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Interactions of Hsp23, FoxO, and ESCRT III-Vps24 in Mediating Proteostasis in *Drosophila melanogaster* Flight Motor Cells

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ABSTRACT

Understanding the molecular mechanisms of cell degeneration, which cause degenerative diseases such as Parkinson's and Alzheimer's, is a key objective in biology and medicine. This complex biological process results from interactions between genetic and environmental factors. We examine these mechanisms using powerful genetic approaches and a newly developed experimental model for stress-induced degeneration in the fruit fly, *Drosophila melanogaster*. Our project focuses on the cellular and molecular mechanisms mediating neuromuscular degeneration induced by environmental stress, which activates the heat-shock response (HSR) in order to maintain quality control of cellular proteins (proteostasis). We examine the flight motor system of *Drosophila melanogaster*, which has shown selective susceptibility of neuronal, glial, and muscle cells to increased temperatures. Our previous research showed an upregulation of one particular chaperone protein, Heat-shock protein 23 (Hsp23), during the HSR response. Additionally, an overexpression of Hsp23 in fly muscle cells was shown to protect muscle and have a non-autonomous protective effect on neurons and glia. Moreover, these muscle cells also showed perinuclear localization of ubiquitinated proteins, which are normally targeted for degradation, and thus reveal a unique aspect of proteostasis. Our current research expands on these findings by exploring potential pathways that lead to cell non-autonomous protection from HS stress, focusing on the FoxO transcription factor, which has been implicated in proteostasis and protection from aging. Furthermore, we take a closer look at patterns of ubiquitination to determine whether proteostasis mechanisms involving clearance of misfolded proteins responsible for cell degeneration are carried out by the proteasome or by selective autophagy as related to the ESCRT III complex. We hope our findings will shed light onto the complex and elusive mechanisms responsible for environmental stress-induced degeneration. Moreover, we

hope these findings can be applied to the development of treatments and gene-targeted therapies that ease the pain of those suffering from degenerative diseases.

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The studies and results presented in the following chapters were obtained through a collaborative research effort amongst lab members. Other members who contributed to this work include Dr. Fumiko Kawasaki and Dr. Richard Ordway as well as former and current undergraduate researchers Annie Lin, Pengshu Fang, Linda Guo, Noelle Koonce, and Catherine Qui. I conducted experiments using fly genetics, heat shock, and Western blots. Dr. Fumiko Kawasaki conducted ICC, electrophysiology, and confocal imaging.

Chapter 1

Introduction

Neurodegenerative Disease

Each year, millions of people worldwide are affected by neurodegenerative disorders, the exact mechanisms of which remain elusive (Sheikh, Haque, and Mir, 2012). The result has been a difficulty in developing targeted therapies that ameliorate the symptoms and eventually cure the patient. Therefore, understanding the molecular mechanisms that cause degenerative diseases, such as Parkinson's Disease and Alzheimer's Disease, is a key objective in biology and medicine. Although these diseases are multi-faceted and multi-symptomatic, there seem to be some similarities amongst the general mechanisms that lead to disease (Soto and Estrada, 2008). This research focuses on the genetic and environmental factors that affect the level and distribution of misfolded proteins that persist due to ineffective degradation. In normal cells, intracellular proteins exist in a dynamic state, constantly being synthesized and degraded (Tanaka and Matsuda, 2013). Failure of this balance causes misfolded proteins to form aggregates that damage the cell and lead to cell death unless they are cleared in a timely and effective manner (Ross and Poirier, 2004). The finding of aggregates in the post-mortem brains of human patients suffering from a range of neurodegenerative diseases implicated them in studies of neural degeneration (Ross and Poirier, 2004). Prior research has also indicated that protein clearance pathways are directly affected by age and fail to work efficiently in older individuals (Tai and Schuman, 2008). Since many neurodegenerative disease are late-onset (Sheikh, Haque, and Mir, 2012), it is reasonable to assume that protein clearance pathways are implicated in the process and our research aims to further our currently incomplete understanding of the mechanisms facilitating protein clearance.

Drosophila melanogaster Flight Motor Model

In order to study mechanisms of neural degeneration and protein clearance, our lab uses powerful genetic approaches to study the high temperature (heat shock; HS) response in the flight motor system of the *Drosophila melanogaster* fly, which shares significant homology with the human genome (Pandey and Nichols, 2011). In fact, 75% of human disease-related genes have functional orthologs in the fly and with a particularly strong physiological, molecular, and genetic conservation of neuromuscular function, *Drosophila* is an ideal model organism to study neural degeneration (Lloyd and Taylor, 2010). We focus on the flight motor system because our previous studies have shown that environmental stress induced by exposing flies to HS produces permanent loss of flight ability. This is caused by selective degeneration of neuronal, glial and muscle cell types, which function in flight and are referred to collectively as the flight motor (Figure 1A-C). These observations can be visualized by noting the selective degeneration observed in the immunocytochemistry (ICC) and confocal microscopy (CF) images of HS flies (Fig. 1F-G) when compared to non-HS flies (Fig. 1D-E). One important feature of this experimental model is that cells of the flight motor, located on the fly thorax, are selectively susceptible to degeneration, in contrast to neuronal, glial and muscle cells which control other motor functions (such as walking), making this a good model of study for degeneration.

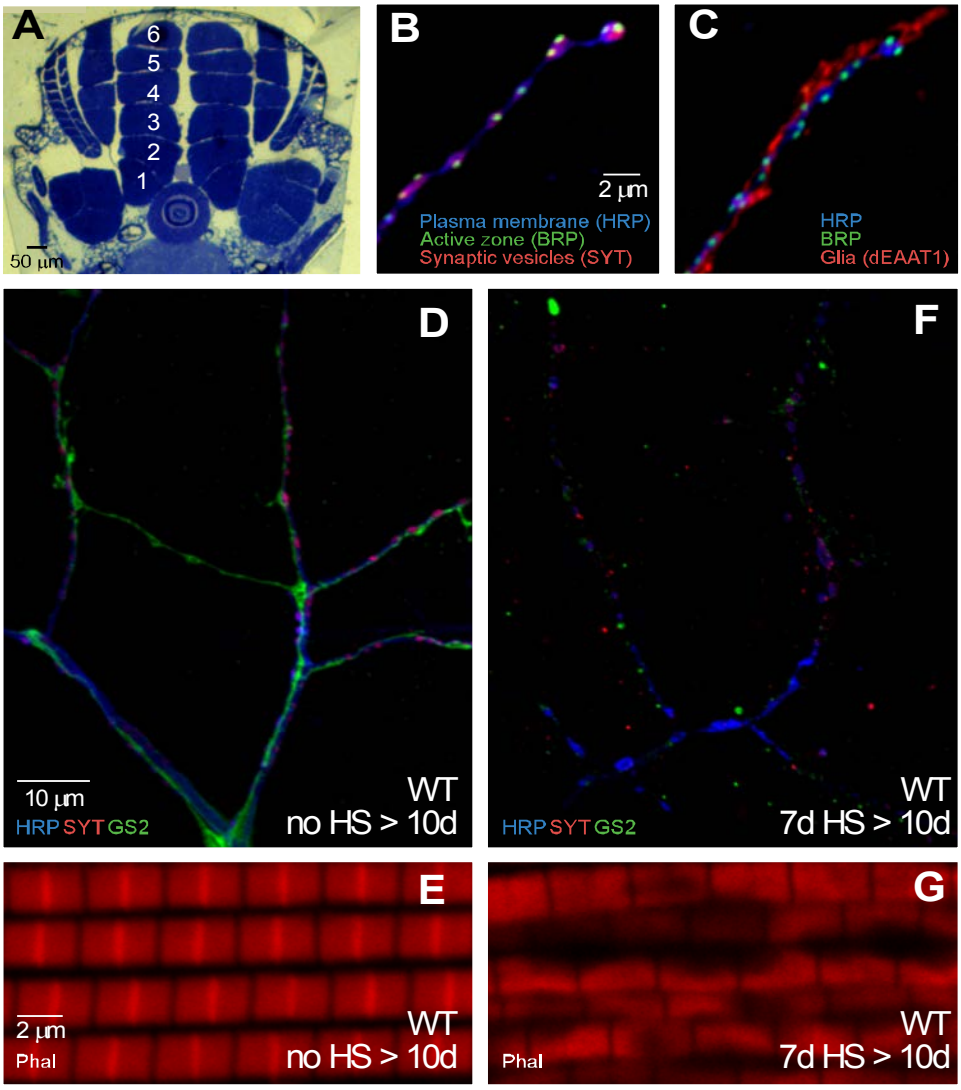

Figure 1. HS stress induces degeneration of motor axons, glia and muscle fibers in the flight motor.

A, morphology of the thorax showing two stacks of six Dorsal Longitudinal Muscle (DLM) fibers in cross section. B,C, axon terminal. Confocal immunofluorescence images of DLM neuromuscular synapses using markers for the neuronal plasma membrane (HRP; blue), active zones (BRP; green), synaptic vesicles (SYT; red in B) and glia (dEAAT1; red in C). D, lower magnification confocal immunofluorescence image of a no HS control fly at 10 d of age (no HS > 10d). The image shows both larger nerve and axon branches (lacking synapses) and synaptic terminal branches as well as associated glial processes (GS2). E, confocal fluorescence image from a no HS fly showing the DLM contractile apparatus stained with Alexa 568-conjugated phalloidin (Phal). F,G, cell degeneration in the flight motor in flies exposed to the 7d HS paradigm (see Methods) and processed for immunocytochemistry at 10d (7d HS > 10d). Confocal immunofluorescence images corresponding to those in D and E, respectively, but from a 7d HS >10d fly. Note the severe degeneration of axons, glia as well as muscle in comparison to the no HS control. WT: wild type. HS: Heat Shock.

