Characterization of Non-apoptotic Roles of Dronc (Caspase-2/9) as a Tumor Suppressor in *Drosophila* Wound-like Tumors



Derek Xu
Linacre College
University of Oxford

Supervisors:
Associate Prof. Luis Alberto Baena-Lopez
Dr. Kenneth M. Yamada

A Thesis Submitted for the Degree of
Doctor of Philosophy

Michaelmas 2020

Abstract

Caspases are proteases best known for their ability to carry out the process of programmed cell death known as apoptosis. Through this function, caspases can act as tumor suppressors, and the evasion of apoptosis and cell death is an established hallmark of cancer. However, caspase activity has also been shown to be involved in a plethora of non-apoptotic physiological processes, and when deregulated, can also contribute to many other aspects of tumor initiation and progression. Because caspase functions are often context-dependent, the functional and mechanistic intricacies between caspase activity and tumorigenesis remain poorly characterized. To investigate the regulation and pleiotropic effects of non-apoptotic caspase activity in tumors, I took advantage of the powerful genetic tractability of the fruit fly, *Drosophila melanogaster*, and a recently described *Drosophila* model of cooperative oncogenesis which is relevant for humans. This tumor model relies upon the upregulation of both the EGFR and JAK/STAT signaling pathways. My results indicate that widespread non-apoptotic activation of the initiator caspase Dronc (orthologous to mammalian caspase-2/9) limits tumor growth. Importantly, this caspase-dependent tumor-suppression occurs independently of apoptosis. Instead, I reveal that the tumor-suppressive function relies on Dronc negatively regulating the JNK-signaling pathway. Furthermore, my data show that JNK-signaling is responsible for several malignant features in the tumor model (e.g. hyperplasia and loss of cell identity) and contributes to the recruitment and potential *in situ* proliferation of tumor-associated macrophage-like cells, which further fuel tumor growth. Interestingly, the tumors bear several similarities to tissues undergoing regeneration, including regenerative signaling profiles as well as the presence of tumor-promoting inflammation. Therefore, my results indicate the suitability of the tumor model to further understand the complex relationship between caspases, cancer, and inflammation in a wound-like tumor. Altogether, my findings regarding novel non-apoptotic tumor-suppressing mechanisms provide details that broaden our understanding of caspase biology and tumors and can eventually lead to improved potential therapeutic strategies against cancer that involve caspase-modulation.

Acknowledgements

Without a doubt, my time as a DPhil student has become something irreplaceable to me. Of course, it has been challenging at times, but it should be that way, and ultimately, it has been an incredibly rewarding experience. It should go without saying that this thesis could not have been completed without the help and support of those that I mention here, and many more beyond those written on this page.

First of all, I would like to thank my two supervisors, Alberto and Ken, for agreeing to take me on four years ago as a student in a brave attempt to establish an entirely new project and collaboration. We all stepped into uncharted waters together, and I am grateful for that opportunity. Thank you both for the unconditional guidance and support I have received through all the ups and downs, twists and turns of this amazing journey. To Ken especially for selflessly continuing to guide my development as a scientist even when it became clear that the original premise of our collaboration would not hold water; and to Alberto especially for his passionate and endless drive, and his sharp and insightful scientific guidance. I believe I have grown tremendously under their mentorship, and there is no way that could have occurred without both their support.

I would be remiss if I did not acknowledge the NIH Oxford-Cambridge Scholars Program, the Intramural Research Program at the National Institute of Dental Craniofacial Research, the Sir William Dunn School of Pathology, and Linacre College. Without the administrative, financial, practical, and social support of these institutions, none of this would have been possible.

On more personal level, I have to thank my family and friends I made during this time. To my fellow members of the Caspase Lab, past and present, there's almost no way I can express how much I enjoyed and benefitted from our time together. I couldn't have learned better alongside, and from, you all. In particular I will always remember fondly the OG crew (Jana Alonso, Alessia Galasso, Lewis Arthurton, and Claire Hill) from when I joined, and together with Sebastian Kittleman, Franz

Wendler, Li Wang, Filipa Vaz, and honorary member Osamu Suyari, I consider myself extremely fortunate to have shared time in the fly room and at the bench together.

To my good friends at Linacre College, at the Dunn School, in the Linacre College Boat Club, and in the NIH OxCam Program – thank you for all the camaraderie, laughter, and endless banter that helped keep me going and sustained me inside and outside the lab. It is impossible to name everyone, or to count, even, but in particular I want to bring attention to some of my adopted family here at Oxford. Nyree and Laur, who helped me establish myself when I first moved. Dan, Abbie, Pascal, Kento, Britt, and Becs, who founded our dynasty at the Hurst Street house (along with our plus ones). Sapna, who along with Dan, completed our trio as the best social secretaries Linacre will ever see.

To Marie, who will forever remain the best discovery I ever made at Oxford, and who, by my side, has brightened my life beyond measure.

And finally, to my parents, my grandmother, and my brother. Without their love and support, I could not be here or achieved any of this.

Thank you all.

Contributions

The DBS-S-QF caspase sensor data in wild-type wing discs (Figures 2.3 and 2.4 C) were collected by Associate Professor Luis Alberto Baena-Lopez. All other experiments were carried out by me.

All figures in Chapter 2, 3, 4, and 5, except for Figures 2.2, 2.4, 4.4, and 4.5., were adapted from the pre-print submission (Xu et al., 2020).

Xu, D.C., Yamada, K.M., and Baena-Lopez, L.A. (2020). Widespread non-apoptotic activation of Drosophila Caspase-2/9 limits JNK signaling, macrophage proliferation, and growth of wound-like tumors. bioRxiv, [Preprint]. https://doi.org/10.1101/2020.1107.1127.223404

Table of Contents

Abstract ... ii

Acknowledgements ... iii

Contributions ... v

List of Figures ... ix

1. Introduction ... 1

Why study caspases and cancer? ... 1

Drosophila as a model to study non-apoptotic functions of caspases in cancer ... 5

Caspase regulation in apoptotic pathways ... 6

Non-apoptotic caspase functions and tumorigenesis in *Drosophila* models ... 14

Tumorigenesis due to mitogenic signaling upon caspase activation ... 15

Caspases and tumor promoting inflammation in $Ras^{V12}/scrib^{-/-}$ tumors ... 17

Non-apoptotic caspase activity promotes invasiveness through JNK signaling ... 18

Context-dependent caspase activity in different tumorigenic backgrounds ... 19

Thesis Outline ... 20

2. Non-apoptotic caspase activity in EJS tumors restricts tumor growth ... 22

Introduction ... 22

The EJS tumor model ... 23

Results ... 26

Induction of EJS tumors in *Drosophila* wing discs ... 26

Caspase Activity detected in EJS tumors using the Drice-based sensor (DBS-S-QF) ... 27

Detection of Widespread Non-apoptotic Caspase Activity in EJS Tumors ... 31

Non-apoptotic Caspase Activity Restricts EJS Tumor Growth ... 34

Non-apoptotic Dronc activity limits proliferation and cell size in EJS tumors ... 39

Discussion ... 43

3. JNK signaling limited by non-apoptotic caspase activity is responsible for malignant features in EJS tumors ... 49

Introduction ... 49

Results ... 54

Non-apoptotic caspase activity limits JNK signaling in EJS tumors ... 54

JNK signaling disrupts cell identity and differentiation in EJS tumors ... 57

JNK pathway activation is likely independent of ROS and conventional TNF alpha ligands in EJS tumors ... 62

Discussion ... 66

4. Tumor associated macrophages and their effect on tumor growth are limited by caspases ... 72

Introduction ... 72

Results ... 75

DTAM presence on EJS tumors is limited by non-apoptotic caspase inhibition of JNK activity ... 75

Functional hemocytes contribute to tumor growth 84
Discussion 87
5. Conclusion and future directions 97
Summary of Thesis 97
Discussion 101
Tumors can be formed entirely of cells with non-apoptotic caspase activity 101
Initiator caspases as tumor-suppressors through mechanisms independent of apoptosis 103
The interplay between caspases, the JNK pathway, and tumor biology is complex and context specific: non-apoptotic caspase inhibition of JNK in EJS tumors limits tumor growth 106
EJS tumors behave like chronic wounds 108
Unpaired ligands: a potential candidate for bidirectional communication between hemocytes and tumor 111
Future Directions 116
Determining the requirement of Dark interaction and catalytic activity for non-apoptotic caspase functions in EJS tumors 116
Determining the origin of JNK activity in EJS tumors 117
Determining if ecdysone production is responsible for developmental delay 118
Determining the relevance of Upd ligands in bidirectional communication between EJS tumors and DTAMs 118
Determining the importance of Diap-1 in EJS tumors and associated hemocytes 120
Determining if there is any potential non-apoptotic activity in EJS associated hemocytes 121
Conclusion 121
6. Materials and Methods 123
Materials 123
Drosophila stocks and husbandry 123
Drosophila genotypes 123
Oligonucleotides 124
Antibodies 125
Methods 125
Tumor induction 125
Dissection of wing discs 126
Immunohistochemistry 126
DHE Labeling 128
TUNEL staining 128
EDU staining 128
Fluorescence microscopy and image analysis 128
Real-time Quantitative PCR 131
Quantification and Statistical Analysis 132
Appendix – Investigating the roles of caspases and Btbd7 as novel regulators of cell shape dynamics 133
Preface 133
Introduction 133
Caspases in migration and motility 134
Btbd7, regulator of cell dynamics and morphogenesis 136
Results 137

Loss of Dronc does not disrupt glial cell dynamics during metamorphosis 137
Loss of caspase activity does not affect glial cells' morphological response to neuronal injury . 138
Dronc is not involved in glial cell clearance of axonal damage 142
Discussion 145
Materials and Methods 147
Drosophila stocks and husbandry 147
Drosophila genotypes 147
Molecular biology, cloning and generation of *D-Btbd7* mutant 148
Live-imaging of peripheral glia during metamorphosis 149
Neuronal injury protocol and adult brain dissection 149
Quantification and Statistical Analysis 150
References 151

List of Figures

1.1 Classical functional and structural classification of caspases 10
1.2 Conservation of the apoptotic pathway in mammals and *Drosophila* 14
2.1 Induction of EJS in *Drosophila* imaginal wing discs 27
2.2 Tracking caspase activity using DBS-S-QF sensor 30
2.3 Lineage tracing of caspase-activating cells in wild-type wing discs 31
2.4 Lineage tracing of caspase-activating cells in EJS tumors 32
2.5 TUNEL staining in EJS tumors reveals small amount of apoptosis 34
2.6 Downregulation of Dronc increases EJS tumor growth 35
2.7 Dronc knock-out EJS tumors increase in size 37
2.8 Dronc-specific non-apoptotic function inhibits EJS tumor cell size 39
2.9 Non-apoptotic activity of Dronc restricts proliferation in EJS tumors 41
2.10 Non-apoptotic Dronc activity restricts EJS tumor cell size 42
3.1 Non-apoptotic Dronc activity limits JNK signaling in EJS tumors 56
3.2 JNK^{DN} suppresses JNK activity in EJS 57
3.3 JNK activity in EJS tumors fuels disorganized growth 59
3.4 JNK activity disrupts cell identity in EJS tumors 60
3.5 Dronc is responsible for ROS production in EJS tumors 63
3.6 ROS is unnecessary for EJS tumor growth and hemocyte attraction 64
3.7 Tumor growth is independent of TNF signaling 66
4.1 Non-apoptotic Dronc activity limits hemocyte presence on EJS tumors 77
4.2 Hemocytes on EJS tumors synthesize DNA and potentially proliferate *in situ* 78
4.3 JNK signaling is necessary only for increased recruitment to *Dronc*-deficient EJS tumors 80
4.4 Proportion of EdU^{+} hemocytes on EJS tumors 81
4.5 Correlation between hemocytes and EdU^{+} hemocytes 83
4.6 Hemocyte-specific overexpression of *reaper* in wild-type larvae ablates vast majority of hemocytes 85
4.7 EJS tumors in *Hml>rpr* backgrounds are significantly smaller 87

5.1 Initiator caspases act as tumor suppressors in a wound-like EJS tumor through negative regulation of the JNK pathway 115
A1 Drosophila peripheral glia display striking morphological dynamics during metamorphosis 138
A2 *Dronc* is transcribed in glial cells surrounding the antennal lobes.....139
A3 Morphological response of glial cells to neuronal injury 140
A4 Loss of caspase function in glial cells does not affect morphological response to neuronal injury.....141
A5 Loss of *Dronc* does not functionally compromise glial injury response.....143
A6 Loss of *D-Btbd7* does not affect glial cells' morphological response to neuronal injury.....145

Chapter 1

Introduction

Why study caspases and cancer?

Despite their seemingly antithetical nature, life and death are inextricably linked in biology. The study of life would not be complete without understanding death—or more specifically, regulated cell death. In contrast to accidental cell death, which is unexpected and biologically uncontrolled, regulated cell death involves a set of genetically encoded molecular mechanisms responsible for purposefully eliminating cells (Galluzzi et al., 2018). In all animals, regulated cell death is critical for proper development and numerous physiological processes, removing unnecessary, damaged, or potentially harmful cells from tissues (D'Arcy, 2019; Elmore, 2007; Galluzzi et al., 2018).

While many forms of regulated cell death exist, such as necroptosis, pyroptosis, and autophagy-dependent cell death, the most studied form of regulated cell death is undoubtedly apoptosis (Fuchs and Steller, 2011; Nagata, 2018; Nhan et al., 2006; Tang et al., 2019). Apoptosis is critically important for normal developmental processes and tissue homeostasis, such as the elimination of interdigital cells and non-functional nerve cells (Elmore, 2007; Fuchs and Steller, 2011; Nagata, 2018). Apoptosis is also induced in response to stress, eliminating unhealthy or pathologically damaged cells (Fulda et al., 2010). Crucially, numerous pathologies are associated with defective apoptosis and insufficient cell death, such as cancer

(Fuchs and Steller, 2011; Galluzzi et al., 2018; Mohammad et al., 2015; Thompson, 1995). Indeed, the evasion of apoptosis and cell death has been widely accepted as a hallmark of cancer (Hanahan and Weinberg, 2000, 2011), and inducing apoptosis to eliminate tumor cells has been suggested and utilized as a strategy against cancer for almost half a century (Carneiro and El-Deiry, 2020; Gerl and Vaux, 2005; Kerr et al., 1972; MacKenzie and Clark, 2008; Thompson, 1995).

The study of apoptosis lead to the discovery of caspases, the family of proteases that, in response to death-inducing stimuli, carry out the proteolytic activity that results in the orderly self-destruction of a cell (Nagata, 2018). Therefore, caspases have been studied extensively over the years, revealing their instrumental roles in the implementation of apoptosis (Budihardjo et al., 1999; Earnshaw et al., 1999; Elmore, 2007; Galluzzi et al., 2018; Nagata, 2018). In addition, these studies have provided strong evidence that caspases have a potent tumor-suppressive ability linked to their apoptotic function (Fernald and Kurokawa, 2013; Hanahan and Weinberg, 2000, 2011; Kelly and Strasser, 2011; Olsson and Zhivotovsky, 2011). Indeed, caspase activation is utilized for therapeutic purposes in cancer treatment (Berthelet and Dubrez, 2013; Hensley et al., 2013; Wen et al., 2012).

However, it is now becoming abundantly clear that a wide variety of non-lethal functions for caspases also exist, which influence a myriad of physiological processes including stem cell function, proliferation, differentiation, inflammation and immunity, motility and migration, stress adaptation, and neuronal remodeling,

learning, and memory formation (Connolly et al., 2014; Hyman and Yuan, 2012; Kuranaga, 2012; McIlwain et al., 2013; Miura, 2012; Mukherjee and Williams, 2017; Nakajima and Kuranaga, 2017; Su, 2020; Unsain and Barker, 2015; Yi and Yuan, 2009). Accordingly, the intersection between caspase and cancer biology appears to not be exclusively defined by apoptosis, and caspases acting in their non-apoptotic capacities can contribute to other hallmarks of cancer (Xu et al., 2018a). Along these lines, caspase activity has been shown to inhibit differentiation and sustain proliferation (Baena-Lopez et al., 2018b; Burgon and Megeney, 2018; Perez-Garijo, 2018), promote tumor-enabling inflammation (Diwanji and Bergmann, 2019), create genomic instability (Cartwright et al., 2017; Liu et al., 2015), and induce invasiveness and metastasis (Connolly et al., 2014; Graf et al., 2014; Zhou et al., 2018). However, the molecular mechanisms by which caspases execute/implement these functions without causing apoptosis remains largely elusive (Arama et al., 2020; Julien and Wells, 2017)

Adding an extra layer of complication, caspase activities have been shown to be highly context-dependent with regard to these emerging non-apoptotic functions. As an illustration of this, they have been shown to either promote or inhibit proliferation and differentiation in bone progenitor cells (Hua et al., 2015; Miura et al., 2004; Mogi and Togari, 2003; Szymczyk et al., 2006), and similar opposing functions can be found regarding their regulation of migration and invasiveness in cancer cells (Brentnall et al., 2014; Helfer et al., 2006; Li et al., 2007; Stupack et al., 2006; Teitz et al., 2013). Thus, the outcome of caspase activity can vary in different contexts,

greatly diversifying the contribution of caspases towards tumorigenesis and tumor progression depending on the specific signaling profile and origin of a tumor.

Attempts to functionally understand the relationship between caspase activity and cancer biology open up exciting research opportunities; however, they also indicate a vast knowledge gap highlighting the fact that we do not yet fully understand how non-apoptotic caspase functions can influence tumor properties. The underlying rationale for this thesis is to shed light upon the intricacies of caspase biology in tumors and uncover the origins of the pleiotropic effects of caspases in different tumor types.

Addressing these biological questions is relevant not only from a perspective of gaining a greater understanding of basic biology but also from a clinical perspective. It is important to recognize that therapeutic strategies involving caspase activity is not as straightforward as inducing apoptosis. It is entirely possible that therapeutic strategies involving activation of caspases could backfire, and instead promote tumorigenesis and malignancy (Zhao et al., 2018). For example, while radiotherapy is able to induce massive amounts of cell death in tumors due to apoptosis, non-apoptotic caspase-3 activity in dying cells has been found to stimulate tumor repopulation from the surviving cells by generating growth signals from the dying cells (Yang and Novack, 2013); accordingly, higher caspase-3 activity in human cancer patients is correlated with greater risks of relapse and deaths following radio- or chemotherapy (Yang and Novack, 2013). This process, dubbed “Phoenix Rising,” is akin to a phenomenon known as apoptosis-induced compensatory proliferation

(Diwanji and Bergmann, 2018), which will be discussed in more detail in a later section.

Additionally, capsase-8 has been shown to both promote and inhibit metastasis in different cancer types (Stupack et al., 2006; Teitz et al., 2013; Torres et al., 2010). Thus, unless the context of the tumor being treated is understood, therapies involving either inhibition or activation of caspases could run the risk of increases the chances of metastasis, the deadliest stage of the disease (Lambert et al., 2017).

Therefore, it is essential to understand the tumorigenic context in which caspases operate, as we may gain the insights necessary to ensure that potential therapeutic strategies do not worsen a prognosis instead of alleviating it.

Drosophila as a model to study non-apoptotic functions of caspases in cancer

Drosophila melanogaster has proven an invaluable model through which to study both non-apoptotic caspase and cancer biology (Kumar and Doumanis, 2000; Mirzoyan et al., 2019; Nakajima and Kuranaga, 2017; Richardson and Kumar, 2002). To begin, flies are inexpensive and easy to maintain in the laboratory; combined with their simpler biology and relatively quick life cycles, they make convenient and practical models (Tolwinski, 2017). Additionally, powerful and sophisticated genetic tools are readily available, allowing for the easy and simple manipulation of gene expression with a high degree of spatiotemporal control (del Valle Rodriguez et al.,

2011). Some of the most commonly used workhorses in *Drosophila* research are the binary gene expression systems: GAL4/*UAS*, LexA/*lexAop*, and QF/*QUAS* (del Valle Rodriguez et al., 2011). Consisting of a transcriptional activator (e.g. GAL4) and a transgene under the control of a corresponding promoter sequence (e.g. *UAS*), these systems can be used to drive gene expression in a cell- and tissue-specific manner; combined with a molecule that can repress these systems (e.g. GAL80), temporal control can also be achieved (McGuire et al., 2003). Finally, due to a high degree of conservation between fly and human genes and signaling pathways (Bier, 2005; Ugur et al., 2016), discoveries made in *Drosophila* have been shown to be highly relevant for understanding human cancer (Elsum et al., 2012; Gonzalez, 2013). Thus, in this thesis I use a recently described model of tumorigenesis in *Drosophila* that is relevant for human cancers as a method to elucidate mechanisms in caspase and cancer biology.

In the following sections of this introduction, I aim to provide a brief overview of the caspase family of proteins and non-apoptotic caspase functions that are directly relevant for tumorigenesis in *Drosophila*. Finally, I will briefly summarize and outline the remainder of the thesis.

Caspase regulation in apoptotic pathways

Named for their specific proteolytic activity, **caspases** are an evolutionarily conserved family of intracellular **c**ysteine-**asp**artic prote**ases** (Alnemri et al., 1996; Lamkanfi et al., 2002). In humans and higher order organisms (mostly vertebrates),

distinct caspases were also renamed according the order in which they were identified (e.g., the first human caspase described, interleukin-1β converting enzyme [ICE], was renamed caspase-1) (Alnemri et al., 1996; Eckhart et al., 2008). Including an erroneously named caspase-13, which in reality is the bovine orthologue of caspase-4 (Koenig et al., 2001), and murine caspase-11, which is orthologous to human caspase-4 and -5 (Lamkanfi et al., 2002; Martinon and Tschopp, 2004), there are currently 18 numbered caspases (Eckhart et al., 2008; Shalini et al., 2015). 13 of these caspases can be found in humans: caspase-1 through -10, -12, -14, and -16 (Crawford and Wells, 2011; Eckhart et al., 2008; Shalini et al., 2015). Reflective of their simpler biology, there are seven caspases in *Drosophila*: Dronc, Dredd, Strica, Drice, Dcp-1, Damm, and Decay (Kumar and Doumanis, 2000).

As some caspases were originally identified for their pro-inflammatory functions (e.g. caspase-1 processing interleukin-1β), caspases have historically been classified as apoptotic (caspase-2, -3, -6, -7, -8, -9, -10) or inflammatory (caspase-1 -4, -5, -11, -12), based upon their functions (Figure 1.1) (Lamkanfi et al., 2002; Li and Yuan, 2008; McIlwain et al., 2013; Shalini et al., 2015). However, this classification is an oversimplification and problematic as several caspases can participate in both processes (McIlwain et al., 2013), and emerging evidence has revealed a multitude of other non-apoptotic and non-inflammatory functions, which I discussed previously. Additionally, since dedicated “inflammatory” caspases seem restricted to vertebrates (Courtiade et al., 2011; Martinon and Tschopp, 2004), it can be more informative to differentiate between caspases using different criteria.

All caspases are synthesized as zymogens that require catalytic processing for activation (Khan and James, 1998). This processing involves cleavage of caspases into short and long subunits that can then interact with each other to form active catalytic pockets (Kumar, 2007). Structural differences between caspases influence the mechanisms and the general order of activation, and based on this, caspases can be divided into two groups: initiator and effector caspases (Figure 1.1).

Initiator caspases (caspases-1, -2, -4, -5, -8, -9, -10, -11, and -12 in humans; Dronc, Dredd, and Strica in flies) have long pro-domains that contain elements for protein-protein interaction (Figure 1.1) (Degterev et al., 2003; Denton et al., 2013; Fuentes-Prior and Salvesen, 2004). These recruitment domains, such as the caspase recruitment domain (CARD) or death effector domains (DEDs), bind with adaptor proteins to create multimeric signaling complexes that greatly increase the efficiency of autocatalytic activation (Degterev et al., 2003; Fuentes-Prior and Salvesen, 2004). Of the fly caspases, Dronc, which is orthologous to caspase-2 and -9 in mammals (Dorstyn and Kumar, 2008), contains a CARD domain and is the primary apoptotic initiator caspase in *Drosophila* (Denton et al., 2013). Dredd, while homologous to the apoptotic initiator caspase-8 containing two DED domains, is not required for apoptosis in *Drosophila* and is instead essential for innate immunity (Leulier et al., 2000; Meinander et al., 2012). Interestingly, the *Drosophila* caspase Strica has an unusual serine/threonine-rich pro-domain of unknown function and appears to be redundant with Dronc in a few context-dependent situations, such as the

programmed cell death in oogenesis and in a specific group of interneurons during metamorphosis (Baum et al., 2007; Doumanis et al., 2001; Lee et al., 2011a; Leulier et al., 2006).

Effector caspases (caspases-3, -6, -7, and -14 in humans; Drice, Dcp-1, Damn, and Decay in flies) have shorter pro-domains without recruiting domains (Figure 1.1), and typically rely on the initiator caspases for processing and activation (Degterev et al., 2003; Denton et al., 2013; Fuentes-Prior and Salvesen, 2004; Riedl and Shi, 2004). Thus, they are generally understood to be downstream of initiator caspases and are the enzymes primarily responsible for the rampant proteolysis observed during apoptosis, with the largest known number of substrates (Crawford and Wells, 2011; Julien and Wells, 2017). However, effector caspases are also known to sometimes be upstream of initiator caspases (Fuchs and Steller, 2011). Of the effector caspases, Drice and Dcp-1 are primarily responsible for executing apoptosis in *Drosophila* and are orthologous to caspase-3 and -7 (Xu et al., 2006). While the two are redundant in some instances, evidence suggests that Drice is the more essential caspase of the two for apoptosis, and Damm and Decay appear to be dispensable for most forms of programmed cell death (Denton et al., 2013).

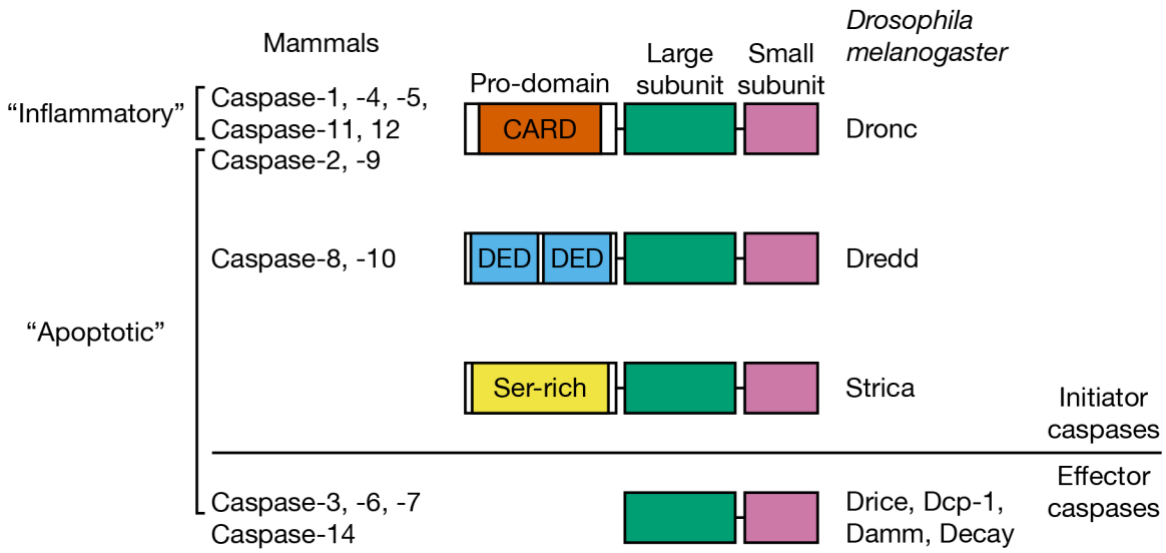


Figure 1.1 **Classical functional and structural classification of caspases.** Simplified diagram indicating the general domain structures of the caspases and types of classification. Functionally, most caspases can be classified as inflammatory (caspases-1, -4, -5, -11, -12) or apoptotic (caspases-2, -3, -6, -7, -8, -9, 10). Structurally, caspases can be divided into pro-domain containing initiator caspases and effector caspases lacking a pro-domain. All caspases can be cleaved into large and short subunits. CARD: caspase recruitment domain; DED: death effector domain; Ser-rich: Serine/threonine-rich domain.

As a result of the immense interest in studying regulated cell death and apoptosis (Budihardjo et al., 1999; Earnshaw et al., 1999; Galluzzi et al., 2018), we now understand a great deal about how caspases' proteolytic activity is tightly controlled and regulated, ensuring that they are only active when needed and not rampantly cleaving over 1300 substrates (Crawford et al., 2013; Galluzzi et al., 2018; Nagata, 2018; Tang et al., 2019). In the absence of apoptotic stimuli, caspase activity can be kept in check through inhibitory post-translational modifications such as ubiquitination and phosphorylation (Kamber Kaya et al., 2017; Kuranaga, 2012; Kurokawa and Kornbluth, 2009). Additionally, interactions with modulatory elements known as inhibitors of apoptosis proteins (IAPs) can lead to direct competitive inhibition of

caspases, or other inhibitory post-translational modifications (Kamber Kaya et al., 2017; Kuranaga, 2012; Mace et al., 2010; Parrish et al., 2013).

There are four IAPs in *Drosophila*: Diap-1, Diap-2, dBruce, and Deterin (Orme and Meier, 2009). Of these IAPs, Diap-1 is considered the most important and essential for cell survival, as it can bind directly with Dronc, Drice, and Dcp-1, inhibiting their activity through ubiquitination (Chai et al., 2003; Meier et al., 2000; Wang et al., 1999; Wilson et al., 2002). This ubiquitination can lead to proteasomal degradation; however, Diap-1 mediated ubiquitination has also been shown to lead to non-degradative inhibition (Ditzel et al., 2008; Lee et al., 2011b).

Additionally, the baculovirus protein P35 has been identified as a potent inhibitor of apoptosis in mammalian cells as well as in *Drosophila* (Hay et al., 1994). The mechanism by which P35 inhibits caspase activity is through competitive inhibition; once cleaved, P35 remains irreversibly bound to the active site, preventing further enzymatic activity by the caspase (Goyal, 2001). Its inhibition is highly specific to caspases, though indiscriminate among them, making P35 extremely useful as a pan-caspase inhibitor (Goyal, 2001; Zhou et al., 1998). Interestingly however, in *Drosophila*, Dronc, Dredd, and Strica have all been shown to be P35 insensitive; thus, only the effector caspases are inhibited by P35 in *Drosophila* (Doumanis et al., 2001; Hawkins et al., 2000; Hay et al., 1994; Kim et al., 2014; Meier et al., 2000).

Though activation of apoptosis can occur through many mechanisms, there are two main pathways: an extrinsic and an intrinsic pathway (Elmore, 2007). The extrinsic pathway generally relies on extracellular stimuli activating death receptors, while the intrinsic pathway generally originates with the mitochondria (Elmore, 2007). Indeed, one of the most potent activators of the mammalian apoptotic pathway is the event of mitochondrial outer membrane permeabilization (MOMP), which is generally considered a “point of no return” in regard to cell death (Chipuk et al., 2006; Kalkavan and Green, 2018). Once triggered by apoptotic stimuli, MOMP results in the diffusion of molecules normally confined between the inner and outer mitochondrial membranes (Chipuk et al., 2006; Kalkavan and Green, 2018). One of these is the co-factor protein cytochrome-c (Liu et al., 1996; Wang and Youle, 2009). Along with the adaptor protein apoptotic protease activating factor-1 (Apaf-1), cytochrome-c forms the apoptosome, a wheel-shaped multimeric signaling complex that mediates intracellularly driven apoptosis (Zou et al., 1997). Through CARD domains in Apaf-1, the apoptosome recruits caspase-9, initiating and amplifying the caspase cascade (D'Arcy, 2019).

In *Drosophila*, however, the existence of MOMP and a mitochondrial-dependent apoptotic pathway is controversial, and cytochrome-c appears to have no crucial role in initiating apoptosis, as this co-factor is not needed for apoptosome formation (Bender et al., 2012; Clavier et al., 2016). Instead, the pro-apoptotic factors Reaper, Hid, Grim, and Sickle are the primary inducers of apoptosis in *Drosophila* (Denton et al., 2013). These proteins, also known as IAP-antagonists, largely function by

promoting the ubiquitination and degradation of Diap-1 (Yoo et al., 2002). Once freed from their negative regulation, Dronc is able to form the *Drosophila* apoptosome through CARD domain interactions with the *Drosophila* Apaf-1 orthologue, Dark (Yu et al., 2006; Yuan et al., 2011). The effector caspases Drice and Dcp-1 are then subsequent cleaved and activated to enact apoptosis (Xu et al., 2009).

While extensive and more comprehensive reviews of caspase regulation can be found elsewhere (D'Arcy, 2019; Julien and Wells, 2017; Nagata, 2018; Parrish et al., 2013; Riedl and Shi, 2004; Tang et al., 2019), a basic overview of the apoptotic program and caspase cascade can be found below (Figure 1.2). Crucially, apoptotic stimuli, either internal or external, induce initiator caspase activation, followed by cleavage and activation of effector caspase activation. In *Drosophila*, Dronc, Drice, and Dark make up the core apoptotic machinery (Leulier et al., 2006). This generally linear scheme is remarkably conserved across species (Nakajima and Kuranaga, 2017) (Figure 1.2), culminating with apoptosis. However, as mentioned before, it is now well known that apoptosis is not the only outcome available from caspase activation, and the study of non-cell-death related caspase functions is a rapidly expanding field (Arama et al., 2020).

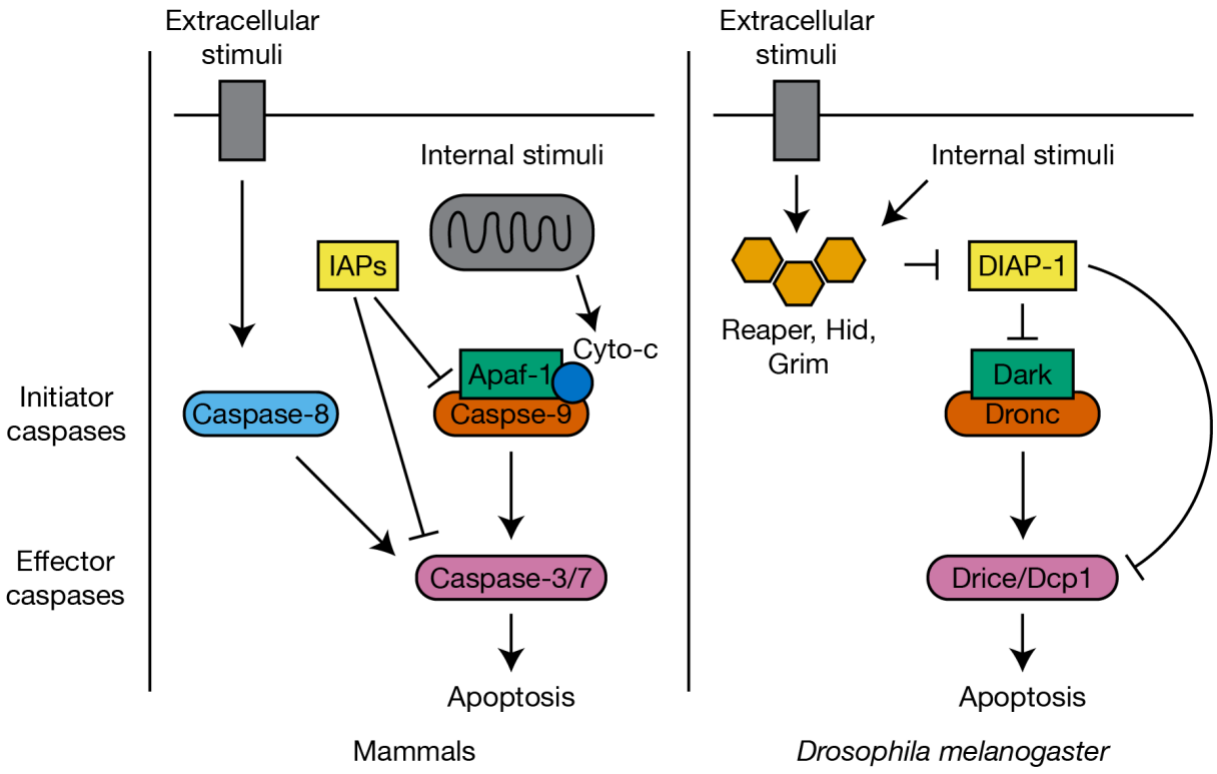


Figure 1.2 **Conservation of the apoptotic pathway in mammals and *Drosophila*.** Diagram illustrating the conservation across species of the core apoptotic pathway in mammals and *Drosophila melanogaster*. In general, apoptotic stimuli (internal or external) stimulate the activity of upstream initiator caspases, which go on to cleave and activate effector caspases. Proteins sharing homology are depicted with same shapes and color schemes. IAPs – inhibitors of apoptosis proteins; Cyto-c – cytochrome-c.

Non-apoptotic caspase functions and tumorigenesis in *Drosophila* models

As mentioned previously, *Drosophila* models have proven useful for studying non-apoptotic caspase functions in tumorigenesis (Xu et al., 2018a), in part due to genetic manipulations that can overcome the technical difficulty of isolating transient non-apoptotic activity from apoptotic activity. Taking advantage of the unique effector-caspase only inhibition by P35 in *Drosophila*, researchers have created scenarios where caspase activity can be highly induced through pro-apoptotic factors (such as *hid* or *reaper* expression or irradiation), but apoptosis is prevented through *P35*

expression (Kashio et al., 2014; Martin et al., 2009; Su, 2015). This state of perpetual pro-apoptotic stimuli combined with effector caspase inhibition results in “undead” cells, where apoptosis is initiated but unable to be completed (Kashio et al., 2014; Martin et al., 2009; Su, 2015). Importantly, these genetically engineered undead cells have contributed greatly to our understanding of non-apoptotic caspase activities, as one of the primary benefits of the “undead” state is that the initiator caspase Dronc can now be persistently active without apoptotic consequences (Diwanji and Bergmann, 2018). Thus, apoptotic and certain non-apoptotic functions of Dronc can be distinguished from each other, and these functions have been shown to be particularly relevant to understanding tumorigenesis as well (Diwanji and Bergmann, 2019; Ryoo and Bergmann, 2012).

Tumorigenesis due to mitogenic signaling upon caspase activation

One of the primary non-apoptotic functions of Dronc that was uncovered through the use of “undead” cells was how Dronc influenced a phenomenon known as apoptosis-induced compensatory proliferation (AiP) (Diwanji and Bergmann, 2018; Fogarty and Bergmann, 2017; Perez-Garijo, 2018; Ryoo and Bergmann, 2012). AiP is a mechanism through which damaged cells undergoing apoptosis influence their uninjured neighboring cells through mitogenic signals to increase proliferation and replace the damaged cells (Mollereau et al., 2013). Part of the regenerative process, this mechanism maintains environment tissue homeostasis, and is conserved across a variety of species from Hydra to mice (Diwanji and Bergmann, 2019; Fogarty and Bergmann, 2017; Fuchs and Steller, 2015; Perez-Garijo and Steller, 2015).

Using the undead system in *Drosophila*, it was discovered that Dronc promoted activation of c-Jun-N-terminal kinase (JNK) signaling to produce several mitogens, such as Wingless (Wg), a WNT family member, Decapentaplegic (Dpp), a bone morphogenetic protein (BMP), and Spitz, an epidermal growth factor homologue, which induce proliferation in neighboring cells (Diwanji and Bergmann, 2018). Exactly how Dronc activated the JNK signaling pathway in undead cells was an open question until recently, when it was discovered that Dronc, when localized to the basal membrane, triggered the release of reactive oxygen species (ROS) into the environment (Amcheslavsky et al., 2018; Fogarty et al., 2016). ROS would then attract *Drosophila* macrophage-like cells, known as hemocytes, to the tissue, and stimulate JNK signaling though the release of Eiger, the *Drosophila* tumor necrosis factor (TNF) homologue (Fogarty et al., 2016; Igaki et al., 2002; Perez et al., 2017). As JNK signaling can amplify the apoptotic process by expressing pro-apoptotic factors *reaper* and *hid*, a strong feedback loop is created, amplifying the supply of mitogenic signals (Diwanji and Bergmann, 2019; Shlevkov and Morata, 2012).

In physiological conditions, apoptotic cells and caspase-derived mitogenic signals only transiently affect their neighboring cells, unlike the “undead” cells which stay in the system. Nonetheless, the mechanistic insights gained from the “undead” model are applicable to “genuine” models of AiP and regeneration involving non-apoptotic caspase activity (Fogarty and Bergmann, 2017; Fuchs and Steller, 2015; Huh et al., 2004; Kondo et al., 2006; Perez-Garijo and Steller, 2015). In damaged tissues, JNK

activity is necessary for regeneration, along with other pathways such as Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling (Ahmed-de-Prado et al., 2018; Bergantinos et al., 2010; Fan et al., 2014; Santabarbara-Ruiz et al., 2015). ROS is also involved in inducing JNK signaling in neighboring cells, though in genuine AiP ROS appears to be intracellular (Diwanji and Bergmann, 2019). However, there are a few distinctions between the two models. For one, hemocytes and Eiger-induced JNK activity are not found in genuine models of AiP, and perhaps the largest difference between the two models is that AiP in genuine situations of regeneration eventually stops as tissue homeostasis is restored (Diwanji and Bergmann, 2019). However, as apoptosis is prevented in undead cells, non-apoptotic caspase signaling perpetuates, and the amplification loop of mitogenic signaling is never stopped. This causes tissues to enter a state of constant "regeneration" and non-stop proliferation, resulting in neoplastic overgrowth (Diwanji and Bergmann, 2019).

