Consequences of cell death in the model organism *Drosophila melanogaster*: potential implications for cancer treatments

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ABSTRACT

The fruit fly *Drosophila melanogaster* shares nearly 75% of known human disease-related genes (Pandey 2011). With its rapid life cycle, simplified genome, and widespread availability, this model organism provides a valuable entry point for cancer research in genetic studies and small-molecule drug screens. In humans, cancer treatments frequently involve the interchange of radiation therapy and chemotherapy in order to avoid overwhelming doses of a single method. Such regimens are administered in intervals to allow a recovery period for a patient's integral, normal cell types. A recent study in *Drosophila* tissues found that these healing intermissions allow dying cells to confer enhanced survival to surrounding cells through a form of non-autonomous cell-signaling protection deemed "The Mahakali Effect" (Bilak 2014). Similar survival mechanisms have been identified in human cancer patients (Sun 2012), and the ability of tumors to acquire resistance to radio and chemotherapy contributes to over 90% of treatment failures in metastatic carcinomas (Longley 2005). The Mahakali Effect, named after a Hindu goddess of death who protects her followers, describes the process by which previously killed or damaged cells protect neighboring healthy cells from subsequent radiation damage. The genes Tie and *bantam* were found to be necessary for this protection (Bilak 2014), and this thesis aims to determine if these genes are employed in the protective mechanism by undead cells. Undead cells are those whose apoptosis (programmed cell death) has been initiated but not completed. This is achieved through the inhibition of executioner and pro-apoptotic caspases (Dischtel-Danjoy 2013). Apoptosis serves the essential function of destroying mutated, precancerous cells, so inhibition of self-destruction makes undead cells more susceptible to tumorigenesis (Don Ryoo 2012). The ultimate goal of this research is to contribute to the ongoing cancer drug development to inhibit the protective effect, so that rounds of cancer

treatments involving cytotoxic drug therapy followed by radiation do not lead to progressively more protected and resistant tumor cells (Yu 2012; Bilak 2014). The results of this research suggest that Tie is not required for the protective effect in undead cells, while *bantam* appears to be important, yet not entirely responsible, for this effect. In this case, *bantam* has a permissive role rather than an instructive role, and the permissive threshold remains to be determined. Further gene candidate testing will be necessary in order to elucidate both the permissive and instructive components of cell-cell protection in undead *Drosophila melanogaster* tissues.

INTRODUCTION

Drosophila melanogaster as a Model Organism

Evolution affords remarkable genetic and physiological preservation between mammals and fruit flies. The invertebrate *Drosophila melanogaster* share nearly 75% of known human disease-related genes, as well as the basic pathways in cell, tissue, and organ development and function (Neckameyer 2012; Pandey 2011). Rapid generation time and a pliable genome allow researchers to rapidly screen *Drosophila* lines for numerous mutations in key physiological processes, as well as to genetically engineer lines carrying specific phenotypic markers.

Fruit flies have several genetic tools that allow the study of gene regulation without affecting the viability of the organism. The lethal mutations can be maintained over a balancer chromosome. Balancer chromosomes are inversions with visible markers that maintain a mutation by preventing crossing over. Yeast site-specific genetic manipulations are also employed in flies through the FLP/FRT and Gal4/UAS systems. FLP is a site-specific recombinase that targets FRT sites to create genetic clones within a tissue (Theodosiou & Xu 1998). To control these mitotic recombination effects, FLP constructs are commonly expressed under the control of a heat-shock promoter and include a marker for FLP/FRT clone identification.

The Gal4/UAS system allows tissue-specific induction and further regulation by Gal80. These lines encode the yeast transcription activator protein Gal4 along with its Upstream Activator Sequence (UAS). When Gal4 binds to its UAS, it specifically activates transcription of the downstream gene (Brand & Perrimon 1993). In order to regulate temporal expression of UAS

promoted gene is to include expression of a temperature sensitive Gal80 yeast protein, which binds to Gal4 and prevents its function. $Gal80^{ts}$ repression of Gal4 is under the control of a temperature shift, allowing control of the onset and duration of expression (Elliot & Brand 2008). This system provides a means for gene expression control by location, concentration, and time of induction (Neckameyer 2012).

Cancer and Apoptosis

Cancer cells are those with regulatory aberrations in proliferation and homeostasis, and evasion of apoptosis is one of the six hallmarks of malignancies (Hanahan 2000). Apoptosis (programmed cell death) provides the essential function of removing damaged or potentially harmful cells. It is critical to cellular homeostasis. Activation of apoptosis occurs in response the DNA damage inflicted by chemo and radiotherapy, but tumor cells may evade this outcome by inhibiting the complete unfolding of their apoptotic program, thus attaining resistance to cancer treatment. Therapy resistance contributes to 90% of treatment failures in metastatic carcinomas (Longley 2005), so it prudent to better understand cell death and the evasion of cell death.

Drosophila melanogaster wing imaginal discs constitute an advantageous tissue system for researching irradiation-induced apoptosis because these tissues are characterized by very little developmentally regulated apoptosis (Perez-Garijo 2004). Additionally, the components of apoptosis in *Drosophila* are homologous to those in mammalian apoptosis (Fig. 1).

In both humans and fruit flies, apoptosis is accomplished through the activation of cysteine proteases called caspases. Caspases are always present in the cell as inactive zymogens, but they are regulated by DIAP1 (Drosophila Inhibitor of Apoptosis Protein 1), which prevents their cleavage and activation. Pro-apoptotic gene products suppress DIAP1 via proteolytic degradation in order to initiate apoptosis, and these are *rpr (reaper), hid (head involution defective), sckl,* and *grim* (Ryoo 2002). There are initiator caspases and effector caspases. Initiator caspases are Caspase-8, Caspase-9, and *Drosophila* Dronc, Dredd, and Strica; effector caspases are Caspase-3, Caspase-7, and *Drosophila* Drice, Dcp-1, Decay, and Damm (Denton 2013). Pro-apoptotic gene products activate initiator caspases, which then cleave and activate executioner caspases to "execute" apoptosis (Denton 2013).