Heat-Shock Response

As mentioned previously, neural degeneration and subsequent cell death are preceded by the accumulation of misfolded intracellular proteins that can form aggregates, or inclusion bodies, which are toxic to the cells (Ross and Poirier, 2004). In order to prevent or rectify aggregate formation, cells use molecular chaperones, a class of proteins responsible for preventing incorrect protein interactions, refolding misfolded proteins, or targeting proteins beyond repair for degradation, amongst a variety of other functions (Muchowski and Wacker, 2005). In order to counteract the effect of stressors, including, but not limited to, temperature elevation, cells activate the heat-shock response (HSR). This mechanism is characterized by an increase in heat-shock proteins (Hsps), a class of molecular chaperones (Muchowski and Wacker, 2005). In order to explore effects of different Hsps, our experiments expose selected flies to increased temperatures for set amounts of time as described in Chapter 2: Materials and Methods. We then test fly behavior for flight paralysis and analyze involvement of specific proteins through Western Blots or immunocytochemistry and confocal microscopy. Thus far, our research has indicated that overexpression (OE) of a small heat shock protein Hsp23, but not Hsp70, induced both a cell autonomous and cell non-autonomous protection from heat shock induced degeneration as shown in Figure 2 (Kawasaki et. al., 2016). That is, muscle-specific overexpression (OE) of transgene UAS-*hsp23* in flies induced protection from heat shock degeneration not only in the muscle cells (autonomous response), but also in neural and glial cells (non-autonomous response). However, muscle-specific OE of Hsp-70 failed to protect any of the three cell types. Our ongoing research explores possible secretory mechanisms, such as the IGF/FoxO pathway discussed later, that could interact with Hsp23 in order to mediate this non-autonomous response.

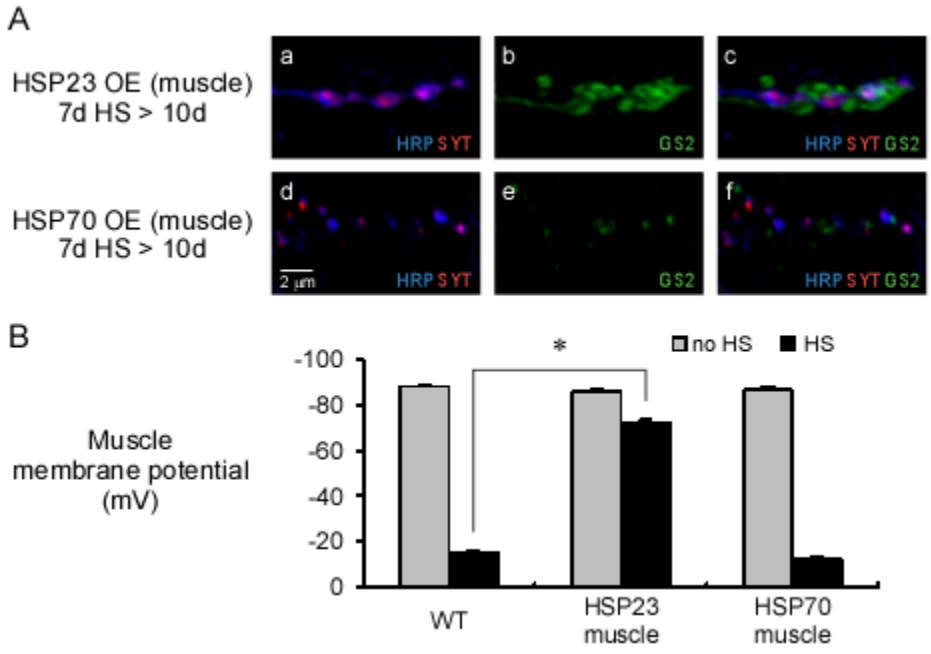

Figure 2. Muscle-specific overexpression of a small heat shock protein confers cell autonomous and non-autonomous protection from HS stress-induced degeneration.

Confocal immunofluorescence images of DLM neuromuscular synapses (A) and DLM muscle fiber membrane potential recordings (B). Muscle-specific overexpression (OE) of Hsp23 provided protection against HS stress-induced degeneration of neurons, glia and muscle (A,a-c and B). Protection of neurons and glia occurred through a non-autonomous mechanism. Protection of muscle is defined as cell autonomous. HSP70 overexpression in muscle did not protect any of the three cell types from HS stress-induced degeneration (A,d-f and B). Error bars indicate SEM and the asterisk indicates a significance difference from WT. Neuronal (HRP), synaptic vesicle (SYT) and glial (GS2) markers are as in Figure 1.

Proteostasis

The dynamic balance between the production and degradation of proteins within a cell is termed protein homeostasis, or more commonly, proteostasis. The constant turnover of proteins, particularly degradation of misfolded proteins, is essential to maintaining cell viability (Tanaka and Matsuda, 2013). Proteostasis is especially important in post-mitotic neural cells, which cannot diffuse the toxic aggregates through cell division and must rely solely on protein clearance mechanisms in order to prevent neuronal degeneration (Muchowski and Wacker, 2005). The process of removing and degrading the majority of intracellular misfolded proteins is mediated by the ubiquitin. Ubiquitin is a small 76-amino acid protein

that is attached to the protein of interest, including misfolded protein aggregates formed under environmental stress, by a covalent linkage between the C-terminus of ubiquitin and the lysine residues of the acceptor protein (Hochstrasser 1996). Moreover, as shown in Figure 3 from our previous study, flies that were exposed to heat-shock stress showed high levels of ubiquitin, further implicating the protein in stress response (Kawasaki et. al., 2016).

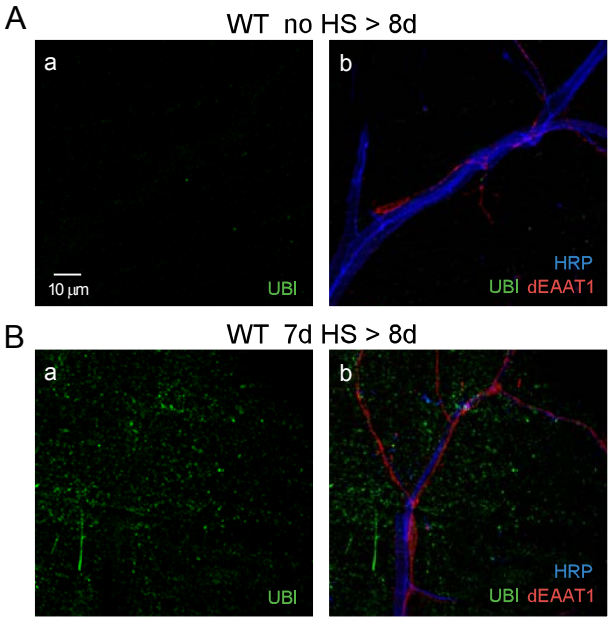

Figure 3. A failure of muscle proteostasis precedes HS stress-induced degeneration in the flight motor.

Confocal immunofluorescence images from no HS > 8d control flies (A) or 7d HS flies dissected at 8d (prior to degeneration; 7d HS > 8d) (B). Immunostaining for ubiquitin (UBI) indicates that muscle exhibits a marked increase in ubiquitin-positive inclusions after HS stress (B,a) and preceding degeneration (note the presence of in tact axonal and glial processes in B,b). Ubiquitin-positive puncta were not evident in axonal and glial processes. Neuronal (HRP) and glial (dEAAT1) markers are as in Figure 1.

Additionally, as shown in Figure 4B-C, flies with an overexpression of Hsp23 in muscle show a unique perinuclear localization pattern of ubiquitinated proteins as opposed to the more scattered distribution in HS flies without Hsp23 OE. Furthermore, a comparison of Figure 4D and 4E shows that flies with Hsp23 overexpression are able to clear the accumulation of perinuclear ubiquitin 3 days after the heat shock induced stress caused their accumulation.

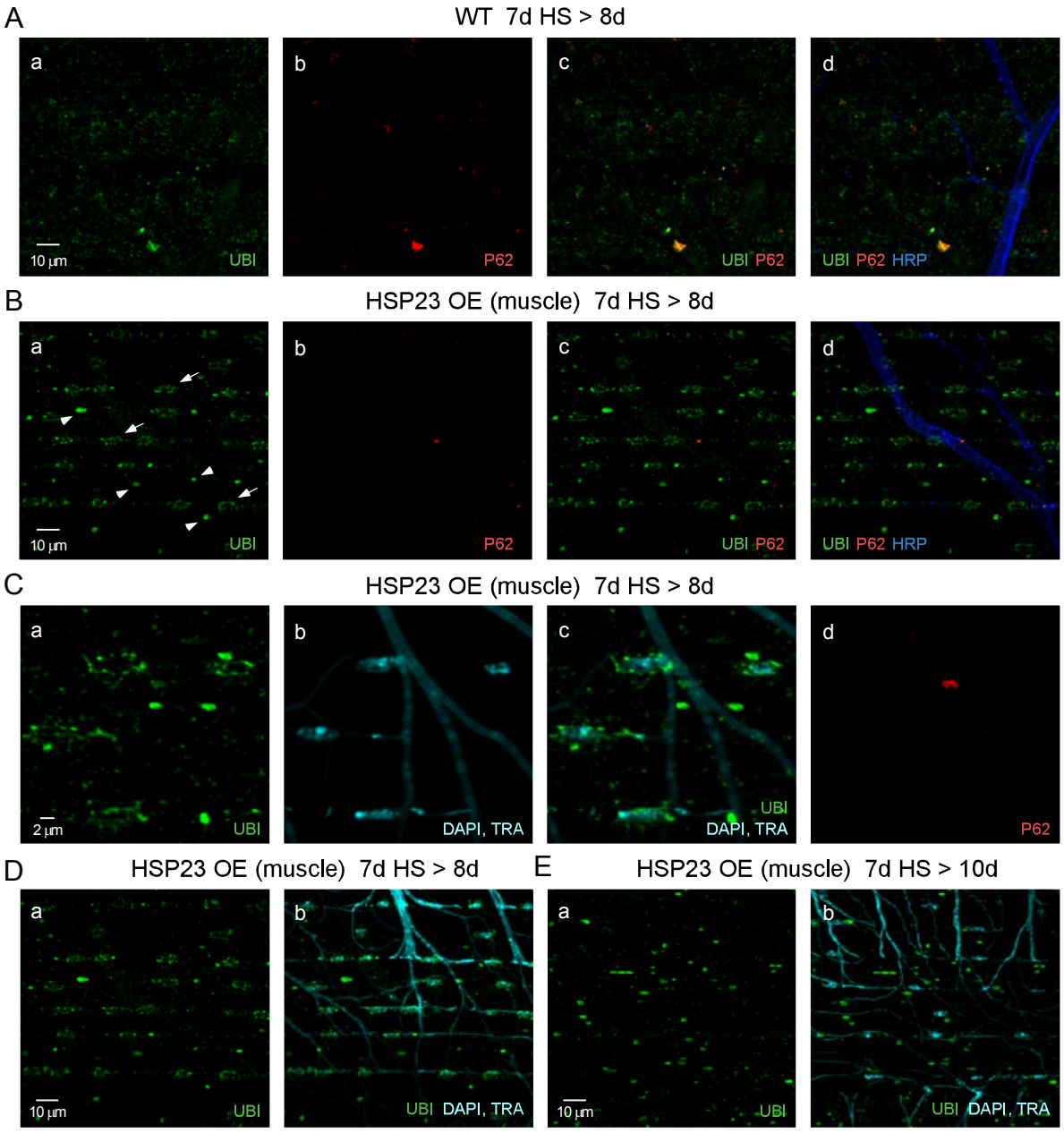

Figure 4. Overexpression of Hsp23 in muscle promotes perinuclear proteostasis mechanisms after HS stress.

