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Establishing a Drosophila Colon Cancer Model to Study Interactions and Therapeutic Targets of Oncogenic Pathways

Kathleen T. McCaslin
University of Dayton

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Honors Thesis

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Abstract

The objective of this project is to develop an in vivo colorectal cancer (CRC) model in *Drosophila melanogaster* to test the role of Ras and Wnt pathways in gastrointestinal cancer as potential therapeutic targets. To do so, we have (a) developed a CRC model in flies, (b) tested the levels of Ras and Wnt pathway activity in this model, and (c) will use drugs to find inhibitors of these pathways.

Using fly mutants and transgenic flies we have created small patches of cancerous cells in the fly intestine in which have activated oncogenic Ras (mutation Ras^{V12}) and dominant negative p53 (mutation $UASp53^{H15N}$) together with loss of function of APC using mosaic techniques. This model allows evaluation of multiple genetic combinations (one-, two-, or three- hit models) to evaluate the induced tumor, its growth profile and the effect of the drug on tumor growth.

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Introduction

Colorectal cancer (CRC) is the third most diagnosed cancer in the world, accounting for 11% of all cancer diagnoses (Rawla, 2019). Among these patients, mutations in genes adenomatous polyposis coli (APC), p53, and the Ras family are frequently found. Inactivating mutations in APC have been reported in 34-70% of cases (Luchtenborg, 2004), and result in activation of Wnt via truncated APC proteins (Zhang, 2017). Oncogenic Ras is observed in approximately 52% of CRC cases, and overactivation results in the constitutive activation of the Ras pathway, which controls cell growth and proliferation (Fernandez-Medarde, 2011). Additionally, Ras is a negative regulator of the Hippo pathway, which regulates tissue size and leads to constitutive activation of transcription factors YAP and TAZ (Barron, 2014). Finally, loss of p53 or mutations causing dominant negative p53 are also associated with CRC that shows increased metastatic or invasive behavior (Li, 2015). P53 is a tumor suppressor gene, so dominant negative activation results in unregulated death and division of the cells.

Although these mutations are frequently reported, the combination of these genetic alterations that generate the tumors that show metastatic growth and cause organ disruption remain poorly understood. Therefore, we have chosen to produce a three-hit model that incorporates these three mutations to affect different aspects of cell proliferation. This model can then be studied in multiple genetic combinations of these affected pathways to isolate the effects of one as a control and study its interactions with the other two mutations.

Literature Review

Colorectal cancer (CRC) is the second most common cause of cancer death in the United States, with an estimated 147,950 new cases in 2020 (Siegel, 2020). Globally, it is the third deadliest cancer, and the fourth most diagnosed (Rawla, 2019).

Those most at risk include those with family history of colorectal cancer; Black, American Indian, and Alaska Native adults; and those with risk factors such as obesity, smoking, diabetes, or unhealthy alcohol use. Worldwide, incidence of CRC is higher in Western countries and has increased steadily in developing countries adopting the "western" lifestyle that features a sedentary lifestyle, red meat consumption, obesity, alcohol, and tobacco (Rawla, 2019). However, age is the most significant risk factor, as 94% of new cases of colorectal cancer occur in adults 45 years or older (US Preventive Services Task Force, 2021). For this reason, the US Preventive Services Task Force (USPSTF) recommends that all adults aged 45 or older be offered regular screening.

Screening in the United States has increased 4.4% since 2018, but still leaves 26% of eligible adults unscreened and 31% overdue (US Preventive Services Task Force, 2021). Most common screening methods are divided into two categories: stool-based tests and direct visualization tests. Stool-based tests include guaiac fecal occult blood test (gFOBT), fecal immunochemical test (FIT), and stool DNA test. Direct visualization tests include colonoscopy, CT colonography, and flexible sigmoidoscopy. To maximize life-years gained and decrease colorectal cancer cases and deaths, the USPSTF recommends screening in one or more of these combinations of intervals: gFOBT or FIT every year, sDNA-FIT every 1-3 years, CT colonography or flexible sigmoidoscopy every 5 years, flexible sigmoidoscopy every 10 years with a FIT every year, and/or colonoscopy screening every 10 years.

