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Signaling Pathways and Genetic Interactions That Lead to Metastatic Cancer in Drosophila melanogaster

Amanda Cole

Signaling Pathways and Genetic Interactions That Lead to Metastatic Cancer in *Drosophila melanogaster*

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

Amanda Cole
Candidate for B.S. Degree, Distinction in Biology,
and Renée Crown University Honors
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Honors Capstone Project in Biology

Capstone Project Advisor:

_____

Francesca Pignoni, Ph.D.

Capstone Project Reader:

_____

John Belote, Ph.D.

Honors Director:

_____

Stephen Kuusisto, Director

Date: May 2, 2012

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1. ABSTRACT

Cancer is a complex and multigenic disease, which is typically initiated by genetic mutations in tumor suppressor genes that regulate homeostatic mechanisms within cells. Oncogenic promoter mutations, like those involved in signal transduction pathways, also have the potential to induce cancer in an otherwise healthy organism. Transformation is highly dependent upon mutations to both tumor suppressor and oncogenes, as neither mutation is exclusive in its ability to generate malignant tumors. In the model organism, *Drosophila melanogaster*, I have generated metastatic cancer through the genetic effect of overactive *Raf* signaling, in conjugation with silencing selected tumor suppressor genes using RNA interference. Metastasis, the uncontrollable migration of cancer to non-adjacent areas within an organism, was analyzed *in vivo*, using Green Fluorescent Protein as an indicator for the presence of mutant tissue. *Scribble* (*scrib*) and *Discs large* (*Dlg*), two genes involved in cell polarity, demonstrated the highest incidence of metastatic cancer when silenced using RNAi. This novel preliminary screen exhibits the influential role of *Raf* signaling and cell polarity genes in generating metastatic cancer.

2. INTRODUCTION

Cancer

As the second leading cause of death in the United States, cancer has become a national as well as global pandemic (Cancer Facts & Figures, 2012). With more than 1,000,000 new national diagnoses and 500,000 plus cancer-related deaths expected to occur in 2012, this disease is aggressive and deadly at its worst (Cancer Facts & Figures, 2012). As such, more pressure is being placed upon researchers to discover the mechanisms that enable cancer to completely alter cellular behavior.

A scientific breakthrough occurred when one researcher, Alfred Knudson, discovered a model to explain the development of cancer, which eventually became known as the "Two-Hit Theory of Cancer Causation" (Knudson's 'Two-Hit' Theory of Cancer Causation). In this model Knudson states that in order for normal cells to transform into cancerous ones, two mutations must occur (Knudson's 'Two-Hit' Theory of Cancer Causation). He argues that the first mutation is genetic, as one inherited chromosome becomes damaged at conception, birth, or a later stage in life (Knudson's 'Two-Hit' Theory of Cancer Causation). When another mutation occurs to that same gene, a "second hit" occurs (Knudson's 'Two-Hit' Theory of Cancer Causation). This hit, in concert with the first mutation, enables the transformation of

cancerous cells (Knudson's 'Two-Hit' Theory of Cancer Causation).

Mutations are able to occur through a variety of mechanisms, like subtle sequence changes, alterations in chromosome number, chromosome translocations, and gene amplifications (Lengauer, Kinzler, and Vogelstein, 1998). In many tumors there has also been a major loss or gain of chromosomes, resulting in different cancerous conditions (Lengauer, Kinzler, and Vogelstein, 1998). For instance in glioblastomas, there is a loss in chromosome 10, which inactivates *Pten*, a tumor suppressor gene (Lengauer, Kinzler, and Vogelstein, 1998).

As such, the stability of tumors is indirectly related to the mutation prevalence among cells (Lengauer, Kinzler, and Vogelstein, 1998). Such instability can result from cellular environmental conditions, like in cell-cell interactions (Lengauer, Kinzler, and Vogelstein, 1998). As tumors develop from continual and uncontrollable cellular proliferation, they are able to be classified as benign or malignant (Understanding Cancer Series). This classification is dependent upon the invasiveness of tumors, or their ability to metastasize (Understanding Cancer Series). Localized tumors are unable to spread to new sites and, as such, are not considered invasive (Understanding Cancer Series). However, cancerous tumors are able to invade neighboring tissues

through metastasis, and even induce blood vessel growth nearby and within the tumor through angiogenesis (Understanding Cancer Series). As such, only malignant tumors are considered to be cancerous (Understanding Cancer Series).

An in-depth study of malignant tumors has shown cancer to possess six fundamental traits: a "self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis" (Hanahan and Weinberg, 2000). My project specifically focuses on two of these fundamental traits – a self-sufficiency in growth signals as well as tissue invasion and metastasis.

Signaling Pathways

Multiple regulatory systems in living organisms are controlled by signaling pathways, which influence cellular growth. These pathways consist of numerous proteins that are triggered by a signal, causing an appropriate response within the cell. Upon ligand reception, a chain reaction occurs so that each protein becomes activated by the previous protein, carrying the signal to the nucleus of the cell. It is in the nucleus that gene expression is affected, causing for the cell to become changed.

Mitogenic growth signals are required for normal cells to change from a dormant state into one that is active and proliferative (Hanahan and Weinberg, 2000). As such, signaling molecules are necessary in order for cell growth to occur. Some oncogenes predispose cells to cancer by mimicking such signals, thereby altering cellular signaling pathways (Hanahan and Weinberg, 2000). GTPase signaling pathways are particularly interesting, as uncontrolled signaling leads to an increase in cellular proliferation and malignant transformation (Reuter, Morgan, and Bergmann, 2000).

As such, these tumor cells exhibit a reduced dependency upon growth signaling in comparison to normal, healthy cells (Hanahan and Weinberg, 2000). Therefore, a signal is no longer required as the cell becomes completely independent of necessary growth signals. This renders inactive such an important homeostatic mechanism in controlling normal cell-like behavior (Hanahan and Weinberg, 2000). In order to induce self-sufficient proliferation, cancerous cells synthesize growth-signaling factors causing a positive feedback-signaling loop within the cell (Hanahan and Weinberg, 2000). Cancerous cells also manipulate growth factor receptors, as these cell membrane receptors receive such growth signals, thereby affecting gene regulation within the cell. However, tumor cells cause overactivity in the tyrosine kinase

activity of growth factor receptors, as well as ligand independent signaling, causing a hypersensitivity to signaling in cancerous cells (Hanahan and Weinberg, 2000).

When mutations like this lead to an overactive signaling pathway, proteins are continually active without any regulatory mechanism. As one of the defining traits of cancer, overproliferation allows cells to become limitless in their replicative potential. As such, mutated proteins involved in overactive signaling pathways are considered oncogenes, because these genes contribute to the initiation or progression of cancer (Hanahan and Weinberg, 2000). In fact, within stomach, brain, and breast tumors, the epidermal growth factor receptor has been proven to be upregulated, affecting the pathogenicity of these cells (Hanahan and Weinberg, 2000).

Epidermal growth factor receptors, or EGFR's, are responsible for initiating two important signaling pathways: the RAS-RAF-MAP kinase and the PDK1-AKT pathways (Benvenuti, et. al, 2012). Cancerous cells also possess different extracellular matrix receptors, or integrins, which promote pro-growth signals (Hanahan and Weinberg, 2000). Such integrins enable the overactivation of the RAS-RAF-MAPK pathway (Hanahan and Weinberg, 2000).

Although it is known that these signaling pathways, when overactive, promote overproliferation, these biological changes are not sufficient to actually cause cancer – only a predisposition to it. However, mutations that induce overproliferation can sometimes, in concert with other mutations, lead to metastasis.

Metastasis

Metastasis occurs when tumourous cells migrate from one organ to another, non-adjacent organ (Understanding Cancer Series). The migration of cancer cells is able to occur through uncontrolled mitosis or by the blood stream and lymphatic system (Understanding Cancer Series). When cancerous cells move to a new location in the body due to metastasis, a secondary tumor, or metastatic site forms (Understanding Cancer Series). As previously noted, benign and malignant cancers differ in their ability to metastasize; metastasis is a hallmark of malignant cancer (Understanding Cancer Series).

Neoplastic growth occurs as cells continue to grow in an uncontrollable manner, causing cells to begin to pile on top of one another (Basler, Toggwiler, Willecke, 2011). With constrained space for growth, cells begin to migrate into new areas and tissues, becoming cancerous (Basler, Toggwiler, Willecke, 2011). Unlike neoplastic growth, hyperplasia results only in the proliferation of non-metastatic cells (Halder and Mills, 2011). Accordingly,

mutations in neoplastic tumor suppressor genes contribute to the invasiveness of tumors (Halder and Mills, 2011).

The ability of cancer to spread to ectopic locations occurs through mutations related to the regulation of cellular processes – particularly those involved in migration and cell-cell adhesion (Hanahan and Weinberg, 2000). Since multiple regulatory systems in living organisms are controlled by signaling pathways, it is no surprise that mutated signaling pathways have been known to contribute to cancer. However, both negative and positive regulatory processes are necessary in order to generate metastasis (Liotta, Steeg, and Stetler-Stevenson, 1991).

Unrestrained growth is unable to individually initiate metastasis, as misregulation of motility and proteolysis is also required to induce tumor invasion (Liotta, Steeg, and Stetler-Stevenson, 1991). Once invasion has occurred, cancerous cells must be able to "arrest at the distant vascular bed, extravasate into the target organ interstitium and parenchyma, and proliferate as a secondary colony" (Liotta, Steeg, and Stetler-Stevenson, 1991).

Tumors exist as a subpopulation of cells with special characteristics; cells become metastatic as they migrate from the original tumor (Liotta, Steeg, and Stetler-Stevenson, 1991). Interestingly enough, it has been shown through the use of genetic

markers that this subpopulation dominates the growth of the primary tumor (Liotta, Steeg, and Stetler-Stevenson, 1991). However, in order for such subpopulations to form, cancerous cells must first pass through the basement membrane, a dense matrix that prevents cellular traversal (Liotta, Steeg, and Stetler-Stevenson, 1991). Only through invasion of the basement membrane is metastases able to occur as cancerous cells enter the blood stream and lymphatics (Liotta, Steeg, and Stetler-Stevenson, 1991). As such basement membrane degradation is a common feature among many carcinomas; this structure remains intact in benign tumors (Liotta, Steeg, and Stetler-Stevenson, 1991). As such, proteolysis, or the degradation of cellular proteins, is also a feature of metastasis (Liotta, Steeg, and Stetler-Stevenson, 1991). When proteolysis is coupled with motility, invasion is able to occur in cancerous cells allowing for the formation of metastatic sites (Liotta, Steeg, and Stetler-Stevenson, 1991).

Drosophila melanogaster

In determining the occurrence of metastasis, *Drosophila melanogaster* was used as my model organism. The use of this organism as a scientific model is very advantageous, due to the fly's small size and genome, with highly conserved sequences shared between the fly and human population.

With a genome consisting of 165 million base pairs, 4 chromosomes, and about 14,000 genes, *Drosophila* genes have easily recognized human homologues (Twyman, 2002). As such, research performed using *Drosophila* is able to contribute to a general understanding of human diseases and disorders.

Additionally, as a small organism, large numbers of *Drosophila* are easily maintained within vials and bottles. This allows for multiple experiments to occur in a research lab regardless of limited space. *Drosophila* also has a short life cycle, which allows for relatively quick results when inducing mutations within the fly. One generation of *Drosophila* takes approximately seven to eight days to reach maturity, transforming from an egg, to larvae, then pupae, and eventually a fly (*See Figure 2.1*). Within any given cross, pending it is at 25°C, progeny appears approximately ten days after crossing. By crossing flies of different genotypes, mutations are easily induced.

As my project utilizes signaling pathways in order to cause malignancy, *Drosophila* was an extremely beneficial organism to use; many known components within signal transduction pathways were originally discovered using the fruit fly (Halder and Mills, 2011). Through *Drosophila*, the flippase system was able to be utilized in order to create homozygous mutant cells, as similarly performed in

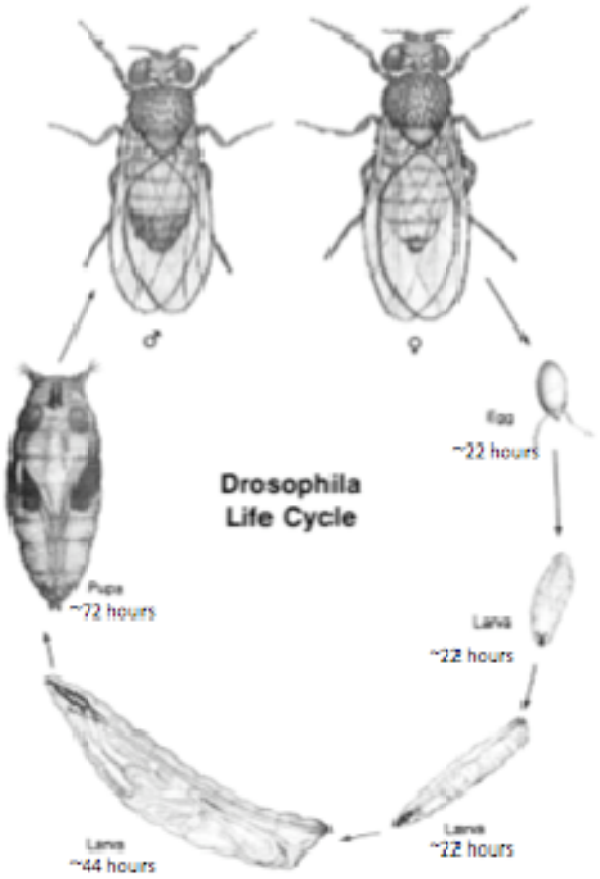


Figure 2.1: A lifecycle of *Drosophila melanogaster* (Powell)

other *Drosophila* genetic screens (Halder and Mills, 2011). Multiple transgenic stocks have also been created carrying different genomes of silenced tumor suppressor genes, which were necessary for my experiment (Halder and Mills, 2011).

