THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

Genetic Analysis of Protection from Environmental Stress-Induced Degeneration: Characterization of a Loss of Function *Drosophila* Mutant for the Cytoplasmic RNA Exosome Component, dSKI2

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SPRING 2018

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree in Biology
with honors in Biology

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ABSTRACT

Given the growing burden of degenerative diseases on human health and healthcare, defining the precise mechanisms that underlie these diseases as well as mechanisms which may mediate protection against cellular degeneration remains a key research objective. Both genetic and environmental factors are known to contribute to the development of degenerative diseases such as Alzheimer's and Parkinson's, however the mechanisms mediating environmental contributions are poorly understood. We are investigating these mechanisms using a simple but powerful experimental model of environmental stress-induced degeneration in *Drosophila*. Previous studies in our laboratory revealed that environmental stress administered to wild-type flies in the form of heat shock (HS) induced a selective loss of flight ability and severe degeneration of flight motor cells. A genetic screen described in this work recovered a new mutant, *pele,* that demonstrates a marked resistance to degeneration and to the loss of flight motor function. Genetic analysis characterized *pele* as an allele of *dski2,* a gene which encodes a required component of the cytoplasmic RNA exosome. Furthermore, this work explores the basis of an interesting infertility phenotype in parallel studies of the *pele* mutant. We anticipate that our investigation of infertility will converge with genetic analysis of degenerative mechanisms, providing further insight into the molecular mechanisms which operate in *pele* to confer protection. By identifying mechanisms that confer protection against environmental stress-induced degeneration, we hope to gain insight that can be applied to the development of gene-targeted therapies.

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ACKNOWLEDGEMENTS

I would like to thank both Dr. Richard Ordway and Dr. Fumiko Kawasaki for welcoming me into the lab and for being a constant source of advice, support, and encouragement in all things throughout my undergraduate career. I have been blessed to have two amazing faculty members who have made themselves available to answer every question and who have taught me the importance of mutual respect, patience, teamwork, and persistence in conducting research.

Along with these mentors, I am grateful for the help of former lab members Noelle Koonce and Linda Guo, who shared their knowledge of lab procedures and formed the basis of a wonderful support system. I also want to thank Jaehee Kim, Priyanka Mariyappa, Jonathan Florian and all current lab members for their contributions to this work. They have been a continuous source of encouragement, laughter and positivity, all of which have made the lab a wonderful community to be a part of and one that has shaped me both as a student and as an individual.

Chapter 1

Introduction

Neurodegenerative Disease

Millions of people are affected worldwide by degenerative diseases and thus the study of such disorders remains critically important to human health and healthcare. Given the growing elderly population in the United States, the burden of these disorders is projected to grow substantially over the next few decades (Kowal et al., 2013). Contributions to the development of degenerative diseases include aging and genetics as well as the environment. Environmental factors make important contributions to degenerative disease including the predominant sporadic forms of major neurodegenerative diseases such as Alzheimer's and Parkinson's (Figure 1) (Levy Noguiera et al., 2016; Burbulla and Kruger, 2011; Paez-Colasante et al., 2015; Daneshvar et al., 2015; Gardner and Yaffe, 2015; Tanner et al., 2014; Lardenoije et al., 2015). Despite substantial progress in defining the molecular basis of degenerative disease, the underlying mechanisms and the contributions of environmental factors remain elusive (Cannon and Greenamyre, 2011). Therefore, gaining a better understanding of the role of environmental stressors and identifying key mechanisms which mediate protection against cellular degeneration remains a major research objective.

Although a broad range of degenerative diseases exists, the failure of cellular proteostasis is a common feature (Labbadia and Morimoto, 2015). The normal disposal of misfolded proteins appears to be disrupted in these disorders, leading to the accumulation of faulty proteins that are

prone to forming toxic aggregates in the cell. Neurons are particularly vulnerable to the effects of abnormal protein aggregation, making neuronal damage a hallmark of neurodegenerative disease (Taylor, Hardy, and Fischbeck, 2002). The shared mechanistic basis among these diseases, also known as proteinopathies, can therefore be exploited in the investigation of how environmental factors promote degenerative processes. A better understanding of these mechanisms is expected to enhance development of effective targeted therapies.

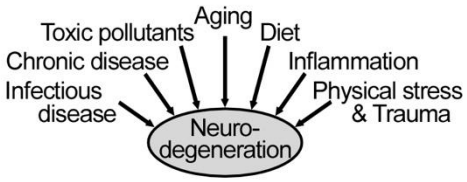

Figure 1. Environmental factors in neurodegenerative disease.

Drosophila Model for Genetic Analysis of HS-Stress Induced Degeneration

To better understand the contributions of environmental stress, our laboratory has developed a powerful but simple *Drosophila melanogaster* model that permits the genetic analysis of degeneration induced by an environmental stressor, in the form of heat shock (HS) stress (Kawasaki et al., 2016). Our studies have focused on the analysis of tripartite neuromuscular synapses of the flight motor, a visible network of neurons, glia, and cells of the dorsal longitudinal flight muscle (DLM) (Figure 2). Importantly, the *Drosophila* tripartite synapse model exhibits functional properties similar to those of mammalian synapses (Danjo, Kawasaki, and Ordway, 2011; Strauss, Kawasaki, and Ordway, 2015; Kawasaki and Ordway, 2009).

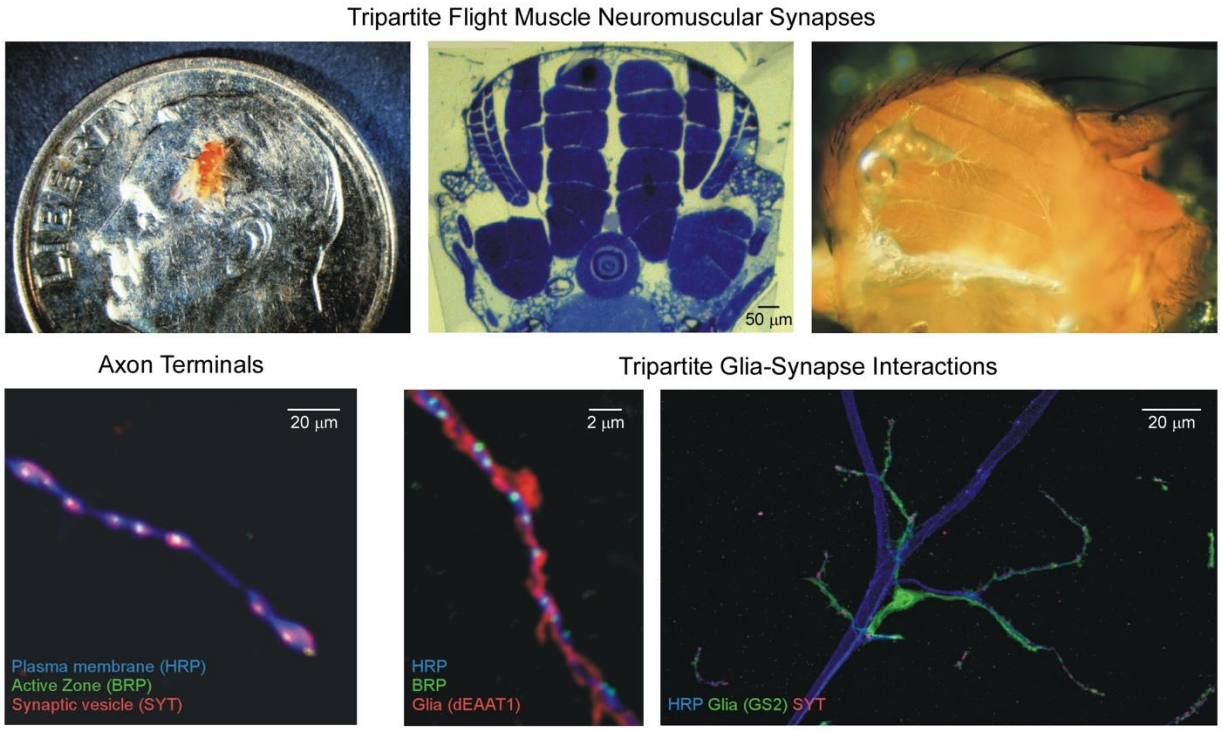

Figure 2. Morphology of Dorsal Longitudinal Muscle (DLM) Neuromuscular Synapses.