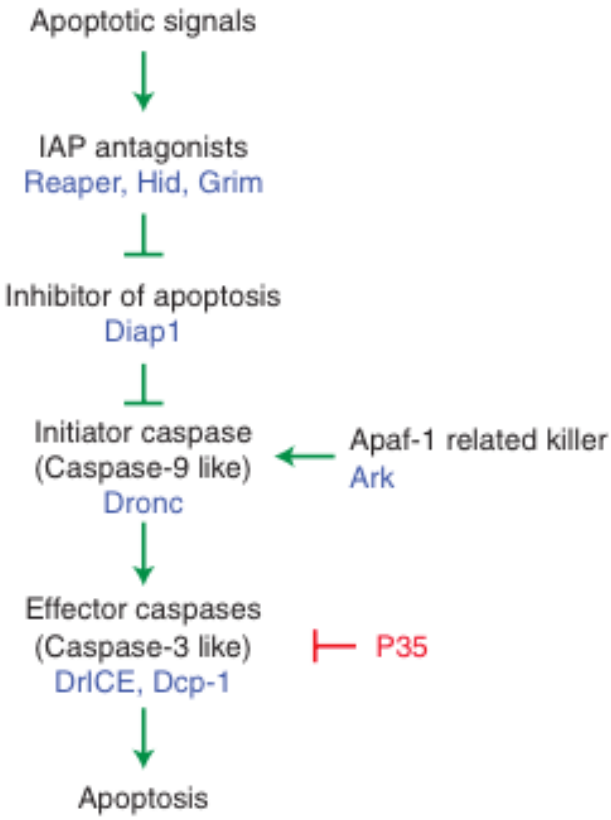


Figure 1. Regulation of apoptotic pathway (Hyung & Bergmann 2012)

p35: inhibitor of apoptosis

In the *Drosophila melanogaster* model, the baculovirus protein p35 inhibits apoptosis by binding executioner caspase Dcp-1 and Drice and inhibiting their function, without affecting Dronc (Fig. 1)(Hay 1994; Hyung 2012). Cells expressing p35 are able to initiate apoptosis, but cannot complete it, and instead exist in an "undead" physiological state. Despite their

abnormalities, undead cells in the wing disc are able to differentiate into proper structures (Perez-Garijo 2004).

Undead cell signaling is both quantitatively and qualitatively distinct from genuine apoptotic cells. For example, undead cells express increased *decapentaplegic (dpp)* and *wingless (wg)*. *dpp* is the mammalian homolog of TGF-β and *wg* is the mammalian homolog of Wnt.

Compensatory Proliferation In *Drosophila* Wing Imaginal Disc

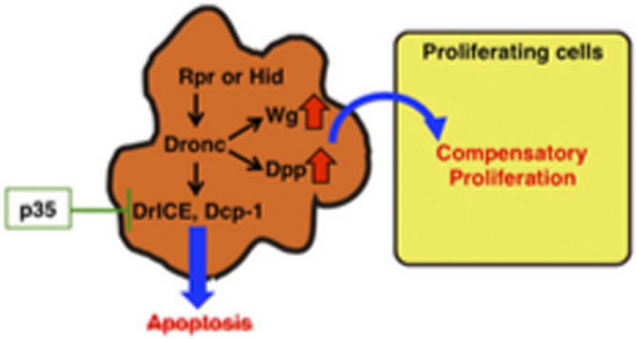


Figure 2. Compensatory Proliferation Model. Inhibition of apoptosis by p35 can lead to "Compensatory Proliferation" through Dronc upregulation of morphogens *wg* and *dpp* in the surrounding cells. (Kashio 2014).

The fly imaginal discs grow inside the larvae and assemble the exterior structures of the adult fly after pupation. The wing imaginal discs develop into adult wings and thorax (Held 2005). When larvae are exposed to the non-lethal stress treatments X-ray irradiation or heat shock, massive cell death is induced in the wing discs. However, normal wings can develop even after nearly half of the wing cells have been eliminated (Perez-Garijo 2009). This regenerative cellular response is known as "Compensatory Proliferation." Some of the studies demonstrating this phenomenon include the co-expression of p35 in the dying cells (Perez-Garijo 2009; Su 2015). When dying cells are kept alive by p35, tissue regeneration results in hyperplastic growths

in the wing disc. In undead cell regeneration, increases in *wg* and *dpp* are required for the induced proliferation as well as for the induction of each other's expression (Perez-Garijo 2009). These mitogens are responsible for the resulting hyperplastic overgrowths. In fully apoptotic cells, compensatory proliferation is a homeostatic process to restore wing disc size after injury. *dpp/wg* signaling in these cells does not lead to hyperplastic growth, and these mitogens are not required for tissue regeneration (Perez-Garijo 2009).

Irradiation

Doses of X-Ray treatment induce chromosomal breaks and cell death in the wing imaginal tissue through pro-apoptotic signals. *Hid* is a known key factor in X-ray induced apoptosis (Brodsky 2004; Burnley 2008).

Apoptotic irradiation responses

The pro-apoptotic gene Hid and the anti-apoptotic microRNA *bantam* are required for the proper growth of larval imaginal discs (organ precursors during embryogenesis) (Brennecke 2003). *bantam* binds to 3'UTR sequences in *hid* mRNA to reduce its stability and/or translation ability (Tang 2005). These two players in the apoptotic pathway help to balance pro- and anti-apoptotic stimuli through signal integration and combinatorial control; high enough concentrations of *bantam* can overcome *hid* activity and vise versa. Exposure to X-ray damage increases *hid* levels and promotes apoptosis, but exposure to X-ray damage also increases *ban* expression, which limits apoptosis and promotes cell survival (Burnley 2008; Bilak 20014). The 21-nucleotide microRNA *bantam* is the first reported microRNA to exhibit functional importance for radiation responses: (1) *ban* mutants suffer more irradiation-induced apoptosis, (2) *ban* is activated by irradiation, and (3) *ban* mutant larvae are more sensitive to killing by irradiation (Burnley 2008; Bilak 2014).

In order to detect the activity of the small, nascent microRNA *bantam* in radiation response experiments, a published fluorescence sensor was utilized (Bilak 2014). The sensor functions through a GFP transgene that is expressed by the tubulin promoter and contains two *bantam* target sequences in its 3'UTR (Bilak 2014). When *bantam* is present, it binds to these sequences on the GFP transgene and represses GFP expression. Thus, the absence of GFP fluorescence indicates the presence of *bantam*.

Induction of apoptosis in *patched* domain of wing imaginal disc

The *patched* (*ptc*) domain is defined by a stripe of anterior compartment cells along the Anterior/Posterior compartment boundary. Using *patched* expression to drive dsRNA against *de2F1* under the control of a UAS-Gal4 system (to be called $ptc>dE2F1^{RNAi}$) reduced the E2F1 protein levels (a transcription factor with a positive role in cell cycle progression) and led to cell autonomous apoptosis (Bilak 2014). Cells with the apoptotic *ptc* domain were then irradiated, and it was found that these wing discs contained decreased caspase staining than control discs with a non-apoptotic *ptc* domain (Fig. 5) (Bilak 2014). It was determined that prior cell death in the *ptc* domain resulted in *ban* activation and protection from IR-induced cell death in the rest of the disc, in a dose-dependent manner. This result was verified by cell killing in regions other than the *ptc* domain (Bilak 2014).