In order to successfully model cancer, it is vital that specific, complex genotypes be created in groups of cells, and that their behavior properly followed (Halder and Mills, 2011). *Drosophila* not only allows for the creation of such genetically modified clones but also enables successful tracking through the use of the Flippase/FRT system (Halder and Mills, 2011).

Project Overview

In my Capstone Project I attempted to generate metastasis within *Drosophila melanogaster* by first combining *Raf*-activated and RNAi transgenes and then crossing them to an eye/antennal epithelium specific Gal4 driver line. This required me to generate a stable stock with an overactive RAS-RAF-MAPK signaling pathway by targeting the *Raf* protein. In the *Raf* gain of function mutation, the signaling pathway was manipulated so that the *Raf* protein was continually phosphorylated, causing for the pathway to become hyperactive. In order to create a stock of flies carrying this genotypic mutation, as well as balancers, multiple crosses were performed.

Since tumorigenesis is multigenic by nature, metastasis could only potentially be induced by also silencing a tumor suppressor gene. A small preliminary screen was performed, in which a total of fifteen tumor suppressor genes were knocked down using an RNA interference mechanism. Flies carrying the genotype containing the silenced genes were mated to those with the hyperactive signaling pathway in order to induce cancer within the progeny.

Theoretically, since the offspring of this cross were carrying both mutations, the formation of malignant, metastatic tumors was more likely in these flies than if their genome had consisted of only one mutation. As such, analysis of the offspring carrying the double mutation was compared to other progeny of the cross that carried only the RNAi or the oncogene.

When studying the Raf^{ACT} flies, it was expected that there would be an overgrowth, but no migration, of GFP positive cells. However, when analyzing the Raf^{ACT} + gene X^{-} (where gene 'X' is knocked out) flies, GFP positive cells will be detected at ectopic locations, whenever the Raf^{ACT} and the knocked out gene caused metastasis together. This stage in my project was important in determining if various interactions between overactive signaling pathways and specific genetic knockouts cause for cancerous cells to metastasize.

All larvae were analyzed *in vivo* using Green Fluorescent Protein as a marker, in order to determine the occurrence of metastasis. The FLP/FRT and UAS/Gal4 systems ensured that the green marker was localized within the developing eye epithelium of the fly, when visualized using the fluorescent microscope. As such, GFP was seen in ectopic locations, areas other than the eye discs, when malignant tumors formed.

In conclusion, my Capstone Project allowed me to identify second site mutations that lead to metastasis. It also enabled me to perform research on a signaling pathway protein that had not previously received significant scientific attention in comparison to the protein *Ras*. Utilizing both mutations, I demonstrated the significance of this protein in causing malignancy, as well as the necessity of cell polarity genes in preventing the formation of malignant tumors.

3. METHODOLOGY

Flipping

Flies are maintained within plastic vials or plastic bottles dependent on the amount of flies in a particular cross or stock. Approximately 20 flies are maintained within vials, while a bottle can sustain more than double this amount. To ensure proper nutrient supply, flies are flipped regularly to new vials and bottles that contain a layer of fresh food- consisting of mainly water, dextrose, yeast, agar, and cornmeal. If less than 10 flies are transferred, certain precautions must be taken to verify the health of the stock. For instance, within any container there must be at least a 3:1 ratio of females to males, with greater than half of these flies appearing to be healthy. Three shakes of dry yeast should also be added to any new vial before transferring. The label from the old vial must also be transferred, with the new vial being dated as well. Rather than disposing of the old vial, it should be taped to the new transfer and placed back in the tray.

Wet Yeast Paste

If flies are particularly unhealthy, wet yeast paste can be added to a vial or bottle. Yeast paste is also reproductively advantageous, as it makes the food more appealing to the females, thereby increasing the likelihood of eggs being laid. Wet yeast paste is made by taking a relative amount of dry yeast and adding

it to a 15 ml tube. Slowly a very minute amount of water is added to the tube, just enough for the yeast to soak up the moisture. A spatula is then used to mix the ingredients. While continuing to stir, water is gradually added again until the mixture becomes a paste. Using the spatula, the desired amount of paste is placed into the vial or bottle by gently placing the substance onto the food and side of the container; the yeast paste is very lightly mixed with the food and spread in a line, a quarter-length, up one wall of the vial or bottle.

Stocks

Stocks are fly lines that are genetically stable through the use of balancers. Balancers ensure the desired genotype by "balancing" the stocks. The most effective balancers suppress genetic exchange along the total length of the chromosome (Greenspan, 2004). By suppressing crossing over of homologous chromosomes, balancers minimize the likelihood of genetic recombination. This is due to the fact that "only those adults doubly heterozygous for the balancer and the lethal-bearing homolog survive" (Greenspan, 2004). A fundamental trait of balancers is the presence of recessive lethal alleles and dominant visible markers, thus homozygous balancer combinations *(See Figure 3.1)* (Greenspan, 2004). Within my project, I used two

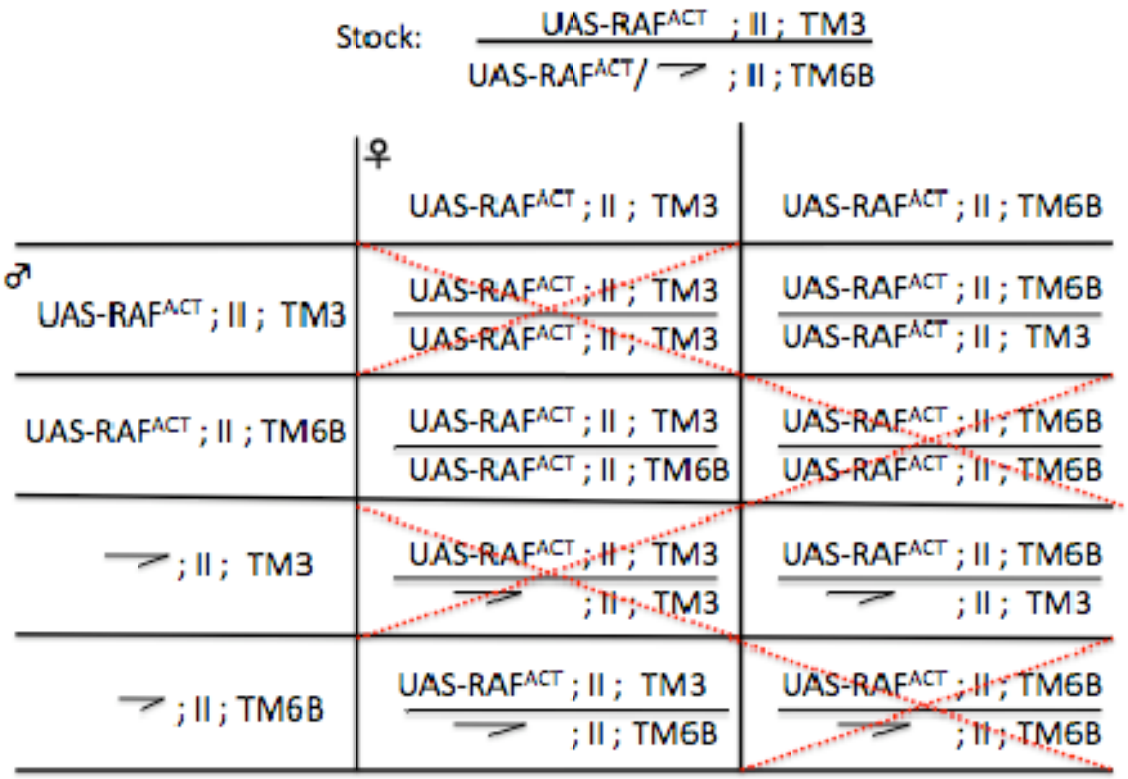


Figure 3.1: Visualization of a punnett square in demonstrating the mechanism of balancers to create stocks

balancers on the third chromosome: TM6B and TM3. TM6B carries dominant *Humeral* (*Hu*) and *Tubby* (*Tb*) along with recessive *ebony* (*e*) (Greenspan, 2004). TM3 instead carries recessive *ebony* (*e*) in addition to *Stubble* (*Sb*) (Greenspan, 2004).

Expansion

Stocks must be expanded when many copies of the same stock are needed for a given experiment. First, flies are flipped to a new vial or bottle, where they must remain until eggs are visible on the surface of the food. This typically takes three days at room temperature. At this point, the adult flies are able to be transferred to another new vial or bottle. Transfers may properly continue, pending the adult flies are given enough time to seed with each transfer. Expansion should end when the necessary amount of flies for the stock is achieved or when the flies are no longer able to sufficiently lay eggs. This typically occurs five days after the first room temperature transfer. Vials are also able to expand into bottle stocks when there are at least 20 flies within a healthy vial.

Bottlenecking

Bottlenecking occurs, as a safety precaution, when only one set of the adult flies remains. It is safe to copy a stock when there is crawling larvae visible within the designated vial. At this point the adult flies can be transferred to a new vial with dry yeast, assuming that there are enough males and females. The new vial

should be labeled and dated appropriately. The old vial should be saved.

Scientific Microscopy

Stereomicroscope

The stereomicroscope is used for sex and phenotypic separation, crossing procedures, virgin collection, and larval analysis. Light intensity is adjusted using the setting on the microscope base. Bulbs are also adjustable. The coarse knob is used to magnify the image, while the fine knob allows for focusing.

Fluorescence Microscope

Samples are analyzed for metastasis using the fluorescence microscope. When using this microscope, the differential interference contrast (DIC) light must be turned off. Next, the BINO/PHOTO filter must be removed. At this point the shutter can then be opened. The color filter should be adjusted appropriately, at FITC/CY2 to allow for GFP visualization. The coarse and fine knobs adjust the discernability of the image. Using the microscope, camera images are able to be obtained.

Confocal Microscope

Pictures of tissues are taken using the confocal microscope. The microscope, camera, and fluorescence box are turned on accordingly. The computer must also be running simultaneously,

so that the LAS AF program can be utilized. Once slides are loaded and clipped, the fifth icon down on the left-most side must be touched. The double arrow or single arrow are used to respectively move the stage up or down. Next the GFP fluorescence is turned on. The 10X objective is used to find the sample. Once the image is focused and centered, the stage must be brought down and a drop of immersion oil must be added to the slide. Switching to the 40X objective, the sample is refocused. Ensuring the strongest intensity of GFP, the confocal software is used to create an image of the sample.

Crosses

Punnett Square

Before performing a cross, a punnett square is made to ensure that the desired progeny will result from mating. As demonstrated in *Figure 3.1*, two axes are drawn with the females shown horizontally and males vertically. In each sector of one axis, all possible alleles, which are genetically transferrable from the parent to offspring, are listed. Therefore each box of the diagram represents possible combinations of both male and female gametes. Each of these combinations signifies the possible genotypes of the offspring. As such, the construction of a punnett square is necessary in order to verify that the desired offspring is produced; it is also used to note the other possible genotypes that could result

from the mating. This allows for specific phenotypes to be selected for further crossing schemes or in the creation of stocks.

Clearing

Vials and bottles must be completely cleared before virgin collection occurs. When clearing a vial or bottle, all adult and non-virgin flies are removed. Clearing is typically synonymous with transferring flies, as a new copy is made while the old is used for collection. However if there are enough copies, then removal occurs by turning the vial or bottle upside down onto a CO_2 pad; the CO_2 gauge level should not exceed to 10-20 ppm. These flies are disposed of in the fly morgue, a flask consisting of ethanol. If flies are still present in the container, then the same procedure can be repeated or a paintbrush can be used to push the remaining flies into the food.

Virgin Collection

The accuracy of a cross is dependent upon proper female virgin collection. On the first day of collection, the designated bottles are cleared and dry yeast is added to the container. A kimwipe is folded into half and gently pushed into the bottom of the food to increase the surface area for crawling larvae. Once all eclosed flies are emptied, the bottles are placed at room temperature. Six to seven hours later, flies are able to be scored on the basis of sex. Newly emerged virgin females are distinguishable

from adults by the presence of the meconium, a dark spot on the abdomen, formed by food eaten during the larval stage (Greenspan, 2004). Females are saved into vials with dry yeast; each vial is labeled with number of virgins, their genotype, and the date. Collection bottles should be double checked to make sure that no flies remain; these bottles are placed at 18°C overnight. Temperature affects the sexual maturity of flies: at 18°C flies take 18 hours to mature, while at 25°C it only takes 8 hours. Therefore, it is important to collect before maturity is reached between each collecting period. As such, flies should be collected no later than 18 hours after they are placed in the 18°C incubator overnight. Collection continues using the outlined procedure until all necessary virgins are obtained for crossing.