Top: Morphology of the thorax showing stacks of six DLM fibers in cross section and a lateral view. Bottom: 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 left and right panels) and glia (dEAAT1; red in center panel).

Several key features make *Drosophila* an attractive model for studying human neurodegenerative disease, including the ease with which rapid genetic manipulation can be performed and the high degree of homology with the human genome. Two-thirds of disease-causing genes identified in humans are estimated to be also present in the fly (McGurk, Berson, and Bonini, 2015). Previous work utilizing this model revealed that after exposure to a HS stress paradigm, a global environmental stress administered under precise temporal control, wild-type (WT) flies exhibited a selective loss of fight ability (Figure 3A). Further analysis by confocal microscopy imaging revealed severe degeneration in all three cell types within the flight motor

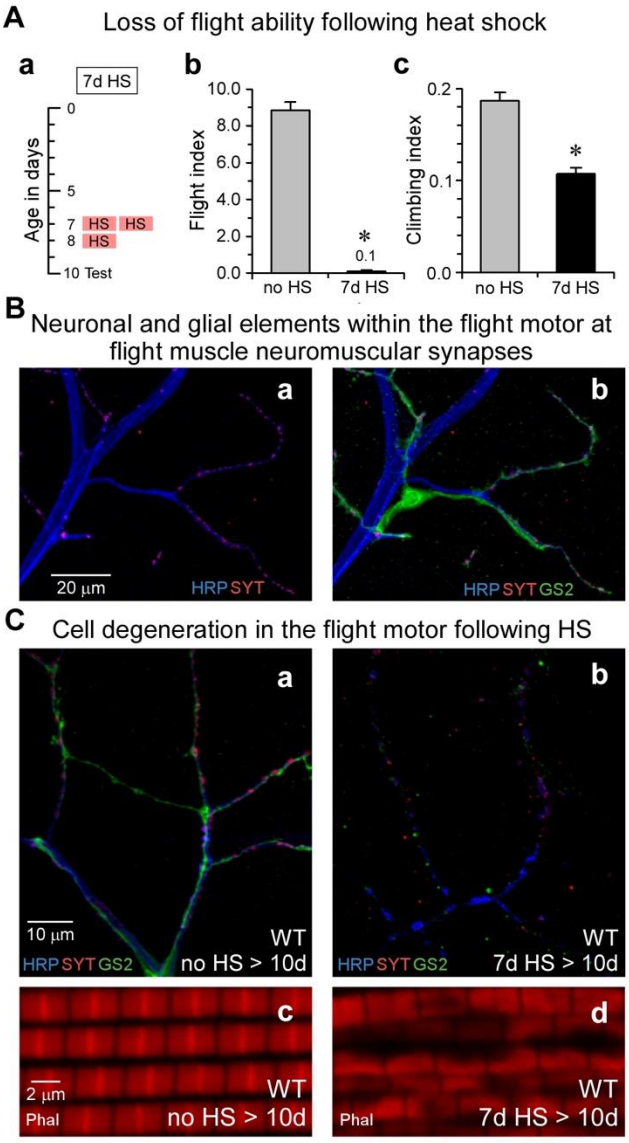

Figure 3. HS stress induces loss of flight ability and degeneration of motor axons, glia and muscle fibers in the flight motor.

(A) a, HS and testing paradigm for HS of 7 day old flies (7d HS). A group of six 7d old flies was exposed to a series of three two-hour HS at 36 °C. Flight and climbing tests were performed at 10 days of age. b, Loss of flight ability in 10d old flies after the 7d HS paradigm in comparison to no HS controls. c, Climbing ability under the same conditions as flight. Error bars indicate SEM. Asterisks denote a significant difference from the no HS control. (B) Confocal immunofluorescence images of DLM neuromuscular synapses. Neuronal plasma membrane (HRP; blue), synaptic vesicles (SYT; red) and glia (GS2; green). (C) a, DLM neuromuscular synapses of a 10d old no HS control. WT: wild type. b, Images corresponding that in a, but from a 7d HS fly prepared for immunocytochemistry at 10d (7d HS > 10d). Note severe degeneration of axons and glia in comparison to the no HS control. c, d, Phalloidin which binds F-actin was used to visualize myofibrils in DLM.

(Figure 3C). These studies made the surprising observation that HS flies retained other motor functions (e.g., walking), and that neuronal, glial, and muscle cells required for leg motor function remained intact. Furthermore, degeneration was age dependent such that young adult flies were resistant to HS stress (Kawasaki et al., 2016). The susceptibility of certain cell types to degeneration and the age-dependence of degeneration are two key features of degenerative disease, making this an ideal model for our studies.

Further characterization of selective loss of flight ability in response to environmental stress revealed the accumulation of ubiquinated protein aggregates, indicative of disrupted proteostasis, in muscle cells of the flight motor but not in those of the leg. Moreover, muscle-specific overexpression of a molecular chaperone protein enhanced muscle proteostasis and conferred protection of all three flight motor cell types against HS stress (Kawasaki et al., 2016).

Genetic Screen for New Mutants

The preceding studies motivated a genetic screen for mutants exhibiting resistance, with the goal of identifying novel mechanisms which can protect against degenerative processes. This forward genetic screen utilized chemical mutagenesis to induce random mutations. Flies displaying a strong resistance to HS stress-induced loss of flight ability, the phenotype of interest, were identified and characterized. The *pele* mutant was recovered from the screen and subjected to further analysis, including exploration of an infertility phenotype exhibited by both male and female mutant flies. As described in Chapter 3, genetic characterization of the *pele* mutant indicated that the mutation mapped to the *tst*/*dski2* gene and thus revealed a novel mechanism of protection from degeneration. In this thesis, this gene will be referred to by its alternate designation, *dski2*.

A Human Homologue of *ski2*, *SKIV2L*

Notably, a homologue of the *dski2* gene exists in humans, although no genetic analysis of SKI2 function in vertebrates has yet been performed. Of particular interest is one known human *SKIV2L* polymorphism that has been significantly associated with age-dependent macular

degeneration (AMD), a degenerative disease which affects approximately 11 million Americans and constitutes a leading cause of blindness (Pennington and DeAngelis, 2016). Although further investigation is required, this variant was associated with protection from AMD in previous research (Shuai et al., 2017). Thus, a protective role for *dski2* mutations may be conserved between flies and humans. Human *ski2* is also associated with a rare genetic disease, Trichohepatoenteric syndrome (THES), caused by recessive loss-of-function mutations. THES is characterized by a number of pleiotropic symptoms, including syndromic diarrhea, increased risk for liver cirrhosis, and hair abnormalities (Zheng et al., 2016).

