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Generation of a deletion of the draper gene in Drosophila melanogaster using CRISPR/C

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Thesis

GENERATION OF A DELETION OF THE *DRAPER* GENE IN *DROSOPHILA MELANOGASTER* USING CRISPR/CAS9

by

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DEDICATION

I dedicate this master's thesis to my family for pushing and encouraging me to finish. Thank you for always believing in me.

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The help and support of many people contributed towards finishing this thesis. I would like to start off, by thanking my advisor Dr. Kimberly McCall for all her guidance in helping me navigating the process of writing a thesis. Thank you for having the patience for answering my many questions that I would always have. Next, I would like to thank Dr. Jeanne Peterson for her knowledge of *Drosophila* especially regarding fly crosses. Then, I would like to thank the McCall lab for always being so supportive and welcoming during my time at BU.

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GENERATION OF A DELETION OF THE *DRAPER* GENE IN *DROSOPHILA MELANOGASTER* USING CRISPR/CAS9

TRUNG LE

ABSTRACT

Cells within the body undergo death on a daily basis and those dead cells are constantly being removed so as to prevent accumulation of dead cells that can further cause negative effects. In *Drosophila,* an important gene necessary for clearance of dead cells is *draper*. This gene encodes a phagocytic receptor essential for clearance of dead cells. This project explored using CRISPR/Cas9 to edit the *Drosophila* genome to delete *draper* to create a mutant. Guide RNAs were designed to produce DNA breakpoints 4.5 kB apart, flanking most of the *draper* coding region. Oligonucleotides with the guide RNA sequences were cloned into an expression vector, and the vectors were injected into Cas9-expressing flies. Candidate mutants were dissected and their ovaries were analyzed for defective cell clearance. Two lines were shown to have defective cell clearance in the ovary and are potential new deletion alleles of *draper*.

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LIST OF ABBREVIATIONS

C……Celsius

Ced-1.....Cell death abnormality protein 1

DAPI……...4', 6-diamidino-2-phenylindole dichloride

diH_2O……deionized water

Drosophila……..……..*Drosophila melanogaster*

drpr…….*draper*

E. coli……..…… *Escherichia coli*

gRNA.…….…… guide RNA

LB……Luria broth

mg……..milligram

mL……milliliter

mM.....millimolar

NEB.……New England Biolabs Buffer

Oligo..……oligonucleotide

PAM.....protospacer adjacent motif

PBS ..... phosphate buffered saline

PBT…….phosphate buffered saline with Triton X-100

PCR……..polymerase chain reaction

pnc.....persisting nurse cell nuclei

SOC …… Super Optimal broth with Catabolite repression

TAE……Tris-acetate-EDTA

TE…….buffer containing Tris-HCl and EDTA

µg ..... microgram

µL……...microliter

µM……...micromolar

CHAPTER ONE

Introduction

1.1 Cell Death Overview

Cell death is an essential process that occurs on a daily basis in order to maintain homeostasis within the body. This process occurs when cells begin to decline in function and cease to be useful (Kroemer et al., 2009). Cell death has many purposes that are essential to life. For example, during development it is used to sculpt the digits while in adulthood it occurs to eliminate dangerous or injured cells to make room for new ones (Jacobson et al., 1997). Keeping this in balance are the many forms of cell death that occur (Figure 1.1).

1.2 Phagocytosis

Following cell death, phagocytes engulf and ingest dead cells and debris. The phagocytic process can be broken into four steps. First, a dying cell emits “find me” signals such as ATP, that spread out and form a concentration gradient. These signals, which are conserved across animals, activate nearby phagocytes who use the concentration gradient as a guide to maneuver towards the dying cell (Galluzzi et al., 2018). After the phagocytes come into close proximity, their receptors can encounter and recognize different signals on the surface of the dying cell. Then if the cell expresses “eat me” signals, such as phosphatidylserine, the phagocyte will undergo cytoskeletal rearrangement to form a phagocytic cup that will surround the dying cell. Finally, once the apoptotic cell is in a phagosome within the phagocyte, it is processed and subsequently degraded by the lysosome (Chekeni & Ravichandran, 2011). This process is

largely conserved between species. Phagocytosis plays an important role in mammals helping to clear out cells that are deemed foreign, harmful, or dead serving as part of the immune system's defenses (Stuart & Ezekowitz, 2005). Besides involvement in the immune system, phagocytosis also plays a role during oogenesis in *Drosophila melanogaster*. One gene responsible for phagocytosis in *Drosophila*, *draper*, is required in follicle cells for nurse cell removal (Timmons et al., 2016). This is important since without it, fertility is reduced.

1.3 *Drosophila* Ovary Overview

The *Drosophila* ovary is a powerful model system in cell biology research, including for the study of phagocytosis. The ovaries of the female *Drosophila* are large organs that occupy much of the region of the abdomen. The ovary consists of strands of egg chambers called ovarioles. Within an ovariole is an egg chamber, consisting of 3 distinct cell types: nurse cells, the oocyte, and follicle cells (Buszczak & Cooley, 2000). The outer somatic region consists of a single layer of small epithelial follicle cells that are easily distinguished from the germline (Buszczak & Cooley, 2000). The ovariole is essentially an egg chamber assembly line. Egg chambers are first produced in the germarium which houses the germline and somatic stem cell niche. Once the egg chamber exits the germarium, it undergoes 14 stages of development before being deposited out the oviduct (Frydman & Spradling, 2001).

1.3.1 Developmental Nurse Cell Death

Developmental nurse cell death can be observed occurring during stages 10 through 14 of egg chamber development (Figure 1.2). Developmental death begins with

the nurse cells transporting their contents into the oocyte. This begins at stage 10 and is finished by stage 12. Degradation of nurse cells occurs as they discard their contents. Simultaneously, stretch follicle cells invade the space surrounding the nurse cells and encapsulate them during stage 13 (Lebo & McCall, 2021). Eventually, the nurse cells become acidified and degrade away. By stage 14, which is identified by the appearance of two dorsal appendages, most, if not all, of the nurse cells should be gone (Timmons et al., 2016).

1.4 *Draper*

Draper, found in *Drosophila*, is a gene that encodes for an engulfment receptor that plays a significant role in *Drosophila* development and homeostasis. Draper, a transmembrane protein, is also a homolog of the Ced-1 protein found in *Caenorhabditis elegans* (Fullard & Baker, 2015). Draper recognizes and responds to signals sent by cells that need to be cleared. These cells are then engulfed and disposed of. This is the process of corpse cell clearance. Mutations or RNAi interference knockdowns of *drpr* lead to accumulation of nurse cells, as there would be not be the usual cell death and clearance processes involving the stretch follicle cells (Timmons et al., 2016). The continual lack of cell death within the egg chambers would eventually disrupt other cellular processes. This disruption in the function of *drpr* can be seen phenotypically in the ovaries and other organs such as the brain (Etchegaray et al., 2016).

1.5 CRISPR/Cas9

To better comprehend the relationships that exist between genotype and phenotype genetic tools are used. In this thesis, the CRISPR-Cas9 system is used to alter

the *Drosophila* genome. This technology was derived from the natural defense mechanisms of bacteria and archaea. These organisms use CRISPR based RNA and Cas proteins to destroy the DNA of foreign genomes (Ratner et al., 2016). Adapting this system from bacteria and archaea allowed manipulation of whole genomes and modification of gene function.

1.5.1 CRISPR/Cas9 Components

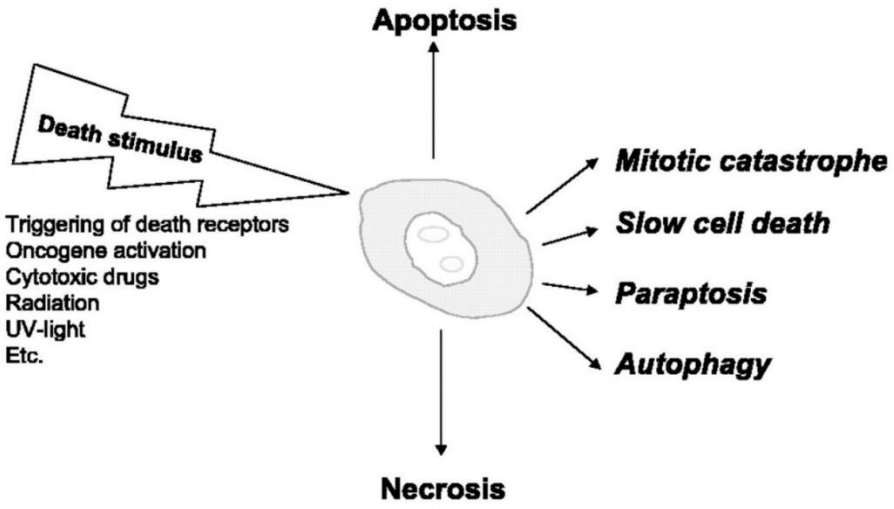
The CRISPR-Cas9 system can be divided into two essential components: the Cas9 endonuclease and guide RNAs (Ratner et al., 2016). The Cas9 endonuclease acts as molecular scissors to cut the genome while the guide RNAs help to guide the "molecular scissors". The guide RNA contains a 20 nucleotide sequence adjacent to a PAM sequence that helps to guide the Cas9 to a specific genomic locus (Bier et al., 2018). It also contains a scaffolding sequence required for the Cas9 to bind. Together the guide RNAs direct Cas9 to the chosen sequence (Port et al., 2014). In this thesis, two gRNAs were designed to guide Cas9 to two positions in the *draper* gene, with the expectation that the DNA would be cut at two positions and repaired using non-homologous end joining to produce a deletion of the sequence between the two gRNAs. This would lead to the creation of a new *drpr* deletion mutant.