Caspases and tumor promoting inflammation in *Ras^{V12}/scrib$^{-/-}$* tumors

While the prevention of apoptosis in "undead" cells is non-physiological and artificially induced through expression of an exogenous protein, similarly apoptosis-refractory cells can occur in very real pathological situations, such as cancer (Mohammad et al., 2015; Wong, 2011). While some studies have shown that overexpression of *Ras^{V12}* induces both autonomous and non-autonomous cell death (Karim and Rubin, 1998), the *Ras^{V12}* oncogene has been shown to confer a degree of resistance to apoptosis in *Drosophila* and mammalian cells (Bergmann et al., 1998; Cox and Der, 2003;

Kurada and White, 1998), and many tumor models in *Drosophila* utilize this constitutively active Ras, such as the well-known model of cooperative oncogenesis, $Ras^{V12}/scrib^{-/-}$ (Mirzoyan et al., 2019; Richardson and Portela, 2018; Rudrapatna et al., 2012). In these over-proliferative and highly invasive tumors (Brumby and Richardson, 2003; Pagliarini and Xu, 2003), both Dronc and effector caspases Drice and Dcp-1 have been shown to be necessary for tumor growth (Perez et al., 2017). Specifically, non-apoptotic caspase activity is required for the production of ROS and the attraction of hemocytes to fuel JNK activity through Eiger release, further stimulating caspases in an amplifying loop that promotes tumor growth (Perez et al., 2017). In this way, the mitogenic and JNK-dependent mechanisms of caspase-dependent overgrowth in $Ras^{V12}/scrib^{-/-}$ tumor model resembles those found in "undead" cells and indicates how tumor promoting inflammation can occur as a result of non-apoptotic caspase activity. Importantly, both these models highlight how many aspects of tumorigenesis reflect an exploitation of the wound-healing process, given the underlying regenerative nature of apoptosis-induced compensatory proliferation (Diwanji and Bergmann, 2019). Interestingly, they also indicate how deregulated caspase activity can play a crucial role in facilitating the switch between physiological regeneration and oncogenic transformation into a wound-like tumor.

Non-apoptotic caspase activity promotes invasiveness through JNK signaling

"Undead" cells have also been useful in uncovering other non-apoptotic functions of caspases that could contribute to malignancy in tumors, such as metastasis (Rudrapatna et al., 2013). Researchers showed that sub-lethal activity of Dronc and

Drice promoted the invasiveness and migration of undead cells into neighboring wild-type tissue (Rudrapatna et al., 2013). This migration was found to be dependent upon JNK activity downstream of caspases in undead cells, leading to the production of matrix metalloproteases (MMPs) which are known to degrade the basement membrane during invasion (Hua et al., 2011). While undead cells are not known to metastasize and invade distant tissues, this study does indicate their ability to migrate, which would be required for metastatic invasion (Diwanji and Bergmann, 2019; Rudrapatna et al., 2013). Just as how caspases in *Ras^{V12}/scrib$^{-/-}$* tumors were shown to promote tumor growth through mechanisms similar to those uncovered in undead cells, it is possible that caspases in *Ras^{V12}/scrib$^{-/-}$* tumors could also enable migration in a similar manner to their undead counterparts. *Ras^{V12}/scrib$^{-/-}$* tumor cells are known to be highly invasive (Wu et al., 2010). Supporting this, mammalian caspases have also been shown to promote migration and metastasis in several models of cancer types (Connolly et al., 2014; Graf et al., 2014; Zhou et al., 2018).

Context-dependent caspase activity in different tumorigenic backgrounds

Thus, the “undead” and *Ras^{V12}/scrib$^{-/-}$* models of neoplastic overgrowth in *Drosophila* have helped elucidate many of the mechanisms through which caspases can exert their non-apoptotic functions in tumorigenesis. However, as mentioned previously, it is important to note that caspase activity can be highly context dependent, and the same caspases have been shown to have opposing effects in different circumstances (Stupack et al., 2006; Torres et al., 2010). This is also true in *Drosophila*, and can happen even in the same tissue and cell type; for example, caspase activity induced

by irradiation non-apoptotically inhibits migration in wild-type versions of the exact same cells in *Drosophila* that previously showed caspase-dependent migration when caspase activity was induced by generating “undead” cells (Gorelick-Ashkenazi et al., 2018). Additionally, in contrast to Dronc promoting tumor growth through ROS and JNK activity in $Ras^{V12}/scrib^{-/-}$ models, the lack of Dronc has been shown to facilitate ROS- and JNK-mediated tumorigenesis in irradiated tissues mutant for Ras^{V12}. It is clear that *Drosophila* models of tumorigenesis can provide the variety of different contexts necessary to study and understand the pleiotropic effects of caspase activation and function. Therefore, I shall use *Drosophila* as a model system to better understand the complex relationship between caspases and cancer and shed light on the functional intricacies of caspase activation and tumorigenesis.

Thesis Outline

In this thesis I set out to explore the non-apoptotic functions of caspases in a newly described tumor model of cooperative oncogenesis in *Drosophila*, with the expectation of broadening our understanding of how caspases can affect tumorigenesis in different contexts. In the following chapters, I will describe a recently developed novel caspase-sensor that can be used to detect non-apoptotic caspase activity (Chapter 2). Using this caspase-sensor, I observed that non-apoptotic caspase activity is ubiquitous throughout my tumor model (Chapter 2). I also observed that caspase activity limits tumor growth through means independent of apoptosis (Chapter 2). I determined that the restriction of tumor growth can be attributed to the non-apoptotic limitation of JNK signaling, which is responsible for

features of the tumor similar to regeneration (Chapter 3). Additionally, I show that caspases limit the presence of the immune system on the tumors, and that these immune cells are potentially contributing towards tumor growth (Chapter 4). As I summarize and discuss in Chapter 5, collectively, these results indicate that this new tumor model behaves like a chronic wound, and that caspase activity can work non-apoptotically to modulate the signaling profile responsible for this characteristic. Ultimately, the work in this thesis contributes to the understanding of context-dependent caspase regulation of autonomous and non-autonomous tumor properties with relevance to mammalian systems.

Chapter 2

Non-apoptotic caspase activity in EJS tumors restricts tumor growth

Introduction

As discussed in Chapter 1, the past two decades has seen the emergence of a large set of non-apoptotic functions for caspases (Connolly et al., 2014; Mukherjee and Williams, 2017; Nakajima and Kuranaga, 2017; Su, 2020; Unsain and Barker, 2015). As a result, their deregulation has also been linked to some of the most well-known features of cancer (Baena-Lopez et al., 2018b; McIlwain et al., 2013; Nakajima and Kuranaga, 2017), expanding their canonical association with cancer as tumor-suppressors through apoptosis (Fernald and Kurokawa, 2013; Hanahan and Weinberg, 2000, 2011; Kelly and Strasser, 2011; Olsson and Zhivotovsky, 2011).

However, our current understanding of this emerging field of non-apoptotic functions is far from complete (Arama et al., 2020; Baena-Lopez et al., 2018b; Bell and Megeney, 2017; Julien and Wells, 2017; Xu et al., 2018a), especially with relation to non-apoptotic functions in tumorous scenarios. As discussed in the previous chapter, this incomplete understanding is in part due to the context-dependent nature of caspase activity. Before we can accurately predict how caspases may be contributing to or inhibiting tumorigenesis, a deeper understanding of both caspase and tumor biology is required. *Drosophila* models of cancer have emerged as valid models with which to understand the molecular basis of human cancers and its relation to

caspases (Gonzalez, 2013; Kumar and Doumanis, 2000; Mirzoyan et al., 2019; Richardson and Kumar, 2002; Xu et al., 2018a). However, many of the tumor models in question share a common feature in that they rely on a well-characterized oncogene to induce tumorigenesis, Ras^{V12} (Mirzoyan et al., 2019; Perez et al., 2017; Rudrapatna et al., 2012). A more complete understanding would necessarily entail the study of non-apoptotic activity in a variety of tumor settings. In this chapter, I attempt to contribute towards this knowledge by investigating the presence and function of non-apoptotic caspase activity in a relatively new *Drosophila* model of tumorigenesis.

The EJS tumor model

In order to study the functional diversity of caspase activities in tumors, I decided to utilize a recently developed *Drosophila* model of cooperative oncogenesis (Herranz et al., 2012). This model relies upon the overactivation of the epidermal growth factor receptor (EGFR) pathway as the main “driver” for oncogenic overgrowth, as EGFR is responsible for a host of critical physiological functions, regulating growth, survival, proliferation, and differentiation (Oda et al., 2005). As such, EGFR activating mutations underlie many different human cancers (Normanno et al., 2006). As an RTK, EGFR phosphorylates tyrosine residues upon binding to its ligands and activation, recruiting a host of proteins and initiating a variety of intracellular signaling pathways, such as the Ras-MAPK and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways (Scaltriti and Baselga, 2006). In this regard it is similar to tumor models

utilizing the *Ras^{V12}* oncogene, as EGFR can activate the same Ras-MAPK pathway that Ras^{V12} constitutively activates (Braicu et al., 2019).

In addition to EGFR overexpression, the model relies on removing the activity of SOCS36E, a suppressor of cytokine signaling (SOCS) protein (Herranz et al., 2012). SOCS proteins inhibit both cytokine and growth factor receptor signaling, and are important in regulating proliferation and differentiation, primarily in immunity and inflammation (Duncan et al., 2017; Trengove and Ward, 2013; Yoshimura et al., 2007). SOCS36E has been shown to negatively regulate both EGFR signaling and the Janus kinase/signal transducer of activators of transcription (JAK/STAT) pathway in *Drosophila* (Almudi et al., 2009; Callus and Mathey-Prevot, 2002). Physiologically, the JAK/STAT pathway integrates a variety of extracellular signals, such as cytokines, to regulate a myriad of processes including stem cell maintenance, hematopoiesis, and inflammatory responses (Owen et al., 2019; Thomas et al., 2015). However, constituitively active JAK/STAT signaling has also been linked to the initiation and progression of numerous human cancers (Brooks and Putoczki, 2020; Owen et al., 2019; Thomas et al., 2015), and JAK/STAT activity is strongly upregulated in *Ras^{V12}/scrib$^{-/-}$* tumors (Wu et al., 2010).

The depletion of SOCS36E, a negative regulator of the JAK/STAT signaling, results in hyperactivation of the pathway. It is this cooperation between overactive EGFR and JAK/STAT which forms the basis for the tumor model. The overactivation is achieved using the Gal4/*UAS* system to overexpress both EGFR and *SOCS36E*-

RNAi (Herranz et al., 2012). Once induced in epithelial cells, the resulting overgrowth is characterized by epithelial disorganization and loss of apicobasal polarity, similar to *Ras^{V12}/scrib$^{-/-}$* characteristics (Herranz et al., 2012). Because this model relies upon the oncogenic cooperation of both the **E**GFR and **J**AK/**S**TAT pathways, hereafter I will refer to this as the **EJS** model.

I decided to use the EJS tumor model for two reasons. Firstly, the EJS tumor model seemed particularly attractive as it has the possibility of providing a favorable background for studying non-apoptotic functions of caspases. This is because the combination of EGFR and JAK/STAT signaling can potentially result in an environment that is able to buffer caspase activation and the apoptotic program through the provision of pro-survival cues. Like Ras^{V12}, EGFR signaling is known to negatively regulate pro-apoptotic signals in *Drosophila* such as the upstream pro-apoptotic factor Hid, and the JAK/STAT pathway can lead to expression of anti-apoptotic factors such as Diap-1 and Bcl-2 (Bergmann et al., 1998; Betz et al., 2008; Fujio et al., 1997; Recasens-Alvarez et al., 2017). Keeping caspase activity levels below the threshold necessary for apoptosis has been suggested as a method for regulating caspases to non-apoptotic functions in both *Drosophila* and mammals (Florentin and Arama, 2012; Gibon et al., 2016). Thus, the negative regulation of caspases through these two pathways has the potential to be able to keep caspase activity at a sub-lethal level in EJS tumor cells, setting the stage for non-apoptotic caspase activity. In this way, the EJS system has the potential to create an apoptosis-refractory environment.

Additionally, the EJS tumor model has strong relevance to human cancers, as the concurrent upregulation of EGFR and the downregulation of SOCS5, the closest mammalian homolog to SOCS36E, has been shown to increase the tumorigenic potential of transformed primary human BJ fibroblast cells beyond that of EGFR/Ras activation alone (Herranz et al., 2012). Additionally, the oncogenic synergy between the EGFR and JAK/STAT pathways has been shown to be important in many other types of cancers, sustaining tumor growth and malignant transformation (Andl et al., 2004; Quesnelle et al., 2007; Sun et al., 2016). More importantly, the chemical inhibition of both signaling pathways can reduce both the invasiveness and proliferation of tumors, including skin and ovarian carcinomas (Bito et al., 2011; Wen et al., 2015).

Results

Induction of EJS tumors in *Drosophila* wing discs

As described previously, I was able to use the Gal4/*UAS* system and a ubiquitously expressed temperature sensitive Gal80 protein ($Gal80^{ts}$) to achieve both spatial and temporal control of EJS induction in *Drosophila* (Herranz et al., 2012). I induced EJS specifically in the dorsal compartment cells of the imaginal wing disc in larvae; these cells eventually form the dorsal side of the wing blade in adult flies. I achieved this spatial control using the *apterous-Gal4* driver (*ap-Gal4*), as *apterous* is only expressed in the dorsal compartment (Blair et al., 1994; Diaz-Benjumea and Cohen, 1993). EJS was induced through the overexpression of EGFR and *SOCS36E-RNAi*

(Figure 2.1), and Gal80^{ts}, which inhibits the Gal4 transcription factor at 18ºC, ensured that EJS induction only occurred when larvae were shifted to 29ºC, the permissive temperature. This allowed larvae to develop physiologically before being subjected to tumor growth. In order to establish a reproducible scheme of tumor induction for experiments, I allowed EJS inducible larvae to develop normally for nine days at 18ºC before shifting them to 29ºC. To track growth over time, EJS tumors were allowed to grow over periods of time ranging from one to five days before dissection (Figure 2.1).

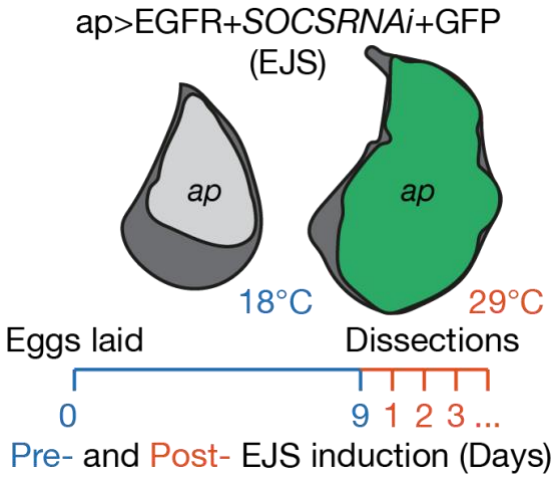


Figure 2.1 **Induction of EJS in *Drosophila* imaginal wing discs.**
Schematic showing the experimental design for inducing tumors based on EGFR and JAK/STAT (EJS) upregulation in imaginal wing discs. The Gal4/*UAS* binary expression system is used to specifically express *UAS-GFP*, *UAS-EGFR*, and *UAS-SOCS36E-RNAi* transgenes in dorsal cells of the wing discs using *apterous-Gal4* (*ap*, light gray in 18 ºC, green in 29ºC). Ubiquitously expressed Gal80^{ts} (*Tubulin-Gal80^{ts}*) prevents Gal4 activity and transcription of transgenes at 18ºC. At 29ºC, Gal80^{ts} is inactivated and EJS induction occurs along with GFP expression. Larvae were kept at 18ºC for 9 days before being shifted to 29ºC to induce tumor formation (blue timeline). Tumor induction occurred for a period of 1 to 5 days, and dissections were carried out at various time points (orange timeline).

Caspase Activity detected in EJS tumors using the Drice-based sensor (DBS-S-QF)

To determine if there is non-apoptotic caspase activity in EJS tumors, I utilized a sensitive tool that was previously developed in the lab to detect caspase activity, the

Drice-based sensor (DBS-S-QF) (Baena-Lopez et al., 2018a). This transgenic reporter works by taking advantage of the physiological processing steps of Drice, a downstream effector caspase in *Drosophila*. Drice is normally cleaved by initiator caspases into two subunits in order to activate its own enzymatic ability (Lannan et al., 2007); the DBS-S-QF sensor is a truncated and membrane-bound version of the Drice protein that replaces one subunit with a QF transcription factor (Baena-Lopez et al., 2018a). Similar to Gal4 in the Gal4/*UAS* system, QF is the transcriptional activator in a parallel binary expression system used in *Drosophila*, QF/*QUAS* (Riabinina and Potter, 2016). DBS-S-QF remains cleavable by initiator caspases, thus, once cleaved by initiator caspases, QF is freed from the membrane and able to transcriptionally activate its target *QUAS* transgenes. It is also ubiquitously expressed under the regulation of an actin promoter (Baena-Lopez et al., 2018a). By pairing the ubiquitously expressed DBS-S-QF tool with transgenes under the regulation of the *QUAS* promoter, initiator caspase activity in any cell can be detected through the caspase-dependent release of QF and subsequent transcription of detectable *QUAS* transgenes to report that activity, such as an HA-tagged Tomato fluorescent protein (*QUAS-Tomato-HA*) (Figure 2.2 A). As Dronc is the primary initiator caspase in *Drosophila*, the reporter mostly detects the physiological activity of Dronc, and indeed, in *Dronc*-deficient conditions, basal activation of the DBS-S-QF reporter is eliminated in most cellular contexts (Baena-Lopez et al., 2018a).

As the QF transcription factor can be used to express any gene of interest, additional *QUAS* transgenes can be added in combination with the DBS-S-QF tool to add

additional layers of information about caspase activity. By incorporating additional *QUAS-Flippase* (FLP) and *actin-FRT-stop-FRT-lacZ-NLS* transgenes into the genetic background, caspase activity and the release of QF from the DBS-S-QF results not only in expression of Tomato-HA, but also of the Flippase protein (Figure 2.2 A). Through FLP-FRT recombination, the Flippase excises a stop cassette placed between an *actin* promoter and a *lacZ* gene with a nuclear localization signal (NLS), thereby inducing permanent expression of a nuclear β-galactosidase (β-gal) (Figure 2.2 A). Thus, even when caspase activity ceases and cells eventually turn over the QF necessary to express QUAS transgenes, cells that had activated caspases in the past can still be identified due to the presence of β-gal, even when Tomato-HA has been lost completely (Figure 2.2 B).

Through use of the DBS-S-QF reporter, cells with expression of Tomato-HA can be identified as cells that have recently experienced initiator caspase activity (Figure 2.2 B). Additionally, cells exhibiting expression of β-gal without the expression of Tomato-HA can be identified as cells that underwent a period of caspase activity in the past and subsequently survived to end that activation (Figure 2.2 B). As β-gal expression is permanently induced after caspase activity, cells with detectable β-gal could also be the progeny of caspase-activating cells that survived and proliferated (Figure 2.2 B). Thus, the presence of β-gal without Tomato-HA to indicate ongoing caspase activity is an unambiguous report that a cell has either undergone non-apoptotic caspase activity in the past or has descended from such a cell.

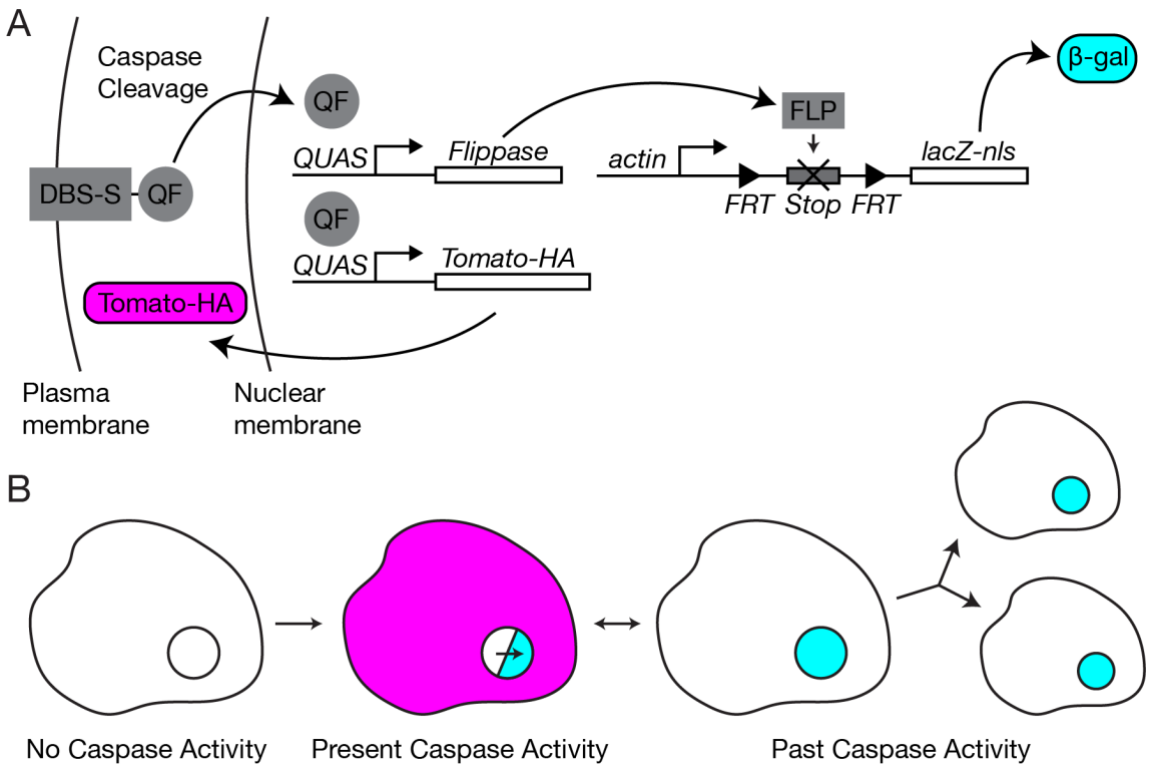


Figure 2.2 **Tracking caspase activity using DBS-S-QF sensor.**
(A) Schematic illustrating the DBS-S-QF sensor system, which consists of the membrane-bound DBS-S-QF, *QUAS-Tomato-HA*, *QUAS-Flippase*, and *actin-FRT-Stop-FRT-lacZ-nls* transgenes. Upon cleavage by an initiator caspase, QF (gray circle) translocates to the nucleus, transiently driving the expression of Tomato-HA (magenta) and Flippase while DBS-S-QF cleavage continues to occur. Flippase (FLP) excises the *Stop* cassette through FLP-FRT recombination, leaving to permanent expression of nuclear localized β-gal (cyan).
(B) Schematic depicting a cell with no caspase activity and no detectable markers. Once caspases are activated, expression of Tomato-HA can be visualized throughout the cell (magenta). β-gal can also be detected in the nucleus, following a slight delay to allow for the secondary step of FLP-FRT recombination (cyan and white). If caspase activity stops without cell death, Tomato-HA eventually turns over, leaving only permanently expressed β-gal in the nucleus (cyan). It is possible cells can go back and forth between these two states, and daughter cells will also be detectable.

When the DBS-S-QF reporter is expressed in wild-type conditions, I observed that a modest fraction of the cells in the wing disc can be labeled with either the marker of transient caspase activity (Tomato-HA), or with the marker of past, non-apoptotic caspase activity (β-gal) (Figure 2.3). This indicates that while caspase activity is present in physiological conditions in *Drosophila* wing discs, the amount of caspase activity is relatively low. This is supported by previous observations in the lab (Baena-

Lopez et al., 2018a), as well as through the use of a separate caspase-sensing tool developed by a different research group (Ding et al., 2016).

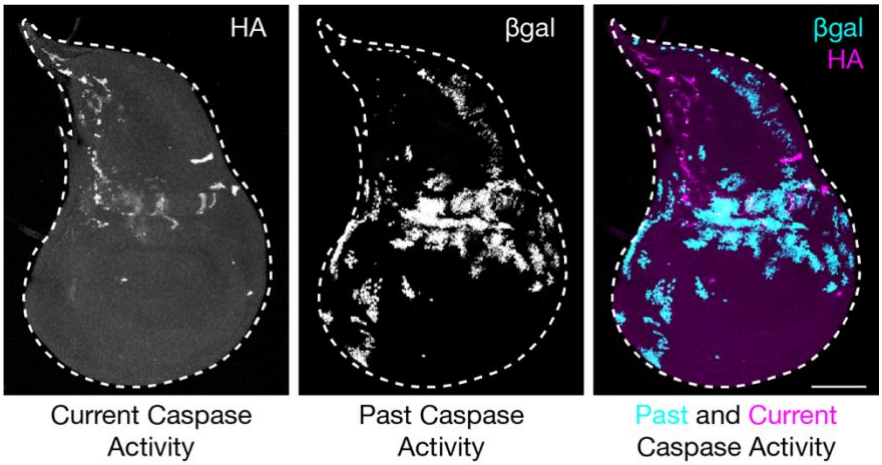


Figure 2.3 **Lineage tracing of caspase-activating cells in wild-type wing discs**. Using the DBS-S-QF caspase sensor, past and present caspase activity can be tracked in tissue. Cells currently experiencing caspase activity express a *QUAS-mTdTomato-HA* transgene and are labeled with anti-HA (gray and magenta). Cells that experienced past caspase activity or descended from such cells permanently express LacZ, and are labeled with anti-β-gal (gray and cyan). Representative maximum projected confocal images of third instar wing discs are shown. The entire wing disc is outlined (white dashes) referencing a DAPI stain (not shown). Scale bar: 150 μm.

Detection of Widespread Non-apoptotic Caspase Activity in EJS Tumors

By combining the DBS-S-QF reporter with the genetic background to induce EJS tumor in wing discs, I aimed to determine if there was any non-apoptotic caspase activity in EJS tumors. After allowing EJS induction for three days, I observed that a large proportion of cells within the tumor showed signs of ongoing caspase activity through the expression of Tomato-HA (Figure 2.4 A). Additionally, and most strikingly, I observed that virtually 100% of EJS tumor cells showed expression of β-gal, indicating that almost all cells in the tumor had either activated caspases in the past and survived, or had descended from such cells (Figure 2.4A). By comparison, only a subset of the residual wild-type cells in the ventral region of the wing disc

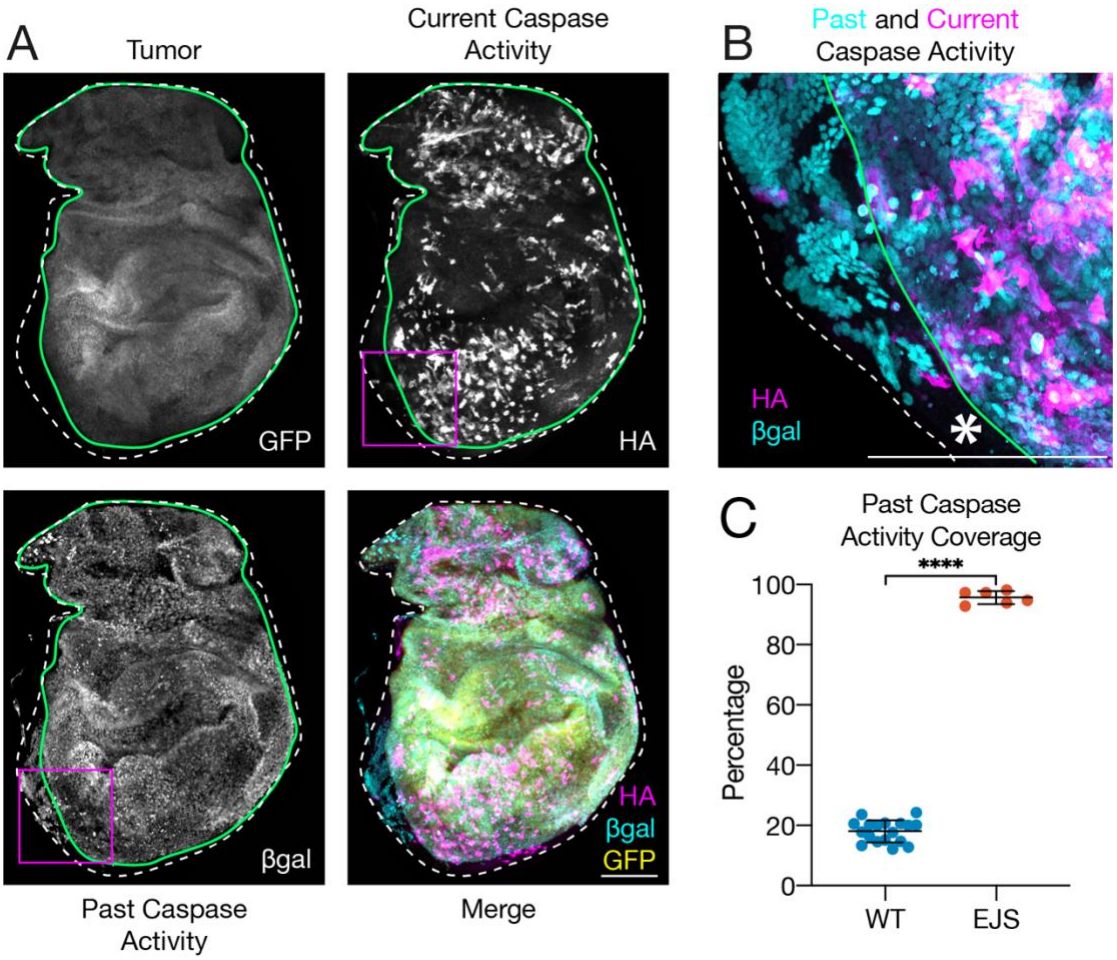


Figure 2.4 **Lineage tracing of caspase-activating cells in EJS tumors.**

(A) As in Figure 2.3, the DBS-S-QF sensor is used to track both past and present caspase activity in EJS tumors. Representative maximum projected confocal images showing EJS tumors after 3 days of EJS induction. EJS tumor cells are labeled with GFP (gray and yellow). Cells with current caspase activity (gray and magenta) and cells with past caspase activity (gray and cyan) are also labeled. The entire wing disc is outlined (white dashes) using a DAPI stain as a reference (not shown). Scale bar: 100 μm.

(B) Representative maximum projected confocal image at higher resolution of the magenta outline in (A). This image indicates the overlap between cells with current caspase activity (magenta) and cells with past caspase activity (cyan). Note also the presence of cells with only the marker of past caspase activity (cyan), as well as the small fraction of cells expressing neither marker (asterisk). Scale bar: 100 μm.

(C) Quantification of the area of wing discs composed of cells expressing the marker for past caspase activity as a percentage of total wing disc area in wild-type wing discs (see Figure 2.3) and EJS tumors. Mean ±SD are plotted. Statistical significance was determined using an unpaired Student's t-test, **** $p < 0.0001$. Numbers of wing discs analyzed for wild-type discs: 18; for EJS tumors: 6.

exhibits β-gal expression, and a number of cells show no signs of either marker of caspase activity (Figure 2.4 B). Additionally, when compared to wild-type discs as a whole, tumorous wing discs exhibit a much larger proportion of cells that can be detected by anti-β-gal (Figure 2.4 C). Taken together, this data indicates that EJS induction results in a large amount of caspase activity in EJS tumors, much of which appears to be non-apoptotic, given the almost universal presence of the permanent marker of past caspase activity. Additionally, the caspase activity detected through the DBS sensor in EJS tumors is specific to Dronc activity, as preliminary data indicates that downregulation of Dronc greatly reduces the number of cells labeled with transient caspase activity (data not shown).

However, the existence of non-apoptotic caspase activity does not preclude the presence of conventional apoptotic activity. To determine if any of the caspase activity detected in EJS tumors corresponded to apoptosis, I used a TUNEL assay, which detects fragmented DNA representative of apoptosis, to identify dying cells. TUNEL staining revealed that a small fraction of cells in EJS tumors were dying from apoptosis, both inside and outside the tumor (Figure 2.5). This suggests that apoptosis does indeed occur within in EJS tumors. The presence of TUNEL positive cells outside the tumor also suggests that cell death can be either induced non-cell autonomously, or that dying cells are able to delaminate and be expelled from the tumor through the normal apoptotic process.

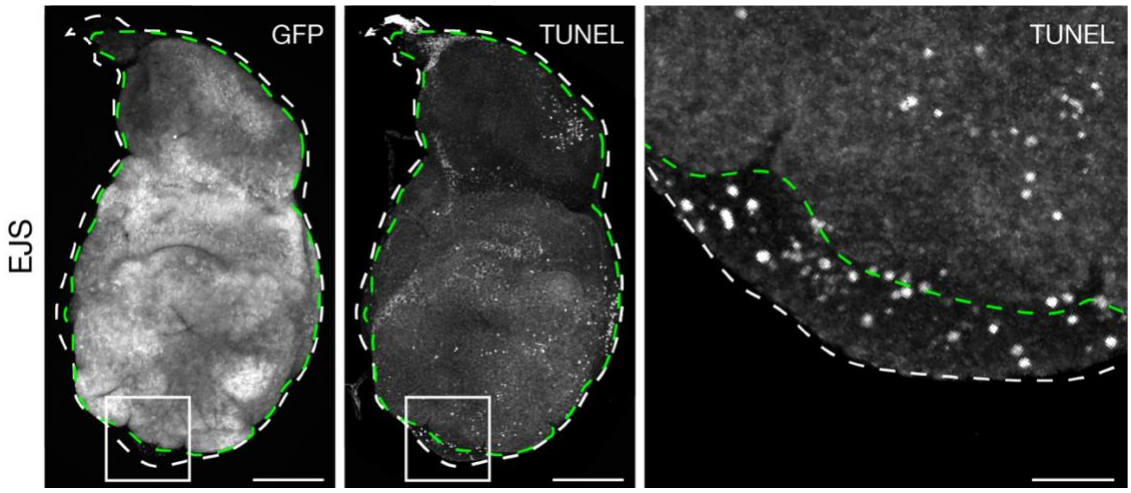


Figure 2.5 **TUNEL staining in EJS tumors reveals small amount of apoptosis.** Representative maximum projected confocal images of EJS tumors (GFP) after three days of EJS induction stained with TUNEL to detect apoptotic cells. A 5x magnification of the area outlined by a white box is shown at the right. The tumor is outlined (dashed green) and the entire wing disc is outlined (dashed white) referencing a DAPI stain (not shown). A modest number of dying cells can be labeled (white puncta), particularly outside the tumor boundary. Scale bars: 100 μm and 20 μm in higher magnification.

Non-apoptotic Caspase Activity Restricts EJS Tumor Growth

As the DBS-S-QF sensor primarily detects activity of apical caspases, I decided to investigate if there was any functional relevance of Dronc in EJS tumors, as Dronc is the primary non-immune system related apical caspase in *Drosophila* (Denton et al., 2013).

I first overexpressed a well-characterized *Dronc-RNAi* transgene to knockdown *Dronc* (Leulier et al., 2006). Initially, *Dronc*-deficient EJS tumors displayed similar outcomes as control EJS tumors (Figure 2.6 A), as before two days of EJS induction and *Dronc* depletion, *Dronc*-deficient tumors remained similar in size to control tumors (Figure 2.6 A). However, after two days had passed, *Dronc*-deficient tumors were noticeably larger than their control counterparts. On the third day, *Dronc*-deficient EJS tumors were consistently about 1.5 times the size of control EJS tumors

(Figure 2.6 B), and capable of enlarging to almost quadruple the size of control EJS tumors by the fifth day when measured by area after being mounted on a microscopy slide (Figure 2.6 A, C).

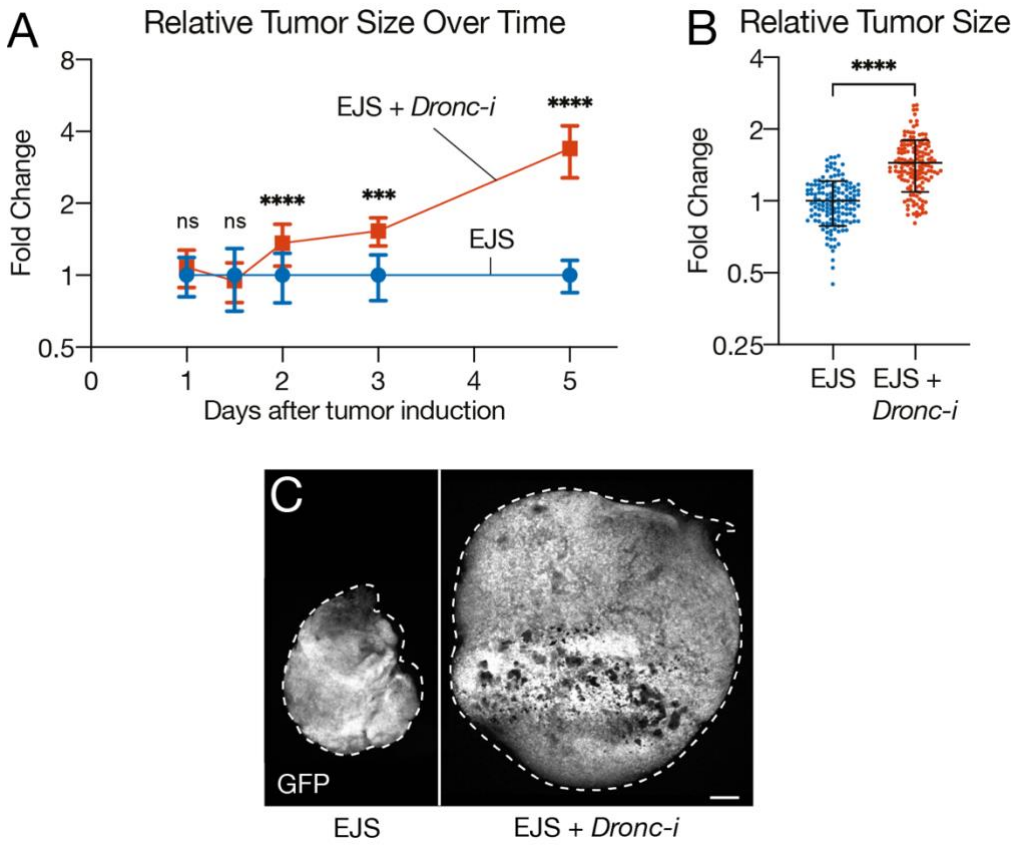


Figure 2.6 **Downregulation of Dronc increases EJS tumor growth.**
(A) Relative sizes of EJS tumors (blue) and *Dronc*-deficient EJS tumors (EJS + *Dronc-i*, orange) over time. Dronc was downregulated through overexpression of a *Dronc-RNAi* transgene. Plotted are the mean ±SD of tumor sizes relative to control EJS tumors at each time point, as measured by area. Control tumors were used for size normalization at each time point. Notice the differences in EJS and EJS + *Dronc-i* tumor sizes two days after EJS induction. One-way ANOVA with Tukey's multiple comparisons tests was used to determine the statistical significance at each time point; ns – not significant $p > 0.05$; *** $p = 0.001$, **** $p < 0.0001$. Numbers of wing discs analyzed for each time point are as follows (**Day**: n[EJS]; n[EJS + *Dronc-i*]): (**1**: 39; 36), (**1.5**: 21; 38), (**2**: 29; 35), (**3**: 18; 7), (**5**: 12; 12).
(B) Quantification of the relative sizes of control EJS and EJS + *Dronc-i* tumors after three days of EJS induction. Control EJS tumors were used for normalization. Statistics were determined by using an unpaired Student's t-test; **** $p < 0.0001$. Numbers of wing discs analyzed for EJS tumors: 145; for EJS + Dronc-i tumors: 154.
(C) Representative confocal images of control EJS and EJS + *Dronc-i* tumors after five days of EJS induction (GFP, gray). The entire wing disc is outlined (dashed white) referencing a DAPI stain (not shown). Scale bar: 100 μm.

To confirm the specificity of these results obtained by RNAi targeting *Dronc* expression, I also employed amorphic and conditional knock-out alleles of *Dronc* (*Dronc*^{KO} and *Dronc*$^{KO\text{-}FRT\ Dronc\text{-}GFP\text{-}Apex\ FRT\text{-}QF}$) previously generated in the lab (Arthurton et al., 2020). The *Dronc*^{KO} allele contains an *attP* integration site at the first exon of *Dronc*'s locus (Arthurton et al., 2020). This was used to reintegrate a "wild-type" *Dronc-GFP-Apex* rescue construct in locus, resulting in the conditional knock-out *Dronc*$^{KO\text{-}FRT\ Dronc\text{-}GFP\text{-}Apex\ FRT\text{-}QF}$ allele (Arthurton et al., 2020). The rescue construct is flanked by FRT recombination sequences that allow the construct to be excised upon exposure to in the Flippase recombinase protein (Arthurton et al., 2020). Using these two knock-out alleles, I observed that EJS tumors homozygous mutant for *Dronc* were also consistently larger than control EJS tumors heterozygous for *Dronc* (Figure 2.7). Given that the suppression of Dronc activity in the development of wild-type wing discs bears no effect on the final size of the wing, a lack of Dronc activity in wing disc tissues does not inherently result in increased tissue growth (Shinoda et al., 2019). Thus, the increased growth observed in *Dronc*-deficient tumors confirmed that Dronc is functionally relevant for EJS tumor growth. In addition, it indicates that Dronc functions as a tumor suppressor, as its deficiency resulted in prominent tumor expansion.

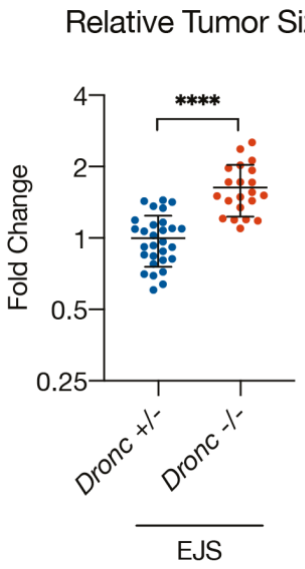


Figure 2.7 **Dronc knock-out EJS tumors increase in size.**
Relative sizes of control (*Dronc*$^{+/-}$) and conditional knock-out EJS tumors (*Dronc*$^{-/-}$) either heterozygous or homozygous mutant for *Dronc*, respectively. Control tumors were used for normalization. Statistical significance was determined by using an unpaired Student's t-test; **** $p < 0.0001$. Numbers of wing discs analyzed for control tumors: 29; for EJS *Dronc*$^{-/-}$ tumors: 21.