Sex Scoring

Distinguishing between males and females is crucial for crossing. Males have sex combs, a rounded abdomen, dark bristles on their genitalia, and dark coloring at the dorsal end of their abdomen. Females have a pointed abdomen with lighter pigmentation. Differences are distinguishable in *Figure 3.2.*

Crossing

When crossing, the following supplies are necessary: an uncapped tray of vials, yeast shaker, bag of cotton, and virgin female flies of the appropriate genotype. Virgin females are

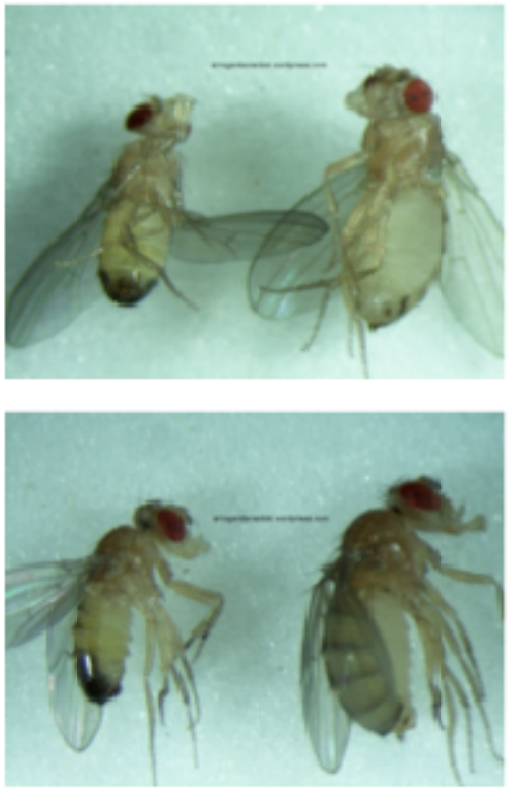


Figure 3.2: Visualization of the phenotypic differences between male (left) and female (right) flies (The Arrogant Scientist)

anesthetized by CO_2. Using a paintbrush, the necessary amount of virgins is added to empty vials containing dry yeast. As virgins are added to each individual vial, they are capped using cotton balls to ensure that no unwanted flies are able to enter the vial. The amount of vials and virgins necessary is dependent on the number of crosses performed. Once finished adding all virgin females, the cross must be completed by adding the appropriate male flies. Once these flies are obtained, they are also anesthetized using the CO_2 apparatus. Although a maximum of 1 male per 7 females is sufficient for a cross, normally 2-3 males are used per 5 or more females. Crosses are completed once males and females have been added to the same vial. All vials must be labeled with the date, cross scheme, and number of males and females in each vial. Vials should be placed at the appropriate temperature.

Maintenance of Crosses

Cross vials are not able to be maintained using the same procedure as stock vials. Three days after a cross is performed, adults should be transferred to a new vial. This should resume at the end of days 4, 5, and 6, with adults being flipped to a new vial. This allows for a single cross to expand approximately five times. Special circumstances arise when there is a scarcity or abundance of flies; transfers can happen as early as day 2 if there is a surplus of flies or as late as day 5 if there are very few flies present. Vials

should be cleared of adult flies when pupae begin to appear, this typically occurs at day 6 or 7 for the original cross vial. In transfer vials, the first pupae will appear approximately five days after the transfer date.

Phenotypic Scoring

Phenotypic scoring is crucial when performing crosses or selecting specific progeny from a desired cross. At the larval stage, male and females are able to be distinguished by the presence of the male testes. The testes are detectable by a small, translucent circle near the posterior end of the male larvae. The TM6B marker is also able to be distinguished at the larval stage by the appearance of tubby larvae; these larvae are shorter and fatter than the wild type. In adult flies TM6B causes the humeral phenotype, as shown in *Figure 3.3*. Humeral flies have greater or less than 2 large bristles on their shoulder, where as wild-type phenotype consists of only 2 macrochaetes.

Larval Dissection

The following supplies are necessary for larval dissection: disposable transfer pipettes, a pair of forceps, glass 9-well plate, plastic 24-well plate, tissue baskets, vial of larvae, 1X PBS, and a dissecting pad. First, the glass wells in the 9-well plate are filled with 1X PBS using a disposable transfer pipette. Next, a drop of

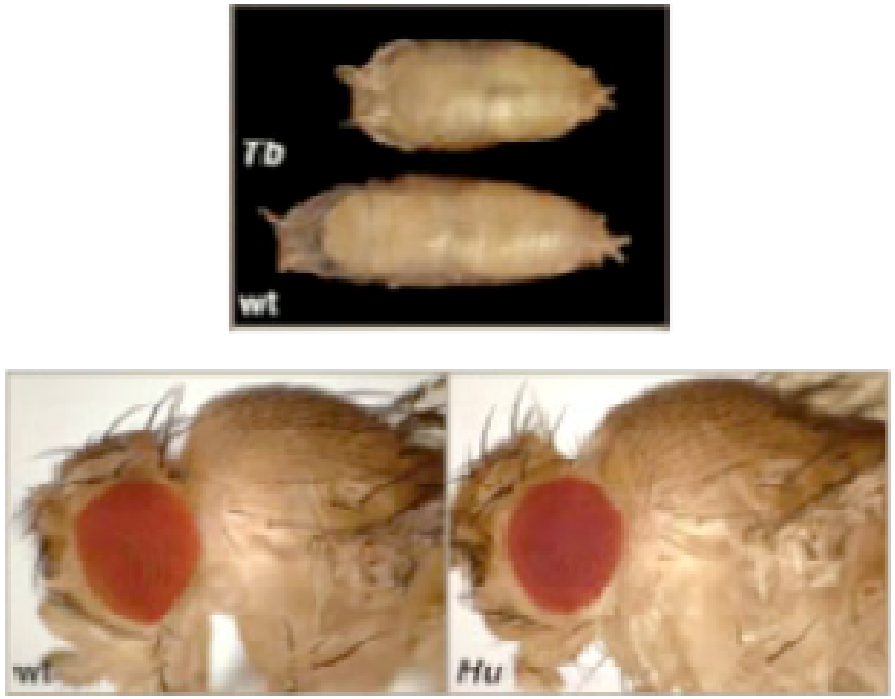


Figure 3.3: Phenotypic expression of the TM6B marker. Dominant *Tubby* (*Tb*) is scorable at the larval level of development (shown above), while *Humeral* (*Hu*) is a bristle phenotype as shown in the figure below (Dehringer, Childress, and Halder)

1X PBS is added on the dissecting pad; each larval dissection requires a separate drop. Using a paintbrush, 10 larvae are removed from the vial and placed in a 1X PBS well. One larvae is transferred to the dissecting pad using the forceps. The dissecting pad is then placed under the light microscope in order to perform the dissection. When dissecting, both forceps should gently pinch some of the larval coating, with the forceps then being pulled in opposite directions; this removes the skin tissue of the larvae. This procedure is repeated until all skin is removed from the larvae, with the skin being disposed of in a designated 1X PBS droplet on the dissecting pad. After the skin is removed, unnecessary tissue must also be eliminated. Both forceps are used to remove all tissue except the ventral nerve cord, brain, antennal discs, eye discs, wing discs, and mouth hooks. Excess tissue is also discarded in a separate droplet. Once the sample is finished, it is transferred to a basket located in 1X PBS in the plastic 24 well-plate. The above procedure is repeated for all larvae, placing all finished samples in the same basket; larvae tissue with different genotypes should not be mixed. Once all tissue samples are obtained and placed in the basket, the basket is transferred to another well plate filled with PLP fixative. Tissues should be fixed in PLP for 15-20 minutes. After this time period has passed the tissue samples are able to be mounted. Dissection supplies are handled accordingly: transfer

pipettes are disposed of in the trash, the glass well plate and dissecting pad are

washed with water followed by an ethanol wash, and 1X PBS is placed back in the 4°C fridge. Forceps are also cleaned with ethanol using a kimwipe, and the vial of larvae is placed back at the appropriate temperature.

Imaginal Disc/Antibody Staining

When necessary, antibody staining occurs after larval dissection. After dissection, the tissue is fixed in PLP for 30 minutes at room temperature. At this same temperature, these samples are washed for 5 minutes in 1X PBS and then twice in 1X PBT, also for 5 minutes each. The primary antibody is then able to be added at the appropriate dilution (in NGS/PBT). After this step, these samples must be placed on the shaker overnight, for at least 10 hours, at 4˚C. The following morning, the well plate and baskets are moved to room temperature, where the tissue is washed in PBT, 3 times for 10 minutes each. Samples are washed for another 3 cycles at 10 minutes each, in NGS/PBT. The secondary antibody is then added in a 1:200 dilution (in NGS/PBT). These samples are shaken again for 2 hours, at room temperature. Wash cycles are then repeated at room temperature: washed in PBT 3 times for 10 minutes each and in PBS at 10 minutes each. Samples are then able to be mounted using mounting solution.

Mounting

Tissue samples are mounted on slides following dissection. In order to mount the following supplies are required: mounting solution, slides, cover slips, 1X PBS, disposable transfer pipettes, and forceps. Before mounting it is necessary that slides are labeled with the name of the sample being mounted, amount of samples mounted, date, and initials. After labeling, one drop of 1X PBS is added towards the end of the slide using a disposable transfer pipette. The designated tissue to be mounted is placed within this drop. Under the light microscope, the tissue is cleaned using a pair of forceps; cleaning separates the tissues just enough so that they can be discerned easily when mounted. Once the tissue is prepared, one drop of mounting solution is added to the center of the slide using a new transfer pipette. The prepped tissue is transferred to the mounting solution and arranged accordingly. This procedure is repeated until all tissue samples are added to the slide, with each slide typically containing five samples. Once all samples are in the mounting solution, a kimwipe is used to remove the drop of 1X PBS. A coverslip is then gently placed over the mounting solution. After a few minutes of drying, the coverslip is sealed using nail polish, by brushing along all four edges of the square.

Solutions

1XPBS

In vitro and *in vivo* larval analysis is performed in 1X PBS. To make 1 L of PBS, 10 ml of 10X PBS is mixed with 90 ml of Millipore water.

1X PBT

This solution is made by adding 1500 µl of 10% Triton (1ml of Triton + 9ml of Millipore water) and 50 ml PBS.

NGS/PBT

This solution is necessary for the antibody staining procedure, mixing: 1 ml of 100% NGS and 600 µl 10% Triton. Using 1X PBS, this solution is then brought to 20 ml.

Mounting Solution

Mounting solution is required in order to preserve tissue samples on slides: 0.40 g of n-propyl gallate, 800 µl of 10X PBS, 2 ml of enzyme-grade glycerol, 1.2 ml of H_20 are combined into a 15 ml tube. The tube is then vortex in order to ensure that the solution is thoroughly mixed. Mounting solution is stored at 4°C.

4. RESULTS AND DISCUSSION

Induced Mutations

Raf^{ACT}

An overactive RAS-RAF-MAPK signaling pathway was induced in order to induce the first mutation within *Drosophila*. Within this signaling pathway a signal is received by EGFR, which triggers the action of *Ras*, a small G-protein (Benvenuti, et. al, 2012). G-proteins, or GTP-hydrolases, are extremely important within this pathway as they enable activation through intermittent conformational changes upon binding to guanosine diphosphate, GDP, and guanosine triphosphate, GTP (Reuter, Morgan, and Bergmann, 2000). When GTP-bound, *Ras* becomes activated from the protein's dormant, GDP-bound state (Reuter, Morgan, and Bergmann, 2000). Guanine nucleotide exchange factors, like SOS, serve as regulatory proteins to control the cycling rate of *Ras* activation through GTP and GDP (Reuter, Morgan, and Bergmann, 2000). SOS stimulates *Ras* by enabling the dissociation of GDP, thereby allowing for GTP incorporation (Reuter, Morgan, and Bergmann, 2000).

When GTP bound, *Ras* activates an effector protein kinase *Raf*, which initiates the mitogen-activated-protein kinase cascade through phosphorylation (*See Figure 4.1*) (Benvenuti, et. al, 2012).

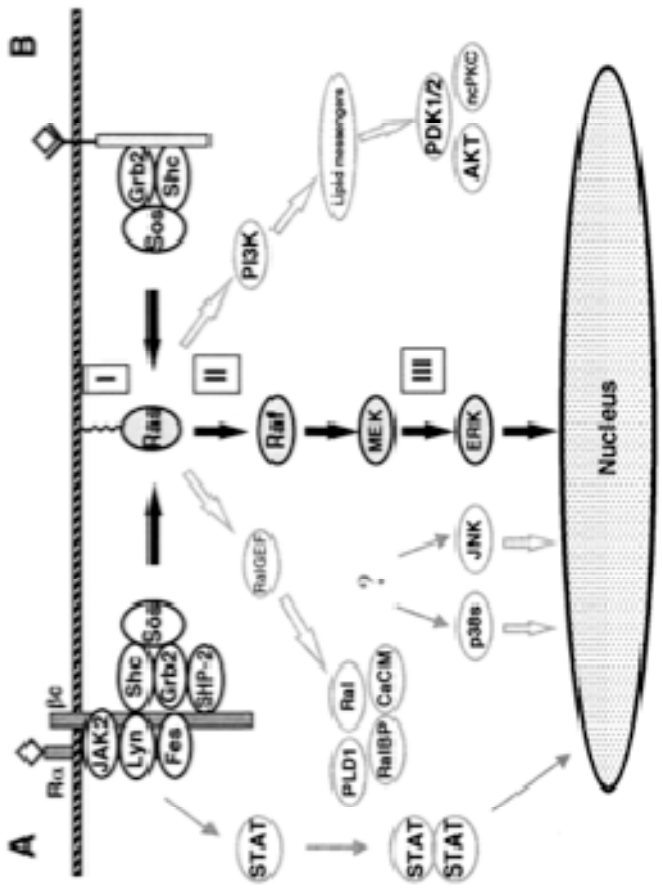


Figure 4.1: A schematic of the RAS-RAF-MAPK signaling pathway (Reuter, Morgan, and Bergmann, 2000)

The intrinsic regulatory mechanism of this signaling pathway is extremely crucial in healthy cells, since hyperactive RAS-RAF-MAPK pathways have been proven to be present in 25% of human tumors (Hanahan and Weinberg, 2000). The presence of *Ras* oncogenes have also been confirmed in approximately half of human colon carcinomas (Hanahan and Weinberg, 2000). In these tumors, the *Ras* protein is mutated so that mitogenic growth signals are continually released, causing habitual cellular stimulation (Hanahan and Weinberg, 2000).