1.6 Thesis Rationale

Programmed cell death and its clearance occurs as part of the natural order of processes within the body. To disrupt that process would bring about disorder within the body. *Drosophila* were chosen since they are inexpensive and have a short lifespan that can be easily studied. In *Drosophila*, *draper* plays a vital role in the clearance of dead

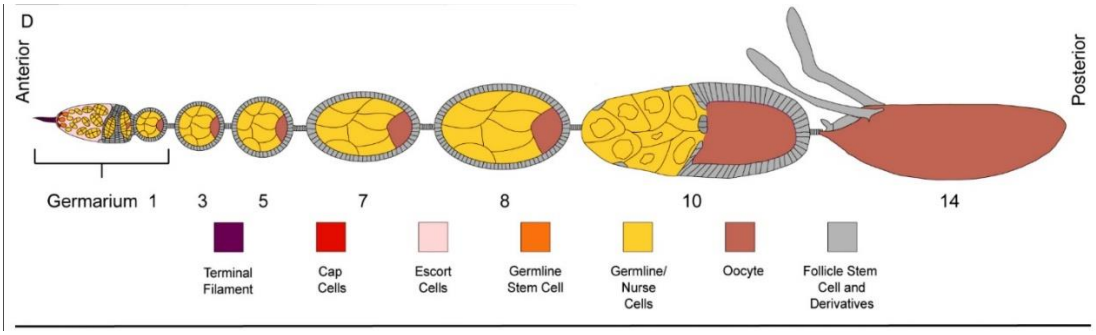
cells and debris formed during development. This project seeks to create a *draper* null mutant through the use of CRISPR/Cas9, as the existing Δ5 allele does not remove the *draper* coding region (Fullard & Baker, 2015) (Figure 1.3). This new mutation will result in the removal of the *draper* coding region ensuring that the gene function is completely disrupted. This will allow us to determine the extent to which engulfment of nurse cells is affected and provide a more complete understanding of *draper* function in the ovary. In future studies, this mutation in *draper* will provide the ability to visualize what effects result because of defective phagocytosis in other tissues.

Figure 1.1 Cell Death



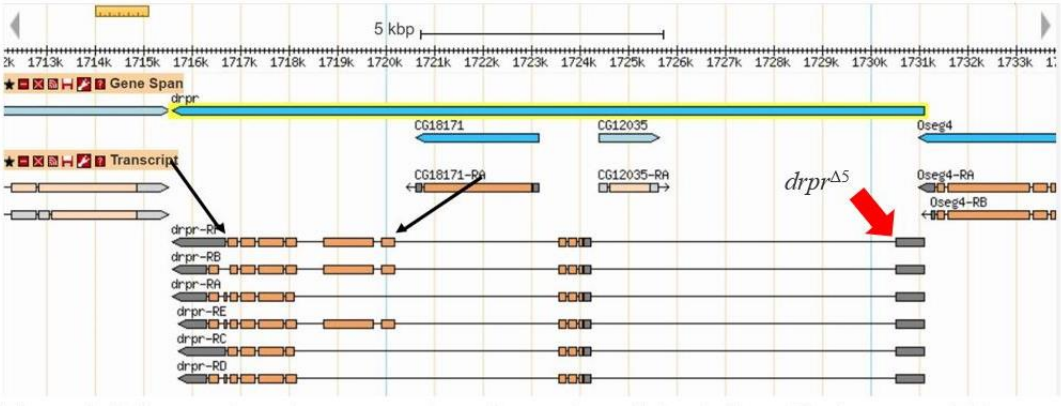
There are a billion cells within the body that become replaced on a daily basis. Cells receive a signal to initiate cell death whether it is because they become old and need to be replaced or they get damaged and stop functioning properly. These cells are disposed of in many ways. Taken from (Broker et al., 2005).

Figure 1.2 Developmental Death in *Drosophila*



There are 14 stages of egg chamber development from which germline stem cells divide leading to the development of nurse cells (yellow) and at the end an egg. Around stage 10, stretch follicle cells (small gray cells surrounding nurse cells) clear away the nurse cells. In the end, at stage 14 the egg chamber is cleared of nurse cells leaving the oocyte (brown). Taken from (Lebo & McCall, 2021).

Figure 1.3 Genomic Organization and Manipulation of *draper*



Shown is the *draper* genomic region (highlighted blue bar) located on the 3rd chromosome. *drpr* is transcribed from right to left relative to the chromosome. Splice variants are shown at the bottom with coding regions in gold, and non-coding regions transcribed in gray. The goal of this experiment is to remove most of the coding region of *drpr* as between the black arrows. This would result in inactivating the gene and result in the creation of a null *drpr* mutant. The widely used *drpr* $^{\Delta 5}$ mutant indicated by the red arrow, affects the upstream non-coding region. Modified from GBrowse (FlyBase).

CHAPTER TWO

Materials and Methods

2.1 Bacteria Cultures

Glycerol stocks made by a previous graduate student (Alla Yalonetskaya) were used. These stocks contain 500 µL of inoculated liquid culture made from bacteria containing a guide RNA expression plasmid (Addgene #49410, plasmid pCFD3:dU6, Figure 2.1). These glycerol stocks were kept at -80°C. After the glycerol stock was thawed slightly, an inoculation loop was used to streak some of this thawed culture onto an LB (Luria Broth) plate with 100 µg/mL Ampicillin. Individual colonies were selected and then used to inoculate 15 mL conical tubes of 2 mL LB broth with 100 µg/mL Ampicillin. These tubes were incubated at 37°C in an incubator shaker overnight.

2.2 Miniprep Protocol

The liquid cultures were miniprepped using Promega's A1460 miniprep kit. 1.5 mL was pipetted into 1.5 mL Eppendorf tubes with the remaining liquid culture saved. These tubes were centrifuged at 4,000 rpm for 5 minutes. Then the pellet was resuspended with 250 µL of Cell Resuspension Solution. 250 µL of Cell Lysis Solution was added to the sample which was then inverted several times to mix. 10 µL of Alkaline Protease Solution was added before the sample was inverted and then incubated at room temperature for 5 minutes. 350 µL of Neutralization Solution was added before inverting. The tube was centrifuged at 4,000 rpm for 10 minutes. The lysate was pipetted into a tube containing a spin column then centrifuged for 30 seconds at 4,000 rpm. After discarding flowthrough, 750 µL of Wash Solution was added before centrifuging at 4,000 rpm for 1

minute. This was repeated twice before centrifuging at 4,000 rpm for 2 minutes. The spin column was transferred to a sterile 1.5 ml Eppendorf. 100 µL of Nuclease-Free Water was added before the tube was centrifuged at 7,000 rpm for 30 seconds. The spin column was discarded, leaving purified DNA.

2.3 Plasmid Restriction, Digestion, and Purification

2.3.1 Preparing the Plasmid

The plasmid was restriction digested to insert gRNA sequences. In a 1.5 mL Eppendorf tube, 10 µL DNA, 1 µL BbsI, 3 µL Buffer 2.1 (NEB Buffer), and 6 µL diH_2O were added for a total of 20 µL. The tube was inverted several times to mix the mixture before being incubated at 37°C for 1 hour.

2.3.2 Gel Electrophoresis Protocol

The agarose gel was prepared by combining in a flask 0.8g (or 1.5 g) agarose and 100 mL 1X TAE buffer (Tris-acetate-EDTA). The mixture was heated for 1 minute in a microwave until the agarose dissolved. The solution was cooled for 10 minutes before 10 µL of SybrSafe (10,000x) was added. The solution was stirred until a uniform orange color was present. A ten-lane comb was placed in the cassette of the gel box before the cooled agarose solution was poured, leaving a small gap between the teeth of the comb and the gel. The gel was hardened for 5 minutes. The comb was removed and 1X TAE buffer poured until the gel was covered by a thin layer of buffer. Then the molecular ladder (NEB) and samples were pipetted into the desired wells. The black and red portions of the lid were connected to their respective colored electrode. The power source was turned on and the gel was run at 100 volts for 40-50 minutes before being shut off.

2.3.3 Gel Staining and Analysis

To visualize the bands of DNA, the gel was placed onto a transilluminator. The gel had SybrSafe added to its mixture, and did not require UV light to be viewed. The transilluminator was turned on, with blue light illuminating the gel from underneath. A photo of the gel was taken and used for analysis (Figure 2.2).

2.3.4 DNA Extraction and Purification

To isolate the completely digested DNA, bands were extracted and purified from the gel. The DNA bands were excised with a razor blade then weighed. Then for each 100 mg of agarose gel 200 µL of Buffer NTI was added (NucleoSpin Gel and PCR Clean-up kit # 740595.150). The gel slice and buffer were added to a 1.5 mL Eppendorf then incubated for 5 minutes at 50°C. The mixture was pipetted into a spin column inserted into a collection tube. The tube was centrifuged for 30 seconds at 11,000 rpm700 µL Buffer NT3 was added and then centrifuged for 30 seconds again before flow through was removed. The tube was then centrifuged for 1 minute at 11,000 rpm then the spin column placed into a new 1.5 mL Eppendorf tube. 30 µL Buffer NE was added and before being incubated at room temperature for 1 minute. Then the tube was centrifuged for 1 minute at 11,000 rpm.

2.4 Cloning

2.4.1 Guide RNA Oligonucleotide Design

Guide RNAs (gRNAs) were designed using several websites. Harvard Medical School's fly RNAi CRISPR tool was used to examine the *drpr* gene map and two gRNAs were chosen to excise the coding regions (Table 2.1). Using FlyBase BLAST analysis,

these two gRNAs were double checked for off-targets. After designing the two gRNAs, 24 base pair oligonucleotide (oligo) sequences were designed using the oligo design protocol from CRISPR Fly Design (Table 2.2). Then they were ordered from Eurofins Genomics.

2.4.2 Annealing and Ligating guide RNA Oligonucleotides

The 24 base pair oligo sequences were resuspended in 100 µM of 1X TE buffer. For annealing, 1.5 µL of both the forward and reverse oligos were mixed with 5 µL of 10X NEB buffer 3.1 as well as 42 µL of diH_2O for a total amount of 50 µL in a PCR tube. In a thermocycler, these samples were incubated at 95°C for 5 minutes, then 70°C for 5 minutes, with the temperature gradually lowering by 5°C every 5 minutes until room temperature.

For ligation to the plasmid, 1 µL of the annealed oligo pair, 3 µL cut plasmid, 2 µL 10X ligase buffer (NEB), 13 µL diH_2O, and 1 µL T4 ligase (NEB) were combined for a total of 20 µL in a PCR tube. The samples were ligated overnight at 16°C in a thermocycler.

2.4.3 Transformation

The transformation protocol for C3019 NEB 10-beta Competent *E. coli* cells was followed. A tube containing competent *E. coli* cells was thawed in a bucket of ice. 5 µL of plasmid DNA was pipetted into the tube which was then flicked several times to mix. The tube was placed on ice for 30 minutes before being heat shocked at 42°C in a waterbath for 30 seconds. Then the tube was placed on ice for 5 minutes. 950 µL of SOC (Super Optimal broth with Catabolite repression) outgrowth medium was pipetted into

the mixture. The tube was then placed at 37°C for 60 minutes and shaken at 250 rpm. 100 µL of the cell mixture was pipetted onto LB plates containing 100 µg/mL Ampicillin. These plates were incubated at 37°C overnight and screened for successful transformants that contained the Ampicillin resistance marker.