Parallel Studies in the *Pele* Mutant

The work described here will focus primarily on two topics, including identification of a new mutant exhibiting resistance to cellular degeneration and the genetic analysis of this mutant. Protection from environmental stress-induced degeneration may involve complex systemic mechanisms including cell-nonautonomous signaling. Thus complementary genetic analysis is focused on an interesting and relatively simple and accessible infertility phenotype observed in the *pele* mutant. We anticipate that these parallel studies of infertility will converge with genetic analysis of degenerative mechanisms and provide further insight into the molecular mechanisms which operate in *pele* and confer protection from environmental stress-induced degeneration.

Chapter 2

Materials and Methods

Heat Shock (HS) Stress Paradigm

As indicated in Figure 3, standard 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 (4d or 7d). The first two heat shocks were started at 10:00 a.m. and 2:00 p.m. on the first day and the third heat shock was started at 10:00 a.m. on the second day. Newly eclosed flies were collected 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. For 4d HS, flies were transferred to fresh vials on Day 3. For 7d HS, flies were transferred to fresh vials on Day 5. 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. Following HS, flies were maintained at 20°C in a 12:12 day: night cycle.

Immunocytochemistry 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 as described previously (Iyer et al., 2013). The same methods were used to examine coxal muscle neuromuscular synapses in the

leg. To visualize myofibrils in the DLM, Alexa 568-conjugated phalloidin, which binds F-actin, was used at 0.13 μM. Images were obtained and processed with Fluoview software (Olympus Optical). Images shown are representative of those obtained from at least 3 different preparations.

Antibodies used: Synaptic vesicles in the presynaptic bouton were labeled using rabbit - Synaptotagmin Dsyt CL1 (Mackler et al., 2002) (α-SYT;1:5000) [Dr. Noreen Reist (Colorado State University, Fort Collins, CO)]. The glutamate transporter dEAAT1, specifically expressed in glial cells, was labeled using Rabbit - dEAAT1 (Rival et al., 2006) (1:2500) [Dr. Serge Birman (Developmental Biology Institute of Marseille, France)]. mAb nc82 anti-BRP was used to label presynaptic active zones (BRUCHPILOT) (Wagh et al., 2006) (1:50) (Developmental Studies Hybridoma Bank, Iowa City, IA). mAb GS-6 anti-Glutamine Synthetase was used to stain a glial cell marker, glutamine synthetase 2 (α-GS2;1:500) (EMD Millipore, Billerica, MA). Neuronal plasma membranes were labeled using goat -HRP-Alexa647 (1:200) (Jackson Immunoresearch Laboratories, West Grove, PA). Actin staining was performed using Alexa Fluor 568 Phalloidin (130 nM) (Invitrogen, Carlsbad, CA) and nuclear staining was performed using DAPI (300 nM) (Sigma-Alrich, St. Louis, MO).

Recovery of a New Mutant in a Forward Genetic Screen

As described in Chapter 3, a new mutant was recovered from a genetic screen utilizing the chemical mutagen ethylmethane sulphonate (EMS). Flies homozygous for the mutagenized third chromosome, ranging in age from 3 to 5 days old, were subjected to HS stress and tested for flight ability. An isogenic 3rd chromosome parental line was used in the screen to facilitate

subsequent molecular characterization of new mutants which retained flight motor function after HS stress. Following recombination mapping used to estimate the approximate location of the affected gene, fly lines possessing nearby third chromosome deficiencies were obtained from the Bloomington *Drosophila* Stock Center and crossed to the mutant. Five candidate genes within a 10.6 kb region of the genome were sequenced.

Cloning and Transformation

Standard cloning procedures were used to generate UAS transgenes for expression of dSKI2. For example, to make the tagged transgene UAS-EGFP-dski2, cloning fragments were generated by PCR from a publicly available cDNA clone (AY061315) using primers containing specific restriction sites and corresponding restriction endonucleases. A construct containing the *dski2* ORF fused with EGFP was inserted into pUAST, a p-element vector that can integrate into the *Drosophila* genome. Restriction enzymes Not I and Kpn I were used to digest the *dski2* ORF and EGFP insert. Using the Not I and Kpn I restriction sites, this construct was ligated into the pUAST vector. The plasmid was transformed into JM109 competent cells and the success of the transformation was evaluated via plasmid preparation, diagnostic digestion, and gel electrophoresis. Transgenic lines were generated by DNA injection into embryos as described previously (Kawasaki, Collins, and Ordway, 2002).

Generation of an Anti-*Pele* Antibody

A rabbit polyclonal antiserum, anti-dSKI2, was generated against a synthesized peptide corresponding to an N-terminal region of the dSKI2 protein (amino acid residues 228-251).

Through a commercial service (Pocono Rabbit Farm and Laboratory, Canadensis, PA), the peptide was synthesized with cysteine appended at the N-terminus and prepared for injection by conjugation with keyhole-limpet hemocyanin (KLH).

Western Blot Analysis

Western blotting was used to analyze levels of dSKI2 protein expressed in wild-type (WT), *dski2*[*pele*]/Df, and MHC-GAL4>UAS-dski2 flies. Collected flies were homogenized in SDS buffer and approximately 0.2 bodies were loaded per lane. Samples were run on a 9% polyacrylamide gel at 15 mA/gel. After 90 minutes, proteins were transferred onto a nitrocellulose membrane overnight. Following incubation with the primary antibody α-dSKI2 (1:5000), the membrane was incubated with a secondary antibody and imaged using a LI-COR Odyssey CLx detection system. To control for loading, the membrane was also incubated with primary antibody α-TUB (1:100,000) and detection was performed.

Generating Fertility Cross Flies with GAL4-UAS System

Cell-type specific genetic manipulation in our fertility studies was achieved by utilizing the GAL4-UAS system. This versatile tool activates the transcription of a downstream gene when a yeast transcriptional activator protein (GAL4) binds to upstream activator sequences (UAS). A genetic cross brings together a “driver” GAL4 line under the control of tissue-specific promoters with the UAS line, containing the downstream gene of interest, thereby activating tissue-specific expression patterns (Brand and Perrimon, 1993). In our fertility studies, test flies

were generated by crossing flies from two different driver lines to two flies from a tagged and untagged UAS-dski2 line.

Table 1. *Drosophila* driver lines used in fertility experiments and their sources.

Name of Line	Source	Site of Expression
w c355-Gal4;; dski2[pele]/TM3, Sb	Bloomington Drosophila Stock Center (#3750)	Somatic follicular cells, posterior
w; mat α4-GAL-VP16 V2H; dski2[pele]/TM3, Sb	Bloomington Drosophila Stock Center (#7062)	Germline cells

Table 2. Additional *Drosophila* lines used in fertility experiments.