Following a positive screening, treatment may vary based on severity of the cancer. If potentially curable, the patient may undergo surgery; in advanced cases, chemotherapy may improve and maintain quality of life (Labianca, 2010). Both treatment options are invasive and target healthy cells as well as cancerous ones. Therefore, it is imperative to find therapeutic targets for less invasive and more effective treatment.

The research question explored by this project is to identify these targets through studying the cooperative interactions between Wnt and Ras resulting in intestinal hyperplasia in *Drosophila melanogaster*. Using the escGAL4 system, a driver specific to

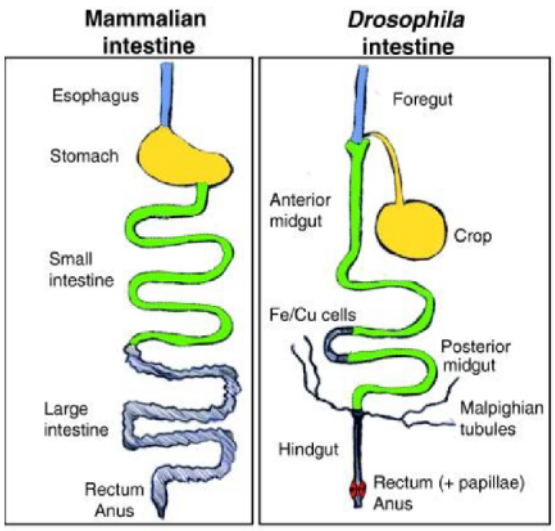

Figure 1: Intestinal structure of mammals and Drosophila. (Apidianakis, 2011)

intestinal stem cells (ISCs), new fly lines were created with manipulated p53 dominant negative and Ras-activated alleles in the intestine.

Drosophila were chosen for this model due to the potential to apply findings to higher vertebrate models because of similarity in intestinal tissue composition and molecular pathway conservation. Like the mammalian intestine, the *Drosophila* adult intestinal epithelium (Figure 1) is highly proliferative because it contains active, inactive, and dormant stem cells. There is also a considerable amount of overlap in the molecular pathways they share with humans. For example, the molecular nature of the Hippo pathway was discovered through genetic screens of *Drosophila*, and this pathway is strongly conserved in humans. (Barron, 2014). In addition, their quick repopulation time, high fecundity, and short life cycle allows for generations of genetic testing and timely results in adult models, as well as the possibility of creating a specific genetic model type. Other benefits include their ability to ingest cancer drugs in vivo, available array of genetic tools, and the ability to manipulate genes in defined cell types. Fruit flies are an effective preclinical model, as some phase I and II clinical trials have been successful based on fly genetic modeling of tumors.

The genes manipulated in this model to induce the constitutive activation of Ras/MAPK and Wnt and negative regulation of Hippo are as follows: p53, to undergo dominant negative mutation $p55^{H159N}$; Ras, to undergo mutation Ras^{v12}; and APC, to undergo mutation 82Bapc1apc2. Gene expression is manipulated with escGAL4. In healthy cells, p53 is a tumor suppressor gene, promoting programmed cell death and division. Inducing loss of function by activating a dominant negative form of this gene will lead to an over proliferation of cells. Normally functioning Ras produces Ras signaling proteins to drive cell growth and proliferation through the Ras pathway (Figure 2), while overactivation will excessively promote stem cell division (Saeed, 2019 and Asha, 2003). Finally, healthy APC is a tumor suppressor gene serving multiple functions, involved in processes related to cell migration, cell adhesion, proliferation, differentiation, and chromosome segregation. Inducing loss of function leads to constitutive activation of the Wnt signaling pathway (Zhang, 2017).