Confocal immunofluorescence images of DLM neuromuscular preparations from WT flies (A) or those overexpressing Hsp23 in muscle (Hsp23 OE; B-E). P62 labels intermediates in autophagy. DAPI labeling of nuclei and autofluorescence from trachea (TRA) appear in the same channel. Ubiquitin (UBI) and neuronal (HRP) markers are as in Figure 4. A-D, flies were exposed to a standard HS stress paradigm and processed before degeneration is observed in WT (7d HS > 8d). In contrast to the diffuse distribution of ubiquitinated protein inclusions in WT muscle (A), Hsp23 OE flies exhibited well-defined and spatially organized ubiquitin-positive puncta including ring-like patterns (arrows in B,a) and a distinct class of larger puncta (arrowheads in B,a). Little colocalization of ubiquitin and P62 is observed in either genotype (A,c and B,c). C, DAPI staining revealed perinuclear rings of ubiquitinated proteins which are clearly distinguishable from a larger class of non-perinuclear puncta. D, E, comparison of ubiquitin-positive puncta in Hsp23 OE (muscle) flies early after HS stress (D, 7d HS > 8d) and later (E, 7d HS > 10d) indicates that perinuclear puncta are cleared whereas non-perinuclear puncta persist.

These findings will be the basis of our future experiments, which investigate how cells dispose of misfolded proteins. While chaperone proteins facilitate proper folding, they also direct unsalvageable protein aggregates to one of two catalytic routes responsible for the degradation of toxic proteins: proteasomal degradation and macroautophagy.

Ubiquitin-Proteasome Pathway

The ubiquitin-proteasome pathway (UPP) is highly conserved amongst eukaryotic species and is an important cell regulatory mechanism due to its substrate specificity and selective protein degradation (Ciechanover 1998). Three enzymes mediate the process: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin-protein ligase (E3). The process begins with the activation and subsequent conjugation of one ubiquitin molecule to the E1 complex, using ATP hydrolysis. The ubiquitin is then transferred to an E2 conjugating molecule. Subsequently, an E3 molecule, which plays the most direct role in substrate specificity, as displayed by the large variety of E3 genes in the eukaryotic genome, transfers the ubiquitin molecule from E2 to the protein being targeted for degradation (Hochstrasser 1996). Additionally, recognition by the E3 ligase often requires post-translational protein modification or interaction of the substrate with chaperone proteins (Ciechanover 1998). Finally, a subclass of the E3 enzymes known as chain elongation factors (E4) catalyzes a chain extension process, resulting in a polyubiquitinated protein. Following the completion of protein polyubiquitination, a number of factors, including chaperone proteins, present this tagged protein for degradation by a 26S proteasome (Hochstrasser 1996). It is important to note that this process is highly specific and used for individual proteins. Interruption to any of these steps can lead to an accumulation of misfolded proteins that form aggregates and result in proteotoxicity that can adversely affect the health of a neuron.

Lysosome-Mediated Macroautophagy

An alternative method of protein degradation is macroautophagy, henceforth referred to simply as autophagy. It is a conserved process in eukaryotes that was initially thought to be used only for non-specific bulk degradation of cytosolic organelles and proteins at times of starvation (Mizushima, 2007). However, it was later discovered that autophagy also constitutively and selectively sequesters and degrades ubiquitinated protein aggregates found within the cytoplasm (Kim et. al., 2008). The discovery was first made in pancreatic cells exposed to oxidative stress and revealed that the clearance of proteins can be accomplished by proteasome degradation as well as autophagy (Kaniuk et. al. 2007). The process of autophagy begins with isolation membrane (phagophore) formation, which encloses the ubiquitinated protein in a double-membrane autophagosome that then fuses with a lysosome and surrenders its contents to degradation (Mizushima, 2007). Several autophagy (Atg) genes control initial phagophore formation; evidence suggests that the membrane is synthesized *de novo* in the cytosol (Tooze and Yoshimori, 2010). The mechanism for selective autophagy of ubiquitinated proteins requires a protein known as p62, which is needed for autophagosome formation as well as degradation by autophagy (Komatsu et. al., 2007 and Pankiv et. al. 2007). Once p62 binds to misfolded protein aggregates, it then interacts with LC3, a mammalian homolog of Atg8 protein, that is incorporated into the phagophore, thus making p62 a necessary protein for selective autophagy (Pankiv et. al. 2007). Once the autophagosome sequesters the ubiquitinated proteins, it fuses either with other autophagosomes or directly with lysosomes, which allows its internalized protein aggregates to be degraded by lysosomal enzymes (Mizushima, 2007).

Insulin and Insulin-like Growth Factors (IGFs) and the FoxO Transcription Factor

In order to understand how Hsp23 overexpression in muscles is able to protect neuronal and glial cells, we explored a secretory pathway related to insulin. Pathways controlling insulin and insulin-like growth factors (IGFs) are highly conserved amongst all species and regulate growth and metabolism. Most

mammals use multiple insulin-like growth factor binding proteins (IGFBPs) to regulate the insulin/ IGF signaling (IIS) pathway. Recently, ImpL2, a secreted protein in *Drosophila melanogaster* that binds insulin and IGFs in order to negatively regulate the IIS pathway, has been elucidated as a homolog for the human IGFBP7 (Honegger et. al. 2008). Interestingly, an increase in ImpL2 induced an increase in the 4E-BP transcript, a target of the Forkhead box transcription factor class O (FoxO) discussed below (Alic et. al. 2011). As shown in Figure 5, insulin and IGFs stimulate Akt to phosphorylate FoxO, promoting its export outside of the nucleus and suppressing its function as a transcription factor (Murphy and Hu, 2013). Since our study focuses on mechanisms facilitating cell non-autonomous protection following the HSR, we are interested in exploring the possible interaction of FoxO and chaperone proteins.

FoxO is a transcription factor that regulates many processes related to stress and longevity (Webb et. al. 2014). They are downstream effectors of the insulin signaling pathway and are conserved factors found in all animals, from *Caenorhabditis elegans*, to *Drosophila melanogaster,* to mammals and humans (Martins et. al. 2015). FoxO has been implicated in proteasome and autophagy control, the two degradation mechanisms responsible for proteostasis (Martins et. al. 2015). Studies have shown that FoxO and one of its targets, 4E-BP, promotes removal of protein aggregates through the autophagy/lysosome system, thus preventing accumulation of toxic proteins and maintaining proteostasis. Additionally, loss of FoxO function has been shown to result in increased protein aggregation (Demontis and Perrimon 2010). Furthermore, the failure of proteostasis that is naturally observed in aging muscle cells can be prevented or delayed by overexpression of FoxO, which decreases susceptibility to degeneration (Demontis and Perrimon 2010). All of these results together indicate that FoxO plays a role in proteostasis, however, our understanding of exact mechanisms remains incomplete. We propose a possible novel role for the FoxO transcription factor in the HSR in conjunction with Hsp23.

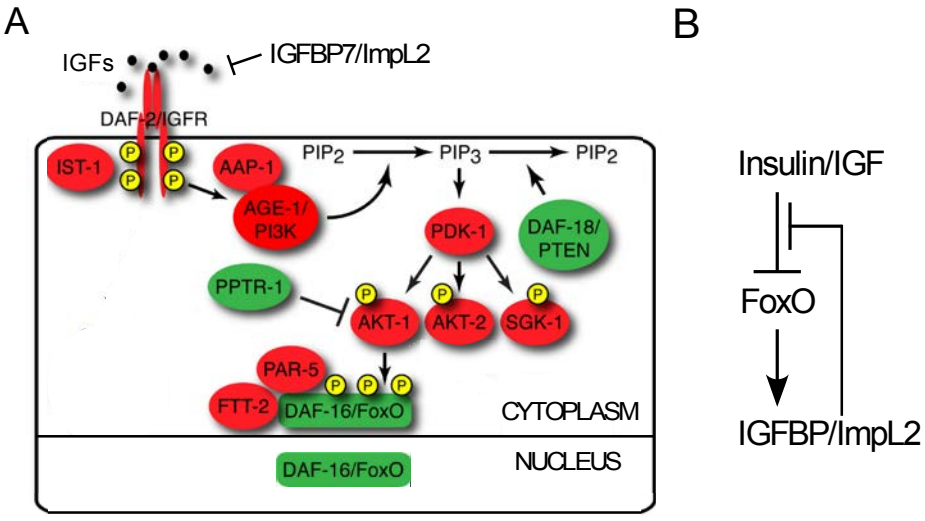

Figure 5. Insulin and Insulin-Like Growth Factor Signaling (IIS)

A. Binding of insulin and insulin-like growth factors (IGFs) to the cell surface receptor (IGFR), which may be inhibited by IGF binding proteins (IGFBP7/ImpL2), begins a cascade with multiple signaling pathways. One pathway is responsible for activating AKT-1, which phosphorylates a transcription factor, FoxO. This prevents FoxO from localizing to the nucleus, and thus inhibits expression of FoxO target genes. B. A simplified scheme for IIS. FoxO activation increases expression of a target gene, IGFBP/ImpL2, which in turn inhibits activation of IIS by binding to insulin/IGF and results in further activation of FoxO.