The tumor-suppressing ability of caspases has long been recognized; conventionally, this has been attributed to their ability to eliminate tumorigenic cells through apoptosis (Hanahan and Weinberg, 2000, 2011). Because the depletion of the major apoptosis initiating caspase would invariably affect the efficiency of the apoptotic program, there was a possibility that the increase in size observed could be unrelated to the loss of Dronc-dependent non-apoptotic activity in EJS tumors. As demonstrated before, apoptosis does occur in a fraction of cells in EJS tumors (Figure 2.5). Therefore, the depletion of *Dronc* and consequently, loss of the ability to eliminate any tumor cells through the apoptotic program could lead to the accumulation of surviving cells, thus, increasing the size of the tumor. In order to determine if this was the case in *Dronc*-deficient tumors, I inhibited the apoptosis program through a variety of different methods without directly affecting Dronc

activity. One such method was to downregulate the upstream pro-apoptotic factors Reaper, Hid, and Grim (RHG) through expression of a microRNA construct targeting all three, *UAS-miR[RHG]* (Siegrist et al., 2010); another method was to overexpress P35, a potent inhibitor of the downstream effector caspases (Hay et al., 1994). In both cases, I determined that these methods of inhibiting apoptosis were effective at blocking cell death in EJS tumors, as assayed by TUNEL staining (Figure 2.8 A). However, despite the lack of cell death, these apoptosis-resistant EJS tumors remained the same size as control EJS tumors, even after three days of EJS induction when *Dronc*-deficient EJS tumors consistently grew to larger sizes (Figure 2.8 B). Interestingly, when both Dronc and the effector caspases are inhibited through the overexpression of the *Drosophila* inhibitor of apoptosis Diap-1, EJS tumors displayed prominent overgrowth as compared to control EJS tumors, similar to *Dronc*-deficient EJS tumors (Figure 2.8 B). Only methods of apoptosis inhibition directly involving Dronc resulted in differences when compared to control tumors. Thus, these results strongly suggest that Dronc, specifically, is acting in a non-apoptotic manner to restrict tumor size.

As caspases are zymogens that require their own activation, in order to gain some mechanistic insight into how Dronc is activated for non-apoptotic functions in EJS tumors, I compromised the conventional activation of Dronc by downregulating Dark, which is the *Drosophila* orthologue of Apaf-1 (Rodriguez et al., 1999). Together, Dark and Dronc form a complex known as the apoptosome, which, like in humans, facilitates the activation of Dronc and the apoptotic caspase cascade (Yu et al., 2006; Yuan et al., 2011). The downregulation of Dark through overexpression of a *Dark*-

RNAi construct in EJS tumors resulted in consistent overgrowth when compared to controls (Figure 2.8 B); however, the overgrowth was of a lesser magnitude compared to other methods of Dronc inhibition (Figure 2.8 B). This suggests that Dark, and thus the formation of the apoptosome, is potentially required for the activation of Dronc for non-apoptotic purposes.

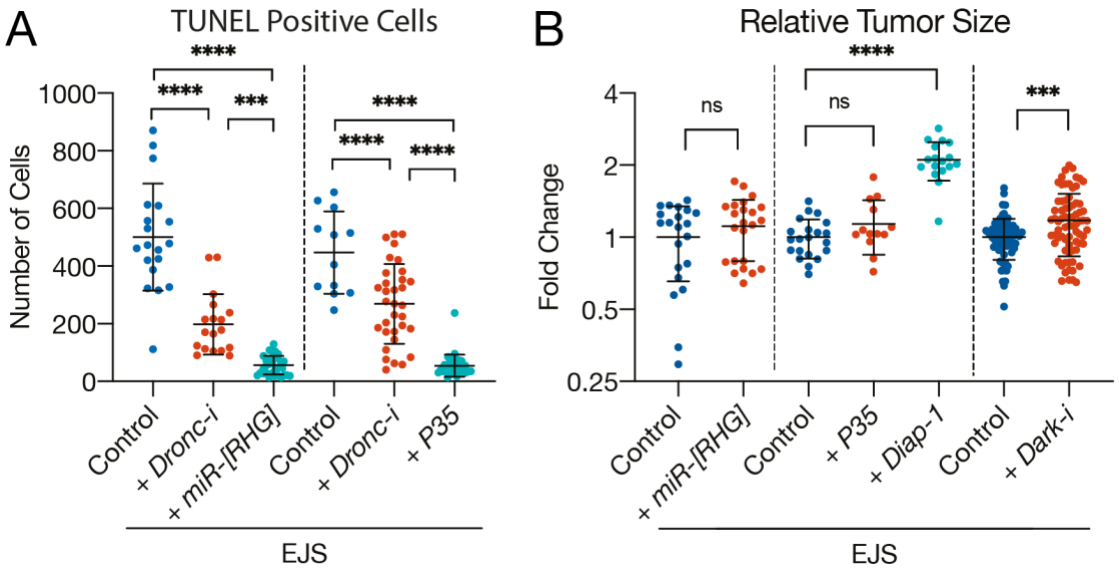


Figure 2.8 **Dronc-specific non-apoptotic function inhibits EJS tumor cell size.** **(A)** Quantification of apoptosis detected by TUNEL in control EJS (Control), EJS + *UAS-Dronc-RNAi* (+ *Dronc-RNAi*), EJS + *UAS-miR[RHG]* (+ *miR[RHG]*), and EJS + *UAS-P35* (+ *P35*) tumors. Mean ±SD of TUNEL positive cells is plotted. Statistical significance was determined by using a one-way ANOVA with Tukey's multiple comparison's test; *** p = 0.0001, **** p < 0.0001. Numbers of wing discs analyzed for EJS tumors: 19 and 12; for EJS + *Dronc-i* tumors: 18 and 33; for EJS + *miR[RHG]* tumors: 33; for EJS + *P35* tumors: 34. **(B)** Relative sizes of control EJS (Control), EJS + *UAS-miR[RHG]* (+ *miR[RHG]*), EJS + *UAS-P35* (+ *P35*), EJS + *UAS-Diap-1* (+ *Diap-1*), and EJS + *UAS-Dark-RNAi* (+ *Dark-i*) tumors. Statistical significance was determined using a Student's t-test to compare EJS/EJS + *miR[RHG]* and EJS/EJS + *Dark-i*. Mean ±SD of relative tumor sizes are plotted. Control (+) tumors were used for normalization. A one-way ANOVA with Tukey's multiple comparisons test was used for EJS/EJS + *P35*/EJS + *Diap-1*. ns – not significant p>0.05, *** p=0.0004, **** p<0.0001. Numbers of wing discs analyzed for EJS tumors: 21, 21, and 65; for EJS + miR[RHG] tumors: 23; for EJS + P35 tumors: 13; for EJS + Diap-1: 17.

Non-apoptotic Dronc activity limits proliferation and cell size in EJS tumors

Because the increase in size of EJS tumors cannot be attributed to an accumulation of cells that are unable to die, I sought to determine if either an increase in

proliferation or cellular size could explain the enlarged size of *Dronc*-deficient EJS tumors. In order to quantify the amount of proliferation occurring in EJS tumors, I immunostained EJS tumors for phospho-Histone-H3 (PH3). As Histone-H3 is only phosphorylated during mitosis for chromosome condensation during metaphase (Kim et al., 2017), PH3 is a reliable marker for cells undergoing mitosis, or proliferative cells. Immunostaining revealed that caspase-deficient tumors contained a slightly greater density of $PH3^{+}$ cells per tumor, suggestive of increased tumor proliferation (Figure 2.9 A, B). I also assayed proliferation by detecting cells undergoing DNA synthesis using a thymidine analogue, 5-ethynyl-2′-deoxyuridine (EdU) (Kaiser et al., 2009). EdU, which is incorporated into newly synthetized DNA during the S-phase of the cell cycle, can be detected using “click” chemistry and specifically marks proliferating cells (Kaiser et al., 2009). Though not statistically significant, a slightly greater proportion of *Dronc*-deficient EJS tumor area was labeled with EdU (Figure 2.9 C), indicating a comparable trend of increased proliferation as was detected by PH3 immunostaining.

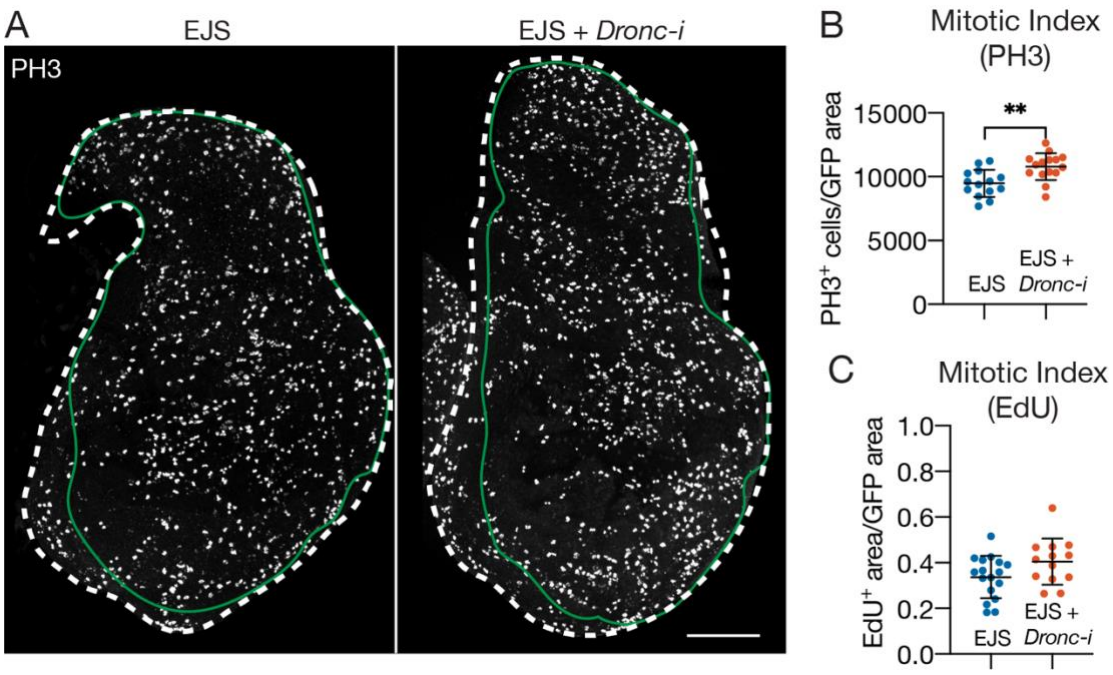


Figure 2.9 **Non-apoptotic activity of Dronc restricts proliferation in EJS tumors.** **(A)** Representative confocal images of control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc*-i) tumors after 1 day of EJS induction showing proliferating cells with a phospho-Histone H3 (PH3) immunostaining (gray). Outline of wing disc (dashed white) and tumor (green) obtained referencing DAPI and GFP, respectively (not shown). Scale bar: 100 μm. **(B, C)** Quantification of $PH3^+$ (B) and EdU^+ (C) cells in control (EJS) and EJS + *UAS-Dronc-RNA*i (EJS + *Dronc-i*) tumors. Using PH3, the number of $PH3^+$ EJS cells per mm^2 of tumor area was calculated. Using EDU, the EDU^+ tumor area was calculated as a fraction of total tumor area. Mean ±SD are also plotted. Statistical significance was determined by an unpaired Student's t-test; $p > 0.05$, ** $p = 0.0025$. Numbers of wing discs analyzed for control tumors: 14 in (B) and 17 in (C); for EJS + *Dronc-i* tumors: 15 in (B) and 13 in (C).

In a parallel set of experiments, I measured the size of EJS tumor cells using their nuclear size as a proxy for cell size (Cantwell and Nurse, 2019), and observed that *Dronc*-deficient EJS tumor cells were significantly enlarged compared to control EJS tumor cells (Figure 2.10 A, B). Additionally, cell nuclei are less densely packed within *Dronc*-deficient EJS tumors, suggesting that individual cells occupy greater space (Figure 2.10 C). Together, these experiments indicated that Dronc limits both EJS tumor cell proliferation and cell size, though these effects may not be fully sufficient to explain the increase in tumor size observed.

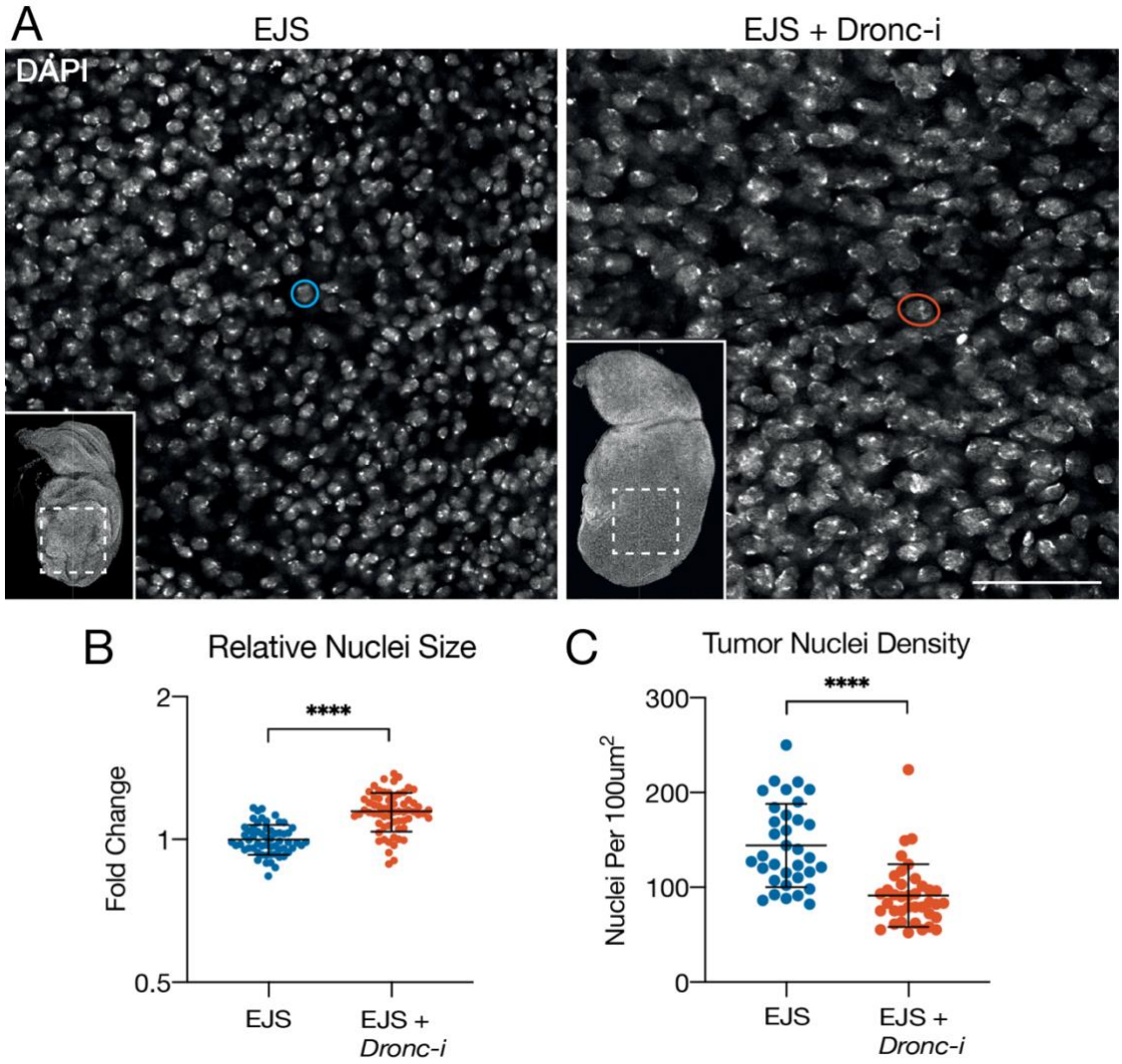


Figure 2.10 **Non-apoptotic Dronc activity restricts EJS tumor cell size.**
(A) Representative confocal image of nuclei in control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) tumors three days after EJS induction. Nuclei were stained with DAPI. Inset depicts the entire tumorous wing disc from with the higher resolution image (dashed white outline) is taken. Example nuclei for size comparison are circled in blue (EJS) and orange (EJS + *Dronc-i*). Scale bar: 50 μm.
(B) Quantification of relative sizes of nuclei in control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) tumors three days after EJS induction. The average nuclei size for each disc was measured. Nuclei in control EJS tumors were used for normalization. Mean ±SD are also plotted. Statistical significance was determined by a Student's t-test; **** $p < 0.0001$. Numbers of wing discs analyzed for control EJS tumors: 59; for EJS + *Dronc-i*: 65.
(C) Quantification of nuclei density in control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) tumors three days after EJS induction, as measure by the number of nuclei per 100μm^2 in each tumor. The mean ±SD is also plotted. Statistical analysis performed by Student's t-test. **** $p < 0.0001$. Numbers of wing discs analyzed for EJS tumors: 34; for EJS + *Dronc-i* tumors: 38.

Discussion

In this chapter, I utilized a recently developed tool, the DBS-S-QF sensor of caspase activity, and a recently described model of co-operative oncogenesis involving upregulation of the EGFR and JAK/STAT signaling pathways (EJS) (Herranz et al., 2012), and established a viable model with which to study the non-apoptotic activity of caspases in a tumor setting. I revealed that not only is there transient caspase activity in EJS tumors, but that virtually all cells with EJS induction either themselves had a history of non-apoptotic caspase activity or descended from cells with caspase activity. Additionally, loss of the major initiator caspase *Dronc* results in increased EJS tumor growth, indicating that Dronc acts as a tumor suppressor in EJS tumors. Importantly, this tumor-suppressing ability is independent of apoptosis. Instead, Dronc activity restricts both cell proliferation and tumor cell size in EJS tumors. Interestingly, while Dronc activity is non-apoptotic in this context, canonical elements of the apoptosis pathway, such as Dark and the apoptosome, appear to facilitate Dronc activation.

While caspase biosensors have been used to detect non-apoptotic caspase activity in *Drosophila* before (Arthurton et al., 2020; Baena-Lopez et al., 2018a; Ding et al., 2016; Tang et al., 2015), and non-apoptotic caspase activity has been functionally described in other tumors (Perez et al., 2017; Perez-Garijo et al., 2005), this is the first report of such widespread existence of non-apoptotic caspase activity in a tumor setting. Since non-apoptotic caspase activity has previously been shown to be influential in regulating tumor characteristics in different tumor backgrounds (Xu et al.,

2018a), it is worth considering that non-apoptotic caspase activity could be widespread in other tumors as well.

Currently, a common perception in the medical field is that high levels of caspase activity or expression indicates a tumor's susceptibility to apoptosis-inducing treatments (Kim et al., 2016; Murphy et al., 2013; Pu et al., 2017). However, in light of this evidence of pervasive non-apoptotic activity, it is perhaps more prudent to also question what non-apoptotic caspase activity could be occurring; this must also be taken into consideration when deliberating caspase activation and apoptosis as part of a treatment plan. For instance, while caspase-3 activation is commonly seen as the endpoint for apoptotic activation (Nagata, 2018) and thus desired for cancer treatment, caspase-3 activity paints a much more complicated picture in colon cancers, where it appears that non-apoptotic caspase-3 activity predominates (Brown et al., 2015; Flanagan et al., 2016; Zhou et al., 2018). In fact, caspase-3 activity is associated with increased invasion and metastasis of colon cancer cells (Zhou et al., 2018), and loss of caspase-3 actually increases sensitivity to therapeutics (Brown et al., 2015; Flanagan et al., 2016; Zhou et al., 2018). Thus, it becomes important to fully understand the potential range of non-apoptotic outcomes of caspase activity in various tumorous backgrounds.

This is made especially more prominent when considering the potential for the context-dependent caspase functions in different tumoral settings. Depending on the cancer, the expression levels and activity of the same caspase can have opposing

influences on the prognosis of a particular tumor patient (Fianco et al., 2017; Kim et al., 2016; Pingoud-Meier et al., 2003). Capase-8 appears to be beneficial for patient survival in ovarian cancer and childhood medulloblastoma (Kim et al., 2016; Pingoud-Meier et al., 2003); however, caspase-8 activity in glioblastomas correlates with worse prognosis (Fianco et al., 2017). Even in the same type of cancer, the same caspase has been found to have opposing context dependent consequences (Pu et al., 2017; Yang et al., 2018). Two recent meta-analysis of patients with breast cancers came to contrasting conclusions towards the associations of caspase-3 expression and patient survivability (Pu et al., 2017; Yang et al., 2018).

Similar themes of context dependency can be found comparing the activity of caspases in EJS tumors with previous descriptions of non-apoptotic caspase activity in *Drosophila* tumors models. Due to the design of the DBS-S-QF senor, the initiator caspase Dronc is the most likely source for the ubiquitous non-apoptotic caspase activity detected (Baena-Lopez et al., 2018a). Additionally, by functionally demonstrating that it is specifically the lack of Dronc that results in extra growth in *Dronc*-deficient tumors, and not just the lack of apoptosis, I have provided strong evidence to suggest that Dronc has a non-apoptotic function in restricting tumor growth. Likely this occurs through Dronc restriction on proliferation and cell size (Figure 2.9, 2.10). However, in a very similar fly model of co-operative tumorigenesis relying on overactivation of the Ras-MAPK pathway downstream of EGFR, the $Ras^{V12}/scrib^{-/-}$ model, it has been demonstrated that the exact same caspase, Dronc, possesses an undeniable function as a tumor promoter (Perez et al., 2017). More

study is needed to comprehend the specific molecular details underlying this disparity, and to understand why these opposing roles of caspases can emerge in different tumoral settings.

Regarding the EJS system, the future priorities would be to determine what aspects of Dronc are necessary for this non-apoptotic, tumor-suppressing activity. Is the catalytic function of Dronc required? Or, as when Dronc interacts with Numb, a Notch signaling regulator, only non-catalytic protein-protein interaction is required (Ouyang et al., 2011). In either case, Dronc's substrate(s) or the proteins interacting with Dronc must be identified. While future experiments will be carried out to resolve these questions, I am able to glean some hints from the experiments already conducted. The inhibition of downstream effector caspases through the overexpression of P35 failed to alter tumor properties, suggesting that this non-apoptotic tumor-suppressing function is specific to Dronc itself, and not indirectly related with the cleavage and subsequent activation of effector caspases. This evidence also echoes previous observations of initiator caspases limiting the potential of transformed cells, indicating that this phenomenon may be more prevalent than previously thought (Ho et al., 2009; Krelin et al., 2008; Puccini et al., 2013).

While it is possible that the catalytic functions of Dronc are not required for the tumor-suppressing effect in EJS tumors, some evidence does suggest that the opposite may be more likely. In order for Dronc to efficiently dimerize and process itself for activation, it forms a complex with Dark, also known as the apoptosome (Yu et al.,

2006; Yuan et al., 2011). Diap1 normally functions to inhibit apoptosis by blocking the interaction between Dronc and Dark (Meier et al., 2000). The similarities between loss of the Dark and inhibition of the Dark-catalyzed self-processing step through Diap1-overexpression with the loss of Dronc suggest that some aspects of the conventional apoptotic pathway are required (Figure 2.8 B). The interaction of Dronc and Dark has previously been shown to be critical for non-apoptotic Dronc functions, albeit for non-catalytic functions (Xu et al., 2005). Interestingly, a recent report also observed a similar non-apoptotic requirement for Apaf-1 in the differentiation of muscle progenitor cells (H. Dehkordi et al., 2020).

As these steps are required to unlock the catalytic activity of Dronc, it is perhaps more likely that the catalytic activity of Dronc is necessary for the non-apoptotic functions described in EJS. However, as the downstream effector caspases are unnecessary for these functions, Dronc must be acting outside the canonical caspase cascade, and instead cleaving some novel substrate. Perhaps this substrate is only made available in EJS tumors, thus imparting specificity for this non-apoptotic function to the EJS system. Novel substrates for Dronc have been described before (Kanuka et al., 2005), and the identification of new substrates in non-apoptotic contexts remains a topic of interest in the community (Arama et al., 2020; Julien and Wells, 2017).

By undertaking these efforts, our understanding of Dronc in this particular scenario could help us understand similar functions in other cancers. Caspase-2 in mice, also

orthologous to Dronc, has been shown to have a tumor-suppressing ability in a model of co-operative oncogenesis also involving Ras (Ho et al., 2009). In this case, caspase-deficiency in transformed mouse embryonic fibroblasts (MEFs) also increased cell proliferation compared to controls (Ho et al., 2009).

Altogether, in this chapter I have concluded that Dronc functions as a tumor suppressor independent of apoptosis in EJS tumors. However, the mechanism by which this occurs remains unclear. In the next chapter, I seek to address this by investigating potential signaling factors in EJS tumors that might be interacting with caspases.

Chapter 3

JNK signaling limited by non-apoptotic caspase activity is responsible for malignant features in EJS tumors

Introduction

In many regards, cancer can be seen as a result of disruption in key cellular signaling pathways (Sever and Brugge, 2015). Due to genetic and epigenetic aberrations, homeostasis is broken and many of the signaling pathways that control cell differentiation, proliferation, migration, and cell death are rewired to result in the over-proliferation and survival of cells that become tumors (Hanahan and Weinberg, 2000, 2011; Sever and Brugge, 2015). Consequently, the regulation of such key signaling pathways are of great interest in understanding the initiation and progression of cancer and how they can be targeted therapeutically (Braicu et al., 2019).

The mitogen-activated protein kinase (MAPK) pathways are examples of such signaling pathways frequently involved in disease and cancer (Braicu et al., 2019; Dhillon et al., 2007; Wagner and Nebreda, 2009). Ubiquitously expressed and evolutionarily conserved, MAPK pathways function as a three-tiered cascade of kinases (Johnson and Nakamura, 2007; Zeke et al., 2016). At the top tier, the most upstream kinases (MAP3Ks) respond to a variety of intra- and extracellular stimuli and phosphorylate the middle tier of kinases (MAP2Ks). MAP2Ks then go on to phosphorylate and activate the MAPKs, which subsequently fulfill their role as

effectors of the MAPK pathway by phosphorylating their own substrates, regulating fundamental cellular processes such as migration, proliferation, and apoptosis (Dhanasekaran and Reddy, 2017; Johnson and Nakamura, 2007; Zeke et al., 2016). While there at least 20 known MAP3Ks in mammals, there are only three main MAPKs and their related isoforms: the extracellular signal-regulated kinases (ERKs), c-Jun-N-terminal kinases (JNKs), and p38 MAPKs (Braicu et al., 2019). Thus, due to the diversity of MAP3K regulation, and funneling nature of the kinase cascade, MAPKs such as JNKs are able to integrate a wide range of intra- and extracellular signals to effect their response (Zeke et al., 2016).

While the MAPK pathways overlap and crosstalk with each other, the ERK pathway is generally thought of as responsive to mitogens and growth factors, while p38 and JNK pathways are considered stress-responsive (Deschenes-Simard et al., 2014; Dhillon et al., 2007; Johnson and Nakamura, 2007; Lee et al., 2020). Primarily affecting cell proliferation and differentiation, the ERK pathway is activated in most cancer types, and activating mutations are found in roughly a 30% – 40% of all human cancers (Dhillon et al., 2007; Guo et al., 2020; Lee et al., 2020; Santarpia et al., 2012). Classical activators of the ERK pathway includes EGFR, a growth factor receptor tyrosine kinase, and subsequent activation of Ras, a downstream GTPase that activates the MAP3K Raf (Braicu et al., 2019; Dhillon et al., 2007). EGFR is of course, a crucial component of the EJS tumor system, and the Ras^{V12} oncogene is a constitutively active version of Ras commonly used in tumor models (Miles et al., 2011).

Intriguingly however, both EGFR and Ras^{V12} overexpression alone results in benign tumors (Herranz et al., 2012; Miles et al., 2011). As is the case with the EJS and *Ras^{V12}/scrib$^{-/-}$* tumor models, secondary oncogenic mutations, such as in polarity genes, are required to transform these tumors into malignant, metastatic neoplasias (Brumby and Richardson, 2003; Herranz et al., 2012; Pagliarini and Xu, 2003). Research has since identified that in *Ras^{V12}/scrib$^{-/-}$* tumors, a critical consequence of the synergistic combination was activation of the JNK pathway, resulting in the enhanced oncogenic transformation in *Drosophila* (La Marca and Richardson, 2020; Pinal et al., 2019). Similarly, JNK activation has been implicated in multiple human cancers, and *Drosophila* has proven a useful model organism in understanding the mechanisms behind which JNK affect tumorigenesis (Hammouda et al., 2020; Tournier, 2013; Wagner and Nebreda, 2009).

In *Drosophila*, the JNK pathway is fundamentally conserved, though simpler than in mammals (La Marca and Richardson, 2020). There is only one JNK, Basket (Bsk), which is orthologous to mammalian JNKs 1/2/3 (Riesgo-Escovar et al., 1996). There are at least two JN2Ks, Hemipterous (Hep) and Map kinase kinase 4 (MKK4), and at least 4 JN3Ks, Slipper (Slpr), Wallenda (Wnd), TGFβ-associated kinase 1 (Tak1), and Apoptotic signal-regulating kinase 1 (Ask1) (La Marca and Richardson, 2020). As in mammalian systems, a complex array of signaling pathways and intra- and extracellular stimuli converge into Bsk activation in order to affect fundamental

cellular processes such as migration, proliferation, and apoptosis in *Drosophila* (La Marca and Richardson, 2020; Zeke et al., 2016).

As part of the stress activated branch of MAPKs (Johnson and Nakamura, 2007), a major function of the JNK pathway is to respond to cellular stresses, such as ionizing radiation and DNA damage, tissue damage, or inflammatory cytokines such as tumor necrosis factor (TNF) (Johnson and Nakamura, 2007; Zeke et al., 2016). For example, under normal physiological conditions in *Drosophila* imaginal wing discs, JNK activity is hardly present (Pinal et al., 2018); however, upon tissue or DNA damage, JNK activity is upregulated and vital for the regeneration process, driving the process of wound healing through cell migration and proliferation (Nikoloudaki et al., 2020; Ramet et al., 2002). Studies have indicated that an initial burst of reactive oxygen species (ROS) released from damaged tissue is important for JNK activation (Santabarbara-Ruiz et al., 2015), and that JNK itself stimulates the release of more ROS, establishing an amplification loops that ensures adequate JNK signaling for the regenerative process (Khan et al., 2017). Along with the JNK pathway, other signaling pathways such as JAK/STAT, are also required to reset the cellular identities of neighboring cells into stem-like states, allowing them to proliferate and restore the damage tissue (Ahmed-de-Prado et al., 2018; Worley et al., 2018).

However, in counterpoint to these pro-proliferative and regenerative qualities, JNK activity is also a potent inducer of cell death, triggering caspase activity to induce apoptosis and eliminate damaged and undesired cells (Dhanasekaran and Reddy,

2017; Igaki, 2009; Liu and Lin, 2005). In *Drosophila*, this occurs through upregulation of the pro-apoptotic genes *reaper* and *hid* (Luo et al., 2007; Moreno et al., 2002). Subsequently, caspase activity can also further enhance JNK activity through feedback loops, ensuring effective apoptosis (Pinal et al., 2019; Shlevkov and Morata, 2012; Wells et al., 2006). Thus, the JNK pathway is a double-edged blade with both pro-apoptotic and pro-proliferative capabilities. Consequently, in different tumoral contexts, JNK activity has been demonstrated to have both anti- and pro-tumorigenic effects (La Marca and Richardson, 2020; Pinal et al., 2019; Tournier, 2013; Wu et al., 2019).

Interestingly, caspase activity can lie at the heart of both ends of JNK's tumorigenic spectrum (Perez et al., 2017; Pinal et al., 2018). Studies in *Drosophila* have revealed that cancerous cells can be eliminated through JNK-mediated apoptosis activated by TNF signaling (Igaki et al., 2009; Li et al., 2019). Neighboring wild-type cells or recruited macrophage-like cells, called hemocytes (Buchon et al., 2014), release Eiger, *Drosophila*'s TNF orthologue, which binds to its receptors Wengen (Wgn) and Grindelwald (Grnd) in tumor cells to activate the JNK pathway and apoptosis (Andersen et al., 2015; Cordero et al., 2010; Igaki et al., 2002; Igaki et al., 2009; Kauppila et al., 2003). However, when apoptosis is prevented from occurring, either from caspase deficiency or from Ras^{V12} expression, JNK activity switches from being anti-tumorigenic to pro-tumorigenic (Diwanji and Bergmann, 2019; La Marca and Richardson, 2020; Pinal et al., 2019). In the case of caspase deficiency, the lack of apoptosis results in perpetual JNK signaling through the ROS feedback amplification

loop, sustaining over-proliferation and tumorigenesis (Pinal et al., 2018). When oncogenic mutations prevent apoptosis from occurring, such as in *Ras^{V12}/scrib$^{-/-}$* tumors, JNK-stimulated caspase activity is also co-opted to sustain a similar feedback amplification loop involving ROS, hemocyte attraction and release of Eiger, and further JNK activation to fuel tumor growth (Perez et al., 2017).

In this regard, caspase and JNK activity and the deregulation of the amplifying feedback mechanisms between them can be fundamental to the malignant transformation of cancer cells; however, we do not yet have a complete understanding of the mechanisms behind the feedback loops between caspase and JNK activity (Pinal et al., 2019). Thus, due to the similar background of cooperative oncogenesis of the *Ras^{V12}/scrib$^{-/-}$* and EJS tumor models and the importance of caspase activity in regulating EJS tumor growth, I decided to investigate the relationship between caspases and JNK signaling in EJS tumors.

Results

Non-apoptotic caspase activity limits JNK signaling in EJS tumors

First, to determine if the JNK pathway is active in EJS tumors, I monitored JNK activity by immunostaining the JNK gene target *Matrix metalloprotease 1* (*MMP1*) (Uhlirova and Bohmann, 2006), as well as a transgenic transcriptional reporter of JNK activity (Tre-RFP) that contains binding sites for a transcription factor activated by JNK (Chatterjee and Bohmann, 2012). While previously though to be functionally unimportant for wing disc development, recent work has shown that localized JNK

signaling regulates growth in developing wild-type discs, (Willsey et al., 2016). However, by later stages of development this activity diminishes, and wild-type wing discs typically show little trace of either readout (Pinal et al., 2018). In constrast, EJS tumors showed strong upregulation of both readouts (Figure 3.1 A, B), indicating that JNK is strongly activated by EJS induction. Additionally, JNK activity in EJS tumors increased even further upon *Dronc* depletion, as indicated by both the increased mean and modal intensity of MMP1 staining after three days of EJS induction (Figure 3.1 C). While the mean intensity of Tre-RFP expressed remained similar, the modal intensity of Tre-RFP expression was significantly higher in *Dronc*-deficient EJS tumors than in control EJS tumors, also indicating additional JNK activity upon *Dronc* depletion (Figure 3.1 D). However, this was only apparent in later stage tumors, as EJS and *Dronc*-deficient EJS tumors showed similar levels of Tre-RFP expression on the first day of tumor induction. Using RT-qPCR, I was also able to confirm that levels of *MMP1* mRNA were increased in *Dronc*-deficient EJS tumors relative to control EJS tumors; additionally, I saw that transcription of the *Unpaired* (Upd) ligands, activators of the JAK/STAT pathway, was also increased (Figure 3.1 E). The Upd ligands are also target genes of the JNK pathway and upregulated in both tumorous and regenerative scenarios in response to JNK stress signaling (Bunker et al., 2015; Jiang et al., 2009; Worley et al., 2018). Altogether, these results strongly suggest that Dronc is acting to limit JNK signaling in EJS tumors.

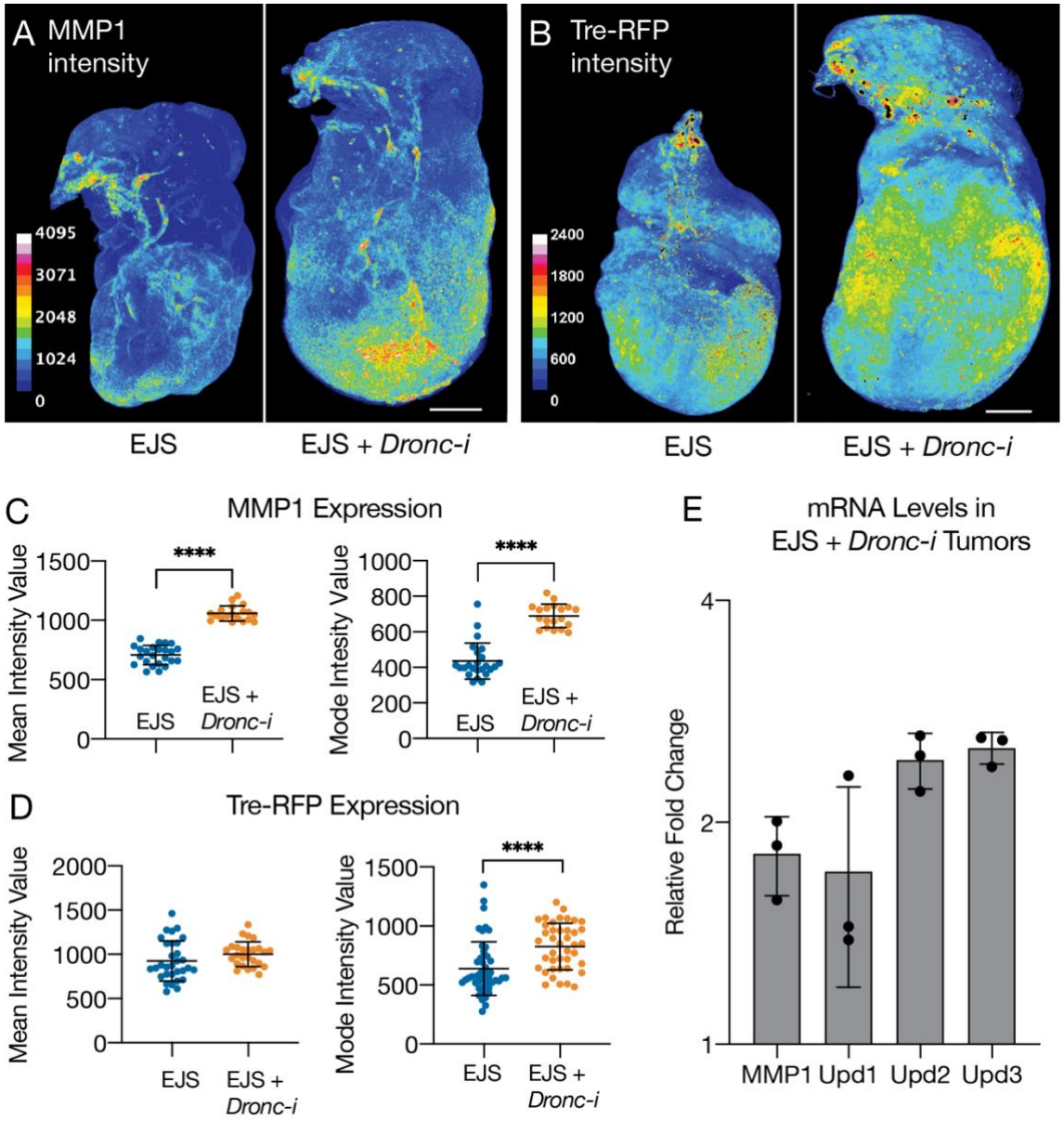


Figure 3.1 **Non-apoptotic Dronc activity limits JNK signaling in EJS tumors.**

(A) Representative maximum projected confocal images of control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) tumors after 3 days of EJS induction stained with anti-MMP1. The images have been false colored to visualize the intensity of MMP1 staining, displaying the full range of pixel intensities from 0 – 4095. Scale bar: 100µm.

(B) Representative maximum projected confocal images of control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) tumors expressing the transgenic construct *Tre-RFP* after 3 days of EJS induction. The images have been false colored to visualize the intensity of Tre-RFP expression, displaying the range of pixel intensities from 0 – 2400 out of the full 4095 to better visualize the differences between control EJS and EJS + *Dronc-i* tumors.

(C) Quantification of the means and modes of MMP1 staining intensity in control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) tumors after 3 days of EJS induction. Mean ±SD are also plotted. Statistical significance was determined by unpaired Student's t-tests; **** $p < 0.0001$. Numbers of wing discs analyzed for control EJS tumors: 24; for EJS + *Dronc-i* tumors: 19.

(D) Quantification of the means and modes of Tre-RFP intensity in control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) tumors after 3 days of EJS induction. Mean ±SD are also plotted. Statistical significance was determined by an unpaired Student's t-test; **** $p < 0.0001$. Numbers of wing discs analyzed for control EJS tumors: 31; for EJS + *Dronc-i* tumors: 26.
(E) Quantification of relative levels MMP1, Upd1, Upd2, and Upd3 mRNA in EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) tumors after 3 days of tumor induction compared to control EJS tumors measured by RT-qPCR. Bars represent the mean of three independent replicates measured and the SDs are also shown.

JNK signaling disrupts cell identity and differentiation in EJS tumors

Given that the JNK pathway is strongly upregulated in EJS tumors, and further activated by the loss of Dronc, I reasoned that the increased JNK activity could be a contributing factor in the increased tumor sizes observed in *Dronc*-deficient EJS tumors. To examine this possibility, I sought to determine the role of JNK activation in control and *Dronc*-deficient EJS tumors. To do this, I utilized a well-characterized dominant-negative form of the sole JNK protein in *Drosophila*, *basket*^{DN} (*bsk*^{DN}, referred to as JNK^{DN}), to remove JNK activity (Adachi-Yamada et al., 1999). Overexpression of this JNK^{DN} abolished detection of Mmp1 (Figure 3.2), indicating the successful suppression of JNK activity.