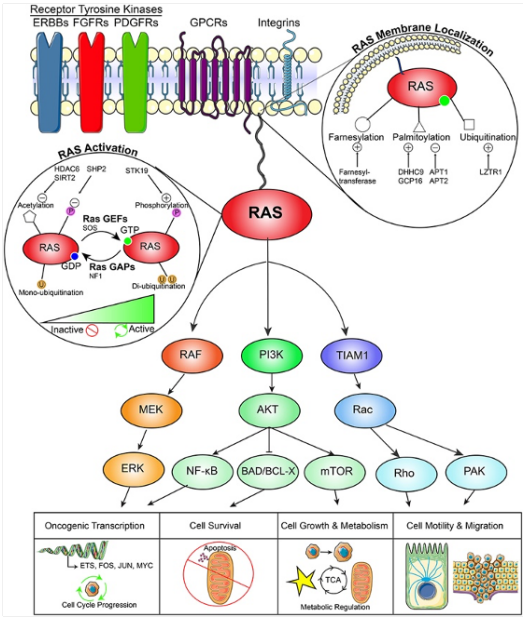

Figure 2: (left) Ras signaling pathway (Gimple, 2019)

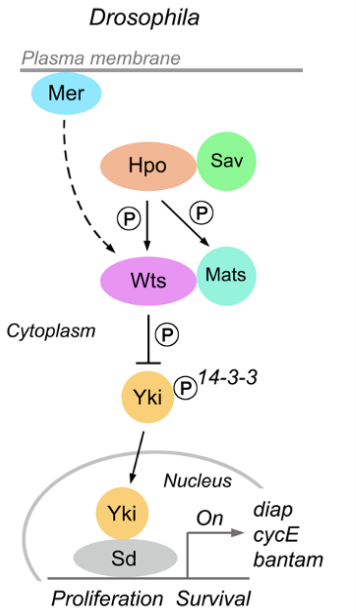

Figure 3: (right) Hippo pathway in *Drosophila* (Wang, 2018)

The Hippo and Wnt pathways are useful targets for their role in homeostasis and regeneration of gastrointestinal tissues. These were mentioned in the introduction as linked to Ras and APC genes respectively. Hippo (Figure 3) is a highly conserved signaling pathway that controls organ size and tissue growth by utilizing YAP and TAZ. The canonical Wnt signaling pathway (Figure 4) maintains embryonic and tissue homeostasis through utilizing B-catenin, a transcriptional coactivator (Majidinia, 2017). Wnt also plays an essential role in the development and regeneration of the intestinal epithelium. Overactivation of this pathway results in constitutive activation of B-catenin, which leads to excessive ISC proliferation. Complex interactions between the Hippo and Wnt pathways result in altered gene expression of multiple protooncogenes and tumor suppressor genes (Majidinia, 2017). Since Wnt signaling is activated through B-catenin, and a transcriptional target of B-catenin is CD44, which has the ability to activate NF2, Wnt signaling can activate the Hippo pathway (Barron, 2014).

Given the intricate roles of these pathways in development and disease, it is important to test which variations play a critical and causal role in colorectal cancer. Therefore, we decided to test the effects of manipulating these pathways in multiple combinations to assess their specific roles in colorectal cancers using Drosophila. The key findings from these initial assessments are described in subsequent sections.

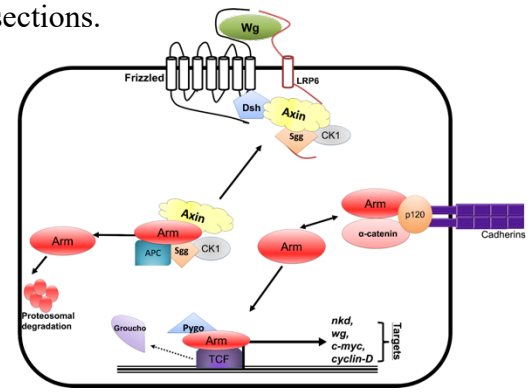

Figure 4: Canonical Wnt signaling pathway (Rai, 2022)