Chapter 2

Materials and Methods

Drosophila Fly Strains

Fly stocks were obtained either from Bloomington Fly Stock Collection or through the generosity of other labs as shown in Table 1 of the appendix. Wild-type flies were Canton-S stocks from our laboratory collection. In order to generate the flies of interest multiple crosses were set-up, followed through, and maintained over the course of weeks. As shown in Figure 6A, we crossed a line of flies with a mutation in the *foxo* gene, termed *foxo*[25], with flies expressing the UAS-*hsp23* transgene marked with *w*+. Since this cross required recombination, we needed to map the UAS-*hsp23* transgene and, as shown in Figure 6B, were able to visualize recombination by looking for the absence of the visual marker *Sb* (*Stubble*). Final lines of flies with likely recombination were confirmed using PCR and sequencing. As shown in Figure 6C, we also generated flies with a new recombinant third chromosome carrying both a deletion of the *foxo* gene [Df(3R) red31] and the MHC-GAL4 driver used for muscle-specific expression of UAS-

transgenes. The preceding recombinants allow muscle-specific overexpression of Hsp23 in a *foxO* mutant background.

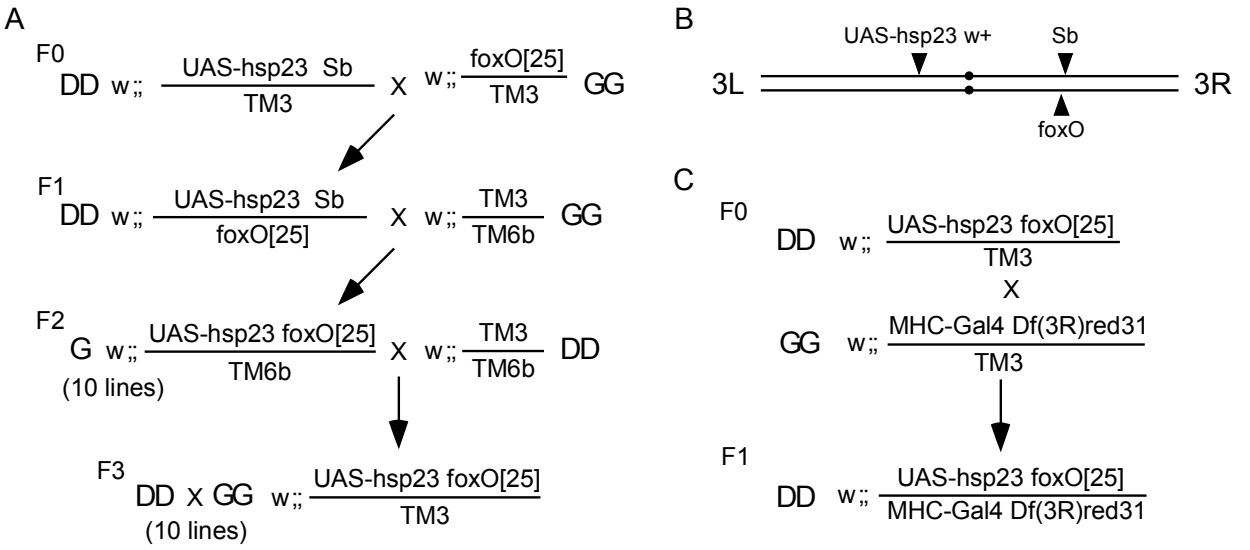

Figure 6. Strategy to achieve muscle-specific overexpression of Hsp23 in a foxO mutant background

A. This strategy used previously generated flies carrying the UAS-hsp23 transgene marked with *white+* (*w+*), and a visible third chromosome marker, *Stubble* (Sb), in *trans* to the balancer chromosome, TM3. These were crossed to flies bearing a *foxO* mutation, *foxO[25]*, in *trans* to TM3. The following crosses were performed in a *white* mutant background and the presence of the UAS-hsp23 transgene was identified by an orange eye color conferred by the *w+* marker. The F1 female progeny carrying UAS-hsp23 and *Sb* in *trans* to *foxO[25]* were crossed to male flies with TM3 in *trans* to another 3rd chromosome balancer, TM6b. Individual F2 male progeny which may carry a recombinant chromosome bearing UAS-hsp23 and *foxO[25]* (but lacking *Sb*) were recovered and crossed back to TM3/TM6b females. Ten lines were established from individual candidate males and each line was tested for the *foxO[25]* mutation by sequencing. B. Chromosomal locations of the UAS-hsp23 transgene marked with *w+*, the *Sb* visible marker and the *foxO* gene on the third chromosome. C. Crosses analogous to those shown in panel A were carried out to generate flies bearing the muscle-specific driver, MHC-Gal4, and a deficiency which removes *foxO*, Df(3R)red31. Muscle-specific overexpression of Hsp23 in a *foxO* mutant background can be achieved in the indicated class of F1 progeny. See Table 1 of the Appendix for more information regarding the sources of the flies.

Mapping of Transgene Insertion by Degenerate PCR

Degenerative PCR is a techniques developed fairly recently for the *Arabidopsis thaliana* plant that was adapted for the purposes of this study (Liu et. al 1996). We used this method to map the location of the UAS-*hsp23* transgene that was inserted into the fly genome using the p-element and supplied to us by Bloomington Fly Stock Collection. The process relied on two separate sets of primers. One set of primers was specific to the pUAST gene within which the *hsp23* transgene was inserted as shown in

Table 2 of the Appendix. The second set of primers was referred to as arbitrary degenerate (AD) primers. These primers were non-specific and contained "wobble regions" that were capable of binding multiple DNA bases as demonstrated in Table 3 of the Appendix. When these samples underwent PCR, the first specific pUAST primer, T1BUAS, was run in combination with each of the three AD primers using samples of genomic DNA obtained via genomic prep. The annealing temperature was raised very slowly to allow the non-specific binding of the degenerate primers in hopes that it would bind to the genomic DNA flanking the pUAST insertion as demonstrated in Figure 7. In order to eliminate any non-specific products, the products of this first round of PCR was rerun using the T2D primer which binds to a region of the pUAST gene that is closer to the genomic flanking region; again in combination with each of the three AD primers. The resulting PCR products were run on a gel to identify which AD primer yielded the most product and another round of PCR was done with this selected AD primer and the T2D primer. The resulting DNA was run on a 1-1.5% agarose gel, extracted, and sent for sequencing.

The resulting DNA sequence was analyzed by first locating the point where the pUAST insertion (containing the UAS-*hsp23* transgene) began. Then the genomic DNA preceding the insertion was entered into a BLAST search in order to find the exact chromosomal location at which the UAS-*hsp23* transgene had inserted.

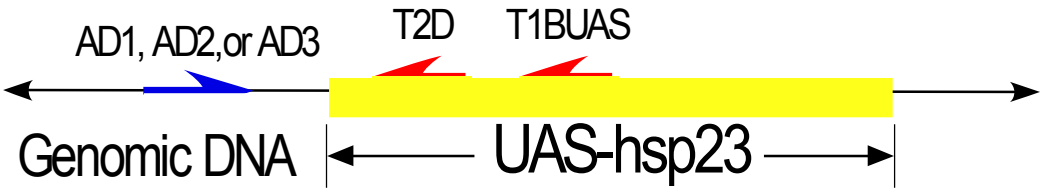

Figure 7. Primers for specific amplification of genomic sequence flanking the UAS-hsp23 transgene insertion by degenerative PCR

T1BUAS and T2D are nested primers, which anneal specifically to the 5' region of the UAS-hsp23 transgene (Table 2). AD1, AD2, and AD3 (Table 3) are three arbitrary degenerate (AD) primers which may anneal non-specifically to a genomic sequence flanking the UAS-hsp23 transgene insertion.

Heat-Shock (HS) Paradigm

As indicated in Figure 8, heat shock paradigms involved a series of three two-hour heat shocks at 36 °C and varied only in the age at which the procedure started (1d or 7d). Flies were collected during a time window between 12:00 p.m. and 3:00 p.m. and stored in standard food vials at 20 °C in a 12 hour day-night cycle. HS was performed by placing standard food vials plugged with rayon in a circulating water bath preheated to 36 °C. Care was taken to ensure the rayon plugs were advanced below the water level. Flies were heat shocked at 36 degrees Celsius 3 times, for 2 hours at a time. The specific HS times were as follows: 10:00am-12:00pm, 2:00pm-4:00pm, and then 10:00am-12:00pm the following day. Following HS, flies were maintained at 20 °C in a 12 hour day-night cycle.

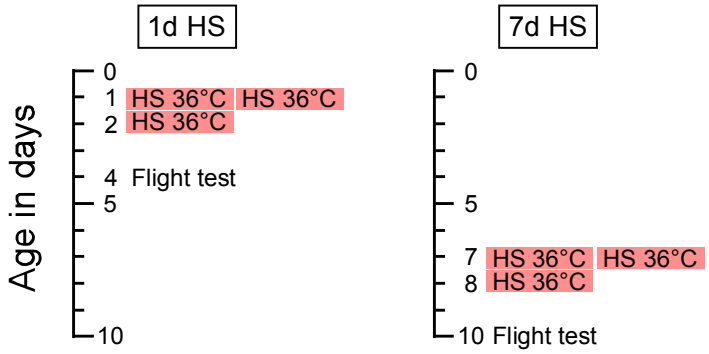

Figure 8. Heat shock (HS) paradigms for 1d or 7d flies.