Name of Line	Source
w UAS-dski2;; Df(3R)BSC 489/TM6c, Sb	Generated via cloning and transformation
w UAS-EGFP-dski2;; Df(3R) BSC 489/TM6c, Sb	Generated via cloning and transformation
w;; Mi $tst^{MB10212}$/TM6c, Sb^{1} (dski2 mutant)	Bloomington Drosophila Stock Center (#29100)

Fertility Cross Procedure

Fly stocks used in the fertility experiments were obtained from Bloomington Fly Stock Collection or generated in our lab, as shown above. Wild-type (Iso3) flies were collected from our own laboratory stock. Crosses to generate test flies of interest were set up and maintained over the course of months at room temperature (RT). Newly eclosed flies were collected during a five-day window from Thursday to Monday. On the fifth day, individual test flies were crossed to three WT flies in standard food vials and a week later, test flies and two WT flies were lightly anesthetized with CO_2 and transferred to fresh food vials. Parents were removed on the second day after the transfer. Hatched eggshells, defined as eggs that were visibly flattened, were counted and recorded on Friday, two days after the vials were cleared.

Chapter 3

Identification of a New Mutant, *Pele*

The studies and results discussed in the following Chapters, 3 and 4, represent collaborative research efforts. Other members who contributed to this work include Dr. Fumiko Kawasaki and Yunzhen Zheng, as well as former and present undergraduate researchers Noelle Koonce, Linda Guo, Jonathan Florian, and Priya Mariyappa. I performed experiments in genetics, molecular biology, imaging, Western blotting and behavioral analysis.

Pele Exhibits Resistance to Cellular Degeneration

Our studies utilized a genetic screen to identify mutants that retain their flight motor ability after HS stress and, accordingly, demonstrate resistance to environmental stress-induced degeneration. Forward genetic screens permit the identification of individuals displaying a phenotype of interest and the genetic analysis of a new mutant provides important information about gene function. In this study, male flies were exposed to the chemical mutagen ethylmethane sulphonate (EMS) and then mated to females with a visible second chromosome marker, Lyra, in *trans* to a balancer chromosome (Figure 4). After a heterozygous stock with the mutagenized third chromosome in *trans* to a balancer was established, F3 flies homozygous for the mutagenized third chromosome were subjected to HS stress.

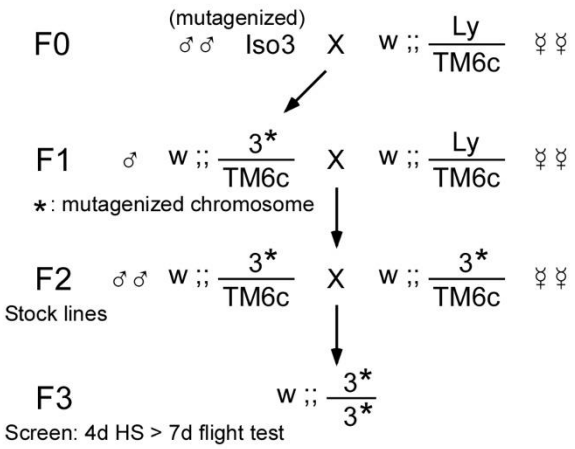

Figure 4. A Genetic Screen for New Mutations Conferring Resistance to Environmental Stress-Induced Degeneration.

Male flies with an isogenized third chromosome (Iso3) were exposed to the mutagen, ethylmethane sulphonate (EMS). Mutagenized males were mated with with females carrying the visible second chromosome marker, Lyra (Ly), in trans to a balancer chromosome, TM6c. The F1 male progeny carrying a mutagenized third chromosome (3*) in trans to TM6c were backcrossed to F0 females. After mating F2 heterozygous siblings, F3 flies homozygous for a mutagenized third chromosome were subjected to HS at 4 days of age and subsequently screened for flight ability.

A subsequent test for flight ability following HS stress recovered a new mutant, *pele*, on the basis that it exhibited protection from HS stress-induced loss of flight ability. While WT flies demonstrated a loss of flight ability, *pele* mutants of four and seven days of age retained their flight ability, with a more marked effect in four day old flies (Figure 5A). This result aligns with our previous work establishing an age-dependent HS stress response, with young flies displaying heightened resistance to HS stress and to the associated loss of flight ability (Kawasaki et al., 2016).

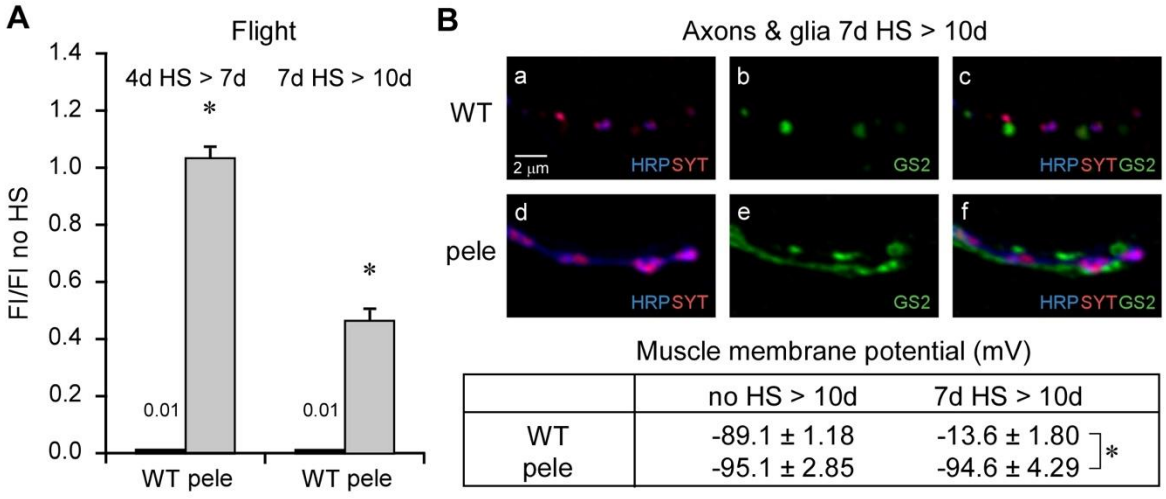

Figure 5. A new mutant, *pele*, exhibits resistance to HS-induced loss of flight ability and cell degeneration.

(A) Analysis of HS-induced loss of flight ability in wild-type (WT) and *pele* mutant flies. *pele* exhibited protection from HS-induced loss of flight ability. (B) Confocal immunofluorescence images and muscle membrane potential recordings from DLM neuromuscular synapses. *pele* exhibited protection from HS-induced degeneration of all three cell types within the flight motor. Data are mean ± SEM.

Further analysis of the *pele* mutant by confocal immunofluorescence imaging revealed protection of the flight motor, indicated by intact constituent neuronal, glial and muscle cells. Degeneration and protection were assessed in 10 day-old flies following exposure to the HS stress paradigm at 7 days (Figure 5B). In addition to fluorescence imaging, the function of the dorsal longitudinal muscle (DLM) was also assessed by recording muscle membrane potentials. In the absence of HS stress, no significance difference was observed between muscle membrane potentials in WT flies and in the *pele* mutant (Figure 5B). In contrast, exposure to HS stress at 7 days of age disrupted muscle membrane potential in 10 day-old WT flies, whereas *pele* mutants possessed a normal membrane potential. Taken together, these results demonstrate the resistance of this new mutant to HS stress-induced degeneration.