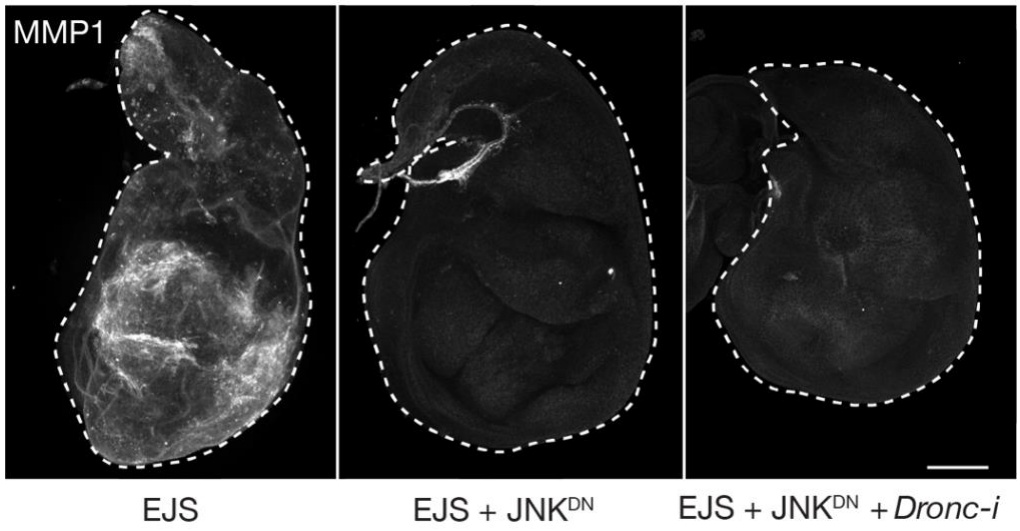


Figure 3.2 **JNK^{DN} suppresses JNK activity in EJS tumors.**

Representative maximum projected confocal images of control tumors (EJS), JNK-deficient EJS tumors (EJS + JNK^{DN}), and JNK- and *Dronc*-deficient EJS tumors (EJS + JNK^{DN} + *Dronc-i*) after 1.5 days of EJS induction stained with anti-MMP1. The same microscope settings were used to capture all three images. Outline of wing disc (dashed white) obtained by tracing DAPI. Scale bar: 100μm.

Interestingly, the loss of JNK resulted in distinct morphological changes in EJS tumors. While still overgrown as compared to wild-type wing discs, JNK^{DN} EJS tumors exhibited a more restrained overgrowth (Figure 3.3). Tumor growth in control EJS tumors results in a complete loss of apicobasal polarity and epithelial organization; EJS tumors grow as a disorganized mass of tissue in a globular shape that increases in size over time (Herranz et al., 2012). However, JNK-deficient EJS tumors retain the archetypical epithelial-bilayer organization, and an overall morphology more similar that that of wild-type discs. Importantly, they retain a flatter two-dimensional shape when compared to control EJS tumors, which balloon outward into a three-dimensional, spherical shape. This makes direct size comparisons (as measured by area) between JNK-deficient and control EJS tumors difficult. JNK-deficient EJS tumors are, however, larger in size than their wild-type counterparts. JNK-deficient EJS tumors also appear to have a greater degree of tissue organization than can be found in EJS tumors. While EJS tumors bear almost no resemblance to wild-type wing discs anymore, specific regions of the wing disc in EJS tumors with suppressed JNK activity can be identified based solely on the topology of these tumors (Figure 3.3). These wing disc regions correspond to the wing pouch and the dorsal hinge, which eventually give rise to the adult wing blade and connecting hinge to the thorax, respectively (Beira and Paro, 2016; Hartl and Scott, 2014). More substantially, the wild-type ventral compartment of the wing disc can still be found in JNK-deficient EJS tumors (Figure 3.3) (Beira and Paro, 2016;

Hartl and Scott, 2014). In EJS tumors, this region is usually almost non-existent, having been overtaken by tumor growth from the dorsal compartment (Figure 3.3).

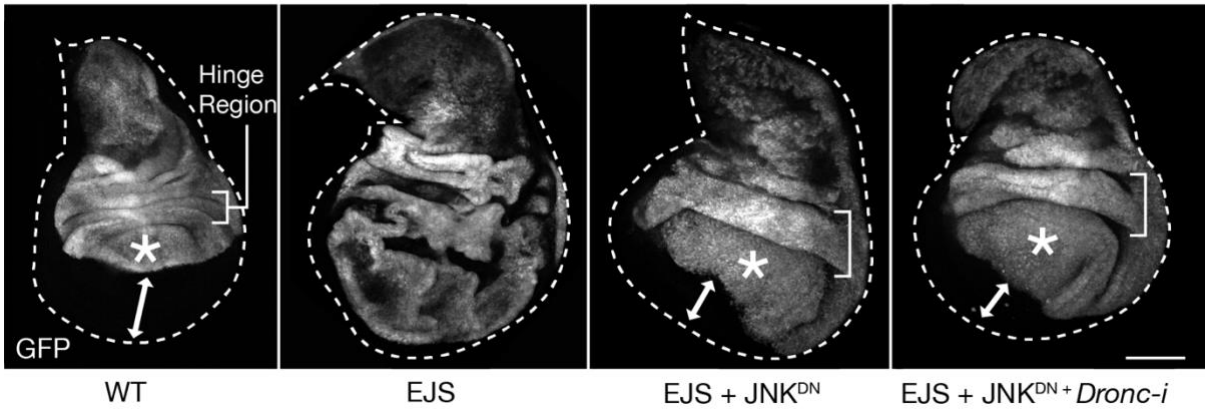


Figure 3.3 **JNK activity in EJS tumors fuels disorganized growth.**
Representative confocal images of wild-type wing discs (WT) and control (EJS), EJS + *UAS-bsk^{DN}* (EJS + JNK^{DN}), EJS + *UAS-bsk^{DN}* + *UAS-Dronc-RNAi* (EJS + JNK^{DN} + *Dronc*-i) tumors after 1.5 days of EJS induction. The earlier time point was chosen as EJS + JNK^{DN} tumors seldom progressed past two days post EJS induction due to larval pupariation. *ap-Gal4* driven expression of GFP (gray) labels cells in the dorsal compartment of the wing discs. Additionally, due to the drastically different growth patterns (control EJS tumors balloon in size in all three dimensions, while JNK-deficient tumors retain a flat epithelial shape), direct size comparisons between different genetic condisions were not made. White double-headed arrows indicate the ventral portion of the wing discs. White asterisks indicate the dorsal portion of the wing pouch region of the wing discs. White brackets indicate the dorsal hinge region of the wing disc. Note the absence of a ventral portion in EJS tumors. The wing pouch and hinge regions also cannot be identified.

Together, these observations suggested to me that the disorganized overgrowth found in EJS tumors is dependent upon JNK activity. To better characterize this observation, I investigated the expression patterns of two well-known cell-identity proteins found in wing discs. In wild-type wing discs these two proteins, Distal-less (Dll) and Wingless (Wg), have very defined and distinct patterns of expression (Figure 3.4 A, B) (Campbell and Tomlinson, 1998; Swarup and Verheyen, 2012). Wg is primarily expressed along the boundary of the dorsal/ventral components of the wing blade, and in two concentric rings (Figure 3.4 A) (Swarup and Verheyen, 2012). The inner ring outlines what becomes the wing blade, while the outer ring frames the

hinge region (Swarup and Verheyen, 2012). The expression in the boundary of the dorsal and ventral compartments contains cells that become the wing margin (Swarup and Verheyen, 2012) (Figure 3.4 A). Dll is symmetrically expressed in the wing blade, forming a double-ended gradient along

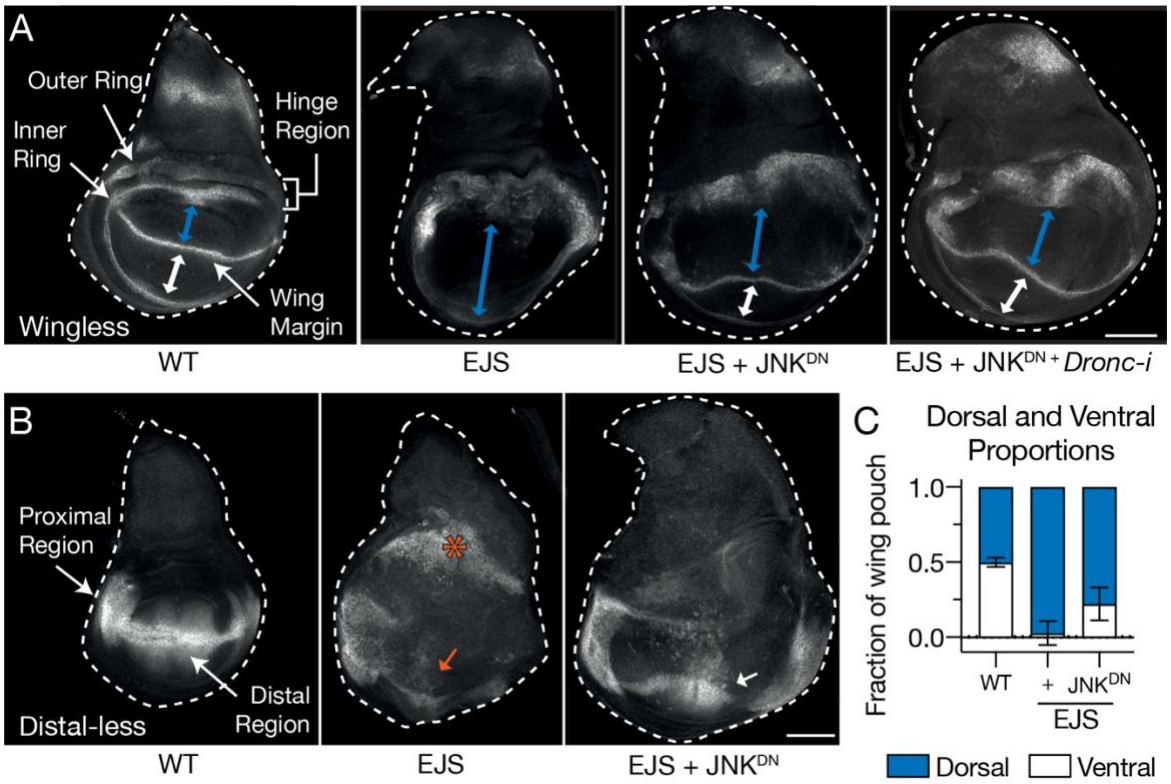


Figure 3.4 **JNK activity disrupts cell identity in EJS tumors.**
(A) Representative confocal images of Wingless (Wg) immunostaining (gray) in wild-type wing discs (WT) and control (EJS), EJS+UAS-*bsk*^{DN} (EJS + JNK^{DN}), EJS + UAS-*bsk*^{DN} + UAS-*Dronc*-RNAi (EJS + JNK^{DN} + *Dronc*-i) tumors after 1.5 days of EJS induction. Dashed white outline of wing discs obtained by tracing DAPI staining (not shown). In WT wing discs, Wg is expressed in an inner and outer ring surrounding the presumptive wing blade, and along the wing margin which divides the wing pouch into dorsal and ventral portions, indicated by blue and white double-headed arrows, respectively. The inner and outer rings also frame the hinge region. Note the lack of a ventral portion in EJS tumors.
(B) Representative confocal images of Distal-less (Dll) immunostaining (gray) in wild-type wing discs and control (EJS), EJS + UAS-*bsk*^{DN} (EJS + JNK^{DN}), EJS + UAS-*bsk*^{DN} + UAS-*Dronc*-RNAi (EJS + JNK^{DN} + *Dronc*-i) tumors after 1.5 days of EJS induction. Dashed white outline of wing discs obtained by tracing DAPI staining (not shown). In WT wing discs, Dll is expressed primarily in the distal region of the wing blade. Note the downregulated Dll in the distal region of the wing pouch in EJS tumors (orange arrow), and the exogenous expression outside the wing pouch (orange asterisk). Dll expression in the distal region is restored in EJS + JNK^{DN} tumors (white arrow).

(C) Quantifications of the dorsal and ventral portions of the wing pouch as fractions of the wing pouch region in WT, EJS, and EJS + JNK^{DN} tumors after 1.5 days of EJS induction. Wingless was used to identify the wing pouch and margin, as in (A). Numbers of wing discs analyzed for wild-type discs: 11; control EJS tumors: 44; for EJS + *Dronc-i* tumors: 26.

the wing margin, with the greatest expression in the center of the wing pouch at what eventually becomes the most distal region of the wing blade (Figure 3.4 B) (Campbell and Tomlinson, 1998). In addition, it is also expressed in a subset of cells of the wing hinge (Campbell and Tomlinson, 1998).The expression patterns of both these proteins are severely disrupted in EJS tumors. The tightly delineated concentric rings of Wg can no longer be distinguished as such, and instead, Wg is expressed in a broad, ill-defined swath of cells around the “hinge” region and the dorsal/ventral boundary (Figure 3.4 A). Expression of Wg in what remains of the adjacent ventral compartment of wild-type tissue is lost completely, as the ventral compartment in tumorous wing discs is eliminated by the overgrowth of dorsal cells (Figure 3.4 C). Dll expression disappears from the prospective wing blade cells and decreases in what remains of the wing margin (Figure 3.4 B). Additionally, Dll is aberrantly expressed in what was the thorax and wing hinge region of the disc (Figure 3.4 B). *Dronc*-deficient EJS tumors also showed similar deviations in Wg and Dll expression (not shown). These results indicated that EJS induction results in severe disruption of cell identity and patterning in affected tissues, both cell- and potentially, non-cell autonomously.

Importantly, both the cell- and non-cell autonomous disruption of patterning and cell identity is rescued by the suppression of JNK in EJS tumors, confirming their dependency upon JNK activity (Figure 3.4 A, B, C). Additionally, this rescue also occurs when JNK is suppressed in *Dronc*-deficient EJS tumors, indicating that the

phenotypes related to *Dronc*-deficiency are JNK-dependent (Figure 3.4 A, B, C). Together, these results suggest that the upregulation of JNK signaling underpins a large extent of the basal neoplastic transformation of EJS tumors, and its exacerbation in *Dronc* mutants intensifies these malignant features.

JNK pathway activation is likely independent of ROS and conventional TNF alpha ligands in EJS tumors

Because JNK signaling is instrumental for the malignant growth of EJS tumors, and increased JNK activity is likely a contributing factor in the exacerbated phenotypes seen in *Dronc*-deficient EJS tumors, I next sought to investigate the origin of JNK activity in EJS tumors.

Reactive oxygen species (ROS), which contribute to oxidative stress in cells, are known to activate JNK (Zeke et al., 2016), and as described in Chapter 1, a recent study has shown that ROS is released in a caspase-dependent manner in $Ras^{V12}/scrib^{-/-}$ tumors in Drosophila (Perez et al., 2017). Attracted by released ROS, hemocytes (*Drosophila* macrophage-like cells) attach to tumors and release Eiger, a TNF orthologue. Eiger binds to its receptors Grindelwald (Grnd) and Wengen (Wgn), activating JNK signaling and promoting tumor growth (Fogarty et al., 2016; Perez et al., 2017). Interestingly, in *Dronc*-deficient conditions, ROS produced as a result of irradiation can lead to persistent JNK activation, further ROS production, and tumor growth through an ROS-JNK feedback loop (Pinal et al., 2018). Given the similarities between the $Ras^{V12}/scrib^{-/-}$ and EJS models of co-operative oncogenesis, and the

exacerbated JNK signaling in *Dronc*-deficient EJS and irradiation conditions, I examined if ROS could have a similar function in EJS tumors to induce JNK.

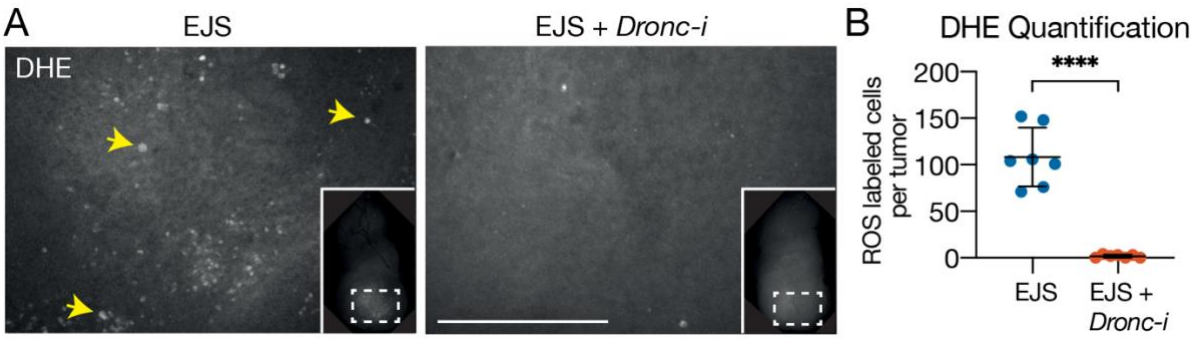


Figure 3.5 **Dronc is responsible for ROS production in EJS tumors.**
(A) Representative maximum projected confocal images of control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) after two day of EJS induction labeled with the ROS indicator DHE. Yellow arrows indicate high intensity DHE puncta in cells. The inset depicts the entire tumorous wing disc from with the higher magnification image shown (dashed white outline) is taken. Scale bar: 100μm.
(B) Quantification of DHE detected in control tumors (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) after two days of EJS induction, measured by counting the number of high-intensity DHE puncta per tumor. Statistical analysis performed by Student's t-test. **** $p < 0.0001$. Numbers of wing discs analyzed for EJS tumors: 7; for EJS + *Dronc-i* tumors: 7.

To assay the levels of ROS in EJS tumors, I used the ROS indicator dihydroethidium (DHE), which detects ROS by reacting with superoxide anions to form a red fluorescent product (Dikalov et al., 2007; Owusu-Ansah et al., 2008). While DHE readily labeled EJS tumors in discrete high-intensity puncta, it was detected far less frequently in *Dronc*-deficient EJS tumors (Figure 3.5 A, B). The loss of ROS detection upon Dronc depletion was in agreement with the previously described caspase-dependent generation of ROS (Perez et al., 2017); however, the continued growth of *Dronc*-deficient EJS tumors suggested that ROS played little role tumor growth in our experimental conditions. To support this conclusion, I examined the functional contribution of ROS to EJS tumors by compromising the production of both extra- and intra-cellular ROS; this was achieved by silencing Duox, an NADPH-oxidase that generates extracellular ROS (Khan et al., 2017), or by overexpressing Catalase, an

ROS-scavenger enzyme that reduces hydrogen peroxide (H_2O_2) to water (H_2O) (Diaz-Albiter et al., 2011). While the downregulation of Duox similarly eliminated ROS detection, the overexpression of Catalase had no noticeable effect in EJS tumors (not shown). Combined with the loss of ROS in *Dronc*-deficient tumors, these results suggested that the main source of ROS in EJS tumors was caspase-dependent and intracellular. Importantly, none of these manipulations of ROS resulted in any discernible changes in EJS tumor formation, strongly suggesting that ROS is unnecessary for EJS tumor initiation, or tumor growth. Additionaly, given the reliance on JNK signaling for EJS tumor growth, these data indirectly suggest that ROS might not be necessary for JNK activitation in EJS tumors as well. Interestingly, loss of ROS did not affect the recruitment of hemocytes to the tumor either (Figure 3.6).

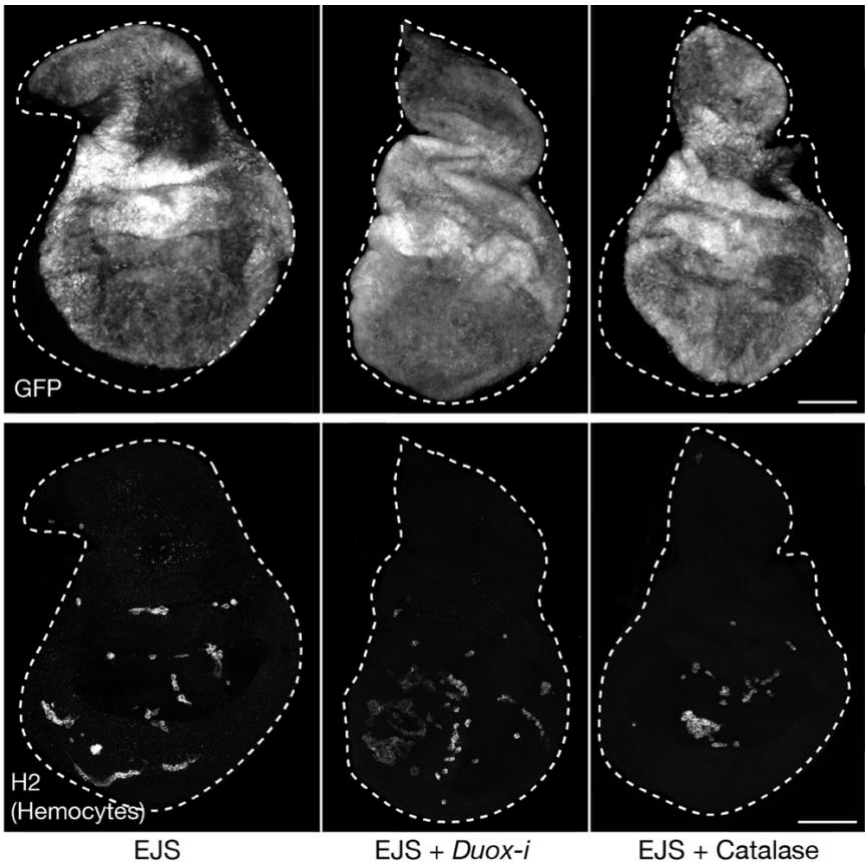


Figure 3.6 **ROS is unnecessary for EJS tumor growth and hemocyte attraction.**

Representative maximum projected confocal images of control tumors (EJS), EJS + UAS-*Duox*-RNAi tumors (EJS + *Duox*-i), and EJS + UAS-Catalase tumors (EJS + Catalase) expressing GFP and with hemocytes (gray) labeled with anti-Hemese (H2). Tumors are induced even in ROS-removing conditions (GFP) and hemocytes are also still present (H2). Scale bar: 100μm.

Surprisingly, this also suggest that ROS is dispensable for the recruitment of hemocytes to EJS tumors.

Given that ROS is unlikely to be the origin of JNK activity in EJS tumors, I examined another well-known source for JNK signaling in Drosophila: TNF-signaling (Figure 3.7 A). As mentioned before, a recent study implicated caspases activity in the recruitment of hemocytes (via ROS) towards tumorous tissue, fueling tumor growth by secreting Eiger, the Drosophila TNF (Perez et al., 2017). Because hemocytes are recruited to EJS tumors—though independently of ROS—I examined the possibility that they could be responsible for the activation of JNK through the secretion of Eiger. As the two characterized TNF Receptors in Drosophila are Wengen and Grindelwald (Andersen et al., 2015; Kauppila et al., 2003), I downregulated both simultaneously in EJS tumors in order to prevent functional redundancy in TNF-dependent activation of JNK signaling. Surprisingly, the downregulation of both receptors did not compromise the growth of EJS tumors (Figures 3.7). Because JNK activity is critical for EJS tumor growth, this data suggests that the JNK activity observed in EJS tumors is largely independent from conventional JNK ligand-receptor complexes, and ROS generation.

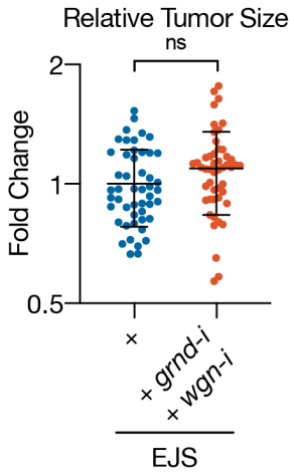


Figure 3.7 **Tumor growth is independent of TNF signaling.**
Quantification of relative sizes of control tumors (EJS) and EJS tumors expressing *Grindelwald-RNAi* and *Wengen-RNAi* to downregulate the TNF-receptors (EJS + *grnd-i* + *wgn-i*) after three days of EJS induction. Graphs show mean ±SD. Statistical analysis performed by an unpaired Student's t-test. ns – not significant p>0.05. Numbers of wing discs analyzed for both EJS and EJS + *grnd-i* + *wgn-i* tumors: 49 each.

Discussion

In this chapter, I establish that there is prevalent JNK activity in EJS tumors, as indicated by the robust immunofluorescence detection of MMP1, a JNK gene target, as well as of a synthetic transcriptional reporter, Tre-RFP. Additionally, transcriptional upregulation of MMP1 and the Upd family of ligands, another set of JNK target genes, was detected through qPCR. I demonstrated that JNK activity is responsible for the malignant features of EJS tumors, as the suppression of JNK rescues EJS tumors from the loss of cell identity and disordered overgrowth. Additionally, I was able to demonstrate that the loss of Dronc results in increased JNK activity, as expression of both Mmp1 and Tre-RFP increased in *Dronc*-deficient EJS tumors; similarly, *Dronc*-deficiency resulted in upregulated transcription of the Upd family of ligands, which also lies downstream of the JNK pathway. Together, these results

suggest that caspases are working to inhibit the JNK signaling pathway in EJS tumors, thus limiting the malignant features dependent upon JNK.

This is not the first time that caspase activity has been shown to moderate MAPK or JNK signaling. A recent study has reported a new non-apoptotic role for caspases in promoting developmental growth in *Caenorhabditis elegans* by limiting a MAPK stress response pathway (Weaver et al., 2020). Additionally, effector caspases have been shown to cleave the JNK Interacting Protein-1 (JIP1) in mammalian cells during apoptosis, attenuating JNK signaling (Vaishnav et al., 2011).

However, in most scenarios, caspase activity and JNK signaling positively regulate each other. In response to cellular stresses, one of the primary functions of JNK signaling is to activate caspases and the apoptotic pathway (Luo et al., 2007; Moreno et al., 2002; Tournier, 2013). In *Drosophila*, it has been shown that Dronc in turn continues to activate or sustain JNK activity, thus ensuring proper apoptosis (Pinal et al., 2019; Shlevkov and Morata, 2012; Wells et al., 2006). Additionally, JNK activation downstream of caspase activity is critical for inducing compensatory proliferation in neighboring cells, a homeostatic mechanism to replace apoptotic cells (Ryoo and Bergmann, 2012; Ryoo et al., 2004). Exactly how Dronc is activating JNK signaling in genuine regenerative scenarios is not yet fully understood (Diwanji and Bergmann, 2019), but recent studies in *Drosophila* tumor models and undead cells indicate that caspase-dependent release of ROS could indirectly amplify JNK signaling (Fogarty et al., 2016; Perez et al., 2017). Moderate, non-apoptotic levels of caspase activation

has also been shown to activate JNK signaling for invasiveness and migration (Rudrapatna et al., 2013).

Thus, to my knowledge, my observations indicate for the first time a negative caspase-dependent regulation of JNK signaling in a tumor setting. It is important to note that this negative regulation occurs independently of apoptosis, as it had previously been shown that persistent JNK signaling could occur in caspase-deficient tissues, leading to tumorigenesis (Pinal et al., 2018); however, this was simply a consequence of cells failing to undergo JNK-induced apoptosis (Pinal et al., 2018), allowing JNK signaling to positively feedback upon itself.

Investigating the specifics behind what allows this non-apoptotic JNK-limiting function for caspases to occur in EJS tumor cells is a future priority. One possibility to explain this is that specific substrates of Dronc within the JNK pathway are only made available to Dronc in the context of EJS induction. While it is unclear what could allow for this differentiation, context-dependent regulation of caspases for specific purposes in different tissues have been shown before; most often, this involves restricting the sub-cellular localization of Dronc for compartmentalized, non-apoptotic activity (Amcheslavsky et al., 2018; Erturk et al., 2014; Kang et al., 2017). My own lab has also recently reported a very specific interaction between Dronc and the Hedgehog (Hh) signaling pathway in the Drosophila germarium, despite caspases and the Hh pathway coexisting in several other tissues (Galasso et al., 2020).

The caspase-dependent negative regulation of the JNK pathway is not the only unique factor in the relationship between JNK and EJS tumors. Interestingly, the activation of JNK signaling in EJS tumors also appears to be distinct from other tumor models. While the majority of other models of tumorigenesis in Drosophila rely on either ROS or Eiger/TNF-signaling for JNK activity (La Marca and Richardson, 2020), JNK-signaling in EJS tumors appears to be independent from either of these two factors. Further investigation will be required to determine the origin of JNK-signaling in EJS tumors; however, these results reinforce the notion that JNK-activation can be tumor-specific (Muzzopappa et al., 2017). It had recently been shown that tumors of different origins (either from Eiger overexpression, chromosomal instability, or polarity-impairment) activate JNK-signaling through distinct mechanisms (Muzzopappa et al., 2017). Interestingly, in wing disc tumor models involving cooperation between Ras^{V12} and either *scrib* or *discs large 1* (*dlg*) mutations (both polarity determinant genes), JNK activation is a result of polarity-impairment (Muzzopappa et al., 2017). Mechanistically, a cytoskeletal regulator, Rho1, acts directly upon a JN3K, Wallenda (Wnd) to activate JNK-signaling (Ma et al., 2016; Muzzopappa et al., 2017). As disruption of apicobasal polarity is also a key feature of EJS tumors (Herranz et al., 2012), it is possible that this could also be how JNK is activated in EJS tumors.

Regardless of how it is activated, JNK signaling in EJS tumors plays an important role in the transformation of cells into EJS tumors. In the course of EJS tumorigenesis, the imaginal wing disc grows into a disorganized mass of cells,

disrupting the normal expression patterns of cell identity proteins. When JNK signaling is inhibited in imaginal wing disc EJS tumors, the resulting wing discs retain their epithelial organization, and for the most part, much of the expression patterns of cell identity proteins. These results suggest that cells in EJS tumors are de-differentiating and losing their cellular identity in a manner similar to the JNK-dependent loss of cell identity observed in wound healing and tissue regeneration (Ahmed-de-Prado et al., 2018; Worley et al., 2018). Additionally, the JNK-dependent overgrowth of the tumor draws further parallels to the wound healing process, as JNK is responsible for the proliferation of regenerating cells (Bergantinos et al., 2010). Thus, as has been demonstrated in other models with JNK-dependent proliferation, the EJS tumor model bears similarities to an open wound attempting to heal (Diwanji and Bergmann, 2019; Dvorak, 2015).

Altogether, in this chapter, I have concluded that caspases act non-apoptotically to limit the JNK activity that is strongly active and instrumental in EJS tumors for malignant tumor properties. Taken together with previous literature, this chapter highlights the complex and context-dependent relationship between JNK signaling and caspase activity, especially in regenerative, wound-like tumors (Diwanji and Bergmann, 2019). Interestingly, while I show that tumor growth (and presumably, JNK signaling) in EJS tumors is independent of two of the best-known activators of JNK signaling in Drosophila tumor models—ROS and TNF-signaling—the most influential source of TNF in Drosophila, hemocytes, can be found in large numbers on EJS tumors. Like in mammalian systems, these macrophage-like immune cells are

capable of inhibiting tumor growth through phagocytosis, or paradoxically, promoting tumor growth through the release of soluble factors (Cordero et al., 2010; Gonzalez et al., 2018; Perez et al., 2017). In the next chapter, I seek to address the role of these macrophage-like cells in either inhibiting or promoting tumor growth, as well as their relationship with caspases.

Chapter 4

Tumor associated macrophages and their effect on tumor growth are limited by caspases

Introduction

The immune system, inflammation, and cancer have been linked together since at least the times of ancient Greece, when the physician Galen first contemplated the possibility that cancers were a product of inflammatory lesions (Trinchieri, 2012). A product of the immune system, acute inflammation is a part of complex biological response to a disruption of tissue homeostasis; namely, harmful stimuli and cellular damage, which can result from either injury or pathogenic invasion (Ashley et al., 2012; Chen et al., 2018; Medzhitov, 2008; Netea et al., 2017). This response involves immune cells and soluble bioactive molecules, such as cytokines or growth factors, creating an inflammatory microenvironment and inducing cell signaling pathways in both damaged tissue and immune cells (Ashley et al., 2012; Chen et al., 2018; Medzhitov, 2008; Netea et al., 2017). The primary physiological function of inflammation is to restore tissue homeostasis, through the removal and clearance of foreign entities and damaged tissue, and induction of wound healing and tissue regeneration (Ashley et al., 2012; Chen et al., 2018; Medzhitov, 2008; Netea et al., 2017). Once the tissue disruption is resolved, inflammation subsides, and homeostasis is restored. However, when inflammation is unresolved and uncontrollably persists, a variety of pathologies can emerge—including cancer, as

overlap exists between the molecular targets and signaling pathways in inflammation and tumorigenesis (Chen et al., 2018; Furman et al., 2019; Landskron et al., 2014).

The first experimental evidence of the relationship between cancer and inflammation originated in 1863 with the German physician-scientist Rudolf Virchow, who observed that inflammatory immune cells could be found infiltrating tumorous tissue (Balkwill and Mantovani, 2001). Since then, ever-growing bodies of evidence have linked cancer and inflammation together, and it has become generally accepted fact that unregulated inflammation in the tumor microenvironment can impact upon almost every aspect of tumorigenesis (Aggarwal et al., 2009; Coussens and Werb, 2002; Gonzalez et al., 2018; Grivennikov et al., 2010; Mantovani et al., 2008; Shalapour and Karin, 2015; Trinchieri, 2012; Wu and Zhou, 2009). It is likely that cancer cells are co-opting the normal inflammatory process for their own purposes (Shalapour and Karin, 2015). This relationship between the two was cemented a decade ago when tumor-promoting inflammation was recognized as a hallmark of cancer (Colotta et al., 2009; Hanahan and Weinberg, 2011). As such, a great deal of effort has been invested in understanding the relationship between cancer and inflammation for potential therapeutic value (Comen et al., 2018; Pandya et al., 2016; Samadi et al., 2015; Zhang et al., 2017).

While *Drosophila* does not have a true adaptive immune system, inflammation is primarily a function of innate immunity, the evolutionarily more ancient branch of the immune system (Newton and Dixit, 2012; Shalapour and Karin, 2015; Xiao, 2017).

There is a great deal of conservation between *Drosophila* and vertebrate innate immunity (Buchon et al., 2014; Hoffmann et al., 1999), and the simplified nature of the system proves to be useful in circumventing the complications associated with mammalian systems and adaptive immunity (Wang et al., 2014).

Drosophila has different types of blood cells circulating in their hemolymph, collectively called hemocytes. Two cell types, lamellocytes and crystal cells, are more specialized cells concerned with the encapsulation and melanization of pathogens too large to be phagocytosed (Vlisidou and Wood, 2015); melanization produced by crystal cells also facilitates efficient wound healing (Vlisidou and Wood, 2015). The main hemocyte type, comprising the vast majority of hemocytes (~95%), are plasmatocytes; these are phagocytic cells similar to mammalian macrophages, monocytes, and neutrophils (Buchon et al., 2014; Honti et al., 2014; Williams, 2007). Like their mammalian counterparts, these macrophage-like innate immune cells recognize pathogen invasion or damage-associated molecular patterns (DAMPs) that are released from compromised cells (Newton and Dixit, 2012; Shaukat et al., 2015). Once recruited and activated by these signals, they trigger inflammation and the release of cytokines that continue shaping the immune response (Newton and Dixit, 2012; Shaukat et al., 2015). Most importantly, hemocytes have also been shown to be associated with *Drosophila* epithelial tumors, where they can have similar tumor-promoting effects through the release of cytokines such as the Unpaired family of ligands (orthologous to IL-6) and pro-inflammatory molecules, such as TNF-alpha

and the activation of JNK signaling (Cordero et al., 2010; Gonzalez et al., 2018; Perez et al., 2017).

In the previous chapter, I demonstrated that TNF-signaling appears unimportant for JNK activation in EJS tumors; however, I did observe strong hemocyte presence on EJS tumors recruited through an ROS-independent mechanism. Therefore, I explored the potential role of caspases in the recruitment of hemocytes to the tumor. Additionally, I sought to determine what purpose these immune cells might serve by functionally testing their contribution to the tumor. As in the previous chapter, I will also refer to hemocytes found on EJS tumors as Drosophila tumor-associated macrophages (DTAMs).

Results

DTAM presence on EJS tumors is limited by non-apoptotic caspase inhibition of JNK activity

To assay the presence of hemocytes on EJS tumors, I used a transgenic marker, *HmlΔDsRed.nls* (*Hml-dsRed*), to visualize the hemocytes (Clark et al., 2011; Makhijani et al., 2011). This marker utilizes the promoter of a hemocyte-specific gene, *Hemolectin* (*Hml*), to drive expression of a nuclear localized RFP specifically in plasmatocytes and crystal cells (Clark et al., 2011; Goto et al., 2003; Makhijani et al., 2011). Hml-dsRed positive hemocytes overlap almost entirely with the same population of hemocytes identified by the hemocyte-recognizing H2 antibody (Kurucz et al., 2003; Makhijani et al., 2011); because of this, I decided to use this marker for

more reliable visualization of DTAMs on EJS tumors, as tightly clustered hemocytes are hard to individually distinguish through antibody staining.

Large numbers of DTAMs can be found on EJS tumors (Figure 4.1 A). Interestingly, I observed that Dronc downregulation resulted in a significant increase of around 75% in the number of hemocytes attached to tumors (Figure 4.1 B). Importantly, the increase in the number of DTAMs was apparent after just one day of EJS induction, even before EJS and *Dronc*-deficient EJS tumors began to differ in size. The density of hemocytes per unit of tumor area was similarly increased in *Dronc*-deficient tumors (Figure 4.1 C), suggesting that caspases can limit the initial recruitment of DTAMs to EJS tumors. Additionally, the excess of DTAMs appeared to further increase over time (Figure 4.1 D).

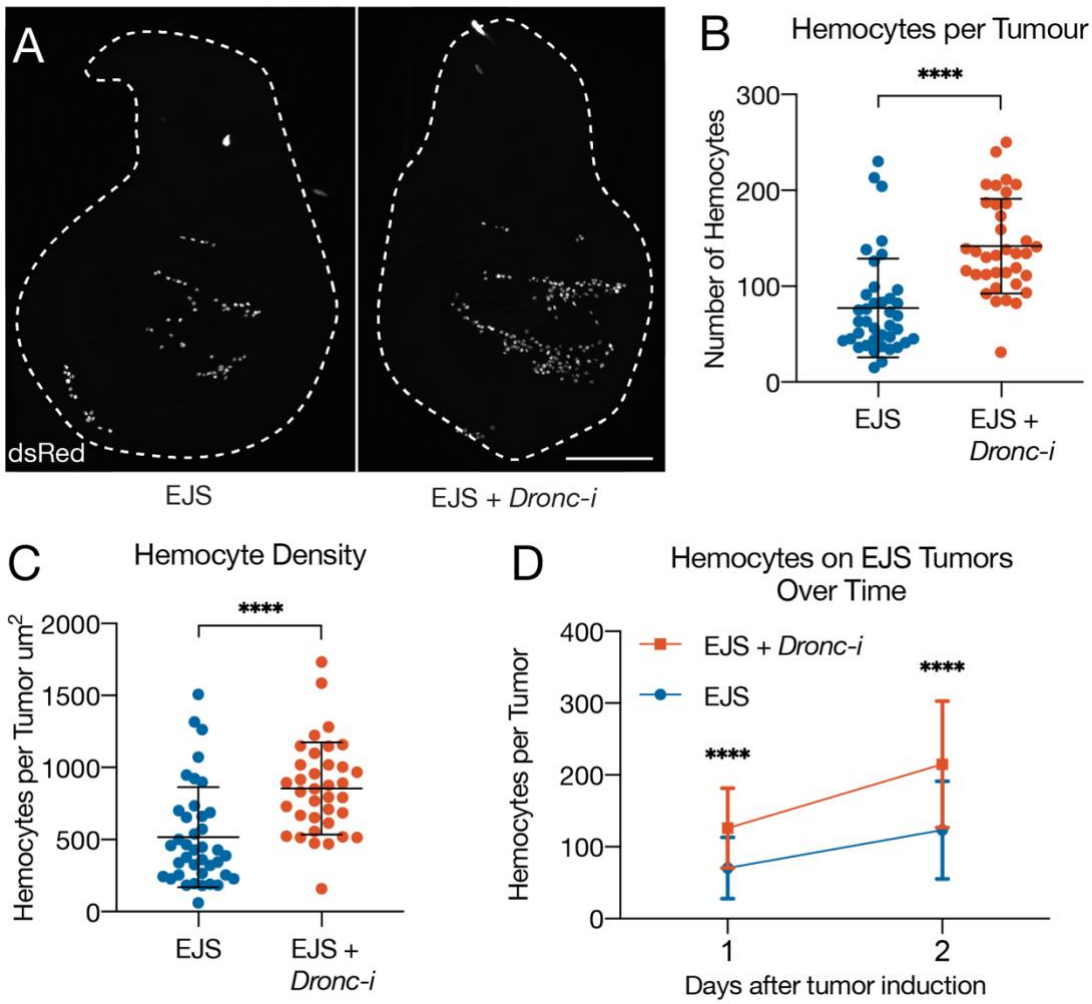


Figure 4.1 **Non-apoptotic Dronc activity limits hemocyte presence on EJS tumors.**
(A) Representative maximum projected confocal images of control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) tumors after 1 day of EJS induction with hemocytes labeled with *hemolectin*-nls-dsRed *(hml*-dsRed). Dashed white outline of wing discs obtained by tracing DAPI staining (not shown). Scale bar: 100μm.
(B) Quantification of number of hemocytes per tumor in control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) tumors after 1 day of EJS induction. The mean ±SD are also plotted. Statistical significance was determined using a Student's t-test; **** $p < 0.0001$. Numbers of wing discs analyzed for EJS tumors: 39; for EJS + *Dronc-i* tumors: 36.
(C) Quantification of the density of hemocytes (calculated as the number of hemocytes per tumor μm^2) in control (EJS) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*) tumors after 1 day of EJS induction. The mean ±SD are also plotted. Statistical significance was determined using a Student's t-test; **** $p < 0.0001$. Numbers of wing discs analyzed for EJS tumors: 39; for EJS + *Dronc-i* tumors: 36.
(D) Time series of the number of hemocytes per tumor in control (EJS, blue) and EJS + *UAS-Dronc-RNAi* (EJS + *Dronc-i*, orange) after 1 and 2 days of EJS induction. Plotted are the mean ±SD. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test; **** $p < 0.0001$. Numbers of wing discs analyzed for each time point are as follows (**Day**: n [EJS]; n [EJS + *Dronc-i*]): (**1**: 82; 78), (**2**: 35; 29).

