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**THE INFLUENCE OF PROTEIN CONTEXT ON POLYGLUTAMINE TOXICITY IN
SPINOCEREBELLAR ATAXIA TYPE 3**

by

JOANNA RAE SUTTON

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

2018

MAJOR: PHARMACOLOGY

Approved By:

Advisor

Date

DEDICATION

To Connor, for sharing this journey with me.

To Mom, Dad, and Jack, for your unconditional love.

And in loving memory of Uncle Dave.

ACKNOWLEDGEMENTS

First, I would like to thank my graduate advisor, Dr. Sokol Todi, for being a wonderful mentor and friend. Through your guidance, patience, and enthusiasm, you taught me how to be a better scientist and individual. I would also like to thank my thesis committee members: Drs. Karin List, Isabela Podgorski, Michael Bannon, and K. Matthew Scaglione. Your mentorship propelled these projects forward and helped me achieve this milestone in my research career. I am grateful to the past and present members of the Todi lab for being great colleagues, teachers, and friends. I extend my gratitude to the faculty and staff of the Department of Pharmacology at Wayne State University School of Medicine. I also acknowledge my funding source from the NIH-NINDS (F31NS095575).

I am so grateful for my family and friends, who were amazing supporters every step of the way. A special thank you goes to my parents and brother, for always believing in me. I would like to thank my cousin, Marian, for being a role model in science and in life. I'm also grateful to Gorica for her advice, support, and friendship during this hectic time.

Finally, I am eternally grateful to my husband, Connor. Words cannot adequately express how you supported me during this experience. Your love, positivity, patience, and selflessness pushed me forward whenever I faltered. If we can both simultaneously pursue PhDs without killing each other, I'm confident we can do anything! Thank you.

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LIST OF ABBREVIATIONS

AR: Androgen receptor

Ataxin-3(SCA3): Pathogenic ataxin-3

CBP: CREB-binding protein

CHIP: C-terminus of Hsc70-interacting protein

DRPLA: Dentatorubral-pallidoluysian atrophy

DUB: Deubiquitinating enzyme

HAT: Histone acetyltransferase

HD: Huntington's disease

HDAC: Histone deacetylase

HNHH: Mutation in VCP-binding site of ataxin-3; replaces the RKRR motif

Htt: huntingtin

MJD: Machado-Joseph disease

N17: the first 17 amino acids of huntingtin

PNKP: polynucleotide kinase 3'-phosphatase

PolyP: polyproline region of huntingtin

PolyQ: Polyglutamine

SBMA: Spinal and bulbar muscular atrophy

SCA: Spinocerebellar ataxia (type 1, 2, 3, 6, 7, or 17)

Ub: Ubiquitin

UbS2: Ubiquitin-binding site 2

UbS2^{Mild}: Mutation in Ubiquitin-binding site 2 of ataxin-3; replaces a tryptophan residue with alanine. Results in a mild reduction in Rad23 binding at this site

UbS2*Strong: Mutation in Ubiquitin-binding site 2 of ataxin-3; replaces three amino acid residues to glutamic acid. Results in a strong reduction in Rad23 binding at this site

UIM: Ubiquitin interacting motif

VCP: Valosin-Containing Protein, also known as p97

CHAPTER 1: INTRODUCTION

1.1 Spinocerebellar ataxia type 3

The polyglutamine (polyQ) disorders are a group of nine genetically inherited diseases caused by CAG triplet repeat expansions in nine distinct genes (Matos et al., 2011). Each disease gene translates into a protein with an abnormally expanded polyQ tract. These mutated proteins have increased propensities for misfolding and aggregation, leading to neurotoxicity and neurodegeneration (Matos et al., 2011). For all polyQ disorders, the length of the polyQ tract is inversely correlated with the age of onset and directly correlated with disease severity (Costa Mdo and Paulson, 2012). Huntington's disease (HD), Spinal-bulbar muscular atrophy (SBMA), Dentatorubral-pallidoluysian atrophy (DRPLA), and six spinocerebellar ataxias (SCA 1, 2, 3, 6, 7, 17) belong to this class (**Table 1.1**). All polyQ disorders are dominantly inherited, except for the X-linked SBMA (Costa Mdo and Paulson, 2012).

SCA3, or Machado-Joseph disease (MJD), is the most common dominantly inherited ataxia (Costa Mdo and Paulson, 2012). The disease is caused by CAG repeat expansion in the *ATXN3* gene, located on chromosome 14 (Takiyama et al., 1993). As a result, the polyQ tract of the ataxin-3 protein increases from a normal range of ~10-40 repeats to a disease range of ~60-80 repeats (Costa Mdo and Paulson, 2012; Matos et al., 2011). The primarily degenerated regions are the cerebellar pathways and nuclei, basal ganglia, midbrain, brainstem, and spinal cord (Costa Mdo and Paulson, 2012; Matos et al., 2011). Onset of SCA3 occurs in middle to late life, with symptoms including progressive ataxia accompanied by complications with speech, swallowing, vision, muscle tone, neuropathy and more (Matos et al., 2011). Over time, the patients will unfortunately succumb to the disease, since there is currently no cure. This introduction will review the normal functions of ataxin-3 and the role of protein context in polyQ

Table 1.1: The Polyglutamine Diseases

Disease	Protein	PolyQ Repeat Length		Regions Most Affected
		Normal	Pathogenic	
HD	Huntingtin (htt)	6-35	36-121	Striatum, cerebral cortex
SBMA	Androgen receptor (AR)	6-36	38-62	Anterior horn, bulbar neurons, dorsal root ganglia
DRPLA	Atrophin 1	3-38	49-88	Pallidum, subthalamic, dentate nuclei and red nucleus
SCA1	Ataxin-1	6-39	41-83	Cerebellar Purkinje cells, dentate nucleus, brainstem
SCA2	Ataxin-2	14-32	34-77	Cerebellar Purkinje cells, brainstem, frontotemporal lobes
SCA3	Ataxin-3	12-40	62-86	Deep cerebellar nuclei, basal ganglia, red nucleus, brainstem, spinal cord
SCA6	CACNA1A	4-18	21-30	Cerebellar Purkinje cells, dentate nucleus, inferior olive
SCA7	Ataxin-7	7-18	38-200	Cerebellum, brainstem, macula, visual cortex
SCA17	TATA-binding protein	25-43	45-63	Cerebellar Purkinje cells, inferior olive

Adapted from: Matos et al., 2011 and Esteves et al., 2017

disorders. Both topics can provide insight into aberrant mechanisms in SCA3 to target for therapeutics.

1.2 Physiological functions of ataxin-3

Ataxin-3 is a deubiquitinating enzyme of the MJD subfamily (Burnett et al., 2003; Winborn et al., 2008). It is found in both the nucleus and the cytoplasm, and is expressed ubiquitously throughout the tissues (Costa et al., 2004; Paulson et al., 1997a; Schmidt et al., 1998; Trottier et al., 1998). As such, ataxin-3 is involved in numerous functions in the cell (Costa Mdo and Paulson, 2012). It has several distinct domains that mediate its interactions with other

proteins (**Figure 1.1**). The cysteine protease domain, called the Josephin domain, is located at the structured N-terminus (Masino et al., 2003; Masino et al., 2004). Two ubiquitin binding sites (UbS1/2) are also located in this region to facilitate the recognition and positioning of ubiquitin (Ub) chains for cleavage (Nicastro et al., 2009; Nicastro et al., 2010). In addition to binding Ub, UbS2 also binds to the proteasomal shuttle protein, Rad23 (Doss-Pepe et al., 2003; Nicastro et al., 2005; Wang et al., 2000). Depending on the isoform, two or three ubiquitin-interacting motifs (UIMs) are located at the flexible C-terminus (Goto et al., 1997; Harris et al., 2010; Schmidt et al., 1998). These motifs are crucial for ataxin-3's binding to polyUb chains, as well as its cleavage specificity (Burnett et al., 2003; Doss-Pepe et al., 2003; Todi et al., 2010). An arginine-lysine rich motif that is essential for binding to the AAA ATPase, VCP, is located between the second UIM and the polyQ tract (Boeddrich et al., 2006; Doss-Pepe et al., 2003). This section will review how ataxin-3's protein-binding domains facilitate its functions in protein quality control, transcriptional regulation, and DNA repair. Potential loss- or gain-of-functions that could contribute to SCA3 will be discussed throughout.

1.2.1 Protein quality control

Ataxin-3 has been implicated in several protein quality control pathways. The most obvious among them is the ubiquitin proteasome system (UPS), due to ataxin-3's DUB activity (Burnett et al., 2003). Ataxin-3 demonstrates cleavage selectivity for chains of 5 or more (Ub) moieties (Winborn et al., 2008). While this DUB can cleave both K48- and K63-linked Ub chains *in vitro*, it shows the greatest catalytic activity towards K63-linkages, especially those within mixed K48-K63 chains (Winborn et al., 2008). Monoubiquitination of ataxin-3, primarily at lysine 117, and sumoylation of ataxin-3 at its N-terminus enhance its DUB activity (Pfeiffer et al., 2017; Todi et al., 2010; Todi et al., 2009). *In vivo*, ataxin-3 likely modulates the

ubiquitination status of many proteins, since total levels of ubiquitination are increased in *atxn3* knockout mouse brains and ataxin-3-depleted human cell lines, while total ubiquitination levels are decreased when ataxin-3 is overexpressed in HEK293 cells (Rodrigues et al., 2010; Scaglione et al., 2011; Schmitt et al., 2007; Winborn et al., 2008).

Ataxin-3 is known to regulate the E3 ligases, C-terminus of Hsc70-interacting protein (CHIP) and parkin (Durcan et al., 2011; Scaglione et al., 2011; Winborn et al., 2008). Through Ube2w-mediated monoubiquitination of CHIP, ataxin-3 is recruited to edit the length of polyUb chains on CHIP substrates (Scaglione et al., 2011). Once polyubiquitinated substrates accumulate, ataxin-3 deubiquitinates CHIP to end the reaction (Scaglione et al., 2011). Initially, ataxin-3 was believed to reduce levels of ubiquitinated parkin via the typical deubiquitination model (Durcan et al., 2011), but further examination suggests that this regulatory mechanism is more complex. Ataxin-3 appears to bind both parkin and the cooperating E2 enzyme (Ubc7), stabilize their interaction, and then “re-route” parkin’s self-monoubiquitination reaction onto ataxin-3 itself (Durcan et al., 2012). This process is still dependent on ataxin-3’s catalytic activity (Durcan et al., 2012). This proposed mechanism is very interesting, since it suggests ataxin-3 is capable of 1) an atypical mechanism of deubiquitination and 2) regulating E2 enzymes, not just E3s. In the cases of both parkin and CHIP, deubiquitination is partially mediated by ataxin-3’s UIMs (Bai et al., 2013; Scaglione et al., 2011). PolyQ-expansion of ataxin-3 seems to heighten both interactions, which correlates with decreased levels of these E3s in an SCA3 mouse model (Durcan et al., 2011; Scaglione et al., 2011). Pathogenic ataxin-3 also enhances parkin’s autophagic degradation in cells, which could explain the decreased levels in the mouse model (Durcan et al., 2011). Ataxin-3’s normal role in autophagy will be discussed later in this section.

Table 1.2: Substrates of Ataxin-3

Substrate	Pathway	Regulation by Ataxin-3	Reference
E3 ligases			
CHIP	Ubiquitination	Ligase activity	Scaglione et al., 2011 Winborn et al., 2008
Parkin	Ubiquitination	Protein stability	Durcan et al., 2011 Durcan et al., 2012
Cell cycle checkpoint mediators			
Chk1	DNA repair	Protein stability	Tu et al., 2017
MDC1*	DNA repair	Interaction with DNA	Pfeiffer et al., 2017
p53	Apoptosis	Protein stability	Liu et al., 2016
Autophagy regulators			
Beclin-1	Autophagy	Protein stability	Ashkenazi et al., 2017

*It has not yet been confirmed that ataxin-3 *directly* deubiquitinates MDC1.

Recently, p53 was discovered to be another substrate of ataxin-3 (Liu et al., 2016). The authors reported that the N-terminus of ataxin-3 is essential for direct binding to p53, and the UIMs assist with binding to the ubiquitinated form. Ataxin-3 deubiquitinates p53, stabilizes the protein by preventing its proteasomal degradation, and promotes transcription of p53-target genes. Interestingly, pathogenic ataxin-3 has increased binding and DUB activity towards p53. This enhances p53 stability, transcriptional activity, and neuronal loss in both zebrafish and mouse brains (Liu et al., 2016). It is worth noting that another lab previously reported that pathogenic ataxin-3 increased p53 transcriptional activation and subsequent cell death, but this was not attributed to an increase in p53 protein levels (Chou et al., 2011b). Nevertheless, the work by Liu et al. is exciting because it demonstrates that ataxin-3 can deubiquitinate proteins other than ligases (**Table 1.2**), and these interactions may contribute to SCA3 progression when the polyQ tract is expanded.

Ataxin-3 physically interacts with polyubiquitinated proteins, the proteasome, and several shuttle proteins, including Rad23, VCP, and ubiquilin-1 (Boeddrich et al., 2006; Doss-Pepe et al.,

2003; Heir et al., 2006; Nicastro et al., 2005; Wang et al., 2000). This prompted scientists to hypothesize that in addition to deubiquitinating substrates, ataxin-3 may facilitate their transfer to the proteasome degradation machinery (Doss-Pepe et al., 2003). Corroborating this idea, multiple labs reported that ataxin-3 cooperates with VCP to regulate the degradation of aberrant proteins through the endoplasmic reticulum-associated degradation pathway (ERAD)(Arrojo et al., 2013; Wang et al., 2006, 2008; Zhong and Pittman, 2006). However, these studies arrived at slightly different conclusions as to how this function is carried out. It remains uncertain if ataxin-3 is mediating substrate translocation across the ER membrane by VCP (Zhong and Pittman, 2006), or if it is modulating the transfer of substrates from VCP to the proteasome (Wang et al., 2006, 2008). Also unclear is if ataxin-3's catalytic activity is involved in this process. In terms of polyQ expansion, one study found that polyQ-expanded ataxin-3 was a greater negative regulator of ERAD substrate degradation than its non-expanded form (Zhong and Pittman, 2006).

In addition to the UPS, ataxin-3 may also monitor substrate degradation by autophagy. Recently, a study reported that ataxin-3 promotes starvation-induced autophagy through polyQ-mediated binding to beclin-1 and subsequent stabilization via deubiquitination (Ashkenazi et al., 2017). The authors also reported that pathogenic ataxin-3 exhibits a dominant-negative effect, whereby it out-competes wild type ataxin-3 for binding to beclin-1, but has reduced catalytic activity towards the protein. The result is reduced levels of beclin-1, presumably through increased proteasomal degradation, and impaired starvation-induced autophagy in cells (Ashkenazi et al., 2017). This seems to contradict Durcan's previous observation that pathogenic ataxin-3 increased degradation of parkin by autophagy (Durcan et al., 2011). It is likely that individual autophagy substrates are affected differently by the polyQ expansion of ataxin-3.

Nevertheless, autophagy impairment and reduced beclin-1 levels have also been reported in other SCA3 models (Chang et al., 2016; Nascimento-Ferreira et al., 2011; Onofre et al., 2016). Furthermore, multiple studies have reported improved phenotypes with autophagy-inducing compounds and restoration of beclin-1 expression (Chang et al., 2016; Jia et al., 2013; Menzies et al., 2010; Nascimento-Ferreira et al., 2013; Nascimento-Ferreira et al., 2011; Onofre et al., 2016; Silva-Fernandes et al., 2014; Wu et al., 2017). However, this could be due to increased clearance of aggregated ataxin-3, rather than a restoration of ataxin-3's potential role in autophagy.

Several lines of evidence suggest a protective role for ataxin-3 against cell stressors. Ataxin-3 is involved in aggresome formation as a means to sequester misfolded proteins (Burnett and Pittman, 2005; Wang et al., 2012). To accomplish this, ataxin-3 may edit the chains of ubiquitinated substrates to signal their transport to the microtubule organizing center. This is supported by ataxin-3's interactions with HDAC6, dynein, and tubulin (Bonanomi et al., 2014; Burnett and Pittman, 2005; Mazzucchelli et al., 2009; Wang et al., 2012). In response to heat shock, ataxin-3 localizes to the nucleus (Reina et al., 2010) and upregulates transcription of Hsp70 (Reina et al., 2012). Notably, ataxin-3 also protects against heat stress in yeast (Bonanomi et al., 2017). When compared to their wild type counterparts, ataxin-3-knockout mouse fibroblasts exhibit increased cell death and decreased hsp70 activation in response to heat shock (Reina et al., 2012; Reina et al., 2010). Interestingly, pathogenic ataxin-3 still accumulates in the nucleus and activates the hsp70 promoter upon cell stress, suggesting that it may retain some of its protective role (Reina et al., 2012; Reina et al., 2010). However, these effects could just be a by-product of expressing a pathogenic protein that has a propensity to misfold and aggregate in the nucleus.

Upon oxidative stress, ataxin-3 translocates to the nucleus and promotes transcription of the antioxidant enzyme, SOD2 (Araujo et al., 2011; Reina et al., 2010). To accomplish this, ataxin-3 binds the transcription factor, FOXO4, and then they jointly bind to the SOD2 promoter. Pathogenic ataxin-3 can bind to FOXO4 at a comparable degree to wild type ataxin-3, but it exhibits increased binding to the SOD2 promoter (Araujo et al., 2011). Ataxin-3's increased binding impairs activation of SOD2 transcription, likely due to blocking FOXO4 binding to the promoter (Araujo et al., 2011). Another study reported that ataxin-3 protects against oxidative stress by regulating the interaction between Bcl-X(L) and Bax (Zhou et al., 2013a). Furthermore, ataxin-3 protected against oxidative stress in a cellular model of Parkinson's disease (Noronha et al., 2017).

Ataxin-3 is also protective in *Drosophila* models of polyQ diseases, which further supports the conclusions drawn from the cell-based experiments. Ataxin-3 reduces toxicity in fly models of SCA1, SCA3, and HD, likely by preventing aggregation of the disease proteins (Tsou et al., 2013; Tsou et al., 2015b; Warrick et al., 2005). However, it is unclear whether proteasome activity is required for this process, since two independent studies arrived at opposite conclusions (Tsou et al., 2015b; Warrick et al., 2005). Both research groups observed that ataxin-3's DUB activity is required for its protective function (Tsou et al., 2013; Tsou et al., 2015b; Warrick et al., 2005). Recently, our lab reported that ataxin-3 reduces toxicity by interacting with Rad23 to upregulate expression of the chaperone protein, DnaJ-1 (Tsou et al., 2015b). Presumably, DnaJ-1's refolding of misfolded polyQ proteins is responsible for their improved solubility in these *Drosophila* disease models.

From the studies outlined above, ataxin-3 clearly reduces toxicity caused by environmental stressors in cells and expanded polyQ in *Drosophila*. However, the significance of

these findings has been questioned, due to contradictory results obtained from mouse models of polyQ diseases. One study coincided with the results from cells and flies: YAC transgenic mice with both an expanded and non-expanded allele for ataxin-3 fared better than mice with only the expanded allele (Cemal et al., 2002). A separate research group also tested the benefit of over expression of non-expanded ataxin-3 in their transgenic SCA3 model, but they did not observe any phenotypic improvement (Hubener and Riess, 2010). Another study observed that knock-out of ataxin-3 resulted in a modest increase in the motor deficiency of HD mice, but otherwise the phenotype was unaffected (Zeng et al., 2013). Additional studies confirm that ataxin-3 knockout mice do not exhibit any obvious adverse effects (Schmitt et al., 2007; Switonski et al., 2011). At this point, it is unclear if ataxin-3's neuroprotective function in flies and cells translates to mammalian systems.

1.2.2 Transcriptional regulation

As mentioned above, ataxin-3's cytoprotective mechanisms include transcriptional activation of Hsp70, SOD2, and DnaJ-1. Alternatively, ataxin-3 is known to act as a transcriptional co-repressor under non-stress conditions. This seems to be mediated, at least in part, by modulating acetylation pathways. Ataxin-3 interacts with the histone acetyltransferases (HATs), CREB-binding protein (CBP), p300, and p300/CBP-associated factor to inhibit their downstream transcription (Chai et al., 2001; Li et al., 2002). In accordance with this, ataxin-3 binds histones H3/H4, and inhibits H4 acetylation by p300 (Li et al., 2002). The N-terminus of ataxin-3 appears to be responsible for the histone-binding function, while the C-terminus facilitates interactions with the HATs (Li et al., 2002). Ataxin-3 also recruits histone deacetylase 3 (HDAC3) to reduce both histone acetylation and transcription by the matrix metalloproteinase-2 promoter (Evert et al., 2006). Pathogenic ataxin-3 is still capable of binding

its interacting partners in the transcriptional pathways tested (Chai et al., 2001; Evert et al., 2006; Li et al., 2002; Lin et al., 2014).

Overall, there is a consensus that ataxin-3's transcriptional regulation is altered when the polyQ is expanded, but there is conflicting evidence as to whether the transcriptional activation or repression contributes to SCA3. For example, there are instances where pathogenic ataxin-3 loses its repressor function, and gains an activating function (Chai et al., 2001; Evert et al., 2006). Conversely, there are studies reporting that pathogenic ataxin-3 represses transcription, and alleviating this improves the phenotype in SCA3 mouse models (Chou et al., 2011a; Chou et al., 2014). In some cases, pathogenic ataxin-3 increases histone acetylation (Evert et al., 2006), but it decreases acetylation in others (Lin et al., 2014). Increasing acetylation levels with HDAC inhibitors often improves the phenotype in SCA3 mouse and *Drosophila* models (Chou et al., 2011a; Chou et al., 2014; Lin et al., 2014; Wang et al., 2018; Yi et al., 2013), but not always (Esteves et al., 2015). Most likely, it varies based on the gene whether a loss or gain of transcriptional repression contributes to SCA3 toxicity. The different disease models in use could also be contributing to the variability in results. Interestingly, ataxin-3's function as a transcriptional repressor may also be relevant in cancer, since it reportedly inhibits transcription of the tumor suppressor, PTEN (Sacco et al., 2014).