Disrupting dSKI2 Confers Protection

To map the mutation underlying this novel phenotype, we utilized meiotic recombination and deficiency mapping. These two traditional genetic approaches rely on the use of recombination with respect to known markers or complementation tests with deficiencies to pinpoint a mutation. Genetic characterization indicated that *pele* is recessive and maps to the right arm of the third chromosome (Figure 6A). Fine scale deficiency or deletion mapping identified five candidate genes and subsequent sequence analysis of protein coding regions demonstrated a molecular lesion in the *Drosophila* ski2 gene (*dski2*) (Figure 6A).

SKI2 was originally identified in yeast as part of the SKI complex and a required component of the cytoplasmic exosome mediating 3'-5' degradation of cytoplasmic RNAs (Zinder and Lima, 2017). Although little functional analysis has been carried out in metazoans, the gene was identified previously in *Drosophila* (Seago, Chernukhin, and Newbury, 2001). SKI2 is the only catalytic component of the SKI complex and functions as an RNA helicase in 3'-5' RNA degradation (Figure 6C). Several functional domains have been identified in SKI2 proteins, including RecA1, RecA2, and a helical domain (Figure 6B). The *pele* mutation introduces a stop codon which truncates the protein after the RecA1 domain and is thus predicted to eliminate helicase activity.

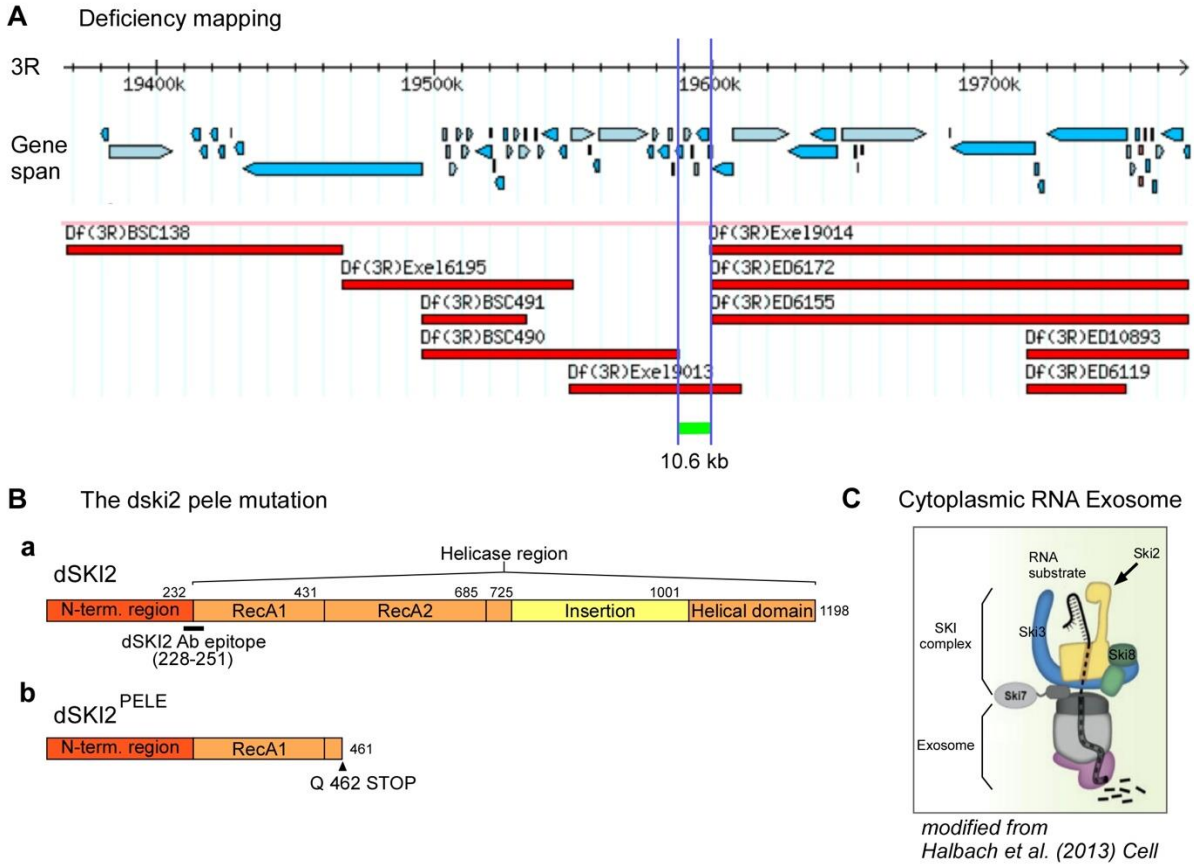

Figure 6. A *Drosophila ski2* mutant.

(A) Meiotic and deficiency (Df) mapping placed the *pele* mutation on the right arm of the 3rd chromosome (3R). Numerical positions (in kb) within the genome are indicated. The deleted region for each Df chromosome is represented by a red bar. *Df(3R)Exel9013* failed to complement *pele* whereas complementation was observed with other small Dfs removing adjacent regions, placing *pele* within a 10.6kb region of the genome. (B) Sequencing of the five candidate genes in this region revealed a single nucleotide deletion in *dski2* resulting in a premature stop codon. Thus, *pele* is an allele of *dski2* and is referred to as *dski2*[*pele*]. The epitope for production of polyclonal antisera against dSKI2 is indicated (solid line in a). (C) SKI2 is an RNA helicase and a subunit of the Ski complex; a required component of the cytoplasmic RNA exosome.

To further characterize the *pele* mutant, an antibody was raised against a dSKI2 peptide (see Materials and Methods) which is located near the N-terminal region of the protein (Figure 6). This antibody was used in Western analysis to demonstrate loss of the full-length dSKI2 protein, which has a molecular mass of 136 kD, in the dSKI2[*pele*]/Df line (Figure 7). In

accordance with our identification of a nonsense mutation in *pele* which results in truncation of the dSKI2 protein, a smaller band of 52 kD was detected specifically in the *pele* mutant. In conjunction with this work, we also developed a transgene to enable the overexpression of the dSKI2 protein as described in the next chapter. Relative to WT flies, higher levels of dSKI2 were expressed in flies when expression of this transgene was driven with a muscle-specific GAL4 driver (MHC-GAL4) (Figure 7).

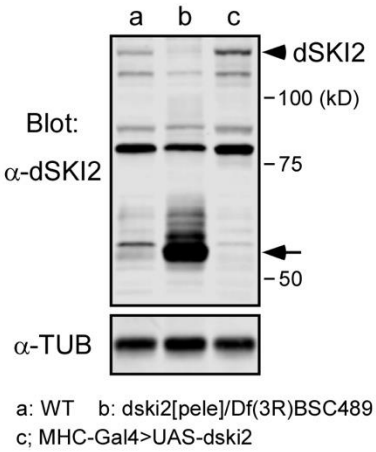

Figure 7. Western blot analysis indicating the absence of full-length dSKI2 protein in *dski2*[*pele*].

Whole fly homogenates were prepared from WT, *dski2*[*pele*] hemizygotes (dski2[pele]/Df(3R)BSC489) as well as WT flies exhibiting overexpression of wild-type dSKI2 in muscle (MHC-Gal4>UAS-dski2). In the *dski2* mutant, a prominent band (arrow) at a molecular mass higher than 50kD is recognized by the anti-dSKI2 antibody. This may represent the truncated dSKI2 protein produced by the *pele* mutation. Tubulin (TUB) was used as an internal loading control.