Within my project, I targeted the *Raf* protein in order to cause overactivity of the RAS-RAF-MAPK pathway. As shown in Figure 4.1, *Ras* triggers multiple pathways by potentially phosphorylating RalGEF, PI3K, or *Raf* (Reuter, Morgan and Bergmann, 2000). Hyperactivity in both the RAS-RAF-MAPK and PI3K pathways have shown to be correlated with tumorigenesis, however only RAF/MAPK pathway overactivity induces metastasis (Janda, et al., 2002). This pathway is also known to be required for initiating transforming growth factor ß epithelial mesenchymal transition, or TGFß EMT (Janda, et al., 2002). EMT is "characterized by spindle-like cell morphology, loss of epithelia markers, and induction of mesenchymal markers" (Janda, et al., 2002). Oncogenic *Raf* has also been shown to prevent TGFß-induced apoptosis as well as create more contact between cells and

their migration (Janda, et al., 2002). As such, I decided to use a mutation of *Raf* that led to hyperactivation of the RAS-RAF-MAPK pathway in order generate malignant tumors within the fly.

RNAi Mechanism

RNA interference was used as a technique for silencing targeted tumor suppressor genes, inducing a second mutation within my organism of study (*See Figure 4.2*). This method utilizes transgenes that encode specific RNAi sequences, which are produced upon transcription of the transgene. Silencing occurs at the post-transcriptional level, interfering with DNA regulation of these specific genes (Hannon, 2002). Double stranded RNA initiates this process upon recognition by the Dicer enzyme (Hannon, 2002). As part of the RNase III ribonuclease family of enzymes, Dicer possess two dicer molecules and five domains that are able to process dsRNA, producing small interfering RNA's (Hannon, 2002). These siRNA's are approximately 22 nucleotides long due to an inactive site on the Dicer enzyme, which shifts the targeted activity of this enzyme to Dicer family members (Hannon, 2002). Small interfering RNA's are received by the RNA-induced silencing complex, which serve as effector nucleases (Hannon, 2002). RISC effectively unwinds the siRNA's through an ATP-dependent process, thereby transforming from a zymogen into an active

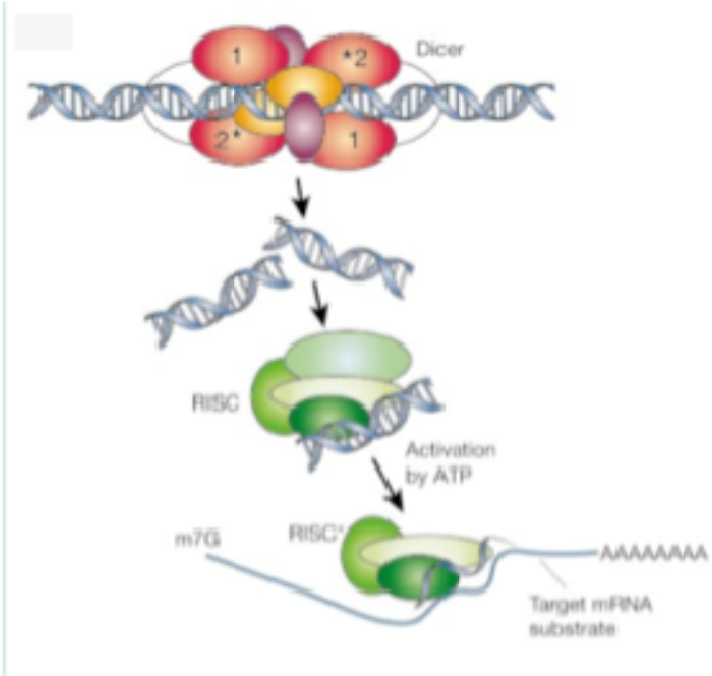


Figure 4.2: The RNA interference mechanism, depicted above, was employed in order to effectively silence targetted tumor suppressor genes (Hannon, 2002)

complex (Hannon, 2002). When activated, the siRNA's guide the RISC complex to homologous substrates, with significant complementation of sequences between the siRNA and mRNA target (Hannon, 2002). Upon recognition, RISC-associated nucleases cleave these mRNA substrates at specific sites thereby inhibiting effective translation (Hannon, 2002). As such, these steps characterize RNAi: "assembly of siRNA with the RNA-induced silencing complex, activation of the RISC, target recognition and target cleavage" (Reynolds, et al, 2003).

By hindering this translational machinery, RNAi was utilized within my Capstone Project to prevent protein synthesis of specific tumor suppressor genes: *dsh, dlg, ce, dsh, arm, shg, skpA, ups7, vps25, scrib, cdc2, pten, cdc27, UASyki, cdc37,* and *tsc1*. It was expected that by silencing these genes that interfere with tumor formation, the development of cancerous cells would be stimulated. As such, this secondary mutation, in conjugation with hyperactive *Raf* signaling, would theoretically lead to the formation of malignant tumors in *Drosophila melanogaster*.

Identification of Ectopic-GFP Cells

FLP/FRT and UAS/Gal4 Systems

Both the FLP/FRT and UAS/Gal4 systems were vital in targeting and identifying the presence of mutant cells. In order to localize cancerous cells in the eye of *Drosophila* and tag these

cells using Green Fluorescent Protein, the following stock was used: ey-Flip; Act>IC>Gal4 UAS-GFP; UAS-dicer2.

The UAS/Gal4 system utilizes two components: a Gal4 driver and a Gal4 responsive UAS expression vector (Rorth, 1998). When in the presence of Gal4, binding sites on the Upstream Activator Sequence become occupied, thereby driving gene expression (Rorth, 1998). Therefore, the presence of Gal4 is necessary in order to activate the transcription of GFP (to mark the cells) and Dicer 2 (an endonuclease that enhances the effectiveness of RNAi) sequences (Duffy, 2002). As such, the absence of Gal4 expression effectively silences these UAS controlled reporter genes (Duffy, 2002). In addition, in the progeny, the UAS-RNAi transgene is also activated in the same cells, such that siRNAs are generated and silence the targeted gene.

The expression of a reporter gene is able to be influenced by cellular localization, timing, sensitivity, and protein and mRNA stability (Duffy, 2002). Most noticeably, Gal4 expression is affected by temperature, as minimal activity occurs below 16˚C (Duffy, 2002). At 29˚C, Gal4 activity is maximal with few effects on fertility and viability (Duffy, 2002).

Temporal and spatial expression of targeted genes is also regulated by the FLP/FRT system, as it directly affects Gal4 expression. The flippase recombinant enzyme, FLP, allows for

genetic recombination between the Flippase Recognition Target sites (Duffy, 2002). As shown in *Figure 4.3*, the FRT sites effectively flank the interruption cassette, which is responsible for terminating transcription (Duffy, 2002). As the interruption cassette is located between the promoter and Gal4 gene, its presence effectively prohibits Gal4 expression (Duffy, 2002). However, ey-Flip allows for the FLP enzyme to remove the cassette, thereby promoting tissue-specific regulation within the *Drosophila* eye tissue (Duffy, 2002). Since the ey-Flip is only expressed in the developing eye-antennal tissue, the flippase localizes GFP within the eye; therefore, metastasis is determined based on the presence of ectopic GFP cells in secondary sites, those other than the eye antennal epithelium. These systems therefore effectively determine the loss of function phenotypes that result from silencing tumor suppressor genes.

Experiments

Ras^{ACT} and Raf^{ACT} Preliminary Test

Before beginning my experimental project, it was crucial to determine which *Ras* and *Raf* lines would be the most effective in generating metastasis. Therefore, a preliminary test was performed, in which each available *Ras* and *Raf* lines within the lab were crossed to the ey-Flip; Act>IC>Gal4 UAS-GFP; UAS-dicer2 stock (*See Figure 4.4*). In the first cross, the ey-Flip line was crossed to

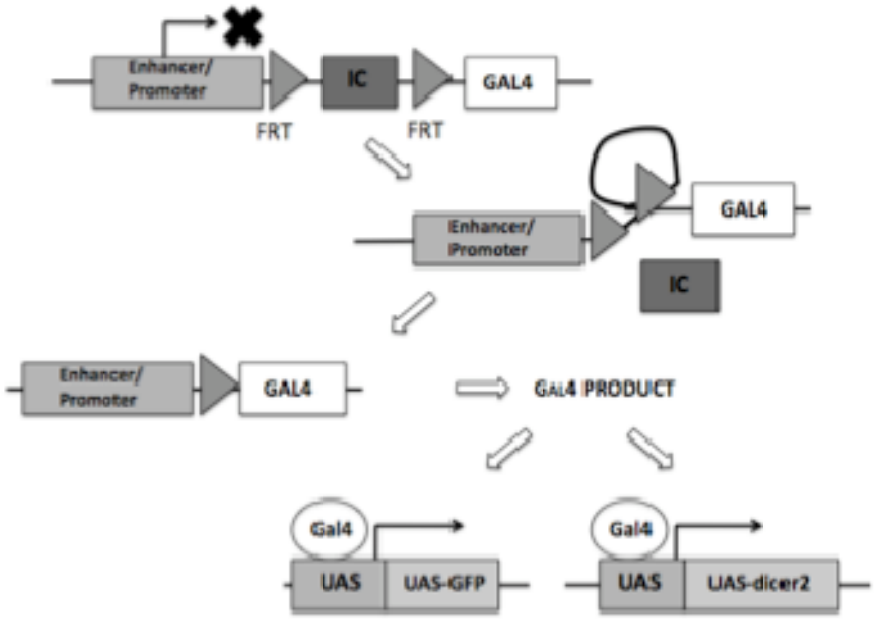


Figure 4.3: FRT/FLP and UAS/Gal4 systems, as used in my Capstone

Figure 4.4: In order to determine which *Ras* and *Raf* lines would be used in the experimental project, each possible line was crossed an Eyflip stock so that the effectiveness of GFP expression could be observed

itself in order to induce double GFP expression. As such, this cross showed the strongest GFP expression, so that all normally expected areas of expression could be noted. Single GFP expression was observed in the second cross, as this cross showed comparable expression to that which was expected in the experimental RNAi project. ey-Flip was also crossed to two *Raf* lines: P{w[+mc]=UAS-hRaf1.gof}ra2. y[1]w[*], encoding an activated form of human *Raf*, and w;FRT82B UAS-Raf^{ACT}, encoding the activated fly *Raf*. A total of three *Ras* lines, all encoding activated fly *Raf*1, were also tested: w; FRT40A UAS-Ras^{v12}/CyO; UAS-Ras^{v12} on 3, and w; UAS-Ras^{v12}/CyO; FRT82B/TM6B.

As indicated in *Figure 4.5*, each of the *Ras* lines produced greater overgrowth in the eye disc, in comparison to *Raf*. There were also more secondary GFP expressing sites in *Ras* than in *Raf*, displayed in *Figure 4.6* as well. Although H-raf^{ACT} (gof) was the only one of these lines to not show extra visualization systems, this cross also produced the fewest progeny resulting in a smaller total sample size in comparison to the other crosses. Ectopic GFP typically appeared in the gut of the Ras^{v12} progeny, while Raf^{ACT} (FRT82B) secondary GFP expression was visible in the haltere discs. Additionally, adult viability was greatest for 2X GFP and 1X GFP expression, but somewhat diminished in the Raf^{ACT} progeny

	Disc Overgrowth	Extra-visual system GFP+ sites
2X-GFP	3	None
1X-GFP	3	None
H-raf$^{\Delta CT}$ (gof)	4	None
Raf$^{\Delta CT}$ (FRT82B)	3	Haltere Discs
Ras^{V12} (FRT82B)	4.5	Genital discs, Fat tissue, Brain, Gut
Ras^{V12} (FRT40A)	4.5	Gut, Haltere Discs
Ras^{V12} on 3	4.5	Gut

Figure 4.5: As noted by the table, *Ras* showed a greater eye disc overgrowth in comparison to overactive *Raf*. Discs were rated on a scale of one to five, based on their size relative to a wild-type disc (one was considered extremely small, with five representing very overgrown discs). Likewise, *Ras* also showed a greater likelihood of containing extra GFP sites.

