Genes regulating cell movement during regeneration in Drosophila melanogaster following radiation damage

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Genes regulating cell movement during regeneration in *Drosophila melanogaster* following radiation damage

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April 5, 2019

Departmental Honors Thesis  
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KEY WORDS

*Drosophila melanogaster*, cell translocation, regeneration, radiation, wing disc, cancer
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Abstract

Each year, 1.7 million people are diagnosed with cancer and 610,000 people die from the disease ("Cancer Statistics," 2018). Ionizing radiation therapy is a commonly used method of treatment for many cancers because it kills cancer cells (Baskar et al., 2012). However, cancer cells that have not been eradicated can proliferate, leading to resistance to treatment (Hanahan & Weinberg, 2011). Drosophila melanogaster is an excellent model organism because they have a short life cycle and share 75% of human disease-causing genes, while the larvae can withstand high amounts of radiation and have regenerative properties. Different genes were investigated to determine if they are required for cell migration during regeneration following radiation damage. Two genes, rab35 and rux, when knocked down using RNA interference and when expressed, respectively, were found to cause reduced cell migration from the hinge to the pouch of the wing disc. However, in order to avoid bias in selecting genes to study, a global approach was attempted to identify genes involved in regeneration by dissociating wing discs to single cells, selecting for regenerative cells, and performing Illumina sequencing. Regenerative single cells were obtained, however, RNA isolated from the cell population was degraded, indicating that methods need to be improved to maintain cell viability and RNA integrity. Identifying genes differentially expressed in regenerating cells of Drosophila larvae will provide candidates to test for roles in regeneration and can be targeted in human cancer for therapeutic potential to delay cancer regrowth and prolong disease-free survival.
Introduction

Cancer and cancer therapeutics

According to the National Cancer Institute, each year, 1.7 million people are diagnosed with cancer and 610,000 people die from the disease (“Cancer Statistics,” 2018). Cancer is a collection of genetic diseases that is characterized by continuous cell growth and division. Cancer can arise from a preexisting mutation in one’s genome or DNA damage caused by environmental factors. Often times, there are gain of function mutations in proto-oncogenes and loss of function mutations in tumor suppressor genes, both affecting the cell cycle and growth. Proto-oncogenes monitor cell growth and division, while tumor suppressor genes inhibit cell propagation, thus a mutation of either genes will lead to unrestricted cell growth and division to form a tumor (Lodish et al., 2000b).

The main cancer treatment methods are surgery, ionizing radiation (IR) therapy, and drug treatment. About 50% of all cancer patients receive IR therapy. The goal of radiation is to solely kill tumor cells while avoiding neighboring healthy tissue with minimal side effects (Baskar et al., 2012). Healthy cells have the proper abilities of DNA repair should the tissue be affected by radiation. Healthy tissue also has a slower rate of cell division than cancerous cells, meaning that cancer cells are more likely to be in S phase at any given moment than non-malignant cells are (Evan & Vousden, 2001). Radiation works by causing doubled stranded breaks (DSB) in DNA (Giglia-Mari et al., 2011). Thus when chromosomes are decondensed during the S phase of the cell cycle, the DNA is more prone to single stranded breaks (SSB) and DSB by radiation (Kastan & Bartek, 2004). The vulnerability of cancer cells being in S phase more frequently is the rationale behind irradiating patients. Cell cycle checkpoints are activated if the DNA has SSB, DSB, or other types of damage. If DNA damage is detected, the cell cycle is stalled or arrested.
by a kinase cascade at the G1–S, intra-S and G2–M cell-cycle checkpoints. This allows for increased time for DNA repair before replication or mitosis ensues. Should DNA repair fail, apoptosis, necrosis, or autophagy will be induced (Kastan & Bartek, 2004; Jackson & Bartek, 2009). If cancerous cells undergo apoptosis, the tumor will stop growing or shrink.

The American Cancer Society defines cancer recurrence as cancer that is found post-treatment, after a period of time where it is undetected. Cancer is more likely to recur if it was initially fast growing, more advanced, and widespread (“What is cancer recurrence?,” n.d.). For example, lung cancer mainly spreads to the liver, brain, and bones (“Metastatic Cancer,” 2015). There are different types of recurrence, local, regional, and distant. Local recurrence refers to cancer coming back in the same location while regional means cancer has come back in lymph nodes near the original site. Distant recurrence, or metastasis, means that the cancer has come back in a different part of the body (“What is cancer recurrence?,” n.d.). Metastatic cells lose their cell-cell adhesion, allowing them to migrate and invade other tissue. Cytoskeletal modifications and extracellular matrix allow for cell motility to occur (Martin et al., 2013). Once metastasis has occurred, it can be harder to treat and control (“Metastatic Cancer,” 2015).

Unfortunately, a large portion of patients receiving radiation therapy experience resistance to treatment (Longley & Johnston, 2005). There are a handful of pathways thought to be involved in radiation resistance, one of which being the DNA damage response pathway. In the DNA damage response pathway, activation of p53 induces cell cycle arrest, DNA repair, and apoptosis, thus when p53 is mutated by a missense mutation, it loses its tumor suppressive abilities, endowing cells with survival advantages and growth and cells become resistant to (radiation induced) apoptosis (Rivlin et al., 2011). It is found that the p53 gene is found to be the most frequently mutated gene in solid tumors (Kim et al., 2015).
There is growing evidence that in order to bypass treatment failure resulting from drug or radiation resistance, a combination of targeted therapies must be used (Al-Lazikani et al., 2012). Currently, combinations of ionization radiation, surgery, and drug treatment are used to treat different types of cancers, such as breast, head and neck, and lung carcinomas. Surgery and radiation therapy are used for static tumors whereas drugs are used for metastatic tumors (Baskar et al., 2012). By using two treatment methods that kill cancer cells by different pathways, it is less likely that resistance will arise. Rather than a universal treatment plan, personalized treatment options will soon become more prevalent (Al-Lazikani et al., 2012). One way to providing effective treatment is to look at genes expressed after radiation damage as a potential therapeutic target. Cells surviving radiation subsequently induce repair and proliferation mechanisms. By understanding what genes are being upregulated or down regulated in cells following IR damage, future therapy methods can be created to target those genes. This would increase the number of adjunct therapy options. Furthermore, inhibiting these repair and proliferation mechanisms might minimize or delay cancer recurrence, potentially allowing for other therapies or the immune system to fully eradicate cancer.