Ongoing and future work will aim to characterize the precise molecular mechanisms operating in the *pele* mutant that confer protection from environmental stress-induced degeneration. This work will include investigating other phenotypes of the *pele* mutant, including a lack of fertility observed in both male and female flies. The latter studies provide a simple and accessible model to examine the molecular mechanisms operating in the *pele* mutant and are expected to provide insights into the mechanisms of protection from environmental stress-induced degeneration. This approach has formed the primary focus of my recent and ongoing thesis research presented in the following chapter.

Chapter 4

Investigation of an Infertility Phenotype

Fertility Experiment Rationale

Although much of our work has focused on identifying the molecular mechanisms that confer protection against HS stress-induced degeneration in the *pele* mutant, we have also made the interesting discovery that both male and female *pele* mutant flies are infertile. As described in the following text, we sought to characterize this infertility phenotype and determine the underlying mechanisms. We anticipate that these parallel studies will converge with those on the novel role of RNA metabolism in the *pele* protection phenotype to reveal underlying molecular mechanisms.

Fertility Defects Observed in dSKI2 Mutants

Initial studies focused on female fertility, which is determined in the ovary, where oogenesis occurs in a series of well-defined developmental stages within egg chamber compartments of the ovariole (Figure 8). To examine the fertility of *pele* mutants, individual mutant male and female test flies ranging from 1 to 5 days old were crossed with WT flies of the opposite sex (Figure 9A). Test crosses were transferred after one week and parents were removed two days later. Fertility was assessed on the 11^{th} day of the procedure and was operationalized as the number of visibly flat eggs, from which larvae hatched. Additionally, the same test crosses were performed with WT control flies to establish a baseline for comparison.

Fertility, which was quantified as the number of hatched eggs per female for each genotype, was then normalized to the WT baseline.

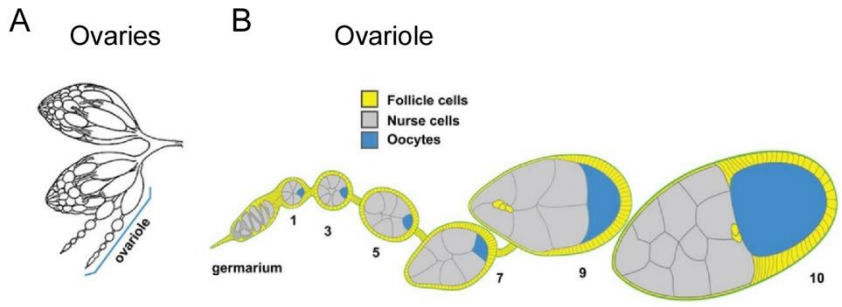

Figure 8. Overview of *Drosophila* oogenesis.

(A) A pair of ovaries with two ovarioles highlighted. (B) An ovariole showing egg chamber morphology at several developmental stages and highlighting key cell types. Modified from Andersen, D. and Horne-Badovinac, S. Development 143, 1375-1387 (2016).

In our investigation, we observed a pattern of infertility among *pele* mutants. First, *dski2*[*pele*]/Df flies, produced markedly fewer progeny than WT flies, with very few hatched eggs observed among female mutants and none observed in crosses of male mutants with wild-type females (Figure 9B). Second, we observed a similar effect among male and female flies carrying a different mutant allele of the *dski2* gene, caused by insertion of a transposable element insertion, *Mi,* into the *dski2* gene. This finding further confirms that loss of dSKI2 function results in loss of fertility among mutants. Along with an infertility pattern, other fertility defects were observed in our investigation. Closer examination of eggs produced by the *pele* mutant revealed morphological changes like the thinning of appendages at the anterior end of the egg and a less prominent eggshell (Figure 9C).

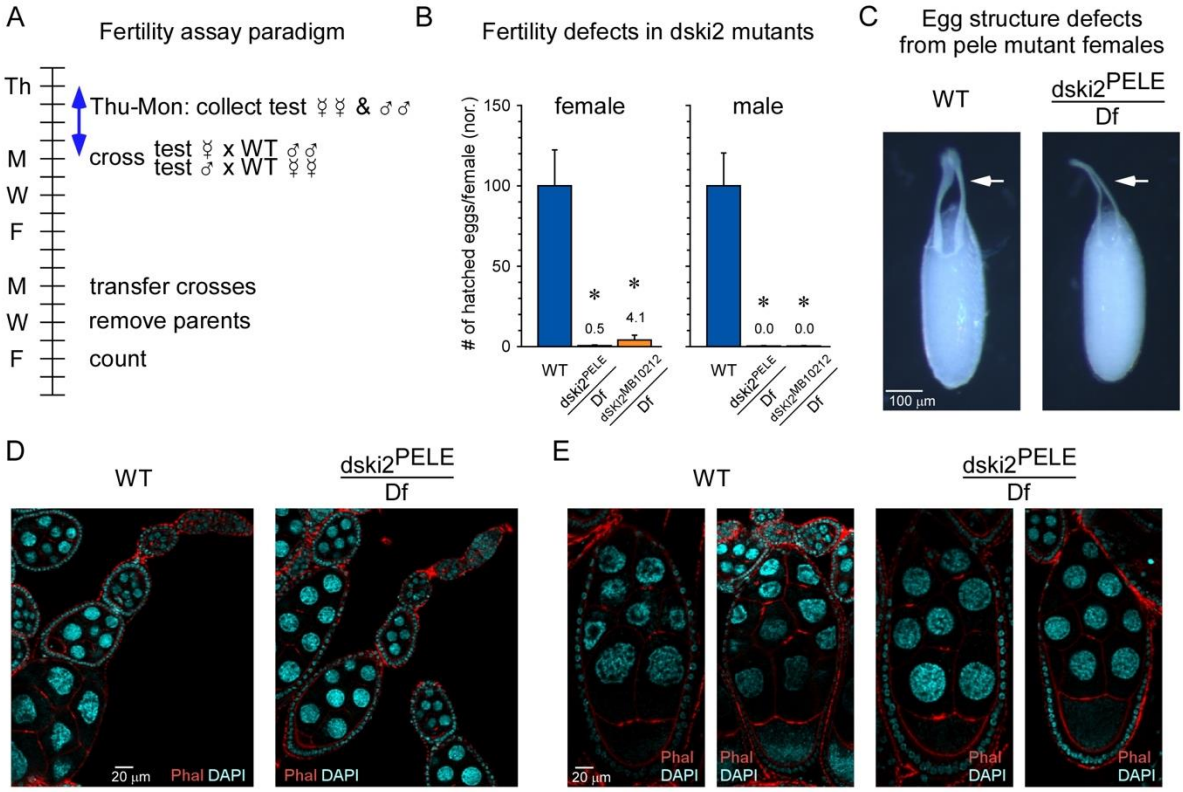

Figure 9. Fertility defects in *dski2* mutants.