Flies were heat shocked at the age of either 1 day or 7 days and tested at the age of either 4 days or 10 days respectively. Each vial of flies underwent 3 rounds of HS at 36°C for 2 hours at a time. Specific times of HS were 10:00am -12:00pm, 2:00pm - 4:00pm, and 10:00am -12:00pm.

Immunocytochemistry (ICC) and Confocal Microscopy

DLM neuromuscular synapse preparations were imaged using an Olympus FV1000 confocal microscope (Olympus Optical, Tokyo, Japan) with a PlanApo 60x 1.4 numerical aperture oil objective (Olympus Optical) and a z-step size of 0.2 µm as described previously (Iyer et. al. 2013). Images were obtained and processed with Fluoview software (Olympus Optical). Images shown are representative of

those obtained from each of at least three different preparations. To visualize myofibrils in DLM, Alexa 568-conjugated phalloidin, which binds F-actin, was used at 0.13 μM.

Antibodies used: Rabbit α-Synaptotagmin Dsyt CL1(α-SYT;1:5000) [Dr. Noreen Reist (Colorado State University, Fort Collins, CO)]; rabbit α-dEAAT1 (1:2500) [Dr. Serge Birman (Developmental Biology Institute of Marseille, France)]; mAb nc82 α-BRP (BRUCHPILOT) (1:50) (Developmental Studies Hybridoma Bank, Iowa City, IA); mAb GS-6 α-Glutamine Synthetase (α-GS2;1:500) (EMD Millipore, Billerica, MA); goat α-HRP-Alexa647, which labels neuronal plasma membranes (1:200) (Jackson Immunoresearch Laboratories, West Grove, PA); mAb FK2 α-Ubiquitin (1:1000) (Enzo Life Sciences, Farmingdale, NY); α-FoxO (1:2000) [Dr. Won-Jae Lee (Seoul National University, Seoul, South Korea)]. Secondary antibodies (Invitrogen, Carlsbad, CA) were generated in Goat: Alexa Fluor 488-conjugated α-mouse IgG (1:200), Alexa Fluor 568-conjugated α-mouse IgG (1:200), Alexa Fluor 488-conjugated α-rabbit IgG (1:200) and Alexa Fluor 568-conjugated α-rabbit IgG (1:200).

Electrophysiology

Current clamp methods were used to record the membrane potential of DLM flight muscle fiber Number 4. These experiments were performed as described previously (Kawasaki and Ordway, 2009). For each condition, four different preparations were analyzed (n=4).

Western Blot and Analysis

Western blots were utilized for the analysis of Hsp23 protein levels for flies of varying ages. Selected fly samples were homogenized, lysed in SDS buffer solution and run on a 9% polyacrylamide gel at l5mA/ gel. Once the bromophenol blue tracking dye reached the bottom of the gel, the proteins were transferred onto a nitrocellulose membrane and incubated with primary antibody overnight. A rabbit α-Hsp23

antibody (Tanguay Lab) was used in a 1:100,000 dilution. Tubulin was the sample loading control detected using a 1:100,000 dilution of mouse monoclonal acetylated α-tubulin antibody (Sigma Technologies). Following primary antibody incubation, the membrane was incubated with secondary antibody and developed using Licor Odyssey detection system.

Chapter 3

Mapping of the UAS-*hsp23* gene

Our studies use the Gal4-UAS system (Brand and Perrimon 1993) to achieve overexpression of specific genes in a cell type specific matter. The UAS-*hsp23* transgenic flies permit overexpression of the Hsp23 due to the insertion of an additional *hsp23* gene via the pUAST vector. When inserted into a fly chromosome, this is referred to as the UAS-*hsp23* transgene. Moreover, we also used an MHC-Gal4 driver in order to specifically overexpress the UAS-*hsp23* transgene in muscle cells. Our previous studies found that the overexpression of small heat shock protein 23, Hsp23, in fly muscle cells produced a cell non-autonomous protection from degeneration due to heat shock stress, which was intriguing because Hsp23 is an intracellular protein. Therefore, we wish to study secretory mechanisms that act alongside Hsp23 in order to mediate this cell non-autonomous protective response. In order to determine protective mechanisms that act in conjunction with Hsp23, we needed to generate new genotypes of flies, which required molecular mapping of the UAS-*hsp23* transgene.

We applied the technique of degenerate PCR as described in Chapter 2: Materials and Methods using modified primers for the pUAST vector. Our sequencing results following degenerate PCR are shown in Figure 9A. The location of the insertion was determined by first finding the location of the pUAST vector that carried the UAS-*hsp23* transgene. Next, the genome sequence upstream of this insertion was put through a BLAST search looking specifically at the *Drosophila melanogaster* genome and the location of the insertion was found to be on the left arm of chromosome 3, at 18626kbp. The

relative location of the insertion was following the aurora borealis (bora) gene and preceding the MYPT-75D gene of *Drosophila melanogaster*.

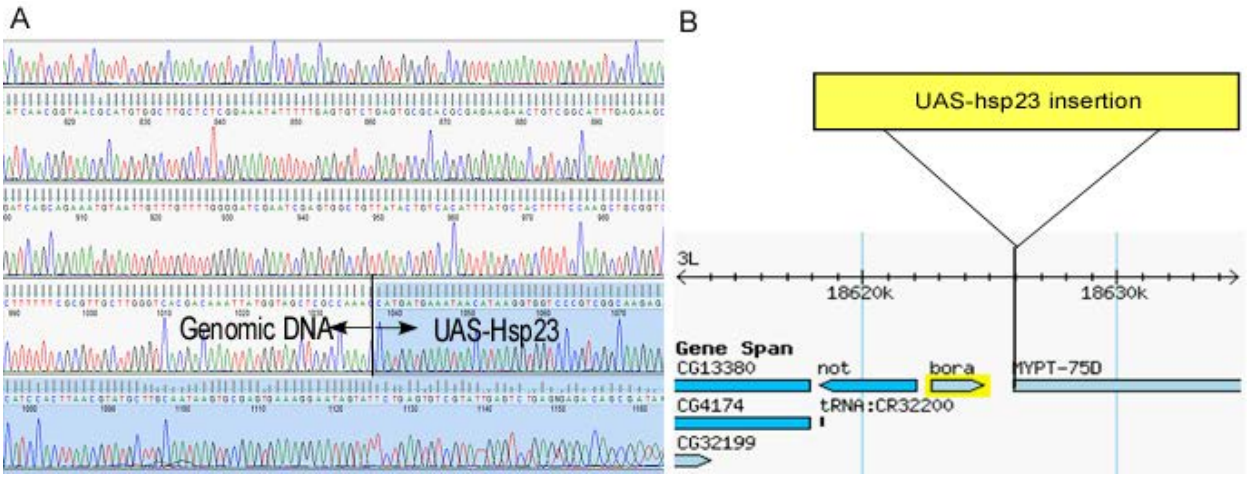

Figure 9. Molecular mapping of UAS-hsp23 insertion by degenerative PCR

A. DNA sequence chromatogram of the degenerative PCR products obtained in mapping the UAS-hsp23 transgene insertion. The 31 base pair repeat of the p-element insertion was located in the sequence data to identify the boundary between the transgene and the flanking genomic DNA sequence. The flanking genomic DNA sequence was used as a BLAST search query to identify its location in the *Drosophila* genome. B. Adapted from Fly Base. The location of the insertion was found to be at position 18626k of chromosome 3L, proximal to the *aurora borealis* (*bora*) gene and distal to the *MYPT-75D* gene.

The mapping of the UAS-*hsp23* transgene was essential to plan the crosses presented in Figure 6. Due to the chromosomal locations of UAS-*hsp23* and *foxO*, the F1 cross of Figure 6A utilized meiotic recombination. Recombination is the process by which two sets of parental chromosomes join and exchange alleles so that the resultant chromosome is a mixture of traits contributed by both parents. As shown in Figure 6A, we used the recombination method to generate F2 flies with the UAS-*hsp23* transgene as well as the *foxo*[25] mutant located on the same chromosome. This is possible because the UAS-*hsp23* transgene and the *foxo*[25] mutation are separated by sufficient chromosomal distance to allow high-frequency recombination. A visible marker, *Stubble* (*Sb*), that is located close to *foxO* was used to further perfect the process. As shown below, if the recombination occurs, the visible marker should no longer be seen in candidate flies. Genetic sequencing was used as the final verification that the desired recombination was obtained. The purpose of the mapping and recombination was to determine the influence that the FoxO pathway may have on Hsp23 mediated cell non-autonomous protection.

Chapter 4

Investigation of the Role of FoxO in the Heat Shock Response

When FoxO is activated, it localizes in the nucleus and activates genes, which control other regulatory mechanisms such as insulin signaling. Specifically, it causes an increase in insulin/insulin-like growth factor binding proteins (IGFBPs), which bind insulin/insulin-like growth factors (IGFs) and inhibit the insulin signaling pathway (IIS). Therefore, FoxO may play a role in conjunction with Hsp23 as a potential mediator of the non-autonomous protection.

In order to explore this possibility, nuclear localization of FoxO as a measure of its activation was compared amongst flies with Hsp23 OE, with and without heat shock (HS), and wild-type (WT) flies, with and without HS. As shown in Figure 10, flies with Hsp23 OE showed nuclear localization of the FoxO transcription factor as opposed to flies with no Hsp23 OE, in which FoxO was spread throughout the cells. The localization of the FoxO transcription factor in the nucleus is an indication that the FoxO pathway is activated in cells with Hsp23 OE, thus implicating FoxO as a potential mechanism by which the cell non-autonomous Hsp23 mediated protection from heat-shock stress may occur. These findings show a novel involvement of FoxO in mediating the HSR. In order to confirm whether FoxO is vital for Hsp23-mediated protection, the crosses, shown in Figure 6C, are being carried out to express a mutated version of FoxO in flies with Hsp23 OE. When these flies are tested under the HS paradigm, we can examine whether they exhibit flight paralysis and neuronal and glial degeneration as expected if protection depends on FoxO.