*Drosophila as a model organism*

*Drosophila melanogaster*, the fruit fly, has characteristics that make it a powerful model organism for human diseases. It is found that in 75% of human disease-causing genes have a homolog in *Drosophila* (Lloyd & Taylor, 2010). The *Drosophila* genome is simpler and more compact, making it easier to study through genetic manipulations. Genetic studies can be performed by mating different flies to produce hundreds of offspring with the desired genes in about 10 days (Jennings, 2011). *Drosophila* larvae are useful due to their imaginal discs, which are formed by a continuous sheet of epithelial cells. Imaginal discs are the precursors for adult
structures, such as legs, wings, and antenna (see Figure 1). The wing disc in the 3rd instar larva stage, which is the focus of this thesis, is subdivided into the notum, hinge, and pouch (see Figure 2A, B). The wing discs also have anterior/posterior, dorsal/ventral, and proximal/distal axes (Butler et al., 2003). The wing discs have highly regenerative capabilities, making them a valuable experimental system (Jennings, 2011). Understanding regeneration in wing discs post-radiation mechanistically is useful because it may be applicable to cancer cells following radiation. Knowing the mechanism of regeneration can help us understand how human tumors form to begin with and how tumors recur following ionizing radiation therapy.
Figure 1: Anatomy of *Drosophila melanogaster*. This diagram provides visuals for what and where certain structures are located in the embryo, larva, and adult. In the larval stage, the wing discs are labeled with ‘8’, which correspond to the wings in the adult structure. The wing discs are of particular interest in this thesis. Modified from (Beira & Paro, 2016).
One method for studying regeneration in larval imaginal discs, particularly the wing disc, is to damage the tissue using IR and determine what genes are expressed during the regeneration process. The genes that are expressed shortly after radiation are presumably important in the regenerative stage after injury. Irradiating larvae with 4000R induces apoptosis throughout the imaginal discs, however, a large percentage of pupae eclosed, producing viable flies (Jaklevic & Su, 2004). It was found by Verghese & Su (2016) that a subpopulation of the wing disc cells has intrinsic resistance to IR-induced apoptosis – the hinge cells (Verghese & Su, 2016). After IR damage, resistant hinge cells will lose their identity, move to areas of high cell death, and

Figure 2: Anatomy of a *Drosophila melanogaster* wing disc. Figure 2A shows a whole wing disc and Figure 2B shows the divisions of the disc. The hinge, yellow, and the pouch, green, are the focus in this thesis.
regenerate the tissue. Hinge cells of the wing disc will move into regions of the pouch to regenerate the tissue following damage (see Figure 3A, B). Two of the known signaling pathways involved in protecting cells from IR-induced apoptosis are STAT92E (STAT) and Wingless (Wg) (Verghese & Su, 2016). STAT, which is part of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, is a transcription factor that activates or represses transcription of target genes. The JAK/STAT pathway stimulates cell proliferation, migration, differentiation, and apoptosis (Rawlings et al., 2004). Wg proteins, on the other hand, regulate cell fate determination, motility, organogenesis, and stem cell renewal (Komiya & Habas, 2008). Since these pathways are involved in IR-resistance, when either of these pathways was inhibited, apoptosis occurred in the hinge following IR damage, thus concluding that they are required for IR resistance (Verghese & Su, 2016). Knowing other pathways involved in regeneration following damage can provide insight into how cancer cells may recur or metastasize and can be targeted for therapeutic potential in the future.

**Figure 3: Hinge to pouch migration following damage.** When larvae are irradiated with 4000R, cell migration from the hinge to the pouch occurs for 72 hours. The hinge cells marked by RFP in Figure A are resistant to radiation damage and help regenerate damage tissue in the pouch. Cells migrating from the hinge into the pouch can be seen in Figure B marked by GFP. Figure C shows an unirradiated wing disc where cell fate is stable indicated by the RFP and GFP overlap in the hinge only.
Not only has the JAK/STAT pathway been studied in *Drosophila*, but it has been found to be active in human solid tumors. Different mutations in JAKs and STATs have been found in different types of cancers. Knowing JAK/STAT is activated in solid tumors has allowed the pathway to be targeted as a new therapy and has shown to be beneficial in preclinical trials (Thomas et al., 2015). Furthermore, Gladstone et al. (2012) performed a drug screen to identify small molecules that increased the effects of radiation in *Drosophila* and discovered 16. Bouvardin in particular, which inhibits the elongation step of translation, preventing protein synthesis, was found to have synergistic effects with radiation in human cancer cells lines (Gladstone et al., 2012). Overall, *Drosophila* studies have provided insight into cancer biology and drug discovery.
Chapter 1: identification of cytoskeletal and cell cycle regulators that influence regenerative behavior

Background

Cell movement during development.

Cell movement during development can occur by cell migration or by directed divisions. Cell migration is fundamental to maintaining cellular organization of multicellular organisms. Both collective cell movement and single cell movement aid in development; for example, collective cell movement of epithelial sheets occurs during gastrulation and single cell movement occurs during nervous system development. In fully formed organisms, cell migration is essential for tissue homeostasis and repair (Trepat et al., 2012). There are two categories within cell migration: directed and random migration. Directed migration requires the input from intrinsic cell directionality or external regulation. If there is a motility stimulus present externally, then guidance and motility machinery will respond, leading to directed migration. However, if a cell has low intrinsic directionality and there are no external signals, random cell migration often ensues (Petrie et al., 2009). On the other hand, directed division can also lead to cell movement. Directed cell divisions specifically places cells within a tissue. A cell's mitotic spindle will orient in such a way that when division occurs, each cell will be directed in a certain direction. During Drosophila wing development, two groups of cells with different growth characteristics become juxtaposed, dividing ‘winner’ cells orient their mitotic spindle in such a way that their daughter cells end up among the ‘losers’ (Amcheslavsky et al., 2009). The experiment present in this chapter is investigating if cell translocation occurs by directed cell division, cell migration, or both.
RNA interference as a scientific tool.

RNA interference (RNAi) is a post-transcriptional mechanism that silences gene expression. RNAi can be used to control gene expression, by preventing translation or degrading mRNA. There are two types of small RNA molecules: microRNA (miRNA) and small interfering RNA (siRNA) (Ambesajir et al., 2012). siRNAs have specific mRNA targets and degrades mRNA whereas miRNAs have multiple targets and inhibit mRNA translation. The experiment presented in this chapter utilized double stranded RNA (dsRNA), which mediates specific silencing of homologous genes, expressed from GAL4-UAS. dsRNA is processed by Dicer which cuts it into siRNA. siRNA will interact with RNA-induced silencing complex to bind to mRNA and cleave the strand, thus silencing the gene. (Lam et al., 2015). Due to their gene silencing abilities, both siRNAs and miRNAs have immense potential for cancer treatments as they could repress oncogenic factors (Bader et al., 2011).

Molecular and cellular functions of enabled, rab35, rab40, and roughex.

Four genes, enabled, rab35, rab40, and roughex (rux) were chosen to be studied due to their molecular and cellular functions. enabled, which encodes an actin binding protein, is thought to mediate cell migrating through cell-cell and cell-matrix adhesion (Kwiatkowski et al., 2003). Both rab35 and rab40 have GTPase activity; when GTP-bound, they mediate vesicular transport through tethering complexes and motor proteins. The Rab proteins coordinate vesicle generation, vesicle motility, and tethering of vesicles to target membranes, all of which assist in cell adhesion and cell migration (Chua et al., 2010, p. 35). Cdk1-CycA activity normally promotes mitosis, but when rux is expressed, it inhibits Cdk1-CycA activity, thus preventing mitosis. rux expression inhibits cell division, however, the rest of the cell cycle still occurs, so the cells’ nuclei become larger due to repeated rounds of S phase (Foley & Sprenger, 2001)
**GAL4 Technique for Real-Time and Clonal Expression.**