1.2.3 DNA repair

In the past few years, researchers have published exciting findings that identify a novel role for ataxin-3 in DNA repair pathways. For example, ataxin-3 interacts with polynucleotide kinase 3'-phosphatase (PNKP), a DNA repair enzyme involved in single-strand break repair and non-homologous end joining (Chatterjee et al., 2015; Gao et al., 2015). Ataxin-3 stimulates PNKP's phosphatase activity so it can signal DNA repair pathways (Chatterjee et al., 2015; Gao

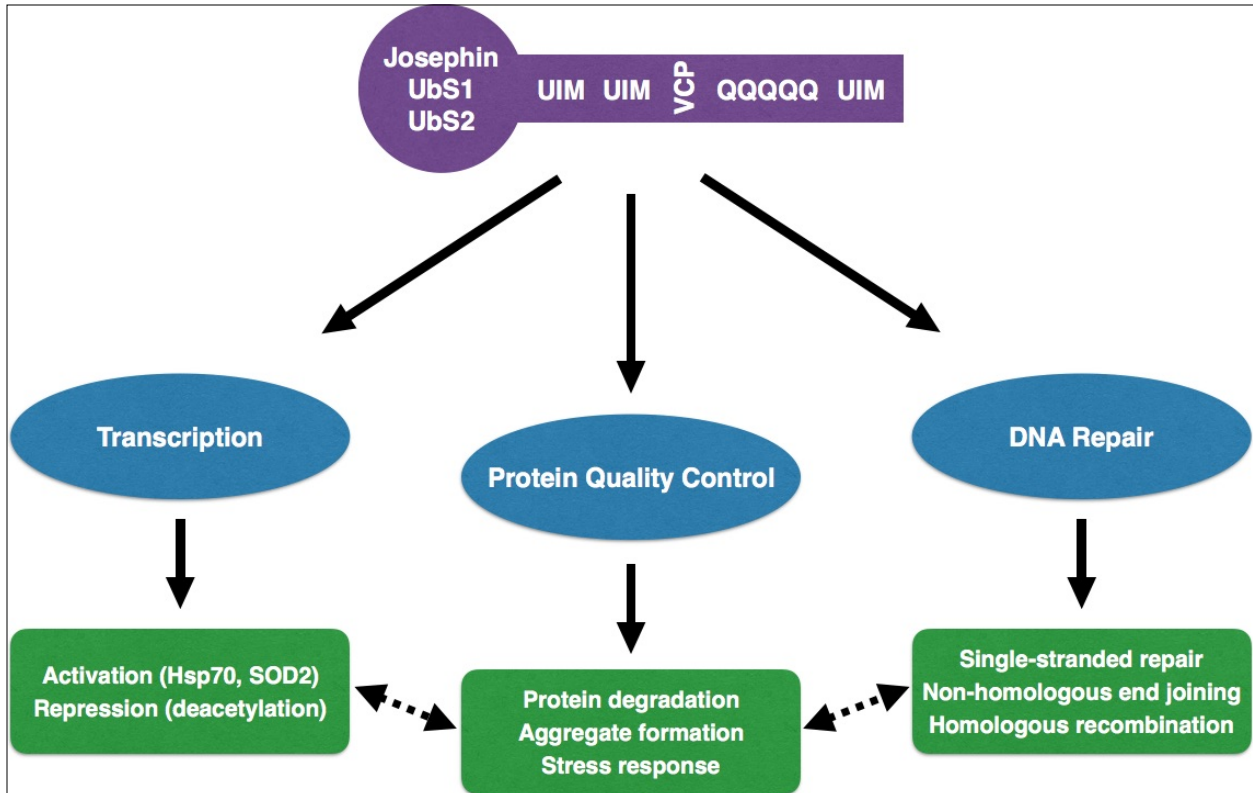


Figure 1.1. The physiological functions of ataxin-3. Ataxin-3 has multiple protein-interacting domains that are essential for performing its functions in protein quality control, transcription, and DNA repair. There is cross-talk between some of these pathways.

et al., 2015). On the other hand, pathogenic ataxin-3 inhibits PNKP's phosphatase activity, increases DNA damage, activates the ATM-mediated damage-response pathway, and triggers p53-dependent apoptosis in cells and SCA3 transgenic mice (Chatterjee et al., 2015; Gao et al., 2015). These findings are in agreement with a role for ataxin-3 in regulation of p53's stability and subsequent transcription of apoptotic genes (Liu et al., 2016). Furthermore, an increase in DNA damage was observed in SCA3 patient brain tissue (Chatterjee et al., 2015; Gao et al., 2015).

Complimentary to these findings, a study using *C. elegans* reported that ataxin-3 localizes to double-stranded break-associated foci, which contain CDC-48(VCP), proteasomes, and the E4 ligase, Ufd-2 (Ackermann et al., 2016). These foci are associated with an increase in DNA

damage-dependent apoptosis. However, the presence of ataxin-3 seems to oppose the formation of these foci, since ataxin-3 mutants exhibit increased formation of foci and apoptotic signaling (Ackermann et al., 2016). These findings raise the possibility that ataxin-3's function in the UPS could also overlap with its regulation of DNA repair pathways.

Supporting this idea, the ubiquitination of Chk1 and MDC1, two DNA damage checkpoint mediators, were found to be regulated by ataxin-3 (Pfeiffer et al., 2017; Tu et al., 2017). In response to DNA damage, ataxin-3 deubiquitinates Chk1 to prevent its proteasomal degradation (Tu et al., 2017). This allows Chk1 to activate the G2/M DNA damage checkpoint and signal for DNA repair mechanisms (Tu et al., 2017). After prolonged DNA stress, ataxin-3 dissociates from Chk1 so it can be degraded, thus resuming the cell cycle. The polyQ-expanded form of ataxin-3 still interacts with and stabilizes Chk1 (Tu et al., 2017). Alternatively, ataxin-3 was also found to localize to sites of DNA damage in a sumoylation-dependent manner (Nishi et al., 2014; Pfeiffer et al., 2017). Importantly, other labs have also gathered evidence for sumoylation of ataxin-3 (Almeida et al., 2015; Guzzo and Matunis, 2013; Shen et al., 2005; Zhou et al., 2013b). Once recruited to the DNA, ataxin-3 counteracts the sumo-targeted Ub ligase, RNF4, to reduce the ubiquitination of MDC1 (Pfeiffer et al., 2017). This is presumably through deubiquitination, since ataxin-3's catalytic activity is required for this effect. In this manner, ataxin-3 increases MDC1's interaction with the DNA and the MDC1-mediated recruitment of other DNA repair proteins (Pfeiffer et al., 2017).

Further supporting these collective findings, loss of ataxin-3 compromises the Chk1-, MDC1-, and PNKP-mediated DNA damage response pathways and sensitizes cells to DNA-damaging agents (Chatterjee et al., 2015; Pfeiffer et al., 2017; Tu et al., 2017). Altogether, these studies demonstrate that ataxin-3 is involved in multiple major DNA repair pathways. Future

work will enhance our understanding of this physiological function of ataxin-3 and how it plays into SCA3.

1.3 Protein context in polyglutamine disorders

Despite the shared mutation in the polyQ tract, each polyQ disorder presents with a distinct set of symptoms and degenerated brain regions (Matos et al., 2011). This selective degeneration is very intriguing, since most of these disease proteins are widely expressed, and many are expressed in the same cell types (Costa Mdo and Paulson, 2012). Furthermore, each disorder has its own threshold for when the polyQ expansion of the disease protein switches from normal to toxic (La Spada and Taylor, 2003). Humans also possess genes encoding polyQ proteins that do not cause disease (Schaefer et al., 2012), leaving scientists to wonder what is so significant about the polyQ proteins that cause this class of neurodegenerative diseases.

For these reasons, the protein context, meaning the normal physiological functions and non-polyQ domains of the disease proteins, are thought to play a major role in pathogenesis (La Spada and Taylor, 2003; Orr, 2012; Robertson and Bottomley, 2010). For example, non-polyQ domains surrounding the polyQ tract, including their post-translational modifications, can influence the aggregation and toxicity of the full-length protein (Adegbuyiro et al., 2017; La Spada and Taylor, 2003; Orr, 2012; Pennuto et al., 2009; Robertson and Bottomley, 2010; Saunders and Bottomley, 2009). Protein-interacting domains that are required for a non-expanded polyQ protein's function can provide clues for toxic mechanisms that occur when the polyQ tract becomes expanded (La Spada and Taylor, 2003; Orr, 2012; Saudou and Humbert, 2016). In some cases, the presence or absence of non-polyQ domains in the full-length protein can determine whether disease pathogenesis occurs, or not (Klement et al., 1998; Tsuda et al.,

2005). This section will highlight the most striking evidence for a link between protein context and polyQ disease pathogenesis.

1.3.1 Huntington's disease (HD)

HD is the most common polyQ disorder, so naturally, it is the most studied. There is a wealth of information supporting protein context in HD. Many post-translational modifications influence huntingtin (htt) toxicity, including phosphorylation (Schilling et al., 2006), acetylation (Jeong et al., 2009), ubiquitination (Jana et al., 2005), sumoylation (Steffan et al., 2004) and caspase cleavage (Graham et al., 2006). Additionally, several protein-interaction pathways may influence HD disease mechanisms (Saudou and Humbert, 2016). Unfortunately, it is unfeasible to adequately address all of the studies on HD protein context in this chapter. Instead, this section will focus on two domains immediately adjacent to the polyQ tract that influence huntingtin (htt) aggregation and toxicity.

Regions flanking the polyQ domain of htt have been found to alter its aggregation propensity. Notably, the first 17 amino acids of htt (N17), located upstream of the polyQ tract, promote its aggregation *in vitro* (Adegbuyiro et al., 2017; Hoffner and Djian, 2014). This is believed to occur through stimulation of htt self-association, since N17 can bind to other N17 domains, as well as the polyQ tract (Hoffner and Djian, 2014; Tam et al., 2009; Thakur et al., 2009). Blocking N17 can reduce aggregation of pathogenic Htt-exon1, as evidenced by binding of the chaperone, TriC, in mammalian cells (Tam et al., 2009). N17 of full-length htt can be post-translationally modified by phosphorylation at serines 13 and 16 (Gu et al., 2009). The significance of this modification has been confirmed *in vivo*; mutation of these residues to alanine prevents motor, behavioral, neurodegenerative, and aggregation phenotypes in BAC transgenic mice expressing full-length, mutant htt (Gu et al., 2009).

Alternatively, the polyproline region (polyP) of htt, located at the C-terminus of the polyQ tract, reduces the aggregation propensity of the HD protein. When polyP is added onto the C-terminus of a pure polyQ tract, the conformation of the polyQ is altered and its aggregation rate is decreased *in vitro* (Bhattacharyya et al., 2006; Thakur et al., 2009). By preventing the polyQ from adopting a β -sheet conformation, polyP protects against htt aggregation (Darnell et al., 2007; Lakhani et al., 2010). In yeast cells, deletion of the proline-rich domain leads to increased aggregation and toxicity of polyQ-expanded htt-exon1 (Dehay and Bertolotti, 2006; Duennwald et al., 2006). Intriguingly, the effects of the polyP domain are position-specific, since addition of polyP to the N-terminus of the polyQ tract does not influence aggregation (Bhattacharyya et al., 2006; Thakur et al., 2009). It is worth noting that the polyP domain is also a site of many protein interactions that could potentially influence HD pathogenesis (Khoshnan et al., 2004; Passani et al., 2000; Steffan et al., 2000).

One shortfall for many HD studies, including several of the publications mentioned here, is that they use a truncated form of the htt protein, containing only exon 1. This is because the htt protein is very large (67 exons; ncbi.nlm.nih.gov/gene/3064), and the polyQ domain is located within the first exon. Ideally, future work should confirm the influence of htt's non-polyQ domains on htt aggregation with the full-length protein in cell-based and *in vivo* systems.

1.3.2 Spinocerebellar ataxia type 1 (SCA1)

Protein context in SCA1 has been firmly established by work conducted in the Zoghbi and Orr labs. Nearly 2 decades ago, they discovered that ataxin-1's nuclear localization signal is essential for pathogenesis in SCA1 mice (Klement et al., 1998). Several years later, they observed that ataxin-1 is phosphorylated at serine 776, and mutating this serine to alanine substantially reduces toxicity in SCA1 transgenic mice (Emamian et al., 2003). This

phosphorylated residue was also found to be required for its interaction with the 14-3-3 protein (Chen et al., 2003). The same study reported that 14-3-3 binding to ataxin-1 enhances polyglutamine toxicity in a *Drosophila* model of SCA1, by slowing degradation of ataxin-1 (Chen et al., 2003). Phosphorylation of serine 776 is also critical for ataxin-1's interaction with the splicing factor, RBM17, and this interaction is promoted by increasing polyQ tract length (Lim et al., 2008). Interestingly, modulating levels of RBM17 influences ataxin-1 toxicity in *Drosophila*; overexpression enhances toxicity, while loss of one RBM17 allele suppresses toxicity (Lim et al., 2008).

The AXH domain of ataxin-1 also influences the SCA1 protein's properties. This domain is the binding site for many of ataxin-1's interactions with other proteins, including its own self-association, and is at least partially responsible for ataxin-1's function as a transcriptional repressor (Chen et al., 2004; de Chiara et al., 2003; de Chiara et al., 2005). Studies conducted by the Orr and Zoghbi labs demonstrated that the AXH domain of ataxin-1 directly binds to the *Drosophila* transcription factor, Senseless, and its mammalian homologue, Gfi-1 (Tsuda et al., 2005). Ataxin-1 negatively regulates the expression levels of Senseless/Gfi-1, but deletion of the AXH domain increases the transcription factor's stability and reduces overall polyQ toxicity (de Chiara et al., 2005; Tsuda et al., 2005). Alternatively, the AXH domain is also the site of ataxin-1's interaction with Capicua, a transcriptional repressor that modulates toxicity in SCA1 (Fryer et al., 2011; Lam et al., 2006). Ongoing research continues to elucidate the mechanisms and outcomes of the Capicua-AXH interaction in this disease (Lu et al., 2017).

1.3.3 Spinal and bulbar muscular atrophy (SBMA)

Protein context also plays a role in SBMA, the only X-linked, recessive polyQ disorder (Costa Mdo and Paulson, 2012). The expanded protein in this case is the androgen receptor

(AR), whose nuclear receptor domains are required for disease progression. For instance, the nuclear localization signal, as well as the ligand-binding domain that binds testosterone, are required for pathogenesis in fly and mouse models of SBMA (Montie and Merry, 2009; Takeyama et al., 2002). Additionally, experiments conducted in *Drosophila* show that the AR's DNA-binding domain and AF-2 interacting domain are also required for toxicity (Nedelsky et al., 2010). Furthermore, altering the levels of testosterone modulates toxicity of AR *in vivo* (Katsuno et al., 2002; Takeyama et al., 2002).

Conversely, a leucine-rich region immediately preceding the AR's polyQ tract may work to prevent toxicity in SBMA; this leucine-rich domain reduces aggregation of an N-terminal AR fragment *in vitro* by maintaining the alpha-helical conformation of the polyQ tract (Eftekharzadeh et al., 2016). On the other hand, another sequence in AR's N-terminus, located downstream of the polyQ tract, appears to enhance AR fibrilization (Oppong et al., 2017). The significance of these domains has not been tested with a full-length AR protein in an *in vivo* setting, but such studies could provide valuable insight into aggregation mechanisms in SBMA.

1.3.4 Spinocerebellar ataxia type 3 (SCA3)

The catalytic Josephin domain of ataxin-3 is known to promote aggregation of the SCA3 protein. Both wild type and mutant ataxin-3 form SDS-soluble aggregates *in vitro* (Chow et al., 2004; Ellisdon et al., 2006; Gales et al., 2005; Masino et al., 2004) and are present in nuclear inclusions *in vivo* (Fujigasaki et al., 2000; Fujigasaki et al., 2001; Lieberman et al., 1999), suggesting that this process can occur independently of an expanded polyQ tract. However, only ataxin-3 with expanded polyQ can form SDS-insoluble aggregates (Ellisdon et al., 2006; Natalello et al., 2011). Notably, aggregation of isolated Josephin occurs in a manner very similar to full-length, non-expanded ataxin-3, where its conformation transitions from an alpha-helix to a

beta-sheet (Ellisdon et al., 2006; Masino et al., 2011; Masino et al., 2004; Natalello et al., 2011). Together, these findings have led to the hypothesis that ataxin-3 aggregation occurs through a two-step mechanism in which aggregation is first initiated by the Josephin domain and then propagated by the polyQ expansion (Ellisdon et al., 2006; Scarff et al., 2015; Silva et al., 2017).

Post-translational modifications to ataxin-3 also appear to influence its aggregation and toxicity. Sumoylation at lysine 356 reduces ataxin-3 aggregation *in vitro* and in rat cortical neurons (Almeida et al., 2015). Intriguingly, this modification also enhances ataxin-3's interaction with the AAA ATPase, VCP/p97 (Almeida et al., 2015), which will be discussed further in Chapter 4. GSK3 β phosphorylates ataxin-3 on serine 256, and ataxin-3's aggregation is increased in mammalian cells when this site is mutated to a non-phosphorylatable alanine (Fei et al., 2007). This is an interesting finding, since the authors also observed that GSK3 β phosphorylates pathogenic ataxin-3 less than the non-pathogenic form (Fei et al., 2007). Additionally, phosphorylation of ataxin-3 at serine 12 by an unidentified kinase was shown to reduce ataxin-3 aggregation in cortical neurons and prevent neuronal loss in a lentiviral rat model of SCA3 (Matos et al., 2016). Moreover, phosphorylation at serine residues 29 (Josephin domain), 340 (UIM3), and 352 (UIM3) increase ataxin-3's nuclear localization (Mueller et al., 2009; Pastori et al., 2010), which is a critical determinant of SCA3 pathogenesis (Bichelmeier et al., 2007; Paulson et al., 1997b). Altogether, these studies indicate that post-translational modifications have the potential to accelerate or attenuate SCA3 progression.

1.4 Conclusions

Although the polyQ disorders have unifying features, it is their mutated proteins that make each disease unique. Each polyQ protein has their own set of physiological functions that can go awry to cause disease. Research into ataxin-3's roles in protein quality control,

transcriptional regulation, and DNA repair elucidates several pathways that are altered in SCA3. Interestingly, some of these functions seem to overlap: transcriptional regulation contributes to protection against cell stress, DUB activities facilitate DNA repair and transcription, etc (**Figure 1.1**). Furthermore, identification of the structural domains that are required for these functions opens the door to the development of targeted therapeutics. Future efforts to merge our understanding of sequence domains and post-translational modifications that modulate toxicity with the normal physiological functions of ataxin-3 will enhance our understanding of SCA3 and how to treat it.

Alternatively, while protein context makes the diseases unique, it is evident that there are some shared mechanisms. Ataxin-3, ataxin-1, htt, AR, each exhibit increased toxicity when localized to the nucleus. All of the proteins have at least one domain that tends to self-associate and promote aggregation. The ability of post-translational modifications to influence polyQ protein aggregation and toxicity is also a unifying feature. Therefore, new discoveries about protein context in one polyQ disorder have the potential to enhance our understanding of the disease class as a whole.

For all of these reasons, my thesis explores the interactions between ataxin-3 and two of its protein-binding partners: Rad23 and VCP. These studies demonstrate that Rad23 and VCP can modulate the toxic properties of ataxin-3 *in vivo*, thus providing novel information about protein context in SCA3.

CHAPTER 2: Materials and Methods

Adapted from:

Published: **Sutton JR**, Blount JR, Libohova K, Tsou W-L, Joshi GS, Paulson HL, Costa MDC, Scaglione KM, Todi SV (2017). Interaction of the polyglutamine protein ataxin-3 with Rad23 regulates toxicity in *Drosophila* models of Spinocerebellar Ataxia Type 3. *Human Molecular Genetics* 26, 1419-1431.

Under review: Ristic GR*, **Sutton JR***, Libohova K, Todi SV (2018). Toxicity and aggregation of the polyglutamine disease protein, ataxin-3 is regulated by its binding to VCP/p97 in *Drosophila melanogaster*. *Neurobiology of Disease*. *- these authors contributed equally to this manuscript.

Antibodies: Anti-Ataxin-3 (mouse monoclonal 1H9, 1:500-1,000; Millipore) (rabbit polyclonal, 1:15,000; (Paulson et al., 1997a)); anti-VCP (rabbit monoclonal, 1:1,000; Cell Signaling Technology); anti-tubulin (mouse monoclonal T5168, 1:10,000; Sigma-Aldrich); anti-HIS (rabbit monoclonal 1:1,000; Cell Signaling); anti-HA (rabbit monoclonal, 1:500-1,000; Cell Signaling Technology); anti-GAPDH (mouse monoclonal MAB374, 1:500; Millipore); anti-lamin (mouse monoclonal ADL84.12-5, 1:1,000; Developmental Studies Hybridoma Bank); peroxidase-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse, 1:5,000; Jackson Immunoresearch).

***Drosophila* lines:** Husbandry was conducted at 25°C on standard cornmeal food at 40%-60% humidity in a regulated diurnal environment. SCA3 flies expressing pathogenic ataxin-3 with 77-80 polyQ repeats were generated by cloning into the pWalium10-moe vector (Perrimon lab). “HNHH”, “UbS2*Mild”, and “UbS2*Severe” mutations were generated on the pathogenic ataxin-3 backbone using the QuikChange mutagenesis kit (Agilent), then cloned into the pWalium10-moe vector. Rad23-overexpressing flies were generated by using HIS⁶-tagged *Drosophila* Rad23 cloned into the pWalium10-moe vector. Injections (Duke University Model Systems) were into y, w; +; attP2. The following stocks from purchased from the Bloomington *Drosophila* Stock

Center: isogenic host strain for GMR-Gal4 (#8121), attP2 (#36303), DnaJ-1 RNAi (#32899), VCP RNAi lines (#32869, #35608), UAS-DnaJ-1 (#30553), and UAS-polyQ⁷⁸ (#8141). Rad23 RNAi (#30498) and its isogenic line (#60000) were purchased from the Vienna *Drosophila* RNAi Center. The following lines were gifts: sqh-Gal4 driver (Dr. Daniel P. Kiehart, Duke University), elav- Gal4(GS) driver (Dr. Robert Wessells, Wayne State University), elav-Gal4 driver (Dr. Daniel F. Eberl, University of Iowa).

Longevity assay: Upon eclosion, approximately 25 male and female adults were collected and aged in conventional cornmeal fly media or RU-486-containing media (Nicholson et al., 2008; Osterwalder et al., 2001; Roman and Davis, 2002; Sujkowski et al., 2015) at 25°C. Flies were transferred to vials with fresh food every 2–3 days. Each vial was checked for death daily, until all flies were dead. Longevity comparisons were restricted to fly lines in the same genetic background, since flies from different backgrounds can have different lifespans (Paaby and Schmidt, 2009; Spencer et al., 2003).

Motility Assay (Negative Geotaxis): Approximately 10 adult flies per vial were forced to the bottom by tapping on the bench. The number of flies that reached the top at 5, 15 and 30 seconds was recorded. The assay was conducted once a week for 4 weeks. Flies were transferred to new vials 1 hour before the assay was conducted, and every 2–3 days for the duration of the experiment.

Ataxin-3 Pulse-Chases in Drosophila: Fly crosses were set up in standard cornmeal media under the conditions described in “*Drosophila* lines”. After eclosion, adult offspring possessing transgenes for the inducible elav-Gal4(GS) driver and UAS-Ataxin-3(SCA3)-Intact or UAS-Ataxin-3(SCA3)-HNHH were transferred to cornmeal media containing RU-486 (Nicholson et al., 2008; Osterwalder et al., 2001; Roman and Davis, 2002; Sujkowski et al., 2015). Expression

of UAS-Ataxin-3(SCA3)-Intact was induced in the RU-486-containing media for 7 or 14 days; expression of UAS-Ataxin-3(SCA3)-HNHH was induced for 10 or 21 days. At the end of the induction period, flies were transferred back to the standard cornmeal media for the chase period. Flies were collected for western blot analysis at day 0, 1, 7, 14, and 21 of the chase period. Motility and western blot analysis were carried out as described above.

Histology: Wings and proboscises were removed from male, adult flies and bodies were fixed overnight in 2% glutaraldehyde/2% paraformaldehyde in Tris-buffered saline with 0.1% Triton X-100. Fixed adults were then dehydrated in a series of 30, 50, 75, and 100% ethanol washes for 1 hour each, then washed in propylene oxide overnight. Dehydrated flies were embedded in Poly/Bed812 (Polysciences) and sectioned at 5 μ m. Sections were stained with toluidine blue.

Western blotting: Three to 5 whole flies, 10 to 15 dissected fly heads, or 10 larvae per group were homogenized in hot lysis buffer (50 mM 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 min. Samples were electrophoresed on 4-20% gradient gels (BioRad). Western blots were developed and quantified using a CCD-equipped VersaDoc 5000MP system and Quantity One software (Bio-Rad). For direct blue staining, PVDF membranes were submerged for 5 minutes 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.

Differential Centrifugation: Five whole flies per group were lysed in 200 μ l of NETN buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40), supplemented with protease inhibitor cocktail (PI, SigmaFast Protease Inhibitor Cocktail Tablets), sonicated, and centrifuged at 20,000 x g for 30 minutes at 4°C. The supernatant was transferred to a new tube and quantified by the

BCA assay. The pellet was resuspended in 200 µl of PBS + 1% SDS, vortexed, and boiled. Ten µg of the supernatant fraction and 7 µl of the pellet fraction were supplemented with 6x SDS, boiled, and loaded onto 4-20% gradient gels for western blot analysis.

Nuclear Cytoplasmic Extraction: Experiments were conducted using the ReadyPrep Protein Extraction Kit ((Cytoplasmic/Nuclear), BioRad) to enrich cytoplasmic and nuclear proteins. Five whole adult flies were lysed in cytoplasmic extraction buffer (Bio- Rad). Nuclei were resuspended in Protein Solubilization Buffer (BioRad). Samples were analyzed by western blot.