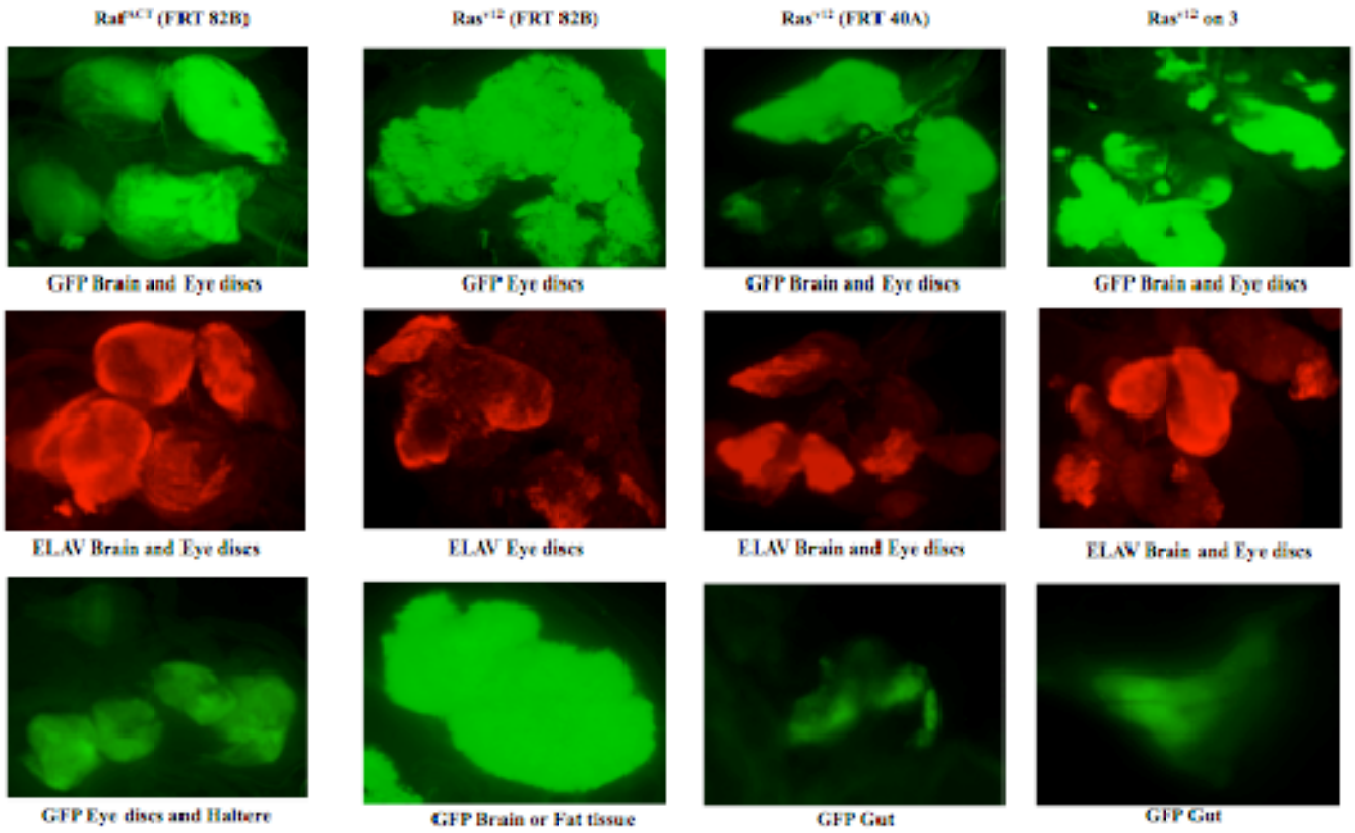


Figure 4.6: As shown in the images above, $Raf^{\Delta CT}$ (FRT82B), Ras^{v12} (FRT82B), Ras^{v12} (FRT 40A), and Ras^{v12} on 3 express GFP within the eye antennal epithelium, when crossed to ey-Flip; Act>IC>Gal4 UAS-GFP; UASdicer2. These samples also all show neuronal differentiation as indiciated by the Cy3 stains, where antibodies against ELAV were used. As seen in the third row of images, ectopic GFP was also present in different sites for each of these lines.

and most severly affected in Ras^{v12} progeny. All *Raf* and *Ras* crosses showed neuronal differentiation when stained with CY3.

The results of this screen indicated that the w; UAS-Ras^{v12}/CyO; FRT82B/TM6B line should be used as the representative line for *Ras* in the experimental RNAi project. This decision was due to the fact that Ras^{v12} (FRT82B) demonstrated the greatest likelihood of producing secondary sites, as extreme overproliferation was consistently observed during three separate trials of testing.

According to this screen, the w; FRT82B UAS-Raf^{ACT} line would have been ideal to use for the experimental screen, as this was the only *Raf* line that considerably proved to generate larger overgrowths. However, in designing a screen to test for metastatic cancer, it was more beneficial for overactive *Raf* to be carried on the first chromosome, rather than the third. In addition, the use of human *Raf* gene rather than fly *Ras* was appealing because any observed genetic interaction would directly apply to the protein found in human cancers. As such, the P{w[+mc]=UAS-hRaf1.gof}ra2. y[1]w[*] stock was bottlenecked in order to increase the viability and health of these flies.

Generating Ras^{ACT} and Raf^{ACT}

Once it was determined which *Ras* and *Raf* lines would be used for the experimental RNAi screen, it was necessary to create

stable, healthy stocks containing these genotypes in order to test for metastasis. The crossing schematic for *Ras* was designed, incorporating the *Ras* genotype with both the TM3 and TM6B markers, as shown in *Figure 4.7*, Rather than using the initial *Ras* stock, it was necessary to create the new w; UAS-RAS^{v12}; TM3/TM6B stock due to the presence of these markers. In performing the experimental crosses, both TM3 and TM6B were necessary in order to ensure that the final experimental flies were carrying the correct genotype, by being scored for, and against, both of these markers.

The first two crosses in the *Ras* schematic were performed at the same time, as the female offspring of the first cross (w; UAS-RAS^{v12}/CyOarmGFP; FRT82B) and male progeny of the second (w; UAS-Ras^{v12}/CyO; FRT82B/TM6B) were then crossed together to generate the final desired genotype (w; UAS-Ras^{v12}; TM3/TM6B).

In order to produce this final stock, it was necessary that the CyO marker was scored against, as the absence of curly wings ensured the presence of homozygous *Ras* on the second chromosome in the final line. Similarly, the presence of the TM3 and TM6B makers confirmed the absence of FRT82B in the ultimate stock, which would have interfered with a necessary mechanism in the experimental cross.

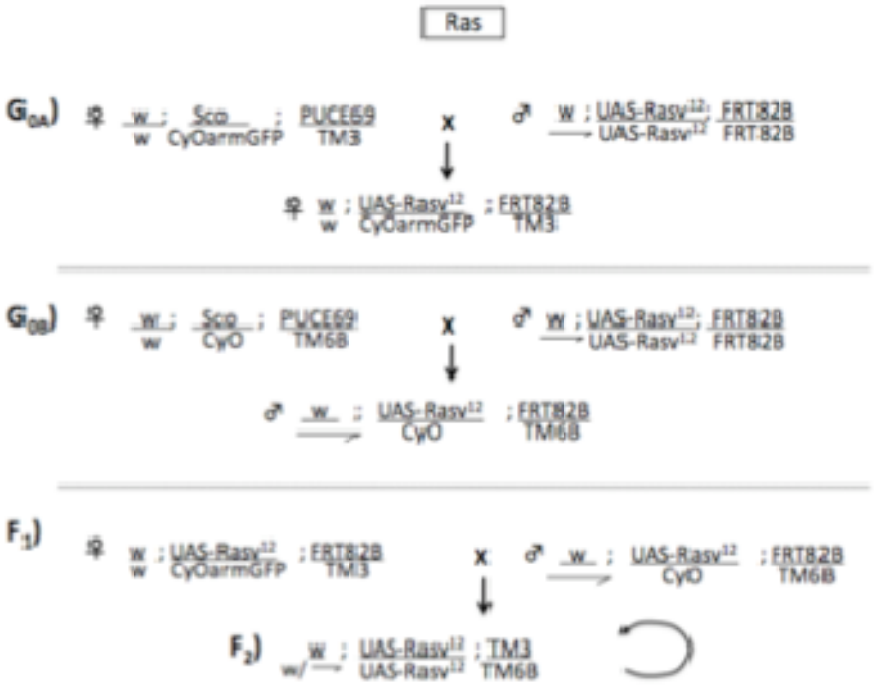


Figure 4.7: The construction of Ras^{ACT}

As the line of *Raf* became more stable through the bottlenecking process, the P{w[+mc]=UAS-hRaf1.gof}ra2. y[1]w[*] line was also able to be used in order to generate the appropriate stock for the experimental screen (*Figure 4.8*). First, this stock was crossed against a line carrying both the TM3 and TIM6B markers; two different males were collected from this cross, with each carrying one of these markers and the overactive *Raf* genotype. Males carrying each of these genotypes were crossed back to the original stock in order to produce females that were homozygous for hyperactive *Raf*, whereas this was not genetically possible in the first cross. These progeny were crossed so that a stock completely homozygous for *Raf* could be produced, also carrying both the TM3 and TM6B phenotypic markers.

However, the *Raf* line also demonstrated an extreme weakness in the presence of both balancers. In order to ensure the viability of the stock, a bottle was made that contained flies that were either TM3 or wild type over TM6B. It was necessary to retain the TM6B marker, as this marker allows for larval scoring, which was necessary in the experimental cross. This bottle was also bottlenecked and expanded; the UAS-Raf^{ACT}; III/TM6B genotype became more dominant over time due to an increased viability in these flies, in comparison to those flies carrying both markers.

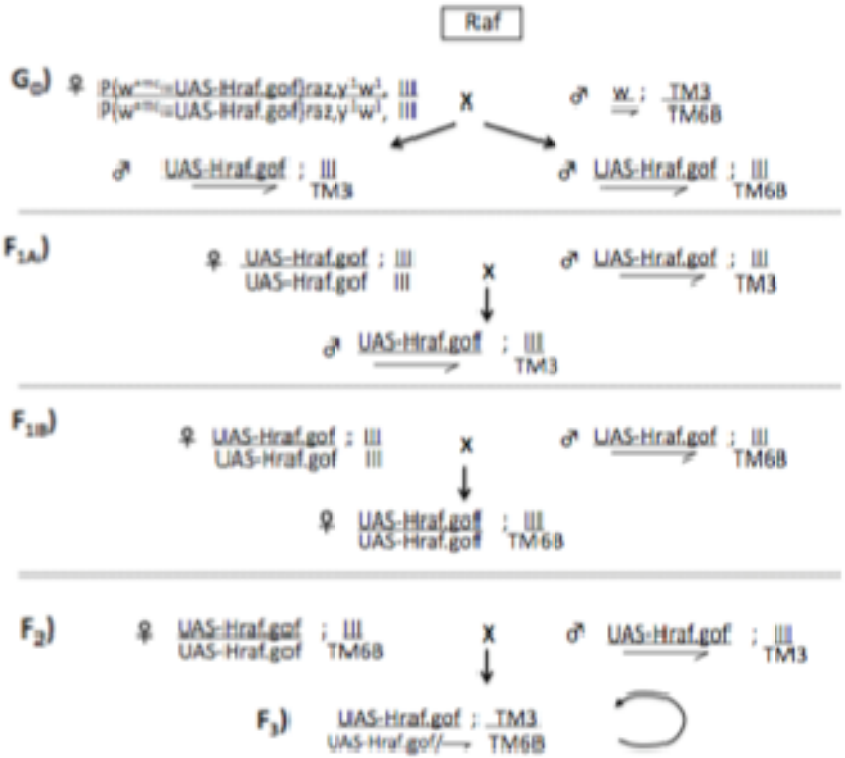


Figure 4.8: The construction of Raf$^{\Delta CT}$

Although the intention was to use both of the newly generated stocks in the experimental screens, the w; UAS-Ras^{v12}; TM3/TM6B stock could not be utilized. Despite using robust crosses, very few flies carrying this genotype were produced, with even a fewer amount of healthy flies. Although similar results occurred in creating the *Raf* stock, a greater among of healthy, viable flies were produced in comparison to *Ras*. As such, the *Raf* line was able to be easily bottlenecked for the final experiment, while *Ras* was not. These flies also exhibited an extreme sensitivity to temperature, as viability drastically decreased with an increase in the environmental temperature. After discovering this fact, these flies were maintained at 18˚C throughout the remainder of the bottlenecking process. However, even in an optimal temperature environment, these flies were too weak to survive, let alone be able to be used for the experimental screen.