GAL4 Technique for Real-Time and Clonal Expression (G-TRACE) is a lineage tracing system to monitor cell translocation from the hinge to the pouch of *Drosophila* wing discs. 30A-GAL4 drives the expression of the G-TRACE system in the hinge. GAL4 drives UAS-RFP (real time marker) and UAS-FLP that catalyzes a recombination event to result in stable green fluorescent protein (GFP) expression (lineage marker) (see Figure 4). In un-irradiated discs, red fluorescent protein (RFP) and GFP mostly overlap, indicating that cell fates are stable. However, after irradiation with 4000R of X-rays, RFP-/GFP+ cells are found in the pouch area (see Figure 4). These are former hinge cells that are expressing the GFP lineage tracker but have lost the hinge fate (became RFP-) and translocated towards the pouch. G-TRACE is schematically explained in Figure 4 (Evans et al., 2009).
In this chapter, I identify genes important for cell migration during regeneration following radiation induced damage using RNA interference. Out of four genes tested, two had significantly less cell migration when compared to their controls. However, in order to identify all genes differentially expressed during cell migration and regeneration, I must take a global, unbiased approach.
Results

In order to identify key regulators of cell migration in *Drosophila* wing discs following irradiation damage, I performed RNA interference (RNAi) on three genes: *enabled*, *rab35*, and *rab40* as well as expressed *rux*, a mitotic division inhibitor. Each RNAi line was made using a KK vector; the empty vector was used as a control to compare percentage of cell migration. The cellular functions of these *enabled*, *rab35*, and *rab40*, and *rux* are: mediate cell migration, play a role in vesicle transport (both *rab35* and *rab40*), and prevent cell division (Chua et al., 2010; Kwiatkowski et al., 2003; Avedisov et al., 2000). Cell migration following damage, was analyzed using G-TRACE and fluorescence microscopy imaging. G-TRACE allows for real time marking using RFP and for lineage tracing using GFP. 30A-GAL4, which is specific to the hinge, was used to drive the G-TRACE system in RNAi lines as well as separately drive the expression of Rux in another fly line. The *Drosophila* line containing the G-TRACE system, 

\[
\frac{30AGAL4 > G-TRACE}{\text{CyOGFP}}; \text{80 ts} \quad \frac{\text{CyOGFP}}{\text{TB}}
\]

was crossed to lines containing the RNAi: *rab35*, *rab40*, and *enabled*, as well as Rux.

In unirradiated wing discs, RFP and GFP mostly overlap in the hinge with little to no expression in the pouch (see Figure 5). When wing discs are irradiated, RFP+/GFP+ cells are still found in the hinge; however, now RFP-/GFP+ cells are found in the pouch (See Figure 6). More specifically, hinge cells, which are radiation resistant, lose their identity following damage, translocate to the pouch, which suffers more IR-induced apoptosis, and participate in regeneration (Verghese & Su, 2016).
Figure 5: Unirradiated wing discs. In unirradiated wing discs, RFP and GFP expression overlaps in the hinge. There is little to no GFP expression in the pouch which is what we expect. Unirradiated samples ensure that there is no migration occurring without radiation. Figure A, B, C, and D represent *enabled*, *rab40*, *rab35*, and *rux*, respectively.
Figure 6: Irradiated wing discs. In the hinge, RFP and GFP overlap, however in the pouch there is GFP expression. GFP expression in the pouch indicates that cells from the hinge lost their identity and translocated into the pouch. Figure A, B, C, and D represent enabled, rab40, rab35, and rux, respectively. Two RNAi lines, rab35 and rux, are statistically significant when compared to their controls. When gene expression was inhibited using RNAi, rab35 and rux, showed less migration into the pouch following damage.
Cell translocation following irradiation was measured by determining the area of RFP-/GFP+ cells in the pouch and RFP+/GFP+ cells in the hinge. In order to obtain a percentage of how many cells migrated, RFP-/GFP+ area in the pouch was divided by RFP+/GFP+ area in the hinge. This percentage indicated the percentage of cells that translocated and were compared to their controls using a two tailed t-test. Of the three RNAi lines tested, only one gene, *rab35*, showed statistically significant (p<0.05) deviation from the control in cell translocation from the hinge to the pouch region of the *Drosophila* wing disc after irradiation (see Table 1.1). *rab35* expression reduced translocation by about 2-fold (21±11% from 39±16% in the KK control; p = 3.1E-2). The *rab35* p-value indicates that the RNAi mostly silenced gene expression in comparison to its control. However, because cell migration was not completely eliminated, all cell translocations cannot be explained solely by vesicle transport.

Directed cell divisions are known to play a role in the final placement of cells within a tissue. It was hypothesized that directed cell divisions could cause cell translocation during regeneration. To address this possibility, we blocked cell divisions in the hinge, by using the same 30A-GAL4 driver to express a mitotic Cdk1 activity inhibitor, Rux. Here, we asked whether expressing Rux prevents the translocation of hinge cells into the pouch. Discs expressing 30A-GAL4>UAS-Rux in the hinge cells show RFP+ cells with enlarged nuclei; this is expected as inhibition of CycA and Cdk1 blocks mitosis but still allows repeated rounds of S phase (Foley & Sprenger, 2001; Sprenger et al., 1997; Verghese & Su, 2017). We found that the hinge cells still translocated into the pouch after irradiation, although less efficiently. Rux expression reduced translocation by about 3-fold (15±10% from 53±18% in GAL4 only controls; p = 6.4E-4). Translocated RFP-/GFP+ cells show reduced nuclear size, which is expected as cells lose their hinge identity and GAL4/Rux expression. The finding that Rux reduced but did not
eliminate cell translocation suggested that we cannot explain all cell translocation by directed divisions. Instead, cell migration may contribute (Myat et al., in press).

**Table 1.1**: Data of each Drosophila line tested showing hinge and pouch area and the percentage of cell that migrated into the pouch from the hinge. It is indicated what two lines were compared using a t-test as well as the p-value returned.

<table>
<thead>
<tr>
<th></th>
<th>AVG RFP/GFP area in hinge</th>
<th>AVG GFP area in pouch</th>
<th>Ratio hinge/pouch area</th>
<th>Standard deviation</th>
<th>T-Test; p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KK +IR</td>
<td>378341.11</td>
<td>131955.22</td>
<td>39%</td>
<td>0.160054</td>
<td></td>
</tr>
<tr>
<td>KK -IR</td>
<td>391847.55</td>
<td>2790</td>
<td>1%</td>
<td>0.027474</td>
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<tr>
<td>Enabled +IR</td>
<td>331010.75</td>
<td>195736.50</td>
<td>29%</td>
<td>0.165323</td>
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<td>32899.22</td>
<td>4%</td>
<td>0.030503</td>
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</tr>
<tr>
<td>Rab35 +IR</td>
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<td>188313.71</td>
<td>21%</td>
<td>0.112956</td>
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<tr>
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<td>0.194813</td>
<td>Rab40/KK+IR p=0.191366</td>
</tr>
<tr>
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<td>Description</td>
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<td>Mean 2</td>
<td>Percent</td>
<td>P-value</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>--------</td>
<td>--------</td>
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<td>---------</td>
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<tr>
<td>Rab40 -IR</td>
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<td>0</td>
<td></td>
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<tr>
<td>Rux +IR</td>
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<td>40585.14</td>
<td>15%</td>
<td>0.105138</td>
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<tr>
<td>Rux -IR</td>
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<td>13097.29</td>
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</tr>
<tr>
<td>GAL4 only</td>
<td>584973</td>
<td>301777.429</td>
<td>53%</td>
<td>0.180147</td>
<td></td>
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</tbody>
</table>