The Mahakali Effect: dying cells protect survivors from radiation-induced cell death

When cells in the *Drosophila* wing imaginal disc were killed, the surviving cells became harder to kill by ionizing radiation. This protection is deemed "The Mahakali Effect" after a Hindu goddess of death who protects her followers (Fig 3. C-H)(Bilak 2014). The Mahakali Effect requires the receptor tyrosine kinase Tie and the microRNA *bantam* for successful induction of protection (Bilak 2014). Tie was identified through a pilot genetic screen for *bantam*

modifiers and radiation sensitizers and encodes a *Drosophila* homolog of mammalian Tie (Tyrosine kinase with Ig and epidermal growth factor homology domain) (Bilak 2014). It was found that both *tie* and caspase activity were required to activate *ban*, but *tie* was not required for caspase activation (Bilak 2014).

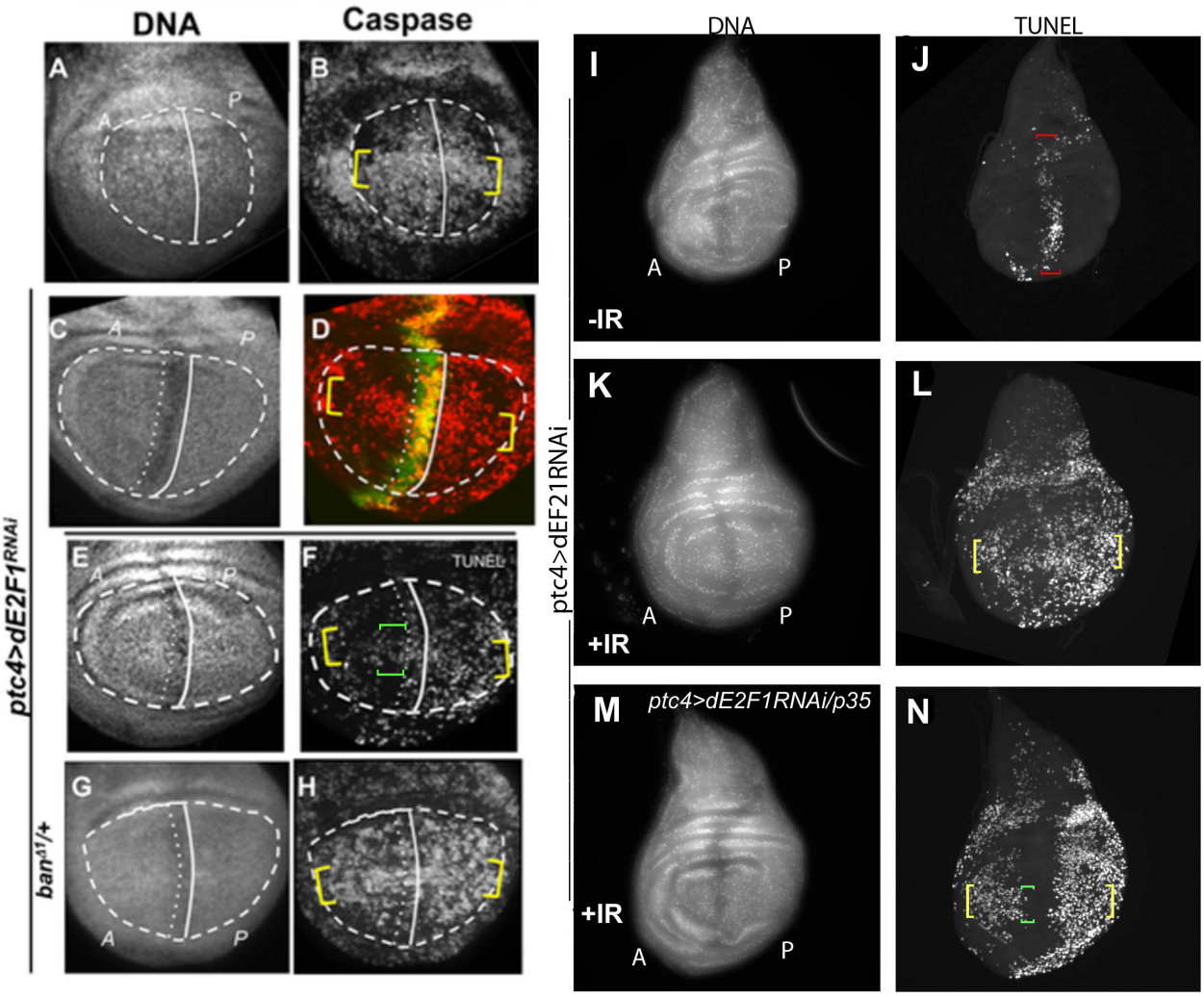


Figure 3. Protective Responses in Wing Discs. A-H from Bilak 2014 published data (Figure 5). I-N Tin Tin Su unpublished data. Dashed lines surround wing pouch and indicate *ptc* boundary. A/P marks Anterior and Posterior wing compartments. Yellow brackets mark D/V dorsal/ventral boundary. Red brackets in (J) mark ptc domain. Green brackets in (F) and (N) compare short-range protection to long-range protection.

Role for p35 in dying cell induced protection

In order to gain a comprehensive understanding of dying cell-induced protection, it was asked if co-expression of p35 in the *ptc* domain induced a signal similar to fully apoptotic cells (T. T. Su, unpublished data). *PE3;UASp35* cells in the wing disc do exhibit reduced caspase staining compared to naïve discs in response to irradiation, but the pattern of caspase reduction is altered. There is a potent, short-range signal (Fig. 3 N). It was then asked if *tie* and *bantam* induce this short-range signal.

METHODS

Apoptosis in the *ptc* domain

The *patched* (*ptc*) domain is defined by a stripe of anterior compartment cells along the Anterior/Posterior compartment boundary. Using *patched* expression to drive dsRNA against *de2F1* under the control of a UAS-Gal4 system (*ptc>dE2F1*^{RNAi}) reduced the E2F1 protein levels (a transcription factor with a positive role in cell cycle progression) and led to cell autonomous apoptosis (Bilak 2014). This pattern of controlled cell death, to be called *PE3*, allows the study of dying cell non-autonomous signals to all surrounding wing imaginal disc tissue types.

Crosses

Flies were crossed by adding virgin females to males in a vial with dry yeast pellets and a strip of 3MM paper (to increase surface area for flies to comfortably reside). Flies mated for two days at room temperature, and were then transferred to a fresh vial every 12-24 hours while the previous vials (containing embryos) were placed at 25°C for three days, then transferred to 29°C for one day.

Irradiation

The larvae were then irradiated in food in a Faxitron Cabinet X-ray System Model RX-650 at 115 kV and 5.33 rad/sec. This resulted in a dose of 4000R, which induced an intermediate level of lethality. Post irradiation, larvae were incubated at 25°C for four hours.