Preliminary Screen

The main purpose of the first preliminary screen was to test the effectiveness of the newly created *Raf* stock in generating metastasis. Although the final *Ras* stock was unable to be used, the experimental screen was still designed and implemented using UAS-Raf^{ACT}; III/TM6B as shown in *Figure 4.9*. These flies were mated to males that carried UAS-RNAi transgenes that when activated would silence tumor suppressor loci by RNAi. Male

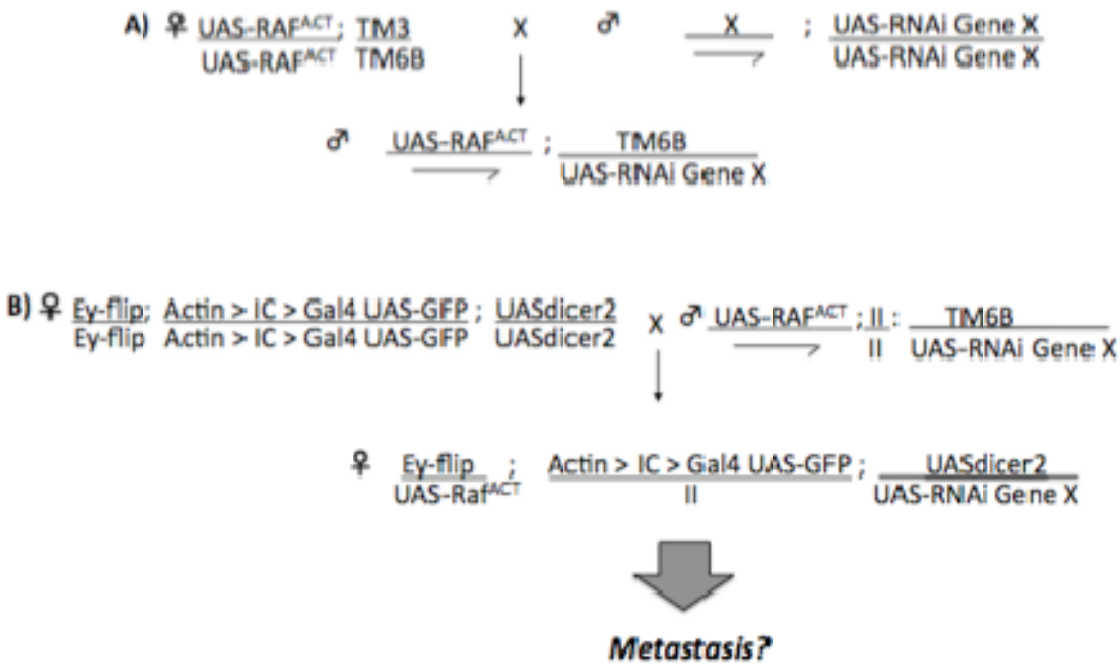


Figure 4.9: The experimental crossing schematic for inducing metastasis in *Drosophila melanogaster*

offspring were then mated to females of the ey-Flip; Act>IC>Gal4 UAS-GFP; UAS-dicer2 genotype. While the first cross combined the two mutations-inducing transgenes within the fly, hyperactive *Raf* and the tumor suppressor gene RNAi, the second ensured the localization and visualization of cancerous cells using Green Fluorescent Protein.

In the final cross, four types of progeny were produced, with only one containing the desired genotype, ey-Flip/UAS-Raf^{ACT}/Actin>IC>Gal4 UAS-GFP; UAS-dicer2/UAS-RNAi (*Figure 4.10*). The other three possible genotypic offspring served as controls with either the tumor suppressor gene being present or hyperactive *Raf* being absent, or with one type of progeny carrying neither. Only the ey-Flip/UAS-Raf^{ACT}; Act>IC>Gal4 UAS-GFP; UAS-dicer2/TM6B progeny were scored, as these determined the phenotype of hyperactive *Raf*.

A subset of RNAi genes were selected for this screen as well, based on their suspected involvement in metastasis development and their proven effectiveness in down regulating the targeted gene based on mutant effects induced in the eye: *vsp25, ce, dsh, arm, shg, skpA, ups7*, and *dlg* (*Figure 4.11*). Larval analysis for *ups7* and *dlg* showed the presence of ectopic cells at both the anterior and posterior, at a respective ratio of 1:7 and 4:11.

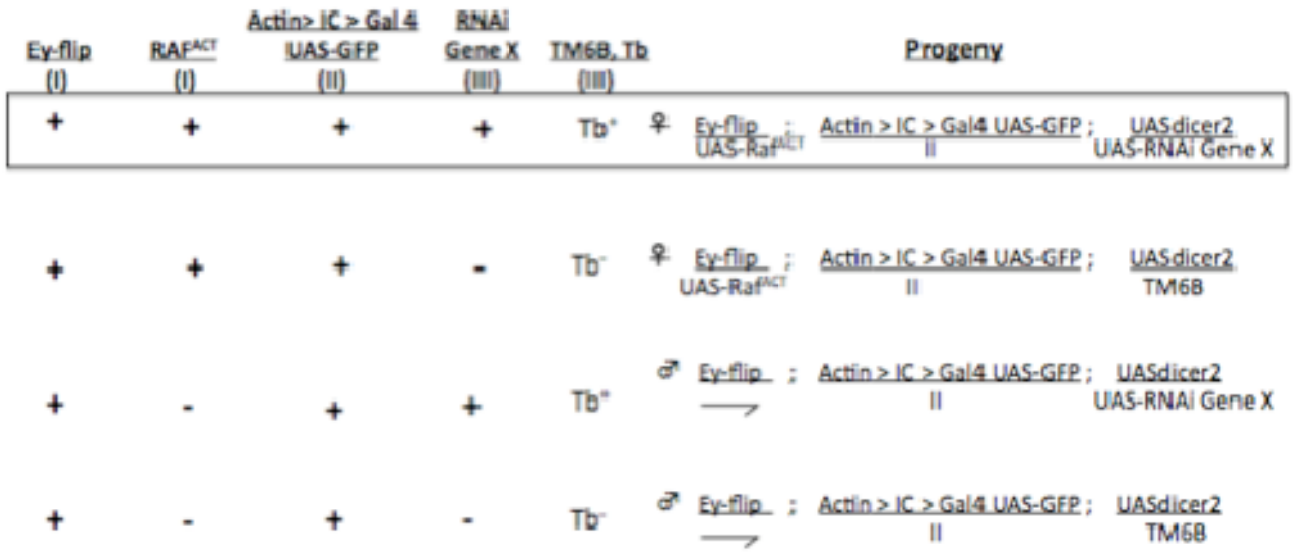


Figure 4.10: Progeny of final experimental cross. The first genotype serves as the experimental, while the remaining 3 possible genotypes act as a control.

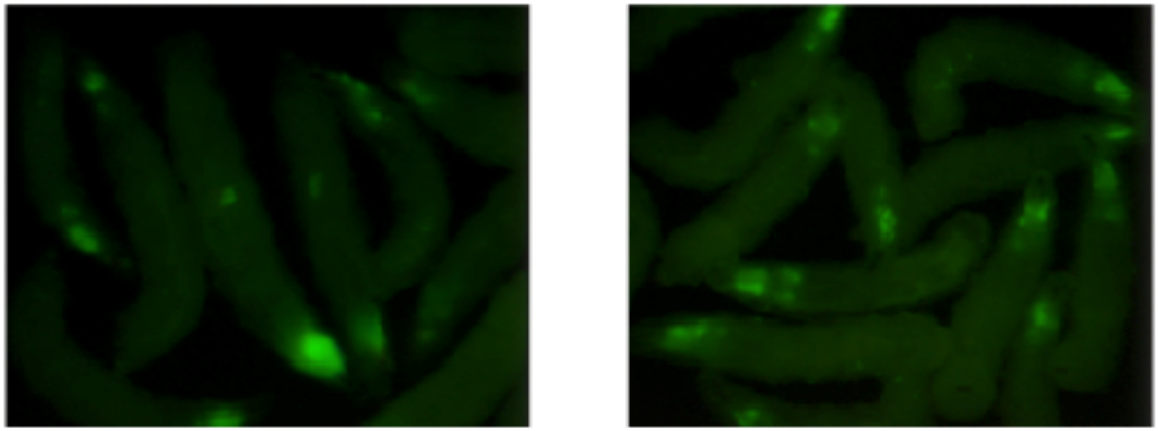
Gene	Gain or Loss of Function	Total Larvae Screened	Neoplastic Growth	Biological Processes	Molecular Function
Vps25	LOF	4	0:4	Vesicle-mediated transport	Unknown
Ce	LOF	3	0:3	Microtuble based movement	Transferase activity
Dsh	LOF	10	0:10	Epithelial cell differentiation	Phosphatidic acid binding
Arm	LOF	8	0:8	Cell adhesion	Kinase binding/ Transcription cofactor activity
Shg	LOF	5	0:5	Cell adhesion	Protein binding
SkpA	LOF	10	0:10	Mitosis	Protein binding
Ups7	LOF	7	1:7	Unknown	Unknown
Dlg	LOF	11	4:11	Cell polarity	Protein binding

Figure 4.11: The preliminary screen tested the knockdown effects of *vps25, ce, dsh, arm, shg, skpA, ups7*, and *dlg*. Neoplastic growth, the presence of cancerous cells at secondary sites, occurred for *dlg* and *ups7*.

As shown in *Figure 4.12*. GFP positive cells were observed in the larval midsection for two *dlg* experimental samples. Two other *dlg* samples (not shown) expressed GFP in the anterior of larvae, with one of the samples also showing ectopic expression in the mid-section as well. Only one (out of seven) *Ups7* larvae was metastatic, expressing green fluorescent protein at the anterior. All controls expressed green fluorescent protein within the eye antennal epithelium and part of the brain lobes, as expected.

Pilot Screen

After it was confirmed that silenced tumor suppressor genes, in combination with Raf^{ACT}, were able to induce metastasis, a larger pilot screen was performed. Since *dlg* and *ups7* generated ectopic GFP cells in the preliminary screen, these genes were re-tested in the secondary screen. Eight other genes were selected based on previous research performed, indicating the involvement of these genes in the regulatory processes relating to metastasis: *scrib, pten, uasyki, dsh, cdc27, cdc2, cdc37,* and *tsc1* (*Figure 4.13*). Specifically, extensive research has been done on the knockdown effects of *scrib, dlg*, and *dsh* in conjugation with hyperactive *Ras*, in causing metastatic behavior. Therefore, it was expected that the silencing of these genes would induce the presence of ectopic cells when combined with overactive *Raf*. These genes did in fact advance the formation of malignant



Eyflp; $Raf^{\Delta CT}$; Act>IC>Gal4 UAS-GFP; UAS-dicer2/RNAi dlg

Eyflp; ; Act>IC>Gal4 UAS-GFP; UAS-dicer2/RNAi dlg

Figure 4.12: In the preliminary screen, *Dlg* knockdown showed the above phenotypes. In the presence of hyperactive *Raf*, silenced *Dlg* resulted in metastasis as shown on the left. However, when in the absence of $Raf^{\Delta CT}$, silenced *Dlg* did not show the presence of ectopic cells (right).

Gene	Gain or Loss of Function	Growth	Biological Processes	Molecular Function
Scrib	LOF	Neoplastic	Cell polarity	Protein binding
Dlg	LOF	Neoplastic	Cell polarity	Protein binding
Pten	LOF	Neoplastic	Negative PI3K pathway regulator	Phosphoprotein phosphatase activity
UASyki	LOF	Neoplastic	Downregulates Hippo tumor suppressor pathway	Transcription factor binding/transcription cofactor activity
Dsh	LOF	Neoplastic	Epithelial cell differentiation	Phosphatidic acid binding
Cdc27	LOF	Hyperplasia	Mitosis	Binding
Cdc2	LOF	Hyperplasia	Cell cycle, Meiosis	Protein binding
Cdc37	LOF	Hyperplasia	Neurogenesis	Unfolded protein binding
Tsc1	LOF	Hyperplasia	Regulation of the insulin receptor pathway	Kinase activity
Ups7	LOF	Hyperplasia	Unknown	Unknown

Figure 4.13: The pilot screen tested the knockdown effects of an assortment of tumor suppressor genes: *scrib, dlg, pten, UASyki, dsh, cdc27, cdc2, cdc37, tsc1*, and *ups7*. Neoplastic growth, the presence of cancerous cells at secondary sites, occurred for *scrib, dlg, pten, UASyki*, and *dsh*.

cancers; *pten* and *UAS-yki*, which caused for the overexpression instead of knockdown of the *Yki* protein, also showed similar results.

As seen in *Figures 4.14/4.15/4.16*, the suppression of *scrib* and *dlg* produced the greatest metastatic rate; suppression of *pten* or *dsh* and the overexpression of *yki* were only able to trigger cancer less robustly, at rates of 5.6%, 5.6%, and 7.1% respectively. Both *tsc1* and *cdc27* had less than a 4% chance of showing metastasis. Within the controls tested, ectopic cells were present in one larvae, generating a 0.5% metastatic rate for the total sample size (*Figure 4.17*).

In order to determine the relationship between larval development and metastatic rate, experimental samples were re-scored as pupae (*Figures 4.18 and 4.19*). While each of these samples contained the desired genotype, none of these larvae contained ectopic GFP cells as larvae. However, these samples exhibited a 7.1% chance of forming malignant tumors at a later stage in development. These samples also consistently exhibited tissue shrinkage from the pupal encasing, interfering with eclosion and thereby causing death. As such, the development of cancer in *Drosophila* mirrors the progression of the disease within humans, strengthening in development over time.