2.5 Midiprep Protocol

Transformed colonies were used to inoculate 50 mL conical tubes containing 25 mL of LB broth with 100 µg/mL Ampicillin to create liquid cultures that were then midiprepped using QIAGEN's midiprep kit 12143. These liquid cultures were centrifuged at 6,000 G for 15 minutes at 4°C. The pellet was resuspended in 4 mL of Buffer P1. 4 mL of Buffer P2 was added before inverting several times to mix. Then the mixture was incubated at room temperature for 5 minutes. 4 mL of Buffer P3 was added before inverting several times to mix. This was incubated on ice for 5 minutes. The conical tube was centrifuged at 20,000 G for 30 minutes at 4°C. The supernatant was transferred to another tub then centrifuged at 20,000 G for 15 minutes at 4°C.

A QIAGEN-tip was equilibrated with 4 mL of Buffer QBT. After the column was equilibrated the supernatant from before was pipetted to the equilibrated column. The column was washed with 10 mL of Buffer QC twice. DNA was eluted into a 15 mL conical tube after pipetting 5 mL of Buffer QF into the column. DNA was precipitated after adding 3.5 mL of room temperature isopropanol. Then the tube was centrifuged at 15,000 G for 30 min at 4°C. The DNA pellet was washed with 2 mL of room temperature 70% ethanol and centrifuged at 15,000 G for 10 minutes. The supernatant was decanted

and the pellet was air dried for 10 minutes. After drying, the pellet of DNA was redissolved in 50 µL 10 mM Tris-HCl, pH 8.5.

2.6 Sequencing

After being midiprepped, the plasmids were restriction digested with BsaAI then analyzed via gel electrophoresis for confirmation of a successful cloning. Then, these transformants were sent to Genewiz for sequencing for further confirmation. The gene sequences from Genewiz were aligned with pCFD3:U6 gene sequence and analyzed via NCBI BLAST to determine if the colonies contained the insert.

2.7 CRISPR/Cas9 *Drosophila* Manipulation

Drosophila melanogaster vas-Cas9 embryos (BDSC stock 51323) were injected by BestGene Inc. with plasmids expressing two 23 nucleotide guide RNAs designed to target regions just outside the coding regions of *drpr*-RF (Figure 1.3). The guide RNA sequences were ACTTAAACTTACGTACCAGTTGG and AGAGAGAGAGAGTGGAGTGTAGG.

2.8 Fly Husbandry

Injected larvae of the genotype *y w M RFP, GFP, vas-Cas9/FM7* were received from BestGene Inc. Balancer fly lines of the genotype *CyO/If*; *TM6B/MKRS* and *Sco/CyO*; *MKRS/TM6B* were obtained from Dr. Jeanne Peterson. These flies were raised on a mixture of cornmeal and molasses food and kept at 25°C in an incubator. These flies were crossed to flies injected with plasmids (G_0), to prevent recombination on the third chromosomes. Individual progeny from these crosses (G_1) were crossed again to the balancer fly line to make individually balanced candidate mutation lines.

2.9 Fly Dissection

Flies were conditioned, for two to three days prior to dissection. They were fed a yeast paste in order to enlarge the ovaries. Flies were then placed onto a fly pad with CO_2 turned on to anesthetize the flies. Then in a glass well filled with 1X PBS (phosphate buffered saline) the fly was positioned under the surface by one set of forceps while another was used to gently pull the abdomen of the fly to tease out the ovaries. The ovaries were extracted and then transferred using a glass pipette into a 1.5 mL Eppendorf tube containing 300 µL of PBS.

2.10 DAPI Staining

To visualize the egg chambers, the ovaries were stained with DAPI (4', 6-diamidino-2-phenylindole dichloride). This process allows fixing of cells as well as adding the ability to fluorescently visualize DNA. In a Eppendorf tube containing the ovaries in PBS, 200 µL heptane and 100 µL 16% paraformaldehyde was added. The tube was inverted several times to mix the solutions. Samples were rotated on a rotator for 20 minutes then removed and the fixative solution disposed of. The ovaries were rinsed twice with PBT (phosphate buffered saline with Triton X-100). New PBT was added before placing samples on a rotator for 20 minutes. Old PBT was removed before new PBT was added. Samples were rotated for 20 minutes. These steps were repeated twice. PBT was removed and then samples rinsed with 1X PBS. 1X PBS was removed and then two drops of Vectashield DAPI were added. Samples were stored at 4°C overnight or left for 1 hour at room temperature before being mounted on a glass slide.

2.11 Mounting Samples

Once the fly ovaries were fixed and stained, they were transferred onto a glass slide with a glass pipette. A glass coverslip was placed over the ovaries. Then nail polish was brushed over the four edges of the coverslip to create a barrier to retain moisture and fasten the coverslip. The nail polish sat and dried for 10 minutes before the glass slide was examined.

2.12 Microscopy

Slides were analyzed under a Olympus BX60 microscope at 10-20X magnification with the DAPI filter applied. The egg chambers and ovarioles were examined to see if there were any persisting nurse cell nuclei (pnc) indicating defective cell clearance.

2.13 DNA Extraction

To analyze flies via PCR, their DNA was extracted. A fly was placed in a 1.5 mL Eppendorf tube filled with a 50 µL aliquot of squish fly buffer taken from a 1X stock made with 953 µL deionized H_2O, 10 µL 1 M Tris pH 8, 2 µL 0.5 M EDTA, and 25 µL 1 M NaCl and 10 µL 20 mg/mL proteinase K. Then the fly was smushed with a pestle. Buffer was removed and the sample incubated at 37°C for 20 min then at 95°C for a few minutes to inactivate Proteinase K. Adapted from (Kim et al., 2014).

2.14 PCR

PCR primers flanking the two gRNAs were designed using Eurofins Genomics PCR Primer Design Tool (Table 2.3). The primers were designed to be about 22 nucleotides long with at least 50% GC content. After the primers were designed, FlyBase BLAST was used to confirm that the primers were far enough upstream and downstream

of the gRNAs. DNA extracted from male flies was used to run PCR. The goal of running PCR was to verify that the CRISPR/Cas9 manipulation resulted in the deletion of *drpr*.

Table 2.1 Guide RNA Sequence

3’ End	ACTTAAACTTACGTACCAGTTGG
5’ End	AGAGAGAGAGAGGGAGTGTAGG

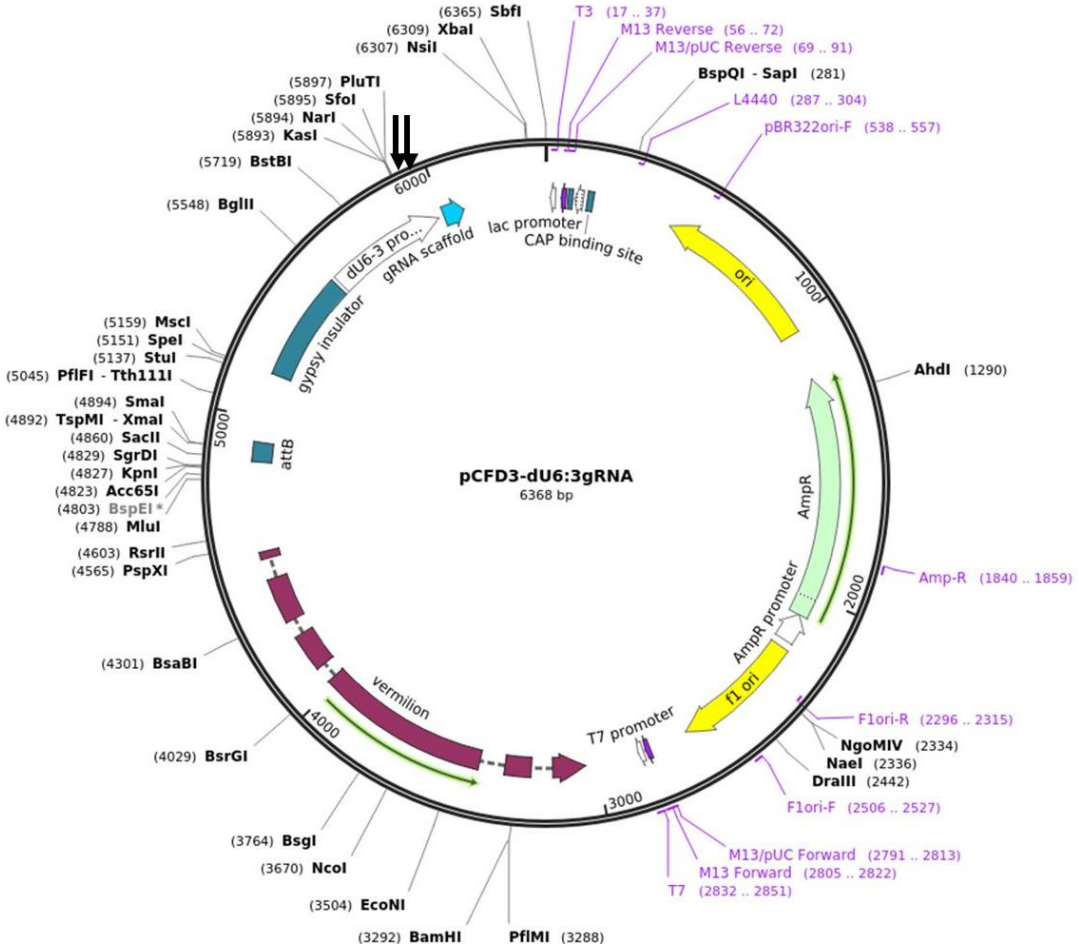
These two gRNAs were designed using FlyRNAi DRSC. They were chosen as points where the Cas9 will come and delete the portion of DNA between the two gRNAs

Table 2.2 Guide RNA Oligos

3’ End gRNA	Sense	GTCGACTTAAACTTACGTACCAGT
	Antisense	AAACACTGGTACGTAAGTTTAAGT
5’ End gRNA	Sense	GTCGAGAGAGAGAGAGTGGAGTGT
	Antisense	AAACACACTCCACTCTCTCTCTCT