Methods

The fly stocks used for this experiment were obtained from Bloomington *Drosophila* Stock Center and cultured on standard yeast-cornemal-agar *Drosophila* food at 20°C in a humidity controlled incubator. Starting by crossing genotypes *UAS p53^{H159N}*(x) with *w:escGAL4;GAL^{80ts}, w:escGAL4 UAS GFP* with *UAS p53^{H159N}*(X), and *w; escGAL4 UAS GFP* with *hsFLP;Sp/CyO;TM3/TM6B*, a team of people was able to generate a large number of crosses to complete progress towards this goal. This involved ensuring that phenotypic markers were present to select for the correct flies, confirming both parent flies had the correct gene on both chromosomes, or including green fluorescent protein (GFP) so that activity can be noted under a microscope.

Flies were maintained by transferring adult flies every third day into a new labeled plastic vial with agar and capped with a cotton ball. All vials were kept for two weeks, resulting in a 5x5 grid of vials featuring a row with adult flies, eggs, larvae, pupae, and new flies, which was then replicated in four identical rows. Using this method, different stages of the Drosophila life cycle can be observed in increasingly older vials.

Mature third instar larvae were collected around day three of the life cycle to undergo intestinal dissection. Dissections were completed in PBS with size 5 and 55 forceps to isolate the intestine before fixing it in 150 μL PBS and 50 μL 16% paraformaldehyde for 20 minutes. Samples then underwent two series of 15 minute washings in 1000 μL 1x PBST. One washing included adding the samples and 1000 μL 1x PBST to a 1.5 mL microcentrifuge tube, rotating it on the nutator for 15 minutes, and carefully removing the 1x PBST via vacuum while leaving the samples in the 1.5 mL microcentrifuge tube. After this series of washings were complete, the samples rested in primary antibody Prospero (dilution 1:100) at 4°C overnight. The amount of antibody added was dependent on the number of larvae dissected, and was equivalent to half the number of samples in microliters. The next morning, Prospero was removed and the samples were again washed twice in 100 μL 1x PBST for 15 minutes each. Secondary antibody mouse Cy3 (dilution 1:250) was then added and the samples were mixed on the nutator covered in tinfoil for two hours at 20°C. After undergoing a final series of washing in 100 μL 1x PBST for 15 minutes, the samples were mounted in Vectashield and useful slides were imaged under a confocal microscope.

Results

The following samples were imaged under a confocal microscope. Red fluorescence indicates Prospero bound to the nuclei of enteroendocrine cells, green fluorescence is due to the presence of green fluorescent protein (GFP) and indicates *escGAL4*-activated gene expression, and yellow fluorescence indicates a combination of the two.