The trend of a further increase in DTAMs located on *Dronc*-deficient EJS tumors over time suggested that other factors besides recruitment could be involved to explain why there are more DTAMs found. Because it has been reported before that tumors in Drosophila are able to stimulate the proliferation of circulating hemocytes in larvae bearing tumors (Pastor-Pareja et al., 2008), I looked at potential proliferation of the hemocytes associated with EJS tumors. Unexpectedly, I was able to detect the incorporation of EdU in hemocytes *in situ* on the tumor, indicating that hemocytes on EJS tumors are entering S-phase. While not direct evidence, the synthesis of new DNA is heavily suggestive of proliferation. This was a striking observation, as proliferation has only been reported previously in circulating hemocytes, or *in situ* in specific hematopoietic pockets (Makhijani et al., 2011).

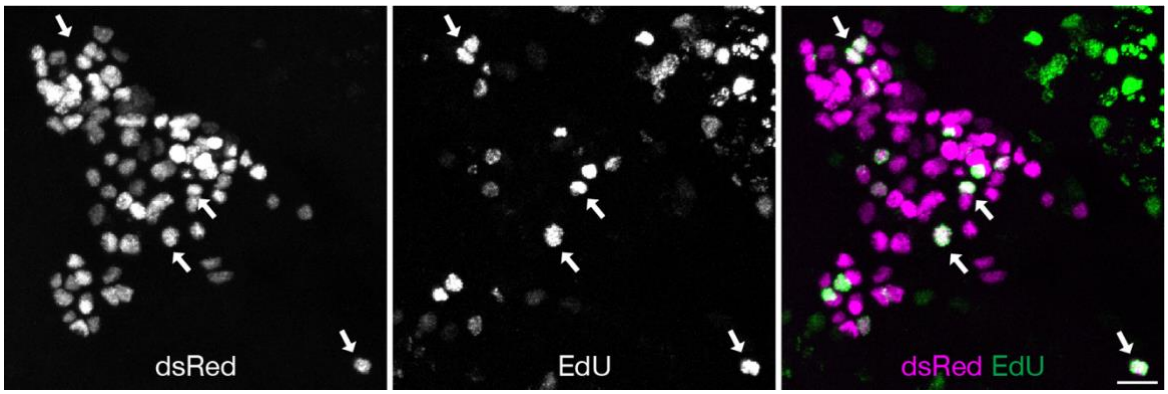


Figure 4.2 **Hemocytes on EJS tumors synthesize DNA and potentially proliferate *in situ*.**
Confocal image of hemocytes labeled with *Hml-dsRed* found on EJS tumors after five days of EJS induction (gray, magenta); notice the colocalization of EdU incorporation (gray, green) with the nuclear *Hml-dsRed* (arrows, white indicates overlap in merged image).

In the previous chapter, I revealed that JNK signaling is necessary for the malignant features of EJS tumors; additionally, the pathway is critical for the phenotypes associated with *Dronc*-deficiency to materialize. To determine if the increased DTAM presence on caspase-deficient EJS tumors had a similar dependency upon JNK

signaling, I assayed the presence of hemocytes on EJS and *Dronc*-deficient EJS tumors with and without suppression of JNK by employing JNK^{DN}. Interestingly, control EJS tumors and JNK^{DN} EJS tumors appear to have similar numbers of hemocytes, indicating that a JNK-independent mechanism is capable of recruiting hemocytes to EJS tumors (Figure 4.3 A, B). Crucially however, I noticed that the inhibition of JNK abolished the increase in hemocytes seen in *Dronc*-deficient conditions (Figure 4.3 A, B). This result indicates that, similar to the previously identified features of *Dronc*-deficient tumors, the excess of DTAMs present in *Dronc*-deficient conditions is most likely the result of an event downstream of JNK signaling. JNK-dependent degradation of the basement membrane (BM) through MMP expression has previously been shown to attract hemocytes (Diwanji and Bergmann, 2020; Pastor-Pareja et al., 2008). However, preliminary data suggest that mechanisms other than the detection of BM disruption recruits hemocytes to EJS tumors, as over-expression of the *Drosophila* tissue inhibitor of metalloproteases (Timp) (Wei et al., 2003) has no effect on the recruitment of hemocytes to EJS tumors (Figure 4.3 C).

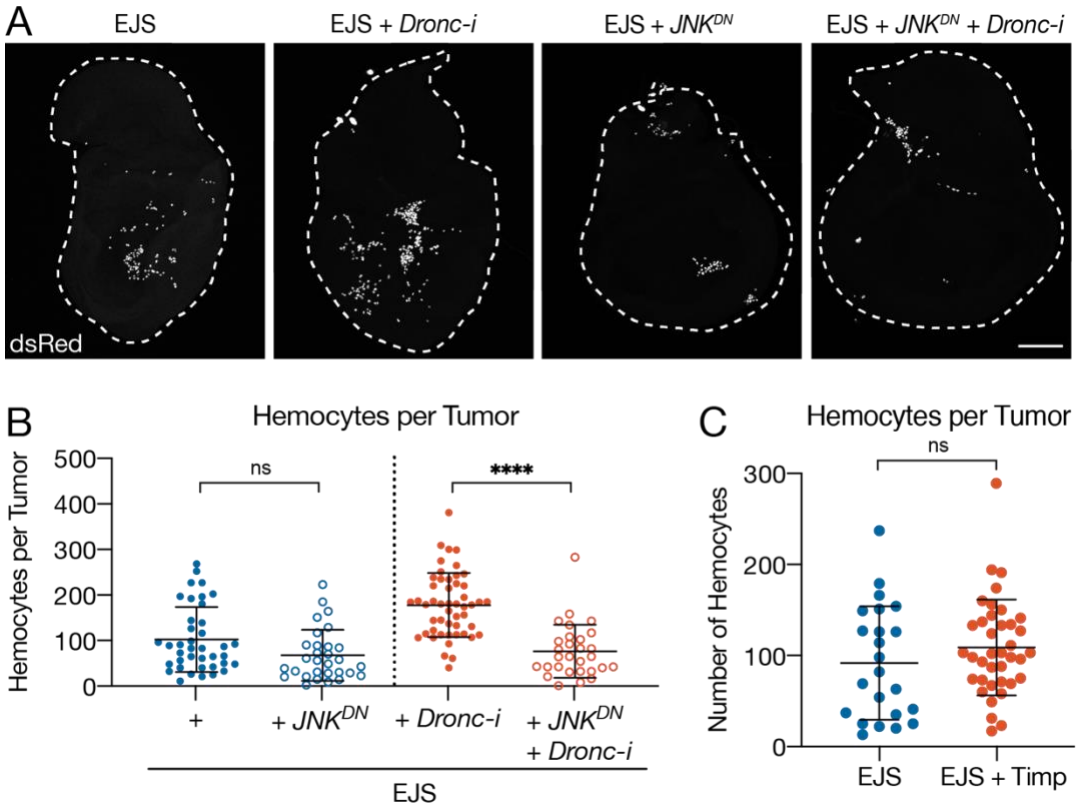


Figure 4.3 **JNK signaling is necessary only for increased recruitment to *Dronc*-deficient EJS tumors.**
(A) Representative maximum projected confocal images of control tumors (EJS), Dronc-deficient EJS tumors (EJS + *Dronc-i*), JNK-suppressed EJS tumors (EJS + JNK^{DN}), and both JNK-suppressed and *Dronc*-deficient EJS tumors (EJS+ JNK^{DN} + *Dronc-i*) after 1.5 days of EJS induction, with hemocytes labeled with *Hml-dsRed* (gray). Dashed white outline of wing discs obtained by tracing DAPI staining (not shown). Scale bar: 100μm.
(B) Quantification of the number of hemocytes per tumor in control (EJS), *Dronc*-deficient EJS (EJS + *Dronc*-i), JNK-suppressed (EJS + JNK^{DN}), and both JNK-suppressed and *Dronc*-deficient EJS tumors (EJS+ JNK^{DN} + *Dronc-i*) after 1.5 days of EJS induction. The mean ±SD are plotted. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons tests. ns – not significant $p > 0.05$, **** $p < 0.0001$. Numbers of wing discs analyzed for EJS tumors: 39; for EJS + *Dronc-i* tumors: 50; for EJS + JNK^{DN} tumors: 30; for EJS + *Dronc-i* + JNK^{DN}: 29.
(C) Quantification of the number of hemocytes per tumor in control (EJS) and EJS + UAS-*Timp* (EJS + Timp) tumors. The mean ±SD are also plotted. Statistical significance was determined using a Student's t-test; ns – not significant $p > 0.05$. Numbers of wing discs analyzed for EJS tumors: 23; for EJS + Timp tumors: 38.

To determine how much of an impact the potential *in situ* proliferation of hemocytes had on the number of DTAMs found on tumors, I examined the relationship between the numbers of DTAMs with EdU incorporation and total DTAM numbers in EJS tumors. After the first day of EJS induction, I found that both EJS and *Dronc*-deficient

EJS tumors showed a similar mean percentage of EdU$^+$ hemocytes, around 10% (Figure 4.4). By the second day of EJS induction, EJS and *Dronc*-deficient EJS tumors again showed a similar mean percentage of EdU$^+$ hemocytes, around 20% (Figure 4.4), though no statistical differences could be found between any comparisons through a one-way ANOVA test (Figure 4.4). While this might suggest that DTAMs found on EJS tumors enter S-phase at similar proportions, regardless of the presence of caspases, this conclusion might be misleading as there is a large amount of variability in the percentage of EdU$^+$ hemocytes (Figure 4.4). Instead, the variability suggests that DNA synthesis and any subsequent proliferation alone may not be the only factor determining the number of hemocytes found on EJS tumors. To further examine the potential influence of proliferation on total DTAM numbers, I examined the correlation between EdU$^+$ DTAMs and total number of DTAMs.

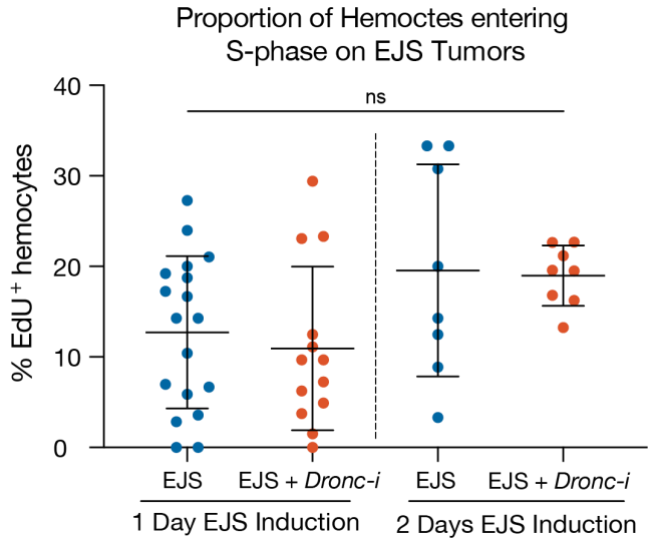


Figure 4.4 **Proportion of EdU$^+$ hemocytes on EJS tumors.**
Quantification of the proportion of hemocytes found on control tumors (EJS) and EJS + *UAS-Dronc-RNAi* tumors (EJS + *Dronc-i*) after one and two days of EJS induction. The mean ±SD are also plotted. Statistical significance was determined by an ordinary one-way ANOVA; ns – not significant $p > 0.05$. Numbers of wing discs analyzed each time point are as follows (**Day**: n [EJS]; n [EJS + *Dronc-i*]): (**1**: 18; 13), (**2**: 8; 8).

This analysis revealed that there is initially a positive correlation (R = 0.6498) between EdU^+ hemocytes and total DTAMs in control EJS tumors (Figure 4.5). This positive correlation remains over time, though it weakens and is no longer statistically significant by the 2nd day of EJS induction (R = 0.3869). Additionally, the coefficient of determination, or R^2 values for early and later time points (R^2 = 0.4222 and .1497, respectively), indicates that only a fraction of variation observed among total numbers of DTAMs can be explained by the number of hemocytes entering S-phase. Taken together with the large variances in percentages of EdU^+ hemocytes, this weak positive correlation suggests that any *in situ* proliferation of hemocytes following DNA synthesis accounts for only a portion of the DTAM presence. Other factors in addition to proliferation, such as the recruitment and adherence of circulating hemocytes, contribute to the amount of DTAMs found on EJS tumors.

However, the situation is different with hemocytes found on *Dronc*-deficient EJS tumors. Initially, there was no correlation between EdU^+ DTAMs and total DTAM number in *Dronc*-deficient tumors, with R approaching zero (R = -0.002231). Additionally, an extremely low coefficient of determination indicates that essentially none of the variation observed among total number of DTAMs can be explained by the number of EdU^+ hemocytes (R^2 = 4.976e-006) (Figure 4.6). By the 2nd day of EJS induction however, there was a drastic change: the number of EdU^+ DTAMs was strongly correlated with the total number of hemocytes found on *Dronc*-deficient tumors, and almost all of the variation observed could be explained by the number of EdU^+ hemocytes (R = 0.97, R^2 = 0.941).

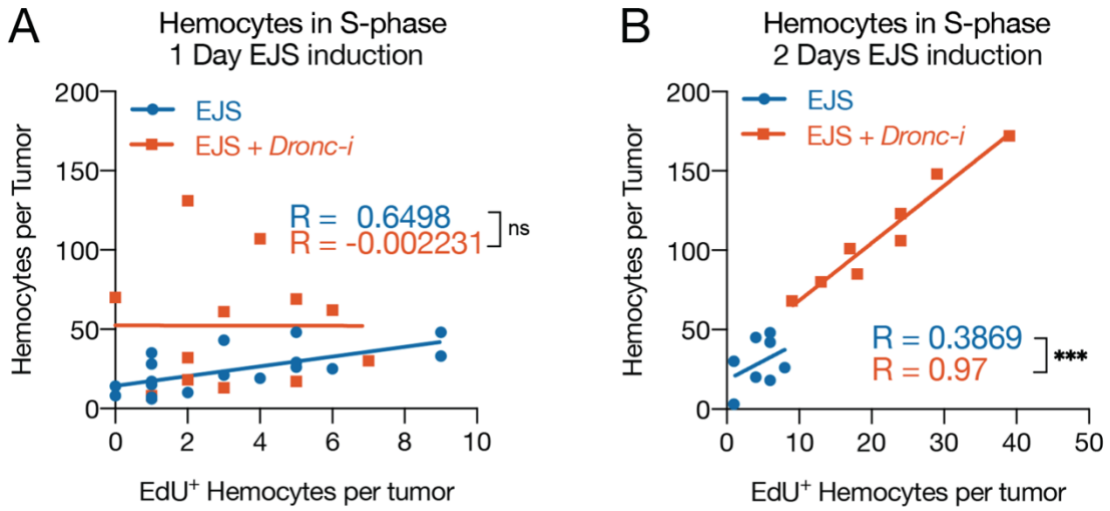


Figure 4.5 **Correlation between hemocytes and EdU⁺ hemocytes**
(A, B) Total number of hemocytes per tumor as a function of EdU⁺ hemocytes per tumor in control (blue, EJS) and EJS + *UAS-Dronc-RNAi* tumors (EJS + *Dronc-i*, orange) after one (A) and two (B) days of EJS induction. Pearson R coefficients displayed. The R values for EJS 1 Day and EJS + *Dronc-i* tumors were significant, ** p = 0.0035 and **** p < 0.0001, respectively. Statistical significance between each day's correlation coefficients was determined by a Fisher-Z-Transformation, via a two-tailed t-test. ns – not significant, p = 0.0569; *** p =.0.0077. Numbers of wing discs analyzed for each time point are as follows (**Day**: n [EJS]; n [EJS + *Dronc-i*]): (**1**: 18; 13), (**2**: 8; 8).

This diverging phenomenon occurring over time led me to two conclusions: first, that the initial recruitment of hemocytes in *Dronc*-deficient tumors is much higher than in control EJS tumors. This is supported by the fact that there is no correlation between DTAMs actively synthesizing DNA for proliferation and total hemocyte numbers on *Dronc*-deficient EJS tumors, suggesting that the increased DTAM numbers observed would have little to do with *in situ* proliferation. Second, the strong correlation found by the second day of EJS induction strongly suggests that after the initial recruitment of hemocytes, potential *in situ* proliferation of DTAMs is a much stronger factor in accounting for the numbers of hemocytes found on EJS tumors. This relationship can also be seen in the relatively tight variation observed in the percentage of EdU⁺ hemocytes found on *Dronc*-deficient EJS tumors after 2 days (Figure 4.4). Altogether,

these observations suggest that increased hemocyte recruitment can explain the initial increase in numbers of DTAMs found on *Dronc*-deficient EJS tumors; additionally, the detection of S-phase hemocytes suggests that *in situ* proliferation could also contribute to the increasing DTAM numbers.

Functional hemocytes contribute to tumor growth

Having determined that caspase-deficiency results in an increased number of hemocytes on EJS tumors, I wanted to investigate the potential influence the hemocytes might have on the tumor. As immune cells, hemocytes could have a negative impact on tumor growth as they attempt to eliminate malignant tissue; hemocytes associated with tumors have previously been shown to restrict tumor growth (Parisi et al., 2014; Pastor-Pareja et al., 2008). However, as discussed earlier, hemocytes have also been shown to promote tumor growth through tumor-promoting inflammation (Cordero et al., 2010; Fogarty et al., 2016; Perez et al., 2017). Could the increased presence of DTAMs on *Dronc*-deficient EJS tumors be an attempt to further restrict EJS tumor growth? Or is the increased growth of EJS tumors a consequence of increased hemocyte numbers?

To resolve these questions, I attempted to eliminate hemocytes from EJS-induced larvae. Different reports have shown the successful ablation of post-embryonic hemocytes in physiological conditions through genetic manipulations (Charroux and Royet, 2009; Defaye et al., 2009; Lam et al., 2010; Shia et al., 2009; Tassetto et al., 2017), all of which employed hemocyte-specific overexpression of pro-apoptotic

factors to induce apoptosis (*Hml-Gal4* and *UAS-reaper*, for example). Because I was already employing the Gal4/*UAS* system to induce EJS in wing discs, hemocyte-specific manipulations would require the use of a different binary expression system to avoid complications. Thus, I decided to use the QF/*QUAS* system to independently manipulate hemocytes without cross-reactivity in EJS tissues, and vice versa (Riabinina and Potter, 2016). By using an *Hml-QF2* driver, which uses a non-toxic variant of the QF transcription factor (Lin and Potter, 2016; Riabinina et al., 2015), I was able to overexpress the pro-apoptotic gene *reaper* specifically in hemocytes (*Hml>reaper*) (Perez-Garijo et al., 2013). As shown in previous literature (Tassetto et al., 2017), I confirmed that the *Hml>reaper* genetic conditions almost completely ablated hemocytes in a wild-type background (Figure 4.6).

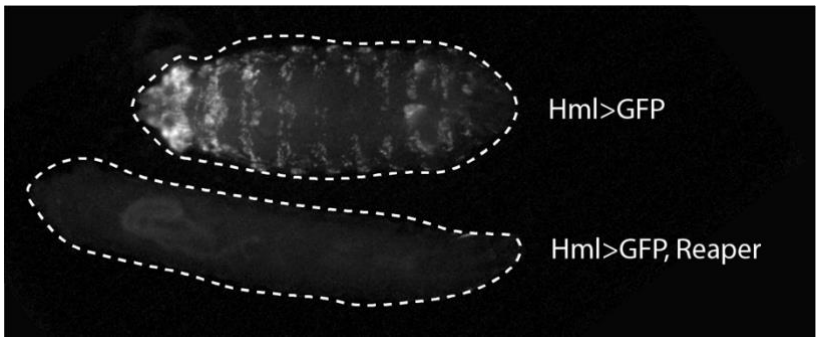


Figure 4.6 **Hemocyte-specific overexpression of *reaper* in wild-type larvae ablates vast majority of hemocytes.**
Top: image of a *Drosophila* larva expressing GFP specifically in hemocytes using the *Hml-QF2* driver. Hemocytes can be easily detected. The larva is in a *Tubby* (*Tb*) background due to the nature of the cross. Bottom: in a sibling larva also expressing *reaper*, no hemocytes can be detected.

Next, I combined *Hml>reaper* with the EJS system to simultaneously induce oncogenic transformation and elimination of hemocytes. Unexpectedly however, I found that the hemocyte-specific expression of *reaper* using *Hml>reaper* did not reduce the presence of hemocytes adhered to EJS tumors. In fact, there was virtually

no effect on DTAM numbers on EJS or *Dronc*-deficient EJS tumors in hemocyte-ablating conditions (Figure 4.7 A). Interestingly, despite the apparent lack of effect on DTAM populations, there was an observable effect on tumor size. EJS tumors induced in an *Hml>reaper* background grew to a smaller size than control EJS tumors over the same period of three days (Figure 4.7 B). Similarly, *Dronc*-deficient EJS tumors in an *Hml>reaper* background were also smaller than their control counterparts (Figure 4.7 C). Exactly how *reaper*-expressing hemocytes manage to survive *reaper*-induced apoptosis is still unknown, nor is it known what changes might be induced in *reaper*-expressing hemocytes. However, it is clear that hemocyte-specific changes result in a reduction of tumor size; this indirectly suggests that hemocytes could be contributing to basal EJS tumor growth, as well as to the increased growth in caspase-deficient EJS tumors. Interestingly, it appears that at the same time that the expression of *reaper* does not manage to eliminate DTAMs in the EJS background, it also does not appear to compromise their ability to be recruited to the tumors or proliferate, as there is no difference in numbers between non-killing conditions and killing conditions.

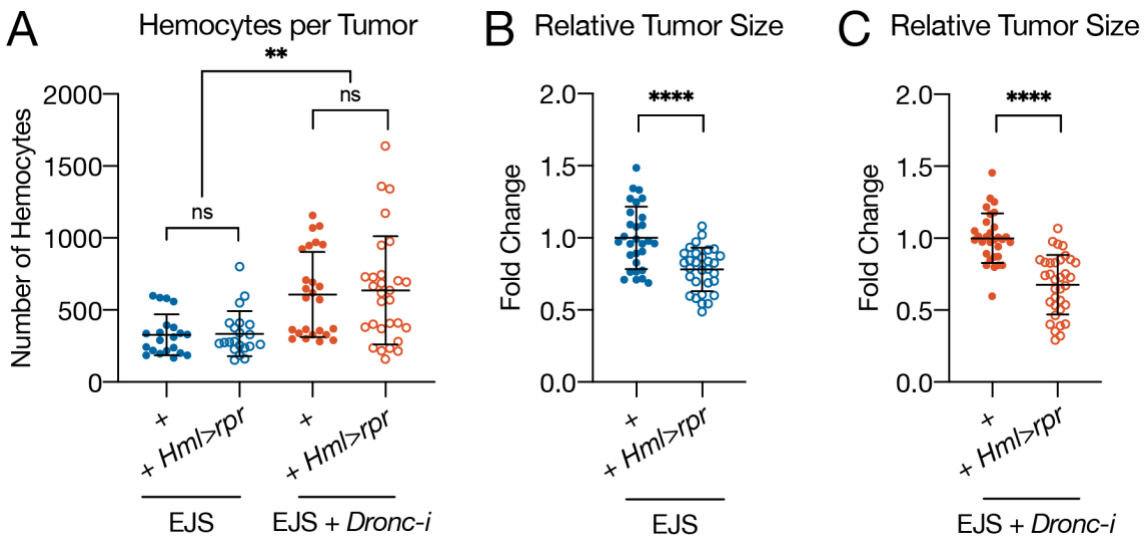


Figure 4.7 **EJS tumors in *Hml>rpr* backgrounds are significantly smaller.**
(A) Quantification of the number of hemocytes per tumor in control and *Dronc*-deficient EJS tumors in a genetic background with either wild-type hemocytes (EJS and EJS + *Dronc-i*) or hemocytes expressing the pro-apoptotic gene *reaper* (EJS + *Hml>rpr* and EJS + *Dronc-i* + *Hml>rpr*) after three days of EJS induction. The mean ±SD are also plotted. Statistical significance was determined by one-way ANOVA with Turkey's multiple comparisons tests. ns – not significant $p > 0.05$. ** $p < 0.01$. Numbers of wing discs analyzed for EJS tumors: 21; for EJS + *Hml>rpr* tumors: 21; for EJS + *Dronc-i* tumors: 25; for EJS + *Dronc-i* + *Hml>rpr*: 29.
(B) Quantification of the relative sizes of control tumors (EJS) and EJS tumors in an *Hml>rpr* background (EJS + *Hml>rpr*) after three days of EJS induction. Control tumors were used for normalization. The mean ±SD are also plotted. Statistical significance was determined using an unpaired Student's t-test. **** $p < 0.0001$. Numbers of wing discs analyzed for EJS tumors: 29; for EJS + *Hml>rpr* tumors: 30.
(C) Quantification of the relative sizes of *Dronc*-deficient EJS tumors (EJS + *Dronc-i*) and EJS + *Dronc-i* tumors in an *Hml>rpr* background after three days of EJS induction. The mean ±SD are also plotted. Statistical significance was determined using an unpaired Student's t-test. **** $p < 0.0001$. Numbers of wing discs analyzed for EJS + *Dronc-i* tumors: 30; for EJS + *Dronc-i* + *Hml>rpr* tumors: 34.

Discussion

The influence of caspases on the tumor microenvironment and the activation of the immune system through presentation of DAMPs has been recognized across different species (Fianco et al., 2018; Legrand et al., 2019). However, the relationship between the two is not fully understood, though the *Drosophila* model is well poised to investigate this interaction (Bangi, 2013; Wang et al., 2014; Xu et al., 2018a). Recent studies have shown that caspases are responsible for the release of ROS

into the tumor microenvironment, recruiting macrophage-like hemocytes to tumorous tissue (Fogarty et al., 2016; Perez et al., 2017). These hemocytes then release Eiger (TNF orthologue), activating JNK signaling and tumor growth (Fogarty et al., 2016; Perez et al., 2017). Additionally, the JNK signaling in these tumorous tissues leads to caspase activation and further ROS release, ultimately resulting in a positive-feedback loop involving hemocytes, ROS, and tumor promoting inflammation (Perez et al., 2017).

However, in this chapter, I show that caspases function in EJS tumors in an almost entirely different manner. Hemocyte recruitment is not only unaffected by a lack of ROS released from EJS tumors (as shown in previous chapter), but also augmented by caspase deficiency, resulting in an increased hemocyte presence on *Dronc-deficient* EJS tumors. As opposed to acting as mediators of tumor-promoting inflammation and fueling tumor growth, caspases in EJS tumors limit the presence of immune cells and tumor growth. Even when hemocyte numbers are normalized for tumor size, there is still a clear increased density of DTAMs found on caspase-deficient EJS tumors than on control EJS tumors (Figure 4.1 C). This suggests that the increased numbers of hemocytes found is not simply scaling with increased tumor size. Instead, *Dronc* depletion in EJS tumors changed an intrinsic property to the tumor itself, resulting in the recruitment of additional hemocytes. Supporting this, I show that the increase in hemocyte numbers on *Dronc*-deficient tumors is dependent upon JNK activity in EJS tumors, similar to other caspase-deficient phenotypes discussed in the previous chapter. As I had previously shown that JNK activity is

limited by caspases in EJS tumors, increased JNK activity in *Dronc*-deficient tumors could be the source of the increased DTAM presence.

Interestingly, I observed that even with heavily suppressed JNK activity, hemocytes can still be recruited to EJS tumors. Presumably, these hemocytes are also proliferating, as the number of hemocytes found on JNK-suppressed EJS tumors are no different from those found on normal EJS tumors, where I have confirmed that *in situ* hemocyte proliferation is at least partially responsible for the number of DTAMs. Independently, this is an interesting observation as to my knowledge, this is the first time that hemocytes have been shown to proliferate *in situ* as part of a response to a tumor. Previously, *in situ* proliferation had only been shown in sessile hemocytes at specific hemopoietic tissues (Leitao and Sucena, 2015; Markus et al., 2009), and it had only been shown that circulating hemocytes proliferate in response to JNK-dependent secretion of cytokines from tumors (Pastor-Pareja et al., 2008). This truly novel observation suggests that even with downregulated JNK activity, EJS are capable of sustaining a basal level of recruitment for hemocytes to EJS tumors, as well as stimulating DTAM proliferation.

However, caspase-limited JNK activity is ultimately responsible for the increased numbers found in caspase-deficient conditions, as suppression of JNK abolishes any increase in hemocytes due to *Dronc*-deficiency. My analyses of the correlations between proliferating DTAMs and the total number of DTAMs found on EJS and *Dronc*-deficient EJS tumors over time suggests that JNK activity increases the

number of hemocytes found on *Dronc*-deficient tumors by affecting at least two factors: the initial recruitment of hemocytes, and subsequently, the entrance into S-phase and the potential *in situ* proliferation of DTAMs.

ROS, produced downstream of JNK activity in wounds and tumors, had previously been shown to be a potent hemocyte attractant (Babcock et al., 2008; Fogarty et al., 2016; Perez et al., 2017; Razzell et al., 2013). However, since I have already established that it is largely dispensable for hemocytes attraction to EJS tumors, I looked towards other JNK-dependent mechanisms that have been shown to affect hemocyte recruitment for explanation. In both injured tissue and invasive tumors, surveilling hemocytes are known to detect and adhere to damaged basement membrane (BM) (Diwanji and Bergmann, 2020; Pastor-Pareja et al., 2008), and JNK-dependent expression of MMPs in $Ras^{V12}/scrib^{-/-}$ tumors has been shown to be responsible for BM degradation (Srivastava et al., 2007; Uhlirova and Bohmann, 2006). Given that *Dronc*-deficient tumors display increased MMP1 expression, it is possible that the resulting increased BM disruption could enhance the attraction of hemocytes to tumors. Preliminary data suggests that BM degradation through MMP activity is not necessary for the basal level of hemocytes found in EJS tumors, as hemocytes can still be found on EJS tumors overexpressing Timp, a *Drosophila* tissue inhibitor of metalloproteases (Wei et al., 2003) (Figure 4.3 C). However, my data suggest that JNK activity is responsible for the additional hemocytes found attached to *Dronc*-deficient tumors. Thus, while a basal level of hemocyte attraction

to EJS tumors could be MMP-independent, it would be interesting to assay the numbers of DTAMs found in *Dronc*-deficient tumors with MMP inhibition.

In addition to the increased initial recruitment of hemocytes, JNK activity in *Dronc*-deficient tumors results in a strong correlation between the number of S-phase DTAMs with the total number of hemocytes found on *Dronc*-deficient EJS tumors after 2 days of EJS induction (Figure 4.5 B). As the correlation between S-phase DTAMs and total number of DTAMs is greater than that found in control EJS tumors, it suggests that proliferation could play a much larger role in accounting for larger numbers of DTAMs on caspase-deficient tumors. How might proliferation be stimulated to such an extent in these tumors? It is possible that the increased expression the Upd ligands (JAK/STAT cytokines) downstream of JNK activity in *Dronc*-deficient EJS tumors (Figure 3.1 E) results in increased proliferation and activation of hemocytes, as has been shown before in hemocyte responses to tumors and wounds (Morin-Poulard et al., 2013). To test this possibility, it would be informative in future experiments to manipulate the JAK/STAT pathway independently in hemocytes. JAK/STAT activity in hemocytes could be downregulated through the expression of a dominant-negative version of Domeless (Dome), the receptor for Upd ligands (Arbouzova and Zeidler, 2006); alternatively, downstream members of the pathway, such as Hop, can be downregulated (Arbouzova and Zeidler, 2006). These manipulations could prevent proliferation in EJS DTAMs.

Finally, in this chapter I examined the functional relationship between DTAMs and EJS tumors. I attempted to remove the hemocyte population from the larval system through hemocyte-specific expression of *reaper*, one of the pro-apoptotic genes in *Drosophila*. While successful hemocyte ablation of most hemocytes has been shown before using this strategy (Charroux and Royet, 2009; Defaye et al., 2009; Lam et al., 2010; Shia et al., 2009; Tassetto et al., 2017), unexpectedly, I observed almost no change in DTAM populations on EJS or *Dronc*-deficient EJS tumors. However, despite their continued presence, EJS tumors induced in *reaper*-expressing hemocyte backgrounds were markedly reduced in size (Figure 4.7 B, C). Thus, expression of *reaper* in hemocytes can have non-cell autonomous effects on EJS tumor growth. A few conclusions can be drawn from this surprising observation.

The first is that hemocytes can potentially play a tumor-promoting role. It should be noted that there is no direct evidence for this given the unsuccessful ablation of hemocytes and the uncertainties about the effects of *reaper*-expression in EJS-associated hemocytes; however, indirect evidence can suggest this possibility. Though not dead, *reaper*-expressing hemocytes are likely functionally compromised, as previous accounts of attempted systemic hemocyte ablation have also reported compromised functionality in the population of hemocytes that managed to survive elimination (Defaye et al., 2009). If hemocytes were functionally restricting tumor growth, or even functionally irrelevant for tumor growth, then presumably their impairment would result in larger tumors or unchanged tumors, which is not the case (Figure 4.7 B, C).

Of course, it is also possible that were hemocytes attempting to restrict tumor growth, *reaper*-expression somehow increases their tumor-limiting potential (or even confers a tumor-inhibiting ability), also resulting in smaller tumors overall. Alternatively, a population shift in the type of hemocyte present on EJS tumors could explain the reduction of tumor size. Hemocytes are already well known to differentiate into different cell types upon stimulation, such as their differentiation into lamellocytes to encapsulate parasitic wasp eggs (Kim-Jo et al., 2019). Additionally, while most hemocytes express the *Hml* gene, there are subpopulations that do not (Goto et al., 2003; Shin et al., 2020), and these subpopulations have been shown to change in response to ablation (Shin et al., 2020). Interestingly, this report of ablation of *Hml*-positive hemocytes (*Hml-Gal4* driving *UAS-hid, reaper*) resulted in an increase of *Hml*-negative hemocytes and the release of cytokines, eventually suppressing systemic growth in larvae (Shin et al., 2020). Thus, it is possible that *Hml-QF2* driven expression of *QUAS-reaper* leads to a similar shift in hemocyte subpopulations, with growth-suppressing effects on EJS tumors. Even so, I would conclude that the original hemocytes present are tumor promoting, or at the very least, less effective at restricting tumor growth. However, given that both the drivers for apoptosis (*Hml>reaper*) and transgenic marker for hemocytes (*Hml-DsRed*) were derived from the same *Hemolectin* promoter region, it is likely that the same subpopulation of hemocytes are present in both normal and *Hml>reaper* conditions. It is clear that further experiments are required to elucidate the true functional relevance of hemocytes with regard to EJS tumor growth.

The second conclusion from this experiment is that *reaper*-expressing hemocytes are still able to receive signals from the tumor and be successfully recruited even while compromised. They are also still capable of increasing in number on *Dronc*-deficient EJS tumors, as the number of DTAMs found on EJS and *Dronc*-deficient EJS tumors in an *Hml>reaper* background is virtually identical to the number of DTAMs found on their corresponding control tumors in a wild-type hemocyte background (Figure 4.7 A).

Last, in order to avoid undergoing apoptosis as their counterparts in wild-type larvae (Figure 4.6), *reaper*-expressing hemocytes must be receiving survival cues from the tumor that prevent their demise. As mentioned before, potential proliferative cues released by EJS tumors are the Upd ligands, activators of the JAK/STAT pathway. As JAK/STAT activity is known to lead to the expression of Diap-1, an apoptosis inhibitor (Betz et al., 2008; Recasens-Alvarez et al., 2017), it is possible that the activation of JAK/STAT through Upd protects hemocytes from apoptosis. Intriguingly, this potential protection from chronic expression of pro-apoptotic stimuli would place these cells in a similar position as the “undead cells” discussed in Chapter 1: cells that initiate apoptosis but are unable to complete the program due to expression of effector caspase inhibitors (Fogarty and Bergmann, 2017; Martin et al., 2009; Ryoo and Bergmann, 2012). Previous study of these undead cells has uncovered non-apoptotic caspase functions, including autonomous promotion of invasion and non-autonomous induction of over-proliferation in neighboring cells (Diwanji and

Bergmann, 2019; Perez-Garijo, 2018; Rudrapatna et al., 2013). It would be extremely exciting to determine if the behavior of “undead” hemocytes observed is also an effect of novel non-apoptotic caspase activity that either imparts tumor-restrictive properties to hemocytes or inhibits their tumor-promoting abilities.

If the hemocytes are indeed tumor-promoting, through what mechanism could they be promoting tumor growth? Previous observations indicate the importance of Eiger released from hemocytes and activation of the JNK pathway through TNF-signaling (Fogarty et al., 2016; Perez et al., 2017). However, in Chapter 3 I have shown that downregulation of the known putative receptors for TNF-signaling resulted in phenotypically normal EJS tumors, suggesting that Eiger is unimportant for JNK activity in EJS tumors. Instead, I hypothesize that hemocytes may be affecting growth of the tumor through the JAK/STAT pathway. Hemocytes are known to secrete Upd ligands which activate the JAK/STAT pathway, and Upds released from hemocytes are already known to fuel stem cell proliferation in the *Drosophila* intestinal system (Chakrabarti et al., 2016; Jiang et al., 2009; Woodcock et al., 2015; Yang et al., 2015). Given that a core component of **EJS** (**E**GFR and **J**AK/**S**TAT) tumor activation is JAK/STAT activity, it is entirely conceivable that the release of JAK/STAT ligands into the tumor microenvironment could further aggravate malignancy in EJS tumors. Stimulated EJS tissue could eventually lead to secretion of more Upd, thus forming a positive feedback loop between hemocytes and tissue.

Altogether, my results in the chapter suggest that caspases regulate the tumor microenvironment, and subsequently, the recruitment and behavior of the immune system in response to the tumor. Additionally, I suggest that the macrophage-like hemocytes found on EJS tumors could potentially contribute towards tumor growth, which parallels the relationship of TAMs found in mammalian systems (Chen et al., 2019). Importantly, this only occurs when DTAMs are not functionally disabled through *reaper* expression. Together, these results support the growing body of evidence positing *Drosophila* as a suitable model organism for studying the somewhat paradoxical nature of immune cells and promotion of tumor growth (Bangi, 2013; Wang et al., 2014). My findings could help the understanding of similar attempts to study caspase functions in human tumors in relation to the immune system.

Chapter 5

Conclusion and future directions

Summary of Thesis

Caspases and their role in regulating apoptosis have been studied extensively since the description of the first apoptotic caspase, Ced-3 in C. elegans (Nagata, 2018). Likewise, their role in inflammation has also been extensively studied as the first human caspase was discovered to be pro-inflammatory (Kostura et al., 1989; Thornberry et al., 1992). However, the past two decades have also uncovered a plethora of non-inflammatory, non-apoptotic functions (Shalini et al., 2015). As a relatively new and continually growing field, many questions are still unresolved, and there is certainly still much more to discover (Arama et al., 2020).

In this thesis I present my contribution to this developing field, uncovering a new role for caspases in cancer biology. Using the EJS tumor model in *Drosophila* (Herranz et al., 2012), I have described a novel tumor-suppressing function for caspases; importantly, this tumor-suppressing ability is truly non-apoptotic in nature, defying the conventional association of caspases and cancer through the evasion of cell death. Additionally, I draw multiple parallels between EJS tumors and the regenerative wound-healing process. With further studies, the EJS tumor model offers the chance of more insights into the complex and context dependent relationship between caspases, cancer and the immune system.

To begin, I first used the recently developed DBS-S-QF caspase sensor to empirically determine that there is widespread non-apoptotic Dronc caspase activity in EJS tumors (Baena-Lopez et al., 2018a) (Chapter 2). To determine the functionality of this caspase activity, I genetically manipulated the major initiator caspase in *Drosophila*, Dronc, using tissue-specific RNA interference (RNAi) or conditional knock-out mutants to downregulate the gene. I observed that *Dronc*-deficient EJS tumors increased in size compared to control tumors over time. I unambiguously demonstrated that the increase in size was not due to a lack of apoptosis, as efficient inhibition of the apoptotic pathway through downregulation of pro-apoptotic factors had no effect on tumor size; similarly, overexpression of inhibitors of the effector/executioner caspases also produced no noticeable effects. Interestingly, while the downstream components of the caspase cascade are unnecessary for inhibition of EJS tumor growth, downregulation of Dark, a major component of the apoptosome, results in increased tumor growth. I also determined that the increase in tumor size is at least partially due to an increase in proliferation and cell size in *Dronc*-deficient EJS tumors.

To understand how caspases might be restricting tumor growth, I examined the relationship between caspases and the JNK pathway in EJS tumors (Chapter 3). I showed that EJS tumors have strong levels of JNK activation, and that downregulation of *Dronc* results in increased JNK activity, indicating that caspases are inhibiting the pathway in EJS tumors. I then functionally demonstrated that JNK activity is necessary in EJS tumors for malignant features, such as disorganized

overgrowth as well as the loss and disruption of cell identity in EJS tumors. Importantly, this is similar to how JNK activity induces the loss of cell identity in tissue regeneration. Additionally, I determined that increased tumor growth in caspase-deficient conditions is dependent upon JNK activity, as there are no discernable differences between JNK-suppressed EJS tumors and *Dronc*-deficient JNK-suppressed EJS tumors. Intriguingly, removing either ROS or the receptors for TNF, two classical activators of the pathway (Igaki et al., 2002; Shen and Liu, 2006), from EJS tumors did not result in any phenotypic changes. This indirectly suggests that the source of JNK activation in EJS tumors is appears to be independent of ROS and TNF. A lack of ROS also appeared to have no effect on the recruitment of macrophage-like hemocytes in either control or *Dronc*-deficient EJS tumors.