Discussion

In this study, I investigated the requirement for cell movement during regeneration of irradiated Drosophila wing discs. Of three RNAi lines tested in wing disc regeneration, only one produced statistically significant effects as well as one other gene tested separately (see Table 1.1). Rux, which inhibits cell division, had strong statistical significance whereas Rab35RNAi did not. Despite Rab35 having weak significance, it is interesting that Rab40 was statistically not significant. They belong to the same family, both aid in vesicle transport, though only Rab35 had an effect on cell migration following IR damage. Rab35 and Rab40 are known to play a role in membrane reshape, but more specifically Rab35 plays a role in cytokinesis and the endocytic recycling pathway while Rab40 plays a role in intra Golgi trafficking (Stenmark, 2009). It is possible that Rab35 and Rab40 have synergistic or redundant functions. When only one gene is knocked out, the other gene may rescue its function, thus still allowing cells to migrate after damage. In order to determine synergistic or redundancy in function, both genes can be knocked
out and cell migration can be quantified post-radiation. Furthermore, although Rux was statistically significant, cell movement was not completely halted; implying that cell translocation after damage is not only mediated by directed division. These results suggest that cell translocation during wing disc regeneration after IR damage has contributions from both cell division (Rux experiments) and cell migration (RNAi experiments).

If we were to have used knockouts rather than RNAi, we would not have obtained the same results. When rab35 is knocked out, invagination of the mesoderm in embryos fails (Jewett et al., 2017). Knocking out enabled causes embryonic lethality (“FlyBase Gene Report: Dmel\ena,” n.d.). Using RNAi allows for the embryos and larvae to develop normally before silencing select genes.

Each gene tested is involved in complex processes requiring other regulators; this potentially explains why the other two RNAi lines tested showed only a partial defect in the wing disc system. Rather than performing a candidate approach to determine the genes involved in regeneration following IR damage, I will now take a global, unbiased approach. I will do this by performing RNA-sequencing on the regenerative cells 0, 24, and 48 hours after irradiation.

Materials and Methods

Drosophila techniques.

The Drosophila lines used were: Enabled\RNAi, Rab35\RNAi, Rab40\RNAi, Rux, KK, and 30AGAL4 > G-TRACE; 80 ts CyOGFP (lineage tracing). KK was used to make the RNAi lines and thus was used as an empty vector control. Larvae were raised in Nutri-Fly Bloomington formula food (Genesse Scientific) at room temperature. The vials were monitored for overcrowding and were split if observed. Drosophila crosses were made by mating stock lineage tracing virgin females
with stock Enabled\textsuperscript{RNAi}, Rab35\textsuperscript{RNAi}, Rab40\textsuperscript{RNAi}, Rux, and KK males. 96-hour old larvae that were treated with the temperature shift protocol shown in Figure 7, were irradiated with 4000R of X-rays and then dissected 72 hours later. Non-irradiated larvae followed the temperature shift protocol in Figure 8. This protocol allowed for larval growth and disc development before activating GAL4 by shifting to 29°C to inactivate GAL80\textsuperscript{ts} (Verghese and Su 2017).

**Figure 7: Irradiation protocol performed.** 24 hour old larvae were treated with this temperature shift protocol, irradiated with 4000R of X-rays, and then dissected. The tissue was then fixed, stained for DNA, and mounted onto glass slides 72 hours after irradiation. Adapted from (Verghese & Su, 2017).

**Figure 8: The protocol for non-irradiated samples performed.** 24 hour old larvae were treated with this temperature shift protocol. Samples were dissected, tissue was fixed then stained for DNA, and mounted onto glass slides 72 h after being shifted to 29°C.
Larvae not expressing GFP under the Leica MZ 125 microscope with EBQ 100 light source were selected for dissection. The larvae were dissected in 1xPBS and then the tissue was fixed using 650uL of ddH2O, 250uL of 16% paraformaldehyde, and 100uL of 10xPBS for 30 minutes on a nutator. After 30 minutes, the solution was removed, 1000uL of 1xPBS was added, and the sample was placed on the nutator for 10 minutes. After 10 minutes, the solution was removed, 0.1% PBTX was added to wash the sample for 5 minutes, then 1% Hoechst 33342 dye was added to stain nuclear DNA for three minutes. Lastly 0.1% of PBTX was used to wash the sample three times. Following fixing and washing the samples, the wing discs were mounted onto a glass slide in Fluoromount-G.

**Imaging and Image Analysis.**

I used the Leica DMR compound microscope, Q-Imaging R6 CCD camera, and Ocular software to image wing discs at 20x magnification under three different wavelengths: 350nm for Hoechst stain, 532nm for RFP expression, and 488nm for GFP expression (“Fluorophore selection - US,” n.d.). I observed that both RFP and GFP are only expressed in the hinge, but upon radiation, GFP only gets expressed in the pouch in addition to the RFP+/GFP+ in the hinge. Cones, which are migrating cells into the pouch, can be seen in irradiated wing discs and were classified as the GFP-only area. The images were processed using ImageJ to quantify cell migration. The RFP+/GFP+ area in the hinge was measured and then the GFP+ only area in the pouch. Both area values were recorded in Microsoft Excel and the ratio of GFP to RFP+/GFP+ area was calculated. This value indicated the amount of cell migration from the hinge to the pouch. For each RNAi line, the average ratio and standard deviation was used to perform a T-test. Irradiated samples were compared to non-irradiated samples to see if the RNAi significantly (p<0.05) disrupted cell migration following damage.
Chapter 2: Optimization of the protocol for gene expression analysis during regeneration

Background

RNA sequencing as a scientific tool.

During development, cells change fate and become specialized through differential gene expression (Costa & Shaw, 2007). When trying to understand diseases, it is important to know not only what genes are expressed, but also how active those genes are. RNA-sequencing (RNA-seq) is a powerful tool for exploring gene expression because it returns both qualitative (gene expression) and quantitative (expression levels) data. RNA-seq is the process by which RNA from cells is made into cDNA, which is then amplified. Fragments are sequenced, and the resulting data are aligned to a reference genome to allow for characterization of the tissue transcriptional profile (Snyder et al., 2009). There are many variations of RNA-seq. One method to study rare cells is to perform single cell RNA-seq, which examines the genome or transcriptome of individual cells (Wu et al., 2014; “RNA Sequencing | RNA-Seq methods and workflows,” n.d.). Another method is total RNA-seq which analyzes both coding and non-coding RNA and can identify known and novel features of the transcriptome. To identify gene expression, mRNA-seq can be done to obtain the coding transcriptome. mRNA-seq is one of the most popular methods of sequencing because it can be used to understand what genes are upregulated or downregulated by comparing mRNA expression to a control (“RNA Sequencing | RNA-Seq methods and workflows,” n.d.). RNA-seq has been performed on a subpopulation of regenerative cells in the Drosophila imaginal discs. Two studies outlined below show the usefulness of RNA-seq in identifying genes involved in imaginal disc regeneration following damage.
The Drosophila Duox maturation factor is a key component of a positive feedback loop that sustains regeneration signaling.