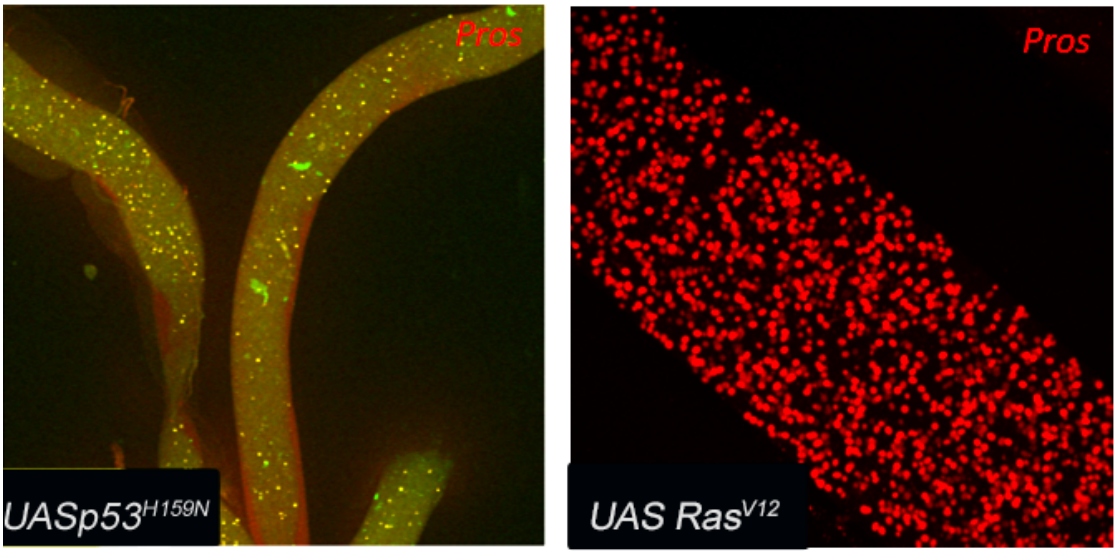

Figure 5: Parental genotypes of UASp55^{H159N} (left) and UAS Ras^{V12} (right).

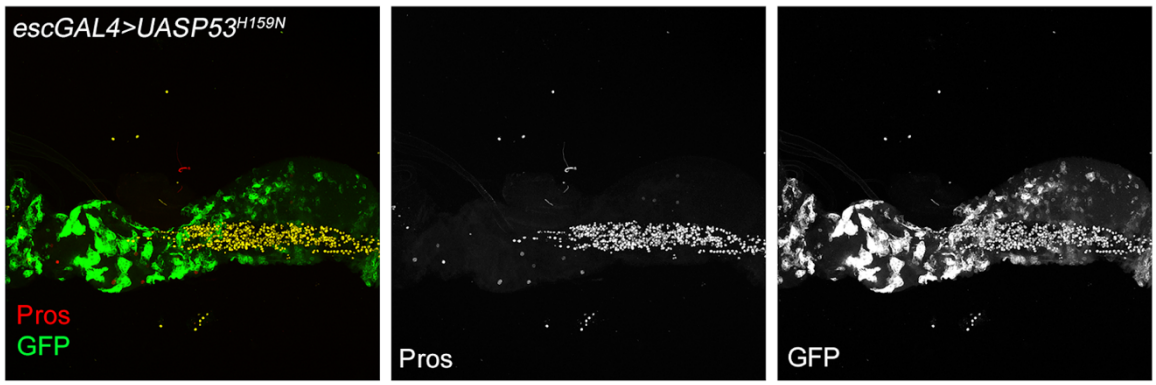

Figure 6: F1 genotype of escGAL4 expressed UAS^{p53H159N}, result of experiment 1. The middle image displays only Prospero fluorescence, the right image displays only GFP+ cells, and the left image displays the two overlapping.

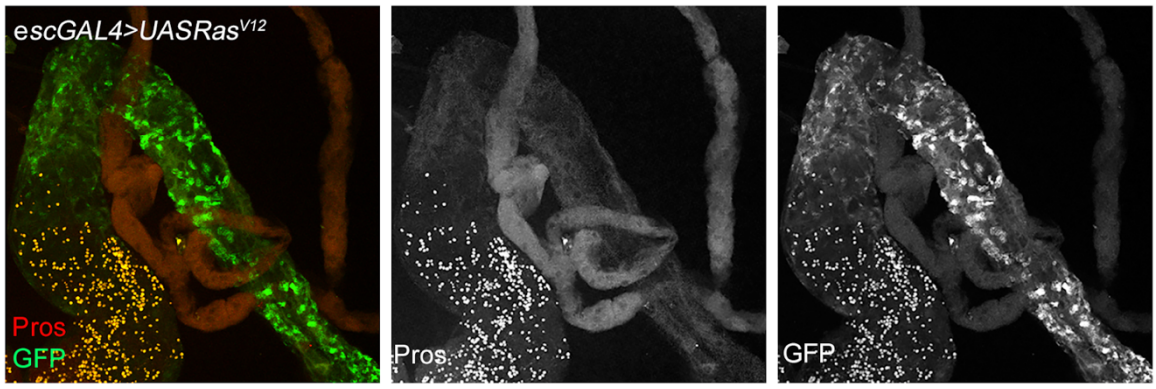

Figure 7: F1 genotype of escGAL4 expressed Ras^{V12}, result of experiment 2. The middle image displays only Prospero fluorescence, the right image displays only GFP+ cells, and the left image displays the two overlapping.