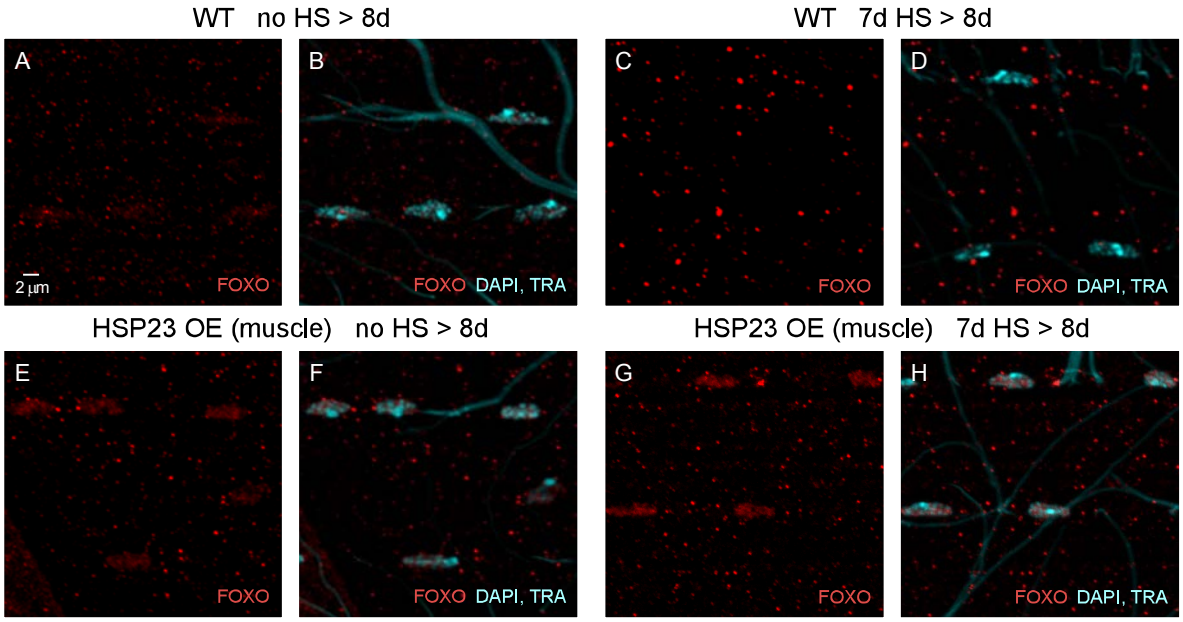

Figure 10. Hsp23 OE in muscle leads to activation of muscle FoxO.

Confocal immunofluorescence images of DLM neuromuscular synapse preparations showing accumulation of nuclear FoxO in muscles overexpressing Hsp23 (Hsp23 OE). A-D, WT control flies (no HS > 8d) or those exposed to a standard HS stress paradigm and processed prior to degeneration (7d HS > 8d) exhibited little localization of FoxO to muscle nuclei. In contrast, Hsp23 OE (muscle) under the same conditions (E-H) exhibited clear nuclear FoxO localization. Markers for nuclei (DAPI) and trachea (TRA) are as in Figure 4.

Chapter 5

Investigation of the Role of ESCRT III- Vps24 in Autophagy

Although much of our investigation was focused on the role that Hsp23 plays in mediating protection from heat shock induced cell degeneration, we also wished to ascertain whether or not similar mechanisms played a role in age-related stress responses. We therefore investigated whether young flies exhibit similar protective mechanisms as flies with Hsp23 overexpression. In order to explore this possibility, a Western Blot was conducted on non-HS WT flies ranging in age from 1 day old to 10 days old. Endogenous levels of Hsp23 were analyzed using an α-Hsp23 antibody and the results, shown in Figure 11C, displayed a progressive decline in Hsp23 with age. That is, 1 day and 2-day old flies showed high levels of Hsp23, which declined to very low levels in 10-day old flies. These findings raise the possibility that age may play a role in protection from stress-induced cell degeneration. This possibility is consistent with the observation that most neurodegenerative disorders are late-onset and take an effect later in life.

These findings were explored further by exposing young 1-day old flies to HS-stress to test the hypothesis that they will show similar mechanisms of protection from degeneration as Hsp23 OE flies. As shown in Figures 11A and 11B, the immunocytochemistry and confocal microscopy images of WT 1-day old HS flies showed similar patterns of perinuclear ubiquitinated proteins as observed in Hsp23 OE 7-day old HS flies (Figure 4). These results indicate that there are similar patterns of protection from HS-induced stress in young flies as there are in older flies with Hsp23 OE, further implicating Hsp23 in protecting cells from stress-induced degeneration. Finally, as shown in Figure 11Bc, young HS flies were also P62- negative, similar to older Hsp23OE HS flies, which also exhibited very low levels of P62.

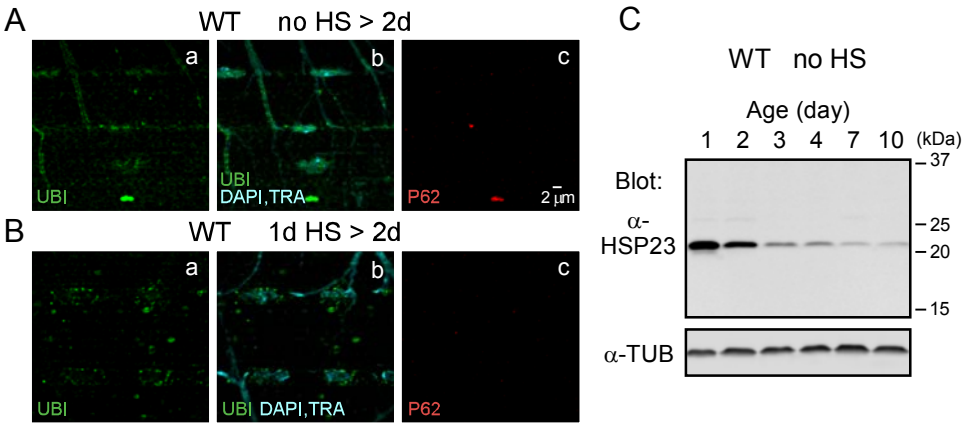

Figure 11. Endogenous protection mechanisms in young flies.

Proteostasis in young WT flies after HS stress resembles that observed in older flies protected by Hsp23 OE in muscle. A,B Confocal immunofluorescence images of DLM neuromuscular synapse preparations. A. In the muscle of control flies (no HS >2d), ubiquitin (UBI) signal was detected within nuclei, however neither perinuclear nor non-perinuclear ubiquitin-positive puncta were observed. B. In contrast, the muscle of flies exposed to a standard HS stress paradigm (1d HS > 2d) exhibited both perinuclear and non-perinuclear ubiquitin-positive puncta which resembled those of older flies protected by Hap23 OE in muscle and, like those, were P62-negative (B,c). C. Western analysis of endogenous Hsp23 expression in homogenates of WT no HS control flies shows that young adults exhibit higher levels of basal Hsp23 expression. Tubulin (TUB) was used as a loading control. Markers for ubiquitin (UBI), nuclei (DAPI), trachea (TRA) and P62 are as described in previous figures.

As explained in Chapter 1: Introduction, P62 plays an essential role in binding polyubiquitinated protein aggregates that are destined for degradation by means of autophagy. Once the aggregates are degraded by autophagy, the P62 is no longer observed. Therefore, the low levels of P62 in both young WT flies and older Hsp23 OE flies that are exposed to HS stress points to a possible upregulation of the autophagy pathway as the likely route of protein aggregate degradation. This mechanism would act to prevent proteotoxicity and degeneration.

Given the possibility that protein aggregates are degraded by the autophagy pathway, it is important to investigate mechanisms contributing to autophagosome formation and autophagosome-lysosome fusion. Therefore, we wished to examine the endosomal sorting complexes required for transport (ESCRTs) that catalyze membrane budding away from the cytoplasm and are necessary for autophagy (Rusten and Stenmark, 2009). ESCRTs play a major role in endolysosomal sorting, where proteins are imported into the lumen of the lysosome instead of being exported out into the cytoplasm. The ubiquitinated proteins are recruited by ESCRT-0 and contained in membrane buds created by the

ESCRT I and II mediated invagination of the endosome membrane. The buds are cleaved and the vesicle membrane closed with the help of the ESCRT III complex and become intralumenal vesicles (ILV). The collection of late ILVs contained within an endosome is known as a multivesicular body (MVB), which fuses with a lysosome for degradation of its contents (Hurley and Hanson, 2010). This entire process is portrayed in Figure 12 below.

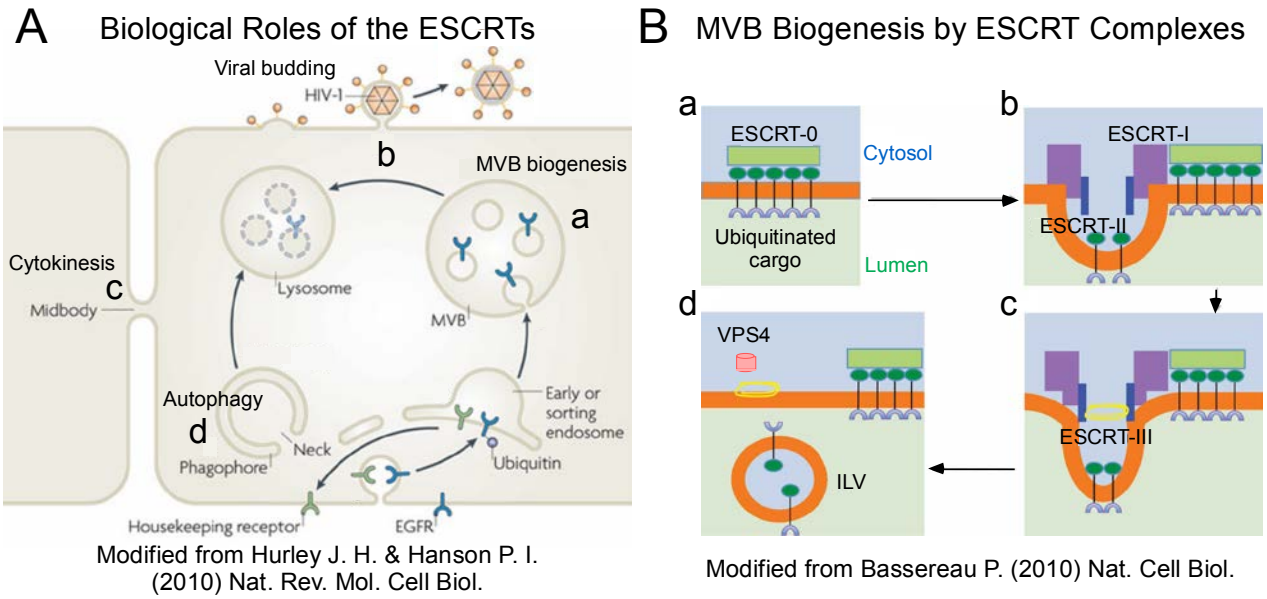

Figure 12. ESCRT (Endosomal Sorting Complexes Required for Transport)