EXPERIMENTAL TOTAL					
Experimental: Female Tb (+) [Yes RNAi, Yes RafACT]					
Gene	GFP in Eye	Ectopic GFP	Speckling	No GFP	# Analyzed
Scrib	32	12	8	4	56
Dlg	40	10	15	3	63
Cdc2	7	0	6	33	40
Pten	32	3	7	2	54
Ups7	27	0	6	2	29
Cdc27	19	1	3	20	39
UASyki	27	3	6	15	42
Cdc37	17	0	11	26	43
Tsc1	27	1	4	0	27
Dsh	33	2	6	3	36
Total analyzed	321	32	72	108	429
Total rate of occurrence	74.8%	7.5%	16.8%	25.2%	

EXPERIMENTAL PROBABILITIES					
Experimental: Female Tb (+) [Yes RNAi, Yes RafACT]					
Gene	GFP in Eye	Ectopic GFP	Speckling	No GFP	# Analyzed
Scrib	92.9%	21.4%	14.3%	7.1%	43
Dlg	95.2%	15.9%	23.8%	4.8%	50
Cdc2	17.5%	0.0%	15.0%	82.5%	40
Pten	96.3%	5.6%	13.0%	3.7%	47
Ups7	93.1%	0.0%	20.7%	6.9%	29
Cdc27	48.7%	2.6%	7.7%	51.3%	36
UASyki	64.3%	7.1%	14.3%	35.7%	42
Cdc37	39.5%	0.0%	25.6%	60.5%	43
Tsc1	100.0%	3.7%	14.8%	0.0%	27
Dsh	91.7%	5.6%	16.7%	8.3%	32

Figure 4.14: The experimental phenotype was scored for ey-Flip/Raf^{ACT}; Act> IC> Gal4 UAS-GFP; UAS-dicer2/UAS-RNAi Gene X. Knockdown of *Scrib* and *Dlg* showed the highest incidence of metastasis, with the suppression of *pten*, *dsh*, and *UASyki* also generating ectopic growth. *Tsc1* and *Cdc27* had less than a 4% chance of developing cancer, when silenced.

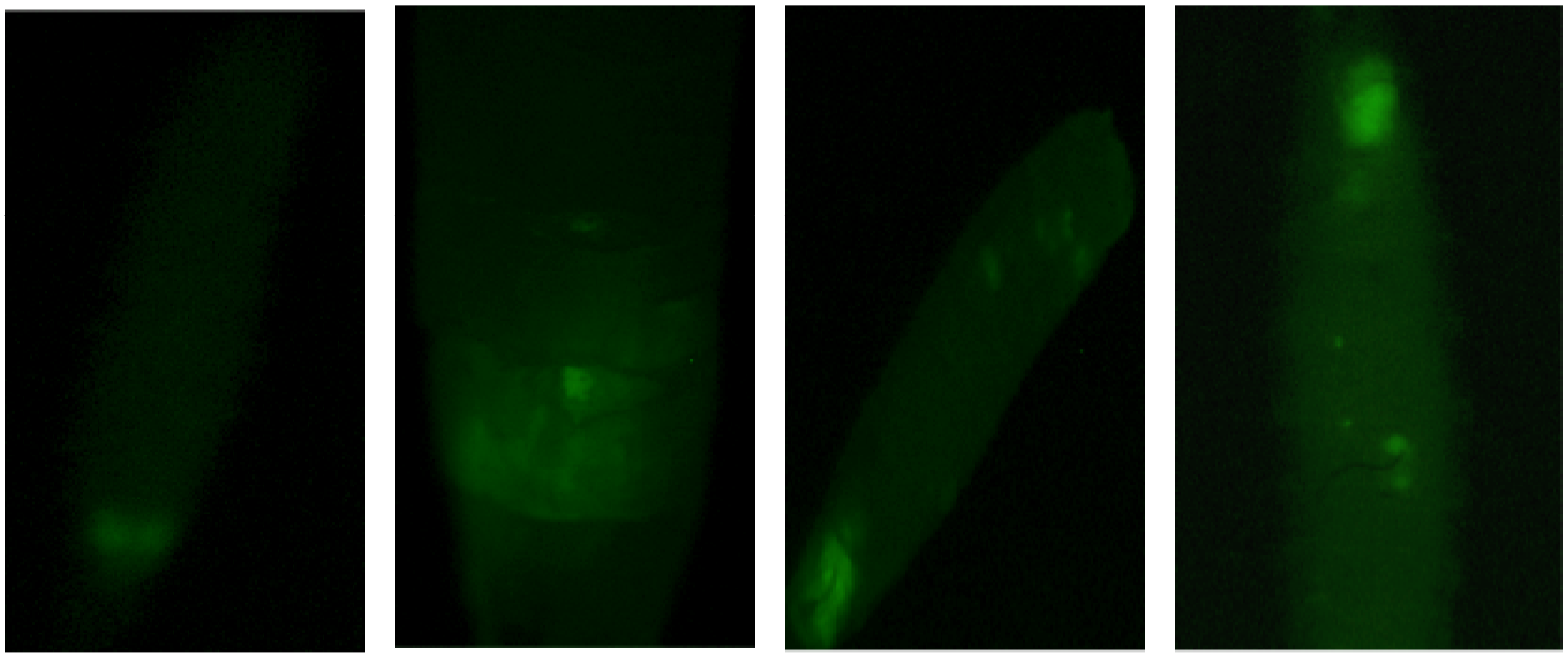


Figure 4.15: Images of ey-Flip/Raf^{ACT}; Act> IC> Gal4 UAS-GFP; UAS-dicer/UAS-RNAi *scrib*. The figure on the left is GFP-positive, as green fluorescent protein is expressed solely in the eye discs. Speckling was observed in the second figure (from the left), with a very small, sharp dot present within the VNC. Ectopic cells are present in the last two figures, which express GFP in the posterior and mid-section respectively. *Scrib* larvae exhibited the highest metastatic rate within the experiment, at 21.4% rate.

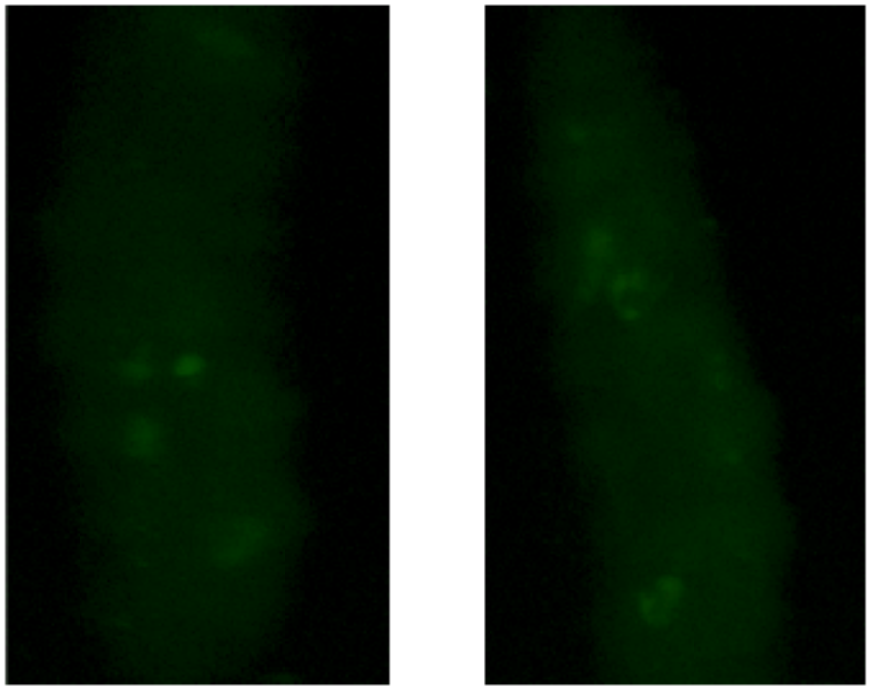


Figure 4.16: Images of ey-Flip/Raf^{ACT}; Act> IC> Gal4 UAS-GFP; UAS-dicer/UAS-RNAi *dlg*. Both larvae exhibit metastatic behavior, as ectopic GFP cells are present throughout the organism. Silenced *dlg* exhibited a 15.9% chance of developing metastasis, when in conjugation with overactive *Raf*.

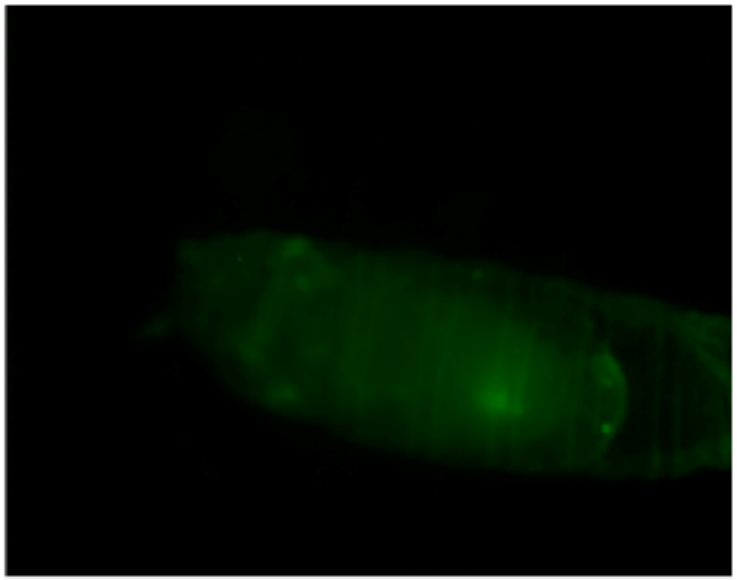
CONTROL TOTAL					
Control: Female Tb (-) [No RNAi, Yes RafACT]					
Gene	GFP in Eye	Ectopic GFP	Speckling	No GFP	# Analyzed
Scrib	41	1	8	6	47
Dlg	14	0	3	2	16
Cdc2	13	0	3	2	15
Pten	16	0	3	2	18
Ups7	30	0	6	0	30
Cdc27	0	0	0	9	9
UASyki	23	0	3	5	28
Cdc37	14	0	6	2	16
Tsc1	10	0	4	0	10
Dsh	5	0	1	0	5
ey-Flip	34	3	0	0	34
wRNAi	12	0	0	0	12
Total analyzed	166	1	37	28	194
Total rate of occurrence	85.6%	0.5%	19.1%	14.4%	

CONTROL PROBABILITIES					
Control: Female Tb (-) [No RNAi, Yes RafACT]					
Gene	GFP in Eye	Ectopic GFP	Speckling	No GFP	# Analyzed
Scrib	87.2%	2.1%	17.0%	12.8%	33
Dlg	87.5%	0.0%	18.8%	12.5%	16
Cdc2	86.7%	0.0%	20.0%	13.3%	15
Pten	88.9%	0.0%	16.7%	11.1%	18
Ups7	100.0%	0.0%	20.0%	0.0%	30
Cdc27	0.0%	0.0%	0.0%	100.0%	9
UASyki	82.1%	0.0%	10.7%	17.9%	28
Cdc37	87.5%	0.0%	37.5%	12.5%	16
Tsc1	100.0%	0.0%	40.0%	0.0%	10
Dsh	100.0%	0.0%	20.0%	0.0%	5
ey-Flip	100.0%	8.8%	0.0%	0.0%	34
wRNAi	100.0%	0.0%	0.0%	0.0%	12

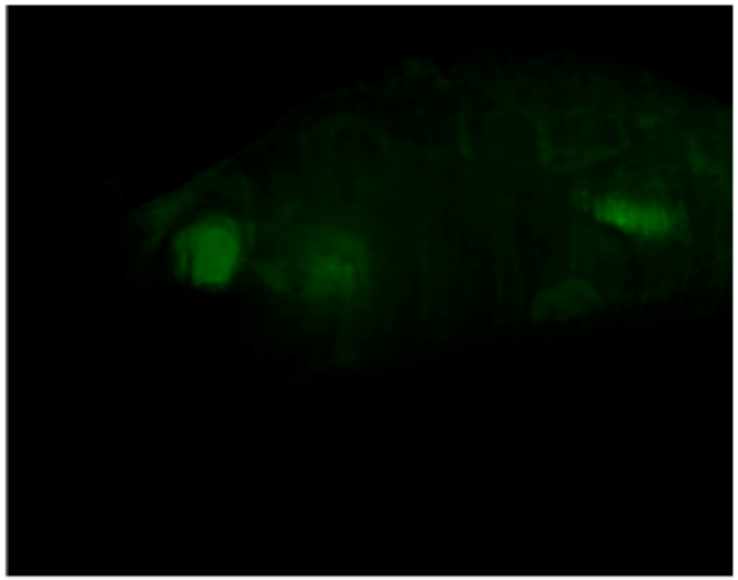
Figure 4.17: The control phenotype was scored for ey-Flip/Raf^{ACT}; Act> IC> Gal4 UAS-GFP; UAS-dicer/TM6B. These controls lacked RNAi expression. However, as depicted in the chart above, metastasis was observed in 0.5% of the samples. These controls also depicted a high rate of the speckling phenotype. *Cdc27* was unique in that no GFP positive cells were observed. The ey-Flip controls that were scored for ectopic GFP fluoresced throughout, due to inaccuracies in the FRT/FLP system.

Gene	Date of larval analysis	Date of pupal analysis	Ectopic GFP previously shown	Experimental	Control
Scrib	2/27	3/2	3:4	1:9	0:2
Scrib	2/28 + 2/29	3/2	4:5	1:13	4:18
Dlg	2/27	3/2	4:4	0:6	2:5
		Total Rate of Occurence	84.6%	7.1%	24%

Figure 4.18: *Scrib* and *dlg* samples that had been previously scored at the larval stage, were rescored as pupae in order to determine the correlation between tissue development and metastatic rate. Larval samples that did not previously contain metastatic sites, exhibited a 7.1% chance of developing metastasis as pupae. Interestingly, control samples showed a higher rate of metastasis; these samples were not expected to develop malignant cancers.