Quantitative RT-PCR: Total RNA was extracted from larvae or adult flies using TRIzol (Life Technologies). Extracted RNA was treated with TURBO DNase (Ambion) to eliminate contaminating DNA. Reverse transcription was carried out with the high-capacity cDNA reverse transcription kit (ABI). Messenger RNA levels were quantified by using the StepOnePlus Real-Time PCR System with Fast SYBR Green Master Mix (ABI). rp49 was used as an internal control. Primers:

SCA3 - F: 5' GAATGGCAGAAGGAGGAGTTACTA - 3'

SCA3 - R: 5' GACCCGTCAAGAGAGAATTCAAGT - 3'

rp49-F: 5' -AGATCGTGAAGAAGCGCACCAAG- 3'

rp49-R: 5' -CACCAGGAACTTCTTGAATCCGG- 3'

Cell lines, plasmids, transfection & chemicals: HeLa and HEK293 cells were purchased from ATCC and cultured in DMEM supplemented with 10% FBS and 5% Penicillin-Streptomycin under conventional conditions. The day before transfection, cells were seeded in 6- or 12-well plates. Cells were transfected using Lipofectamine LTX (Invitrogen) as directed by the manufacturer. Twenty-four hours after transfection, cells in 12-well plates were lysed in boiling SDS lysis buffer for western blot analysis. Cells in 6-well plates were harvested in PBS, pelleted

by centrifugation at 500 x g for 5 minutes at 4°C, and frozen at -80°C for immunoprecipitation experiments. Non-pathogenic ataxin-3 constructs were in pcDNA3.1-HA. Pathogenic ataxin-3 constructs were in pFLAG-CMV6a. VCP construct was in pEGFP. Cycloheximide (A.G. Scientific) dissolved in ultra-pure water, was used at 50 mg/ml.

Co-Immunoprecipitation: For co-IPs from fly lysates, 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 at 4°C, and supernatant was transferred to 30 µl slurry of anti-HA bead-bound antibody (Sigma- Aldrich). Beads with lysates were tumbled at 4°C for 4 hours. For co-IPs from cell lysates, HEK293 or HeLa cells were transfected and harvested as described under “Cell Lines, Plasmids, and Transfections”. Cell pellets were lysed in 500 mL of NETN+PI, vortexed briefly every 5 minutes for a total of 30 minutes, and centrifuged at 12,000 x g for 15 minutes at 4°C. Supernatants were transferred to 30 µl slurry of anti-FLAG bead-bound antibody (Sigma-Aldrich). Beads with lysates were tumbled at 4°C for 4 hours. For *in vitro* pulldowns, 1.5 mL of bacterial lysate containing GST, GST-Ataxin-3(SCA3)-Intact, or GST-Ataxin-3(SCA3)-HNHH was tumbled with 20 µl of glutathione sepharose slurry for 30 minutes. Both Ataxin-3(SCA3) constructs contained 80 polyQ repeats. GST-Atax- in-3(SCA3)-Intact and Ataxin3(SCA3)-HNHH supernatants were replaced with 1.5 mL of fresh bacterial lysate before tumbling all lysates for an additional 30 minutes. Forty whole flies were homogenized in 500 µl of NETN + PI, sonicated, and centrifuged at 15,000 x g for 15 minutes at 4°C. The bacterial lysate was removed from the beads, beads were rinsed with NETN + PI, the fly lysate was distributed evenly among the 3 experimental groups, and samples were tumbled for 2 hours at 4°C. Bead-bound complexes from all co-immunoprecipitations were

rinsed thrice with NETN + PI, then eluted with heat and Laemmli buffer.

Protein Purification: Glycerol stocks of BL21 cells transformed with pGEX6p1, pGEX6p1-Ataxin-3(SCA3)-Intact, or pGEX6p1-Ataxin-3(SCA3)-HNHH were used to inoculate 10 mL of LB + 50 ug/mL ampicillin. Both Ataxin-3(SCA3) constructs contained 80 polyQ repeats. Cultures were grown overnight at 37°C, then used to inoculate 500 mL of LB + ampicillin, which was grown for an additional 1.5 hours to OD₆₀₀=.60. Protein expression was induced with 0.5 mM of isopropyl-1-β-D-galactopyranoside (A. G. Scientific) for 4h at 30 °C. Bacterial cells were pelleted by centrifugation at 4,500 x g for 10 minutes at 4°C. Pellets were lysed in NETN buffer, sonicated, centrifuged, aliquoted, and frozen at -80°C.

CHAPTER 3: Interaction of the polyglutamine protein, ataxin-3, with Rad23 regulates toxicity in *Drosophila* models of Spinocerebellar ataxia type 3

Published: **Sutton JR**, Blount JR, Libohova K, Tsou W-L, Joshi GS, Paulson HL, Costa MDC, Scaglione KM, Todi SV (2017). Interaction of the polyglutamine protein ataxin-3 with Rad23 regulates toxicity in *Drosophila* models of Spinocerebellar ataxia type 3. *Human Molecular Genetics* 26, 1419-1431.

3.1 Introduction

Polyglutamine (polyQ)-dependent diseases comprise a class of neurodegenerative disorders characterized by abnormal CAG nucleotide repeat expansions in the disease gene (Costa Mdo and Paulson, 2012). These repeats translate into an abnormally long polyQ tract, resulting in subsequent misfolding and aggregation of the disease protein in neurons and, ultimately, neuronal dysfunction and death. Among these disorders, Spinocerebellar Ataxia Type 3 (SCA3, or Machado-Joseph Disease), is the most common dominantly inherited ataxia in the world (Costa Mdo and Paulson, 2012; Matos et al., 2011). Onset occurs in mid to late life, with degeneration observed in cerebellar pathways and nuclei, dentate nuclei, pontine nuclei, and basal ganglia. Symptoms include ataxia, dysarthria, dystonia, ophthalmoplegia, spasticity, and neuropathy (Costa Mdo and Paulson, 2012). There is no cure for SCA3.

SCA3 is caused by expansion of a CAG tract in *ATXN3* from 22–42 repeats to a disease range of 52–84, leading to the translation of a pathogenic form of the ataxin-3 protein with expanded polyQ (Costa Mdo and Paulson, 2012; Matos et al., 2011). Ataxin-3, a deubiquitinating enzyme, edits ubiquitin chains on substrates (Scaglione et al., 2011; Winborn et al., 2008); it has been reported to function in protein quality control and transcriptional regulation (Costa Mdo and Paulson, 2012; Reina et al., 2012; Sacco et al., 2014; Scaglione et al., 2011; Todi et al., 2010), and is neuroprotective in *Drosophila melanogaster*, where it suppresses degeneration from various polyQ proteins (Burr et al., 2014; Tsou et al., 2013; Tsou et al., 2015b; Warrick et

al., 2005). Ataxin-3 also protects against various stressors in mammalian cell culture (Chatterjee et al., 2015; Reina et al., 2012; Reina et al., 2010; Zhou et al., 2013a).

The N-terminal catalytic domain of ataxin-3 contains two ubiquitin-binding sites that can bind mono-ubiquitin (Nicastro et al., 2009; Nicastro et al., 2010) Ubiquitin-binding site 2 (UbS2) also binds to the proteasomal shuttle proteins, Rad23/hHR23A and B (Nicastro et al., 2009; Nicastro et al., 2005; Nicastro et al., 2010; Wang et al., 2000). According to our earlier published work, proteasomal degradation of ataxin-3 is regulated by the direct interaction between UbS2 and Rad23 (Blount et al., 2014). When Rad23 is bound to UbS2, ataxin-3 is protected from degradation (**Figure 3.1A**). Disrupting this interaction decreases the steady state levels of ataxin-3 in the cell. While this previous work showcased the importance of the ataxin-3-Rad23 interaction for the SCA3 protein's turnover, it did not directly test whether this interaction is critical for ataxin-3-dependent toxicity. In other words, does UbS2 of ataxin-3 directly modulate SCA3 pathogenicity? More importantly, should UbS2 be targeted to protect against SCA3, since it regulates overall cellular levels of ataxin-3?

Here, we show that disrupting UbS2 of disease-causing ataxin-3 decreases the levels of the pathogenic ataxin-3 protein, but greatly enhances its toxicity *in vivo*. Further investigations indicate that this increase in toxicity is due to a reduction in ataxin-3's neuroprotective function against itself, which is mediated by the UbS2-Rad23 interaction and depends on the chaperone partner, DnaJ-1. Our data demonstrate a critical role for the interaction of ataxin-3 with Rad23 in the regulation of this polyQ protein's abundance, function, and pathogenicity.

3.2 Results

3.2.1 Modulating levels of Rad23 influences ataxin-3's toxicity in Drosophila

To examine the function of the interaction between UbS2 of ataxin-3 and Rad23, we used

a transgenic fly model of SCA3 that we recently generated by using the phiC31-targeted integration system (Groth et al., 2004). The phiC31 system yields a similar expression of transgenes among different fly lines, due to site-specific integration (Groth et al., 2004), in our case into attP2 on the third chromosome of the fly. This method allows for direct comparisons among the different transgenic lines we generated (**Figure 3.1B**). Our *Drosophila* model of SCA3 expresses untagged, full-length, human pathogenic ataxin-3 under the control of the Gal4/UAS system. We also generated a control line that contains the empty host vector inserted into attP2 (Tsou et al., 2015a) and another line that expresses wild-type, human ataxin-3, inserted at a different site than attP2 (Tsou et al., 2013).

When pathogenic ataxin-3 (Ataxin-3(SCA3)) is expressed throughout the fly, it leads to some lethality at late pharate adult stages and during eclosion from the pupal case (**Figure 3.1C**). Those flies that mature into adulthood only live up to 30 days, as opposed to the control flies that live up to 100 days (**Figure 3.1D**).

Previously, we reported that reducing the levels of endogenous Rad23 through RNAi by approximately 50%, as determined through qRT-PCR, or by removing one copy of its gene, decreased ataxin-3 protein levels and noticeably improved degeneration in *Drosophila* eyes expressing pathogenic ataxin-3 with an intact Ubs2 ((Blount et al., 2014); other supporting data not shown). We recapitulated this effect by reducing endogenous Rad23 levels in all the tissues of the fly through RNAi. We observed improved adult longevity compared to sibling SCA3 flies without Rad23 knockdown (**Figure 3.1E**). Based on our earlier work with various Gal4 drivers and different polyQ and non-polyQ constructs (Blount et al., 2014; Ristic et al., 2016; Tsou et al., 2013; Tsou et al., 2015a; Tsou et al., 2015b), improved longevity from pathogenic ataxin-3 when Rad23 is knocked down through UAS-RNAi is not due to a dilution effect of the Gal4 driver co-

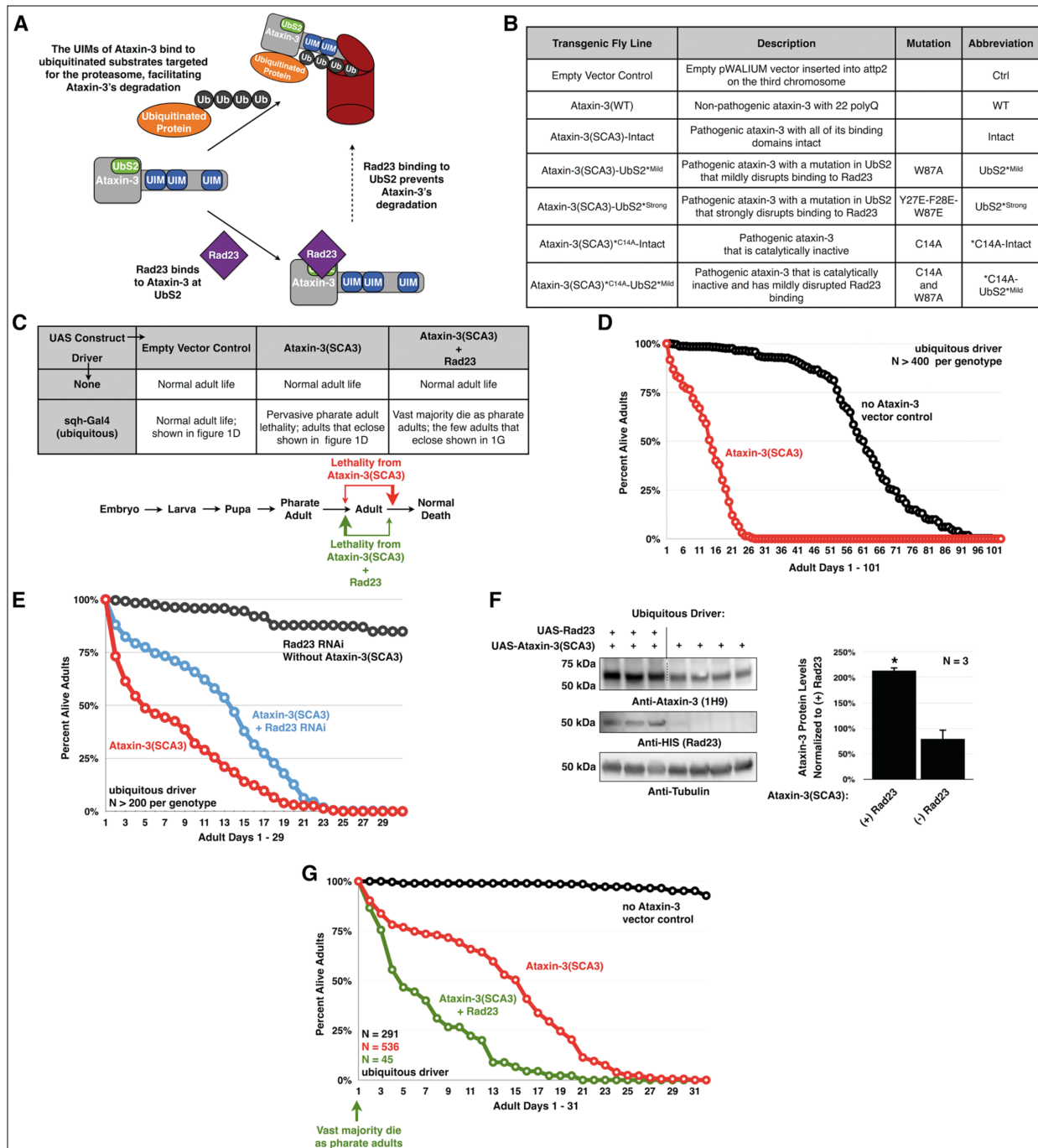


Figure 3.1. Rad23 influences Ataxin-3(SCA3)-dependent toxicity. (A) Model depicting how Rad23 influences ataxin-3's degradation, based on studies from Blount et al., 2014. The ubiquitin-interacting motifs (UIMs) of ataxin-3 bind ubiquitin chains on proteasome substrates, facilitating the interaction of ataxin-3 with this machinery. Once at the proteasome, in the absence of interaction with Rad23, ataxin-3 is degraded. If ataxin-3 interacts with Rad23 through UbS2, it is rescued. (B) Summary of transgenic *Drosophila* lines used. (C) Observations with ubiquitous expression of pathogenic ataxin-3 with or without Rad23 overexpression. In this panel and rest of the figures, unless otherwise noted, flies were heterozygous for all transgenes. (D) Longevity of flies ubiquitously expressing pathogenic ataxin-3 or the empty host vector inserted into attP2. (E) Longevity of flies ubiquitously expressing pathogenic ataxin-3 without or with UAS-RNAi targeting Rad23. A control line was included that expresses Rad23 RNAi in the absence of ataxin-3. Flies were siblings from the same crosses and on a different background (*y-w*) than flies in the rest of this figure (*w¹¹¹⁸*). (F) Western blots from independent lysates of whole flies ubiquitously expressing the specified transgenes. Means \pm SD. Asterisks: $P < 0.05$ from Student's *t*-tests compared to UAS-Ataxin-3(SCA3) without exogenous Rad23. When quantifying ataxin-3 protein levels for (-)Rad23 flies, lane 4 was set to 100%. Lane 5 was not quantified, due to the bubble disrupting the ataxin-3 band. (G) Longevity of flies ubiquitously expressing pathogenic ataxin-3 with or without Rad23 overexpression.

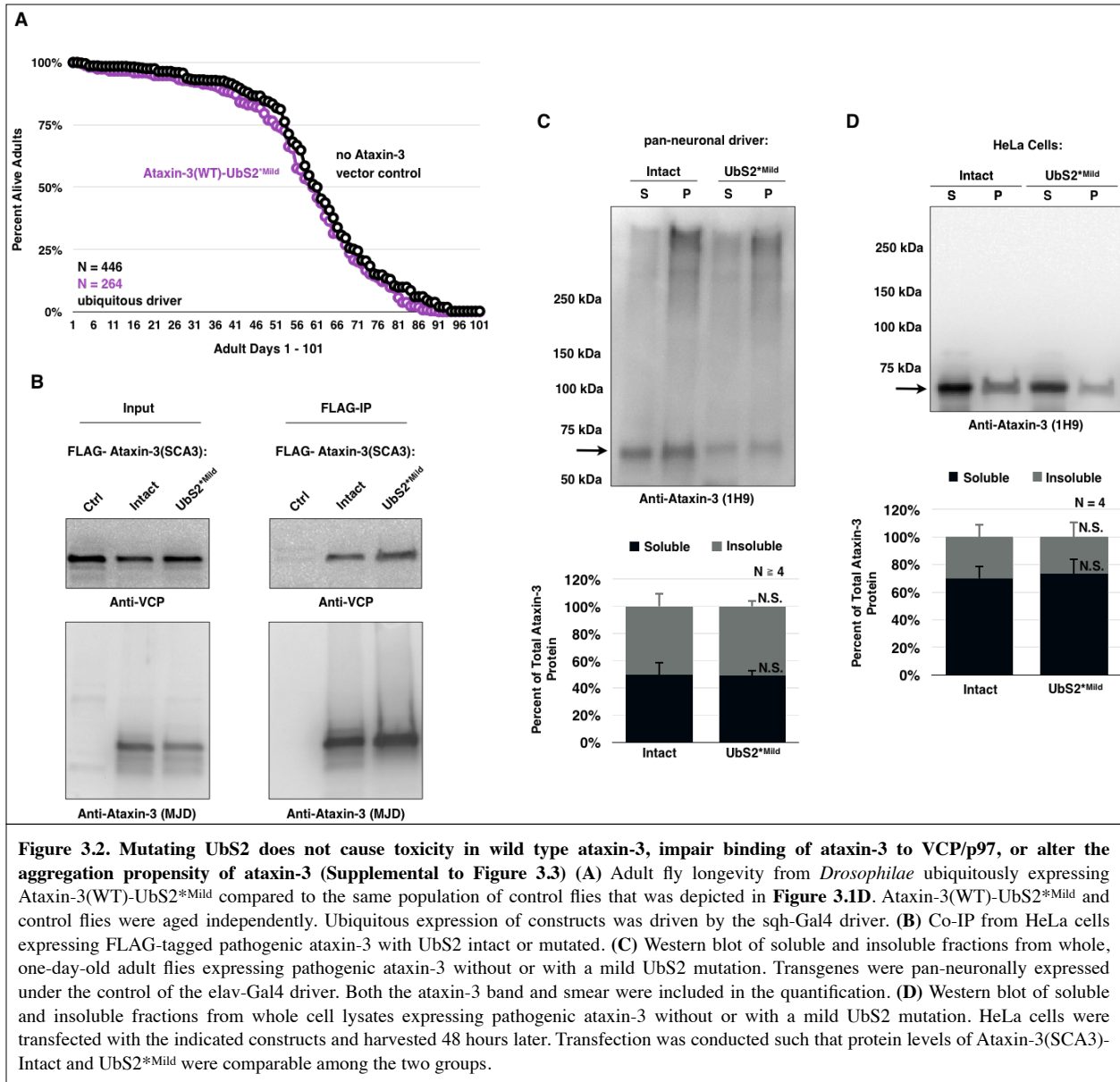
expressing ataxin-3 and the knockdown construct.

Conversely to Rad23 knockdown, exogenous Rad23 noticeably increases Ataxin-3(SCA3) protein levels (**Figure 3.1F**). This increase in protein levels coincides with higher lethality (**Figure 3.1C**). Almost all of the SCA3 flies that co-express Rad23 die as pharate adults, before they eclose from the pupal case. Very few adult flies co-expressing Rad23 and Ataxin-3(SCA3) eclose successfully (**Figure 3.1C**). Those flies that survive to adulthood exhibit reduced longevity compared to the SCA3 flies that are not expressing exogenous Rad23 (**Figure 3.1G**).

Flies expressing exogenous Rad23 or expressing RNAi targeting Rad23 in the absence of ataxin-3 were kept as healthy stocks for over a year without clear signs of reduced fecundity or of toxicity (**Figure 3.1E and data not shown**). These collective data from the perturbation of Rad23 levels support the idea that this proteasome-associated protein regulates pathogenic ataxin-3 levels and subsequent toxicity. These results led us to next investigate if disturbing the binding site of this protein on ataxin-3 is beneficial *in vivo*.

3.2.2 Mutating UbS2 reduces ataxin-3 protein levels, but greatly enhances its toxicity

Based on the results from our experiments with altered Rad23 levels, we hypothesized that reducing binding of UbS2 to Rad23 would lower Ataxin-3(SCA3) protein levels and subsequently alleviate its toxicity. We generated additional transgenic flies that express Ataxin-3(SCA3) with a mutation in UbS2 that was previously shown by us and others to impair Rad23 binding (Blount et al., 2014; Nicastro et al., 2009; Nicastro et al., 2010), in this case by approximately 50 percent (Blount et al., 2014). This mutation (referred to as UbS2*^{Mild}) replaces a critical tryptophan residue on UbS2 with an alanine. It does not alter ataxin-3's cellular distribution, or the overall structure of the isolated catalytic domain; it does not negate the ability



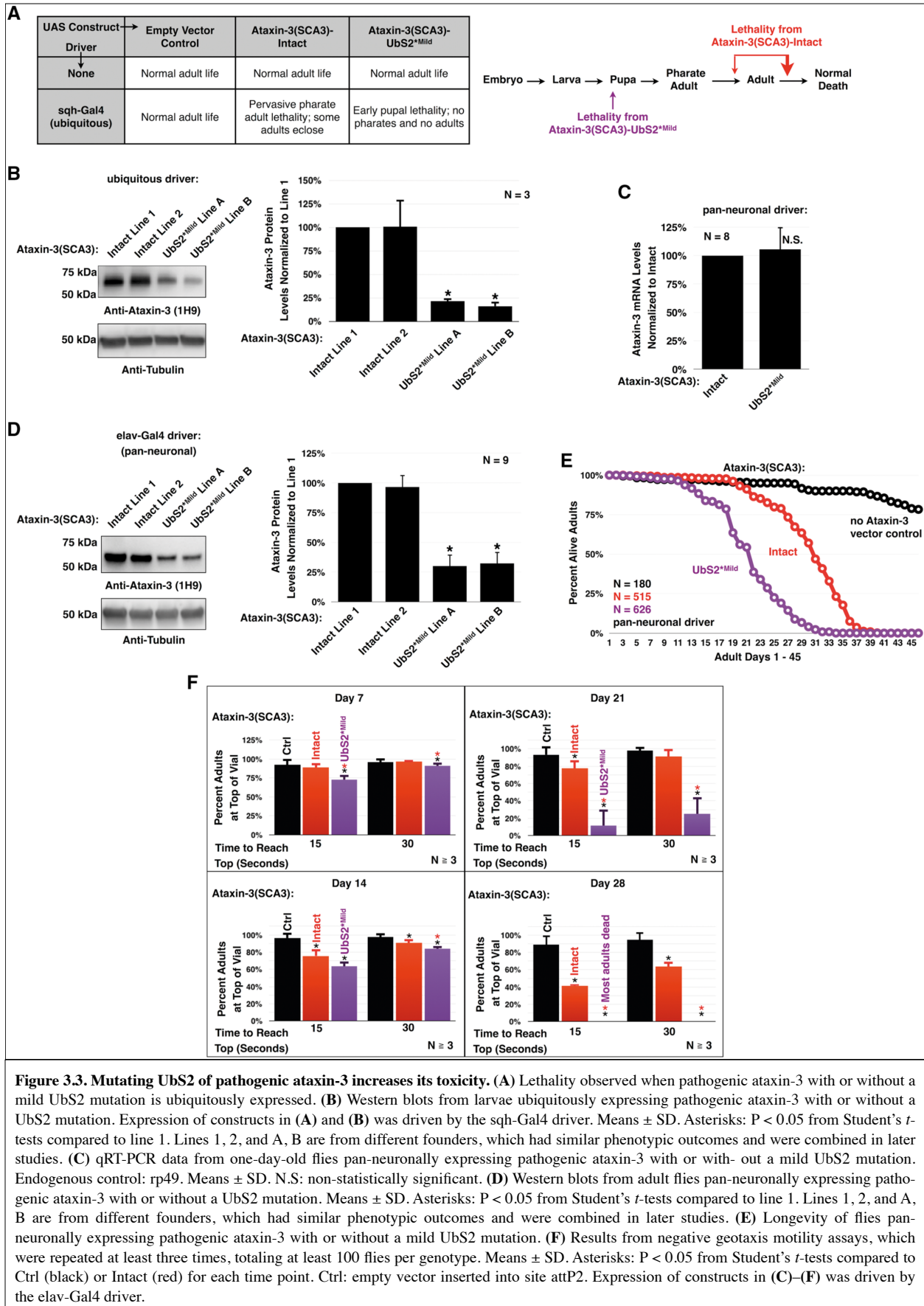
of the full-length protein to become ubiquitinated and catalytically activated; it does not abrogate the catalytic activity of full-length ataxin-3 ((Blount et al., 2014; Nicastro et al., 2010; Todi et al., 2010) and data not shown); and, in the context of ataxin-3 with a normal polyQ repeat, this mutation does not impact fly longevity (Figure 3.2A). This mutation also does not perturb the ability of ataxin-3 to bind another of its partners, VCP/p97 (Figure 3.2B). Together, these findings led us to conclude that the conformation of the overall catalytic domain of ataxin-3 is

not detrimentally impacted by the UbS2*^{Mild} mutation.