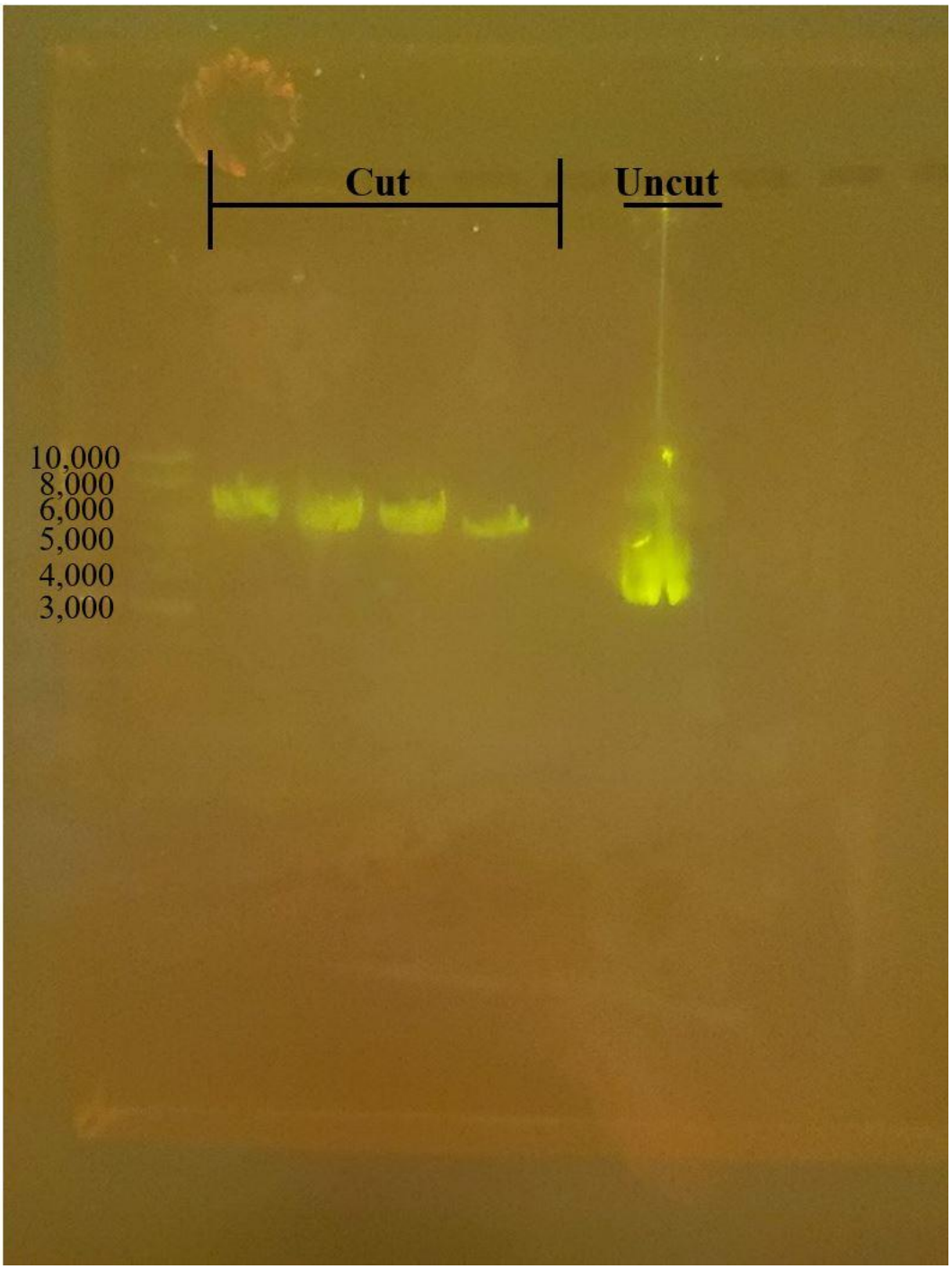
These oligo sequences for the gRNAs were designed using NCBI BLAST. They were designed following protocol from Addgene.

Figure 2.1 Plasmid Map



The arrows on the plasmid map indicate the Bbs1 cut sites, where after restriction digestion, the insert is added. This process will allow ligation of the annealed oligo pairs and the cut plasmid. Taken from (Port et al., 2014)

Figure 2.2 Vector Restriction Digestion



Agarose gel with Bbs1 digested pCFD3:dU6. Left lane - 1 kb DNA ladder. Lanes 1-4, cut DNA. The four bands of cut DNA are more condensed compared to the uncut DNA, and they run more slowly at the predicted size of 6.3 kb

Table 2.3 PCR Primers

3’ End gRNA	Forward	CGACAACACGAACACCAACC
	Reverse	TGCAAGACAAATGCTACGCTAC
5’ End gRNA	Forward	CCAAAGATCTGCACGTAGTGTC
	Reverse	TTTGGTGGTGGGGATTAGTGAC

The primer sequences were designed using Eurofins Genomics Primer Design Tool. These primers are used to amplify the genomic region surrounding the gRNA target sites.

CHAPTER THREE

Results

In *Drosophila*, the gene *draper* is essential for phagocytosis in many cell types. The genetic analysis of *draper* has relied on the Δ*5* allele which does not disrupt the coding region and could have residual activity. The goal of this project was to create a *draper* null mutant through the use of CRISPR/Cas9.

3.1 Guide RNAs

3.1.1 gRNA Identification

Two gRNAs were designed to be far enough upstream (5') and downstream (3') of the coding region of *draper* without disrupting other genes that map to the large introns of *drpr* (Figure 1.3). Included in the gRNA sequence in Table 1.1 are the PAM sequences which are adjacent to the genomic regions homologous to the gRNAs. These gRNAs were also screened for off target sites. After verifying that the target region was specific for the target of interest, 4 oligos to facilitate the cloning of the gRNAs were ordered from Eurofins Genomics. These oligos corresponded to two pairs corresponding to the 3' gRNA and the 5' gRNA.

3.1.2 Annealing and Ligating

The oligos were annealed and ligated into pCFD3:dU6 (see Methods). This process took several attempts to achieve results. In the first few attempts to transform cells using the prepared plasmid, there were no colonies that grew, but after plating a higher concentration of cells, enough colonies grew for analysis.

3.1.3 Screening

The oligos corresponding to the 5' gRNA had a BsaAI restriction site, which was helpful in identifying the correct clones. Four BsaAI cut sites were identified in pCFD3:dU6 (Figure 3.1) A 5th new cut site would appear if the plasmid insert was cloned correctly (Figure 3.1). The size and number of the DNA bands were predicted for colonies that had the insert or did not contain the insert (Figure 3.2). To determine if any of the clones had the insert, a restriction digest was performed with BsaAI then gel electrophoresis analysis performed (Figure 3.3). Sixty percent of the colonies were found to have bands matching the prediction, indicating that the insert was successfully cloned.

3.1.4 Sequencing

The DNA was purified and sent to Genewiz for sequencing. For the 3' gRNA, there were no diagnostic restriction digests possible, so eight individual colonies were analyzed directly by sequencing. The sequences were aligned by BLAST to the starting plasmid sequence. BLAST analysis revealed mismatches between pCFD3:dU6 and the cloned plasmid indicating that the correct gRNA sequence was cloned (Figure 3.4 and 3.5). There were confirmed to be 8 clones that had the correct insertion: 2 clones for the 3' gRNA plasmid and 6 clones for the 5' gRNA plasmid.

3.2 Injection

After confirmation that the cloned plasmid had the correct insertion, midiprep plasmid preparations were generated and plasmids were sent to BestGene Inc. for injection of *Drosophila* embryos. *Drosophila Cas9*-expressing embryos were simultaneously injected with 2 plasmids containing the 5' and 3' gRNAs in order to

create a deletion of *draper* between the two gRNAs. Out of the 200 injected embryos, 21 successfully matured into adults. From those 21 adults, 12 were sterile while 9 were fertile and able to be crossed. Those crosses provided many progeny that were analyzed for a *draper* mutation (Table 3.1).

3.3 Drosophila Cross Scheme

The 9 injected flies that were fertile were crossed following different schemes for G_0 males (Figures 3.6 and 3.6.1) and for G_0 females (Figure 3.7 and 3.7.1). Both male and female flies were crossed for several generations with flies that contained balancer chromosomes to prevent recombination of the third chromosome. Doing so would prevent loss of any newly generated mutations. After several generations of mating, the final generation of flies would have balanced chromosomes. This means that they would be stable enough for use as maintaining a future stock of a fly line as well as examining for the mutation of interest in homozygous flies.

3.4 Dissection Results

To determine if the fly lines carried a *draper* mutation, ovaries from homozygous females were dissected and analyzed by fluorescence microscopy for persisting nurse cell nuclei, a readily apparent phenotype that is seen in $drpr^{\Delta 5}$ mutants. Many flies were dissected and their ovaries were fixed, stained and examined for a *draper* phenotype. Out of the 86 fly lines that were examined via dissection (Table 3.1), two fly lines: M3-19M and M3-A were found to contain ovaries that displayed numerous amounts of persisting nurse cell nuclei suggesting that there was defective cell clearance (Figure 3.8). This evidence indicates that a *draper* mutant was created. Three other lines: M6-1F/2F, F6-

2M, and M3-C showed ovary phenotypes that were distinct from *drpr* and should be further characterized (Figure 3.9). These fly lines are a promising future direction to focus on.