A. Multivesicular body (MVB) biogenesis (a) requires all the ESCRTs. Whereas, HIV-1 budding (b) and cytokinesis (c) require ESCRT-I, ESCRT-III and VPS4. Autophagy (d) requires all the ESCRTs. B. (a) ESCRT-0 clusters ubiquitinated cargoes. (b) ESCRT-I and II together form membrane invaginations and are localized inside the bud neck. Cargoes are sequestered in the bud. (c) ESCRT-III assembles at the neck of the bud, colocalizing with ESCRT-I and II. (d) Vesicle scission by ESCRT-III leading to the formation of Intralumenal Vesicle (ILV). The ATPase VPS4 is recruited to dissociate ESCRT-III oligomers.

It was earlier discovered that in yeast, the formation of vacuoles (equivalent to mammalian lysosomes) is regulated by vacuolar protein sorting (vps) genes (Raymond et. al., 1992). In particular, a subgroup of the vps genes known as class E genes, are involved in Multivesicular body (MVB) creation and encode Vps proteins that are subunits of the ESCRT complexes (Hurley and Hanson, 2010). An autophagosome is essentially similar to a MVB with a single Interlumenal Vesicle (ILV) and it is known that ESCRT III has a function in the autophagy (Rusten and Stenmark, 2009 and Lee and Gao, 2008). As explained in Chapter 1: Introduction, autophagosomes are formed *de novo* in the cytoplasm. Selective

autophagy of protein aggregates requires the presence of P62 bound to the polyubiquitinated protein aggregates (Komatsu et. al., 2007 and Pankiv et. al. 2007). Therefore, we created a transgene to allow further evaluation of the Vps24 subunit of ESCRT III in mediating selective autophagy of protein aggregates. This transgene will permit expression of Vps24 bearing a GFP tag that can be used to determine its subcellular localization and biochemical interactions.

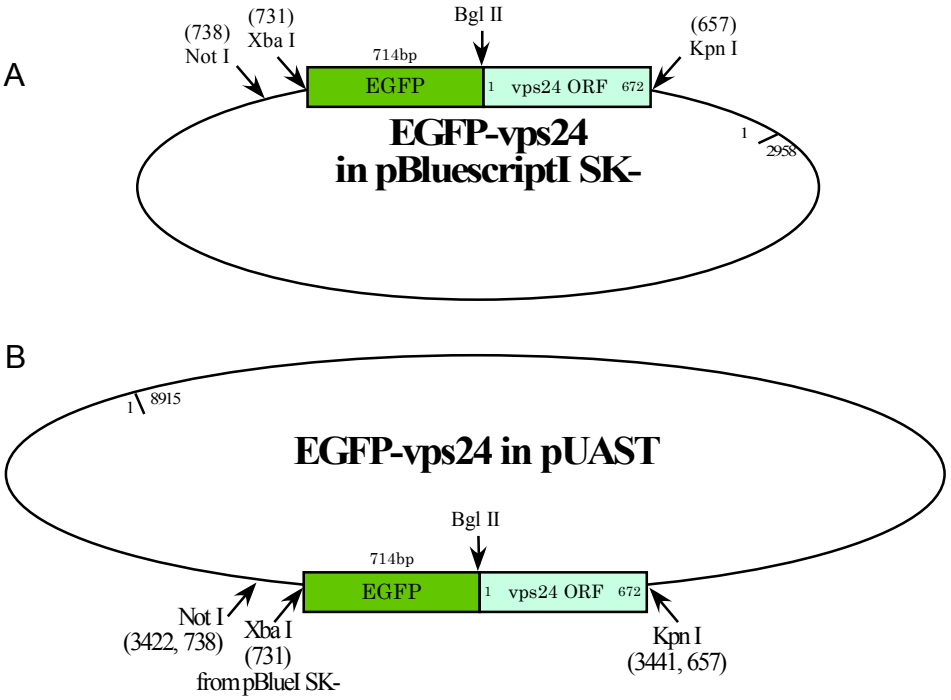

Figure 13. EGFP-vps24 construct in pBluescript SK- and pUAST

A. The labeled Bgl II and Kpn I restriction sites were used to ligate the vps24 ORF into the pBluescript SK- vector with a preexisting EGFP sequence. B. The Xba I and Kpn I restriction sites were used to ligate the EGFP-VPS24 ORF sequence into the pUAST vector.

A transformation construct containing the vps24 open reading frame (ORF) fused with Enhanced Green Fluorescent Protein (EGFP) on its N-terminus was inserted into a p-element vector known as pUAST which can integrate into the *Drosophila melanogaster* genome and generate transgenic fly lines. We chose to insert fuse the GFP protein on the N-terminus of VPS24 because the C-terminus has a domain called MIT which is essential for ESCRT III function and we did not wish to disrupt its functionality (Obita et. al. 2007). The fusion with GFP was necessary because there are currently no

antibodies specific for the fly VPS24 protein. Therefore GFP fusion will not only allow for fluorescent imaging of VPS24 in live cells, but also permits biochemical analysis of VPS24 using the anti-GFP antibody for Western Blot analysis and immunoprecipation experiments. The vps24 cDNA clone was obtained from the Drosophila Genomics Resource Center in a pOT2 vector. The VPS24 ORF insert, including the Bgl II and Kpn I restriction sites, was amplified via PCR using PFU polymerase. Enzymes Bgl II and Kpn I were used to sequentially digest the VPS24 ORF insert as well as a cloning vector, pBluescriptI SK- (pBlue), which already contained the EGFP sequence. Using the Bgl II and Kpn I restriction sites, the VPS24 ORF insert was ligated into the pBlue vector containing EGFP as shown in Figure 14. Next, this EGFP-vps24 segment and the pUAST expression vector were sequentially digested by Not I and Kpn I. The EGFP-vps24 insert was then ligated into the pUAST as shown in Figure 14. The plasmid was transformed into JM109 competent cells. The success of ligation and transformation was checked using plasmid preparation, diagnostic digestion, and gel electrophoresis.

Once the construct was prepared, the DNA was purified and injected into the posterior end of fly embryos in order to increase the chances of the construct's integration into fly germ cells. Integration into fly gametes ensures that the transgene can be passed on to future generations of flies. Flies that survived the injection were crossed to flies that had balancers for the second and third chromosomes. Flies with successful transgene integration were identified in the next generation by their orange eye color since transgenic flies carried a white+ marker present in the pUAST vector. We are currently in the process of identifying these transgenic lines and hope it will further characterize the role of the VPS24 subunit of ESCRT III in mediating selective autophagy of ubiquitinated protein aggregates, thus protecting cells from degeneration.

Chapter 6

Conclusions and Future Directions

Our previous research had found that muscle-specific overexpression of Hsp23, a chaperone protein induced during the heat shock response, was able to protect muscle cells as well as neuronal and glial cells of the *Drosophila melanogaster* flight motor (Kawasaki et. al., 2016). Since Hsp23 is an intracellular protein, it was important to look into intercellular signaling mechanisms that mediate this cell non-autonomous response. The relationship between the conserved, ubiquitous, and secreted insulin/IGF pathway and its connection to the transcription factor FoxO led us to investigate this pathway in relation to the heat shock response. We observed a very distinct nuclear localization of the FoxO transcription factor in flies with muscle-specific Hsp23 overexpression, which was not observed in wild-type flies. This evidence raises the possibility that the FoxO pathway plays a role in protection from HS stress-induced degeneration mediated by Hsp23.

In order to test this possibility using genetics, it was necessary to map the UAS-*hsp23* transgene, which was found to be on the left arm of the third chromosome following the *aurora borealis* gene. With these findings in hand, we were able to design a genetic scheme (Figure 6) that would produce loss of *foxO* in conjunction with overexpression of Hsp23. These fly crosses are currently in progress and the hypothesis is that these flies will not be protected from HS-induced cell degeneration. Since our current findings suggest that the FoxO transcription factor is necessary for cell non-autonomous protection from HS-induced degeneration, we predict that if the *foxO* gene is non-functional and does not produce the FoxO transcription factor, the cells of the flight motor will be susceptible to HS stress, despite the overexpression of Hsp23. We will test this hypothesis by subjecting the candidate flies to the HS paradigm as mentioned in Chapter 2: Materials and Methods, and then test their flight ability. Further testing using immunocytochemistry and confocal microscopy may be used to ascertain the extent of

degeneration as well as monitor changes to ubiquitination patterns in flies with a mutated *foxO* gene and HSP23 overexpression.

An additional goal of this thesis was to determine whether the protective mechanisms, induced by Hsp23 overexpression in older flies, were observed in young wild-type flies. Since most neurodegenerative disorders are late-onset, we tested whether we would see similar protective mechanisms in young flies when compared to older protected Hsp23 overexpression (OE) flies. Using the methods of heat-shock and immunocytochemistry that had been used with Hsp23 OE flies, we observed that young HS flies exhibit a similar distribution of perinuclear ubiquitinated proteins as older Hsp23 OE flies. Furthermore, we noticed that both Hsp23OE and young flies that underwent heat shock showed minimal levels of the P62 molecule. Since this molecule is responsible for binding to polyubiquitinated protein aggregates destined for autophagosomal degradation, we hypothesized that selective autophagy, as opposed to proteasomal degradation, plays the primary role in the clearance of toxic protein aggregates responsible for causing cellular degeneration.