Because hemocytes could be found in significant numbers on EJS tumors, I assessed their influence on the growth of EJS tumors as well as their relationship with caspase activity (Chapter 4). Downregulation of caspases resulted in an increased number of hemocytes associated with EJS tumors suggesting that caspases non-apoptotically limit the presence of hemocytes (also referred to as DTAMs) on EJS tumors. The numbers of additional hemocytes on *Dronc*-deficient tumors also increased over time. Using EdU incorporation to detect entrance into S-phase and potential proliferation, I was able to determine that DTAMs replicate their DNA *in situ* on the tumor, suggesting that they are in a proliferative state. Importantly, from the correlations of EdU^{+} DTAMs to total number of DTAMs over time, I concluded that the initial increase in hemocytes found on *Dronc*-deficient EJS tumors

is a result of increased recruitment to the tumor. Additionally, this increased recruiting capacity relies on JNK activity, as suppression of JNK signaling in *Dronc*-deficient EJS tumors abolished the increase in numbers of hemocytes. However, after a day of EJS induction, a strong correlation between EdU^+ DTAMs and total DTAM number suggests that, if the hemocytes are proliferating, that the *in situ* proliferation accounts for the higher numbers of hemocytes found on *Dronc*-deficient EJS tumors. Interestingly, control EJS and JNK-suppressed EJS tumors have similar numbers of DTAMs, suggesting that a JNK-independent mechanism is responsible for the basal level of hemocytes found on the tumor.

To determine the functionality of DTAMs associated with EJS tumors, I attempted to systemically eliminate hemocytes from the animal through hemocyte-specific overexpression of the pro-apoptotic factor *reaper* (Chapter 4). Interestingly, I was unable to fully eliminate hemocytes in EJS-induced larvae, and the numbers of DTAMs found on both EJS and *Dronc*-deficient EJS tumors were unchanged. However, despite my unsuccessful ablation, both EJS and *Dronc*-deficient EJS tumor sizes were reduced in backgrounds with *reaper*-expressing hemocytes. This surprising observation led me to conclude that hemocytes potentially had a tumor-promoting effect on EJS tumors, which was compromised upon *reaper* expression; alternatively, *reaper*-expression could lead to an enhancement, or gain-of-function, of tumor-suppressive abilities. The survival of *reaper*-expressing hemocytes in animals with EJS tumors also suggests a model of bidirectional communication between EJS tumors and hemocytes, whereby EJS tumors also provide survival and proliferation

cues to DTAMs, overriding apoptosis in these cells. This is especially compelling as the numbers of hemocytes found on EJS tumors were unchanged, regardless of hemocyte-specific *reaper* expression, indicating that even functionally compromised, hemocytes are just as capable of surviving and increasing in number, potentially through proliferation *in situ*.

In the remainder of this chapter, I will attempt to place the relevance of these results in the context of the field of caspases and tumor biology at large, while also proposing future experiments that could resolve questions and better understand the mechanism behind how caspases act as tumor suppressors independently of apoptosis.

Discussion

Tumors can be formed entirely of cells with non-apoptotic caspase activity

Non-apoptotic caspase activity has previously been shown to be widespread at an organism level (Baena-Lopez et al., 2018a; Ding et al., 2016; Tang et al., 2015), and as discussed before in this thesis, non-apoptotic caspase activity is known to be present in cancer cells. However, my analysis in EJS tumors using a novel caspase sensor is the first indication that nearly all cells in a tumor can undergo activation of caspases without completing the apoptosis program, or, alternatively, be derived from caspase-activating cells that are not eliminated (Chapter 2).

The presence of such previously undescribed ubiquitous non-apoptotic caspase activity in certain tumor types can have huge implications for the field of cancer biology, especially in the domain of caspase- and apoptosis-based therapies for cancer treatment. Modulation of caspase activity to reinstate apoptosis in cancer cells for this purpose is already a subject of much study (Hensley et al., 2013; Kim et al., 2015; MacKenzie and Clark, 2008); however, one must be cautious of assuming that any caspase activity is beneficial caspase activity (Zhao et al., 2018). This thesis alone demonstrates the ability of caspases to regulate cell-signaling pathways along with the tumor microenvironment (Chapters 3 and 4). Given the wide range of other non-apoptotic functions available to caspases, the potential for caspase activation to actually do more harm than good has been recognized (Jager and Zwacka, 2010). Indeed, the activation of caspases in current radiotherapies and chemotherapies have already shown the potential for negative consequences (Cheng et al., 2015; Lee et al., 2017; Liu et al., 2015; Rahmanian et al., 2016; Sofia Vala et al., 2010; Xu et al., 2018b), and a recent study has indicated that only a combination of radiation with inhibition of both caspase-9 and a negative immune regulator leads to effective control of tumor cells (Han et al., 2020). Though some attribute the potential relapse and increased malignancy after therapy to subpopulations of cancer cells that survive the apoptotic stress or are apoptosis-resistant (Safa, 2016), my observation of ubiquitous non-apoptotic caspase activity suggests that potential for negative consequences upon caspase activation are higher than previously thought. Though in this thesis I have described a tumor-suppressing role for the widespread caspase activity, the context-dependent nature of caspase activity guarantees that this will not

always be the case. Thus, it becomes important to understand the context of caspase activation in individual tumors to determine the suitability of potential therapies; indeed, there are ongoing efforts along these lines (Murphy et al., 2013).

Initiator caspases as tumor-suppressors through mechanisms independent of apoptosis

The tumor-suppressing abilities of caspases has long been linked with their almost synonymous roles of executing apoptosis and cell death (Hanahan and Weinberg, 2000, 2011). However, my findings in Chapter 2 unambiguously demonstrate that caspases can carry out non-apoptotic functions that result in tumor inhibition. Instead of cell death, this non-apoptotic caspase activity has significant effects in the signaling profile of cancer cells, limiting pathways such as JNK that compromise cell fate commitment and promote tumor growth (Chapter 3). Simultaneously, caspase activity also alters the microenvironment and the interplay between tumors and the immune system to limit tumor growth (Chapter 4). Importantly, these observations fall in line with the growing consensus in the field of caspase biology that these proteins can affect numerous physiological processes beyond apoptosis, such as intracellular signaling; accordingly, they can influence varying aspects of tumor biology (Jager and Zwacka, 2010; Shalini et al., 2015).

For example, there is evidence to suggest that caspase activity can inhibit metastasis and the invasiveness of tumor cells (Connolly et al., 2014; Jager and Zwacka, 2010).

At the same time, my observations draw attention to the fact that caspase activity can have opposing functions in different contexts. For example, the same caspases responsible for promoting invasiveness and migration, can also inhibit the same processes in different environments (Brentnall et al., 2014; Helfer et al., 2006; Li et al., 2007; Stupack et al., 2006; Teitz et al., 2013); for an example more directly relevant to my observations, a recent study had previously indicated a pro-tumorigenic role for caspases in a similar model of co-operative oncogenesis also dependent upon JNK signaling, the *Ras^{V12}/scrib$^{-/-}$* model (Perez et al., 2017). These differences highlight the importance of understanding mechanisms and the context in which caspases act.

While a complete understanding of the molecular basis behind caspase-mediated EJS tumor suppression remains to be elucidated, my work in Chapter 1 suggests that the initiator caspase Dronc appears to be fully responsible for restricting the proliferation and growth of EJS tumors; tumor suppression in this case does not employ the standard caspase cascade involving the effector caspases. This was demonstrated when inhibition of effector caspases failed to alter tumor properties and elicited no extra growth as loss of *Dronc* did. Interestingly however, at least some elements of the apoptotic machinery appear to be required, as downregulation of the *Drosophila* orthologue of Apaf-1, Dark, results in a less-penetrant but appreciable increase in tumor size. It is possible that the particular RNAi used to downregulate *Dark* was not very effective; alternatively, despite the reduced expression of the protein, there is still enough Dark to form apoptosomes and catalyze the activation of

Dronc. Regardless, this result suggests that the assembly of the apoptosome complex is responsible, at least to some extent, for the non-apoptotic caspase-mediated effects observed in EJS tumors. Dronc's ability to interact with Dark has previously been shown to be important for other non-apoptotic functions as well (Kamber Kaya et al., 2017), and similar non-apoptotic functions for the mammalian equivalent have also recently been documented in the differentiation of muscle progenitor cells (H. Dehkordi et al., 2020). Altogether, these findings indicate that novel non-apoptotic tumor-suppressing functions can be attributed to initiator caspases, adding to a body of evidence that initiator caspases can limit tumorigenic potential (Krelin et al., 2008; Puccini et al., 2013).

However, a distinguishing characteristic of my current work is that the initiator caspase Dronc appears to actively inhibit ongoing tumor growth; in contrast, previous depictions of caspases as tumor suppressors can be described as more preemptive in nature, where the lack of initiator caspases in the face of oncogenic stress simply facilitates cellular transformation and tumorigenesis (Krelin et al., 2008; Puccini et al., 2013). Thus, my results represent the first observations of an entirely new facet of non-apoptotic caspase functions that result in tumor suppression. Importantly, these functions relate to caspase-mediation of both intrinsic cell-signaling pathways such as JNK, as well as extrinsic modulation of the tumor microenvironment and interactions with tumor-associated hemocytes, or macrophage-like cells.

The interplay between caspases, the JNK pathway, and tumor biology is complex and context specific: non-apoptotic caspase inhibition of JNK in EJS tumors limits tumor growth

The JNK pathway and caspase activity are strongly linked together, if primarily through the induction of apoptosis. In this context, caspases and JNK can also positively feedback on each other, working in tandem to ensure effective cell death (Pinal et al., 2019; Shlevkov and Morata, 2012; Wells et al., 2006); additionally, JNK activity downstream of apoptosis/caspase activation release mitogenic signals that induce compensatory proliferation in neighboring cells (Ryoo and Bergmann, 2012; Ryoo et al., 2004). In the absence of apoptosis, the combination of this positive feedback mechanism and JNK's pro-proliferative effects can have disastrous consequences, resulting in tumorigenesis (Diwanji and Bergmann, 2019; Perez-Garijo, 2018; Ryoo and Bergmann, 2012). Studies in *Drosophila* have also established that reactive oxygen species (ROS), immune cells (hemocytes) and TNF (Eiger) are critical factors in this process (Diwanji and Bergmann, 2019; La Marca and Richardson, 2020; Pinal et al., 2019). In a $Ras^{V12}/scrib^{-/-}$ based tumor model, caspases, activated by JNK signaling, are required to mediate the release of ROS; this in turn attracts hemocytes, which release Eiger to stimulate JNK and completes a tumor-promoting feedback amplification loop (Fogarty et al., 2016; Perez et al., 2017).

Strikingly, instead of a similar process occurring in EJS tumors, I observe almost a complete reversal of these requirements: caspases inhibit JNK signaling; ROS is

unnecessary for tumor growth and the recruitment of hemocytes; tumor growth is not dependent upon either ROS or Eiger released from hemocytes. This is a stark contrast to previous depictions where hemocytes are attracted either by chemoattractants such as ROS (Perez et al., 2017) or cleaved Tyrosyl-tRNA synthetase (TyrRS) (Casas-Tinto et al., 2015), or by disruption of the basement membrane (BM) through matrix metalloproteinase (MMP) activity (Diwanji and Bergmann, 2020; Pastor-Pareja et al., 2008). Importantly, all these mechanisms of attraction can be found downstream of JNK activity (Casas-Tinto et al., 2015; Diwanji and Bergmann, 2020), and I have shown that ROS and MMP activity (which in addition to disrupting the BM, also cleaves TyrRS) are inconsequential to the DTAM presence on EJS tumors (Figures 3.6 and 4.3 C). In fact, due to the unchanged presence of hemocytes in JNK-suppressed EJS tumors, it appears that the basal attraction of hemocytes is entirely JNK-independent. Despite these differences, there are a few similarities: EJS tumors also display a caspase-dependent release of ROS, and JNK activity is ultimately responsible for the attraction of additional hemocytes observed in *Dronc*-deficient EJS tumors, which, as discussed in Chapter 4, are tumor-promoting. The specific molecular mechanism by which hemocyte attraction occurs, JNK-dependent or not, remains to be uncovered, along with the context-specific details that explain the differences between Dronc activities in EJS and $Ras^{V12}/scrib^{-/-}$ tumors. As discussed in Chapter 3, it is possible that unique substrates for Dronc only become available in EJS tumors; this could occur through EJS-specific expression of certain genes, or it could occur through the localization of Dronc into different sub-cellular compartments in both tumor models.

Altogether, this is the first description of caspases acting as negative regulators of the JNK pathway in tumoral settings, outside of silencing JNK activity through apoptosis (Pinal et al., 2018; Vaishnav et al., 2011). The striking contrast between the activity of caspases in EJS tumors as non-apoptotic negative regulators of the JNK pathway and tumorigenesis with the pro-tumorigenic function in different tumor models again highlights the complex and context-dependent nature of caspase activities. As discussed in Chapter 3, understanding the specific biological details shaping the relationship between caspases and JNK signaling in EJS tumors requires significant further study.

EJS tumors behave like chronic wounds

The paradigm of tumors behaving as wounds that do not heal has been advocated for decades (Dvorak, 2015), given the multiple similarities between tumors and the regenerative process of wound healing (MacCarthy-Morrogh and Martin, 2020). *Drosophila* has long been used as a model to study the mechanisms of wound response and inflammation (Ahmed-de-Prado and Baonza, 2018; Belacortu and Paricio, 2011; Tsai et al., 2018), and more recently, several studies have shed light into the immune and inflammatory response to tumors in *Drosophila* as well (Cordero et al., 2010; Hauling et al., 2014; Muzzopappa et al., 2017; Parisi et al., 2014; Pastor-Pareja et al., 2008), positioning the model organism as a means to study the intersection (Bangi, 2013; Wang et al., 2014; Xu et al., 2018a). The results I discuss in Chapter 4 detailing the attraction of the immune system to EJS tumors (DTAMs)

and their influence on the tumor complement these studies, strengthening the viability of the model. Importantly, previous studies have emphasized the more “damaged” qualities of wound-like tumors: stress-induced JNK signaling and release of cytokines activating the immune system (Pastor-Pareja et al., 2008); the attraction of the immune system due to basement membrane degradation (Diwanji and Bergmann, 2020; Parisi et al., 2014; Pastor-Pareja et al., 2008).

While these elements are certainly at play in EJS tumors (though the impact of BM degradation due to MMPs on the immune system appears negligible), my observations now document aspects of the regenerative branch of wound-healing as well. The simultaneous activation of both the JAK/STAT and JNK signaling pathways has been shown to be critical for tissue regeneration during wound healing in *Drosophila*, with the two pathways cooperating to elicit de-differentiation and proliferation of cells in the vicinity of the wound (Ahmed-de-Prado et al., 2018; Jiang et al., 2009; Santabarbara-Ruiz et al., 2015; Worley et al., 2018). As discussed in Chapter 3, I have shown that in EJS tumors, the co-operative oncogenesis of EGFR and JAK/STAT upregulation leads to hyperactivation of the JNK pathway as well, mimicking the signaling profile of regenerating tissue. Additionally, my data indicate that JNK activity in EJS tumors is necessary for the extra proliferation observed as well as the perturbation of cell identities, paralleling the activity of JNK in wound healing (Ahmed-de-Prado et al., 2018; Ramet et al., 2002).

Further increasing the similarities between EJS tumors and regenerating tissue is the prevention of pupariation observed in animals with EJS tumors (Herranz et al., 2012). The delay of pupariation and development has been previously shown to occur as a result of tissue damage, giving time for damaged tissue to recover (Colombani et al., 2012; Garelli et al., 2012; Hackney et al., 2012; Halme et al., 2010; Smith-Bolton et al., 2009). The mechanism by which this occurs involves the inhibition of ecdysone (Hackney et al., 2012), a key hormone that triggers developmental progression in *Drosophila* (Yamanaka et al., 2013). Tumors have previously been shown to impose a similar delay on development by modulating ecdysone (Colombani et al., 2012; Cordero et al., 2010; Garelli et al., 2012), and recent work has also shown that high JNK activity in Ras^{V12} expressing tissues reduces ecdysone levels (Ray and Lakhotia, 2019). Given that suppression of JNK signaling in EJS tumors rescues the prevention of pupariation, thus allowing developmental progression (data not shown), it is possible that EJS tumors are similarly inducing ecdysone inhibition through JNK activity. While this mechanism would need to be confirmed, the developmental delay induced by EJS tumors is reminiscent of that found from regenerating tissue.

Crucially, in Chapter 3 I have established that caspases work non-apoptotically to limit the JNK signaling responsible for many of the wound-like characteristics in EJS tumors, preventing the exacerbation of malignant properties. As also discussed in Chapter 4, the increased JNK signaling resulting from downregulated caspases is responsible for the increased presence of the immune system on EJS tumors. Interestingly, the increased JNK signaling and immune presence observed with the

downregulation of caspases resembles a perspective that caspases are primarily regulators of inflammation (Martin et al., 2012; McArthur and Kile, 2018). In this regard the traditionally “apoptotic” caspases are activated to sequester and extinguish proinflammatory signals (DAMPs) (Martin et al., 2012; McArthur and Kile, 2018); without caspase activity or apoptosis, cells undergo necrosis, release these signals, and attract the immune system (Martin et al., 2012; McArthur and Kile, 2018). Additionally, caspase activity generates “tolerate-me” signals in apoptotic cells that induce anti-inflammatory states in immune cells (Voll et al., 1997); in this state immune cells promote wound-healing (Ellis et al., 2018; Martin et al., 2012). While the perspective is not completely aligned with my observations in EJS tumors, namely that the prevention of apoptosis does not result in any increased immune presence (Chapter 2), it is possible that caspases in EJS tumors are carrying out similar anti-inflammatory roles. If fact, as discussed in the next section, through the downregulation of JNK signaling, Dronc indirectly decreases the expression of the *Unpaired* cytokines orthologous to inflammation-associated cytokine IL-6 (Jiang et al., 2009). In any case, it is clear that with the perpetual induction of a signaling profile similar to that of a regenerating tissue, and the presence of the immune system, EJS tumors behave much like a chronic wound.

Unpaired ligands: a potential candidate for bidirectional communication between hemocytes and tumor

While the interaction of the immune system and cancer is well known, less certain is their functional impact on tumor system. As expected, these protective cells can be

tumor-restrictive and indeed, many new therapies are premised on bolstering the immune system's response to tumors (Esfahani et al., 2020; Waldman et al., 2020); equally well established, however, is cancer's ability to co-opt immune cell functions to promote tumor growth (Gonzalez et al., 2018; Wang et al., 2019). In Chapter 4, I determined that hemocytes attracted to EJS tumors have the potential for a tumor-promoting function. In this regard, EJS-associated hemocytes could be similar to hemocytes found on $Ras^{V12}/scrib^{-/-}$ based models, which are also tumor-promoting (Perez et al., 2017).

However, as discussed previously and in Chapter 3, there are obvious differences between the two models. The mechanism by which hemocytes are recruited to EJS tumors is ROS-independent, unlike their recruitment to $Ras^{V12}/scrib^{-/-}$ tumors. Additionally, in $Ras^{V12}/scrib^{-/-}$ tumors, JNK activity and tumor growth are fueled by Eiger (*Drosophila* TNF) released from hemocytes (Perez et al., 2017); in EJS tumors, JNK activity appears independent from TNF-signaling. In this regard, EJS tumors are similar to other *Drosophila* tumor models that have shown Eiger-independent and tumor-intrinsic mechanisms for JNK activation (Muzzopappa et al., 2017); however, because of this intrinsic JNK activity, hemocytes in these tumor systems appear to have no appreciable role in tumor growth (Muzzopappa et al., 2017).

To explain the difference in outcomes between tumor and hemocyte interactions, it is possible that hemocytes associated with EJS tumors are behaving in an entirely different manner than previously described DTAMs. However, an alternative

explanation is that the genetic background of the tumor is the primary factor that determines this difference. An example of such disparity can be found between two tumor models that differ in only a single gene, *scrib*$^{-/-}$ and *Ras*^{V12}*/scrib*$^{-/-}$: in *scrib*$^{-/-}$ based tumors, Eiger released from hemocytes induces apoptosis through JNK signaling (Pastor-Pareja et al., 2008); however, in *Ras*^{V12}*/scrib*$^{-/-}$ tumors, JNK signaling ends up actually promoting tumor growth (Perez et al., 2017).

Thus, one possibility is that the genetic background of EJS tumors is contributing to the differential outcomes of hemocyte interactions with tumors. **EJS** tumors are induced through the upregulation of **EGFR** and **JAK/STAT** activity (Herranz et al., 2012); hyperactivation of the JAK/STAT pathway is achieved through the downregulation of a negative regulator of the pathway, SOCS36E (Herranz et al., 2012; Stec et al., 2013). The JAK/STAT pathway in *Drosophila* is rather simple and straightforward, with a single family of ligands, the Unpaired ligands (Upd1, 2, and 3), a single receptor, Domeless (Dome), a single JAK protein (Hopscotch, Hop), and a single STAT protein (STAT92E) (Arbouzova and Zeidler, 2006). The activity of the Dome receptor is critical for EJS tumors (Herranz et al., 2012), and Upd ligands are known to be released by hemocytes in response to various stresses, including JNK activation (Chakrabarti et al., 2016; Pastor-Pareja et al., 2008). Upd released by hemocytes can activate JAK/STAT in tissues (Woodcock et al., 2015; Yang et al., 2015), and in stem cells, promote proliferation (Chakrabarti et al., 2016; Jiang et al., 2009). Thus, it is entirely possible that the potential tumor promoting function of hemocytes described in Chapter 4 could occur through the release of Upd ligands;

upon binding to Dome receptors in EJS tumor cells, hemocyte-derived Upds would promote JAK/STAT activity, one of the core components of EJS tumors driving tumor growth (Figure 5.1).

Interestingly, evidence suggests that Upd ligands are important not only for EJS tumors, but are also key components of bidirectional communication between EJS tumors and hemocytes. In Chapter 4, I discuss how the detection of S-phase EJS-associated hemocytes suggests that hemocytes proliferate *in situ* on EJS tumors, and how hemocytes' entrance into S-phase is stimulated in caspase-depleted EJS tumors. Upd ligands produced by JNK activity in tumors have already been shown to promote proliferation in circulating hemocytes (Pastor-Pareja et al., 2008), and in Chapter 3 I demonstrated that *Dronc*-depleted EJS tumors display significant upregulation of all Upd ligands; thus, it is possible that Upds produced by JNK activity in EJS tumors are responsible for the increased presence of hemocytes found in caspase-deficient EJS tumors. Upd released from EJS tumors could recruit hemocytes, and also stimulate their proliferation. In this way, a positive feedback loop is established, when Upds released by hemocytes further stimulate EJS tumors (Figure 5.1).

Additionally, as discussed in Chapter 4, Upd ligands produced by EJS tumors could potentially explain why *reaper*-expressing hemocytes do not undergo apoptosis in the presence of EJS tumors. As an inhibitor of apoptosis, Diap-1 produced downstream of JAK/STAT activity could confer a protective effect in *reaper*-expressing hemocytes

(Betz et al., 2008; Recasens-Alvarez et al., 2017). Reciprocally, Diap-1 expression in EJS tumors could help modulate caspase activity, allowing for the sub-lethal and non-apoptotic caspase activity that ultimately regulates the crosstalk between tumor and immune cells, through their inhibition of JNK activity and consequently, Upd production (Figure 5.1) (Chakrabarti et al., 2016; Jiang et al., 2009). Thus, it would be interesting to assay the expression of Diap-1 in both tumors and hemocytes to see if this were the case.

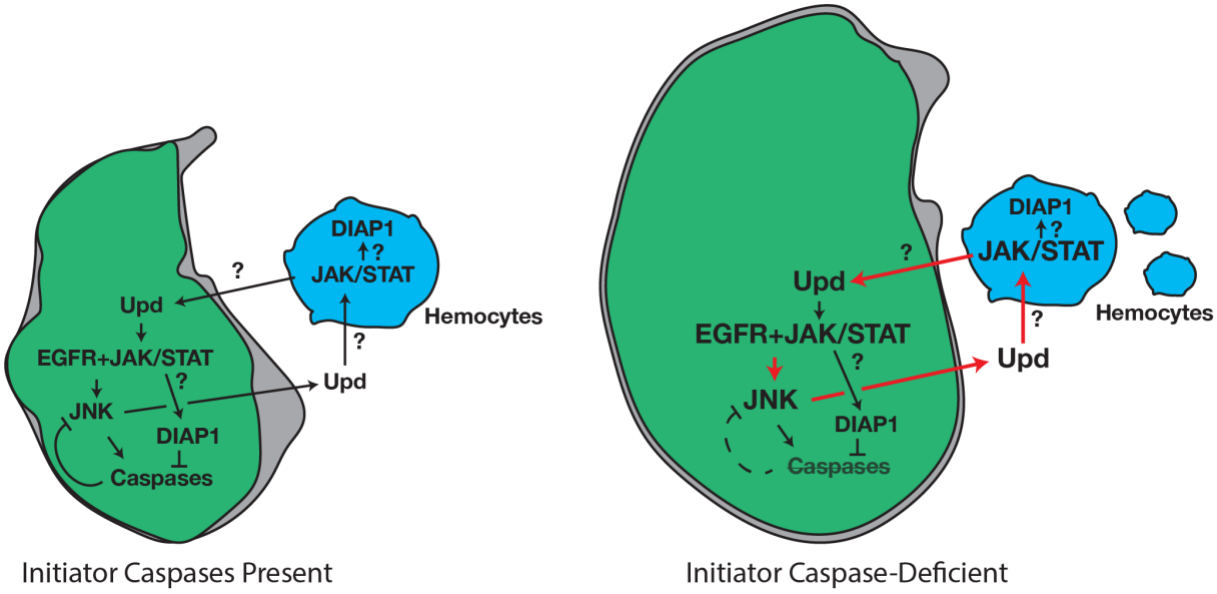


Figure 5.1 **Initiator caspases act as tumor suppressors in a wound-like EJS tumor through negative regulation of the JNK pathway**

Diagram summarizing the results of this thesis as a model. The signaling profile, growth, and hemocyte interactions in EJS (left) and *Dronc*-deficient EJS tumors (right) are depicted. Caspases interrupt a tumor-promoting inflammatory feedback loop through inhibition of the JNK pathway; upon loss of caspase activity, a positive feedback loop is reinforced that promotes JNK signaling, hemocyte proliferation, and tumor growth. The Unpaired ligands (Upd) are potential candidates for bi-directional communication between EJS tumors and hemocytes. Potential Diap-1 upregulation downstream of JAK/STAT activity could enable hemocytes to resist apoptosis and restrict caspase activity to non-lethal levels in EJS tumors.

Ultimately, considering the crosstalk between immune cells and EJS tumors (Figure 5.1), and the conservation of the pro-proliferative and pro-survival effects of EGFR and JAK/STAT signaling in human tumors as well (Fisher et al., 2014), my findings

could help the understanding of similar attempts to study caspase functions in human tumors in relation to the immune system.

Future Directions

While my work has yielded a number of insights into the biological activities of caspases in tumorigenic scenarios, a number of questions still remain regarding the specific molecular mechanisms through which caspases enact their tumor-suppressive function. The specific details of EJS tumors which allow for the caspases to act non-apoptotically in this context are yet to be uncovered as well. Additionally, the effect of *reaper* expression in the hemocytes' ability to functionally affect the EJS tumor system raises interesting questions about caspase activity in hemocytes as well. Clearly, there is more work that could be done, and I have detailed here possible future directions for experimental work.

Determining the requirement of Dark interaction and catalytic activity for non-apoptotic caspase functions in EJS tumors

While I have determined that Dronc carries out a non-apoptotic function in EJS tumors, the question still remains as to whether or not the catalytic activity of Dronc is required, as both catalytic and non-catalytic non-apoptotic functions have been described (Kamber Kaya et al., 2017; Nakajima and Kuranaga, 2017; Ouyang et al., 2011). As discussed in Chapter 2, if catalytic activity is required, Dronc is likely cleaving a novel substrate, as the effector caspases downstream in the caspase cascade are not necessary for EJS tumor inhibition. To address this question,

catalytically inactive Dronc can be expressed in a *Dronc* mutant EJS background. Additionally, I have determined that Dark and the formation of the *Drosophila* apoptosome likely play a role in activating Dronc for this tumor-inhibiting function. Thus, it would be informative to perform additional experiments to determine to what extent the interaction between Dronc and Dark influences Dronc's tumor inhibition. This can be achieved through expression of a CARD-less variant of Dronc to inhibit Dark/Dronc interactions. Additionally, expression of a Dronc mutant that cannot be monoubiquitinated at a specific residue that inhibits Dark/Dronc interactions could bolster tumor inhibition through increased activation of Dronc (Kamber Kaya et al., 2017).

Determining the origin of JNK activity in EJS tumors

As discussed in Chapter 3, indirect evidence suggests that JNK activity in EJS tumors does not appear to be reliant on either ROS or Eiger/TNF-signaling. This is because the elimination of either does not affect tumor growth, which depends on JNK activity. Tumor-specific origins of JNK activity have been described before in *Drosophila* models, stemming from mechanisms downstream of ROS, Eiger, and polarity-impairment (Muzzopappa et al., 2017). Interestingly, in tumors of different genetic backgrounds, three different JN3Ks in *Drosophila* is responsible for activating JNK in different contexts (Muzzopappa et al., 2017). While the JN3Ks Ask1 and Tak1 correspond to ROS- and Eiger-dependent JNK-signaling, respectively, the JN3K Wallenda (Wnd) is activated by polarity-impairment to activate JNK (Muzzopappa et al., 2017). Thus, because loss of apicobasal polarity is also a prominent feature of

EJS tumors, it would be interesting to remove Wnd function through expression of a kinase-dead version of the protein (Collins et al., 2006), and examine the effect on JNK activity and EJS tumor growth.

Determining if ecdysone production is responsible for developmental delay

As discussed above, the inhibition of ecdysone though increased JNK signaling in EJS tumors could be a wound-like factor that prevents developmental progression in larvae with EJS tumors. To determine if this is the case, it would be interesting to see if restoration of ecdysone can rescue the giant larvae phenotype observed; this can be achieved through ectopic feeding of ecdysone to larvae (Hackney and Cherbas, 2014), or by inhibiting *Dilp8*, a gene responsible for inhibiting ecdysone production (Colombani et al., 2012). Doing so could further strengthen the similarities between EJS tumors and open wounds.

Determining the relevance of Upd ligands in bidirectional communication between EJS tumors and DTAMs

As it stands now, the Upd ligands are a lynchpin in my current understanding of the EJS tumor system and its interactions with the immune system. While direct modulation of the JAK/STAT pathway in EJS tumors could be counter-productive, as the Dome receptor is required for EJS induction (Herranz et al., 2012), manipulation of the hemocytes could shed light onto the true relevance of Upd in EJS tumors and associated DTAMs. It would be interesting to express a dominant-negative form of the Dome receptor ($Dome^{DN}$) specifically in hemocytes (Brown et al., 2001), thus

preventing the potential activation and proliferation of hemocytes receiving Upd ligands produced by EJS tumors. A similar effect of downregulating the JAK/STAT pathway can be achieved through the use of mutants for *hop*, the JAK protein in *Drosophila* (Binari and Perrimon, 1994). Additionally, RNAis for the Upd ligands can be expressed specifically in hemocytes to help elucidate the mechanism through which hemocytes promote EJS tumor growth.

Unfortunately, the experiments I have just described are not immediately possible due to the necessity of creating new transgenic lines to specifically manipulate hemocytes without affecting EJS induction (*QUAS-Dome*^{DN}, for example). Thus, it could be possible to take advantage of the requirement for functional hemocytes to affect EJS tumor growth; Upd production can be assessed in both wild-type and *reaper*-expressing hemocytes associated with EJS tumors using existing reporters (Bunker et al., 2015; Tsai and Sun, 2004). If Upd production is decreased in *reaper*-expressing hemocytes, which are associated with smaller tumors than their wild-type counterparts, then Upd ligands are likely the mechanism by which hemocytes promote EJS tumor growth.

Alternatively, if it is revealed that Upds are not important to EJS tumors, at least in relation to hemocyte recruitment, then there are other possibilities to pursue. While I have determined that basement membrane degradation is not responsible for hemocyte attraction to normal EJS tumors, it is possible that the increased MMP production resulting from increased JNK activity in *Dronc*-deficient EJS tumors could

provide the additional boost necessary to recruit extra hemocytes. Similarly, additionally cleavage of Tyrosyl-tRNA synthetase (TyrRS) by MMPs could be responsible for increased DTAM presence. Thus, it would be interesting to interfere with MMP activity in conjunction with Dronc downregulation, either through downregulation of MMP1 and MMP2 in *Drosophila*, or expression of the MMP inhibitor TIMP (Wei et al., 2003).

Determining the importance of Diap-1 in EJS tumors and associated hemocytes

Two unresolved questions regarding non-apoptotic caspase activity in EJS tumors and the ability of EJS associated hemocytes to survive *reaper*-induced apoptosis revolve around the same protein: Drosophila Inhibitor of Apoptosis 1 (Diap-1). Expressed downstream of JAK/STAT activity (Betz et al., 2008; Recasens-Alvarez et al., 2017), Diap-1 expression could contribute towards controlling the sub-lethal and non-apoptotic activity of Dronc in EJS tumors. Similarly, Diap-1 expression in hemocytes could provide the protective environment necessary to prevent apoptosis, explaining their survival when *reaper* is overexpressed in hemocytes. Monitoring Diap-1 expression in EJS tumors through a Diap-1-lacZ reporter could provide an explanation for the context-specific nature of the non-apoptotic caspase activity in EJS tumors (Wu et al., 2008); in parallel, I could also monitor Diap-1 expression in EJS DTAMs, shedding light onto how they survive apoptosis.

Determining if there is any potential non-apoptotic activity in EJS associated hemocytes

In Chapter 4, I discussed the possibility that hemocytes associated with EJS tumors are tumor promoting, as well as the (perhaps less likely) possibility that *reaper* expression could enhance any tumor-inhibitory function in DTAMs, or even confer a tumor-inhibitory function to hemocytes. While I do find this scenario extremely unlikely, it is possible that the prevention of *reaper*-induced apoptosis through JAK/STAT signaling and Diap-1 expression renders EJS-associated hemocytes in a similar position to previously studied “undead cells” – cells which have been instrumental in uncovering novel non-apoptotic functions of caspases in the past (Diwanji and Bergmann, 2019; Perez-Garijo, 2018). Therefore, it would be extremely interesting to determine if similarly novel non-apoptotic functions can be uncovered in these cells. Of course, a full investigation would be far outside the scope of this thesis or immediate follow-ups; however, preliminary experiments that can be conducted include the modulation of caspase activity in hemocytes through expression of P35, or various caspase-RNAis. At the very least, these experiments can inform the community on whether or not non-apoptotic functions exist in hemocytes.

Conclusion

In this thesis I have shown that independently of apoptosis, caspases in EJS tumors can have tumor-suppressing functions. They perform this function by limiting JNK signaling that is critical for malignant features in EJS tumors, as well as the presence of tumor-associated macrophage-like hemocytes that can potentially fuel tumor

growth. Through this limitation, caspases prevent the exacerbation of tumor features such as increased proliferation and defective cell differentiation; importantly, I also show that non-apoptotic caspase activity can be found in virtually all EJS tumors cells, indicating the potential for ubiquitous non-apoptotic caspase activity in other tumors. In addition, I show that the combination of JNK and JAK/STAT signaling in EJS tumors results an environment that is very similar to that found in regenerating tissues during wound healing, with JNK signaling being critical for disrupted cell identity and proliferation. A strong immune presence on the tumor further parallels EJS tumors to a physiological response to damaged or injured tissue. From this, I conclude that EJS tumors represent a *bona fide* wound-like tumor. Finally, my work also highlights the complex and context-dependent relationship between caspase function and tumorigenesis. While many questions still remain, altogether my findings improve our knowledge of caspase biology in tumors and how they can restrict tumorigenesis through novel, non-apoptotic mechanisms; additionally, the EJS tumor model presents a useful and highly relevant system that allows for the study of the intersection between caspases, cancer, and the immune system.

Chapter 6

Materials and Methods

Materials

Drosophila stocks and husbandry

All *Drosophila* stocks were maintained at 20°C or 18°C in vials of *Drosophila* culture medium (0.77% agar, 6.9% maize, 0.8% soya, 1.4% yeast, 6.9% malt, 1.9% molasses, 0.5% propionic acid, 0.03% ortho-phosphoric acid, and 0.3% nipagin). Additional yeast was also added to each vial before flies were added. Crosses were made at 25ºC unless otherwise indicated for temperature sensitive experiments.

Drosophila genotypes

Genotype	Description	Source	Figures used
Actin-DBS-S-QF, QUAS-mTdTomato-HA; QUAS-Flippase; Actin5C FRT-stop-FRT lacZ-nls/SM6A-TM6B	DBS-S-QF sensor	(Arthurton et al., 2020)	2.3, 2.4
ap-Gal4, UAS-GFP, UAS-SOCS36E-RNAi/CyO; UAS-EGFR, tub-Gal80^{ts}	EJS	Gift from H. Herranz (Herranz et al., 2012)	All figures from 2.4 onwards except for 2.7, 4.6, and 5.1
UAS-Dronc-RNAi	UAS-Dronc-RNAi	Gift from Pascal Meier (Leulier et al., 2000)	2.6, 2.8 – 3.5, 4.1, 3.3 – 3.5, 3.7
tub-Gal80ts, UAS-histone-RFP, DroncKO/TM6B	Dronc^{KO}	(Arthurton et al., 2020)	2.7
UAS-EGFR, UAS-SOCS36E-RNAi/CyO	EJS (alternate)	Gift from H. Herranz	2.7
UAS-flippase, Dronc$^{KO\text{-}FRT\text{-}Dronc\text{-}GFP\text{-}Apex\text{-}FRT\ QF}$/TM6B	Conditional Dronc^{KO}	(Arthurton et al., 2020)	2.7
UAS-miRNA[Reaper,Hid,Grim]	UAS-microRNA-[RHG]	Gift from I. Hariharan (Siegrist et al., 2010)	2.8
UAS-P35/Cyo; UAS-P35/TM6B	UAS-P35 (2x)	(Arthurton et al., 2020)	2.8

y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00870}attP2	UAS-Dark-RNAi	Bloomington Stock Center: 33924	2.8
w[*]; P{w[+mC]=UAS-DIAP1.H}3	UAS-Diap-1	Bloomington Stock Center: 6657	2.8
w[*]; P{y[+t7.7] w[+mC]=TRE-DsRedT4}attP16	TRE-RFP	Bloomington Drosophila Stock Center: 59012	3.1
w[1118] P{w[+mC]=UAS-bsk.DN}2	UAS-JNK^{DN}	Bloomington Drosophila Stock Center: 6409	3.2 – 3.4, 4.3
RFP$^{Hml.\Delta.Tag:NLS(tra)}$/TM6B	Hml-dsRed	Gift from B. Lemaitre (Makhijani et al., 2011)	4.1 – 4.5, 4.7
y[1] w[*]; P{w[+mC]=Hml-QF2.Delta.L}2; P{w[B1-12]=lacW}mirr[B1-12]/TM6B, Tb[1]	Hml-QF2	Bloomington Drosophila Stock Center: 66468	4.6, 4.7
UAS-QS, QUAS-Reaper/TM6B	QUAS-Reaper	Gift from A. Baonza (Perez-Garijo et al., 2013)	4.6, 4.7
w^{1118}	Wild-type (WT)	Bloomington Drosophila Stock Center: 3605	2.3, 3.3, 3.4
y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00678}attP40	UAS-Duox-RNAi	Bloomington Drosophila Stock Center: 38907	3.6
w[1]; P{w[+mC]=UAS-Cat.A}2	UAS-Catalase	Bloomington Drosophila Stock Center: 24621	3.6
w^{1118}; P{GD12580}v43454	UAS-Grnd-RNAi	Vienna Drosophila Resource Center: 43454	3.7
y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GLC01716}attP2	UAS-Wgn-RNAi	Bloomington Drosophila Stock Center: 50594	3.7
y[1] w[*]; PBac{y[+mDint2] w[+mC]=10XQUAS-6XGFP}VK00018/CyO, P{Wee-P.ph0}Bacc[Wee-P20]	QUAS-GFP	Bloomington Drosophila Stock Center: 52264	4.6

Oligonucleotides

Name	Sequence	Source
RPL32 qPCR Forward	ATGCTAAGCTGTCGCACAAATG	(Gomez-Lamarca et al., 2018)
RPL32 qPCR Reverse	GTTCGATCCGTAACCGATGT	(Gomez-Lamarca et al., 2018)
MMP1 qPCR Forward	AGGACTCCAAGGTAGACACAC	(Jia et al., 2014)
MMP1 qPCR Reverse	TTGCCGTTCTTGTAGGTGAACGC	(Jia et al., 2014)
Unpaired1 qPCR Forward	CAGCGCACGTGAAATAGCAT	DRSC Fly Primerbank: PD70143
Unpaired1 qPCR Reverse	CGAGTCCTGAGGTAAGGGGA	DRSC Fly Primerbank: PD70143
Unpaired2 qPCR Forward	ACGAGTTATCAAGCGCAAGCA	(Ahmed-de-Prado et al., 2018)
Unpaired2 qPCR Reverse	ATATCTTGGTATTCGCTCATCGTG	(Ahmed-de-Prado et al., 2018)

Unpaired3 qPCR Reverse	ACAGATTCCTGCCCCGTCT	(Ahmed-de-Prado et al., 2018)
Unpaired3 qPCR Reverse	GGTCGCGATGGGCGT	(Ahmed-de-Prado et al., 2018)

Antibodies

Antibody	Source	Catalogue #
Chicken polyclonal Anti-Beta Galactosidase	Abcam	Cat# ab9361 RRID:AB_307210
Rabbit monoclonal Anti-HA-tag (clone C29F4)	Cell Signaling	Cat# 3724 RRID:AB_1549585
Rabbit polyclonal Anti-Phospho-Histone H3 (Ser10)	Cell Signaling	Cat# 9701S RRID:AB_331535
Mouse monoclonal Anti-MMP1 Supernatent	Developmental Studies Hybridoma Bank	Cat# 3A6B4 RRID:AB_579780
Mouse monoclonal Anti-Wingless Supernatent	Developmental Studies Hybridoma Bank	Cat# 4D4 RRID:AB_528512
Goat polyclonal Anti-Distal-less	Santa Cruz	Cat# Sc15858 RRID:AB_639128
Mouse monoclonal anti-Hemese (H2)	Gift from I. Andó (Kurucz et al., 2003)	N/A
Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific	Cat# A-21449 RRID:AB_2535866
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	Thermo Fisher Scientific	Cat# A-31572 RRID:AB_162543
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	Thermo Fisher Scientific	Cat# A-31570 RRID:AB_2536180
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	Thermo Fisher Scientific	Cat# A-21432 RRID:AB_2535853

Methods

Tumor induction

For experiments involving induction of tumor formation during larval stages, Gal80^{ts} was used to temporally control overexpression of oncogenes. Flies were reared and crossed at 18°C, inhibiting Gal4 activity. 30–40 virgins were crossed to 10–15 males for each genotype and experiment. Crosses were flipped twice a day (morning and evening) into fresh vials of food, to ensure larvae used in experiments were of similar age. If necessary, eggs could be scooped out to ensure similar larval densities between experimental genotypes and to prevent overcrowding. One day before

larvae entered the wandering third instar stage (9 full days in our fly incubators and experimental environment), larvae were shifted to 29°C for up to 5 days, depending upon the experiment.