Ey_flip/ Raf^{ACT}; Act>IC>Gal4 UAS-GFP; UAS-dicer2/UAS-RNAi GeneX



Ey-flip/ Raf^{ACT}; Act>IC>Gal4 UAS-GFP; UAS-dicer2/TM6B

Figure 4.19: In approximately 7% of experimental pupae, ectopic GFP cells were observed at the anterior tissue (left). Pupae that exhibited signs of metastasis also characteristically were affected by tissue shrinking from the pupal encasing. This interfered with eclosion and resulted in death. A larger sample of control larvae also exhibited signs of metastasis (right).

However, some larvae also exhibited no GFP expression; this was most likely due to interference by the RNAi mechanism; RNAi expressing cells possibly may have died thereby causing an absence of GFP expression. It should also be noted that control samples containing Raf^{ACT}, which did not previously contain ectopic GFP cells, later indicated metastasis with a 24% metastatic rate. As demonstrated in the *Ras* vs. *Raf* screen, hyperactive signaling sometimes resulted in ectopic GFP expression. While this result was not expected, its occurrence is most likely correlated with inefficiencies in the FLP/FRT and UAS/Gal4 systems. An inefficient flippase enzyme would have been unsuccessful in its role to localize GFP expression to the epithelial tissue of larvae.

Larvae of the original ey-Flip; Act>IC> Gal4 UAS-GFP; UAS-dicer2 were analyzed under the fluorescence microscope in order to test this theory. Some larvae exhibited GFP expression throughout all tissues, thereby substantiating the imperfections within the ey-Flip stock.

5. FUTURE DIRECTION

Important Cellular Processes

As noted by the experimental results, suppressed *scribble (scrib)* and *discs large (dlg)* produced the most significant rate of metastasis, in concert with hyperactive *Raf*. Extensive research has proven both of these genes to be involved in the processes of cellular polarity; mutations in both *scrib* and *dlg* have been known to cause overgrowth phenotypes and defects in epithelial monolayer formation (Pagliarini, 2003). It has been suggested that the involvement of these genes in "the abrogation of inter-cellular junctions or the mislocalization of plasma membrane-targeted signaling molecules" prevents the formation of NONinvasive tumors (Pagliarini, 2003). Likewise, the conserved scaffolding protein that is encoded by *scrib* and *dlg* is also necessary in maintaining cell polarity (Halder and Mills, 2011). Therefore the existence of these genes within the *Drosophila* genotype is necessary in order to ensure proper apical-basal cell polarity and consequently normal, healthy epithelial cells (Halder and Mills, 2011).

Future Connections

By identifying the cellular processes that are related to cancer, like cell polarity and epithelial monolayer formation, more advances can be made in the field of oncology. By understanding

such connections, the nature of cancer can be further questioned through analyzing the initiating effects of various molecular and cellular processes in cancer development. For instance, it is quite possible that the characteristics of this disease could vary based on whether suppression occurs in cell polarity or vesicular trafficking proteins. By identifying the biological role of specific proteins, questions like these can be addressed and the inherent qualities of cancer can be further understood.

Development of Screen

Based on the pilot screen, a larger-scale screen could be generated to further test the metastatic effects of UAS-Raf^{ACT}; III/TM6B. Tumor suppression genes that consistently lead to green offsites could also be analyzed using different hyperactive signaling pathways, like with Notch or EGFR. By comparatively studying the interaction of these pathways with the mutated genes, it can be determined if all tumor cells are inherently the same or not in terms of which second site hits best promote metastasis.

As such, the newly generated *Raf* stock, from my Capstone, could be useful in determining the roles and regulatory processes of specific genes, as well as helping researchers to further understand malignant growth.

6. REFERENCES

Basler, K, Toggwiler, J, Willecke, M. (2011). Loss of PI3K Blocks Cell-Cycle Progression in *Drosophila* Tumor Model. *Oncogene* (e-pub ahead of print; doi:10.1038/onc.2011.125).

Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F, et al. (2007). Oncogenic Activation of the RAS/RAF Signaling Pathway Impairs the Response of Metastatic Colorectal Cancers to Anti-Epidermal Growth Factor Receptor Antibody Therapies. *Cancer Research* **67**: 2643-2648.

Childress, J, Behringer, R, and Halder, G. 2005. Learning to Fly: Phenotypic Markers in *Drosophila*. Poster. *Genesis*.

"Cancer Facts & Figures 2012." *American Cancer Society*. 2012. 23 Feb. 2012. <http://www.cancer.org/Research/CancerFactsFigures/CancerFactsFigures/cancer-facts-figures-2012 >.

Duffy, J. 2002. GAL4 System in Drosophila: A Fly Geneticist's Swiss Army Knife. *Genesis* **34**: 1-15.

Greenspan, Ralph J. *Fly Pushing: The Theory and Practice of Drosophila Genetics*. 2nd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 2004.

Halder, G, Mills, GB. (2011). *Drosophila* in Cancer Research: to Boldly Go Where No One Has Gone Before. *Oncogene* (e-pub ahead of print; doi:10.1038/onc.2011.128).

Hanahan, D, Weinberg, R. (2000). The Hallmarks of Cancer. *Cell* **100**: 57-70.

Hannon, G. 2002. RNA interference. *Nature* **418**: 244-251.

Janda, E, Lehmann, K, Killisch, I, Jechlinger, M, Herzig, M, Downward, J, Beug, H, Grünert, S. (2002). Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *Cell* **152**: 299-313.

Knudson, Alred G. Two genetic hits (more or less) to cancer. 2001. Review. *Nature* **1**: 157-162.

"Knudson's 'Two-Hit' Theory of Cancer Causation." *Fox Chase Cancer Center*. <http://www.fccc.edu/research/areas/advisors/knudson/twoHit.html >.

Lengauer, C, Kinzler, K.W.and Vogelstein, B. (1998). Genetic Instabilities in Human Cancers. *Nature* **396**: 643–649.

Liotta LA, Steeg PS, Stetler-Stevenson WG. (1991). Cancer Metastasis and Angiogenesis: An Imbalance of Positive and Negative Regulation. *Cell* **64**: 327-336.

Pagliarini, R, *et al*. (2003). A Genetic Screen in *Drosophila* for Metastatic Behavior. *Science* **302**: 1227-1231.

Powell, Jeffery R. 1997. *Progress and Prospects in Evolutionary Biology: The Drosophila Model*. New York: Oxford Univ. Press. 562 pp.

Reuter CW, Morgan M, Bergmann L. (2000). Targeting the RAS Signaling Pathway: a Rational, Mechanism-based Treatment in Hematological Malignancies. *Blood* **96**: 1655-1669.

Reynolds, A, Leake, D, Boese, Q, Scaringe, S, Marshall, W, and Khvorova A. 2003. Rational siRNA Design for RNA Interference. *Nature Biotechnology* **22**: 326-330.

Rorth, P. 1998. Gal4 in the Drosophila female germline. Mechanisms of Development 78: 113-118.

The Arrogant Scientist. "The Differences Between the Sexes." Blog. 6 Mar. 2009. *Word Press*. 26 Mar. 2012. <http://arrogantscientist.wordpress.com/2009/03/06/the-differences-between-the-sexes/>.

Twyman, Richard. "Model Organisms: The Fruit Fly." *The Human Genome*. 29 Aug. 2002. <http://genome.wellcome.ac.uk/doc_WTD020807.html>.

"Understanding Cancer Series". *National Cancer Institute at the National Institutes of Health*. <http://www.cancer.gov/cancertopics/understandingcancer/cancer/page9>.

7. SUMMARY

Cancer is an extremely complex disease, as tumorigenesis requires mutations in tumor suppressor and oncogenes (Basler, Toggwiler, and Willecke). However, over the years, oncogenic research has proved there to be six fundamental traits of cancer: a "self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue evasion and metastasis" (Hanahan and Weinberg). While each of these identified qualities contribute to the lethality of the disease, my Capstone project specifically focuses on the topic of tissue evasion and cancer metastasis.

Both benign and malignant tumors possess the first five fundamental traits of cancer, only differing in their ability to evade tissues and thereby infect new areas of the body. Cancer is described as being benign when the disease is localized to a specific area within an organism, demonstrating no outward growth from the disease's initial area of formation. In contrast, malignant cancer is extremely dangerous as it visibly moves to new locations within the body, creating metastatic sites.

Metastatic sites form when an area that was previously unoccupied by cancerous cells, exhibits the presence of new, cancerous cells (Hanahan and Weinberg). By migrating from the

original location of the disease to a non-adjacent area within the body, cancerous cells cause for new sites to become infected by the disease (Hanahan and Weinberg). There are multiple mechanisms that enable such movement of cancerous cells, like uncontrolled mitosis or angiogenesis (Hanahan and Weinberg). Angiogenesis enables cancer cells to utilize the blood stream or lymphatics in order to metastasize, while mitosis refers to the uncontrollable division of cancerous cells (Hanahan and Weinberg). Both of these qualities are dependent upon the other fundamental traits of cancer.

As proposed by Dr. Knudson, there is also a simplified model of the development of cancer termed the "two-hit" theory (Knudson's 'Two-Hit' Theory of Cancer Causation). Knudson suggests that the causation of cancer is heavily based on multiple chromosomal mutations (Knudson's 'Two-Hit' Theory of Cancer Causation). While the first "hit" makes an organism more susceptible to developing the disease, the second "hit" is most likely to cause for cancer (Knudson's 'Two-Hit' Theory of Cancer Causation). This theory is reflected in my Capstone Project, as I utilized the effects of two separate mutations, in both tumor suppressor and oncogenes, as a leading cause to metastasis in *Drosophila melanogaster*.

The first mutation in my experiment was in a specific signaling pathway, since overactive signal transduction pathways

have also been known to contribute to the formation of cancer. Within this pathway, a signal or ligand is received by a growth factor receptor, initiating an appropriate cellular response within the cell. Phosphorylation allows for the activation of proteins through the addition of a phosphate molecule, regulating the desired activation level within a pathway. As such, the behavior of overactive signaling pathways is as if the proteins within the transduction pathway are continually phosphorylated, or activated. Manipulation of signaling pathways can also mimic the effects of the constant presence of a ligand or signal, which would also cause for the pathway to be overactive.

The pathway that I specifically manipulated was an MAPK signaling pathway called the RAS-RAF pathway (Research BRAF). In this pathway there are two types of protein kinases, *Ras* and *Raf*, which are responsible for relaying the extracellular signal within the cell (Research BRAF). Mutations to the RAS-RAF pathway can cause changes in cell differentiation, proliferation, and growth – three processes that this pathway is responsible in controlling for normal cell function (Research BRAF). In order to determine the most effective hyperactive *Ras* and *Raf* lines to use, each available stock was analyzed to distinguish which caused the greatest proliferation within larval eye tissues. Originally, two stable stocks for hyperactive *Ras* and *Raf* were created; however,

due to a substantial weakness in the *Ras* line, it was eliminated from the experimental project. Therefore, in my project, I used a *Raf* mutant protein in order to create a hyperactive GTPase signaling pathway within a specific line of flies.

These tester flies were mated to a separate line of flies that carried a transgene with the ability to silence targeted genes, using RNA interference. By decreasing the activity of specific genes, RNA interference enabled me to create a second mutation in my project, causing the progeny flies to be more susceptible to developing cancer.

The offspring carried a genome that had an overactive as well as a "knocked-out" gene and consequently were analyzed for the occurrence of metastasis. Analysis was possible due to the UAS/Gal-4 and FLP/FRT systems and by the presence of the protein marker, green fluorescent protein, which localized expression of GFP in the eye tissue of normal, or wild type, larvae. As such, the occurrence of metastasis was verified when GFP positive cells were detected in locations other than the developing head epithelium.

Originally larvae were analyzed *in vitro*, however later the procedure changed to an *in vivo* approach due to a better visualization of metastasis within living, intact larvae. A preliminary screen was performed in order to test the effectiveness

of the final overactive *Raf* stock, by crossing this line of flies to a subset of genes: *vps25, ce, dsh, arm, shg, skpA, ups7*, and *dlg*. Ectopic behavior was observed in *dlg* and *ups7*; once it was confirmed that the newly constructed *Raf* line was able to induce metastasis, in conjugation with the suppression of specific genes, a pilot screen was designed. In this final screen a variety of genes were also tested including *scrib*, *dlg*, *cdc2*, *pten*, *ups7*, *cdc27*, *UASyki*, *cdc37*, *tsc1*, and *dsh*. Ectopic expression of GFP and the occurrence neoplastic growth was most significant in *scrib* and *dlg*.

In the scientific community, extensive research has been performed on both *scrib* and *dlg*, using a mutant *Ras* protein. This research has indicated the biological processes of both *scribble* and *discs large* to be involved in cell polarity (Pagliarini, 2003). Mutations in these genes also caused for defects in epithelial monolayer formation (Pagliarini, 2003). As such both cellular polarity and epithelial monolayer formation are critical in preventing the metastasis of cancer (Pagliarini, 2003).
In performing a novel screen, it was discovered that *scrib* and *dlg*, in combination with RAF^{ACT}, led to metastatic cancer. This indicates that an overactive *Raf* pathway, in conjugation with these silenced cell polarity genes, is also able to cause defects in the normal functioning of cells. As such, both of these mutations are necessary in order to induce metastatic behavior.

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