Selection, Dissection, Staining

Genotype selection was informed by a GFP reporter and Tb phenotype. Selected larvae were then dissected in PBS (Phosphate Buffered Saline) and fixed for 30 minutes at room temperature. Tissues were blocked in 5% normal goat serum before immunostaining with Dcp-1 rabbit antibody (1:100 microL in block) followed by anti-rabbit rhodamine secondary antibody

(1:500 microL in block). Blocking the tissues with goat serum helps prevent non-specific antibody binding as the natural antibodies in the goat serum bind to high affinity proteins. β-Galactosidase staining of *dppLacZ* tissues were stained with β-Galactosidase (1:100 microL in block) followed by anti-mouse rhodamine secondary antibody (1:100 microL in block).

Tissue DNA was also stained with Hoechst 33258 to provide a localized context for caspase staining. Stained wing discs were removed, mounted in Fluoromount G, and analyzed via fluorescence imaging on a confocal microscope or a fluorescence microscope. For confocal images, 25 Z-axis slices where imaged with 1 micron steps. The amount of Dcp-1 staining was interpreted to indicate cell death, and the extent of the staining informed the strength of the protective effect.

Genotypes

PE3 = ptc-GAL4>UAS-dsRNA against *dE2F1 (2R)*

CyO-GFP = Curly of Oster (curly wings), Green Fluorescent Protein

Tb = tubby phenotype

TM6B = third multiple 6, tubby phenotype and more than two humoral bristles, chromosomal inversion (FBba0000197)

$ban^{\Delta 1}$ = null bantam allele 3L

ban^{L1170a} = transposable element insertion site 3L

tie^{7925} = *PBac{RB5.WH5}Exel9028* (Bloomington #7925)

p35 = caspase inhibitor p35 on chromosome three

PE3;ban^{L1170a}*/UASp35*

PE3;$ban^{\Delta 1}$ */UASp35*

PE3;tie^{7925}*/UASp35*

PE3/dppLacZ;UASp35/ $Gal80^{ts}$

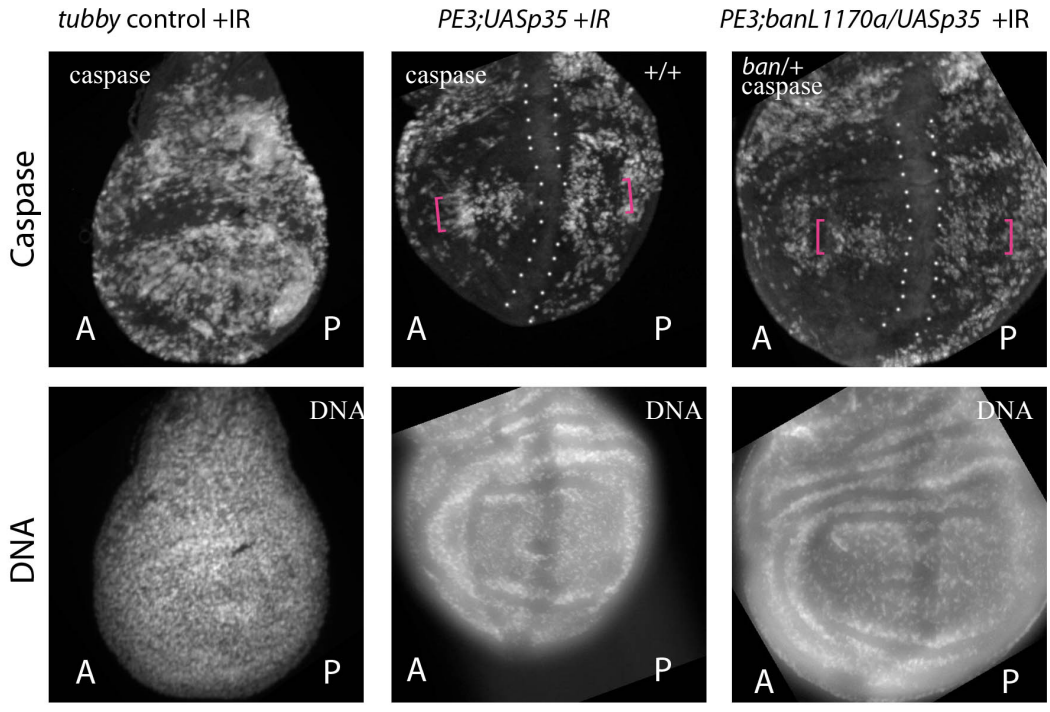
RESULTS

tie and *bantam* are required for long-range protection by apoptotic cells in response to irradiation. One goal of this thesis was to determine if they are also required for short-range protection by undead cells. Another goal was to observe *dpp* transcript levels in response to p35 co-expression in *PE3* domain of death in order to observe if this compensatory proliferation mitogen is implicated in the *PE3* wing disc experimental model.

Effect of *bantam* mutants on undead cell-induced protection

To address the role for *bantam* in short-range protection, p35 was co-expressed in the *PE3* domain of death. Then, two mutant *bantam* alleles were tested to observe the impact on caspase staining upon reduction of *ban* gene dosage. $ban^{\Delta 1}$ mutants are generated via chromosomal deletion, while ban^{1170} mutants are the result of a p-element insertion (Hipfner 2002).

Figure 4. Effect of ban^{1170} heterozygosity on caspase staining. All tissues irradiated with 4000 rads. Pink brackets demarcate D/V boundary. White dotted lines around *ptc* domain.