*Drosophila melanogaster* is a powerful model organism as they possess an important model system for tissue repair and regeneration. Investigating changes in gene expression in regenerating tissue is a powerful approach to identifying necessary genes for regeneration. Khan et al. (2017) used genetic tissue ablation and deep-sequencing techniques to generate a complete transcriptional profile of regeneration in imaginal discs. They identified novel regulators of regeneration as well as mechanisms that ensure growth through sustained regeneration signaling. By whole-genome transcriptional profiling of regenerating tissue, they identified changes in gene expression that control a key regenerative mechanism. The mechanism for regeneration is initiated by damage, causing activation of the Jun N-terminal Kinase (JNK) signaling pathway, leading to upregulation of the gene *moladietz (mol)*. *mol* encodes for NADPH dual oxidase (Duox) which generates reactive oxygen species (ROS), a key tissue damage signal. When *mol* is highly expressed, ROS are continuously produced during regeneration. This cascade is a positive feedback loop, allowing for sustained regeneration at the site of damage.

Ablation of the pouch of the wing disc was induced by expressing pro-apoptotic gene *reaper (rpr)* specifically in the pouch using *rn-GAL4* to drive UAS-rpr expression. GAL4 activity was temporally controlled with a temperature sensitive repressor GAL80. This allowed them to ablate the pouch for a specific period of time, then shut down rpr, allowing regeneration to occur. The *nubbin (nub)* gene is a wing determinant found in wing pouch cells during both natural development and new pouch cells that form during regeneration. *Nub-GFP* expression was used to identify blastema cells in damaged discs as green fluorescent protein (GFP) could be expressed simultaneously under its control. These GFP positive cells were selected for using
fluorescence-activated cell sorting (FACS) once they were removed from the wing disc tissue. mRNA was isolated from the GFP+ cells and subject to Illumina sequencing. Nubbin-GFP+ cells from ablated discs were compared to nubbin-GFP+ cells from non-ablated samples to determine which genes were differentially expressed in the pouch during regeneration. Khan et al. (2017) identified 660 statistically significant (p<0.05) differentially expressed genes, 504 of which were upregulated, 156 which were downregulated in the regeneration blastema. To ensure the genes identified were differentially regulated in the blastema, they used antibodies and qPCR.

During wing imaginal disc regeneration, dying cells release ROS which are taken up by live cells at the wound edge after physical damage or tissue ablation. Interestingly, two NADPH oxidases were found that produce ROS: NADPH Oxidase (Nox) and Dual Oxidase (Duox). Nox was upregulated during regeneration, while Duox remained the same. However, Duox-maturation factor (NIP), encoded by mol, showed high levels of induction after damage. ROS are reduced by superoxide dismutases (Sods) and Catalase (Cat); both were found to be downregulated in the regeneration blastema. Thus, after damage, regeneration in the blastema could be explained by the upregulation of Nox and mol/NIP and downregulation of Sods and Cat.

Not only is mol required for ROS propagation, but also for sustained JNK signaling. Khan et al. (2017) found that JNK signaling is required for mol upregulation leading to ROS production which maintains JNK signaling during regeneration. This positive feedback loop allows for full regeneration. Furthermore, Khan et al. (2017) found that ROS persist for at least 24 hours after tissue ablation in regenerating cells, suggesting that there is an active mechanism sustaining ROS production throughout regeneration.
Regeneration involves various cellular processes and pathways to repair and replace damaged tissue. Growth, proliferation, and patterning are needed for regeneration. Khan et al. (2017) identified a key pathway that allows cells to regenerate ablated tissue. More specifically, they identified mol as a critical regulator for sustained ROS production and JNK signaling (Khan et al., 2017).

**During Drosophila disc regeneration, JAK/STAT coordinates cell proliferation with Dilp8-mediated developmental delay.**

To identify regulatory networks involved in Drosophila imaginal disc regeneration, Katsuyama et al. (2015) investigated the transcriptome of regenerative cells of the leg disc following fragmentation. The regenerative cells were tagged with GFP using puc-GAL4 and UAS-GFP; puckered (puc) is expressed during the Jun N-terminal kinase (JNK) signaling pathway allowing for easy identification of regenerative cells. GFP positive cells were selected for under a fluorescent stereomicroscope using a fine tungsten needle at set timepoints after damage and culturing. RNA was then extracted and subjected to Illumina-sequencing.

Katsuyama et al. (2015) found that both wound healing and regenerative cell proliferation are dependent on the JNK signaling pathway. Upon fragmentation, unknown signals activate JNK, which initiates regeneration at the wound site. During regenerative cell proliferation, JNK signals to at least two downstream signaling pathways: JAK/STAT signaling and Wg signaling. They found that Upd, a cytokine which activates immune cell proliferation, is downstream of the JNK signaling pathway. Upd goes on to activate the JAK/STAT signaling pathway, which regulates regenerative cell proliferation in cooperation with Wg signaling. JAK/STAT is transcriptionally controlled by upd, and Wg is transcriptionally controlled by wg. Input from both JAK/STAT and Wg signaling is required to induce the cells near the damage site to reenter
the cell cycle for proliferation. Their results suggest that both signaling pathways, JAK/STAT and Wg, are required for regenerative cell proliferation initiation. They also found that *Drosophila insulin-like peptide 8 (dilp8)* expression, which controls timing of pupariation, is controlled by JAK/STAT. Activation of Dilp8 postpones pupariation to allow for recovery after damage (Katsuyama et al., 2015).

In this chapter, I will describe my attempts to perform RNAseq on regenerative cells of wing discs after radiation. My results indicate that I can isolate single cells that are appropriately marked with RFP and GFP, indicating their regenerative status. However, I need to improve methods of preserving cell viability and RNA integrity in order to complete this project.