(A) Fertility assay paradigm. Individual 0- to 4-day-old male or virgin female test flies were mated with wild-type males or virgin females. After 7 days, each cross was transferred to a fresh vial and parents were discarded in 2 days. Further 2 days later, each vial was visually examined to record the number of hatched and unhatched eggs. (B) Mean number of hatched eggs per female with respect to WT control. The *dski2*[*pele*] mutant exhibits fertility defects in both females and males. Furthermore, an independently generated mutant allele, *dski2*[MB10212], showed a fertility defect as well. (C) Example eggs from WT and *dski2*[*pele*] females. The *dski2*[*pele*] mutant egg has substantially thinner dorsal appendages (arrows) and appears to have a morphologically abnormal eggshell in comparison to that from WT. (D, E) Confocal immunofluorescence images of early (D) and late (E) developmental stages of egg chambers from WT and *dski2*[*pele*] mutant females. Oogenesis appears to be normal in *dski2*[*pele*] mutant.

Despite the observed differences in fertility levels, confocal microscopy imaging revealed few structural differences between WT and mutant ovarioles (Figure 9D). This result suggests that the mutant egg chamber undergoes normal development. However, one interesting morphological feature was noted among nurse cell nuclei found in late-stage egg chambers, which were distinctly round in the mutant compared to those in WT (Figure 9E).

Rescue of Fertility with *c355-GAL4* Driver

In order to explore the mechanistic basis of infertility, we investigated whether restoring dSKI2 expression in a cell-type specific manner would be sufficient to rescue fertility, with the goal of gaining insight into where dSKI2 function is critical for successful gamete production. Cell type-specific rescue experiments were performed to identify the cell types in which dSKI2 protein function is required for fertility. This was achieved using the GAL4-UAS system (see Materials and Methods) to drive cell type-specific expression of wild-type dSKI2 protein in a *pele* mutant background.

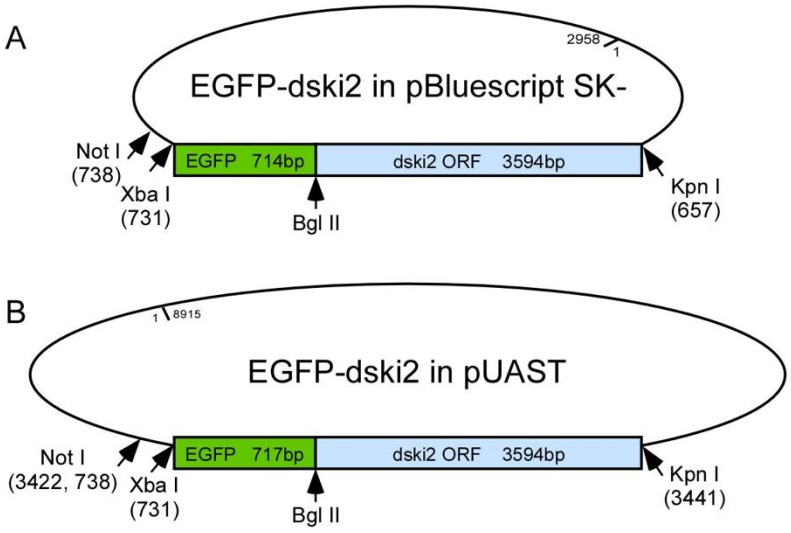

Figure 10. *EGFP-dski2* construct in pBluescript SK- and pUAST.

(A) The labeled Bgl II and Kpn I restriction sites were used to ligate the *dski2* ORF into the pBluescript SK- vector with a preexisting EGFP sequence. (B) The Not I and Kpn I restriction sites were used to ligate the *EGFP-dski2* ORF sequence in the pUAST vector.

Two UAS-transgenic lines were used in the rescue experiments, including UAS-dSKI2 and UAS-dSKI2 EGFP (tagged with EGFP). To make the tagged transgene, cloning and transformation were performed according to the procedure detailed in Chapter 2 (Figure 10). Expression of the wild-type dSKI2 protein was achieved by crossing these flies with flies from two GAL4 driver lines expressed in the ovary, either in somatic follicle cells or germline oocyte and nurse cells. For fertility rescue experiments, test flies possessing both the driver and the

transgene in a *pele* mutant background were crossed with WT flies. Flies carrying only one component of the GAL4-UAS system (the UAS transgene or the GAL4 driver) and thus should not express wild-type dSKI2 were also included as controls.

When expression of the tagged and untagged dSKI2 proteins was restored to somatic follicular cells using the *c355-GAL4* driver, a rescue effect was observed, with female fertility levels approaching those observed in WT flies (Figure 11A). In contrast, when dSKI2 was expressed in the female germline using the *matα4-GAL-VP16 V2H* driver in the *pele* mutant background, low to zero levels of fertility were observed and thus germline expression did not produce rescue.

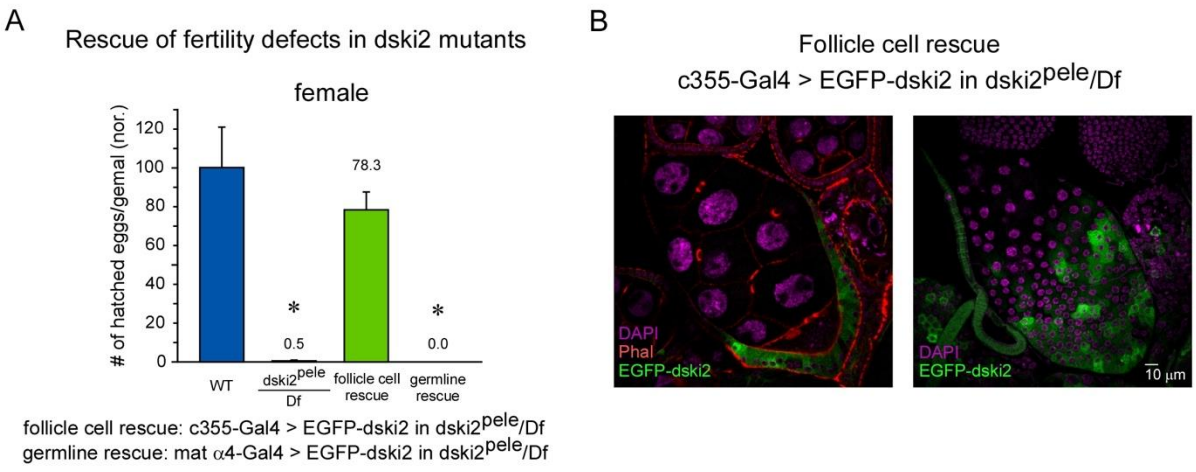

Figure 11. Transgenic expression of wild-type dSKI2 in follicle cells rescues the female infertility of *dski2*[*pele*].

(A) Mean number of hatched eggs per female in comparison to WT control. Data for WT and the *dski2*[*pele*] mutant are the same as those shown in Figure 9. The fertility defect in the dski2[pele] mutant was rescued by expressing a transgene encoding EGFP-tagged wild-type dSKI2 in follicle cells (follicle cell rescue), In contrast, expression of the same transgene in germline cells (germline rescue) failed to rescue. *c355-GAL4* is a GAL4 driver that expresses UAS-transgenes in all follicle cells over the oocyte, whereas *mat a4-Gal4* drives expression in germline cells. (B) Confocal immunofluorescence and native EGFP fluorescence images of an egg chamber from dski2[pele] mutant female expressing the EGFP-tagged wild-type dSKI2 in follicle cells. Transverse (left) and surface (right) views of the same egg chamber showing EGFP-dSKI2 signal in follicle cells at the posterior region of the egg chamber. In the right panel, autofluorescence from trachea was also seen in the GFP channel.