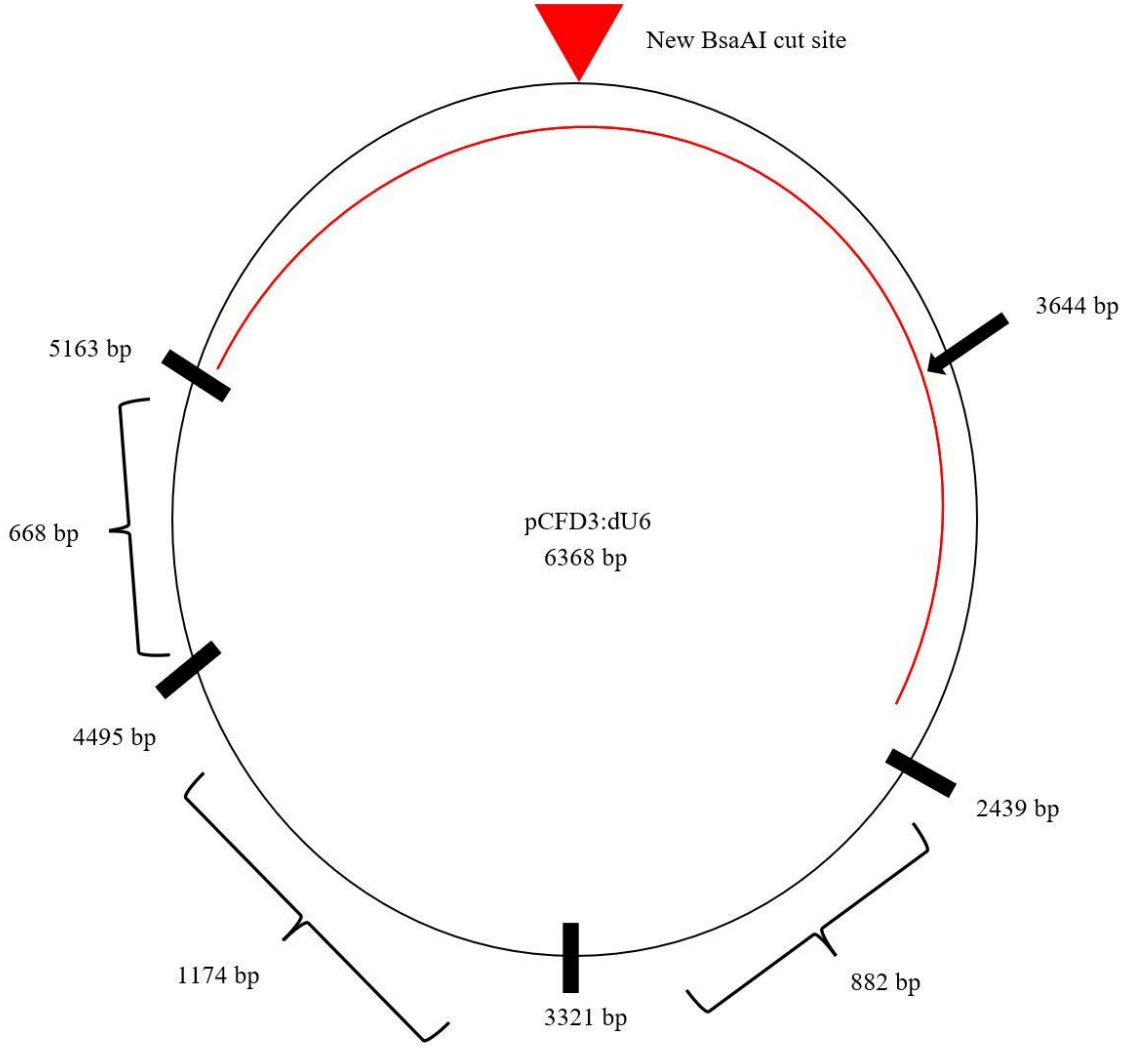
3.5 Draper Gene Sequence

Fly lines that were found to have persisting nurse cell nuclei will be analyzed further by PCR to determine if the *draper* deletion was generated. The sequence of the *draper* genomic region, and the sites of gRNA targeting is shown in Figure 3.8. When the gRNAs were expressed in the fly embryos, they dictated to the Cas9 where to target. The region in between the two gRNAs would be deleted and a mutation created as a result. For our *draper* gRNAs, that result would be a region the size of 4.5 kb (Figure 3.10). A deletion this size would ensure that the *draper* null mutation would be successful. However, it is possible that smaller deletions would also be generated.

3.6 PCR Results

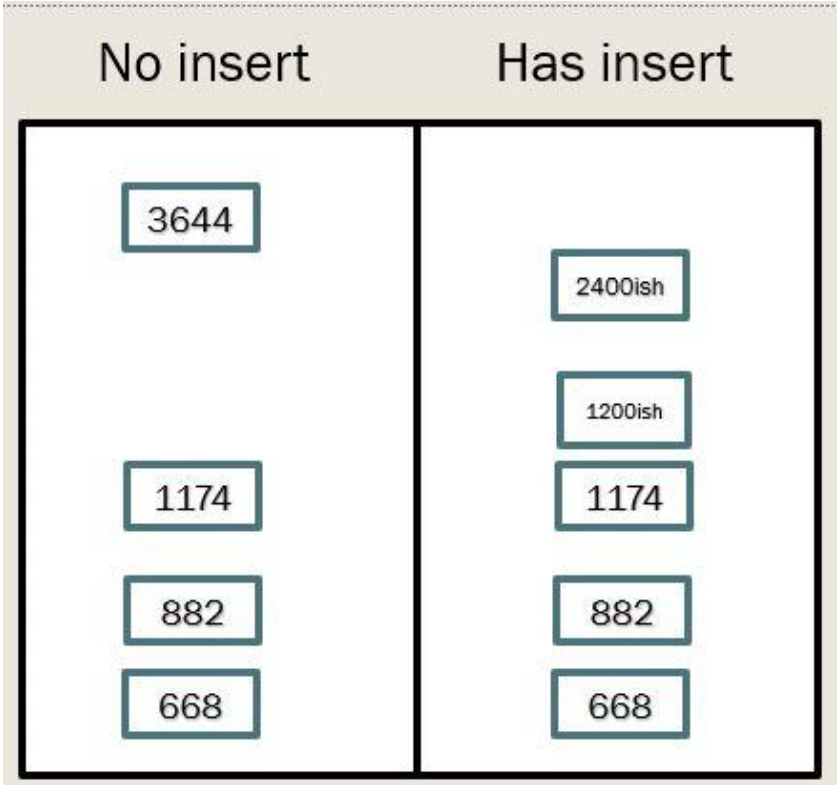
The M3-19M line that was found to contain persisting nurse cell nuclei was analyzed via PCR to identify mutations. PCR primers were designed to detect small deletions/insertions flanking the gRNA sites, or alternatively the pair of outside primers could be used to detect a larger deletion (Figure 3.10). There were no obvious deletions in the M3-19M line (Figure 3.11). Surprisingly, in the control line (M3-20M) tested by PCR, there appeared to be a small deletion. However, this is only apparent in the set of primers that correspond to the 5' end of *drpr*. This would indicate that a partial deletion occurred instead of the whole 4.5 kb deletion.

Figure 3.1 BsaAI Cut Sites



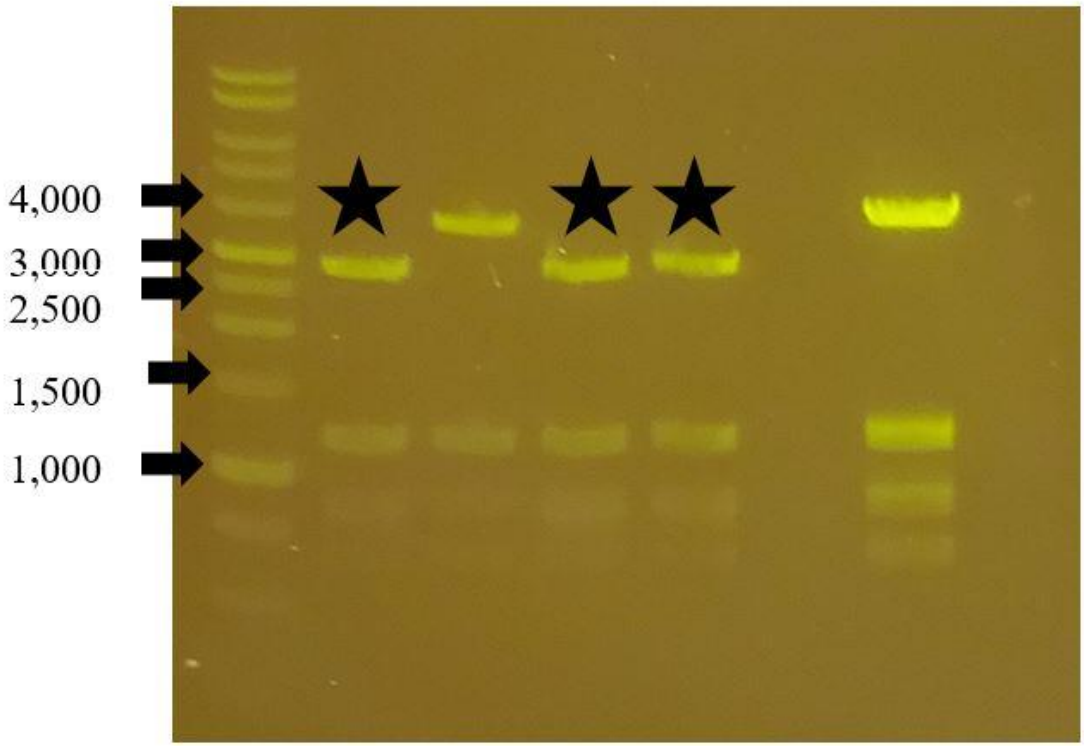
This plasmid map indicates where the cut sites of BsaAI are located in the pCFD3:dU6 plasmid. When the 5' gRNA insert is cloned into the plasmid, there is another cut site added at the location indicated by the red inverted triangle.

Figure 3.2 Predicted DNA Band Sizes



Transformed colonies of *E. coli* that contained the plasmid insert, were predicted to contain 5 bands after being restriction digested. Those that do not should only have 4 bands. Additionally, the 3.6 kb band will be cut and reduced to 2.4 kb if the cloning is successful.

Figure 3.3 Screening for the Plasmid Insert



Gel electrophoresis of BsaAI-digested colonies after transformation with oligos corresponding to the 3’ gRNA. Left lane, DNA ladder. Digested DNA from 5 colonies shown. The DNA in lanes 1, 3, and 4 indicated by the star symbol were found to contain the insert, whereas lanes 2 and 6 do not. The size of their DNA bands match the predicted sizes.