**Results**

Following the protocol outlined in Khan et al. (2017), with adjustments, I obtained single cells from whole *Drosophila* wing discs. Fluorescence Activated Cell Sorter (FACS) results showed that 71% of the events that passed in front of the laser were live cells in the unirradiated sample, where 89% were single cells (see Figure 9). Conversely, for the irradiated sample, 58% of the events that passed in front of the laser were lives cells, 95% being single cells (see Figure 10). Observing more dead cells in the irradiated sample is to be expected as irradiation kills cells. Furthermore, I mounted part of a single cell suspension sample onto a glass slide and looked at it under a fluorescent microscope. I observed single cells as well as small cell clumps (<5 cells) and larger cell clumps (10-20 cells), despite passing it through a cell strainer. These clumps were selected against in FACS.
In order to identify the regenerative cells of the wing disc, the G-TRACE system was used to label them with RFP and GFP. I dissociated the wing disc tissue to obtain single cells and in order to isolate the RFP+/GFP+ cells, the single cell suspensions were initially sent to the BioFrontiers FACS core. The single cells were separated into two populations RFP+/GFP+ and RFP-/GFP- (these cells were used to practice RNA isolation on later). We expected 20% of the single cells to be double positive based off of previous microscopy results of whole wing discs (data not shown), however roughly only 0.1% of the single cells were reported as double positive. Compensation was then implemented to account for RFP and GFP bleed over using single color controls and then 11% were reported as double positive for non-irradiated samples (see Figure 11). However, in irradiated samples, less than 2% of the single cells were reported as double positive (see Figure 12). I then sent later single cell suspension samples to the FACS core on the CU Boulder main campus; compensation was also established. In un-irradiated samples, roughly 15% were identified as double positive, but in irradiated samples, only 9% were double positive (see Figures 9 and 10). When both -IR and +IR wing discs, 72 and 80 discs, respectively, were dissected, about 40,000 and 23,000 cells, respectively, were collected. Multiple sorts were performed in order to obtain an adequate number of cells for RNA isolation.
Figure 9: -IR sample FACS profile from main campus core. 72 wing discs were dissected and dissociated. 70% of the sample is live cells and 89% of those cells are single cells. About 16% of the single cells are double positive. R1 represents all events and R2 represents all selected single cells. R3 represents the number of cells expressing GFP and R4 represents the number of cells expressing RFP. R5 represents double positive cells and R6 represents double negative cells. Gates were manually set using single color (RFP and GFP only) controls and a negative (no fluorescence) control.
Figure 10: +IR sample FACS profile from main campus core. 80 wing discs were dissected and dissociated. 58% of the total sample are live cells, 95% of which are single cells. About 9% of the single cells are double positive. R1 represents all events and R2 represents all selected single cells. R3 represents the number of cells expressing GFP and R4 represents the number of cells expressing RFP. R5 represents double positive cells and R6 represents double negative cells. Gates were manually set using single color (RFP and GFP only) controls and a negative (no fluorescence) control.
Figure 11: -IR sample FACS profile from the BioFrontiers core. 65 wing discs were dissected and dissociated. 70% of the sample is live cells and 89% of those cells are single cells. About 16% of the single cells are double positive. P2 represents RFP positive cells and P3 represents GFP positive cells. Gates were manually set using single color (RFP and GFP only) controls and a negative (no fluorescence) control.
I performed RNA isolation on the double negative cells for practice before isolating RNA from the sample of interest. Using TRIzol reagent, I isolated RNA from a little less than half a million double negative cells. The RNA concentration was 35 ng/μL and the purity (260/280 ratio) was 1.55. A clean sample should have a 260/280 ratio close to 2.0 (Matlock, 2015). In order to determine if the dissociation protocol affected RNA concentration, I also isolated RNA from whole wing discs (one, three, and five wing discs were used). For one, three, and five wing discs, the concentration and 260/280 ratio were 56 ng/μL and 1.57, 68 ng/μL and 1.74, and 88 ng/μL and 1.83, respectively.
Gel electrophoresis was performed on the isolated RNA to specifically view rRNA because it makes up 80% of the cells RNA (Lodish et al., 2000a). mRNA is less stable and smaller, but if rRNA can be seen, one can infer that mRNA is also present (“Agarose Gel Electrophoresis of RNA - US,” n.d.). If there are no rRNA bands, there is definitely no mRNA. An agarose gel can also determine degradation of an RNA product, so to visualize possible degradation, I ran the RNA extracted from the double negative cells on a 1.2% agarose gel. In order to determine if the dissociation protocol caused RNA degradation, I ran the RNA isolated from whole wing discs alongside the RNA isolated from the double negative cells. Each sample was dividing into three amounts of RNA (100ng, 300ng, and 800ng). Upon imagining the gel, I observed inconsistent amounts of RNA loaded into each well indicated by band brightness. In Figure 13, the first three lanes are properly represented, brighter bands signify more RNA. However, there are also bands below the dye front; this is most likely degradation. The next three lanes show consistent brightness, implying similar RNA amounts, suggesting that the Nanodrop spectrophotometer is not always accurate. The next three lanes appear to have no distinct bands but have dull bands below the dye front, likely degradation product. The last three lanes further suggest that the Nanodrop is not always accurate and the concentration it returned is likely from contaminants from the TRIzol extraction method. No rRNA band present in the last three lanes indicates that there is no mRNA. Since there is no intact RNA in the double negative cells, there is a high likelihood that the double positive cells also do not contain intact rRNA, thus no mRNA that can be sequenced.
Figure 13: Gel visualization of RNA isolated from three and five whole wings discs and single cells. Each sample was visualized at three different concentrations based off of Nanodrop results. RNA from three and five wings discs show amounts of RNA, however only the five wing discs show increased brightness with increased concentration. The single cell sample has no RNA and all three samples show degradation.
Discussion

Since Khan et al. (2016) used wing discs and dissociated the tissue rather than using leg discs and individually selecting for cells using a tungsten needle as Katsuyama et al. (2015) did, my estimate of the projected number of cells needed will be in accordance with what Khan et al. (2016) obtained. Khan et al. (2016) cell population of interest was 2-5% of the cells in a wing disc. In order to obtain adequate amounts of single cells and RNA for Illumina sequencing, they dissected 400 wing discs. To acquire ample RNA, the RNA extracted from multiple rounds of dissection and sorts was pooled. Because my cell population of interest, according to FACS results, is 9%, irradiated, and 15%, unirradiated, of the wing disc, I am aiming for 200 wing discs for my irradiated sample and 150 wing discs for my unirradiated sample. 200 and 150 wing discs will provide sufficient RNA for Illumina sequencing following the Khan et al. (2016) protocol.

In order to maximize single cell yield, I tested different amount of trypsin for dissociation, the number of times I pipetted every five minutes and at the end of the 15 minute trypsinization period, and the temperature of the trypsin used. I found that using 500uL of trypsin, rather than 100uL, gave a higher yield of single cells; this was determined by microscopy. Furthermore, in order to aid in cell clump-dissociation, I pipetted a select number of times every five minutes as well as at the end of the 15 minute trypsinization period. I found that when I pipetted more than 50 times, there was not a noticeable increase in the number of single cells. Furthermore, I found that pipetting 25 times throughout the incubation period killed about 5% of the cells whereas pipetting 25 times throughout the incubation period and a further 20 times after killed 22% of the cells. I also found that putting the cells through a 25uM filter caused another 3% of the cells to die. I used acridine orange stain and microscopy to determine the number of single cells and dead cells under each pipetting condition. Another aspect of the
protocol that I changed was the temperature of the trypsin used – room temperature versus 37°C. I found that when I used heated trypsin, cell dissociation increased by an average of 40%. The final protocol adapted from Khan et al. (2017) I ended up following used 500uL of heated trypsin at 37°C while pipetting five times every 5 minutes, and 15 times after the 15 minutes incubation period was complete.

In order to have my samples sorted by FACS, they had to be resuspended in basic sorting buffer (BSB). In order to do this, I centrifuged my sample for 1 minute at 1,500 g, removed the supernatant which was then centrifuged itself at 6,000 x g, and both samples were resuspended in the BSB for FACS. The viability of cells after centrifugation was not determined, however the centrifugation of the sample could have led to compromised membranes.

Overall the adapted protocol is effective at dissociating tissue into single cells. The discrepancy between results returned by the two FACS cores could be due to different instruments or different operators. Since the results from the core on main campus gave results most similar with what I was expecting, I continued sending samples to that core. Furthermore, I decided to only use one core to eliminate the unknown variation between cores. In addition, the core on main campus required significantly less travel time, so there is lower chance of cells dying. Khan et al. (2017) reported that they saw 26% more dead cells when the samples sat for 30 minutes. In order to minimize cell death, cells were immediately taken to the main campus FACS core for sorting following the dissociation protocol. However, both +IR and -IR samples were sorted sequentially in one session. Sorting took roughly one hour per sample, so the second sample was sitting for an hour before getting sorted. This likely led to significant cell death, especially because Khan et al. (2017) reported 26% of their cells died in half an hour. In the
future, only one sample will get sorted during a session in order to minimize cell death from sitting.