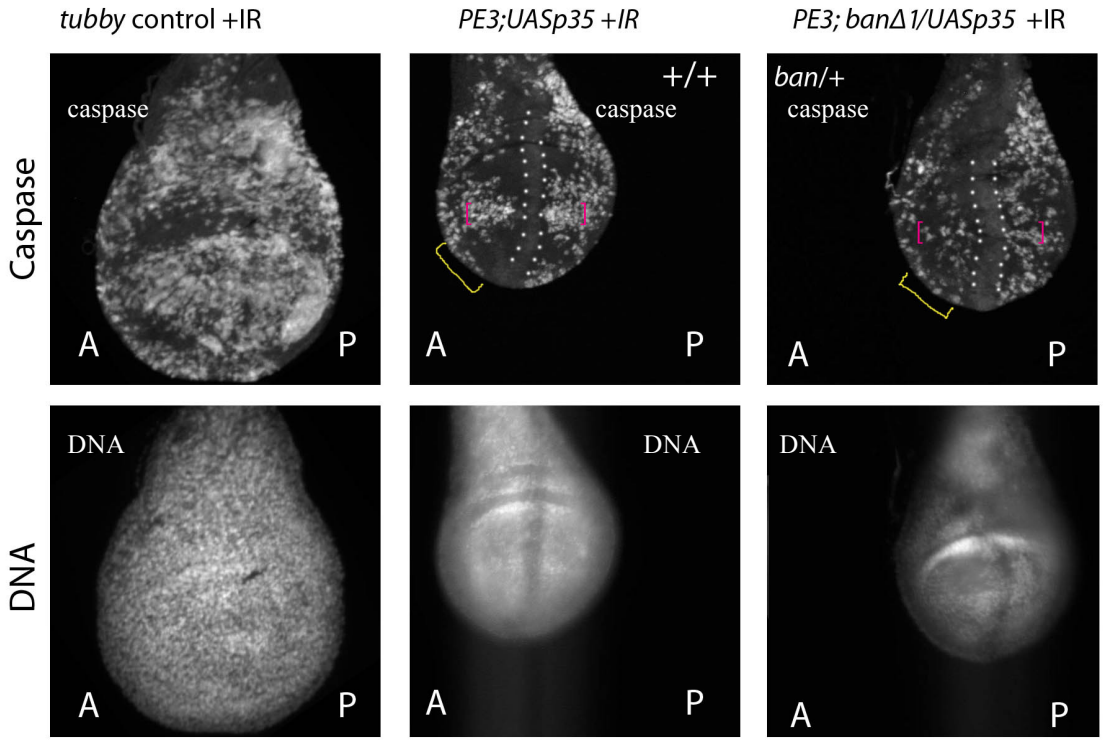


Figure 5. Effect of *ban*$^{\Delta 1}$ heterozygosity on caspase staining. All tissues irradiated with 4000 rads. Pink brackets demarcate D/V boundary. White dotted lines around *ptc* domain. Yellow bracket indicates region of increased caspase staining in *ban*$^{\Delta 1}$/+wing discs.

DV boundary cells consistently have higher levels of caspase staining (Fig. 4 and 5). The undead cells in the *ptc* domain of the DV boundary interrupt the solid axis of DV caspase stained cells. *ban*$^{\Delta 1}$/+ wing discs have increased caspase staining and are smaller compared to *ban*^{1170} /+ and *ban* +/+ tissues (Figs. 4 and 5).

The mean intensity of caspase staining was measured in a 5-cell radius from the *ptc* domain in the Anterior compartment and the Posterior compartment of each disc. The A:P ratio was then calculated and the student's t-test was utilized to determine the statistical significance of the A:P ratio in heterozygous mutants versus homozygous *Tb* phenotypic discs. The average

caspase intensity ratio in *ban*^{1170} /+ wing discs was 0.82(±0.06):1 and the average in +/+ wing discs was 0.70(±0.09):1. The p-value for these data was 0.0028 with 17 degrees of freedom.

The average caspase intensity ratio in *ban*$^{\Delta 1}$/+ wing discs was 0.90(±0.07):1 and the average ratio in +/+ wing discs was 0.70(±0.04):1. The p-value for these data was 0.0003 with 9 degrees of freedom. The difference in caspase staining between the A:P ratios for these genotypes is far more statistically significant than the difference calculated for *ban*^{1170} data sets.

Effect of *tie* heterozygous mutant on undead cell-induced protection

To address the role for *tie* in short-range protection, p35 was co-expressed with *PE3* domain of death. The heterozygote loss of function mutant *tie*^{7925} (Bloomington #7925) was then tested to observe the impact on caspase staining with a 50% reduction in gene dosage. Caspase staining remained unchanged in *tie*^{7925} heterozygous mutant wing discs compared to *tie* +/+ wing discs (Fig. 6).

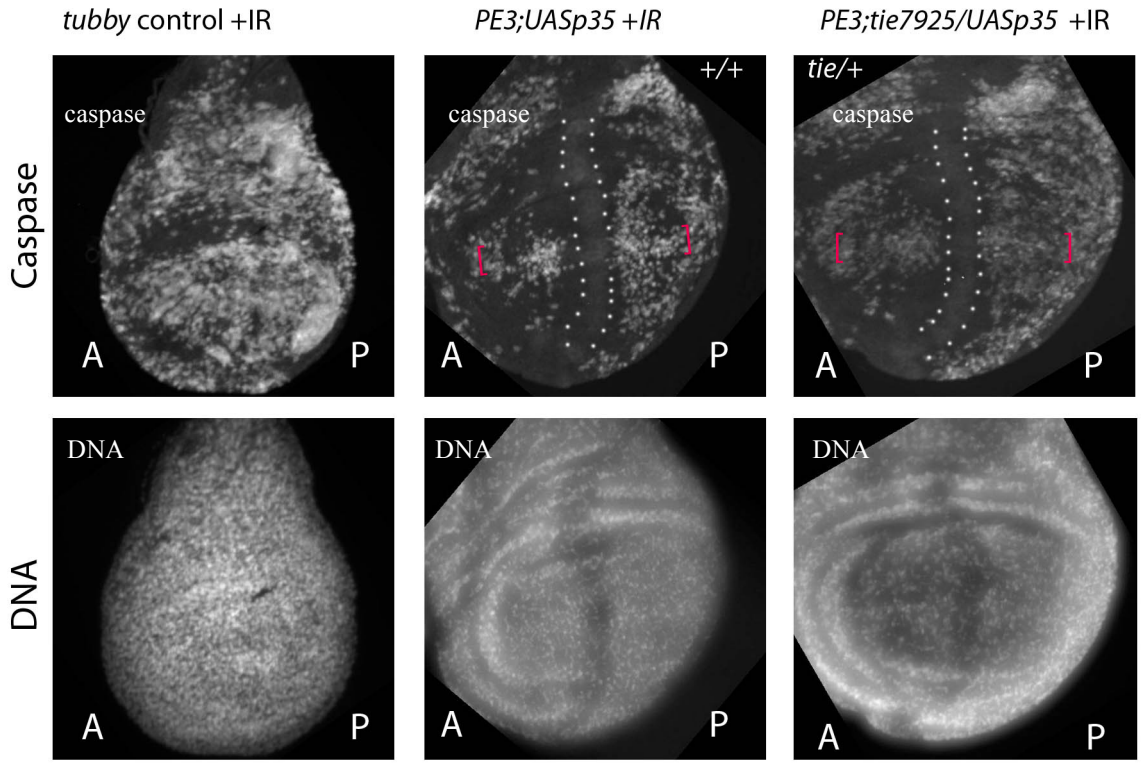


Figure 6. Effect of tie gene dosage reduction on caspase staining. All tissues irradiated with 4000 rads. Pink brackets demarcate D/V boundary. White dotted lines around *ptc* domain.

The mean intensity of caspase staining was measured in a 5-cell radius from the *ptc* domain in the Anterior compartment and the Posterior compartment of each disc. The A:P ratio was then calculated and the student's t-test was utilized to determine the statistical significance of the A:P ratio in heterozygous mutants versus homozygous *Tb* phenotypic discs. The average caspase intensity ratio in *tie*^{7925} /+ wing discs was 0.78(±0.06):1 and the average in +/+ wing

discs was 0.68(±0.05):1. The p-value for these data was 0.018 with 15 degrees of freedom, making the difference between these ratios insignificant.