Surprisingly, we found that expression of UbS2*^{Mild} throughout the fly causes lethality at early pupal stages (**Figure 3.3A**). Western blots from larval lysates demonstrated that mutating UbS2 reduces Ataxin-3(SCA3) protein levels (**Figure 3.3B**), confirming our earlier data that UbS2 is critical for ataxin-3 protein levels. Nevertheless, the lethality phenotype is markedly worse than what we observe in the flies expressing Ataxin-3(SCA3) with UbS2 intact, which reach pharate adult and adult stages (**Figure 3.3A**).

Since these results failed to support our above hypothesis, we pan-neuronally expressed the Ataxin-3(SCA3) constructs to more thoroughly characterize the phenotype. Western blotting revealed that mutating UbS2 leads to lower Ataxin-3(SCA3) protein levels (**Figure 3.3D**), similar to what was observed with ubiquitous expression in larvae (**Figure 3.3B**). qRT-PCR indicates that this is not due to differences at the mRNA level (**Figure 3.3C**). To examine if mutating UbS2 alters the aggregation propensity of Ataxin-3(SCA3) before the onset of pathology, we performed differential centrifugation of one-day-old flies pan-neuronally expressing Ataxin-3(SCA3)-Intact or UbS2*^{Mild}. We did not observe a noticeable difference in soluble/insoluble fractions of Ataxin-3(SCA3)-Intact or UbS2*^{Mild} in flies (**Figure 3.2C**), or in mammalian cell culture (**Figure 3.2D**). When Ataxin-3(SCA3)-Intact is expressed pan-neuronally, flies exhibit reduced longevity and motility (**Figure 3.3E and F**). However, expression of UbS2*^{Mild} triggers greater impairment in longevity and motility than Ataxin-3(SCA3)-Intact, supporting our results from ubiquitous expression of these two proteins (**Figure 3.3A**).

Drosophila eyes are a popular tool for investigating neurodegenerative diseases, as degeneration can be reliably visualized at the external level or internally through histological



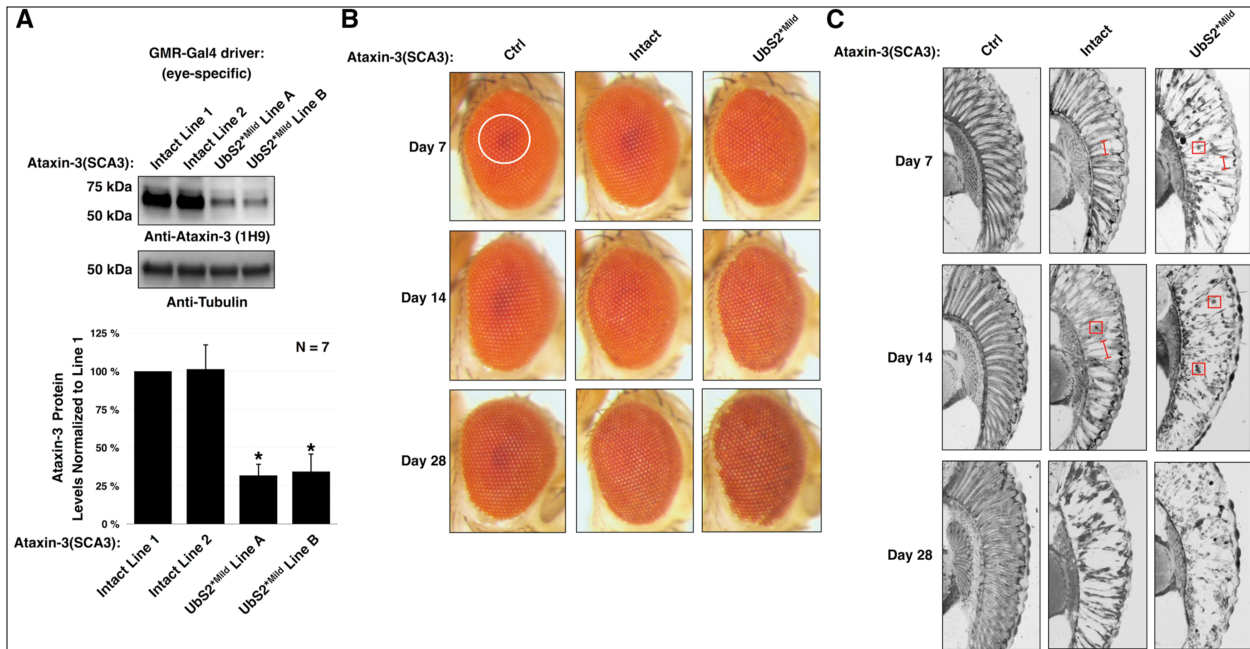


Figure 3.4. Mutating Ubs2 increases pathogenicity of ataxin-3 in fly eyes. (A) Western blots from fly heads expressing pathogenic ataxin-3 without or with a Ubs2 mutation. Means \pm SD. Asterisks: $P < 0.05$ from Student's t -tests compared to line 1. Lines 1, 2, and A, B are from different founders, which had similar phenotypic outcomes and were combined in studies in the other panels of this figure. (B) External photos and (C) histological sections of fly eyes expressing pathogenic ataxin-3 without or with a mild Ubs2 mutation. Ctrl: empty vector inserted into attP2. White circle in (B): example of the pseudo-pupil. Red boxes in (C): examples of densely-staining aggregates. The precise subcellular localization of the aggregates is not clear from this assay. Red bracketed lines in (C): examples of disruption of the ommatidial boundaries. Expression of constructs was driven by the GMR-Gal4 driver.

sectioning. Using the GMR-Gal4 driver, we drove the expression of pathogenic ataxin-3 transgenes in *Drosophila* eyes to characterize the subsequent degeneration. Once again, we witnessed a significant reduction in ataxin-3 protein in dissected fly heads expressing Ubs2^{*Mild}, as compared to ones expressing the intact variant (Figure 3.4A). Expressing Ataxin-3(SCA3)-Intact in fly eyes leads to a slight depigmentation of the retina, which is visible by day 14 and increases with age (Figure 3.4B). On day 14, the dark, central pseudo-pupil is also lost, signifying a compromise of the underlying photoreceptor cells. In the fly eyes expressing Ubs2^{*Mild}, depigmentation and loss of the pseudo-pupil is observed as early as day 7 and progresses more markedly than with Ataxin-3(SCA3)-Intact (Figure 3.4B).

Next, we performed histology to obtain a more detailed look at the structure of the internal eye. A healthy *Drosophila* eye is characterized by an organized ommatidial array with

distinct boundaries between the ommatidia, the functional unit of the eye. With Ataxin-3(SCA3)-Intact, a slight disruption of the ommatidial array is visible at day 7 (**Figure 3.4C**). By day 14, darkly staining structures, which were shown previously to contain the pathogenic ataxin-3 protein (Warrick et al., 2005), are visible. At 28 days, the ommatidial array is significantly degenerated. Compared to the effect of Ataxin-3(SCA3)-Intact, UbS2*^{Mild} causes markedly increased disorganization of the ommatidial array and the presence of densely-staining structures as early as day 7. By day 28, the ommatidial boundaries are no longer distinguishable. The data from **Figures 3.3** and **3.4** demonstrate that pathogenic ataxin-3 with mutated UbS2 causes significantly more degeneration than intact Ataxin-3(SCA3) in various fly tissues.

Our working model predicted that reducing Rad23 binding to Ataxin-3(SCA3) by mutating UbS2 would decrease Ataxin-3(SCA3) protein levels and lower its toxicity. Characterization of flies expressing Ataxin-3(SCA3)-Intact or Ataxin-3(SCA3)-UbS2*^{Mild} under the control of different drivers revealed that mutating UbS2 reduces Ataxin-3(SCA3) protein levels, but greatly enhances the toxic phenotype. In the fly eyes, this toxicity is concomitant with the presence of densely-staining aggregates on an earlier time scale with UbS2*^{Mild} than with Ataxin-3(SCA3)-Intact. The exact type of aggregated species formed by pathogenic ataxin-3 *in vivo* is likely to be dependent on cellular context and type of tissue. These results led us to next probe how disrupting the interaction between Ataxin-3(SCA3) and Rad23 increases the pathogenicity of the Ataxin-3(SCA3) protein.

3.2.3 Mutating UbS2 increases ataxin-3 toxicity by compromising its autoprotective function

Wild-type ataxin-3 is protective in *Drosophila* models of polyQ diseases (Burr et al., 2014; Tsou et al., 2013; Tsou et al., 2015b; Warrick et al., 2005). These findings were supported by studies in mammalian cell culture where ataxin-3 also protects against various stressors

(Chatterjee et al., 2015; Reina et al., 2012; Reina et al., 2010; Zhou et al., 2013a). Work published by the Bonini lab (Warrick et al., 2005) and ours (Tsou et al., 2015b) reported that wild-type ataxin-3 requires both its catalytic activity and its binding to Rad23 to protect against polyQ-dependent toxicity in *Drosophila*. Collectively, these earlier results from flies presented the possibility that pathogenic ataxin-3 retains the protective function against itself (**Figure 3.5A summarizes the model**). We posited that when we mutate Ubs2 to reduce Rad23 binding, we also compromise ataxin-3's autoprotective function. Without its autoprotection, the Ubs2*^{Mild} protein is more toxic than Ataxin-3(SCA3)-Intact, even though there is less protein present (**Figure 3.5A; also see Figure 3.8A**).

To first test whether mutating Ubs2 impairs the protective function of Ataxin-3(SCA3), we co-expressed a polyQ peptide with 78 glutamine repeats (polyQ⁷⁸) alongside full-length, pathogenic ataxin-3 transgenes in *Drosophila* eyes. PolyQ⁷⁸ is the isolated C terminus of the ataxin-3 protein without UIMs or the N terminus (Warrick et al., 1998). Fly eyes expressing two copies of polyQ⁷⁸ exhibit severe depigmentation of the retina (**Figure 3.5B**). Indentations are also visible on the external eye, indicative of the collapse of internal structures. Flies heterozygous for polyQ⁷⁸ and the empty host vector for Ataxin-3(SCA3) transgenes display milder, but clearly visible external depigmentation. Expression of wild-type ataxin-3 ameliorates this phenotype, as also shown previously (Burr et al., 2014; Tsou et al., 2013; Tsou et al., 2015b; Warrick et al., 2005). When Ataxin-3(SCA3)-Intact is co-expressed with polyQ⁷⁸, the level of degeneration is significantly less than what is observed in flies expressing two polyQ⁷⁸ alleles. However, Ubs2*^{Mild} is unable to suppress polyQ⁷⁸ toxicity, supporting the notion that Ataxin-3(SCA3) retains some protective function, and that mutating Ubs2 results in at least some loss of this role.

To further examine the importance of an intact Ubs2 on the pathogenicity of the SCA3

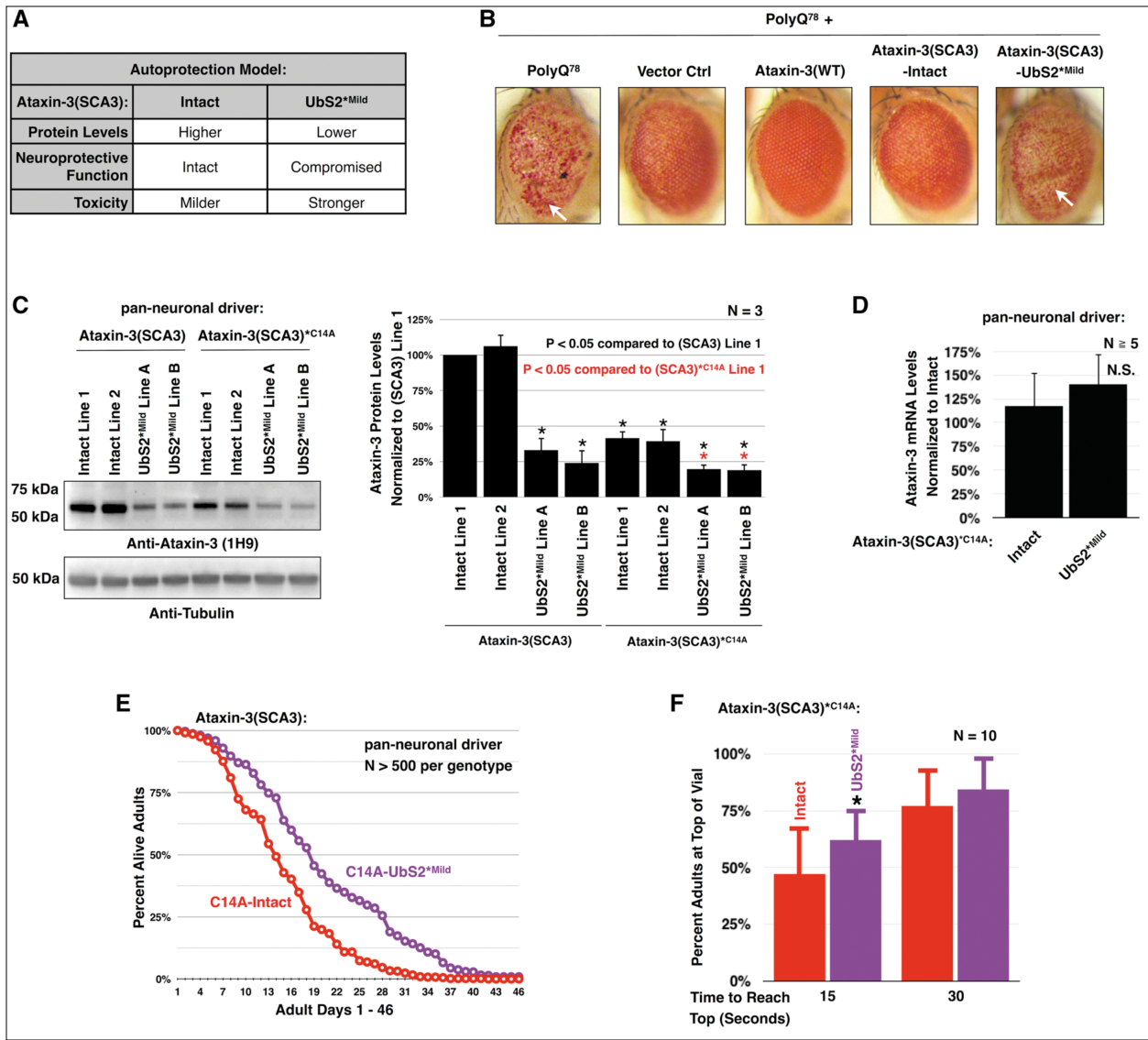


Figure 3.5. Mutating UbS2 of catalytically inactive, pathogenic ataxin-3 reduces its protein levels and toxicity. (A) Table summarizing the working model for the role of ataxin-3's UbS2 in toxicity. (B) Photos of external eyes expressing a truncated polyQ fragment of ataxin-3 with 78 repeats and co-expressing the indicated lines. Flies were heterozygous for all transgenes, except for flies homozygous for polyQ⁷⁸ (far left). Constructs were expressed using the GMR-Gal4 driver. White arrows: examples of indentation. (C) Western blots from adult flies pan-neuronally expressing pathogenic ataxin-3 that is catalytically active or inactive. Means \pm SD. Black asterisks: $P < 0.05$ from Student's t -tests compared to catalytically active ataxin-3 line 1. Red asterisks: $P < 0.05$ from Student's t -tests compared to catalytically inactive ataxin-3 line 1. Lines 1, 2, and A, B are from different founders, which had similar phenotypic outcomes and were combined in later studies in this figure. (D) qRT-PCR data from one-day-old flies expressing pathogenic, catalytically inactive ataxin-3 with or without a mild UbS2 mutation. Endogenous control: rp49. Means \pm SD. N.S: non-statistically significant. (E) Longevity data from flies expressing pathogenic, catalytically inactive ataxin-3 without or with a mild UbS2 mutation. (F) Motility of flies expressing pathogenic, catalytically inactive ataxin-3 without or with a mild UbS2 mutation. Flies were 7 days old. $N = 10$, totaling at least 100 flies per genotype. Asterisk: $P < 0.05$ from Student's t -tests compared to C14A-Intact.

protein, we generated additional transgenic fly lines that express catalytically inactive, pathogenic ataxin-3 either with all of its domains intact (Ataxin-3(SCA3)^{C14A}-Intact) or with UbS2 mutated (Ataxin-3(SCA3)^{C14A}-UbS2^{Mild}) (Figure 3.1B). To render Ataxin-3(SCA3)

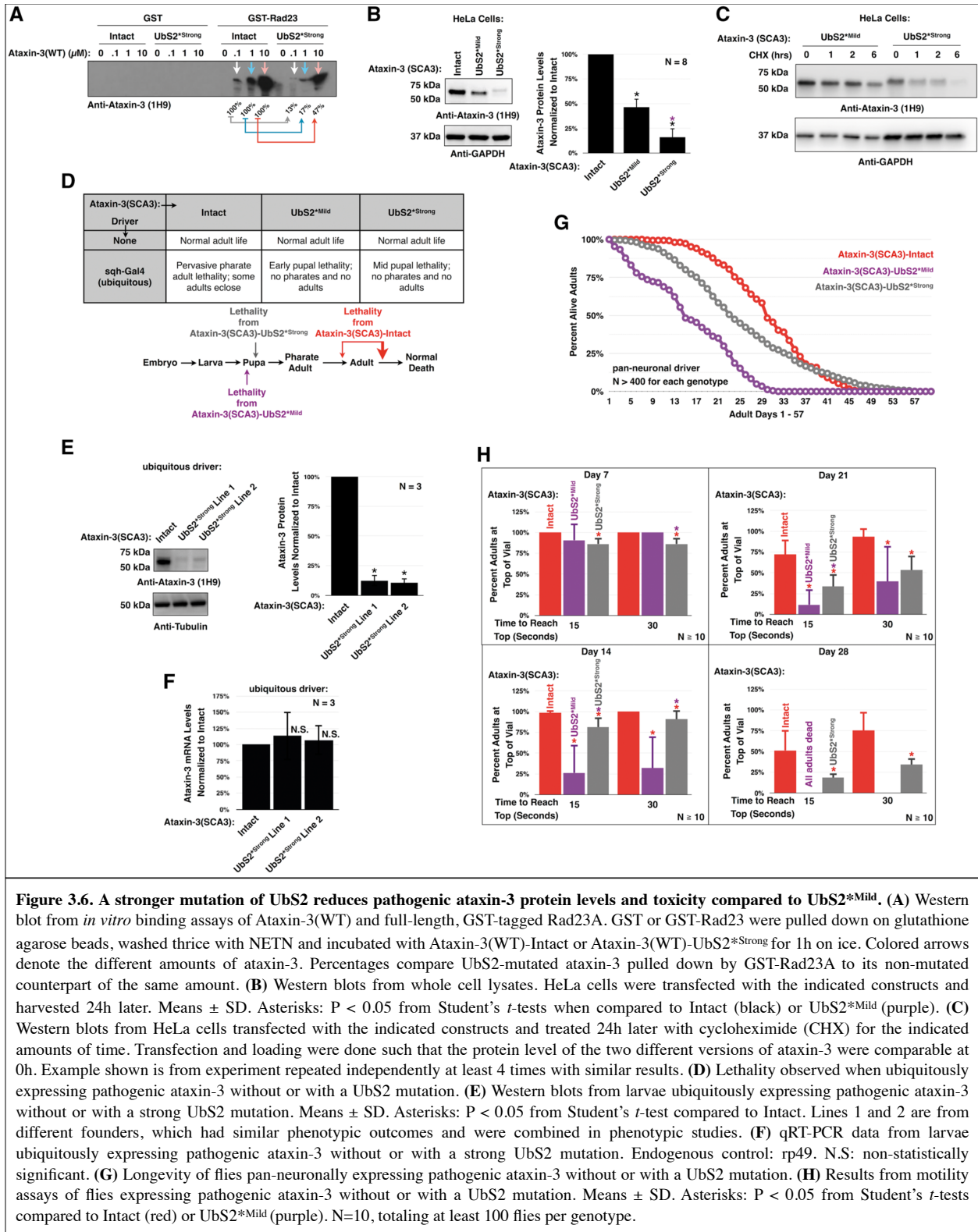
catalytically inactive, we mutated the critical cysteine in its protease domain to an alanine, which compromises the protein's ability to protect against polyQ toxicity (Todi et al., 2007; Todi et al., 2010; Tsou et al., 2013; Winborn et al., 2008). These transgenic lines would help us to determine if mutating Ubs2 mitigates ataxin-3-dependent toxicity when its autoprotective role is no longer a factor.

When these catalytically inactive transgenes are pan-neuronally expressed in *Drosophila*, the C14A-Ubs2*^{Mild} mutation reduces ataxin-3 protein levels, independently of transcriptional levels (**Figure 3.5C and D**). The protein levels of catalytically inactive Ataxin-3(SCA3) are also significantly lower than catalytically active Ataxin-3(SCA3), indicating that ataxin-3's catalytic activity regulates its steady state levels *in vivo*. Importantly, flies expressing C14A-Ubs2*^{Mild} exhibit improved adult longevity compared to the flies with C14A-Intact (**Figure 3.5E**). Motility is also significantly improved in C14A-Ubs2*^{Mild} flies on day 7 (**Figure 3.5F**).

Overall, these results indicate that reducing ataxin-3 protein levels can indeed decrease the toxicity of pathogenic ataxin-3, as long as its protective function is not a factor. We conclude that Ubs2*^{Mild} increased the toxicity of Ataxin-3(SCA3) in our earlier experiments because the mutation impaired its autoprotective function.