The amount of double positive cells that were sorted is lower than expected, but this could be due to double positive cells remaining in clumps and thus being caught by the filter or selected against during sorting. It is understandable that there are fewer double positive, single cells from the irradiated samples because irradiation causes more cells to die, therefore there are less cells from the start. Obtaining less double positive cells from a sort means that more sorts will need to be done to acquire an adequate number of cells for RNA isolation and sequencing.

Comparing the Nanodrop and gel electrophoresis results, there is a clear discrepancy. According to the Nanodrop, there is RNA in the sample, but on the gel, there is little to no RNA. This could mean that the Nanodrop is not very accurate, the concentration is mainly contaminants especially since the purity was not high, or the amount is too little to see on a gel. A Qubit and a Tapestation, run by the sequencing facility, can be done on a sample to detect smaller amounts of RNA and determine a more accurate concentration and purity level. If a Qubit is needed to detect small amounts of RNA in the sample, then it is highly unlikely that there is enough RNA for sequencing. However, once I know how much RNA comes from a known number of cells, I can calculate how many wing discs and sorts will need to be done to obtain the desired amount of RNA. The Next-Gen Sequencing Facility at BioFrontiers needs a minimum of 50ng of RNA to sequence, but 500ng is ideal.

Since the single cells obtained from sorting had no RNA, the next step will be to determine where the RNA degradation is occurring. Since degradation can be seen even in whole wing discs, this means that some degradation is occurring even before dissociation and sorting. I will start from the beginning and determine if it is the solutions used to wash and dissected
larvae in that causes the degradation. I will do this by using whole wing discs and letting them sit in different solutions, then I will isolate RNA, perform gel electrophoresis, and visualize for degradation. From there, the next step will be to determine if the dissociation protocol is causing RNA degradation. I will dissociate whole wing discs, isolate RNA, perform gel electrophoresis, and then look for RNA degradation. I plan to modify the solutions used as well as the amounts to pin point what and how much is causing the degradation. Furthermore, I will not pipet every five minutes as this could potentially be shearing cells or causing compromised membranes; I will only pipet three times at the end of the 15 minute incubation period according to Khan et al. (2017).

I suspect that the RNA degradation is occurring from dead cells getting sorted into the live cell population. Freezing the cells causes them to lyse, thus upon thawing, the RNA may become degraded by the RNases released by the lysed dead cells. In order to prevent this from happening, the FACS gates will be set more conservatively to ensure dead cells are not sorted into the desired sample. Another way to ensure dead cells are removed is to use the Hoechst 33258 stain which is 10 times more permeable to dead than live cells. The stained cells can be sorted out of the population and then all the RFP+/GFP+ cells that are Hoechst negative can be collected. This will minimize the number of dead cells that are sorted into the desired population, thus reducing the amount of RNase that can degrade the RNA.

Once intact RNA is successfully isolated, it will be subject to Illumina sequencing. High output 75-cycle mRNA sequencing with 20-30 million reads will be performed with a polyA enrichment library prep. I will compare gene expression in RFP+/GFP+ cells in the hinge at 0, 24, and 48 hours after irradiation to (1) non-irradiated samples and to (2) RFP-/GFP- cells in irradiated discs. Gene expression in irradiated RFP+/GFP+ cells will be considered significant if
a p-value of less than 0.05 is obtained using a t-test against non-irradiated RFP+/GFP+ cells. Also, only genes that changed expression by 2-fold or greater will be considered further. At the completion of the project, I will have identified a list of important genes being expressed in hinge cells of the wing disc following damage.

**Materials and Methods**

**Stock amplification.**

I used the fly stock $3^{0A GAL4 > G-TRACE}_{SMS}$ because it contains the G-TRACE system and the SM5 balancer chromosome to prevent recombination of chromosomes, thus preserving the desired genetic background. Flies were transferred into new bottles with fresh Nutri-Fly Bloomington formula food (Genesee Scientific) daily. Flies and larvae were kept at 25°C. The fly food in old bottles was kept moist to allow larvae to grow. Bottles were surveyed for mold growth and were thrown out if growth was observed.

**Irradiation.**

Embryos were collected for 24 hours at 25°C and aged for 96 hours from the end of collection to reach the desired larvae age. Half of the larvae were the unirradiated control, while the other half were irradiated with 4000R (Verghese & Su, 2017). Next, the wing discs were dissected off 24 and 48 hours after irradiation. Based on previous findings, at 24 hours, not much has happened; however, at 48 hours cell movement begins, and by 72 hours, hinge cells have already migrated into the pouch and changed fate (Verghese & Su, 2016).

**Larval washing.**

Larvae are placed in a small petri dish with 70% ethanol to remove food particles from the larvae. The ethanol is removed and dH$_2$O is added and swirled to remove food. Larvae are placed in 1x PBS to remove remaining food on larvae and then placed in Supplemented
Schneider’s Medium (SSM) (10% fetal bovine serum, 2% penicillin/streptomycin, 0.02 mg/ml insulin, Schneider’s Medium) for dissection.

**Wing disc dissociation.**

Once the wing discs were removed, the extracellular matrix was degraded using trypsin, which breaks down proteins, dissociating the cells. The wing discs were incubated in glass dishes at 37°C in 500uL of heated, undiluted trypsin for 15 minutes for trypsinization. Every five-minute interval, pipetting was performed on the sample to ensure maximum dissociation. At the end of the 15 minutes, the reaction was stopped by quenching it with 800uL of SSM, which halts trypsinization. The sample was pipetted vigorously and then centrifuged at 1,500 x g. The supernatant was transferred to a 1.5ml tube, centrifuged at 6,000 x g, and then its supernatant was discarded. Both pellets were resuspended in 250uL of basic sorting buffer (BSB) (1 x PBS, 1mM EDTA, 25mM HEPES, 1% fetal bovine serum). Both suspensions were passed through a cell strainer to remove any remaining large cell lumps. The dissociation protocol is outlined in Figure 14 (Khan et al., 2016).
Figure 14: Dissociation protocol. This schematic shows the different steps of the dissociation protocol used in this thesis minus a few adjustments. Wing discs are dissected off and incubated in Trypsin at 37°C in a metal bead bath. The trypsin reaction is stopped using SSM and the wing discs are pipetted three times before putting through a cell strainer. Single cell suspensions are then sent to FACS. Adapted from (Khan et al. 2016).
Fluorescence Activated Cell Sorter.

The single cell suspensions were sent to the Fluorescence activated cell sorter (FACS) facility at the University of Colorado Boulder Molecular, Cellular, Developmental Biology department. The single cells where were sorted for RFP+/GFP+ and RFP-/GFP- into BSB. Based off of previous microscopy experiments, I expected roughly 20% of the cells be to RFP+/GFP+.

Once the cells were sorted, they were centrifuged, and the supernatant was removed to a final volume of 100uL. The samples were flash-frozen using liquid nitrogen and then stored at -80°C.

RNA Isolation.