Dissection of wing discs

Both male and female larvae crawling along the walls of the vial (or otherwise not submerged in culture medium) from an experimental cross were used for dissections. Larvae were collected in PBS at room temperature in a glass 9-well dissection plate. Excess food was washed off by rinsing the larvae 3 times in PBS. Suitable larvae for dissection were identified under fluorescent stereomicroscope and collected by genotype in petri dishes. A glass watch glass on ice with ice cold PBS was used for dissection. Wing discs were dissected according to standard protocols: larvae were inverted inside-out in PBS on ice, and extraneous tissues such as the fat bodies and salivary glands were removed carefully, leaving the wing discs attached to the carcass. Samples were then collected immediately in 4% formaldehyde in 1x PBS on ice to prevent potential hemocyte dissociation from the tissue. After as many dissections per genotype could be performed within a 20-minute period, samples were then moved to room temperature for 20 minutes with shaking for additional fixation. Discs were washed in PBS-TX (1x PBS, 0.3% Triton X-100).

Immunohistochemistry

After dissection, fixation, and washing, wing discs were blocked in blocking solution (3% BSA and 0.5% Sodium Azide in PBS-TX) for 1 hour at room temperature.

Discs were incubated while shaking overnight at 4°C with primary antibodies in blocking solution. The following primary antibodies and concentrations were used in this thesis: chicken anti-Beta-galactosidase (1:500, Abcam, RRID:AB_307210), rabbit anti-HA-tag (1:1000, Cell Signaling, RRID:AB_1549585), rabbit anti-Phospho-histone H3 (1:100, Cell Signaling, RRID:AB_331535), mouse anti-MMP1 (1:200, Hybridoma Bank, RRID:AB_579780), mouse anti-Wingless (1:200), Hybridoma Bank, RRID:AB_528512), goat anti-Distal-less (1:100, Santa Cruz, RRID:AB_639128).

After washing, discs were incubated with conjugated secondary anti-bodies for 2 hours at room temperature. The following secondary antibodies were used in this thesis, all from Thermo Fisher Scientific and used at a concentration of (1:200): Goat anti-chicken Alexa Fluor 647 conjugated, Donkey anti-rabbit, -mouse, and -goat Alexa Fluor 555 conjugated. DAPI (1:1000) was added to a 15-minute washing step after secondary antibody incubation. After washing, discs were rinsed with 1x PBS, and then incubated in a 50% glycerol solution in 1x PBS for 1 hour. Discs were then incubated in an 80% glycerol solution in PBS for at least one hour. Discs were removed from the carcasses and mounted in 80% glycerol. Samples were then covered with a 1.5H coverslip, secured with nail polish, and imaged immediately or stored at 4°C. Control and experimental samples were mounted on the same slide to control for samples being pressed between the slide and coverslip.

DHE Labeling

DHE labeling of ROS was conducted according to a previously described protocol (Owusu-Ansah et al., 2008), including the optional fixation in 4% formaldehyde in 1x PBS.

TUNEL staining

As for immunohistochemistry, wing discs were dissected in ice-cold PBS and fixed in 4% formaldehyde in PBS for 20 minutes. *In situ* detection of fragmented genomic DNA was then performed according to methods established previously (Galasso et al., 2020 Preprint), using the DeadEnd Colorimetric TUNEL (Terminal transferase-mediated dUTP nick-end labeling) system (Promega).

EDU staining

For EDU staining, larvae were selected and dissected as described above. Instead of fixative, discs were collected in ice cold PBS. Dissection times were kept under 20 minutes. After dissection, the PBS was replaced with a 0.1mg/mL solution of EdU in PBS for a 5-minute incubation. After incubation, discs were rinsed with PBS and then fixed in 4% formaldehyde in 1x PBS for 20 minutes. Subsequent steps followed instructions according to the manufacturer (Thermo Fisher, Click-iT Edu Kit).

Fluorescence microscopy and image analysis

Images were acquired using an inverted FV1200 Olympus microscope, using 10x air, 20x air, or 60x oil objective lenses, depending on the experiment. Unless otherwise

indicated, the entire wing disc was imaged in all three dimensions, using the optimal step size determined by the Olympus Fluoview software. For images where relative intensities were being measured, a constant laser power was used throughout genotypes and experiments. Post-acquisition imaging processing was performed using Fiji (Schindelin et al., 2012) and CellProfiler image analysis software (CellProfiler).

To measure EJS tumor sizes, maximum intensity projections of each z-stack were generated using Fiji to produce 2D images. Tumor sizes were measured using CellProfiler's IdentifyPrimaryObjects module.

To measure nuclei sizes and density in EJS tumors, the midpoint of a disc was determined in the z-axis, and a 60x image of the DAPI channel was acquired in a similar location amongst all discs, in the ventral compartment. Using CellProfiler, nuclei sizes were segmented and measured using the IdentifyPrimaryObjects module. To measure nuclei density, images were shuffled and blinded to the investigator for quantification. A 100μm^2 area was selected in the center of all images and the number of nuclei were counted manually using Fiji.

To quantify the numbers of hemocytes present on EJS tumors, maximum intensity projections of each z-stack were generated using Fiji to produce 2D images. After a tumor was identified and outlined using CellProfiler, a mask was applied on the channel containing hemocyte nuclei (visualized using Hml-dsRed) to restrict counting

to hemocytes present only on the wing disc. Individual hemocyte nuclei were segmented, identified, and counted using CellProfiler's IdentifyPrimaryObjects module.

To quantify proliferation in EJS tumors, maximum intensity projections of each z-stack were generated using Fiji to produce 2D images. For PH3 based measurements, after a tumor was identified and outlined using CellProfiler, a mask was applied on the channel containing PH3 staining to restrict counting to cells in the EJS tumor. Individual hemocyte nuclei were segmented, identified, and counted using CellProfiler's IdentifyPrimaryObjects module. For EdU based measurements, thresholding in Fiji was used to determine both EJS tumor area and EdU^+ area.

To measure the intensity of *Tre-RFP* and antibody staining for MMP1 in EJS tumors, maximum intensity projections of each z-stack were generated using Fiji to produce a 2D image. Mean intensities in EJS tumors were measured using Fiji. To measure the modal intensity for MMP1 and *Tre-RFP*, individual nonzero pixel values were extracted from each image using the Save XY Coordinates function in Fiji and imported into R Project (R Core Team, 2020) using RStudio. Pixel intensity values below a certain background value (determined using the Threshold function in Fiji) were removed from the data set. A density curve of pixel intensities was calculated for each disc, along with the modal intensity using the ggridges package (Wilke, 2020). The standard deviation of the modes for each genotype was also calculated using R.

Real-time Quantitative PCR

Larvae were collected and identified in the same manner as for immunostaining. Larvae were dissected in one well of a 9-well dissection plate on ice, and inverted carcasses were collected in a separate well containing PBS. Once 15–20 larvae were collected, wing discs were carefully separated from the carcass, disposing of the carcass. Dissection time was limited to 20–30 minutes. Cleaned discs were then transferred to a clean 0.5mL Eppendorf tube, using a P20 micropipette tip. Cut the tip off the P20 micropipette tip and coat the tip by pipetting the contents of the remaining carcasses up and down several times to prevent discs from sticking to the tip. After the discs were transferred, the PBS was replaced with 100µl of RNA lysis buffer. Tissue was lysed by short bursts of vortexing using a tabletop vortex mixer. Samples of similar genotypes can be frozen at this point in liquid nitrogen and pooled together if necessary. RNA was subsequently extracted using an RNA Easy Plus kit following the manufacturer's instructions (Qiagen, 74034). 500ng of total RNA of each sample was then used for reverse transcription, according to manufacturer instructions (Thermo Fischer Scientific Maxima First Strand cDNA Synthesis Kit – K1671). Q-PCR was then performed using a QuantiNova SYBER Green PCR Kit (Qiagen, Cat# 208054) and a Rotor-Gene Q Rea-time PCR cycler (Qiagen). Data was analyzed using the comparative CT method (Livak and Schmittgen, 2001) with *RPL32* used as a housekeeping gene for internal control. Primers used for qPCR can be found in the above in the Materials section.

Quantification and Statistical Analysis

All data were processed and analyzed using R, GraphPad Prism 8, and Microsoft Excel. Unpaired Student's t-tests, or one-way ANOVA with Tukey's multiple comparisons test were used to compare values between genotypes for univariate data. To compare Pearson correlation coefficients for multivariable data, a Fisher-Z-Transformation two-tailed test was used (Diedenhofen and Musch, 2015). Figures were generated using Adobe Illustrator CC.

Appendix

Investigating the roles of caspases and Btbd7 as novel regulators of cell shape dynamics

Preface

While the major focus of this thesis is on investigating the non-apoptotic roles of caspases in pathological contexts (e.g. cancer), caspases are known to have functions in physiological scenarios, such as regulating cell shape and motility (Connolly et al., 2014). Prior to the work described previously in this thesis involving caspases and EJS tumors, I was involved in a project investigating a potential role of caspases as regulators of cell shape dynamics in *Drosophila* glial cells. Additionally, as part of a collaboration, I investigated the potential for another modulator of cell shape dynamics, Btbd7, to regulate glial cell dynamics. As is the case in much of scientific research, these endeavors ultimately did not bear fruit, as I did not find any substantial evidence supporting the conclusion that either caspases or Btbd7 had a role in regulating glial cell shape dynamics. Consequently, the main focus of my graduate research shifted to the project described in the previous chapters. For the sake of recording even negative results (Nimpf and Keays, 2020), I include this section in my thesis.

Introduction

For many cells, life is constantly in motion, involving dynamic cell shape rearrangements and coordinated movements at both the single- and multi-cellular

level. While these morphological cell changes are critical for many developmental and physiological processes, such as tissue morphogenesis and wound healing (Mayor and Etienne-Manneville, 2016; Scarpa and Mayor, 2016; Shaw and Martin, 2009), abnormal cell dynamics and motility also enable tumors to invade and metastasize (Lambert et al., 2017). As metastasis and the spread of cancer to secondary tissues is the most dangerous aspect of the disease (Lambert et al., 2017), it is important to understand the underlying mechanisms of cell shape dynamics and migration in both physiological and pathological conditions. Though the mechanisms of cell shape remodeling and migration have been studied for decades, we still lack a complete understanding. This section of the thesis focuses on two regulators of cell shape dynamics—caspases and Btbd7—and their roles in established models of cellular shape dynamics.

Caspases in migration and motility

As described in Chapter 1, the caspase family of proteases, in addition to apoptosis, have been linked to a wide range of cellular functions, including cellular remodeling and migration (Connolly et al., 2014; Crawford and Wells, 2011). In some regards, this non-apoptotic outcome from caspase activity stands to reason, as one of the classical outcomes of apoptosis is the dynamic remodeling of the cytoskeleton (Povea-Cabello et al., 2017). Cytoskeletal proteins are known caspase substrates (Connolly et al., 2014; Crawford and Wells, 2011), and as cell motility and shape changes are dependent upon the reorganization of the cytoskeleton, it is not unsurprising that non-lethal caspase activity could mediate cell motility.

Indeed, many examples of caspases affecting cytoskeletal dynamics in migratory or motility related contexts have been described. The ability of immune cells to migrate is important for proper pro-inflammatory responses (Luster et al., 2005), and caspase-11 has been shown to interact with actin-interacting protein 1 (Aip1) at the leading edge of migratory macrophages to promote actin depolarization and cell migration (Li et al., 2007; Li et al., 2008). In the nervous system, the downstream effector caspase-3 has also been implicated in cell migration. A wave of caspase-3 activity unrelated to cell death can be detected in cells of the rat forebrain that go on to divide and migrate to the olfactory bulb (Yan et al., 2001), and in *in vitro* models of neuronal differentiation, caspase-3 has been shown to cleave the neuronal microtubule-stabilizing protein Tau (Rohn et al., 2004). In this way, caspase activation allows for the cytoskeleton disassembly necessary to properly disperse and migrate towards their destinations (Rohn et al., 2004).

Drosophila has also proven useful in understanding caspase regulation of cytoskeletal dynamics and migration. A commonly used model system to study cell migration involves a special group of cells within the *Drosophila* egg chamber, known as the border cells (Prasad et al., 2015). In physiological conditions, the Rho family GTPase Rac is required in the leading border cells to regulate actin rearrangement and guide the collective migration of these cells (Ridley, 2015). Interestingly, the overexpression of Diap-1, the inhibitor of apoptosis, rescued border cell migration defects in loss-of-function Rac-mutant cells (Geisbrecht and Montell, 2004). Evidence

indicates non-apoptotic Dronc activity negatively regulates Rac-dependent cell motility, however, the exact mechanism by which Dronc regulates actin dynamics is still unclear (Geisbrecht and Montell, 2004). Additionally, Dronc has been implicated in regulating actomyosin dynamics in migratory cells during developmental thorax closure and wound healing (Fujisawa et al., 2019).

Btbd7, regulator of cell dynamics and morphogenesis

Similarly, Btbtd7 is a regulatory factor affecting cell adhesion and migration that regulates branching morphogenesis, but whose mechanisms remain largely unclear (Onodera et al., 2010). Providing a mechanistic link between the extracellular environment and the cellular rearrangement required for the development of many branched organs, such as the lungs and salivary glands, Btbd7 expression is induced by the matrix protein fibronectin (Onodera et al., 2010). Once expressed, Btbd7 induces expression of *Snail2*, a cell-dispersal gene necessary for pro-migratory epithelial-mesenchymal transition (EMT) and decreases cell-cell adhesion by reducing E-cadherin levels (Onodera et al., 2010). Btbd7 also appears to be required for invasion and migration in many types of cancers, promoting EMT and invasiveness in cultured tumor cells (Luo et al., 2015; Tao et al., 2013; Yang et al., 2017). As Btbd7 contains two BTB domains, Btbd7 could also affect actin cytoskeleton remodeling allowing for migration (Chen et al., 2009). However, as with caspases, the characterization of the mechanisms by which Btbd7 affects cell shape dynamics and migration is still incomplete.

Thus, to study the contribution of caspases and Btbd7 in cell shape dynamics, I decided to study *Drosophila* glial cells, which are morphologically dynamic during development and also exhibit prominent morphological changes in response to neuronal injury. As glial dynamics in migration and engulfment of neuronal debris requires extensive actin and cytoskeletal remodeling (Sepp and Auld, 2003; Winfree et al., 2017), I hypothesized that either caspases or Btbd7 could affect glial cell dynamics through potential cytoskeletal regulation.

Results

Loss of Dronc does not disrupt glial cell dynamics during metamorphosis

Glial cells display striking morphological dynamics during physiological behaviors (Sild et al., 2013; Tremblay et al., 2009). During metamorphosis, peripheral glial cells in *Drosophila* are particularly active, extending and retracting elaborate cellular processes (Figure A1 A). Interestingly, high levels of caspase activity have been observed during this period of remodeling (Darren Williams, personal communication). To characterize the dynamics of peripheral glial, I specifically expressed LifeActGFP in glial cells using *repo-Gal4* and the Gal4/*UAS* system to visualize actin dynamics during live time-lapse microscopy (Figure A1 A). During this time, wild-type glial cells undergo intense ramification and remodeling, extending and retracting filopodia several times a minute (Figure A1 A). To determine if caspases had any functional during this period, I first tested the effects of silencing caspase activity specifically in glial cells by expressing a *Dronc-RNAi* transgene. I found no obvious or immediately apparent morphological or dynamic differences between wild-

type and *Dronc*-deficient glial cells (Figure A1 B). This suggested that the initiator caspase Dronc plays no essential role in this particular developmental process.

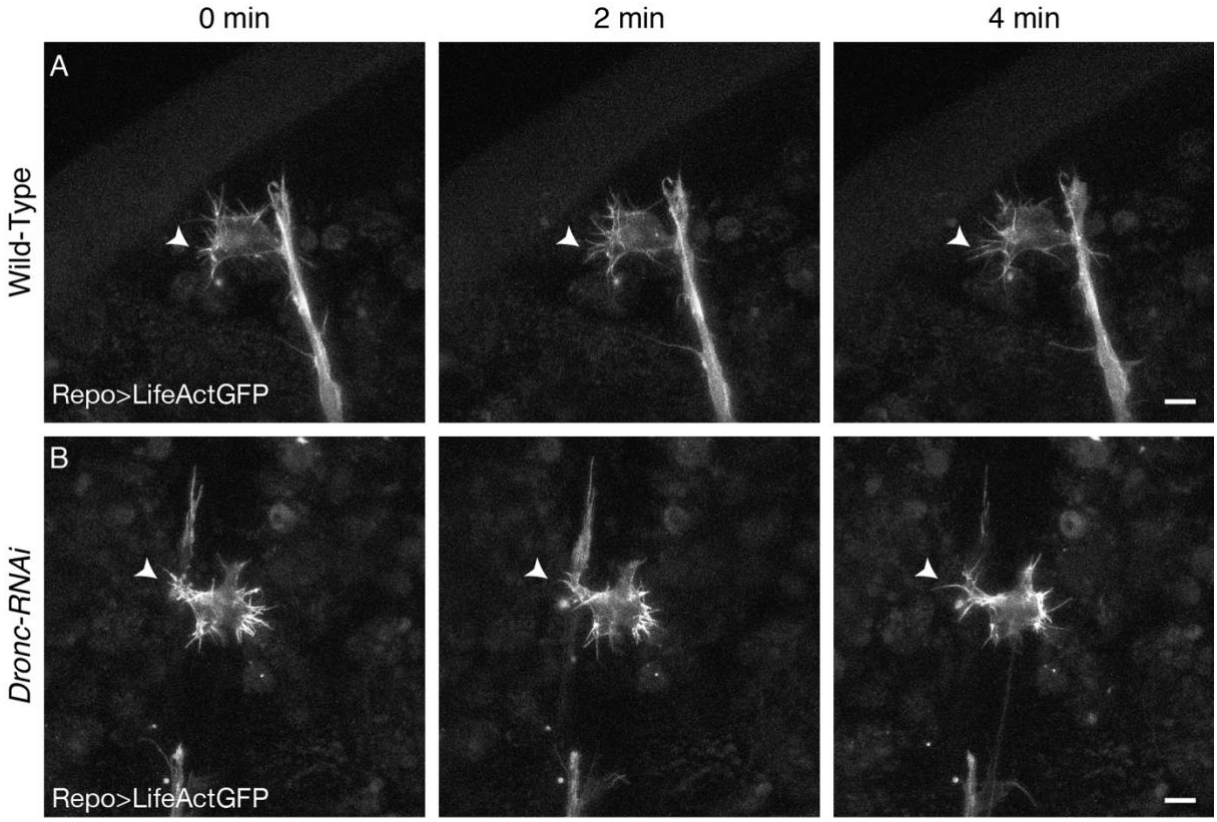


Figure A1. ***Drosophila*** **peripheral glia display striking morphological dynamics during metamorphosis.**
(A, B) Wild-type peripheral glia and peripheral glia expressing *Dronc-RNAi* to silence *Dronc* both continually extend and retract filopodia during metamorphosis. White arrowheads indicate filapodial projections present at the 4-minute mark not present at the beginning of recording. Glia projections are labelled using LifeActGFP using the Gal4/UAS system (*repo-Gal4* and *UAS-LifeActGFP*), and images were acquired every 20 seconds for a 20-minute period. Two-minute intervals are displayed here. Scale bar represents 10μm.

Loss of caspase activity does not affect glial cells' morphological response to neuronal injury

Outside of normal development, *Drosophila* glial cells also display dramatic morphological and transcriptional changes in response to neuronal damage during adulthood (Freeman, 2015). Much like macrophages, in response to injury, glial cells

extend cellular processes for the phagocytic engulfment of neuronal debris (Freeman, 2015). Because we observed non-lethal transcription of *Dronc* in *Drosophila* glial cells in the adult brain (Figure A2), I decided to investigate if there was a role for caspases in the extensive morphological changes in glia responding to injury. To do this, I used an established model of neuronal injury and glial response in the *Drosophila* olfactory system (MacDonald et al., 2006).

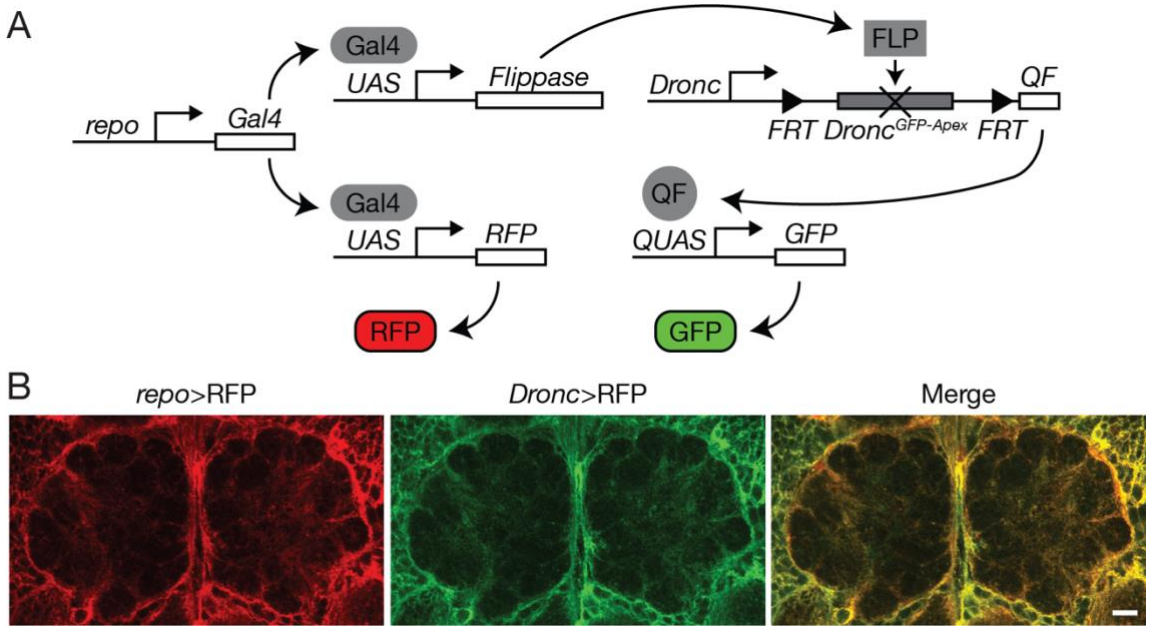


Figure A2. ***Dronc* is transcribed in glial cells surrounding the antennal lobes.**
(A) Schematic illustrating the genetic method used to detect the transcription of *Dronc* specifically in glial cells. Glial-cell specific expression of *Gal4* (*repo-Gal4*) induces expression of RFP and Flippase. RFP labels the glial cells, while Flippase excises an inserted rescue construct in the *Dronc* locus ($Dronc^{GFP\text{-}Apex}$), resulting in QF expression under the control of the endogenous *Dronc* promoter region. *Dronc* transcription results in GFP expression through QF/*QUAS*.
(B) Representative confocal images of wild-type uninjured antennal lobes, with glial cells labeled with RFP using the Gal4/*UAS* system. *Dronc*-expressing glial cells are labeled in GFP using the QF/*QUAS* system, where QF is expressed under the physiological regulation of the *Dronc* promoter. A merged image shows overlap between glial cells and *Dronc* expression. Scale bar: 10μm.

Consistent with previous findings (Lu et al., 2017; MacDonald et al., 2006), glial cells increase their surface area and thickness around the antennal lobes in the brain in

response to antenna ablation and damage to the olfactory receptor nerves (ORNs) found there (Figure A3 A, B).

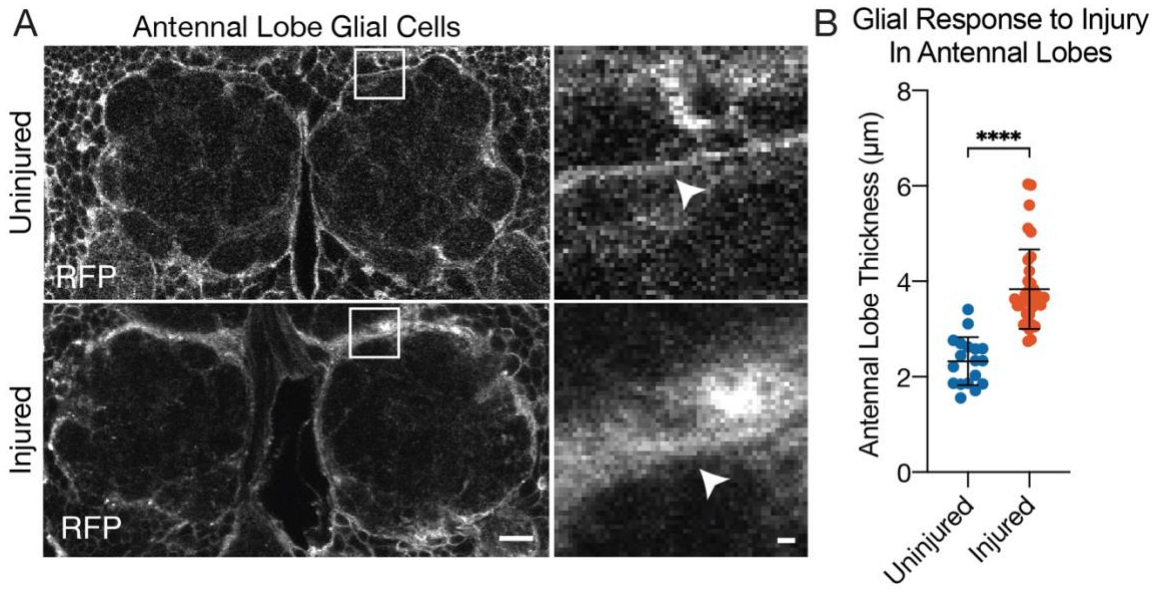


Figure A3. **Morphological response of glial cells to neuronal injury.**
(A) Confocal images of glial cells surrounding *Drosophila* antennal lobes in uninjured (no antennal ablation) and injured (antennal ablation) conditions 1 day after injury. The white box outlines the higher magnification image to illustrate the thickness of the glial cell processes. White arrowheads indicate the glial cell processes around the antennal lobes. Note the increased presence and thickness in injured conditions. Scale bars: 10µm in low magnification images, 1µm in higher magnification image.
(B) Quantification of the mean thickness of wild-type glial cell processes surrounding antennal lobes in injured and uninjured conditions 1 day after injury. Mean ±SD is also shown. Statistical significance was determined using an unpaired Student's t-test; **** $p < 0.0001$. Numbers of antennal lobes analyzed for uninjured conditions: 18; for injured conditions: 35.

To test the involvement of caspases in this process, I inhibited or removed caspase activity through a variety of different methods. In the same manner as described in Chapter 2, glial cells homozygous mutant for *Dronc* (*Dronc*$^{-/-}$) were generated using *Dronc* knockout (*Dronc*^{KO}) and *Dronc* conditional-knockout (*Dronc*$^{KO\text{-}FRT\ Dronc\text{-}GFP\text{-}Apex\ FRT\text{-}QF}$) alleles. Effector caspase activity was inhibited through glial cell specific over-expression of P35, and both initiator and effector caspases were inhibited through over-expression of Diap-1. Additionally, using animals entirely homozygous mutant

for *Dredd*, an initiator caspase primarily involved in immunity (Leulier et al., 2000; Meinander et al., 2012), I was able to remove Dredd activity as well. Ultimately however, I observed that all these various manipulations of caspase activity in glial cells had no interpretable effect on the morphological changes in glial cells responding to axonal injury (Figure A4). In all conditions, glial cells increased the presence of their cellular processes at the injured antennal lobe (Figure A4), suggesting that loss of caspase function had little impact on the ability of glial cells to react to damage.

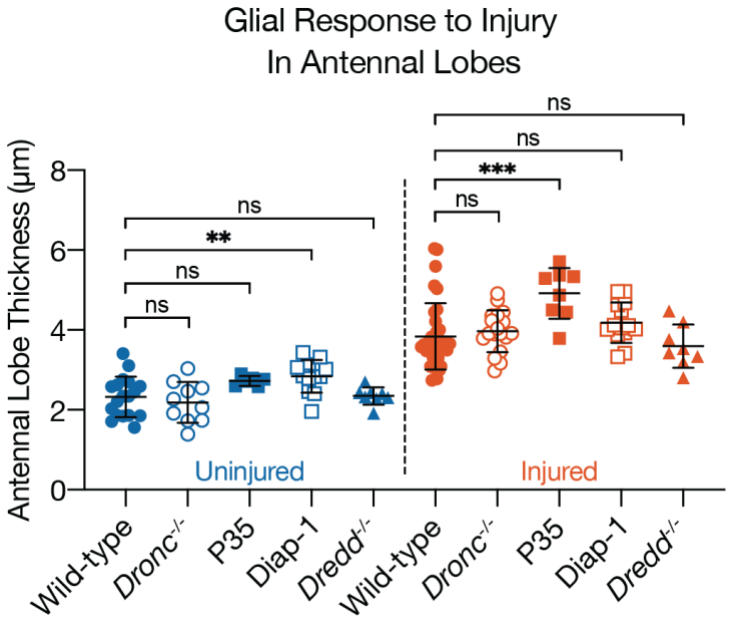


Figure A4. **Loss of caspase function in glial cells does not affect morphological response to neuronal injury.**
Quantification of the mean thickness of glial cell processes surrounding antennal lobes in injured and uninjured conditions 1 day after injury in wild-type and different caspase-inhibiting or caspase mutant genetic backgrounds. *Dronc*$^{-/-}$ mutants, P35 overexpression and Diap-1 overexpression were specific to glial cells. *Dredd*$^{-/-}$ mutants were homozygous mutant in all cells. In all genotypes, in increase in glial membrane thickness around the antennal lobes can be measured a day after injury. Mean ±SD is also shown. Statistical significance was determined by one-way ANOVA with Sidak's multiple comparisons tests; ns – not significant, ** p = 0.0082, *** p = 0.0005. Numbers of antennal lobes analyzed for all conditions (**genotype**: uninjured, injured) – **wild-type:** 18, 35; ***Dronc*$^{-/-}$:** 10, 18; **P35:** 6, 8; **Diap-1:** 12, 14; ***Dredd*$^{-/-}$:** 7, 7.

Dronc is not involved in glial cell clearance of axonal damage

In addition to investigating the morphological effects of the loss of caspase function in glial cells, I examined the possibility that caspase activity could be involved in the functional aspects of glial injury response. These functions include the phagocytosis and clearance of neuronal debris in a process similar to Wallerian degeneration in mammals (MacDonald et al., 2006). During this process, glial cells recruited to the damaged neurons upregulate the glial engulfment receptor Draper, which is required for glial cells to clear the degenerating severed axons resulting from injury (MacDonald et al., 2006). In the case of damage to maxillary olfactory receptor neurons (ORNs) by ablation of the maxillary palps, axonal fibers in the antennal lobes can be cleared in 3 – 5 days (MacDonald et al., 2006).

Consistent with these descriptions, in wild-type flies I found that Draper was consistently upregulated in glial cells upon antennal ablation, as detected by Draper immunolabeling (Figure A5 A, B). Similarly, I was able to label a specific subset of maxillary ORNs with GFP using the *OR85e-Gal4* driver, and observe that GFP labeled axonal fibers were cleared in 3 days upon maxillary palp ablation (Figure A5 C, D). However, *Dronc* mutant glial cells also consistently upregulated Draper expression, and were also capable of clearing axonal fibers by 3 days after maxillary palp ablation (Figure A5 A, B, C, D). These results suggest that Dronc is not required for functional glial cell response to neuronal damage. Altogether, my findings suggest that caspases in general are not essential for dynamic morphological remodeling of glial cells during development or injury response.

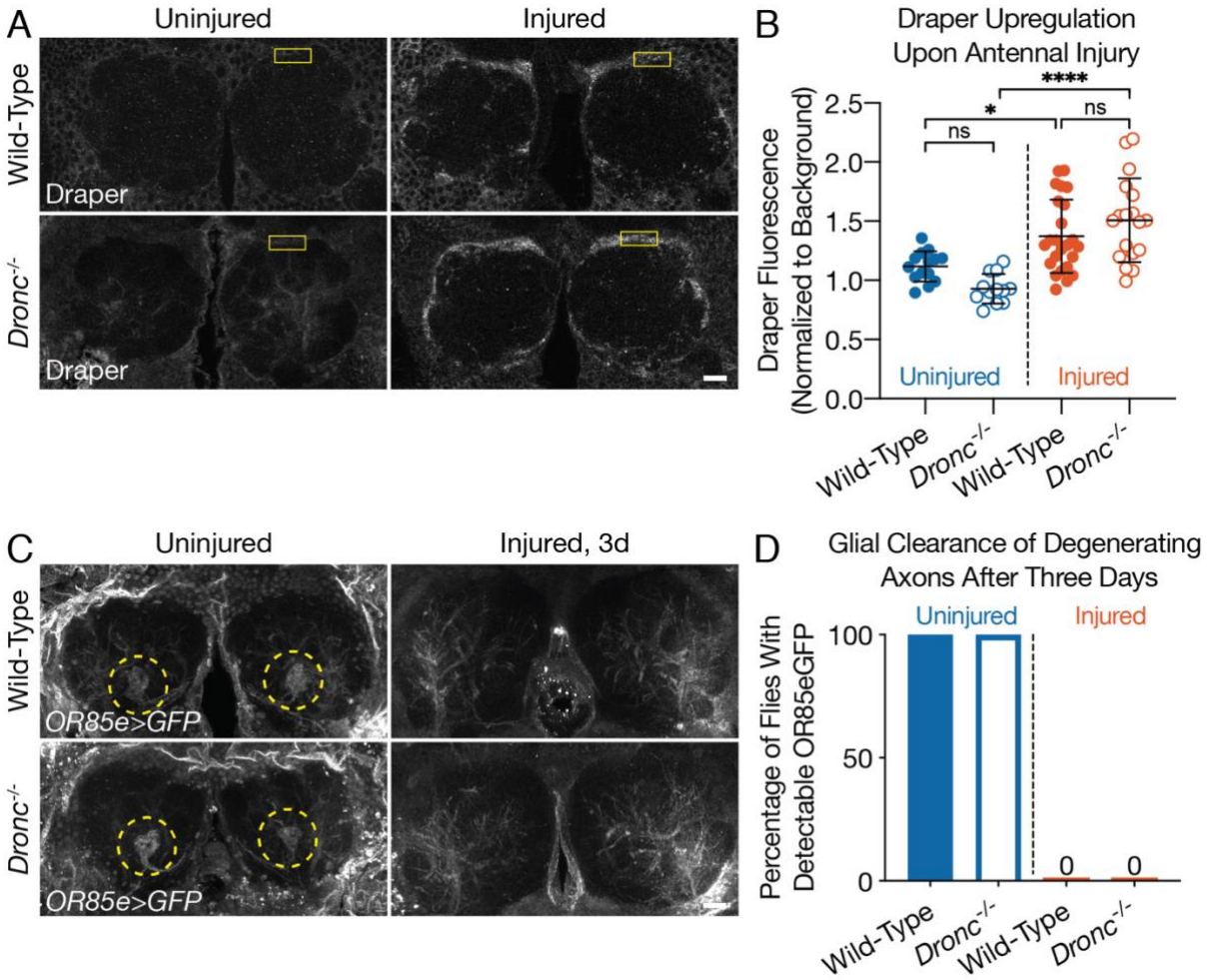


Figure A5. **Loss of *Dronc* does not functionally compromise glial injury response.**
(A) Representative confocal images of *Drosophila* antennal lobes immunolabeled for Draper in uninjured and injured conditions 1 day after injury. *Dronc*$^{-/-}$ represents animals with glial cells homozygous mutant for *Dronc*. Yellow rectangle indicates the area where Draper expression was measured. Scale bar: 10µm.
(B) Quantification of Draper expression in wild-type and *Dronc*$^{-/-}$ glial cells surrounding uninjured and injured antennal lobes 1 day after injury. Measurements were normalized to background intensities in each quantified image. Mean ±SD is also shown. Statistical significance was determined using one-way ANOVA with Tukey's tests for multiple comparisons. * $p = 0.338$, **** $p < 0.0001$. Numbers of antennal lobes analyzed for all conditions (**genotype**: uninjured, injured) – **wild-type:** 14, 24; ***Dronc*$^{-/-}$:** 12, 18.
(C) Representative confocal images of *Drosophila* antennal lobes with a subset of maxillary ORNs labeled with GFP (*OR85e>GFP*) in uninjured and injured conditions 3 days after injury. *Dronc*$^{-/-}$ represents animals with glial cells homozygous mutant for *Dronc*. In uninjured conditions, GFP^{+} OR85e axonal fibers can be located in antennal lobes (yellow dotted circles). 3 days after injury, axonal fibers can no longer be located.
(D) Quantification of the percentage of flies with detectable OR85e axonal fibers in wild-type animals and animals homozygous mutant for *Dronc* in glial cells (*Dronc*$^{-/-}$) in uninjured and injured conditions three days after maxillary palp ablation. Numbers of antennal lobes analyzed for all conditions (**genotype**: uninjured, injured) – **wild-type:** 12, 8; ***Dronc*$^{-/-}$:** 12, 11.

Loss of function of Btbd7 does not compromise glial cell shape remodeling

As described before, the mouse and human gene *Btbd7* has been associated with cell shape remodeling, migration, and metastasis. An orthologue to the gene can also be found in *Drosophila*, identified as *CG16952*. Hereafter I will refer to this gene as *D-Btbd7*. Because high transcriptional levels of *D-Btbtd7* can be found in the central nervous system and brain (Gramates et al., 2017), I decided to also evaluate its potential roles during glial cell remodeling in development and injury. To better evaluate the gene's functional importance, I created a null mutant allele of *D-Btbd7* by combining RISPR/Cas9 technology with homologous recombination (Baena-Lopez et al., 2013). I replaced most of the gene's coding region on the X chromosome with an *attP* genomic "landing site," which could allow the integration of customized exogenous DNA sequences at the locus (Baena-Lopez et al., 2013; Knapp et al., 2015). Homozygous null mutants of *D-Btbd7* were both viable and fertile, and showed no obvious morphological phenotypes of developmental defects under standard fly husbandry conditions. As with caspases, *D-Btbd7* null mutant animals exhibited a biologically normal glial response to olfactory nerve injury as measured by the thickness of glial cell processes at the antennal lobe, suggesting no major role in this biological process (Figure A6 A, B).

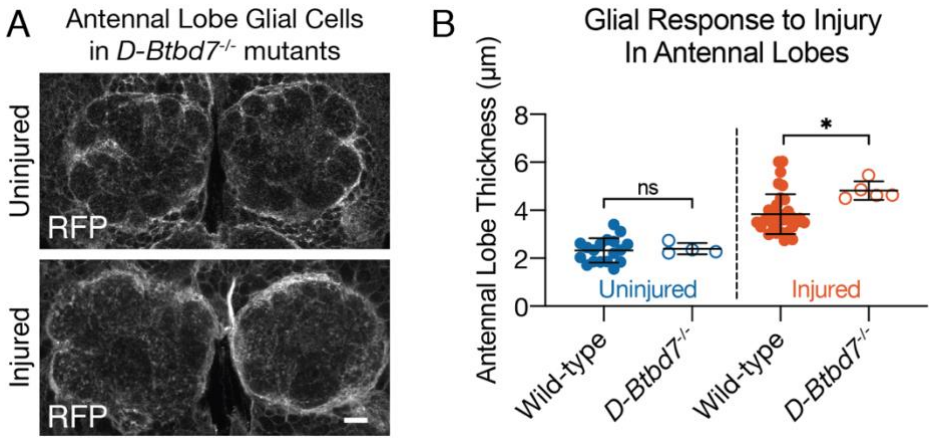


Figure A6. **Loss of *D-Btbd7* does not affect glial cells' morphological response to neuronal injury.**
(A) Representative confocal images of glial cells surrounding *Drosophila* antennal lobes in uninjured (no antennal ablation) and injured (antennal ablation) conditions 1 day after injury in *D-Btbd7* mutants. Scale bar: 10µm.
(B) Quantification of the mean thickness of glial cell processes surrounding antennal lobes in injured and uninjured conditions 1 day after injury in wild-type animals and *D-Btbd7* mutants. Mean ±SD is also shown. Statistical significance was determined using a one-way ANOVA with Tukey's tests for multiple comparisons; ns – not significant, * p = 0.0259. Numbers of antennal lobes analyzed for uninjured conditions: 18 wild-type, 4 *D-Btbd7*; for injured conditions: 35 wild-type, 5 *D-Btbd7*.