3.2.4 Strong disruption of pathogenic ataxin-3's Ubs2 is less toxic than mild disruption

Mild disruption of Rad23 binding to Ubs2, which decreases binding of ataxin-3 to Rad23 *in vitro* by about 50% (Blount et al., 2014), yielded a more toxic protein. We next reasoned that a stronger disruption of Rad23 binding to Ubs2 might reduce the protein's toxicity by nearly eliminating the ataxin-3 protein. To test this possibility, we mutated three amino acid residues of Ubs2 to glutamic acid (**Ataxin-3(SCA3)-Ubs2*^{Strong}; Figure 3.1B**). *In vitro* binding assays indicate that Ubs2*^{Strong} greatly reduces binding to Rad23 (**Figure 3.6A**). When we express



pathogenic ataxin-3 with the UbS2^{Strong} mutation in HeLa cells, we notice a reduction in Ataxin-3(SCA3) levels more profound than with UbS2^{Mild} (Figure 3.6B). The turnover rate of

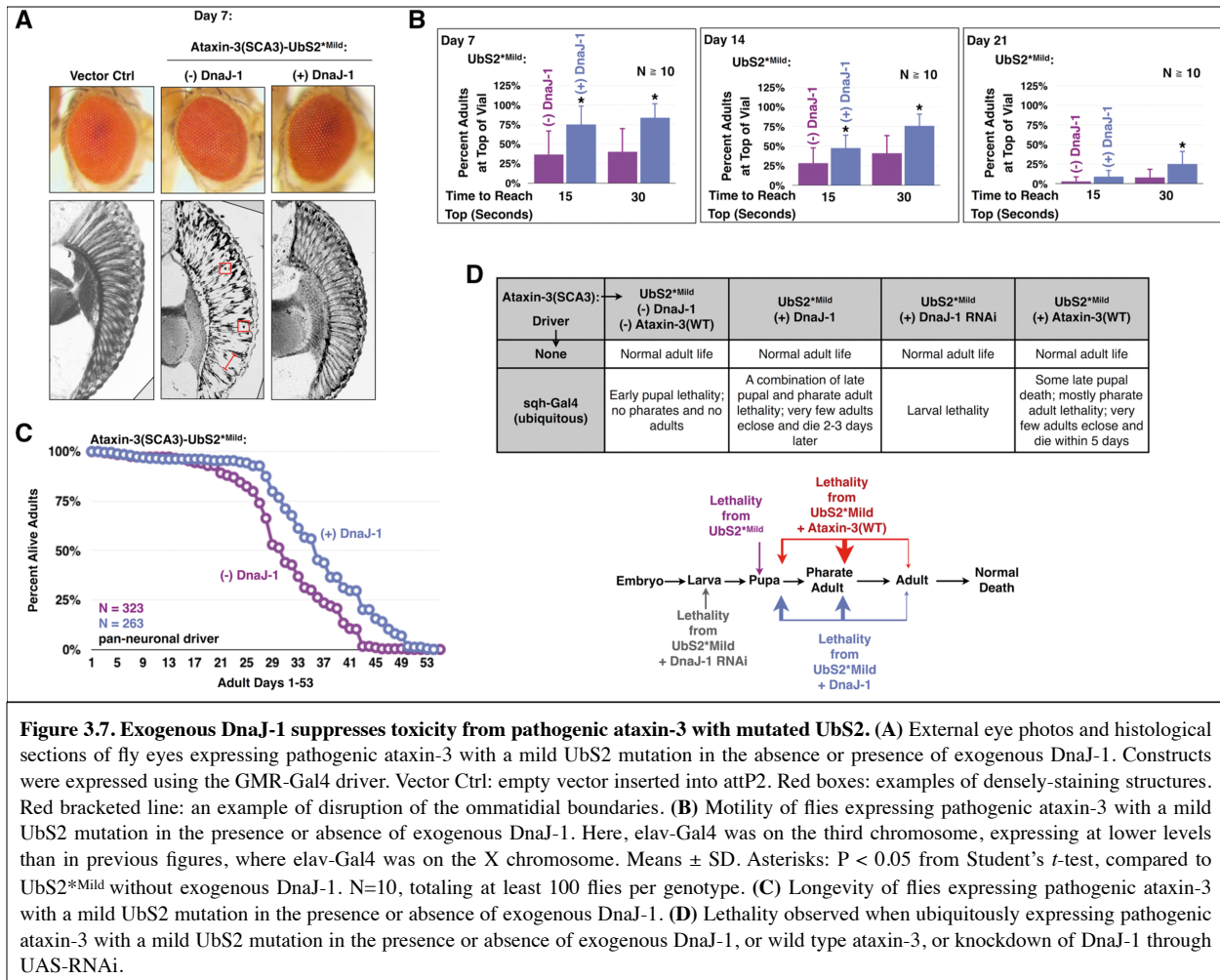
UbS2*Strong is also higher than that of UbS2*Mild (**Figure 3.6C**).

We generated additional fly lines that express pathogenic ataxin-3 with the UbS2*Strong mutation. Ubiquitous expression of UbS2*Strong elicits developmental lethality primarily in the mid pupal stage, which represents a slight improvement from what was seen earlier with the UbS2*Mild flies (**Figure 3.6D**). Western blotting of larvae expressing UbS2*Strong reveals that ataxin-3 protein levels are reduced by approximately 90% (**Figure 3.6E**). qRT-PCR indicates that the decrease in the UbS2*Strong version is not due to transcriptional changes (**Figure 3.6F**).

We continued our analyses of the SCA3 phenotype by pan-neuronally expressing UbS2*Strong. When expressed in neurons, the protein levels of this version of ataxin-3 are also markedly lower than those of the intact counterpart (data not shown), similar to what was observed with ubiquitous expression in larval lysates (**Figure 3.6E**). These adults live longer and perform better in the motility assay than the flies expressing UbS2*Mild (**Figure 3.6G and H**). Still, in general they do not perform as well as flies expressing Ataxin-3(SCA3)-Intact. Collectively, these data further support the role of UbS2 in the pathogenicity of ataxin-3, and stress the importance of this site on both the toxicity and protein levels of the SCA3 protein.

3.2.5 DnaJ-1 suppresses toxicity from Ataxin-3(SCA3) with mutated UbS2

Previously, we reported that wild-type ataxin-3 (denoted here as Ataxin-3(WT)) suppresses polyQ toxicity in *Drosophila* by binding Rad23 and leading to an increase in endogenous DnaJ-1 at mRNA and protein levels (Tsou et al., 2015b). A version of ataxin-3 with the UbS2*Mild mutation did not have the same effect on DnaJ-1 (Tsou et al., 2015b). DnaJ-1 is a member of the J/HSP40 family of co-chaperone proteins that maintain the substrate specificity and stimulate the ATPase activity of HSP70 chaperones (Kampinga and Craig, 2010; Koutras and Braun, 2014). Since our data are consistent with the idea that Ataxin-3(SCA3) with mutated



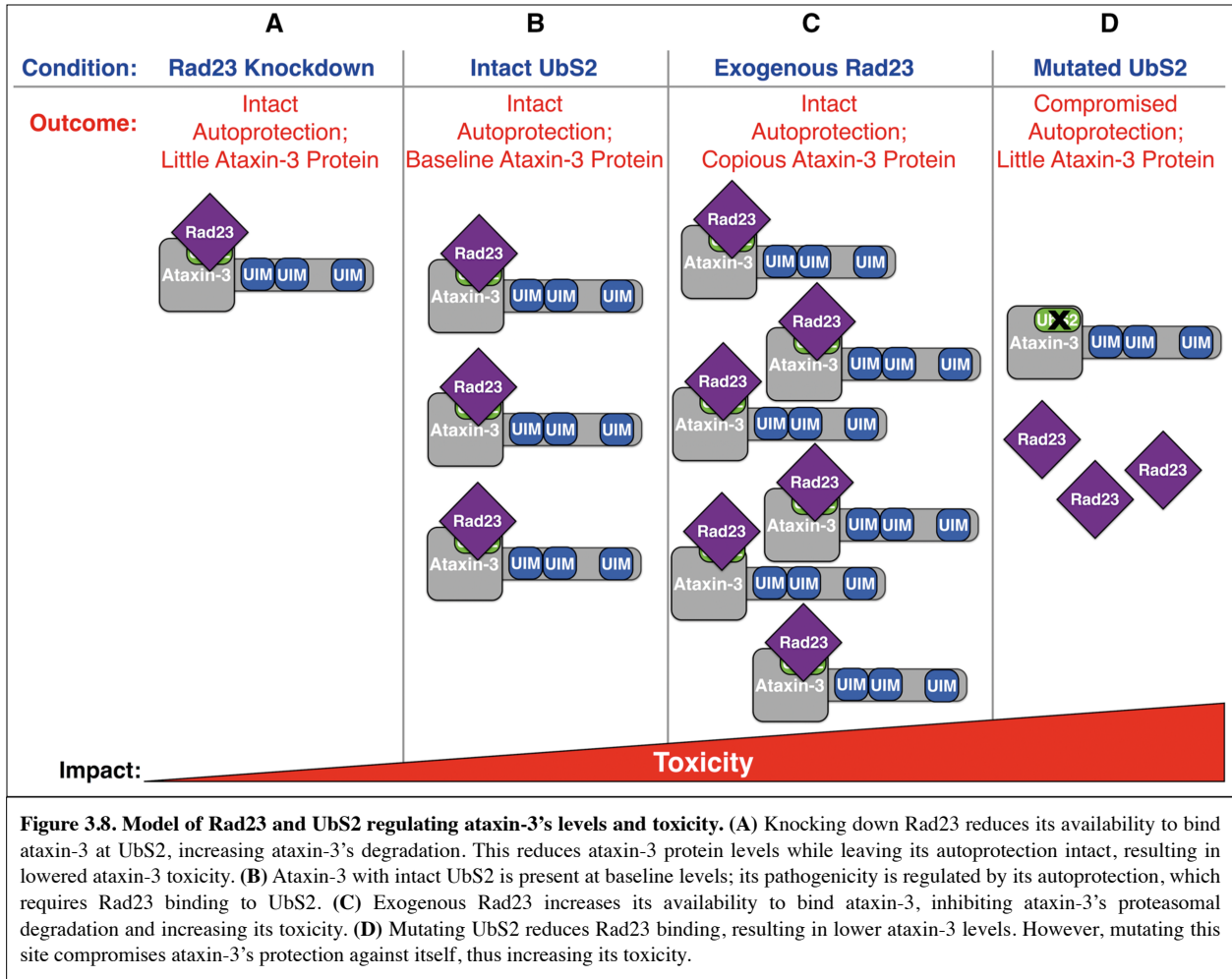
UbS2 is more pathogenic due to impaired autoprotection, we tested whether DnaJ-1 can suppress this toxicity.

When we co-express DnaJ-1 with UbS2^{Mild} in fly eyes, we observe great improvement in the internal retinal structure: the boundaries and the organization of the ommatidia are restored (**Figure 3.7A**). External photos of DnaJ-1 eyes display the reappearance of the pseudo-pupil, consistent with a healthy underlying structure (**Figure 3.7A**). Pan-neuronal expression of DnaJ-1 alongside UbS2^{Mild} also improves adult longevity and motility (**Figure 3.7B and C**). Moreover, exogenous DnaJ-1 reduces the rate of developmental lethality in UbS2^{Mild} flies when the toxic protein is expressed everywhere (**Figure 3.7D**). Instead of dying in the early pupal stage, these

flies exhibit late pupal and pharate adult lethality. A small number of adults even successfully eclose and survive for two or three days. Importantly, RNAi-dependent knockdown of DnaJ-1 causes the UbS2^{*Mild} flies to die much earlier (**Figure 3.7D**).

Lastly, we examined the protective role of Ataxin-3(WT) against its pathogenic variant with UbS2 mutated. As summarized in **Figure 3.7D**, expression of non-pathogenic ataxin-3 has a suppressive effect towards UbS2^{*Mild} that closely mirrors the protective effect of exogenous DnaJ-1. Ataxin-3(WT) delays lethality from pathogenic ataxin-3 with a mutated Rad23-binding site from early pupal to late pupal and pharate adult stages. A handful of adults eclose and survive for a few days. Altogether, the results in **Figure 3.7** lend additional support to the hypothesis that pathogenic ataxin-3 with mutated UbS2 is more toxic due to a perturbation of its autoprotective role.

Wild-type ataxin-3 has a protective effect against various polyQ proteins in *Drosophila* (Burr et al., 2014; Tsou et al., 2013; Tsou et al., 2015b; Warrick et al., 2005). This role of ataxin-3 in the fly depends on its catalytic activity, on an intact UbS2, and on the presence of Rad23 (Tsou et al., 2015b; Warrick et al., 2005). According to those findings, ataxin-3, in a manner dependent on Rad23, leads to an increase in the transcription of DnaJ-1 and a subsequent rise in its protein levels. This co-chaperone then functions with inducible heat shock proteins to reduce the aggregation and toxicity of polyQ species in flies. The data in **Figure 3.7**, together with the results from prior figures where we perturbed Rad23 levels, mutated UbS2 of ataxin-3, and incapacitated the DUB activity of pathogenic ataxin-3, collectively indicate a similar mechanism at work on autoprotection from pathogenic ataxin-3.



3.3 Discussion

Our prior work showed a critical role for the SCA3 protein interaction with the proteasome-associated protein, Rad23 in the proteasomal turnover of ataxin-3 (Blount et al., 2014). Whether the site of this interaction was directly important for the toxicity of the SCA3 protein, or if targeting this interaction could be beneficial against SCA3, was unclear. We found that mutating Ubs2 on ataxin-3 to disrupt this interaction substantially reduces steady state levels of the SCA3 protein *in vivo*, but greatly enhances its toxicity. According to our work, ataxin-3's binding to Rad23 regulates this polyQ protein's levels, autoprotective function, and, consequently, its pathogenicity. The data presented here showcase a previously unreported role

for the interaction between the SCA3 protein and Rad23 as a regulator of ataxin-3's own pathogenicity in an intact, model organism (**Figure 3.8**). Our results bring together a new understanding of the role of ataxin-3's domains and its interactions with partners in its toxicity. They also highlight the importance of protein context in the biology of this polyQ disease, and the need to carefully consider it when devising strategies for suppressive avenues.

Ataxin-3 can also bind ubiquitin through Ubs2 (Nicastro et al., 2009; Nicastro et al., 2010). One might surmise that binding of ubiquitin, or other similar proteins, could potentially impact ataxin-3 levels and toxicity through their interaction with Ubs2. In earlier work, we found that knockdown of ubiquitin-like proteins did not impact ataxin-3 levels in *Drosophila* (Blount et al., 2014). Our genetic manipulations of Rad23 levels in this study and in other, previously published work (Blount et al., 2014) make a strong case for a specific and significant role from the binding of ataxin-3 to Rad23 in the toxicity and levels of the SCA3 protein.

Ataxin-3's interaction with Rad23 might have a cellular role by assisting with the degradation of select poly-ubiquitinated substrates destined for the proteasome ((Buchberger et al., 2010; Dantuma et al., 2009). Binding of ataxin-3 to Rad23 hinders the degradation of the SCA3 protein (Blount et al., 2014). This interaction between Rad23 and ataxin-3, however, need not impact the ability of these two ubiquitin-binding proteins to deliver cargo to the degradative machinery, which depends on the transfer of poly-ubiquitinated substrates from shuttles to the 19S component of the proteasome (Ristic et al., 2014).

Our findings raise the question why we observe decreased ataxin-3-dependent toxicity when we reduce Rad23 levels through RNAi, but not when we mutate Ubs2 to reduce Rad23 binding to ataxin-3. Rad23 knockdown reduces its levels and thus ataxin-3 protein levels as a result of its increased degradation (**Figure 3.1A; (Blount et al., 2014)**), but the remaining Rad23

and ataxin-3 proteins can still interact since Ubs2 is intact. In this scenario, ataxin-3 is likely less toxic because some of the pathogenic protein is eliminated, but the remaining SCA3 protein can still bind Rad23 and exert its unperturbed autoprotective role. When we mutate Ubs2, we reduce the protective ability of all of the ataxin-3 protein present as a result of compromised binding to Rad23 (**Figure 3.8**). Ataxin-3 with mutated Ubs2 is unable to elevate expression of the co-chaperone DnaJ-1 (Tsou et al., 2015b). Thus, it is not surprising that when we increase the levels of DnaJ-1, we observe a reduction in the pathogenicity of ataxin-3 with mutated Ubs2.

Various studies have reported that reducing levels of disease proteins improves the pathology in SCA3 and other polyQ disorders (Alves et al., 2010; Harper et al., 2005; Pedersen and Heegaard, 2013; Tsou et al., 2011; Williams et al., 2009; Williams and Paulson, 2008; Xia et al., 2004). Our work demonstrates that the means by which pathogenic protein levels are reduced can influence its toxicity. Since Ubs2 is important for both ataxin-3's protein levels and autoprotective function, genetically mutating this site to prevent Rad23 binding proved inadequate for simultaneously reducing ataxin-3 levels and its toxicity. Thus, pathogenic protein levels cannot be reduced indiscriminately; the functionality of the protein binding domains and its interactions must be considered before targeting it therapeutically.

Our results show that mutating Ubs2 exacerbates, rather than alleviates, ataxin-3-dependent toxicity in *Drosophila*. The question remains whether Ubs2 could be a potential therapeutic target for SCA3 in mammalian systems. The toxicity from mutating Ubs2 in flies results from disruption of ataxin-3's protective role. While a protective role for ataxin-3 in flies and mammalian cells is clear (Burr et al., 2014; Chatterjee et al., 2015; Reina et al., 2012; Reina et al., 2010; Tsou et al., 2013; Tsou et al., 2015b; Warrick et al., 2005; Zhou et al., 2013a), there is conflicting evidence for a similar role for ataxin-3 in mice. In one study, SCA3 transgenic mice

that were heterozygous for pathogenic ataxin-3 and possessed one copy of wild-type ataxin-3 exhibited a milder phenotype than mice expressing pathogenic ataxin-3 alone (Cemal et al., 2002). Alternatively, co-expression of wild-type ataxin-3 in a different SCA3 mouse model did not ameliorate the phenotype (Hubener and Riess, 2010). Yet another study reported that complete loss of endogenous, wild-type ataxin-3 in a mouse model of Huntington's disease exacerbated a motor phenotype, but it did not affect other aspects of the pathology (Zeng et al., 2013). If ataxin-3 does not play a significant protective role in intact mammals, mutating Ubs2 *in situ* could be a potential therapeutic approach for SCA3, as this should reduce ataxin-3 protein levels without causing much additional toxicity. Since ataxin-3's neuroprotective role in mammals remains to be fully clarified, our work suggests that the ideal approach for SCA3 therapy should aim to reduce ataxin-3 levels while leaving its autoprotection intact.

In conclusion, the interaction of ataxin-3 with Rad23 through the Ubs2 domain appears to serve dual roles on the SCA3 protein by regulating its levels and inherent toxicity. Our findings provide novel clues towards the understanding of the biology of disease in SCA3, and caution against arbitrarily reducing the levels of a toxic protein for the purposes of therapy. The next chapter will investigate the interaction of ataxin-3 with another one of its binding partners, VCP.

CHAPTER 4: Toxicity and aggregation of the polyglutamine disease protein, ataxin-3, is regulated by its binding to VCP/p97 in *Drosophila melanogaster*

Under review: Ristic G*, Sutton JR*, Libohova K, Todi SV (2018). Toxicity and aggregation of the polyglutamine disease protein, ataxin-3 is regulated by its binding to VCP/p97 in *Drosophila melanogaster*. *Neurobiology of Disease*. *- these authors contributed equally to this manuscript.

4.1 Introduction

The previous chapter explored ataxin-3's interaction with Rad23, an interaction partner that is linked to ataxin-3's neuroprotective function. Another protein quality control partner of ataxin-3 is Valosin-Containing Protein (VCP, also known as p97), a member of the AAA (ATPases Associated with diverse cellular Activities) family of proteins (**Figure 4.1A-B**) (Meyer et al., 2012). VCP functions as a homo-hexamer, converting chemical energy harvested from ATP hydrolysis to mechanical energy to exert force on its substrates. It is an essential, ubiquitously expressed protein involved in numerous cellular processes, including protein degradation (Xia et al., 2016).

Ataxin-3 binds VCP directly (Doss-Pepe et al., 2003; Hirabayashi et al., 2001; Wang et al., 2006) through a region on its C-terminal half (**Figure 4.1A**) (Boeddrich et al., 2006). According to biochemical and cell-based experiments from an elegant study by the Wanker and Bonini labs, ataxin-3 binds to the N-terminal portion of VCP through an arginine-rich area (amino acid sequence RKRR) (Boeddrich et al., 2006) (**Figure 4.1A**). Mutating the VCP-binding site on ataxin-3 disrupts the interaction between these two proteins (Boeddrich et al., 2006). The same study also reported that VCP stimulates fibrillogenesis/aggregation of pathogenic ataxin-3 in a dose-dependent manner in *in vitro*, reconstituted systems. These results suggested a seeding role for VCP: the hexamer binds multiple ataxin-3 proteins, increasing their chances of interacting together and enhancing their initial aggregation (Boeddrich et al., 2006). Exogenous

VCP mitigated, to some extent, degeneration caused by full-length, pathogenic ataxin-3 in one tissue tested: the eyes of fruit flies (Boeddrich et al., 2006). These compelling data highlight the need for additional examinations to understand the interaction between ataxin-3 and VCP *in vivo*. Direct comparison between pathogenic ataxin-3 that can bind VCP and a version that has the VCP-binding site mutated—expressed similarly in various tissues— is necessary to appreciate the interplay between these proteins in SCA3. It would also help overall understanding of SCA3 biology to examine if VCP regulates the aggregative propensity of pathogenic ataxin-3 in an intact animal, beyond *in vitro* settings. Lastly, examining the relationship of the VCP-ataxin-3 interaction to the temporal aggregation and pathogenicity of the SCA3 protein in an intact animal would be helpful both to further our knowledge of SCA3 disease mechanisms and to examine if this interaction could be of therapeutic benefit. These were the various aspects that we set out to tackle in this work, by using *Drosophila* as a model organism.

To accomplish this, we generated isogenic lines of *Drosophila melanogaster* that express, in a Gal4-UAS-dependent manner (Brand et al., 1994; Brand and Perrimon, 1993), full-length, human, pathogenic ataxin-3 with a normal or mutated VCP-binding site. We found that pathogenic ataxin-3 with a mutated VCP-binding site is markedly less toxic in flies, even though the protein is present at levels similar to its VCP-binding counterpart. Initially, flies that express the SCA3 protein with impaired VCP-binding are indistinguishable from controls that do not express any pathogenic ataxin-3. This coincides with markedly lower levels of aggregated ataxin-3 species. Altering the levels of endogenous VCP protein modulates the toxicity of the SCA3 protein in a manner that supports the idea of a nucleation role for the aggregation of ataxin-3 by this AAA ATPase. Our data highlight an important physiological role for VCP on ataxin-3-dependent degeneration and underscore the importance of non-polyQ regions of this

DUB to the toxicity of its expanded repeat.