RNA isolation was practiced on the RFP-/GFP- cells as well as whole wing discs using TRIzol Reagent and chloroform. TRIzol was added in a 3 to 1 ratio for lysis for each sample then vortexed at room temperature for 5 minutes. 0.2mL of chloroform was added (per 1mL of TRIzol) to each sample then vortexed for 3 minutes at room temperature. The samples were centrifuged for 15 minutes at 16,000 x g at 4°C. The samples separated into three layers: phenol-chloroform, interphase, and aqueous phase. The aqueous phase was removed using a pipet and transferred to a new 1.5mL tube. 1uL of glycogen and 0.5mL of 100% isopropanol (per 1mL of TRIzol) was added to the aqueous sample and incubated at room temperature for 10 minutes. The sample was centrifuged for 10 minutes at 16,000 x g at 4°C. The supernatant was discarded and then 1mL of ethanol (per 1mL of TRIzol) was added and centrifuged for 5 minutes at 16,000 x g at 4°C. The supernatant was discarded, and the pellet was airdried for 5-10 minutes. The pellet was resuspended in 15uL of 1 x TE and heated at 65°C for 5 minutes, then placed on ice ("TRIzol Reagent - Thermo Fisher Scientific," n.d.). Sample purity and concentration was determined using a Nanodrop spectrophotometer, then stored at -20°C.
Gel electrophoresis.

The rRNA size for *Drosophila* is 2 kb, so I used a 1.2% agarose gel to visualize my RNA samples’ purity (Ausubel, 1987; “Ribosomal RNA Sizes - US,” n.d.). The gel was made by adding 1.2 grams of agarose power to 100mL of 1 x TAE and boiled until no particulates remained. 5uL of SyBr Gold was added to the semi-cooled gel solution, and then was poured into a gel box at 4°C and a 10-well comb was placed. 250ng of RNA, which was calculated using the concentration value obtained from the Nanodrop, was added to 10uL DNA Loading Buffer, 0.08uL of SyBr Gold, and water to obtain a final total volume of 20uL. The samples were heated at 95°C for 10 minutes then cooled on ice for 5 minutes before loading into each well. The GeneRuler 1kb Plus DNA Ladder was used. The gel was run at 4°C for 15 minutes at 70 volts and then increased to 115 volts for 30 minutes. To image the gel, I used the Gel Logic 200 Imaging System and Kodak Molecular Imaging software.
Discussion

Parts of the *Drosophila* wing disc have been identified to be ionizing radiation (IR) resistant and help to regenerate damaged tissue (Verghese & Su, 2016). The hinge cells specifically are resistant to IR and translocate to regenerate the damaged pouch. In Chapter 1, I investigated the requirement for cell movements during regeneration following irradiation damage using three RNA interference (RNAi) lines and one other gene. One RNAi line, *rab35*, and the other gene, *rux*, produced statistically significant effects when compared to their controls. *rux* inhibited cell migration to a greater extent than *rab35*, but in both cases cell migration was not completely inhibited. Therefore, the results suggest that both cell division (*rux* experiment) and cell migration (RNAi experiment) are necessary during wing disc regeneration following radiation induced damage, but there are likely other factors aiding in regeneration.

Rather than performing a candidate approach to determine the genes involved in regeneration following IR damage, I then attempted a global, unbiased approach in Chapter 2 by dissociating wing disc tissue, isolating regenerative single cells, and submitting isolated RNA for Illumina sequencing.

In Chapter 2, I followed a dissociation protocol published by Khan et. al (2016). I spent time optimizing dissociation conditions, such as trypsin volume and temperature and the number of times the sample was pipetted. I determined single cell yield using microscopy and Hoechst stain 33342. Cell viability was also determined using microscopy and acridine orange stain for different amounts of pipetting and filtering through a cell strainer. Increased pipetting led to more dead cells and filtering the sample also increased dead cell count. The final adapted protocol I ended up following used 500uL of heated trypsin at 37°C while pipetting five times
every 5 minutes, and 15 times following the 15 minutes incubation period. Overall the adapted protocol was effective at dissociating tissue into single cells.

I decided to only use the main campus FACS core to eliminate the unknown variation and because it required significantly less travel time, so less cells die during the transit time. Both +IR and -IR samples were immediately taken to the main campus FACS core for sorting following the dissociation protocol and were sorted in one session. Sorting took roughly an hour per sample, so the second sample was sitting for one hour before getting sorted, likely causing significant cell death. In the future, only one sample will get sorted during a session in order to minimize cell death from sitting.

We expected about 20% of the cells to be double positive since microscopy has shown that the hinge comprises about 20% of the wing disc. However, the lower than expected amount of double positive cells could be due to cell clumps getting caught in the strainer or being selected against in FACS. Irradiated samples had a lower overall yield of single cells, and therefore a lower double positive yield, but this is expected since radiation induces apoptosis. Acquiring less double positive cells will mean that more sorts will be to be performed to obtain an adequate number of cells for RNA isolation and sequencing. To obtain ample RNA for eventual Illumina sequencing, I am aiming for 200 wing discs for my irradiated sample and 150 wing discs for my unirradiated sample according to my FACS results: 9% were double positive for the irradiated and 15% for the unirradiated sample.

Isolating RNA from sorted single, double negative cells yielded no RNA on an agarose gel. When the sample was analyzed on the Nanodrop however, it indicated that there was RNA in the sample. The sample was likely comprised mainly of contaminants with little RNA, since no bands appeared on the agarose gel. Furthermore, since the double negative cells did not have
RNA it is highly likely that the double positive cells do not contain RNA either. The Next-Gen Sequencing Facility at BioFrontiers needs a minimum of 50ng of RNA to sequence, but 500ng is ideal, so the next step will be to determine where degradation is occurring.

I suspect that the RNA degradation is occurring from dead cells getting sorted into the live cell population and pipetting frequently is causing more cell death. When the cells are lysed by freezing, RNases from the dead, lysed cells are released, degrade RNA from live cells. The FACS gates will be set more conservatively to ensure dead cells are eliminated from the desired sample. Another way to ensure dead cells are not selected for is to specifically stain dead cells and actively deselected those cells from the population. This will minimize the number of dead cells that are sorted into the desired population, thus reducing the amount of RNase that can degrade the RNA.

Once intact RNA is successfully isolated, it will be subject to Illumina sequencing. High output 75-cycle mRNA sequencing with 20-30 million reads will be performed with a polyA enrichment library prep. I will compare gene expression in RFP+/GFP+ cells in the hinge at 0, 24, and 48 hours post-irradiation to (1) non-irradiated samples and to (2) RFP-/GFP- cells in irradiated discs. Gene expression in irradiated RFP+/GFP+ cells will be considered significant if a p-value of less than 0.05 is obtained using a t-test against non-irradiated RFP+/GFP+ cells. Also, only genes that changed expression by 2-fold or greater will be considered further.

The next step in this project after identifying genes of interest will be to validate hits, genes involved in regeneration after damage, identified in the RNA-sequencing experiment. In order to validate significant genes, real-time polymerase chain reaction (RT-PCR) can be done to measure gene expression. RT-PCR would still require dissociation, FACS, and RNA isolation, but once the RNA is isolated, necessary primers can be added along with nucleotides and Taq
polymerase to amplify selected genes of interest. From there, mutant fly lines can be obtained to see what occurs when the set of genes are knocked out or knocked in. Genes that prove to have a significant effect on regeneration after irradiation damage can be investigated in human cancer cells or other mammals. If said genes are identified in humans and other mammals, then they can be targeted for therapeutic potential.
References


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