These findings suggest that the normal function of the dSKI2 protein in follicle cells is critical to female fertility. This result was further explored by visualizing the expression of dSKI2 tagged with EGFP using confocal fluorescence microscopy to confirm the reported expression pattern of the *c355-GAL4* driver. Consistent with our expectations, the GFP signal was primarily confined to the posterior region of late-stage egg chambers where somatic follicle cells are located. From the interior, expression is visible in follicle cells surrounding the developing germline but not within the germline. Notably, fluorescence was not visible in early-stage egg chambers, indicating that dSKI2 expression in somatic follicle cells at later stages of egg development is critical for female fertility (Figure 8B). Investigation of the role of dSKI2 and the contributions of disrupted RNA metabolism to infertility is ongoing, and we plan to continue this analysis with additional cell type-specific genetic manipulation.

Chapter 5

Discussion and Future Directions

Our previous work established an attractive *Drosophila* experimental model for the genetic analysis of degeneration induced by environmental stress in the form of heat shock. When exposed to a global stress, WT flies exhibited a selective loss of flight motor ability. Therefore, identifying mutants with resistance to HS stress-induced cellular degeneration was a key objective. Through the genetic screen described in Chapter 3, we identified one such mutant with a protection phenotype. Further analysis by genetic mapping determined that *pele* is a recessive nonsense mutation within the *dski2* gene located on the right arm of the third chromosome. Loss of *dski2* function disrupts the larger SKI complex responsible for degradation of cytoplasmic mRNAs, of which SKI2 is a required catalytic component. These findings indicate that the protection mechanisms in the *pele* mutant may involve alterations in RNA metabolism.

After discovering that *pele* mutant flies were infertile, we initiated parallel fertility studies that represent a major ongoing component of my thesis research. We began by characterizing the mutant infertility phenotype by determining that crosses with WT flies exhibited low to zero levels of both male and female fertility. Further examination of eggs produced by *pele* mutants revealed subtle morphological changes compared to WT. Specifically, alterations were suspected in the chorion, the outer layer of the *Drosophila* eggshell made up of proteins synthesized and secreted by follicle layers surrounding the oocyte (McLaughlin and Bratu, 2015). This layer appears to be less robust in the mutant egg, and early evidence suggests

that expression of genes encoding components of this membrane may be dampened in *pele* mutants. Future studies will focus on the basis of these morphological changes and how they relate to disruptions in cytoplasmic RNA metabolism.

To identify where dSKI2 expression and normal function is important for fertility, we used cell-type specific drivers to restore the wild-type dSKI2 protein in two locations within the ovary: (1) somatic follicle cells and (2) the germline. A rescue effect was observed when dSKI2 was expressed in follicle cells but not in the germline—a finding that suggests dSKI2 and SKI complex function in the somatic follicle cells is critical for fertility. The expression pattern of the *c355-GAL4* driver was confirmed by subsequent imaging of the ovarioles of rescued flies, which showed that expression is limited to follicle cells at late stages of oogenesis. Ongoing studies seek to identify the precise role of dSKI2 within follicle cells in maintaining fertility. In addition, we plan to investigate mechanisms underlying the infertility observed in male mutants by characterizing male reproductive function and performing rescue experiments utilizing other cell-type specific drivers.

We expect that our findings regarding the basis of the *pele* infertility phenotype will converge with those on molecular mechanisms which confer protection against environmental stress. The results presented in this thesis identify a novel and interesting role of cytoplasm RNA degradation as a common mechanistic basis of both resistance to degeneration in response to environmental stress and fertility. Improving our understanding of the cellular mechanisms that lead to environmental stress-induced degeneration may facilitate the development of gene targeted therapies for patients suffering from neurodegenerative disease.

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RESEARCH EXPERIENCE

2015-2018 The Pennsylvania State University | Dr. Richard Ordway & Dr. Fumiko Kawasaki
Undergraduate Researcher in Molecular Neuroscience and Genetics
Goal: Utilize *Drosophila* to investigate how genetic and environmental factors interact in degenerative disease

- Helped generate transgenic lines for use in a genetic model of heat shock stress-induced degeneration
- Gathered data that could be used in future targeted gene therapies for neurodegenerative disorders like Parkinson's disease

Skills obtained: Transgenic line generation, Western blot techniques, Drosophila care and maintenance, and molecular biology techniques including PCR, gel electrophoresis, and DNA extraction, purification, and digestion.

Poster Presentation & Publication:

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Kawasaki, F., Koonce, N.K., Guo, L., Fatima, S., Qiu, C., **Moon, M.T.,** Zheng, Y., Ordway, R.W. (2016). Small heat shock protein-mediated cell-autonomous and -nonautonomous protection in a Drosophila model for environmental stress-induced degeneration. *Dis. Model Mech.* 9:953-64. doi: 10.1242/dmm.026385.

TEACHING EXPERIENCE

Fall 2017 Learning Assistant, General Biochemistry I (BMB 401)
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- Facilitated active learning and problem-solving activities in lectures
- Co-led BMB 398B supplemental course with ~60 students
- Met weekly with course instructor and helped lead exam reviews

Fall 2016 Teaching Assistant, Honors Biology: Molecules and Cells (BIOL 230M)
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- Led selected lab discussions and graded all lab assignments
- Taught molecular biology techniques to a section of 20 undergraduate students
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HONORS AND AWARDS

2014-2018 Schreyer Academic Excellence Scholarship, Penn State Provost Scholarship
2017 Evan Pugh Scholar Senior Award for being in upper 0.5% of class
2016-2017 Edward C. Hammond Jr. Memorial Scholarship for academic achievement
2016 J. Ben and Helen D. Hill Memorial Scholarship Fund, awarded on basis of nomination for research achievement
2016 Louis A. Martarano Endowment for Education Abroad, awarded by the Eberly College of Science for French embedded study abroad
2015 President's Freshman Award for outstanding freshman earning a perfect 4.0 GPA

SERVICE & LEADERSHIP

2015-2018 Executive and Brigade Member | Global Brigades Public Health & Medical

- Elected to organize fundraising efforts and educate members about global health disparities
- Traveled on two weeklong brigades to Panama and Honduras
- Helped run medical clinics with local physicians and build infrastructure to reduce transmission of preventable diseases

2016-2017 Patient Floors & Emergency Department Volunteer | Mt. Nittany Medical Center

- Discharged patients and delivered medications on all patient floors
- Transported patients for CT and MRI scans in the Emergency Department

2014-2016 Pen Pal Chair | Penn State Dance Marathon Hospitality Committee

- Largest student-run philanthropy culminating in 46-hour dance marathon
- Raised $9,770,332 in 2016 for pediatric cancer treatment and research
- Acquired and distributed food and meals to dancers and THON families

2013-2017 Summer Counselor | Royal Family Kids Camp New Castle

- Camp to provide kids in local foster care system with week of positive experiences in a safe, supportive environment
- Relief and full-time trained counselor responsible for care of two assigned campers ages six to twelve

INTERNATIONAL EXPERIENCES

July 2015 PSU-Peking University Study Abroad Exchange | Beijing, China

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March 2016 France and the Holocaust in Film & Literature Study Abroad | Paris, France

- Met and interviewed French Holocaust survivors and members of French Resistance efforts, who hid vulnerable individuals or aided in escape