4.2 Results

4.2.1 Mutating the VCP-binding site of ataxin-3 disrupts its interaction with VCP in flies

We began our journey into the role of the VCP-ataxin-3 interaction in SCA3-like degeneration in *Drosophila* by confirming that the VCP-binding site mutation, “RKRR” to “HNHH”, disrupts the DUB’s interaction with fly VCP. This mutation was shown before to disrupt binding of recombinant ataxin-3 to mammalian VCP (Boeddrich et al., 2006). We generated recombinant, pathogenic ataxin-3 with 80 polyQ in bacterial cells. This repeat is within patient range. We produced two versions of the DUB: one that has the intact VCP-binding site and one whose site is mutated from “RKRR” to “HNHH”. We then incubated bead-bound, GST-tagged, recombinant ataxin-3 with whole fly lysates and probed for the ability of human ataxin-3 to interact with fly VCP. As shown in **Figure 4.1C**, the pathogenic version of ataxin-3 co-precipitates fly VCP, whereas the version with impaired VCP-binding has a drastically reduced capacity to bind fly VCP; the western blot signal was barely above non-specific background that was non-specifically brought down by GST alone (**Figure 4.1C**). This disruption in interaction between VCP and ataxin-3 is similar to what we observe in mammalian cells: pathogenic ataxin-3 with impaired VCP-binding is unable to co-IP endogenous, human VCP beyond non-specific background, whereas pathogenic ataxin-3 with intact VCP-binding co-IPs this interactor (**Figure 4.1D**). In **Figures 4.1C and D**, we used mild conditions that brought down some VCP non-specifically, which helped us to not exclude the possibility of residual VCP interaction with VCP-binding-mutated ataxin-3. We did not observe clear and specific interaction of VCP with pathogenic ataxin-3 that has its VCP-binding site mutated. Lastly, as shown in **Figure 4.1E**, mutating the RKRR region on pathogenic ataxin-3 removes its ability to bind fly VCP *in vivo*. In this last

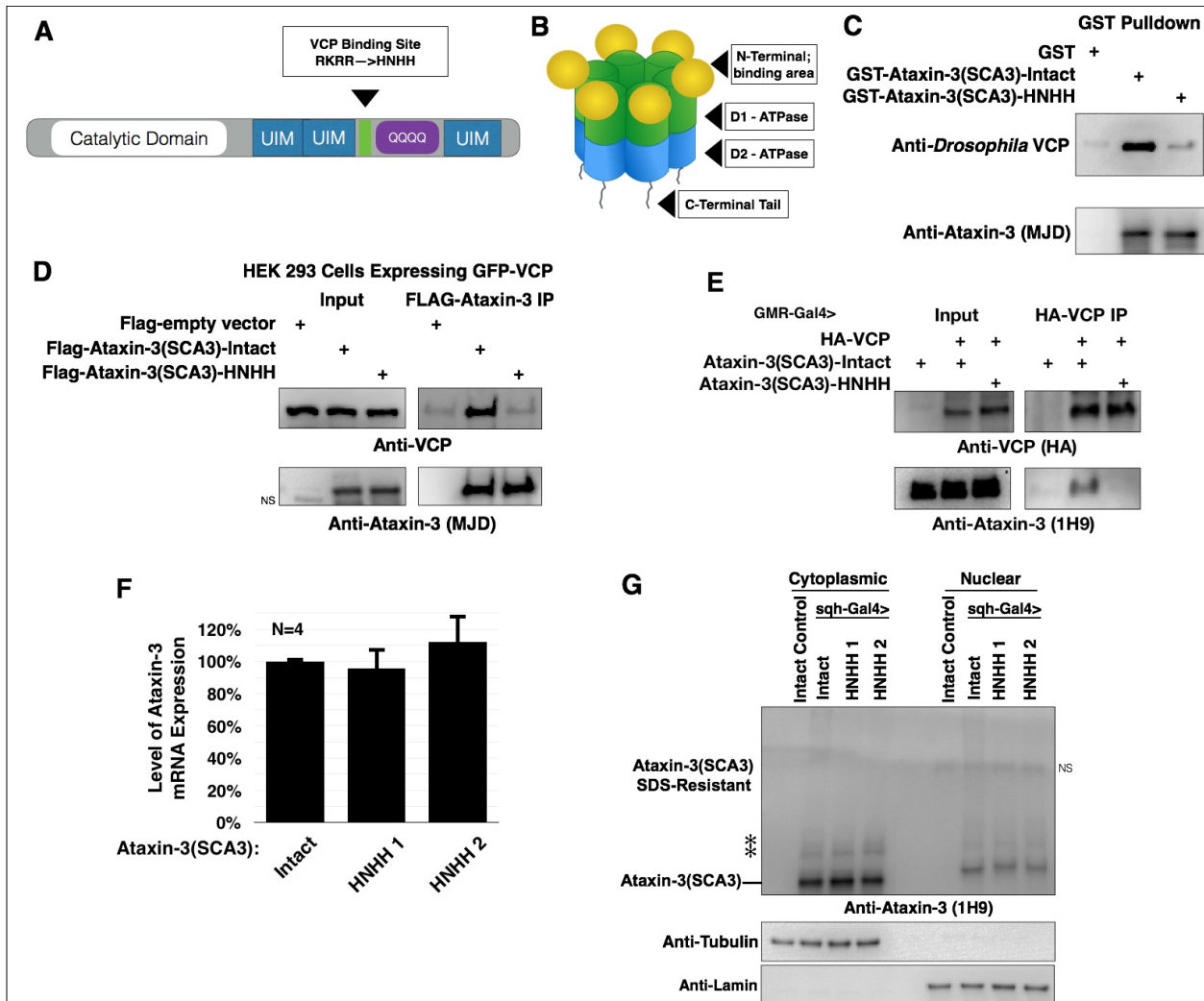


Figure 4.1. Pathogenic ataxin-3 with a mutated VCP-binding site does not bind *Drosophila* VCP. (A) Diagrammatic representation of ataxin-3. The catalytic domain of ataxin-3, Josephin Domain, is located at the structured N-terminal half of the protein. The inherently unstructured C-terminal portion contains three Ubiquitin Interacting Motifs (UIMs), the polyQ tract (which is abnormally expanded in SCA3 patients), and the VCP-binding site. Box shows this site was mutated from “RKRR” to “HNHH” to disrupt the interaction with VCP. (B) Diagrammatic representation of VCP. VCP functions as a homohexamer. Each VCP protein contains an N-terminal domain (through which it binds to most of its co-factors, including ataxin-3), two tandem ATPase domains, and a short C-terminal tail. (C) Western blot from *in vitro* pull-downs with whole fly lysates and immobilized, recombinant GST, GST-Ataxin-3(SCA3)-Intact, or GST-Ataxin-3(SCA3)-HNHH. Both Ataxin-3(SCA3) constructs contained 80 polyQ repeats. Blots are representative of experiments repeated independently three times with the same outcomes. (D) Western blot of co-immunoprecipitations from HEK293 cells transiently transfected with the indicated constructs. Blots are representative of experiments repeated independently three times with the same outcomes. NS=Nonspecific band we often observe with the polyclonal ataxin-3 antibody. (E) Western blot of co-immunoprecipitations from dissected heads of transgenic flies expressing the indicated constructs. Transgenes were expressed through the eye-specific GMR-Gal4 driver. Blots are representative of experiment repeated independently three times with the same outcomes. In this panel and the rest of the figures, flies were heterozygous for all drivers and transgenes, unless otherwise noted. (F) qRT-PCR results from whole adult flies expressing pathogenic ataxin-3 with an intact or mutated VCP-binding site. Ataxin-3(SCA3)-Intact or Ataxin-3(SCA3)-HNHH was driven by the sqh-Gal4 ubiquitous driver. No significant differences in the mRNA expression levels were observed when the VCP-binding site is mutated to HNHH. N=4 independent repeats. In this panel and the rest of the figures, HNHH 1 and 2 are independently derived transgenic lines. Endogenous control: rp49. Means \pm SD. P values were calculated by two-tailed Student’s *t*-tests, comparing HNHH 1 and HNHH 2 to Intact. (G) Western blot of fly lysates after cytoplasmic/nuclear centrifugation. Transgenic adult flies expressed pathogenic ataxin-3 with or without the VCP-binding site mutated from “RKRR” to “HNHH”. Ataxin-3(SCA3)-Intact or Ataxin-3(SCA3)-HNHH were expressed under the control of the sqh-Gal4 driver. “Intact Control” flies were Ataxin-3(SCA3)-Intact-encoding flies without a driver. Asterisks: ubiquitinated ataxin-3. NS=Nonspecific band. Blots are representative of experiments repeated independently three times with the same outcomes.

setup, we co-expressed HA-tagged, fly VCP alongside ataxin-3 in fly eyes and precipitated VCP. HA-tagged, *Drosophila* VCP is able to co-IP pathogenic ataxin-3 with an intact VCP-binding site, but not the pathogenic version with the “RKRR to HNHH” mutation (**Figure 4.1E**). We conclude that this specific mutation on pathogenic ataxin-3 effectively disrupts the interaction of the SCA3 protein with *Drosophila* VCP.

4.2.2 Mutating the VCP-binding site of ataxin-3 does not alter its protein or mRNA levels, or its subcellular localization

At hand, we have flies that express pathogenic ataxin-3 with an intact or mutated VCP-binding site. We generated the full-length, human, pathogenic ataxin-3 for earlier work (Chapter 3). For the present work, we created new transgenic *Drosophila* lines expressing ataxin-3 with 80 polyQ repeats with the “RKRR” to “HNHH” mutation. The new lines were constructed 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; Tsou et al., 2016). These flies are referred to as “HNHH” 1 and 2, as we utilized two independently-derived lines, which ultimately led to the same results that were combined in most figures. The “HNHH” lines are isogenic to the “Intact” SCA3 line; the constructs are each inserted into attP2, in the same orientation and as a single copy (Groth et al., 2004).

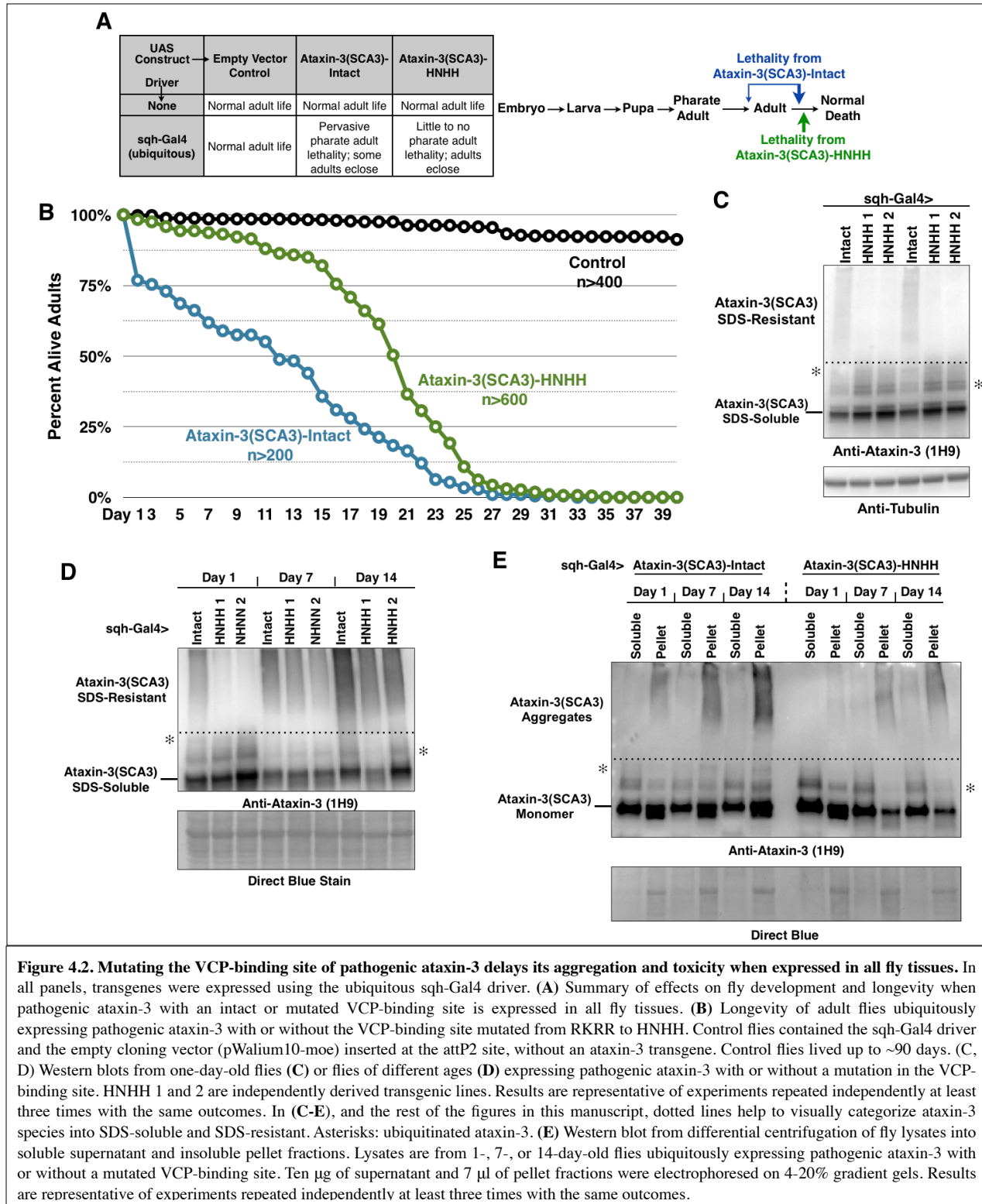
First, we wanted to confirm that any difference in phenotype we might observe among the different lines is not due to a change in ataxin-3 transgene expression levels. We examined mRNA expression levels of ataxin-3 by qRT-PCR when the protein was expressed in the whole fly using the ubiquitous driver sqh-Gal4 (**Figure 4.1F**). The “HNHH”-1 and -2 lines were compared to the line expressing pathogenic ataxin-3 with an intact VCP-binding site. There is no

statistically significant difference in the expression levels of ataxin-3 among the three fly lines. Our lab also showed previously that ataxin-3 turnover is not affected when the VCP-binding site was mutated in mammalian cells (Blount et al., 2014) or in *Drosophila* (Tsou et al., 2015b).

The VCP-binding site on ataxin-3 is an arginine-rich region located at the C-terminus of ataxin-3. Previously, this domain was thought to encode a nuclear localization signal (Albrecht et al., 2004). We examined whether mutating the VCP-binding site affects the localization of pathogenic ataxin-3 in the fly. We used centrifugal fractionation 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 VCP-binding site is mutated (**Figure 4.1G**). We conclude that mutating the VCP-binding site from "RKRR" to "HNHH" does not impact the cellular localization of pathogenic ataxin-3 in the fly.

4.2.3 Mutating the VCP-binding site of pathogenic ataxin-3 reduces its toxicity in Drosophila

To physiologically assess how mutating the VCP-binding site affects the toxicity of ataxin-3 in *Drosophila*, we conducted longevity studies. As we reported previously (**Chapter 3**), pathogenic ataxin-3 is highly toxic to the fly when it is expressed ubiquitously. We observe death during late pharate adult stages, during eclosion from the pupal case, and shortly following eclosion (**Figure 4.2A**). Those flies that successfully eclose survive for about 30 days (**Figure 4.2B**); as a frame of reference, flies not expressing pathogenic ataxin-3 can live up to ~90 days (**Chapter 3**). "HNHH" mutant flies all reached adulthood (**Figure 4.2A**). We did not observe death during development as we had with flies encoding ataxin-3 with the intact VCP-binding site. From longevity data (**Figure 4.2B**), we observed a clear separation in the rates of death



early on: the "HNHH" mutant-expressing flies appear healthier and more flies remain alive in the earlier days of life than their "Intact" ataxin-3-expressing counterparts (Figure 4.2B). Whereas

about 25% of flies that express Ataxin-3(SCA3)-Intact are dead before day 5, 25% of flies expressing the “HNHH” counterparts are dead by day 17. As more flies die, we observe that about 50% of flies expressing “Intact” pathogenic ataxin-3 are gone by day 13, compared to “HNHH” mutant-expressing flies which reach 50% death around day 21. When compared with the control flies that do not express pathogenic ataxin-3, both “Intact” and “HNHH” live a lifespan that is markedly shorter (**Figure 4.2B**). Control flies in this experiment contain the cloning vector (pWalium10-moe; inserted at the attP2 site), without any ataxin-3 transgenes, and the sqh-Gal4 driver. Still, the “HNHH” mutation has a dramatic, early impact on the longevity of these flies when the transgene is expressed in all tissues of the fruit fly.

Next, we assessed by western blotting the expression of ataxin-3 protein in one-day-old, whole-fly lysates where pathogenic ataxin-3 with an intact or mutated VCP-binding site was expressed using sqh-Gal4. Flies which harbor pathogenic ataxin-3 with intact VCP-binding show higher-molecular weight species, indicative of ataxin-3 SDS-resistant aggregates as early as one day after eclosion, whereas flies with a mutated VCP-binding site do not show this high molecular weight smear (**Figure 4.2C**).

In **Figure 4.2C** and throughout the rest of this work, you will observe several ataxin-3-positive bands in western blots. The band highlighted as SDS-soluble species contains the unmodified version of this protein (bottom-most band) and what could be a phosphorylated form of ataxin-3 (immediately above it in lighter exposures in some instances; this band is not always visible) (Fei et al., 2007; Kristensen et al., 2017; Matos et al., 2016). The asterisks denote ubiquitinated species of ataxin-3, based on our earlier studies with this protein *in vitro*, in mammalian cells and in *Drosophila* (Todi et al., 2010; Todi et al., 2009; Tsou et al., 2013). We have drawn a dotted line to separate what we know are unmodified and ubiquitinated species of

ataxin-3 from the higher-molecular weight species. The smear above the dotted line is consistent with SDS-resistant ataxin-3 species, based on our earlier work with this protein (Blount et al., 2014; Scaglione et al., 2011; Todi et al., 2009; Tsou et al., 2013; Tsou et al., 2015b). In our extensive work with this protein *in vitro*, in mammalian cells, in *Drosophila* and in mouse models of this disease, we are confident that our delineation by the dotted line is a reasonable classification of ataxin-3 species that are SDS-soluble vs. not. The SDS-resistant species may contain higher-order aggregates of the SCA3 protein.

As already noted, SCA3 is an age-related, progressive neurodegenerative disease. While aging the flies in the longevity experiment, we collected adults for western blot analysis after eclosion on day 1, 7 and 14 to examine the state of SDS-resistant and SDS-soluble species as flies age (**Figure 4.2D**). We observe that on day 1, SDS-resistant species are already clearly present in flies that express the “Intact” pathogenic protein, whereas, with “HNHH” mutant flies, we see clear accumulation of SDS-resistant protein species on day 7. On day 1, “HNHH” flies have noticeably less SDS-resistant species than “Intact” counterparts. Both “Intact” and “HNHH” flies show SDS-resistant species with age, but this accumulation is delayed in the “HNHH” flies.

To further examine the accumulation of insoluble aggregates of ataxin-3 with an intact or mutated VCP-binding site, we utilized differential centrifugation to separate whole fly lysates into a soluble, supernatant fraction and an insoluble, pellet fraction (**Figure 4.2E**) (Kim et al., 2011; O'Rourke et al., 2013; Santarriaga et al., 2015; Yang et al., 2014). We noticed a trend similar to what we saw with the SDS-resistant species in the previous panel, confirming our categorization of those species as aggregated ataxin-3. Flies expressing ataxin-3 with intact VCP-binding contain insoluble ataxin-3 aggregates in the pellet fraction as early as day 1, and

accumulate over time. Flies expressing pathogenic ataxin-3 with impaired VCP-binding do not exhibit marked insoluble aggregates in the pellet until day 7. Accumulation of these species occurs with time, but is delayed compared to ataxin-3 with intact VCP-binding, similar to what we observe with SDS-resistant species in earlier blots.

Altogether, these experiments indicate that when the VCP-binding site of pathogenic ataxin-3 is mutated, there is a delay in the accumulation of aggregated protein species in flies as compared to their “Intact”, pathogenic ataxin-3-expressing counterparts. Disrupting the interaction between pathogenic ataxin-3 and VCP leads to markedly lower toxicity from pathogenic ataxin-3 during development and early on in the life of adult flies. These results prompted us to next explore the physiological impact of this mutation on the nervous system, the primary site of pathology in SCA3.

4.2.4 Pathogenic ataxin-3 with mutated VCP-binding is less toxic in fly neurons

Ataxin-3 is expressed in all tissues and cells in the human body. However, the polyQ disease SCA3 manifests as a neurodegenerative disorder with degeneration specifically in the brainstem and the cerebellum (Costa Mdo and Paulson, 2012). With fly genetics, we can direct the expression of ataxin-3 only in the nervous system using a neuronal-specific driver, *elav-Gal4*, and examine its effects in the fly.

Western blot analysis from one-day-old whole-fly lysates where pathogenic ataxin-3 with an intact VCP-binding site is expressed pan-neuronally shows the presence of SDS-resistant ataxin-3 as early as day one in adults (**Figure 4.3A**). Comparing the impaired VCP-binding flies to those expressing the “Intact” protein, the high molecular weight smear is largely absent. The control flies used in this experiment were ones which contain the “Intact” pathogenic ataxin-3 transgene without the pan-neuronal driver; thus, there was no ataxin-3 expression in these flies.

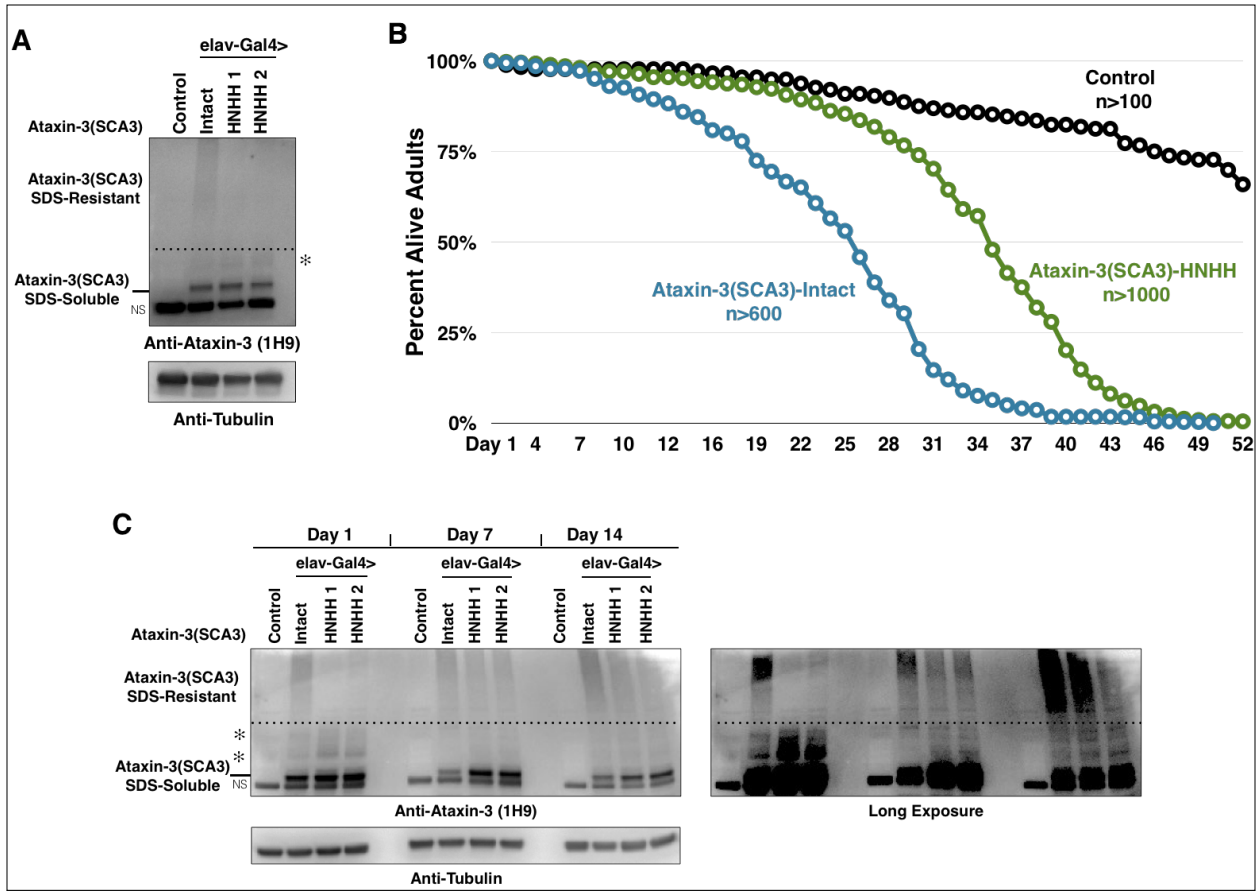


Figure 4.3. Mutating the VCP-binding site of pathogenic ataxin-3 delays its aggregation and toxicity when expressed pan-neuronally.

In all panels, transgenes were expressed using the pan-neuronal elav-Gal4 driver. (A, C) Western blots from one-day-old flies (A) or flies of different ages (C) when pathogenic ataxin-3 with or without a mutation of its VCP-binding site is expressed in the nervous system. Control flies contained the Ataxin-3(SCA3)-Intact transgene, but without a Gal4 driver. Blots are representative of experiments repeated independently five times with the same outcomes. NS: nonspecific band we observe sometimes with the monoclonal anti-ataxin-3 antibody when this transgene is expressed pan-neuronally. (B) Longevity assay from adult *Drosophila* pan-neuronally expressing pathogenic ataxin-3 with an intact or mutated VCP-binding site. Control flies contained the empty pW^{alium10-moe} vector, without a transgene inserted at attP2, in the presence of the elav-Gal4 driver. Control flies lived up to ~90 days.

