without RU-486. Once flies eclose from the pupal case, they would be moved to RU468-containing food to activate expression of pathogenic ataxin-3. These flies would be aged on RU-486 food and flies collected at different time points to monitor expression of ataxin-3 by western blot of whole fly lysates. From our previous experiment with RU-486 inducible expression of pathogenic ataxin-3, we saw that accumulation of aggregates starts around day 10 of the fly life; we could use this as a starting point to stop ataxin-3 expression by moving flies from RU-486 containing food back to regular media. We would also monitor how long it takes for ataxin-3 expression and aggregation to be eliminated by collecting flies for lysates at multiple times after they are moved to regular fly media,. For flies expressing HNHH mutant ataxin-3, following the same logic as above and stopping ataxin-3 expression at a point when we first began to observe protein aggregation, a

good starting point to move the flies to food lacking RU-486 would be around day 21. We would also conduct longevity studies to see if the lifespan of flies is prolonged after ataxin-3 expression is stopped. This experiment has the potential to answer various questions regarding ataxin-3 aggregation and its toxicity *in vivo*, including the temporal relation between the two; it would also give more clues into whether removing a mutant protein from the organism would have a positive outcome and further decrease toxicity in the fly. This point could be explored for other polyQ diseases and a potential therapeutic strategy.

In conclusion, our studies provided new insights on the involvement of DUBs in the maintenance of ubiquitin homeostasis and in disease. Starting from a cellular function perspective, we examined the role of USP5 function *in vivo* in *Drosophila*, in contrary to earlier notions, we found that USP5 functioned to hydrolyze unanchored poly-Ub chains and not as a recycler of mono-Ub. Recent publications about USP5 support the idea that USP5 plays a role in maintaining normal function of the proteasome by acting on unanchored poly-Ub chains to prevent them from blocking proteasome substrates destined for degradation (Ling et al., 2017; Wang et al., 2017). Perhaps most intriguing of all, we showed that this enzyme could deubiquitinate a substrate in an *in vitro* reaction, substrate specificity would most likely be able to explain the developmental death observed when USP5 is knocked down in the fly. From a disease standpoint, we studied the ataxin-3 and VCP interaction role in SCA3 pathogenesis. Our work built on previously published work that discovered this interaction and show that VCP has the ability to modulate pathogenic ataxin-3 toxicity in an *in vivo* disease model. We showed that disrupting the interaction between these two proteins results in the delay of protein aggregation

and toxicity. This interaction offers a potentially promising therapeutic target to further investigate for SCA3 treatment.

DUBs serve an important role in the cell, as highlighted in the studies above. There are over 90 DUBs in humans and we are just skimming the surface in terms of understanding their roles, functions and substrates in the cells. This field offers many exciting possibilities that need to be further investigated.

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ABSTRACT**DEUBIQUITINASES IN UBIQUITIN HOMEOSTASIS AND DISEASE**

by

GORICA RISTIC**August 2017****Advisor:** Dr. Sokol V. Todi**Major:** Pharmacology**Degree:** Doctor of Philosophy

Protein quality control (PQC) is indispensable for normal cellular functions, ensuring proteins are properly folded and removing those proteins that are functioning aberrantly. Perturbations in PQC can lead to various malignancies, neurodegeneration and neurological diseases. The Ubiquitin-Proteasome Pathway (UPP) is one important pathway in PQC, it relies on ubiquitination-dependent post-translational modification to selectively degrade misfolded or short-lived protein. Ubiquitination is reversed by the action of proteases known as deubiquitinating enzymes (DUBs). By hydrolyzing ubiquitin linkages, DUBs are responsible for cleaving and activating newly produced ubiquitin molecules, editing poly-Ub chains, removing ubiquitin from a substrate protein and recycling mono-Ub. Here, I investigated two proteins critically involved in the UPP: one that is important for normal cellular function (USP5), and another whose mutations cause neurodegeneration (ataxin-3).

Ubiquitin Specific Protease 5 (USP5) is a member of the USP class of DUBs. Based on structural and *in vitro* results, it was long thought to function as a recycler of mono-Ub in the cell, by hydrolyzing unanchored poly-Ub chains. We studied the function of USP5 *in vivo* in

Drosophila melanogaster. Knockdown of DmUSP5 in the whole fly results in death during developmental stages and in an increase in poly-Ub chains, but does not result in a depletion of mono-Ub in the fly, contrary to long-held opinion. Based on our data, lethality from USP5 knockdown is not due to depletion of mono-Ub, but likely resulted due to accumulation of unanchored poly-Ub chains and disruption of proteasomal degradation. Our results also suggest that USP5 has specific substrates.

The other DUB investigated, ataxin-3, is the disease causing protein in the age-related, poly-glutamine (polyQ) dependent disease Spinocerebellar Ataxia Type-3 (SCA3). Through its different interacting partners, ataxin-3 is involved in protein quality control pathways. One such protein is the AAA ATPase Valosin Containing Protein (VCP). Ataxin-3 interacts directly with VCP through a VCP Binding Motif (VBM). Through biochemistry, *Drosophila* genetics and physiological assays, we showed that pathogenic ataxin-3 with a mutated VBM is markedly less toxic than its intact pathogenic counterpart. Mutating the VBM of ataxin-3 caused a delay in the aggregation of ataxin-3 *in vivo*, in various tissues tested. We propose that VCP acts as a nucleation center to increase local concentrations of ataxin-3 proteins and increase their likelihood of interacting and fibrilization. Our work suggest this interaction as a potential therapeutic target for SCA3.

AUTOBIOGRAPHICAL STATEMENT*EDUCATION***- Wayne State University, Detroit, MI**

Bachelor of Science, Biology (Magna Cum Laude)- December 2011

-Wayne University School of Medicine, Detroit, MI

Ph.D in Pharmacology Graduate Program - August 2012-present; Expected graduation
08/2017

PUBLICATIONS

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