Effect of p35 co-expression and ionizing radiation on *dppLacZ* transcription in *ptc*

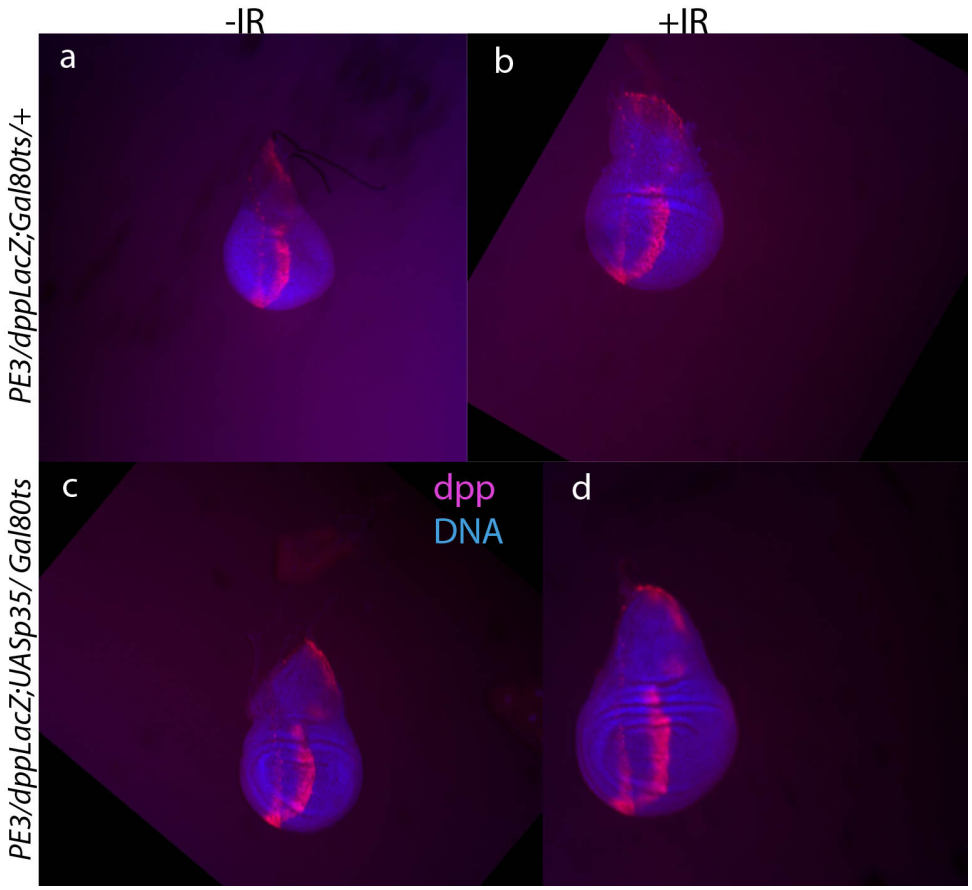


Figure 7. Effect of ionizing radiation on *dpp-lacZ reporter.* expression in undead cells. Signal intensity for (a) and (b) 1145.5, signal intensity for (c) and (d) 1577.5 by image J quantification. Intensity quantified by selecting *ptc* cells in the wing disc and calculating the average intensity in that region.

To determine if *dpp* transcription increases in undead cells, the *PE3* domain of death was co-expressed with p35 in wing discs containing a *dppLacZ* allele. Wing discs were then stained with an antibody against β-Galactosidase in order to observe transcript expression in wing discs with and without p35 co-expression, and both exposed and unexposed to radiation. The intensity of the *dppLacZ* transcript signal increases in p35 undead cells compared to fully apoptotic cells: fully apoptotic cell signal intensity averaged 1126.3 (standard deviation 53.22, n=9), while undead cell signal intensity averaged 1574.5 (standard deviation 81.68, n=11). The p-value for

two-tailed student's t-test is 4.88×10^{-11} and the p-value for one-tailed student's t-test is 2.44×10^{-11}, making the difference between these data samples extremely significant. The intensity of the *dppLacZ* signal is unaltered by radiation exposure in both p35 undead cells and fully apoptotic cells (Fig. 7).

DISCUSSION

The aim was to determine the effect of *tie* and *ban*tam heterozygous mutants on the short-range protection induced by undead cells, and to observe *dpp* transcription levels via β-Gal LacZ reporter in undead cells compared to fully apoptotic cells. It was found that *bantam* heterozygous null mutants exhibit less protection, tie heterozygous mutants have no impact on protection, and *dpp* transcription increases in *PE3* undead cells.

ban$^{\Delta 1}$ has dose-dependent role in undead cell-induced protection

While *bantam* is required for the Mahakali Effect, it seems to only be a factor in undead cell-induced protection. There was little to no effect on the undead cell-induced protection in *ban*^{1170} heterozygous mutants (Fig. 4), but there was a distinct decrease in protection in *ban*$^{\Delta 1}$ mutants as indicated by increased caspase staining (Fig. 5).

It is likely that the chromosomal deletion in *ban* $^{\Delta 1}$ creates a more severe or complete loss of *bantam* function, while the p-element insertion in *ban*^{1170} may allow limited or variable *bantam* expression. This indicates a dose-dependent role for *bantam* in undead cell-induced protection. The *bantam* dosage disparity between the two mutants must be very minor, indicating the sensitivity of undead cell-induced protection to even slight *bantam* concentration changes.

ban $^{\Delta 1}$ mutant wing discs are also significantly smaller than other genotypes of the same age (Figs. 4, 5, 6). *ban* $^{\Delta 1}$ growth is inhibited by their chromosomal deletion (Hipfner 2002). Further experiments with an increased experimental time course will be required in order to generate *ban* $^{\Delta 1}$ wing imaginal discs of comparative size to the other experimental wing discs. Similarly, wing imaginal discs of the other genotypes must be tested at earlier time points, thus

providing a size-equivalent spectrum of caspase staining in wing discs of all tested genotypes and determine the impact of variable wing disc size on caspase staining.

Undead cell-induced protection is constant in +/+ and *tie*^{7925}/+

tie^{7925} heterozygous mutants did not effect short-range protection in the wing discs (Fig. 6). This reduction in *tie* signaling may not have been severe enough to reduce *bantam* levels in undead cells and subsequently reduce protection. Homozygous null mutants will need to be tested in order to confirm if tie is required for undead cell-induced protection. It will also be useful to quantify *bantam* in *tie*^{7925} heterozygous mutants to observe impact of *tie* gene dosage.