Next, we aged adults to investigate how ataxin-3 expression in the nervous system affects their lifespan. Similarly to results we obtained when ataxin-3 forms are expressed in all tissues (Figure 4.2B), pan-neuronal expression of the VCP-binding site mutant delays lethality in adults compared to the “Intact” form (Figure 4.3B). A higher percentage of flies remain alive early on; we observed a 50% loss of flies expressing pathogenic ataxin-3 with intact VCP-binding by day 25 whereas this reduction occurs around day 35 in flies expressing pathogenic ataxin-3 with impaired VCP-binding. However, even though in earlier time points more mutant “HNHH” flies remain alive, both fly lines have an average life span of 45 to 50 days (Figure 4.3B). Flies pan-

neuronally expressing either variant of pathogenic ataxin-3

live longer than those expressing the same constructs ubiquitously with the *sqh-Gal4* driver. The difference in toxicity observed in this case is likely due to expression in all tissues of the fly as compared to only in the nervous system.

We examined by western blotting whole fly lysates at three points: day 1, day 7, and day 14 (**Figure 4.3C**). Similar to the results with ubiquitous expression of ataxin-3, (**Figure 4.2D**), SDS-resistant pathogenic ataxin-3 species are evident at day one in flies expressing the protein with intact VCP-binding (**Figure 4.3C**). Conversely, in those flies expressing pathogenic ataxin-3 with impaired VCP-binding, high molecular weight species are not prominent until day 7 (**Figure 4.3C**).

SCA3 is a movement disorder, marked by progressive ataxia followed by death (Costa Mdo and Paulson, 2012). We tested neuronal function of flies when the pathogenic ataxin-3 proteins were expressed

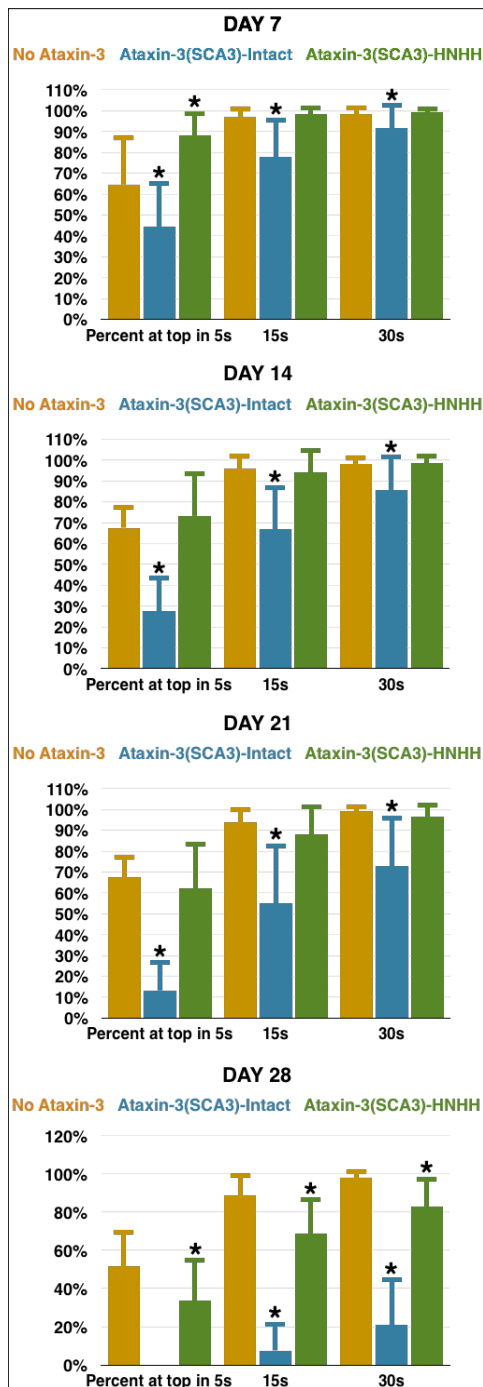


Figure 4.4. Flies that express pathogenic ataxin-3 with a mutated VCP-binding site are indistinguishable from non-ataxin-3 flies early on in motility assays. Results from a negative geotaxis assay used to assess the motility of flies expressing pathogenic ataxin-3 with or without the VCP-binding site mutation from “RKRR” to “HNHH”. Ataxin-3 transgenes were expressed using the pan-neuronal *elav-Gal4* driver. Histograms show the mean \pm SD, expressed as a percentage, of flies able to reach the top of the vial at the indicated time points. Asterisks: $P < 0.05$, compared to “No Ataxin-3” control flies. P values are from two-tailed Student’s *t*-tests, comparing “Intact” and “HNHH” to “No Ataxin-3”. “No Ataxin-3” flies contained the *elav-Gal4* driver and the empty pWalium10-moe vector inserted into the attP2 site. N=5 independent repeats, totaling at least 50 flies per genotype.

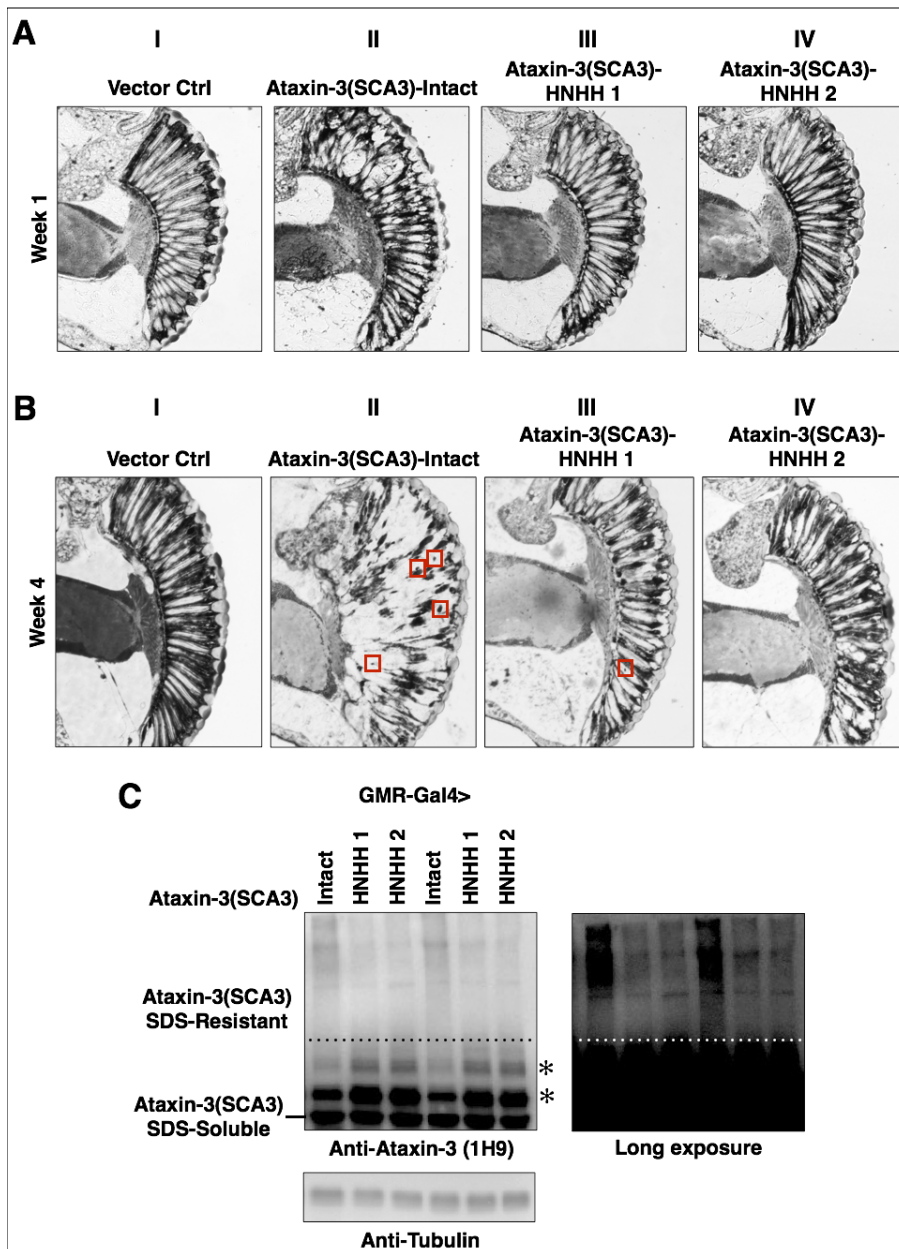
specifically in the nervous system by examining their motility against the gravitational index. We assessed how many flies reach the top of the vial within 5, 15 and 30 seconds. Adult flies were maintained over a period of four weeks and motility was assessed weekly (**Figure 4.4**). As early as day seven, flies expressing the “Intact” pathogenic protein are slower than control flies containing the empty vector across all three time points. This difference increases markedly with age, and at four weeks of age the majority of flies do not reach the top of the vial even during the longest time interval, 30 seconds. Conversely, flies expressing pathogenic ataxin-3 with a mutated VCP-binding site perform as well as control counterparts for up to three weeks of age (**Figure 4.4**). We did not notice statistically significant reduction in the motility of these flies early on, a testament to the reduced toxicity of this protein in neurons, compared to the “Intact” counterparts. Finally, at four weeks of age, the “HNHH” flies also show slower motility compared to non-ataxin-3-expressing controls, but still perform markedly better than flies expressing pathogenic ataxin-3 with intact VCP-binding (**Figure 4.4**).

In conclusion, when the VCP-binding site of ataxin-3 is mutated, accumulation of SDS-resistant species in neurons is delayed, as also observed with ubiquitous expression of the respective proteins. Flies pan-neuronally expressing pathogenic ataxin-3 with a mutated VCP-binding site perform significantly better than their “Intact” pathogenic ataxin-3-expressing counterparts in the negative geotaxis motility assay, comparable to control flies until three weeks of age. Reduced performance by the flies expressing pathogenic ataxin-3 with a mutated VCP-binding site occurs after noticeable accumulation of SDS-resistant species by western blotting (**Figure 4.3C**), indicating a temporal relationship between aggregation and toxicity.

4.2.5 Ataxin-3 with a mutated VCP-binding site is less toxic in *Drosophila* eyes

Drosophila eyes serve as a widely accepted model to study degeneration in polyQ

diseases and other proteotoxic disorders (Bonini and Fortini, 2003; Kim et al., 2018). Histological sections of the fly eye allow visualization of degeneration and accumulation of proteinaceous aggregates. Using the GMR-Gal4 driver, we expressed the Ataxin-3(SCA3) transgenes in fly eyes and conducted histological assays at different time points to characterize



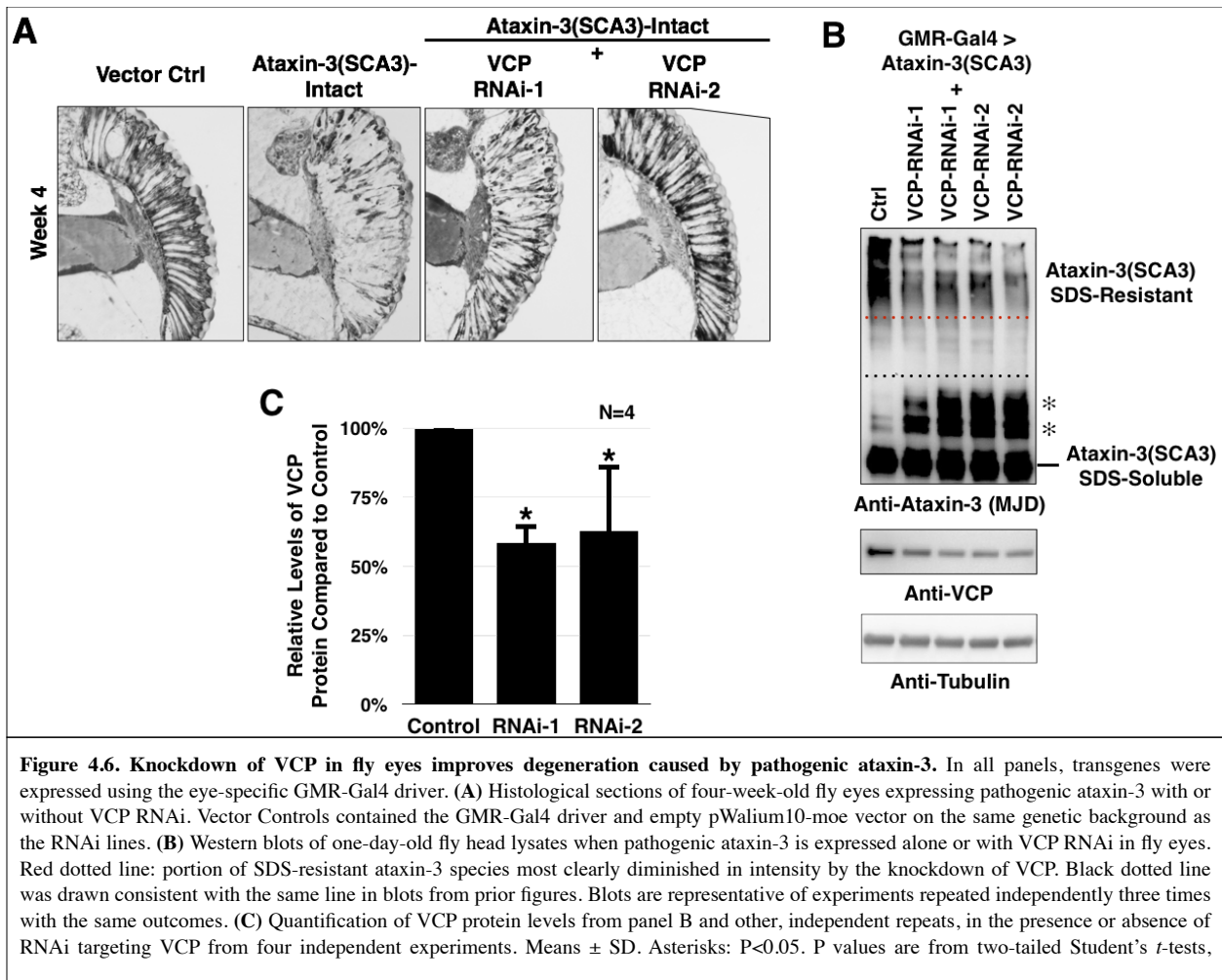
the degeneration caused by expression of “Intact” pathogenic ataxin-3 and the effects of mutating the VCP-binding site on the phenotype.

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

Figure 4.5. Disrupting the interaction between ataxin-3 and VCP reduces toxicity in fly eyes. In all panels, transgenes were expressed using the eye-specific GMR-Gal4 driver. (A, B) Histological sections of fly eyes expressing pathogenic ataxin-3 with or without a mutation in the VCP-binding site at one week (A) or four weeks (B) of age. Vector Control flies contain the GMR-Gal4 driver, as well as the empty pWalium10-moe vector inserted at attP2. Red boxes: example of densely-staining structures. (C) Western blots from lysates of fly heads dissected from one-day-old flies. Fly eyes expressed pathogenic ataxin-3 with an intact or mutated VCP-binding site. Blots are representative of experiments repeated independently at least 5 times with the same outcomes.

model to easily and reliably observe perturbations arising from toxicity (Bonini and Fortini, 2003). Histological sections from one-week-old flies expressing pathogenic ataxin-3 with intact VCP-binding show that there is already some disruption of the internal ommatidial array of the eye compared to the control flies which did not express any ataxin-3 (**Figure 4.5A, panel II compared to panel I**). Note the vacuolization of retinal structures when “Intact” pathogenic ataxin-3 is expressed in the fly eye. By comparison, eyes expressing the “HNHH” version of pathogenic ataxin-3 closely resemble control eyes, with a well-arranged ommatidial array and no visible signs of degeneration or aggregated structures (**Figure 4.5A, panels 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 is the case for four-week-old fly eyes expressing the “Intact” pathogenic ataxin-3 protein, where we see substantial degeneration and loss of internal eye structure (**Figure 4.5B, panel II**). We also observe densely-staining structures, indicative of protein aggregates (red boxes; (Warrick et al., 2005; Warrick et al., 1998)). In “HNHH” eyes, we also begin to observe degeneration and some densely-staining structures (**Figure 4.5B, panels III and IV**). However, the ommatidial array is still clearly present and remains intact overall through the course of the examinations.

We coupled histological assays with western blotting from dissected fly heads. Western blots from one-day-old fly heads show the presence of ataxin-3 SDS-resistant species, as indicated by the high molecular weight smears, when we probe with ataxin-3 antibody (**Figure 4.5C**). We do not observe these species as prominently when the “HNHH” pathogenic ataxin-3 is expressed in the fly eyes (**Figure 4.5C**). We conclude that mutating the VCP-binding site of pathogenic ataxin-3 delays the formation of SDS-resistant species and slows the degeneration of fly eyes, similar to what we observe when these ataxin-3 forms are expressed in other tissues.



Altogether, the studies conducted in the various fly tissues with the different forms of pathogenic ataxin-3 lead us to conclude that its binding to VCP influences the aggregation and ensuing toxicity of pathogenic ataxin-3. To further probe into this model, we turned to endogenous VCP.

4.2.6 Knockdown of VCP improves degeneration caused by pathogenic ataxin-3

The results that we obtained with our experiments above indicate that pathogenic ataxin-3 protein is less toxic when its VCP-binding site is mutated. We wanted to confirm these differences in phenotype 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, but knockdown of VCP in fly eyes is well tolerated (our own, unpublished observations). Thus, we examined whether knockdown of VCP in fly eyes expressing pathogenic ataxin-3 with an intact VCP-binding site impacts the structure of the eye.

From histological sections at four weeks of age, as shown above (**Figure 4.5B**), expression of “Intact” pathogenic ataxin-3 in the eyes using GMR-Gal4 is toxic and causes the breakdown of the ommatidial array (**Figure 4.6A**). We used two different RNAi lines targeting fly VCP. As shown in **Figure 4.6C**, knockdown of VCP by each RNAi line leads to statistically significant reduction in VCP protein levels in the fly head. An important note here is that the blots underestimate the extent of VCP reduction. This is because we target RNAi to the *Drosophila* eyes, but, unable to isolate only the eyes, we examined protein levels from intact, dissected fly heads, as is customary in fly studies.

Knockdown of VCP in fly eyes leads to improvement of pathology caused by ataxin-3. As shown in **Figure 4.6A**, knocking down VCP with either RNAi-1 or -2 reduces ommatidial disarray in the presence of pathogenic ataxin-3. VCP RNAi-1 is not as effective as RNAi-2 at suppressing the degenerative phenotype. This could be due to the extent of VCP knockdown. RNAi-1 targeting VCP consistently provides reduction of VCP protein based on western blots, whereas the extent of knockdown with RNAi-2 is not as consistent, as indicated by the standard deviation bars in **Figure 4.6C**. There may be a fine line between beneficial knockdown of VCP to suppress ataxin-3-dependent pathology and too much suppression, which might be problematic, on and of itself, to the eye since we are targeting a crucial gene. RNAi-1 might be achieving this level of some improved pathology but also inherent toxicity due to low VCP levels. Nevertheless, both RNAi lines present with improved pathology compared to pathogenic ataxin-3 with intact VCP-binding in the absence of RNAi, supporting our model that the

interaction between VCP and ataxin-3 is important for the toxicity of the SCA3 protein *in vivo*.

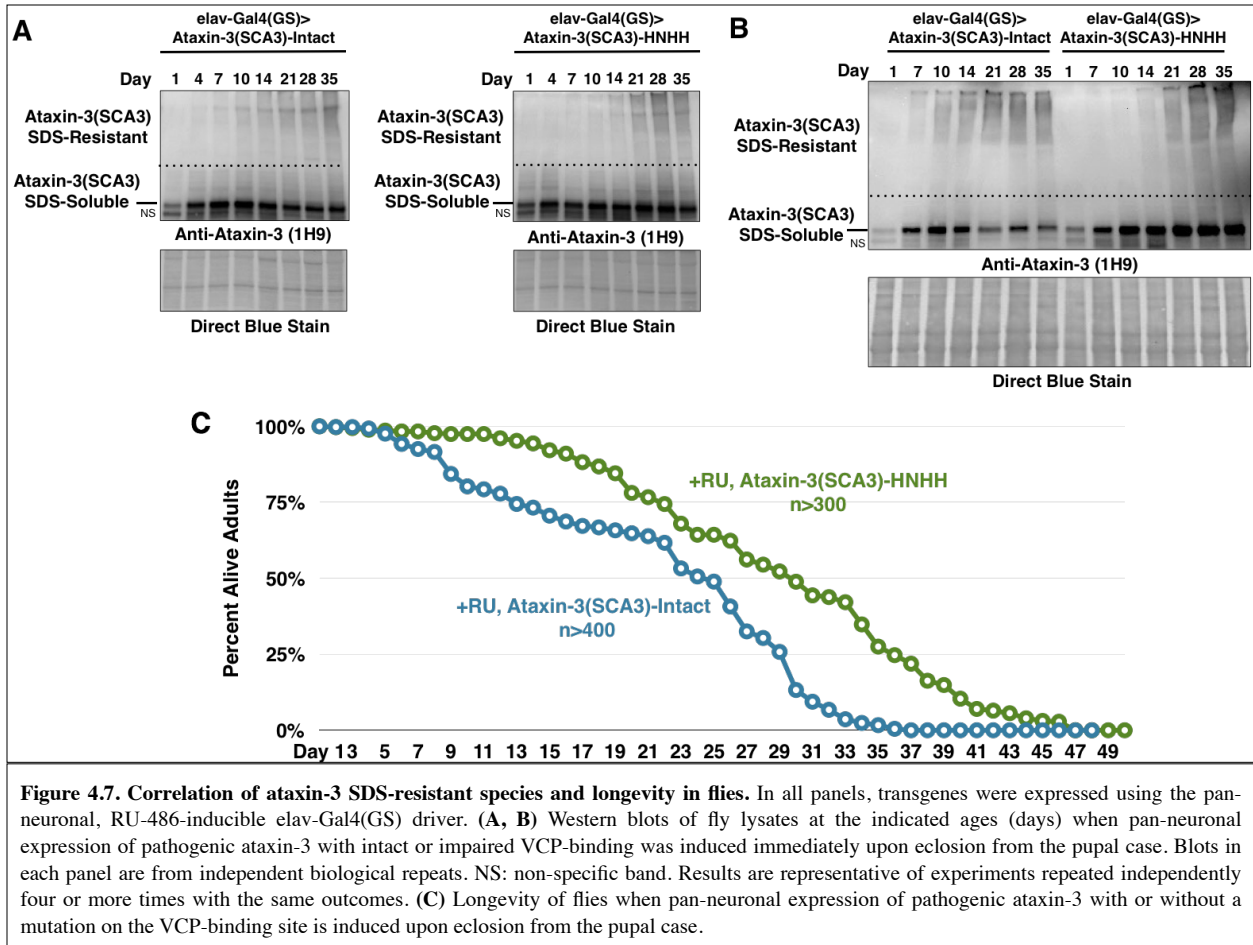
Concomitant with improved pathology, we observe reduced levels of SDS-resistant species of ataxin-3 when VCP is knocked down (**Figure 4.6B**). In **Figure 4.6B**, and to a lesser extent in earlier figures, a higher proportion of SDS-soluble ataxin-3 seems to be ubiquitinated in flies when VCP is knocked down or the VCP-binding site is mutated. It is possible that when ataxin-3 proteins bind to VCP they are brought in close proximity and deubiquitinate each other.

In conclusion, we observe decreased toxicity from ataxin-3 when VCP is knocked down in fly eyes, indicating that VCP enhances ataxin-3 aggregation and toxicity.