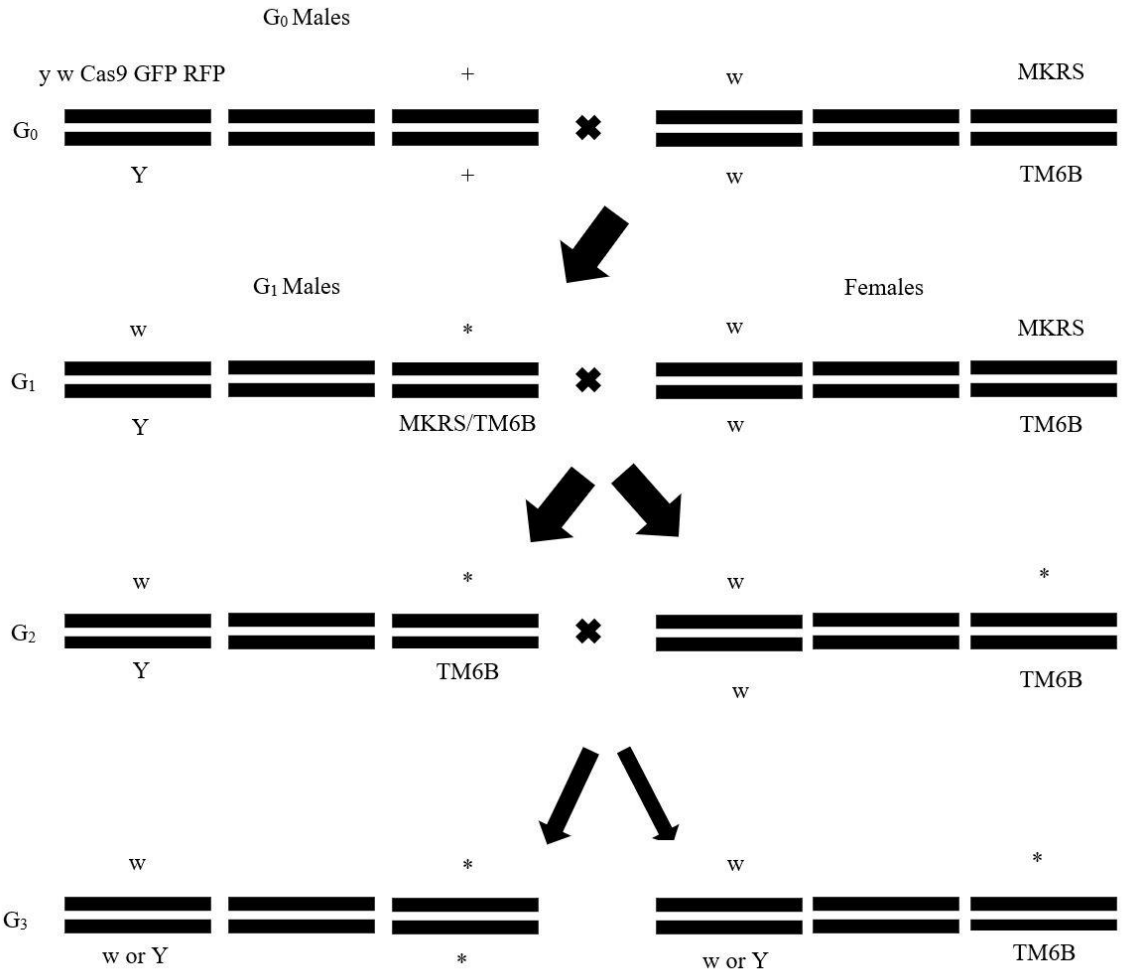
Figure 3.4 BLAST Analysis of 3’ gRNA Cloning

The subject is the DNA sequence while the query is the plasmid pCFD3:dU6. The underlined region is the gRNA. The cloned plasmid and original plasmid sequence were BLAST analyzed to ensure successful cloning. The mismatch region shows that the correct gRNA sequence was cloned.

Figure 3.5 BLAST Analysis of 5’ gRNA Cloning

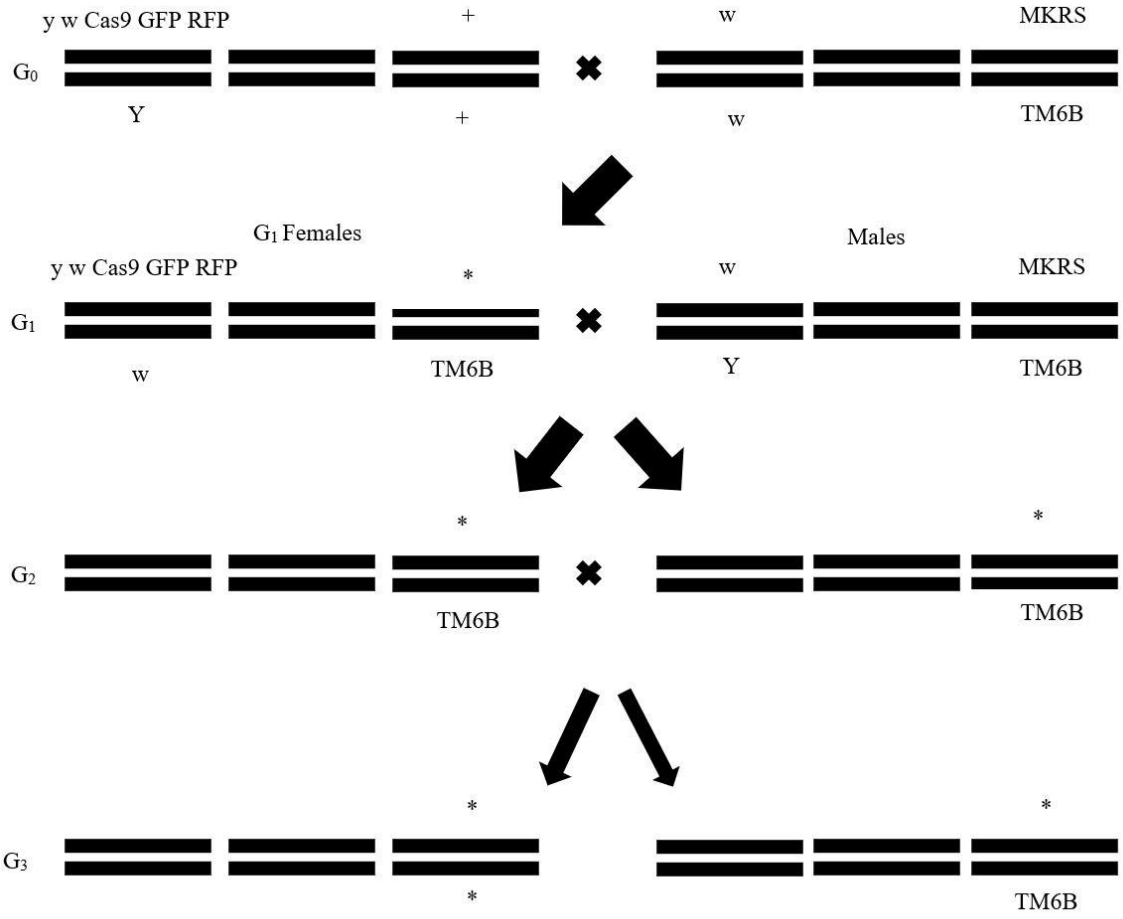
The subject is the DNA sequence while the query is the plasmid pCFD3:dU6. The underlined region is the gRNA. The cloned plasmid and original plasmid sequence were BLAST analyzed to ensure successful cloning. The mismatch region shows that the correct gRNA sequence was cloned.

Figure 3.6 G_0 Male *Drosophila* Cross Scheme



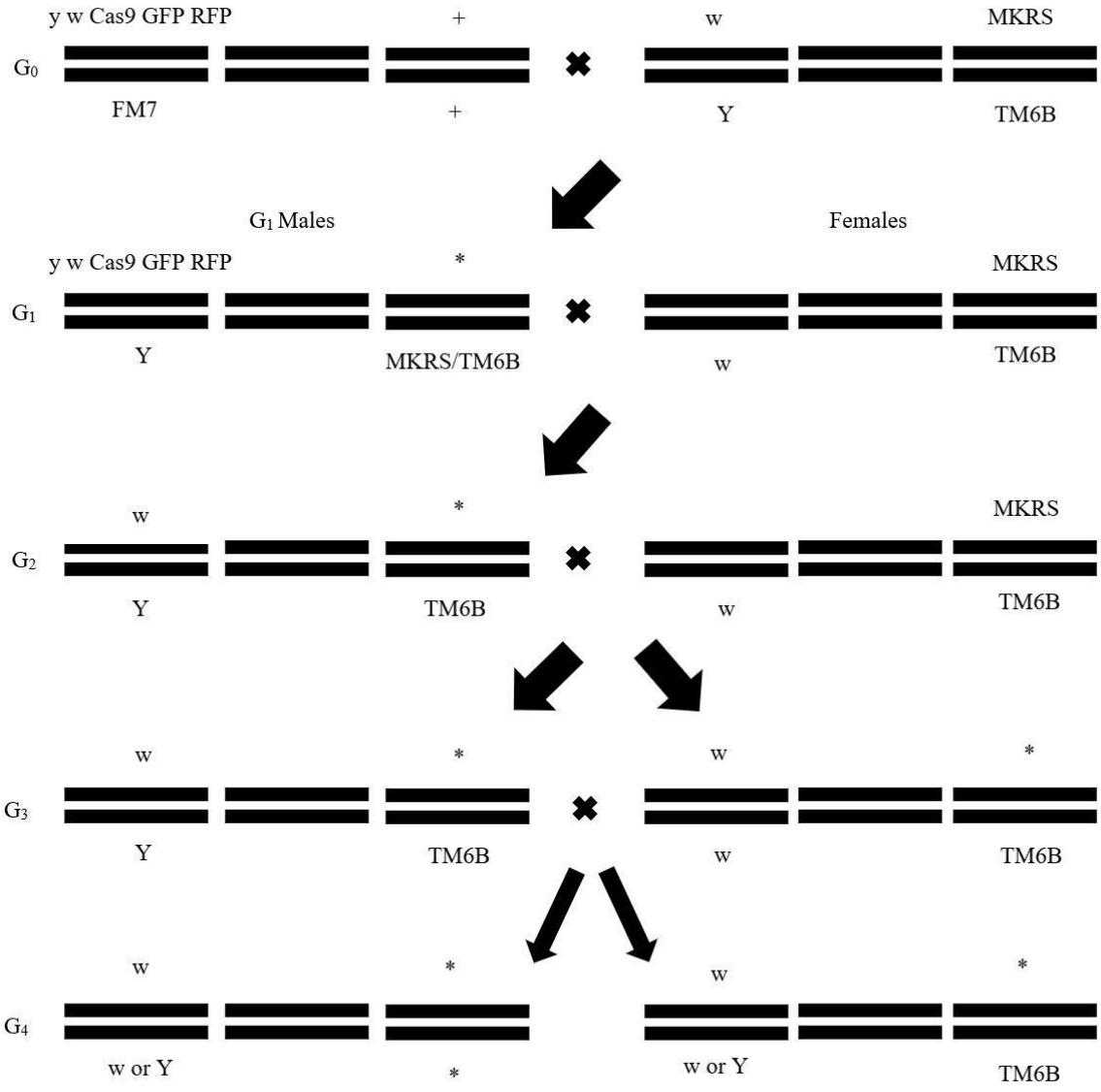
With the G_0 male fly cross scheme, there are several generations to produce the G_3. G_1 male flies were crossed with female flies with the *w/w; MKRS/TM6B* genotype. The G_2 flies were crossed with other progeny of G_2 to produce the G_3. The TM6B balancer chromosome was used to prevent recombination of the chromosomes and provide a dominant marker to identify the Cas9-targeted chromosome. The first set of bars with w/Y are the 1st chromosome. The second set of bars that are empty are the 2nd chromosome. The third set of bars with the * and other genotypes are the 3rd chromosome.