***dppLacZ* transcription increases in undead cells**

The intensity of the *dppLacZ* reporter did not increase in any wing disc cells in response to irradiation. Co-expression of p35 in the *ptc* domain did increase the *dppLacZ* signal intensity compared to controls without p35, which is consistent with known compensatory proliferation responses in undead cells (Figs. 7, 2). This contributes to *PE3;UASp35* experimental model's reliability in the context of other undead cell studies in the wing imaginal disc.

Results confirm increased transcription of *dpp* in undead cells (Fig. 7), but in order to determine if *dpp* protein diffuses into surviving cells, discs need to be stained with a *dpp* antibody.

Inhibition of apoptosis by p35 creates a sustained *dpp* signal, which is known to be necessary for compensatory proliferation (Perez-Garijo 2009). These results suggest that *dpp* transcription does not increase in response to irradiation, although it does increase when co-expressed with p35. *dpp* could initiate a protective signaling cascade in survivors or diffuse 3-5 cells away from *ptc* undead cells. Undead *dpp* mutants will also need to be tested in order to confirm its potential role in undead cell-induced protection.

Non-autonomous undead cell-induced protection is distinct from the Mahakali Effect

Altogether, these results indicate that there may be signaling differences between Mahakali Effect protection and undead cell-induced protection. *bantam* has a dose-dependent protective role, while reducing *tie* gene dosage by half does not effect protection. Increased *dpp* is a known requirement for undead cell-induced proliferation, and *dpp* transcription increased in *PE3* undead cells and remained constant in response to irradiation. These results indicate that the non-autonomous protective cell signaling by undead cells remains to be elucidated. Detailed study of gene dosages in survivors 3-5 cells away from *PE3;UASp35* will provide a better understanding of the specific threshold requirements for undead cell-induced protection.

These results in the context of the field

Differences in the A/P compartments

The results of undead cell-induced protection and the results for the Mahakali Effect demonstrate the juxtaposition of the protective potency in the Anterior cells versus the Posterior cells. Each compartment contains its own principal set of signaling cascades, and it will be important to parse out which signals in the Anterior are responsible for increased protection, and/or which signals in the Posterior are responsible for decreased protection. There is a current understanding in the field that each compartment is arrested at a different G-phase of the cell cycle, and this may help illuminate their regenerative or protective potency, but the fact that protection is still seen in both the Anterior and Posterior cells indicates that cell cycle phase is not the primary determinant for protective capability.

The results reported in this thesis reinforce the recognition that the Anterior compartment houses the peak of regenerative potency in the wing imaginal disc (Held 2005). However, it

remains unclear whether the results of these non-autonomous protection experiments are due to conferred protection from dying cells or from pro-proliferative signals from dying cells.

Cell non-autonomous protection is distinct from Hormesis

Hormesis is a physiological factor in which low exposure to cell damage results in greater resistance to subsequent high exposure to the same cell damage. For example, mice exposed to non-lethal doses of radiation were more resistant to sub-lethal doses of radiation compared to completely unexposed mice (Miyachi 2000). In undead cell-induced protection, the exposure to cell damage in the *ptc* domain is not a non-lethal or low dose, it is strong enough to completely kill the cells and is simply inhibited by p35. Additionally, the subsequent induction of cell damage did not employ the same killing agent: the *ptc* stripe of cell death was induced by protein depletion and the protection was conferred to surrounding cells after ionizing radiation exposure (Su 2015). The increased resistance to cell death as seen in the Mahakali Effect and in undead cell-induced protection is competent regardless of the method of cell insult. The protected cells are not “immune” or “resistant” to a specific treatment, they become ubiquitously harder to kill.

Analogous Mammalian Processes

Cancer treatment resistance

Levels of caspase activity are a diagnostic feature in cancer patients, and it has been identified that the level of active Caspase 3 or Caspase 3 mRNA pretreatment correlates with tumor recurrence after radio or radio-chemotherapy or relapse during therapy (Huang 2011; Fennell 2005). A genome-wide analysis of transcriptional responses to genotoxic stress in prostate fibroblasts also identified a role for the up-regulation of WNT16B acting in a cell non-autonomous manner to promote the survival of cancer cells after cytotoxic therapy. WNT16B transcript level increased 33.7-fold as a result of chemotherapy, and higher WNT16B

immunoreactivity in prostate stroma post-treatment was associated with a significantly greater likelihood of cancer recurrence. A decrease in the amount of cleaved Caspase-3 was also positively correlated to WNT16B levels in this study (Sun 2012), reinforcing the importance of caspase activity in non-autonomous protective responses. Mammalian Caspase 3 and WNT16B are homologs to Drosophila Dcp-1 and Wg, respectively. These homologous gene products and their importance in tumor resistance reinforce the utility of *Drosophila melanogaster* as a model organism for identifying methods to increase apoptotic sensitivity.

The baculovirus protein p35 contains baculovirus inverted repeat domains that are also found in the mammalian Inhibitor of Apoptosis Proteins (Hay 1994). Evasion of apoptosis is almost pervasive in metastatic cancer types (Holohan 2013) and there is increasing recognition that cancer cells are "addicted" to a small number of anti-apoptotic proteins for survival (Holohan 2013; Letai 2008). Antagonists of IAPs are being identified as clinical candidates for cancer treatments (Flygare 2012; Huang 2015; Mckee 2015), which underscores the importance of characterizing undead cell signaling processes.

Future Aims

The *bantam* threshold needs to be determined for undead cell-induced protection. FISH is an appealing method for measuring *bantam* levels, as long as it can provide a precise enough signal to distinguish even minor changes in *bantam* levels, i.e. between ban^{1170} and $ban^{\Delta 1}$.

Wing imaginal disc size also needs to be equivalent among tissues of differing genotypes for improved caspase staining comparison. Further experiments with a longer experimental time course will be required in order to generate $ban^{\Delta 1}$ wing imaginal discs of comparative size to the other experimental wing discs. Similarly, wing imaginal discs of the other genotypes will be

tested at earlier time points, thus providing a size-equivalent spectrum of caspase staining in wing discs of all tested genotypes.

The goal is to identify genes with instructive roles in undead cell-induced protection. *wg* and JNK will be tested due to their known roles in compensatory proliferation, and *dpp* mutants will also be tested in order to identify whether or not it is required for protection. Wing discs should also be antibody stained for *dpp* protein to observe its potential diffusion.

The screen for *bantam* modifiers and radiation sensitizers that indentified Tie will be completed in order to identify any additional genes involved in the Mahakali Effect or in undead cell-induced protection.

CITATIONS

Bilak, A., Uyetake, L., Su, T. T. (2014). Dying Cells Protect Survivors from Radiation-Induced Cell Death in Drosophila. *PLOS Genetics, 10*, 1-15.

Burnley, J., Uyetake, L., Wichmann, A., Bilak, A., English, C., & Su, T. T. (2008). Modulation of ionizing radiation-induced apoptosis by *bantam* microRNA in *Drosophila*. *Developmental Biology, 320*, 122-130.