4.2.7 Correlating ataxin-3 SDS-resistant species to fly longevity

Our results with the ubiquitous and pan-neuronal drivers show the presence of SDS-resistant ataxin-3 species with the “Intact” version before we observe marked lethality in flies. We sought to further examine the temporal relationship between the SDS-resistant species and death. In our previous experiments, expression of pathogenic ataxin-3 proteins (intact and impaired VCP-binding) was driven throughout development and adulthood. In the next round of experiments, we used fly genetics to initiate ataxin-3 expression after eclosion and for specific points of time during adulthood to examine the correlation between fly death and the accumulation of SDS-resistant ataxin-3 species.

We used the tissue specific, inducible GeneSwitch Gal4 (Nicholson et al., 2008; Osterwalder et al., 2001; Roman and Davis, 2002; Sujkowski et al., 2015). Using this system, the pan-neuronal driver, *elav-Gal4(GS)* does not yield expression of ataxin-3 constructs until flies are fed RU-486, the compound which activates the driver. In our setup, we reared flies that harbor the driver and ataxin-3 constructs in fly media without RU-486, and then switched them to RU-486-containing food on day 1 as adults. Offspring were then aged on RU-486-containing



media and collected for western blotting on day 1, 4, 7, 10, 14, 21, 28 and 35. During this time, we also examined their longevity.

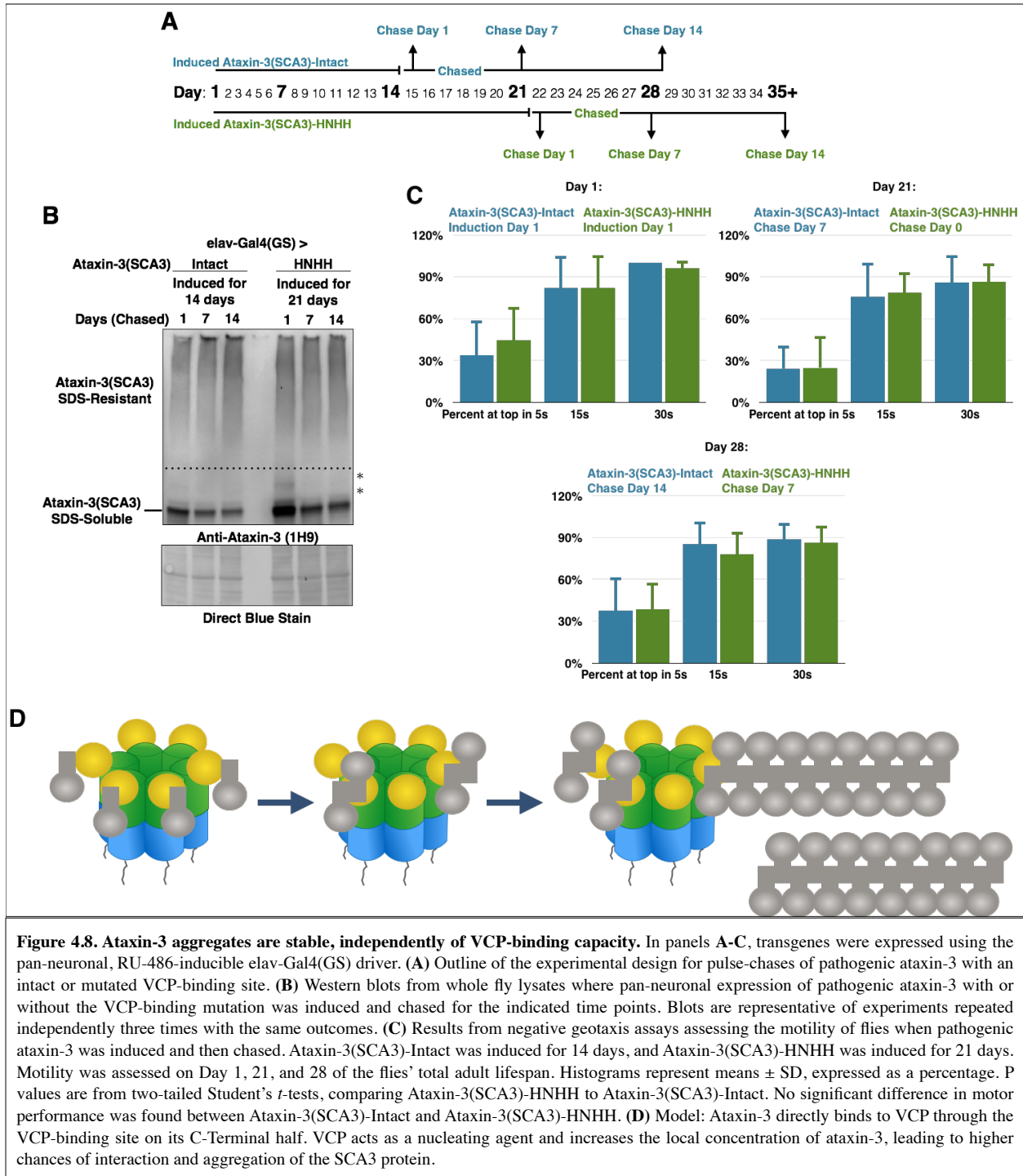
When pathogenic ataxin-3 with intact VCP-binding is driven by the RU-486-inducible elav-Gal4(GS) driver, we begin to more clearly observe SDS-resistant species around days 4-7; the intensity of these smears increases over time (Figure 4.7A). When expression of pathogenic ataxin-3 with the “HNHH” mutation is induced, we observe SDS-resistant species more clearly around days 10-14. These smears also increase in intensity over time (Figure 4.7A). When the samples are compared side-by-side, on the same PVDF membrane, the difference in the timing of appearance of SDS-resistant species among the two forms of ataxin-3 becomes noticeable (Figure 4.7B). We observe comparable levels of SDS-resistant species between the two forms of

pathogenic ataxin-3 around days 7 and 14 (“Intact” vs. “HNHH”, respectively) and later, around days 14 and 21 (“Intact” vs. “HNHH”, respectively). Based on longevity curves, (**Figure 4.7C**), temporally-induced expression of pathogenic ataxin-3 with impaired VCP-binding leads to lethality at later time points than the version with intact VCP-binding, correlating with delayed appearance of SDS-resistant species with the “HNHH” version of the SCA3 protein. These results support our findings from various tissues that the “HNHH” mutation delays the appearance of higher-molecular weight, SDS-resistant, ataxin-3-positive species in western blots, as well as the toxicity of this protein, compared to the “Intact” version.

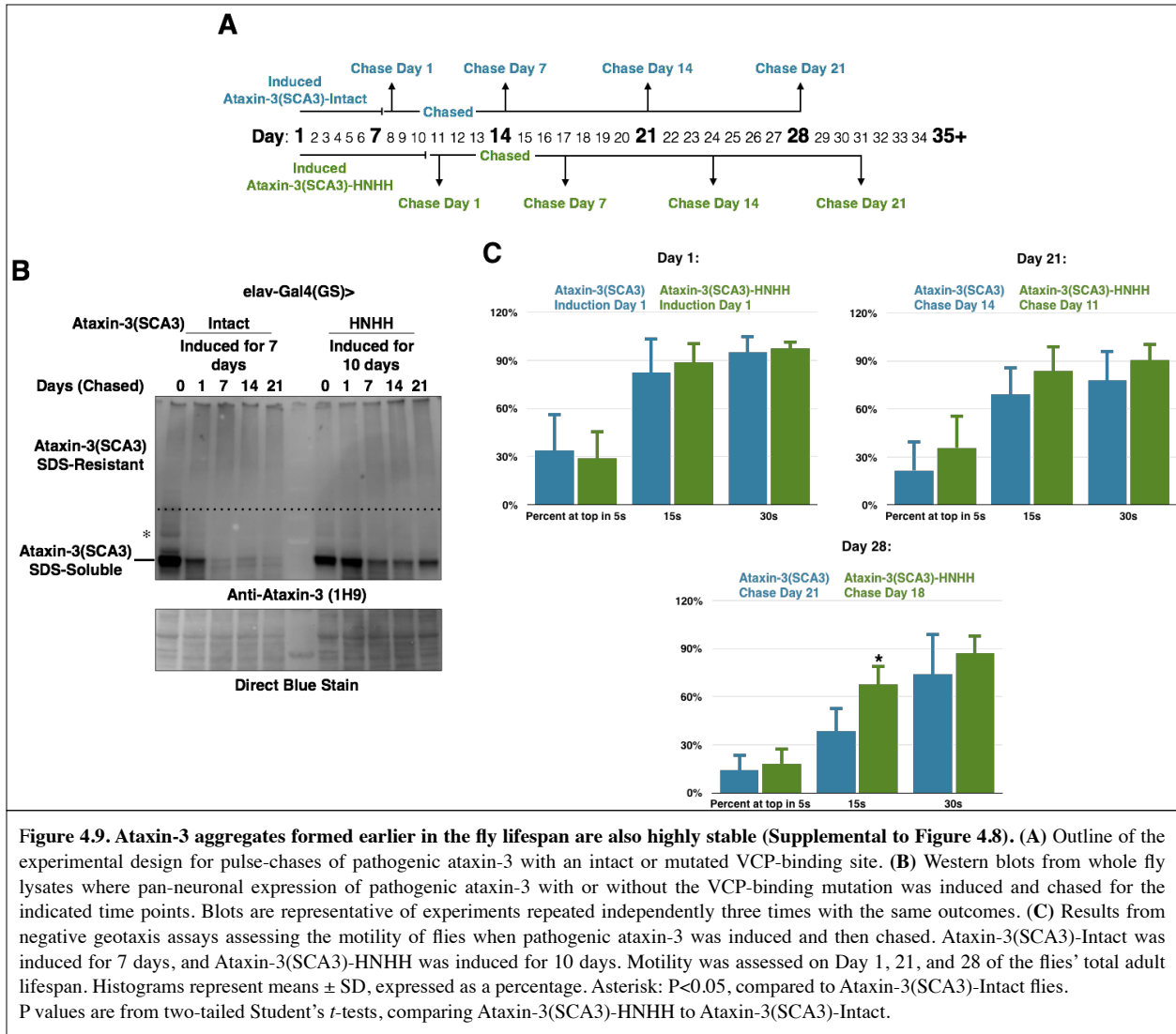
4.2.8 SDS-resistant species of pathogenic ataxin-3 are stable once formed

Lastly, we utilized the power of the RU-486-dependent elav-Gal4(GS) driver to examine how stable the SDS-resistant species of pathogenic ataxin-3 are *in vivo*. We wondered if eliminating the production of additional ataxin-3 could lead to time-dependent dissipation of the protein itself, and if mutating its VCP-binding site influences this process. We have shown before that ataxin-3 is a stable protein in the cellular environment (Todi et al., 2007). Thus, we chased ataxin-3 for 14 days after we stopped its production by removing RU-486 from the fly media. To begin with comparable levels of the SDS-resistant species, we induced pathogenic ataxin-3 with an intact VCP-binding site for 14 days and the “HNHH” mutant form for 21 days in adult flies, based on results from **Figure 4.7B**. Flies were reared in media without RU-486. They were switched to media with the inducer on day 1 as adults (**Figure 4.8A**).

As shown in **Figure 4.8B**, the SDS-resistant species are stable for the 14 days that we conducted our chases for both pathogenic ataxin-3 species. Whereas the SDS-soluble species diminish in intensity over time, the intensity of the SDS-resistant species does not decrease. Lastly, we tested if the physiological phenotypes of “Intact” and “HNHH” flies differ when the



aggregated species are present at comparable levels, following the same induction and temporal parameters described above. In motility assays, we find no statistically significant difference between the performance of flies expressing either version of pathogenic ataxin-3 (Figure 4.8C).



This suggests that the difference in motility phenotypes for the “Intact” and “HNHH” flies, which we observed in **Figure 4.4**, is dependent on the different amounts of aggregated species, rather than the intrinsic nature of the “Intact” vs “HNHH” proteins themselves. We also conducted a similar experiment, with similar results, where we induced expression of pathogenic ataxin-3 with an intact VCP-binding site for 7 days and mutated VCP-binding site for 10 days, to address the possibility that aggregated species formed earlier and present at lower levels might be more easily dispelled. This was not the case (**Figure 4.9**).

Altogether, the *in vivo* chase studies indicate highly stable aggregated species of ataxin-3

and lack of a role for the VCP-binding site of ataxin-3 in their dissipation over time.

4.3 Discussion

We investigated the interaction of the SCA3 protein, ataxin-3, with its direct binding protein, VCP, and the effect of this interaction in SCA3-like degeneration in *Drosophila*. Ataxin-3 was reported previously to function with VCP to regulate degradation of ERAD substrates (Wang et al., 2006; Zhong and Pittman, 2006). Interestingly, while ataxin-3 has been ascribed various roles in cell culture-based studies, different knockout mouse lines that targeted the *atxn3* gene failed to show that this protein is a necessary entity in an intact mammal (Schmitt et al., 2007; Switonski et al., 2011). Based on the knockout lines, we hypothesized some time ago that targeting the ataxin-3 protein to enhance its degradation might have therapeutic benefits. In those studies, we found that the VCP-ataxin-3 interaction did not impact the levels or turnover rate of ataxin-3 degradation in cultured mammalian cells or *in vivo* in *Drosophila* (Blount et al., 2014). We also found that the VCP-binding capability of ataxin-3 was dispensable for its role in the fly, where the SCA3 protein serves a neuroprotective role (Tsou et al., 2015b). The VCP-ataxin-3 interaction was described in detail by the Wanker and Bonini groups over a decade ago. In that work, Boeddrich et. al. reported that ataxin-3 and VCP bind each other directly through a VCP-binding site on the C-terminus of ataxin-3 (Boeddrich et al., 2006). A model emerged where binding of multiple ataxin-3 proteins by a single VCP hexamer brings SCA3 proteins in close physical contact, thus expediting, or nucleating, early steps of their fibrillization. Intrigued by that work and by our own findings that the VCP-ataxin-3 interaction does not impact the degradation of ataxin-3 or its function in the fly, we investigated how this binding impacts ataxin-3 aggregation and toxicity *in vivo* over time and in different tissues.

We found that accumulation of SDS-resistant species and insoluble aggregates occurs

faster in flies expressing pathogenic ataxin-3 with an intact VCP-binding site than in flies expressing pathogenic ataxin-3 with a mutated VCP-binding site. The SDS-resistant species that we observe in western blots likely consist of lower- and higher-order multimeric species of pathogenic ataxin-3. The insoluble pellet fractions we obtained from differential centrifugation could contain higher-order ataxin-3 multimers and oligomers (O'Rourke et al., 2013). Coincident with a delay in pathogenic ataxin-3 aggregation when its VCP-binding site is mutated (or when VCP is knocked down in fly eyes), we noticed reduced toxicity from this version of the SCA3 protein, compared to the “Intact” form. In each of the studies we conducted, the form of ataxin-3 with impaired VCP-binding led to reduced toxicity, especially early on in the life of the flies. In fact, at early stages, fly motility was entirely non-impacted by pathogenic ataxin-3 with impaired VCP-binding. Later on, these flies did perform poorly when compared to flies not expressing the SCA3 protein, but still much better than the “Intact” flies. Thus, while interrupting the interaction between ataxin-3 and VCP had an ameliorative effect early on, in later stages the disease protein still proved toxic. This is an important finding, as it suggests that VCP enhances, but does not play a vital role, in the overall toxicity of the SCA3 protein. Also, from a therapeutic point of view, interrupting the interaction of ataxin-3 with VCP might have beneficial effects, but perhaps would not reach “curative” properties. The latter statement is further supported by the data that inducing the expression of ataxin-3 only in adults still leads to premature death in flies, and that mutating the VBM delayed lethality, but did not fully suppress it.

Collectively, our studies support a role for VCP in the early stages of ataxin-3 aggregation, consistent with a nucleation role for the hexamer put forth based on *in vitro* work (Boeddrich et al., 2006) (**Figure 4.8D**). 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 (Ellisdon et al., 2006; Masino et al., 2011; Masino et al., 2004). Ataxin-3 that does not contain an expanded polyQ tract is thought to first dimerize through the catalytic Josephin Domain in the structured N-terminus. 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, although in that instance highly stable, SDS-resistant aggregates form *in vitro* (Ellisdon et al., 2006; Masino et al., 2011). We propose that VCP acts as a nucleating agent in initial ataxin-3 fibrillization. Ataxin-3 directly binds to VCP through its VCP-binding site. A VCP hexamer could bind up to six ataxin-3 proteins (Boeddrich et al., 2006; Xia et al., 2016). 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 4.8D**). Inhibiting this interaction, or reducing levels of VCP available for this step decreases aggregation and ensuing toxicity.

There was a recent study by the Loll lab that contradicts the model presented above. Utilizing wild type (non-pathogenic) ataxin-3 and VCP proteins produced in bacteria, their *in vitro* biochemical and electron microscopy work indicated that each VCP hexamer is bound by a single, wild type ataxin-3 (Rao et al., 2017). Work by Wanker and Bonini hinted at a stoichiometry of 4:1 ataxin-3:VCP (Boeddrich et al., 2006). Our own data in *Drosophila* are consistent with a stoichiometry different than 1:1. It could be that the VCP:ataxin-3 stoichiometry is 1:1 for wild type ataxin-3, but different for pathogenic ataxin-3. Also, unlike in an isolated system, it is possible that additional factors impact the stoichiometry between these two proteins *in vivo*. This is an area that requires additional investigation *in vivo*.

In summary, the VCP-binding site of ataxin-3 has a significant role on the aggregation and toxicity of the SCA3 protein, underscoring the importance of non-polyQ domains in this disease and pointing to the ataxin-3-VCP interaction as a potential therapeutic target. The model for VCP-mediated fibrillization may extend beyond SCA3, since VCP also interacts with other polyQ proteins (Fujita et al., 2013).

CHAPTER 5: CONCLUSIONS

SCA3 is a devastating neurodegenerative disease for which the causative biology is currently unknown. As such, ataxin-3's non-polyQ domains and physiological functions can provide us with valuable context for what mechanisms go awry in SCA3 and how to ameliorate them. The Josephin domain, sumoylation at K166, and phosphorylation at several residues are known to modulate toxicity of ataxin-3, but otherwise not much is known about how protein context plays into SCA3. For this reason, we explored how ataxin-3's interactions with Rad23 and VCP at specific binding sites can influence its disease properties.

We found that Rad23-binding at UbS2 regulates ataxin-3's stability, autoprotective function, and overall toxicity *in vivo*. Since reducing levels of polyQ proteins can be beneficial in these diseases (Esteves et al., 2017; Keiser et al., 2016; Wang, 2017), lowering ataxin-3 stability by targeting the Rad23-UbS2 interaction seemed like a reasonable therapeutic approach. However, our results demonstrate the complexity of the Rad23-UbS2 interaction, since it controls several of ataxin-3's properties. Until more is known about how Rad23 regulates ataxin-3's neuroprotective function, this interaction is likely not a viable therapeutic target for SCA3. As such, future work should focus on elucidating the mechanism for ataxin-3 and Rad23's upregulation of DnaJ-1. Experiments testing whether ataxin-3 and Rad23 bind directly to the DnaJ-1 promoter, or influence the activity/stability of transcriptional regulators (HATs, HDACs, transcription factors, etc.) would be an excellent place to start. Moreover, the discrepancies between cell/fly and mouse studies emphasize the need to confirm these potential protective mechanisms in mammalian systems. If ataxin-3 is not neuroprotective in humans, then disrupting the Rad23-UbS2 interaction could be reconsidered as a potential therapeutic avenue.

Our work with VCP indicates that this protein can also influence ataxin-3 toxicity, but by

a different mechanism. Our data suggests that VCP acts as a seeding factor for ataxin-3 aggregation, likely by bringing multiple ataxin-3 proteins in closer contact and increasing their chances of aggregation. Mutating the VCP-binding site reduces ataxin-3 aggregation, which is intriguing from a therapeutic perspective, since increasing solubility of polyQ proteins could improve disease outcomes (Esteves et al., 2017; Wang, 2017). Decoy peptides that mimic the N-terminus of VCP may be a way to reduce both ataxin-3's binding to endogenous VCP and subsequent aggregation *in vivo*. To accomplish this, future experiments will aim to identify the region of VCP's N-terminus that most efficiently binds ataxin-3, while remaining stable in an *in vivo* environment and without causing adverse toxicity. It will also be important to identify the physical nature of the binding interaction between ataxin-3 and VCP, since the nucleation hypothesis has not been formally confirmed.

The physiological purpose of ataxin-3's interaction with VCP is not fully understood. Past studies have linked ataxin-3 and VCP as potential partners in ERAD and the UPS, but the molecular mechanisms remain to be clarified (Doss-Pepe et al., 2003; Wang et al., 2006; Zhong and Pittman, 2006). Recently, VCP has been linked to defects in DNA repair in multiple polyQ disorders (Fujita et al., 2013). Considering VCP and ataxin-3 are already known to co-localize at sites of DNA damage (Ackermann et al., 2016), it would be very interesting to investigate if VCP and ataxin-3 co-regulate DNA repair pathways. Moreover, Rad23 is also an established entity in DNA repair (Dantuma et al., 2009). It is possible that these proteins might function as a complex at the DNA, similar to their proposed role in the UPS (Doss-Pepe et al., 2003). Overall, additional studies to elucidate the interplay between VCP, ataxin-3, and Rad23 will enhance our appreciation for the physiological significance of these interactions in normal and disease states.

Ataxin-3 knockout studies report that the mice do not exhibit a decrease in viability or

fecundity (Schmitt et al., 2007; Switonski et al., 2011). However, a complete characterization of these models under stress and non-stress conditions remains to be conducted. Such studies could reveal an essential role for ataxin-3 that was previously undetected. It is also possible that another DUB is compensating for the loss of ataxin-3 in these models. The generation of catalytically inactive ataxin-3 knock-in mice could enhance our understanding of ataxin-3's significance in protein quality control, DNA repair, and other pathways.

Finally, there are some notable similarities between our results and findings related to other polyQ disorders, which were discussed in Chapter 1. Interestingly, the influence of UbS2-Rad23 on ataxin-3's stability is remarkably similar to the relationship between 14-3-3 and ataxin-1 turnover (Chen et al., 2003). Moreover, the ability of the VCP-binding site to enhance ataxin-3 aggregation is in agreement with multiple studies that demonstrate the ability of domains flanking the polyQ tract to modify its solubility (Adegbuyiro et al., 2017; Nozaki et al., 2001; Robertson and Bottomley, 2010). Thus, our findings related to SCA3 could have implications for other polyQ diseases, and vice-versa. Undoubtedly, efforts to unravel the complexities of protein context will be invaluable to understanding the biology of these diseases and how to treat them. Moving forward, an appreciation for both the similarities and differences between the polyQ disorders will facilitate new collaborative discoveries in the field.

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ABSTRACT**THE INFLUENCE OF PROTEIN CONTEXT ON POLYGLUTAMINE TOXICITY IN SPINOCEREBELLAR ATAXIA TYPE 3**

by

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The polyglutamine (polyQ) disease family is composed of nine neurodegenerative diseases caused by CAG trinucleotide repeat expansions in the associated genes. Although these diseases are caused by similar mutations, the polyQ proteins are otherwise structurally and functionally unique. Thus, the protein domains surrounding the polyQ tracts, as well as their interacting partners, are thought to be critical to the pathogenesis of these clinically distinct diseases. Spinocerebellar ataxia type 3 is caused by polyQ expansion of ataxin-3, a deubiquitinating enzyme implicated in protein quality control, transcriptional regulation, and DNA repair. Rad23 and VCP are two of ataxin-3's protein quality control partners that bind at its Ubiquitin-binding site 2 and VCP-binding site, respectively. To learn more about protein context in SCA3, we generated new transgenic *Drosophila* lines that express pathogenic ataxin-3 with these protein-binding sites intact or mutated. Through a battery of physiological and biochemical assays, we observed that ataxin-3's interaction with Rad23 regulates the stability, autoprotection, and toxicity of the SCA3 protein. We also find that VCP can modulate the aggregation propensity and subsequent toxicity of ataxin-3. Altogether, our findings provide insight into SCA3 biology and potential avenues for therapeutic intervention.

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