In order to investigate this hypothesis, we turned our attention to the endosomal sorting complexes required for transport (ESCRTs). These complexes function in the formation and fusion of the autophagosomes that engulf protein aggregates tagged with P62. We take a particular interest in ESCRT III because it mediates the fusion of the two developing ends of a new phagophore and is thus directly involved in autophagosome formation, without which protein aggregate compartmentalization is disrupted. Since ESCRT III is a large complex made up of multiple subunits, we wished to examine one particular subunit, Vps24. Our goal was to first determine whether Vps24 is colocalized with uniquitinated protein aggregates in the cell as expected if the ESCRT III complex functions in their clearance. Unfortunately, there are currently no antibodies specific to the Vps24 protein. In order to overcome this challenge, our plan was to use a transgenic construct that fuses a green fluorescent protein (GFP) to the Vps24 subunit of ESCRT III as shown in Figure 13. The fusion of GFP to the *vps24* gene, will allow us to use the antibodies, such as α-GFP, which target the GFP fusion protein, to monitor

Vps24. Therefore, we will be able to use immunocytochemistry to determine the subcellular distribution of Vps24.

While we have been successful in creating and injecting the EGFP-*vps24* transgene into flies, we are currently waiting to identify the successful injections as well as to ascertain the locations of the transgene insertion. Once we have this information, we will proceed to explore a possible role for Vps24 by examining its subcellular localization. We will first examine flies which are protected from HS stress, including older flies overexpressing Hsp23 as well as young flies, and accumulate ubiquitinated protein aggregates in the perinuclear region. If autophagy is the primary method of protein aggregate clearance, we expect to see EGFP-Vps24 colocalized with protein aggregates. This future experiment will be able to further characterize the role of selective autophagy in mediating the clearance of proteins responsible for causing cell degeneration, particularly in post-mitotic cells such as the neuron, which lacks the ability to diffuse toxic aggregates through cell division (Sheikh, Haque, and Mir, 2012). Furthermore, if our hypothesis proves to be true, we can then proceed to elucidate whether or not the role of Vps24 is vital to protein aggregate clearance by introducing mutations into the *vps24* gene with the prediction that such a mutation would cause HS-stress induced degeneration even in flies with Hsp23 OE.

The results presented within this thesis help elucidate some of the mechanisms mediating HS stress-induced degeneration. We found that FoxO plays a role in mediating the intracellular non-autonomous protection against HS-stress and hypothesize that selective autophagy, not proteasomal degradation, is the primary process that mediates the clearance of ubiquitinated protein aggregates via interactions between P62 and autophagosomes. Our goal is to continue building on these findings in order to thoroughly understand the processes leading to degeneration. This will provide a basis for the development of therapies to treat patients suffering from neurodegenerative conditions such as Parkinson's and Alzheimer's Disease.

Appendix A

Additional Information Relevant to Methods

Table 1. Sources for the strains of *Drosophila* needed to set up crosses and conduct experimentation.

Drosophila Strains Utilized for Experimentation	
Name of Strain	**Source**
yw;; dFoxO[25]/TM6b	Hafen Lab
w;; MHC-Gal4	Brodie Lab
w;;UAS-*hsp23*	Bloomington Fly Stock Collection (#30541)
W;; UAS-*hsp70*	Robertson Lab
w;;UAS-FoxO	Bloomington Fly Stock Collection (#9575)
Df(3R)red31/TM6b, Tb[1]	Bloomington Fly Stock Collection (#1917)

Table 2. Sequences of primers that were used in degenerate PCR that were specific to the pUAST insertion of Hsp23

Specific Primers	
Primer Name	**Primer Sequence**
T1BUAS	5'-GCCAAGCTTTGCGTACTCGC-3'
T2D	5'-ATTCAAACCCCACGGACATG-3'

Table 3. Sequence of primers that were used in degenerate PCR that were non-specific and anneal to genomic DNA.

Arbitrary Degenerate(AD) Primers	
Primer Name	**Primer Sequence**
AD1	5'-NTCGASTWTSGWGTT-3'
AD2	5'-NGTCGASWGANAWGAA-3'
AD3	5'-WGTGNAGWANCANAGA-3'
Mixed bases: S=G/C, W=A/T, N=A/T/G/C	

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Anticipated Date of Graduation: May 2016
Minor: Theater
Honors: Biology

RESEARCH EXPERIENCE

2012 - 2016 The Pennsylvania State University | Dr. Fumiko Kawasaki and Dr. Richard Ordway
Undergraduate Researcher in Neuroscience and Molecular Genetics

- Goal: Deduce the mechanisms of neural degeneration using *Drosophila melanogaster*
- Assisted in transgenic line generations for behavioral testing following heat shock
- Gathered data that can be used for future targeted gene therapy for neurodegenerative diseases such as Parkinson's disease and Amyotrophic lateral Sclerosis (ALS)
- Skills obtained:
  - Transgenic line design and experimentation
  - DNA extraction, purification, and digestion
  - Gene amplification through PCR and gel electrophoresis
  - Western blot techniques
  - Larval fly preparations and fluorescence microscopy
  - Drosophila care and maintenance

Poster Presentations

Fatima, S., Perez, R., Kawasaki, F. (2014) *Genetic Analysis of Synaptic Transmission in Drosophila.* The Pennsylvania State University Undergraduate Research Exhibition Presentation

Perez, R., Cao, K., Baker, E., Fatima, S., Kawasaki, F. (2013) *A Novel Role for the ESCRT Pathway in Synaptic Transmission*. The Pennsylvania State University Undergraduate Research Exhibition Presentation

CLINICAL EXPERIENCE

2013 – 2015 University Health Services | Pennsylvania State University
Clinic Intern | 4 hours/week

- Performed intake of clinic patients, including vital signs and history
- Trained on electronic medical records (EMR)
- Observed outpatient procedures under Dr. Lewis Logan

June 2013 Summit Health | 160 total hours
Summer Work Experience | 40 hours/week

- Observed a variety of procedures including:
  - Angioplasty using stent placement
  - Total knee replacement

- Colonoscopy
- Caesarean section

- Shadowed a variety of physicians in various medical fields including:
  - Pediatrics
  - Oncology
  - Radiology

TEACHING EXPERIENCE

Spring 2016 — Teaching Assistant, Medical Embryology (BIOL 411)
The Pennsylvania State University
Course Director: Dr. James Strauss
- Give selected class lectures when needed and hold office hours
- Position given to 4 students a year

Fall 2015 — Teaching Assistant, Mammalian Physiology (BIOL 472)
The Pennsylvania State University
Course Director: Dr. James Strauss
- Give selected class lectures when needed and hold office hours
- Position given to 4 students a year

Spring 2015 — Lab Teaching Assistant, Function and Development of Organisms (BIOL 240W)
The Pennsylvania State University
Course Director: Dr. Kimberlyn Nelson
- Taught lab techniques to a section of 20 undergraduate students
- Lessons included:
  - miRNA isolation and amplification using qRT -PCR
  - Heart anatomy and physiology using sheep hearts
  - Ultrasound use to locate the carotid artery
- Emphasized scientific writing and article analysis

Fall 2015 — Lab Teaching Assistant, Function and Development of Organisms (BIOL 240W)
The Pennsylvania State University
Course Director: Dr. Carla Hass and Dr. Denise Woodward
- Taught introductory techniques to 24 primarily first-year undergraduate students
- Lessons included:
  - Microscopy and fungi reproductive scoring
  - Micropipetting, PCR, and gel electrophoresis
  - Biodiversity activity and research

HONORS AND AWARDS

2016	Christopher R. Dyckman and Susan Scotto Scholarship in Biology presented by the Eberly College of Science at Pennsylvania State University
2012 – 2015	Dean's List
2015	Phi Beta Kappa Honor Society Invitation
2014	Bayard D. Kunkle Faculty Senate Scholarship given on the basis of academic merit and extracurricular involvement
2014	President Sparks Award for outstanding sophomores earning a perfect 4.0 GPA after taking a minimum of 36 credits
2013	President's Freshman Award for outstanding freshman earning a perfect 4.0 GPA after taking a minimum of 12 credits

2012 Eternal Grand Master H.U. Lee Scholarship awarded to 1 student annually by the American Taekwondo Association recognizing academic and taekwondo excellence

SERVICE & LEADERSHIP

2013 – 2016 Security Leader | Penn State Dance Marathon Rules and Regulations Committee
- Biggest student-run philanthropy that culminates in a no-sitting dance marathon
- Raised over $12,000,000 each year for pediatric cancer
- Educated and led committee members alongside captains during THON weekend (2015 and 2016) and ensured they were comfortable in their positions

2015 – 2016 Gateway Peer Mentorship Founder | Schreyer Honors College
- Found a mentorship program, which paired current and new Gateway students
- Eased new students' transition into the Schreyer Honors College

2015 Gateway Orientation Director | Schreyer Honors College
- Selected to organize and implement an orientation for 250 new honors students
- Held team leader meetings and collaborated with staff to develop new ideas

2014 Childcare Volunteer | South Central Pennsylvania Community Action Program
- Help underprivileged individuals rise above their situations and fight poverty
- Selected to take care of children when adults convened to discuss plans

2014 Service Leader | Council of Lionhearts | Penn State University
- Led a student-wide State Day of Service trip to repaint a house for the disabled

2013 – 2014 President and Recruitment Director | Paws of Friendship | Penn State University
- Dedicated to raising money to buy stuffed animals for children in need and fostering relationships between college students and the local community
- Elected to oversee and develop new fundraising and service activity
- Events included bake sales, afterschool programs, and library arts and crafts

HOBBIES & SKILLS

CPR certified; Fluent in Hindi; Taekwondo black belt and former Pennsylvania State Champion; National Latin Exam silver medalist