Brand, A. & Perrimon, N. (1993). Targeted gene expression as a means of altering cells fates and generating dominant phenotypes. *Development, 118*, 401-415.

Brennecke, J., Hipfner, D.R., Stark, A., Russell R.B., Cohen, S.M. (2003). Bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell, 113*, 25-36.

Carroll, S.B., Grenier, J.K., and Weatherbee, S.D. (2001). From DNA to Diversity: Molecular Genetics and the Evolution of Animal Design. Blackwell Science, Malden, Mass.

Denton, D., Aung-Htut, M.T., Kumar, S. (2013). Developmentally programmed cell death in Drosophila. *Biochimica et Biophysica Acta, 1833*, 3499-3506.

Dichtel-Danjoy, M. L., Ma, D., Dourlen, P., Napoletano, F., Robin, M., Corbet, M., …& Mollereau, B. (2013). *Drosophila* p53 isoforms differentially regulate apoptosis and apoptosis-induced proliferation. *Cell Death and Differentiation, 20*, 108-116.

Elliot, D.A. & Brand, A.H. (2008). The Gal4 System: a versatile system for the expression of genes. *Methods in Molecular Biology, 420*, 79-95.

Fan, Y., Bergmann, A. (2008). Distinct mechanisms of apoptosis-induced compensatory proliferation in proliferating and differentiating tissues in the *Drosophila* eye. *Developmental Cell, 14*, 399-410.

Flygare, J. A., Beresini, M., Budha, N., Chan, H., Chan, I. T., Cheeti, A.,…& Fairbrother, W. J. (2012). The Discovery of a Potent Small-Molecule Antagonist of Inhibitor of Apoptosis (IAP) Proteins and Clinical Candidate for the Treatment of Cancer (GDC-0152). *Journal of Medicinal Chemistry, 55*, 4101-4113.

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

Hay, B.A., Wolff, T., Rubin, G.M. (1994). Expression of bculovirus P35 prevents cell death in Drosophila. *Development, 120(8)*, 2121-2129.

Held, L. I. (2005). Imaginal Discs: complete figures & legends. *Cambridge University Press*, sbdonline.org.

Hipfner, D.R., Weigmann, K., Cohen, S.M. (2002). The bantam gene regulates Drosophila growth. *Genetics, 161*, 1527–1537.

Huang, J., Lyu, H., Wang, J., & Liu, B. (2015). MicroRNA regulation and therapeutic targeting of surviving in cancer. *American Journal of Cancer Research, 5*, 20-31.

Huang, Q., Li, F., Liu, X., Li, W., Shi, W., Liu, F. F.,… Li, C. Y. (2011). Caspase 3-mediated stimulation of tumor cell repopulation during cancer radiotherapy. *Nature Medicine, 17*, 860-867.

Hyung, D. R., Bergmann, A. (2012). The Role of Apoptosis-Induced Proliferation for Regeneration and Cancer. *Cold Spring Harbor Perspectives in Biology*, 1-17.

Kasai, Y. & Cagan, R. (2010). *Drosophila* as a tool for personalized medicine: a primer. *Personalized Medicine, 7*, 621-632.

Kashio, S., Obata, F., Miura, M. (2014). Interplay of cell proliferation and cell death in *Drosophila* tissue regeneration. *Development, Growth & Differentiation, 56*, 368-375.

Longley, D. B. & Johnston, P. G. (2005). Molecular Mechanisms of drug resistance. *Journal of Pathology, 205*, 275-292.

Martin, F.A., Perez-Garijo, A., Morata, G. (2008). Apoptosis in Drosophila: compensatory proliferation and undead cells. *The International Journal of Developmental Biology, 53,* 1341-1347.

McKee, C. M., Ding, Y., Zhou, J., Chunrui, L., Huang, L., Xin, X.,…& Xu, D. (2015). Protease nexin 1 induces apoptosis of prostate tumor cells through inhibition of X-chromosome-linked inhibitor of apoptosis protein. *Oncotarget, Advanced Publications*, 1-13.

Mollereau, B., Perez-Garijo, A., Bergmann, A., Miura, M., Gerlitz, O., Ryoo H. D. & Morata, B. (2013). Compensatory proliferation and apoptosis-induced proliferation: a need for clarification. *Cell Death and Differentiation, 20*, 181.

Moberg, K. H. & Hariharan, I. K. (2003). Big things from a little RNA. *TRENDS in Cell Biology, 13*, 455-457.

Neckameyer, W. S. & Argue, K. J. (2012). Comparative approaches to the study of physiology: *Drosophila* as a physiological tool. *American Physiological Society, 304*, 177-188.

Pandey, U.B., Nichols, C.D. (2011). Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery. *Pharmacological Reviews, 63*, 411-436.

Perez-Garijo, A., Matin, F.A., Morata, G. (2004). Caspase inhibition during apoptosis causes abnormal signaling and developmental aberrations in *Drosophila. Development, 131*, 5591-5598.

Perez-Garijo, A., Shlevokov E., Morata, G. (2009). The role of Dpp and Wg in compensatory proliferation and in the formation of hyperplastic overgrowths caused by apoptotic cells in the *Drosophila* wing disc. *Development, 136*, 1169-1177.

Postlethwait, J.H. (1975). Pattern formation in the wing and haltere imaginal discs after irradiation of *Drosophila melanogaster* first instar larvae. *W. Roux' Arch.* 178, 29-50.

Rusek, A.M., Abba, M., Eljaszewicz, A., Moniuszko, M., Niklinski, J. & Allgayer, H. (2015). MicroRNA modulators of epigenetic regulation, the tumor microenvironment and the immune system in lung cancer. *Molecular Cancer, 14*, 14-34.

Saint Phalle, B. (2004). Immunostaining of Whole-Mount Imaginal Discs. *Drosophila Cytogenetics Protocols: Methods in Molecular Biology, 247*, 373-387.

Su, T. T. (2015). Non-autonomous consequences of cell death and other perks of being a metazoan. *AIMS Genetics, 2*, 54-69.

Sun, Y., Campisi, J., Higano, C., Beer, T. M., Porter, P., Coleman, I., True, L., & Nelson, P. S. (2012). Treatment-induced damage to the tumor microenvironment promoted prostate cancer therapy resistance through WNT16B. *Nature Medicine, 18*, 1359-1368.

Tang, G. (2005). siRNA and miRNA: an insight into RISCs. *Trends in Biochemical Sciences, 30*, 106-114.

Theodosiou, N.A., Xu, T. (1998). Use of FLP/FRT system to study Drosophila development. *A Companion to Methods in Enzymology, 14*, 355-365.

Wilson, T. R., Johnston, P.G. & Longley, D.B. (2009). Anti-Apoptotic Mechanisms of Drug Resistance in Cancer. *Current Cancer Drug Targets, 9*, 307-319.