Figure 3.6.1 G_0 Male *Drosophila* Cross Scheme



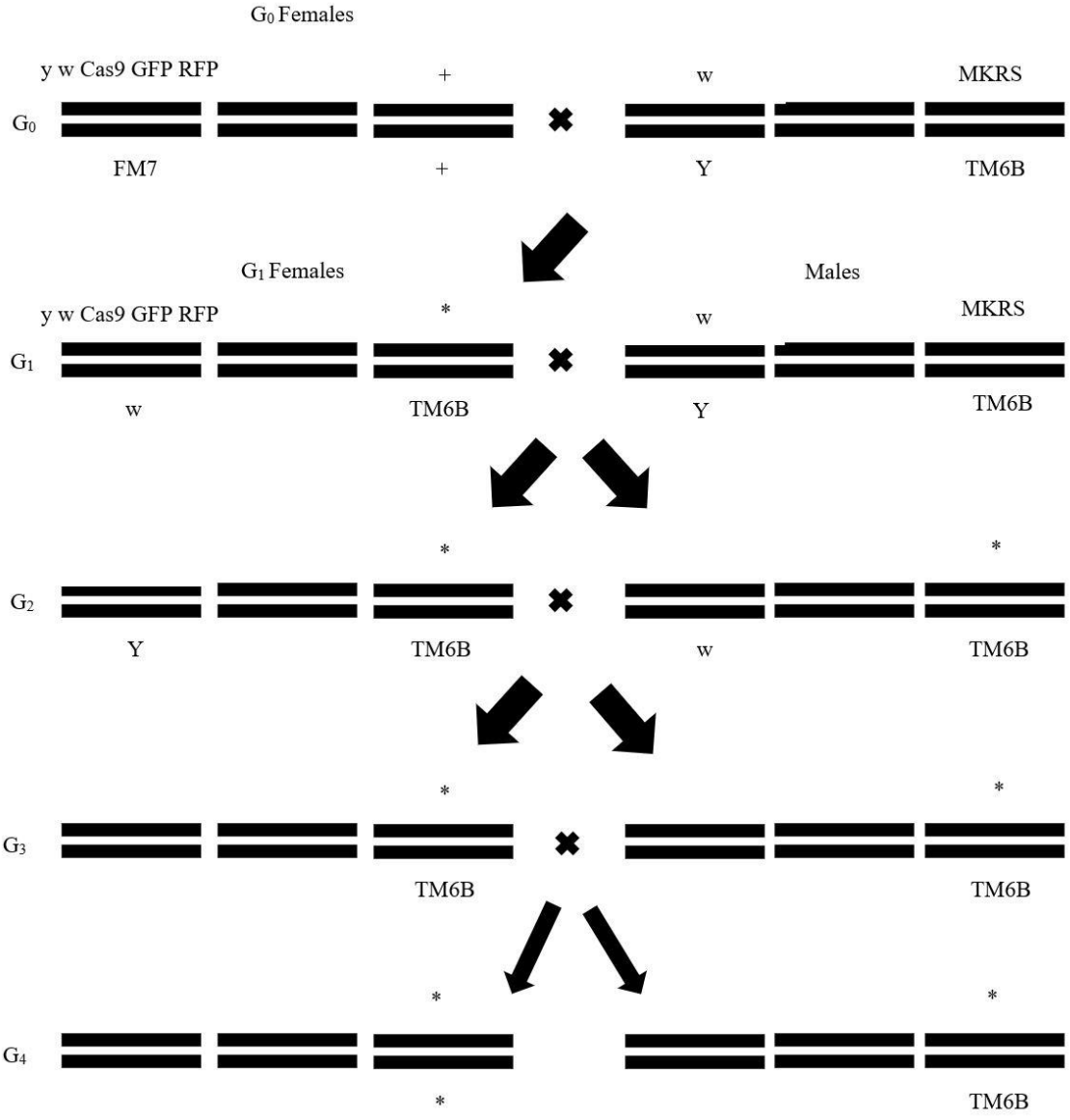
With the G_0 male fly cross scheme, there are several generations to produce the G_3. G_1 female flies were crossed with males flies of the genotype *w/Y; MKRS/TM6B* to produce the G_2 flies. Those G_2 flies were then crossed with other G_2 flies to produce the G_3. The resulting G_3 flies had balanced chromosomes and a dominant marker to identify the Cas9-tageted chromosome.

Figure 3.7 G_0 Female *Drosophila* Cross Scheme



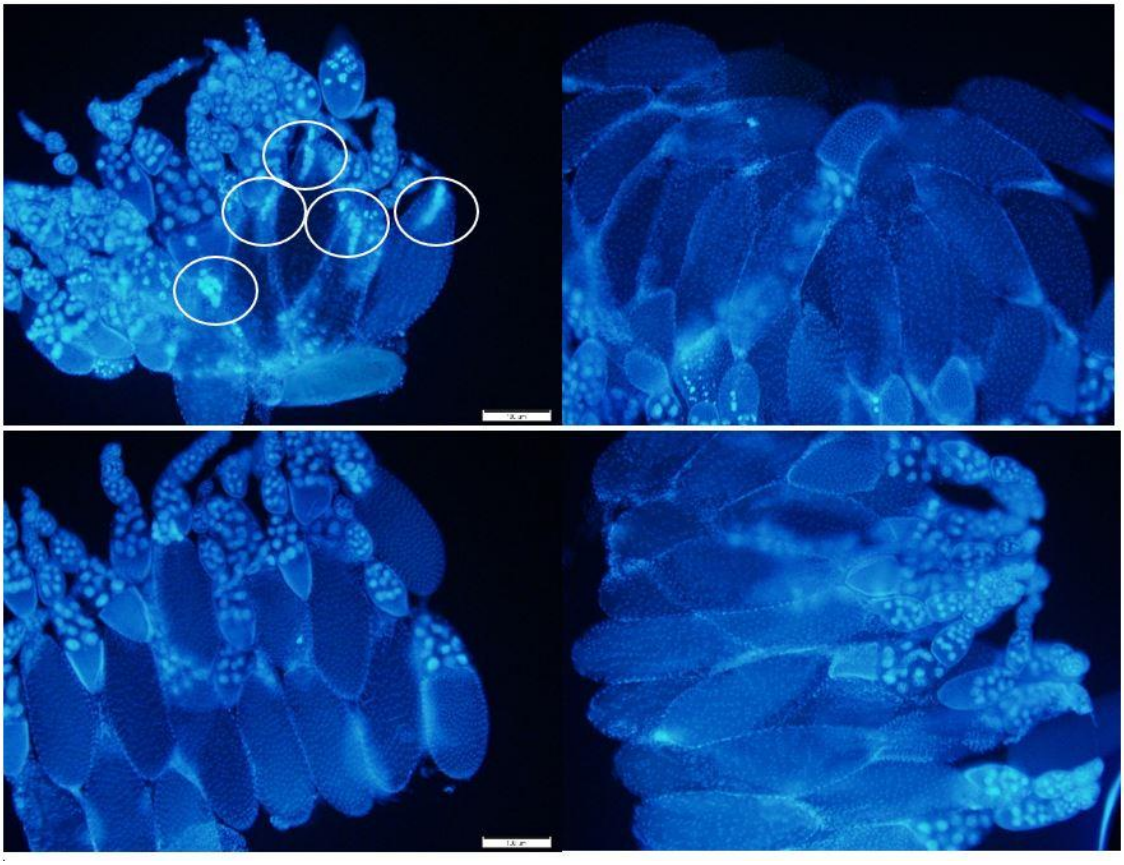
With the G_0 female fly cross scheme, there are several generations to produce the G_4. Flies with balancer chromosomes were used to prevent recombination of the chromosomes. The first set of bars with *w/Y* are the 1st chromosome. The second set of bars that are empty are the 2nd chromosome. The third set of bars with the * and other genotypes are the 3rd chromosome. The extra generation was included to remove the Cas9-carrying chromosome.

Figure 3.7.1 G_0 Female *Drosophila* Cross Scheme



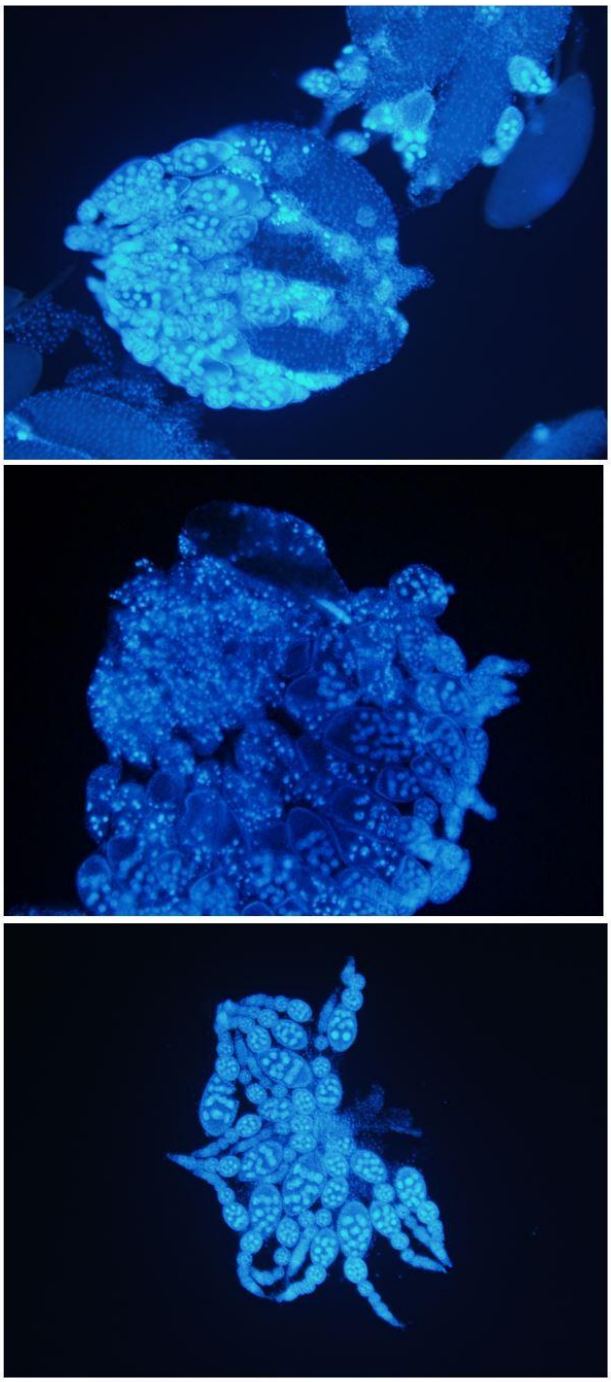
With the G_0 female fly cross scheme, there are several generations to produce the G_4. G_1 female flies were crossed with male flies of the genotype *w/Y;MKRS/TM6B* to produce G_2 flies. These flies were crossed with other G_2 flies to produce G_3 flies which were then crossed to G_3 flies to produce G_4 flies. The G_4 flies would have balanced chromosomes and a dominant marker to identify the Cas9-tageted chromosome.