Discussion

Because intestinal stem cells (ISCs) have the potential to differentiate into any other type of cell, they are often dysregulated in cancers and therefore a useful target for identifying and measuring tissue proliferation. The epithelium of the colon contains four different cell lineages: enterocytes, goblet cells, endocrine cells, and Paneth cells (Munro, 2018). These all arise from ISCs at the base of crypts (Figure 8), known as crypt base columnar cells (CBCs). Once these cells lose control of replication and differentiation, they become cancer stem cells (CSCs), and lead to tumorigenesis. This increased unregulated ISC proliferation can eventually result in intestinal polyposis and colon cancer (Majidinia, 2017).

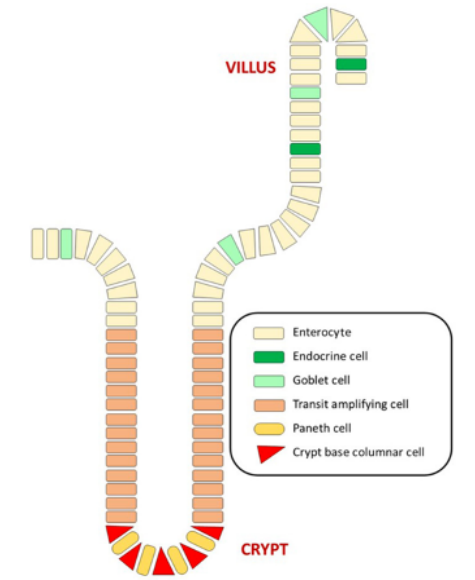

Figure 8: Anatomy of the intestinal epithelium (Munro, 2018)

Drosophila ISCs are distributed evenly throughout the intestine, but with variable proliferation capacity in different zones. ISCs self-renew and form a transient committed progenitor cell called enteroblasts (EBs). The EBs differentiate into absorptive enterocyte (ECs) and enteroendocrine (ee) cells.

The antibody Prospero used in this experiment binds to the nuclei of ee cells, making it an excellent marker for intestinal stem cell proliferation. Analysis of concentration of Prospero in controls and progeny shows an increased concentration and density of distribution of Prospero in p53 progeny and a slightly decreased concentration and density of distribution of Prospero in RasV12 progeny. This could be due to the area of the intestine that was imaged, as there is some variability between the midgut and hindgut.

GFP+ clusters also indicate areas of intestinal stem cell proliferation. Since clusters of GFP are wider in diameter than spots of Prospero, they could indicate a denser area composed of multiple ISCs. Significant levels of GFP in the progeny that are not present in the parent generation indicate successful escGAL4-controlled expression of dominant negative p53 and RasV12, meaning that it can be used as a measure of p53 and RasV12 activity.

In p53 progeny, yellow autofluorescence is sourced from food in the gut. This can indicate luminal space and help assess the thickness of the intestinal epithelium in future experiments and dissections. Colocalization could also indicate the nuclei of ISCs that Prospero has bound to.

Conclusion

Significant findings from this project include the creation of a model utilizing escGAL4-controlled expression of dominant negative p53 and RasV12. Also noteworthy are the increased levels of Prospero in RasV12 progeny, decreased levels in p53 progeny, and clusters of GFP+ cells in both progeny. This means that ISC proliferation occurs in response to escGAL4 expression and results in clusters in the intestine of the fly.

Next steps for this project could include combining the progeny of experiment 1 and experiment 2 with the APC mutated flies to finalize the three-hit model. We could then dissect the combinations of one-, two-, and three-hits integrating the APC mutation. After studying the survival and progression of these flies throughout the life cycle, we could test the effect of tumor size, shape, and proliferation in larvae and later as adults. We could then complete the third aim to identify drugs that inhibit the Ras and Wnt pathways, and feed larvae target drugs over a range of concentrations.

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