Figure 3.8 Fluorescence Microscopy of G_0 Progeny



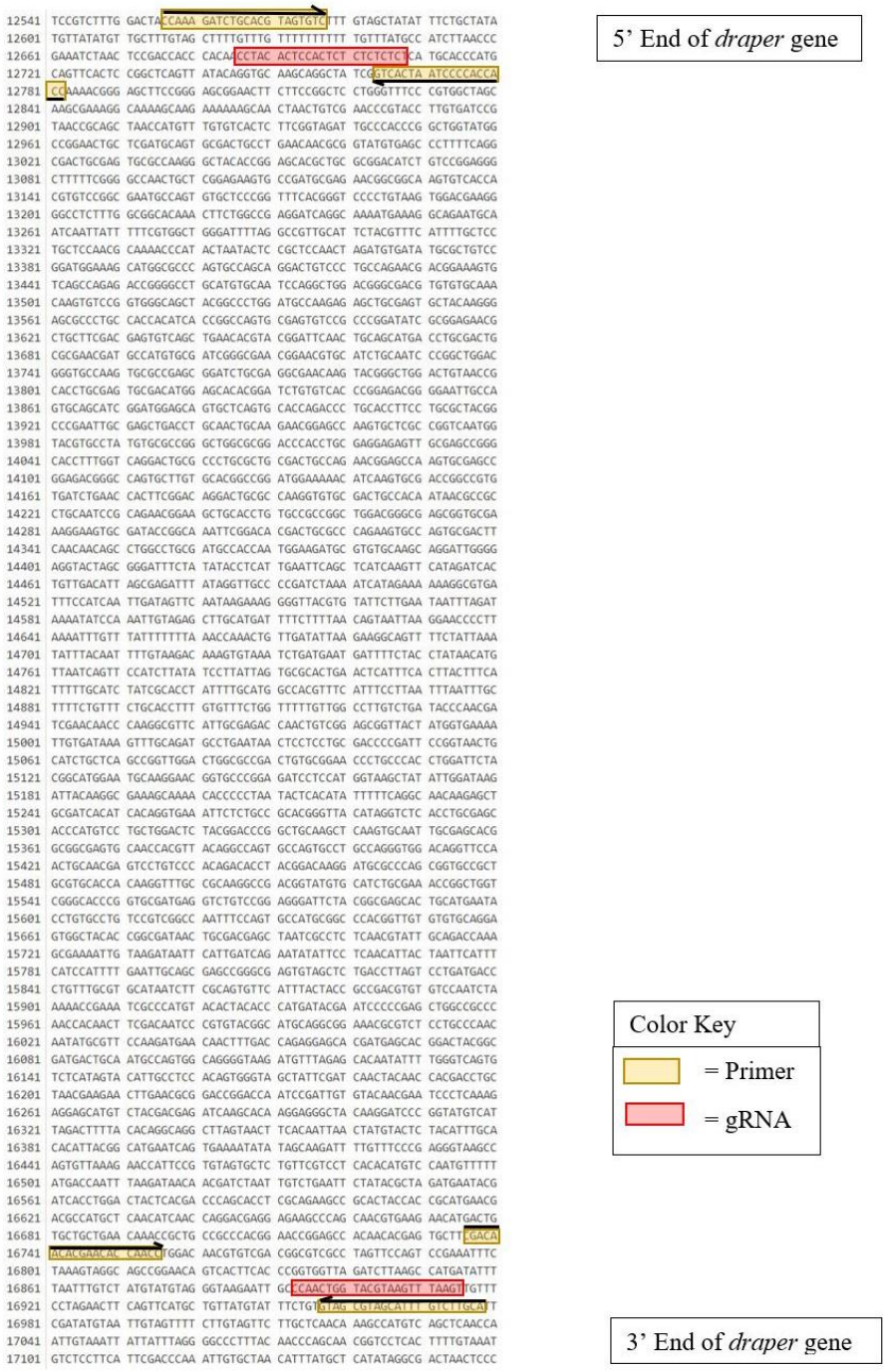
Progeny of the male G_0 fly were dissected and DAPI stained and then examined for persisting nurse cell nuclei. Many fly lines did not produce any *draper* mutants. One fly line, the M3-19M had some egg chambers with pnc, indicated by the white circles

Figure 3.9 Fluorescence Microscopy of Other Promising Fly Lines



Three fly lines: M6-1F/2F, F6-2M, and M3-C showed ovary phenotypes that were distinct from *drpr*. The different phenotypes displayed by these fly lines are a promising direction for future investigation.

Figure 3.10 *draper* Gene Sequence



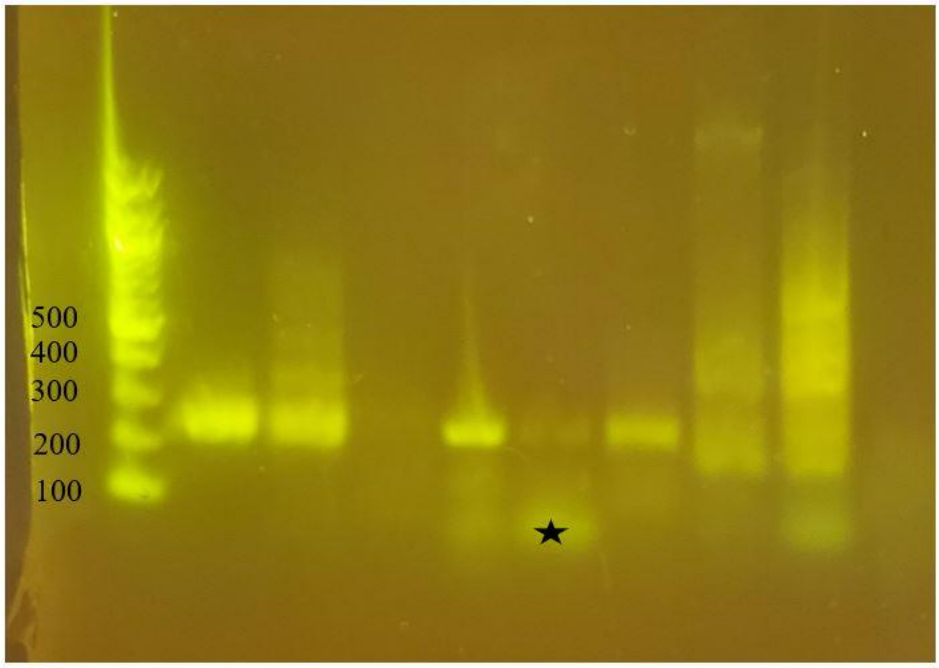
The sections highlighted in yellow are the primers that will define the region of DNA including the gRNA that will be amplified during PCR. The sections highlighted in red indicate the gRNA. In total, the deletion of *draper* will amount to 4.5 kb.

Table 3.1 Fly Lines Recovered and Analyzed

Fly Line	Crosses	Survived	Analyzed
M1	1	0	0
M2	1	0	0
M3	39	17	17*
M4	1	0	0
M5	1	0	0
M6	17	17	17*
M7	1	0	0
F1	15	6	6
F2	25	10	10
F3	1	0	0
F4	14	13	13
F5	33	14	14
F6	2	1	1*
F7	1	0	0
F8	1	0	0
F9	1	0	0
F10	13	5	5^
F11	1	0	0
F12	20	8	8
F13	1	0	0
F14	1	0	0

Many flies were crossed over several generations to remove the Cas-9 carrying chromosome. Many of the embryos that were injected with the two plasmids were infertile, with only some yielding fertile crosses. * indicates lines with phenotypes, while ^ indicates lines that still need to be analyzed

Figure 3.11 PCR Analysis of a Potential *drpr* Mutant



PCR analysis was performed to provide further evidence that the mutant seen during fluorescence microscopy was caused by a deletion in *drpr*. This is seen in lane 6, with the star marking the band that would appear if there was a deletion. A 1.5% agarose gel was used to visualize the PCR products.

CHAPTER FOUR

Discussion

This project was started with several goals in mind. The main goal was to create a *draper* null mutant through the use of CRISPR/Cas9. The second goal after this was to visualize the effects of this *draper* mutation, focusing on the potential defects to phagocytosis mainly in the *Drosophila* ovaries. The third goal was to create a stable fly line that could be kept and maintained for obtaining future generations.

The creation of a *draper* null mutant was suggested through fluorescence microscopy analysis of the fly ovaries. This was partly confirmed via PCR, however it was a small or partial deletion, not the whole 4.5 kb deletion. The first signs of confirmation indicated by the presence of persisting nurse cell nuclei are promising, as wild-type ovaries of *Drosophila* typically contain very few or no pnc. The presence of numerous pnc as well as signs of phagocytosis defects are strong evidence that the main goal was accomplished.

From 86 fly lines that were screened so far, mutant phenotypes were observed in several fly lines. In the ovaries of two fly lines, there were numerous pnc that were seen during fluorescence microscopy analysis. There were several other promising fly lines that appeared to contain different phenotypes from *drpr*. These three distinct alleles contained different phenotypes such as more immature oocytes and cell debris clustering rather than pnc typically associated with a typical *drpr* mutant.

The discovery of other fly lines that contain different mutations has promising future directions and applications that may be applied in other experiments. The M3-19M

mutant fly line that was created can be used in future experiments, although the *draper* allele should be confirmed by DNA sequencing. There are phenotypes that occur in other organs of *Drosophila* of as result a *draper* mutation that can be studied. This new mutation can be studied to see how defective phagocytosis can affect other tissues.

Throughout several of these protocols that were followed, there were periods of troubleshooting involved. During the process of preparing the plasmid there were numerous attempts where the agarose gel did not produce any visible or sometimes faint bands. Cloning the plasmid also took numerous attempts as well; colonies streaked onto LB plates containing Ampicillin did not produce transformed colonies initially.

These troubleshooting moments however frustrating they were, provided valuable opportunities to learn from mistakes. They allowed me to retrace my steps and gain a deeper understanding of each and every protocol that was followed. Ultimately though, I was able to complete the goals of the project.

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