Discussion

In this section, I sought to investigate the mechanisms behind which caspase and Btbd7 influence cell morphology and motility. Glial cells are known to be especially morphologically dynamic, rapidly remodeling their dense ramifications, and are highly motile during development and in their response to injury (Freeman, 2015; Tremblay et al., 2009). I decided to study the function of these proteins in *Drosophila* glial cells, as *D-Btbd7* had been shown to be highly expressed in the central nervous system (Gramates et al., 2017). Non-apoptotic caspase activity had been demonstrated in neuronal remodeling before, as caspases are instrumental for dendrite modeling in neurons in many organisms, including *Drosophila* (Mukherjee and Williams, 2017). Additionally, high levels of caspase activity can be detected in peripheral glial cells

during metamorphosis, a period of intense reorganization as glial cells remodel themselves to adapt from larvae to adult life (Darren Williams, personal communication).

However, my data suggest that neither caspases nor Btbd7 are involved in regulating glial cell dynamics. In the case of glial cell dynamics during metamorphosis, I found that downregulation of the main initiator caspase Dronc in glial cells resulted in no obvious phenotypes that differed from wild-type glial cells. It is possible that the *RNAi* transgene used to downregulate *Dronc* was insufficient to deplete enough of the gene to affect function in glial cells. Alternatively, *Dronc* perdurance could explain the lack of an effect of downregulation, as maternally deposited *Dronc* mRNA is sufficient for *Dronc* homozygous mutants to survive and otherwise lethal mutation (Xu et al., 2005). However, in light of the results regarding glial cell response to axonal injury, where I inhibited caspase function through a variety of methods to no effect, it is more likely that caspases simply play no role in these processes.

Similarly, D-Btbd7 appears to have no functional role in glial cell dynamics either, as null mutants appear phenotypically normal in the contexts I investigated. It is possible that the high expression of *D-Btbd7* observed in the brain and central nervous system corresponds not to glial cell expression of the gene, but neuronal expression. Thus, it would be interesting to investigate the tissue-specific expression of *D-Btbd7* in the nervous system to better inform the planning of future experiments. This can be

achieved by the insertion of a *Gal4* into the *D-Btbd7* gene locus through the use of the *attP* site I inserted.

At a later date, it could be possible to investigate further the role of caspases and Btbtd7 in regulating cellular remodeling and cell shape dynamics using different techniques, or in different contexts. Ultimately however, in the interest of focusing on the role of caspases in EJS tumors as described in the preceding chapters, I decided to put this topic aside.

Materials and Methods

Drosophila stocks and husbandry

All *Drosophila* stocks were maintained at 20°C in vials of *Drosophila* culture medium (described in Chapter 6). Additional yeast was also added to each vial before flies were added. Crosses were made at 25ºC and then moved to 29ºC if necessary, for temperature sensitive experiments.

Drosophila genotypes

Genotype	Description	Source	Figures used
Repo-Gal4, UAS-LifeActGFP	Glial cell marker	Gift from D. Williams	A1
Repo-Gal4, TubGal80^{ts}, UAS-RFP^{myr}	Glial cell marker	Gift from S. Casas-Tinto	A2- A6
Repo-Gal4, TubGal80^{ts}, UAS-RFP^{myr}, Dronc^{KO}/TM6B	Dronc^{KO} Glial cell marker	Baena-Lopez lab	A4, A5
UAS-Flippase, Dronc$^{KO\text{-}FRT\text{-}Dronc\text{-}GFP\text{-}Apex\text{-}FRT\ QF}$/TM6B	Conditional Dronc^{KO}	(Arthurton et al., 2019)	A2, A4, A5
UAS-Dronc-RNAi	UAS-Dronc-RNAi	Gift from Pascal Meier (Leulier et al., 2000)	A1
Dredd^{KO}	*Dredd* null mutant	Baena-Lopez lab	A4
w[*]; P{w[+mC]=UAS-p35.H}BH1	UAS-P35	Bloomington Drosophila Stock Center: 5072	A4

w[*]; P{w[+mC]=UAS-DIAP1.H}3	UAS-Diap-1	Bloomington Stock Center: 6657	A4
OR85e-mcd8GFP	Maxillary ORN marker	Gift from T. Fulga	A5
D-Btbd7	CG16952 mutant	This Thesis	A6

Molecular biology, cloning and generation of *D-Btbd7* mutant

Standard molecular biology techniques and protocols were used. The following primers were used to amplify 5' and 3' homology arms of *D-Btbd7*:

FP 5': `atttatagcggccgctggtgtaggtgcttctatgcaggTCCTGCGAGTTTTTTACATGTGAGG`

RP 5': `ttaattaggtaccAGGACACGCACAGTGGGTAATATCTTTAAATTTCATGC`

FP 3': `attattaactagtGGGCATTATTTTAGCATGCC`

RP 3': `attatattaccggtttcgagtggcacccgatcacgggGACAAGTACAAGTTTGATGATGG`

Homology arms were then integrated into a modified PTV4 vector containing a Pax-RFP motif instead of *mini-white* (Baena-Lopez et al., 2013). The Not1, Kpn1, SpeI, and Age1 restriction enzymes were used. The following guide primers were designed using the following tool (http://tools.flycrispr.molbio.wisc.edu/targetFinder/) and subcloned into the pCFD3 vector (Baena-Lopez et al., 2013) using the BbsI restriction enzyme:

FP Guide 1: `gtcgTGGTGTAGGTGCTTCTATGC`

RP Guide 1: `gtcgTTCGAGTGGCACCCGATCAC`

FP Guide 2: `aaacGCATAGAAGCACCTACACCA`

RP Guide 2: `aaacGTGATCGGGTGCCACTCGAA`

Gene editing of the *D-Btbd7* locus was then performed as described previously (Baena-Lopez et al., 2013) to generate a null-allele of the gene with an *attP* site at the locus.

Live-imaging of peripheral glia during metamorphosis

To examine peripheral glia dynamics during metamorphosis, pupae were staged and collected 24 hours after pupariation. Pupal cases were then carefully removed using Dumont no. 5 forceps, and pupae were mounted onto slides in a similar fashion as previously described (Hellerman et al., 2015). Peripheral glial cells were then imaged using an inverted Zeiss 880 confocal microscope using a 60x oil objective. Approximately 20-30 z-stacks were captured every 20 seconds or 1min for 20 minutes. Movies were then processed using Fiji.

Neuronal injury protocol and adult brain dissection

Crosses were kept at 25°C and then moved to 29°C after eclosion to fully repress Gal4 inhibition by $Gal80^{ts}$ for at least 5 days before injury. Neuronal injury was inflicted upon adult male *Drosophila* as described (MacDonald et al., 2006). Flies were returned to 29°C for one day, and brains were then dissected, stained, and mounted as described (Wu and Luo, 2006). The mouse anti-draper (Developmental Studies Hybridoma Bank 5D14, 1:200) primary antibody was used, along with the Alexa Fluor secondary antibody secondary 488-anti-mouse (Life Technologies, 1:500). Images were collected using an Olympus Fluoview FV1200, making sure to collect the entirety of antennal lobes. Images were processed in Fiji, and antennal

lobe thickness measurements were taken manually. 12 measurements around the circumference of each lobe (spaced roughly every 30°) were averaged to measure antennal lobe thickness at a single z-plane at the center of the antennal lobe. Intensity of Draper fluorescence was also measured on maximum projected images of antennal lobes using Fiji. Background intensities were measured and used to normalize intensities of Draper expression in each image quantified. Draper measurements were taken at similar positions across all antennal lobes.

Quantification and Statistical Analysis

All data was processed and analyzed using GraphPad Prism 8. Unpaired Student's t-tests were used to compare values between genotypes for univariate data. Figures were generated using Adobe Illustrator CC.

References

Adachi-Yamada, T., Nakamura, M., Irie, K., Tomoyasu, Y., Sano, Y., Mori, E., Goto, S., Ueno, N., Nishida, Y., and Matsumoto, K. (1999). p38 mitogen-activated protein kinase can be involved in transforming growth factor beta superfamily signal transduction in Drosophila wing morphogenesis. Mol Cell Biol *19*, 2322-2329.

Aggarwal, B.B., Vijayalekshmi, R.V., and Sung, B. (2009). Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe. Clin Cancer Res *15*, 425-430.

Ahmed-de-Prado, S., and Baonza, A. (2018). Drosophila as a Model System to Study Cell Signaling in Organ Regeneration. Biomed Res Int *2018*, 7359267.

Ahmed-de-Prado, S., Diaz-Garcia, S., and Baonza, A. (2018). JNK and JAK/STAT signalling are required for inducing loss of cell fate specification during imaginal wing discs regeneration in Drosophila melanogaster. Dev Biol *441*, 31-41.

Almudi, I., Stocker, H., Hafen, E., Corominas, M., and Serras, F. (2009). SOCS36E specifically interferes with Sevenless signaling during Drosophila eye development. Dev Biol *326*, 212-223.

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., and Yuan, J. (1996). Human ICE/CED-3 protease nomenclature. Cell *87*, 171.

Amcheslavsky, A., Wang, S., Fogarty, C.E., Lindblad, J.L., Fan, Y., and Bergmann, A. (2018). Plasma Membrane Localization of Apoptotic Caspases for Non-apoptotic Functions. Dev Cell *45*, 450-464 e453.

Andersen, D.S., Colombani, J., Palmerini, V., Chakrabandhu, K., Boone, E., Rothlisberger, M., Toggweiler, J., Basler, K., Mapelli, M., Hueber, A.O.*, et al.* (2015). The Drosophila TNF receptor Grindelwald couples loss of cell polarity and neoplastic growth. Nature *522*, 482-486.

Andl, C.D., Mizushima, T., Oyama, K., Bowser, M., Nakagawa, H., and Rustgi, A.K. (2004). EGFR-induced cell migration is mediated predominantly by the JAK-STAT pathway in primary esophageal keratinocytes. Am J Physiol Gastrointest Liver Physiol *287*, G1227-1237.

Arama, E., Baena-Lopez, L.A., and Fearnhead, H.O. (2020). Non-lethal message from the Holy Land: The first international conference on nonapoptotic roles of apoptotic proteins. FEBS J.

Arbouzova, N.I., and Zeidler, M.P. (2006). JAK/STAT signalling in Drosophila: insights into conserved regulatory and cellular functions. Development *133*, 2605-2616.

Arthurton, L., Nahotko, D.A., Alonso, J., Wendler, F., and Baena-Lopez, L.A. (2020). Non-apoptotic caspase activation preserves Drosophila intestinal progenitor cells in quiescence. EMBO Rep, e48892.

Ashley, N.T., Weil, Z.M., and Nelson, R.J. (2012). Inflammation: Mechanisms, Costs, and Natural Variation. Annual Review of Ecology, Evolution, and Systematics *43*, 385-406.

Babcock, D.T., Brock, A.R., Fish, G.S., Wang, Y., Perrin, L., Krasnow, M.A., and Galko, M.J. (2008). Circulating blood cells function as a surveillance system for damaged tissue in Drosophila larvae. Proc Natl Acad Sci U S A *105*, 10017-10022.

Baena-Lopez, L.A., Alexandre, C., Mitchell, A., Pasakarnis, L., and Vincent, J.P. (2013). Accelerated homologous recombination and subsequent genome modification in Drosophila. Development *140*, 4818-4825.

Baena-Lopez, L.A., Arthurton, L., Bischoff, M., Vincent, J.P., Alexandre, C., and McGregor, R. (2018a). Novel initiator caspase reporters uncover previously unknown features of caspase-activating cells. Development *145*.

Baena-Lopez, L.A., Arthurton, L., Xu, D.C., and Galasso, A. (2018b). Non-apoptotic Caspase regulation of stem cell properties. Semin Cell Dev Biol *82*, 118-126.

Balkwill, F., and Mantovani, A. (2001). Inflammation and cancer: back to Virchow? Lancet *357*, 539-545.

Bangi, E. (2013). Drosophila at the intersection of infection, inflammation, and cancer. Front Cell Infect Microbiol *3*, 103.

Baum, J.S., Arama, E., Steller, H., and McCall, K. (2007). The Drosophila caspases Strica and Dronc function redundantly in programmed cell death during oogenesis. Cell Death Differ *14*, 1508-1517.

Beira, J.V., and Paro, R. (2016). The legacy of Drosophila imaginal discs. Chromosoma *125*, 573-592.

Belacortu, Y., and Paricio, N. (2011). Drosophila as a model of wound healing and tissue regeneration in vertebrates. Dev Dyn *240*, 2379-2404.

Bell, R.A.V., and Megeney, L.A. (2017). Evolution of caspase-mediated cell death and differentiation: twins separated at birth. Cell Death Differ *24*, 1359-1368.

Bender, C.E., Fitzgerald, P., Tait, S.W., Llambi, F., McStay, G.P., Tupper, D.O., Pellettieri, J., Sanchez Alvarado, A., Salvesen, G.S., and Green, D.R. (2012). Mitochondrial pathway of apoptosis is ancestral in metazoans. Proc Natl Acad Sci U S A *109*, 4904-4909.

Bergantinos, C., Corominas, M., and Serras, F. (2010). Cell death-induced regeneration in wing imaginal discs requires JNK signalling. Development *137*, 1169-1179.

Bergmann, A., Agapite, J., McCall, K., and Steller, H. (1998). The Drosophila gene hid is a direct molecular target of Ras-dependent survival signaling. Cell *95*, 331-341.

Berthelet, J., and Dubrez, L. (2013). Regulation of Apoptosis by Inhibitors of Apoptosis (IAPs). Cells *2*, 163-187.

Betz, A., Ryoo, H.D., Steller, H., and Darnell, J.E., Jr. (2008). STAT92E is a positive regulator of Drosophila inhibitor of apoptosis 1 (DIAP/1) and protects against radiation-induced apoptosis. Proc Natl Acad Sci U S A *105*, 13805-13810.

Bier, E. (2005). Drosophila, the golden bug, emerges as a tool for human genetics. Nat Rev Genet *6*, 9-23.

Binari, R., and Perrimon, N. (1994). Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. Genes Dev *8*, 300-312.

Bito, T., Sumita, N., Ashida, M., Budiyanto, A., Ueda, M., Ichihashi, M., Tokura, Y., and Nishigori, C. (2011). Inhibition of Epidermal Growth Factor Receptor and PI3K/Akt Signaling Suppresses Cell Proliferation and Survival through Regulation of Stat3 Activation in Human Cutaneous Squamous Cell Carcinoma. J Skin Cancer *2011*, 874571.

Blair, S.S., Brower, D.L., Thomas, J.B., and Zavortink, M. (1994). The role of apterous in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of Drosophila. Development *120*, 1805-1815.

Braicu, C., Buse, M., Busuioc, C., Drula, R., Gulei, D., Raduly, L., Rusu, A., Irimie, A., Atanasov, A.G., Slaby, O.*, et al.* (2019). A Comprehensive Review on MAPK: A Promising Therapeutic Target in Cancer. Cancers (Basel) *11*.

Brentnall, M., Weir, D.B., Rongvaux, A., Marcus, A.I., and Boise, L.H. (2014). Procaspase-3 regulates fibronectin secretion and influences adhesion, migration and survival independently of catalytic function. J Cell Sci *127*, 2217-2226.

Brooks, A.J., and Putoczki, T. (2020). JAK-STAT Signalling Pathway in Cancer. Cancers (Basel) *12*.

Brown, M.F., Leibowitz, B.J., Chen, D., He, K., Zou, F., Sobol, R.W., Beer-Stolz, D., Zhang, L., and Yu, J. (2015). Loss of caspase-3 sensitizes colon cancer cells to genotoxic stress via RIP1-dependent necrosis. Cell Death Dis *6*, e1729.

Brown, S., Hu, N., and Hombria, J.C. (2001). Identification of the first invertebrate interleukin JAK/STAT receptor, the Drosophila gene domeless. Curr Biol *11*, 1700-1705.

Brumby, A.M., and Richardson, H.E. (2003). scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila. EMBO J *22*, 5769-5779.

Buchon, N., Silverman, N., and Cherry, S. (2014). Immunity in Drosophila melanogaster--from microbial recognition to whole-organism physiology. Nat Rev Immunol *14*, 796-810.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999). Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol *15*, 269-290.

Bunker, B.D., Nellimoottil, T.T., Boileau, R.M., Classen, A.K., and Bilder, D. (2015). The transcriptional response to tumorigenic polarity loss in Drosophila. Elife *4*.

Burgon, P.G., and Megeney, L.A. (2018). Caspase signaling, a conserved inductive cue for metazoan cell differentiation. Semin Cell Dev Biol *82*, 96-104.

Callus, B.A., and Mathey-Prevot, B. (2002). SOCS36E, a novel Drosophila SOCS protein, suppresses JAK/STAT and EGF-R signalling in the imaginal wing disc. Oncogene *21*, 4812-4821.

Campbell, G., and Tomlinson, A. (1998). The roles of the homeobox genes aristaless and Distal-less in patterning the legs and wings of Drosophila. Development *125*, 4483-4493.

Cantwell, H., and Nurse, P. (2019). Unravelling nuclear size control. Curr Genet *65*, 1281-1285.

Carneiro, B.A., and El-Deiry, W.S. (2020). Targeting apoptosis in cancer therapy. Nat Rev Clin Oncol *17*, 395-417.

Cartwright, I.M., Liu, X., Zhou, M., Li, F., and Li, C.Y. (2017). Essential roles of Caspase-3 in facilitating Myc-induced genetic instability and carcinogenesis. Elife *6*.

Casas-Tinto, S., Lolo, F.N., and Moreno, E. (2015). Active JNK-dependent secretion of Drosophila Tyrosyl-tRNA synthetase by loser cells recruits haemocytes during cell competition. Nat Commun *6*, 10022.

Chai, J., Yan, N., Huh, J.R., Wu, J.W., Li, W., Hay, B.A., and Shi, Y. (2003). Molecular mechanism of Reaper-Grim-Hid-mediated suppression of DIAP1-dependent Dronc ubiquitination. Nat Struct Biol *10*, 892-898.

Chakrabarti, S., Dudzic, J.P., Li, X., Collas, E.J., Boquete, J.P., and Lemaitre, B. (2016). Remote Control of Intestinal Stem Cell Activity by Haemocytes in Drosophila. PLoS Genet *12*, e1006089.

Charroux, B., and Royet, J. (2009). Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the Drosophila immune response. Proc Natl Acad Sci U S A *106*, 9797-9802.

Chatterjee, N., and Bohmann, D. (2012). A versatile PhiC31 based reporter system for measuring AP-1 and Nrf2 signaling in Drosophila and in tissue culture. PLoS One *7*, e34063.

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., and Zhao, L. (2018). Inflammatory responses and inflammation-associated diseases in organs. Oncotarget *9*, 7204-7218.

Chen, Y., Song, Y., Du, W., Gong, L., Chang, H., and Zou, Z. (2019). Tumor-associated macrophages: an accomplice in solid tumor progression. J Biomed Sci *26*, 78.

Chen, Y., Yang, Z., Meng, M., Zhao, Y., Dong, N., Yan, H., Liu, L., Ding, M., Peng, H.B., and Shao, F. (2009). Cullin mediates degradation of RhoA through evolutionarily conserved BTB adaptors to control actin cytoskeleton structure and cell movement. Mol Cell *35*, 841-855.

Cheng, J., Tian, L., Ma, J., Gong, Y., Zhang, Z., Chen, Z., Xu, B., Xiong, H., Li, C., and Huang, Q. (2015). Dying tumor cells stimulate proliferation of living tumor cells via caspase-dependent protein kinase Cdelta activation in pancreatic ductal adenocarcinoma. Mol Oncol *9*, 105-114.

Chipuk, J.E., Bouchier-Hayes, L., and Green, D.R. (2006). Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell Death Differ *13*, 1396-1402.

Clark, R.I., Woodcock, K.J., Geissmann, F., Trouillet, C., and Dionne, M.S. (2011). Multiple TGF-beta superfamily signals modulate the adult Drosophila immune response. Curr Biol *21*, 1672-1677.

Clavier, A., Rincheval-Arnold, A., Colin, J., Mignotte, B., and Guenal, I. (2016). Apoptosis in Drosophila: which role for mitochondria? Apoptosis *21*, 239-251.

Collins, C.A., Wairkar, Y.P., Johnson, S.L., and DiAntonio, A. (2006). Highwire restrains synaptic growth by attenuating a MAP kinase signal. Neuron *51*, 57-69.

Colombani, J., Andersen, D.S., and Leopold, P. (2012). Secreted peptide Dilp8 coordinates Drosophila tissue growth with developmental timing. Science *336*, 582-585.

Colotta, F., Allavena, P., Sica, A., Garlanda, C., and Mantovani, A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis *30*, 1073-1081.

Comen, E.A., Bowman, R.L., and Kleppe, M. (2018). Underlying Causes and Therapeutic Targeting of the Inflammatory Tumor Microenvironment. Front Cell Dev Biol *6*, 56.

Connolly, P.F., Jager, R., and Fearnhead, H.O. (2014). New roles for old enzymes: killer caspases as the engine of cell behavior changes. Front Physiol *5*, 149.

Cordero, J.B., Macagno, J.P., Stefanatos, R.K., Strathdee, K.E., Cagan, R.L., and Vidal, M. (2010). Oncogenic Ras diverts a host TNF tumor suppressor activity into tumor promoter. Dev Cell *18*, 999-1011.

Courtiade, J., Pauchet, Y., Vogel, H., and Heckel, D.G. (2011). A comprehensive characterization of the caspase gene family in insects from the order Lepidoptera. BMC Genomics *12*, 357.

Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860-867.

Cox, A.D., and Der, C.J. (2003). The dark side of Ras: regulation of apoptosis. Oncogene *22*, 8999-9006.

Crawford, E.D., Seaman, J.E., Agard, N., Hsu, G.W., Julien, O., Mahrus, S., Nguyen, H., Shimbo, K., Yoshihara, H.A., Zhuang, M.*, et al.* (2013). The DegraBase: a database of proteolysis in healthy and apoptotic human cells. Mol Cell Proteomics *12*, 813-824.

Crawford, E.D., and Wells, J.A. (2011). Caspase substrates and cellular remodeling. Annu Rev Biochem *80*, 1055-1087.

D'Arcy, M.S. (2019). Cell death: a review of the major forms of apoptosis, necrosis and autophagy. Cell Biol Int *43*, 582-592.

Defaye, A., Evans, I., Crozatier, M., Wood, W., Lemaitre, B., and Leulier, F. (2009). Genetic ablation of Drosophila phagocytes reveals their contribution to both development and resistance to bacterial infection. J Innate Immun *1*, 322-334.

Degterev, A., Boyce, M., and Yuan, J. (2003). A decade of caspases. Oncogene *22*, 8543-8567.

del Valle Rodriguez, A., Didiano, D., and Desplan, C. (2011). Power tools for gene expression and clonal analysis in Drosophila. Nat Methods *9*, 47-55.

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

Deschenes-Simard, X., Kottakis, F., Meloche, S., and Ferbeyre, G. (2014). ERKs in cancer: friends or foes? Cancer Res *74*, 412-419.

Dhanasekaran, D.N., and Reddy, E.P. (2017). JNK-signaling: A multiplexing hub in programmed cell death. Genes Cancer *8*, 682-694.

Dhillon, A.S., Hagan, S., Rath, O., and Kolch, W. (2007). MAP kinase signalling pathways in cancer. Oncogene *26*, 3279-3290.

Diaz-Albiter, H., Mitford, R., Genta, F.A., Sant'Anna, M.R., and Dillon, R.J. (2011). Reactive oxygen species scavenging by catalase is important for female Lutzomyia longipalpis fecundity and mortality. PLoS One *6*, e17486.

Diaz-Benjumea, F.J., and Cohen, S.M. (1993). Interaction between dorsal and ventral cells in the imaginal disc directs wing development in Drosophila. Cell *75*, 741-752.

Diedenhofen, B., and Musch, J. (2015). cocor: a comprehensive solution for the statistical comparison of correlations. PLoS One *10*, e0121945.

Dikalov, S., Griendling, K.K., and Harrison, D.G. (2007). Measurement of reactive oxygen species in cardiovascular studies. Hypertension *49*, 717-727.

Ding, A.X., Sun, G., Argaw, Y.G., Wong, J.O., Easwaran, S., and Montell, D.J. (2016). CasExpress reveals widespread and diverse patterns of cell survival of caspase-3 activation during development in vivo. Elife *5*.

Ditzel, M., Broemer, M., Tenev, T., Bolduc, C., Lee, T.V., Rigbolt, K.T., Elliott, R., Zvelebil, M., Blagoev, B., Bergmann, A.*, et al.* (2008). Inactivation of effector caspases through nondegradative polyubiquitylation. Mol Cell *32*, 540-553.

Diwanji, N., and Bergmann, A. (2018). An unexpected friend - ROS in apoptosis-induced compensatory proliferation: Implications for regeneration and cancer. Semin Cell Dev Biol *80*, 74-82.

Diwanji, N., and Bergmann, A. (2019). Two Sides of the Same Coin - Compensatory Proliferation in Regeneration and Cancer. Adv Exp Med Biol *1167*, 65-85.

Diwanji, N., and Bergmann, A. (2020). Basement membrane damage by ROS- and JNK-mediated Mmp2 activation drives macrophage recruitment to overgrown tissue. Nat Commun *11*, 3631.

Dorstyn, L., and Kumar, S. (2008). A biochemical analysis of the activation of the Drosophila caspase DRONC. Cell Death Differ *15*, 461-470.

Doumanis, J., Quinn, L., Richardson, H., and Kumar, S. (2001). STRICA, a novel Drosophila melanogaster caspase with an unusual serine/threonine-rich prodomain, interacts with DIAP1 and DIAP2. Cell Death Differ *8*, 387-394.

Duncan, S.A., Baganizi, D.R., Sahu, R., Singh, S.R., and Dennis, V.A. (2017). SOCS Proteins as Regulators of Inflammatory Responses Induced by Bacterial Infections: A Review. Front Microbiol *8*, 2431.

Dvorak, H.F. (2015). Tumors: wounds that do not heal-redux. Cancer Immunol Res *3*, 1-11.

Earnshaw, W.C., Martins, L.M., and Kaufmann, S.H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem *68*, 383-424.

Eckhart, L., Ballaun, C., Hermann, M., VandeBerg, J.L., Sipos, W., Uthman, A., Fischer, H., and Tschachler, E. (2008). Identification of novel mammalian caspases reveals an important role of gene loss in shaping the human caspase repertoire. Mol Biol Evol *25*, 831-841.

Ellis, S., Lin, E.J., and Tartar, D. (2018). Immunology of Wound Healing. Curr Dermatol Rep *7*, 350-358.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. Toxicol Pathol *35*, 495-516.

Elsum, I., Yates, L., Humbert, P.O., and Richardson, H.E. (2012). The Scribble-Dlg-Lgl polarity module in development and cancer: from flies to man. Essays Biochem *53*, 141-168.

Erturk, A., Wang, Y., and Sheng, M. (2014). Local pruning of dendrites and spines by caspase-3-dependent and proteasome-limited mechanisms. J Neurosci *34*, 1672-1688.

Esfahani, K., Roudaia, L., Buhlaiga, N., Del Rincon, S.V., Papneja, N., and Miller, W.H., Jr. (2020). A review of cancer immunotherapy: from the past, to the present, to the future. Curr Oncol *27*, S87-S97.

Fan, Y., Wang, S., Hernandez, J., Yenigun, V.B., Hertlein, G., Fogarty, C.E., Lindblad, J.L., and Bergmann, A. (2014). Genetic models of apoptosis-induced proliferation decipher activation of JNK and identify a requirement of EGFR signaling for tissue regenerative responses in Drosophila. PLoS Genet *10*, e1004131.

Fernald, K., and Kurokawa, M. (2013). Evading apoptosis in cancer. Trends Cell Biol *23*, 620-633.

Fianco, G., Contadini, C., Ferri, A., Cirotti, C., Stagni, V., and Barila, D. (2018). Caspase-8: A Novel Target to Overcome Resistance to Chemotherapy in Glioblastoma. Int J Mol Sci *19*.

Fianco, G., Mongiardi, M.P., Levi, A., De Luca, T., Desideri, M., Trisciuoglio, D., Del Bufalo, D., Cina, I., Di Benedetto, A., Mottolese, M.*, et al.* (2017). Caspase-8 contributes to angiogenesis and chemotherapy resistance in glioblastoma. Elife *6*.

Fisher, D.T., Appenheimer, M.M., and Evans, S.S. (2014). The two faces of IL-6 in the tumor microenvironment. Semin Immunol *26*, 38-47.

Flanagan, L., Meyer, M., Fay, J., Curry, S., Bacon, O., Duessmann, H., John, K., Boland, K.C., McNamara, D.A., Kay, E.W.*, et al.* (2016). Low levels of Caspase-3 predict favourable response to 5FU-based chemotherapy in advanced colorectal cancer: Caspase-3 inhibition as a therapeutic approach. Cell Death Dis *7*, e2087.

Florentin, A., and Arama, E. (2012). Caspase levels and execution efficiencies determine the apoptotic potential of the cell. J Cell Biol *196*, 513-527.

Fogarty, C.E., and Bergmann, A. (2017). Killers creating new life: caspases drive apoptosis-induced proliferation in tissue repair and disease. Cell Death Differ *24*, 1390-1400.

Fogarty, C.E., Diwanji, N., Lindblad, J.L., Tare, M., Amcheslavsky, A., Makhijani, K., Bruckner, K., Fan, Y., and Bergmann, A. (2016). Extracellular Reactive Oxygen Species Drive Apoptosis-Induced Proliferation via Drosophila Macrophages. Curr Biol *26*, 575-584.

Freeman, M.R. (2015). Drosophila Central Nervous System Glia. Cold Spring Harb Perspect Biol *7*.

Fuchs, Y., and Steller, H. (2011). Programmed cell death in animal development and disease. Cell *147*, 742-758.

Fuchs, Y., and Steller, H. (2015). Live to die another way: modes of programmed cell death and the signals emanating from dying cells. Nat Rev Mol Cell Biol *16*, 329-344.

Fuentes-Prior, P., and Salvesen, G.S. (2004). The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J *384*, 201-232.

Fujio, Y., Kunisada, K., Hirota, H., Yamauchi-Takihara, K., and Kishimoto, T. (1997). Signals through gp130 upregulate bcl-x gene expression via STAT1-binding cis-element in cardiac myocytes. J Clin Invest *99*, 2898-2905.

Fujisawa, Y., Kosakamoto, H., Chihara, T., and Miura, M. (2019). Non-apoptotic function of Drosophila caspase activation in epithelial thorax closure and wound healing. Development *146*.

Fulda, S., Gorman, A.M., Hori, O., and Samali, A. (2010). Cellular stress responses: cell survival and cell death. Int J Cell Biol *2010*, 214074.

Furman, D., Campisi, J., Verdin, E., Carrera-Bastos, P., Targ, S., Franceschi, C., Ferrucci, L., Gilroy, D.W., Fasano, A., Miller, G.W.*, et al.* (2019). Chronic inflammation in the etiology of disease across the life span. Nat Med *25*, 1822-1832.

Galasso, A., Iakovleva, D., and Baena-Lopez, L.A. (2020). Non-apoptotic caspase activity sustains proliferation and differentiation of ovarian somatic cells by modulating Hedgehog-signalling and autophagy. bioRxiv, [Preprint]. https://doi.org/10.1101/722330722330.

Galluzzi, L., Vitale, I., Aaronson, S.A., Abrams, J.M., Adam, D., Agostinis, P., Alnemri, E.S., Altucci, L., Amelio, I., Andrews, D.W.*, et al.* (2018). Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death Differ *25*, 486-541.

Garelli, A., Gontijo, A.M., Miguela, V., Caparros, E., and Dominguez, M. (2012). Imaginal discs secrete insulin-like peptide 8 to mediate plasticity of growth and maturation. Science *336*, 579-582.

Geisbrecht, E.R., and Montell, D.J. (2004). A role for Drosophila IAP1-mediated caspase inhibition in Rac-dependent cell migration. Cell *118*, 111-125.

Gerl, R., and Vaux, D.L. (2005). Apoptosis in the development and treatment of cancer. Carcinogenesis *26*, 263-270.

Gibon, J., Unsain, N., Gamache, K., Thomas, R.A., De Leon, A., Johnstone, A., Nader, K., Seguela, P., and Barker, P.A. (2016). The X-linked inhibitor of apoptosis regulates long-term depression and learning rate. FASEB J *30*, 3083-3090.

Gomez-Lamarca, M.J., Falo-Sanjuan, J., Stojnic, R., Abdul Rehman, S., Muresan, L., Jones, M.L., Pillidge, Z., Cerda-Moya, G., Yuan, Z., Baloul, S.*, et al.* (2018). Activation of the Notch Signaling Pathway In Vivo Elicits Changes in CSL Nuclear Dynamics. Dev Cell *44*, 611-623 e617.

Gonzalez, C. (2013). Drosophila melanogaster: a model and a tool to investigate malignancy and identify new therapeutics. Nat Rev Cancer *13*, 172-183.

Gonzalez, H., Hagerling, C., and Werb, Z. (2018). Roles of the immune system in cancer: from tumor initiation to metastatic progression. Genes Dev *32*, 1267-1284.

Gorelick-Ashkenazi, A., Weiss, R., Sapozhnikov, L., Florentin, A., Tarayrah-Ibraheim, L., Dweik, D., Yacobi-Sharon, K., and Arama, E. (2018). Caspases maintain tissue integrity by an apoptosis-independent inhibition of cell migration and invasion. Nat Commun *9*, 2806.

Goto, A., Kadowaki, T., and Kitagawa, Y. (2003). Drosophila hemolectin gene is expressed in embryonic and larval hemocytes and its knock down causes bleeding defects. Dev Biol *264*, 582-591.

Goyal, L. (2001). Cell death inhibition: keeping caspases in check. Cell *104*, 805-808.

Graf, R.P., Keller, N., Barbero, S., and Stupack, D. (2014). Caspase-8 as a regulator of tumor cell motility. Curr Mol Med *14*, 246-254.

Gramates, L.S., Marygold, S.J., Santos, G.D., Urbano, J.M., Antonazzo, G., Matthews, B.B., Rey, A.J., Tabone, C.J., Crosby, M.A., Emmert, D.B.*, et al.* (2017). FlyBase at 25: looking to the future. Nucleic Acids Res *45*, D663-D671.

Grivennikov, S.I., Greten, F.R., and Karin, M. (2010). Immunity, inflammation, and cancer. Cell *140*, 883-899.

Guo, Y.J., Pan, W.W., Liu, S.B., Shen, Z.F., Xu, Y., and Hu, L.L. (2020). ERK/MAPK signalling pathway and tumorigenesis. Exp Ther Med *19*, 1997-2007.

H. Dehkordi, M., Tashakor, A., O'Connell, E., and Fearnhead, H.O. (2020). Apoptosome-dependent myotube formation involves activation of caspase-3 in differentiating myoblasts. Cell Death Dis *11*, 308.

Hackney, J.F., and Cherbas, P. (2014). Injury response checkpoint and developmental timing in insects. Fly (Austin) *8*, 226-231.

Hackney, J.F., Zolali-Meybodi, O., and Cherbas, P. (2012). Tissue damage disrupts developmental progression and ecdysteroid biosynthesis in Drosophila. PLoS One *7*, e49105.

Halme, A., Cheng, M., and Hariharan, I.K. (2010). Retinoids regulate a developmental checkpoint for tissue regeneration in Drosophila. Curr Biol *20*, 458-463.

Hammouda, M.B., Ford, A.E., Liu, Y., and Zhang, J.Y. (2020). The JNK Signaling Pathway in Inflammatory Skin Disorders and Cancer. Cells *9*.

Han, C., Liu, Z., Zhang, Y., Shen, A., Dong, C., Zhang, A., Moore, C., Ren, Z., Lu, C., Cao, X.*, et al.* (2020). Tumor cells suppress radiation-induced immunity by hijacking caspase 9 signaling. Nat Immunol *21*, 546-554.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Hartl, T.A., and Scott, M.P. (2014). Wing tips: The wing disc as a platform for studying Hedgehog signaling. Methods *68*, 199-206.

Hauling, T., Krautz, R., Markus, R., Volkenhoff, A., Kucerova, L., and Theopold, U. (2014). A Drosophila immune response against Ras-induced overgrowth. Biol Open *3*, 250-260.

Hawkins, C.J., Yoo, S.J., Peterson, E.P., Wang, S.L., Vernooy, S.Y., and Hay, B.A. (2000). The Drosophila caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. J Biol Chem *275*, 27084-27093.

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

Helfer, B., Boswell, B.C., Finlay, D., Cipres, A., Vuori, K., Bong Kang, T., Wallach, D., Dorfleutner, A., Lahti, J.M., Flynn, D.C.*, et al.* (2006). Caspase-8 promotes cell motility and calpain activity under nonapoptotic conditions. Cancer Res *66*, 4273-4278.

Hellerman, M.B., Choe, R.H., and Johnson, R.I. (2015). Live-imaging of the Drosophila pupal eye. J Vis Exp, 52120.

Hensley, P., Mishra, M., and Kyprianou, N. (2013). Targeting caspases in cancer therapeutics. Biol Chem *394*, 831-843.

Herranz, H., Hong, X., Hung, N.T., Voorhoeve, P.M., and Cohen, S.M. (2012). Oncogenic cooperation between SOCS family proteins and EGFR identified using a Drosophila epithelial transformation model. Genes Dev *26*, 1602-1611.

Ho, L.H., Taylor, R., Dorstyn, L., Cakouros, D., Bouillet, P., and Kumar, S. (2009). A tumor suppressor function for caspase-2. Proc Natl Acad Sci U S A *106*, 5336-5341.

Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., and Ezekowitz, R.A. (1999). Phylogenetic perspectives in innate immunity. Science *284*, 1313-1318.

Honti, V., Csordas, G., Kurucz, E., Markus, R., and Ando, I. (2014). The cell-mediated immunity of Drosophila melanogaster: hemocyte lineages, immune compartments, microanatomy and regulation. Dev Comp Immunol *42*, 47-56.

Hua, H., Li, M., Luo, T., Yin, Y., and Jiang, Y. (2011). Matrix metalloproteinases in tumorigenesis: an evolving paradigm. Cell Mol Life Sci *68*, 3853-3868.

Hua, P., Liu, J., Tao, J., Liu, J., and Yang, S. (2015). Influence of caspase-3 silencing on the proliferation and apoptosis of rat bone marrow mesenchymal stem cells under hypoxia. Int J Clin Exp Med *8*, 1624-1633.

Huh, J.R., Guo, M., and Hay, B.A. (2004). Compensatory proliferation induced by cell death in the Drosophila wing disc requires activity of the apical cell death caspase Dronc in a nonapoptotic role. Curr Biol *14*, 1262-1266.

Hyman, B.T., and Yuan, J. (2012). Apoptotic and non-apoptotic roles of caspases in neuronal physiology and pathophysiology. Nat Rev Neurosci *13*, 395-406.

Igaki, T. (2009). Correcting developmental errors by apoptosis: lessons from Drosophila JNK signaling. Apoptosis *14*, 1021-1028.

Igaki, T., Kanda, H., Yamamoto-Goto, Y., Kanuka, H., Kuranaga, E., Aigaki, T., and Miura, M. (2002). Eiger, a TNF superfamily ligand that triggers the Drosophila JNK pathway. EMBO J *21*, 3009-3018.

Igaki, T., Pastor-Pareja, J.C., Aonuma, H., Miura, M., and Xu, T. (2009). Intrinsic tumor suppression and epithelial maintenance by endocytic activation of Eiger/TNF signaling in Drosophila. Dev Cell *16*, 458-465.

Jager, R., and Zwacka, R.M. (2010). The enigmatic roles of caspases in tumor development. Cancers (Basel) *2*, 1952-1979.

Jia, Q., Liu, Y., Liu, H., and Li, S. (2014). Mmp1 and Mmp2 cooperatively induce Drosophila fat body cell dissociation with distinct roles. Sci Rep *4*, 7535.

Jiang, H., Patel, P.H., Kohlmaier, A., Grenley, M.O., McEwen, D.G., and Edgar, B.A. (2009). Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell *137*, 1343-1355.

Johnson, G.L., and Nakamura, K. (2007). The c-jun kinase/stress-activated pathway: regulation, function and role in human disease. Biochim Biophys Acta *1773*, 1341-1348.

Julien, O., and Wells, J.A. (2017). Caspases and their substrates. Cell Death Differ *24*, 1380-1389.

Kaiser, C.L., Kamien, A.J., Shah, P.A., Chapman, B.J., and Cotanche, D.A. (2009). 5-Ethynyl-2'-deoxyuridine labeling detects proliferating cells in the regenerating avian cochlea. Laryngoscope *119*, 1770-1775.

Kalkavan, H., and Green, D.R. (2018). MOMP, cell suicide as a BCL-2 family business. Cell Death Differ *25*, 46-55.

Kamber Kaya, H.E., Ditzel, M., Meier, P., and Bergmann, A. (2017). An inhibitory mono-ubiquitylation of the Drosophila initiator caspase Dronc functions in both apoptotic and non-apoptotic pathways. PLoS Genet *13*, e1006438.

Kang, Y., Neuman, S.D., and Bashirullah, A. (2017). Tango7 regulates cortical activity of caspases during reaper-triggered changes in tissue elasticity. Nat Commun *8*, 603.

Kanuka, H., Kuranaga, E., Takemoto, K., Hiratou, T., Okano, H., and Miura, M. (2005). Drosophila caspase transduces Shaggy/GSK-3beta kinase activity in neural precursor development. EMBO J *24*, 3793-3806.

Karim, F.D., and Rubin, G.M. (1998). Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in Drosophila imaginal tissues. Development *125*, 1-9.

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

Kauppila, S., Maaty, W.S., Chen, P., Tomar, R.S., Eby, M.T., Chapo, J., Chew, S., Rathore, N., Zachariah, S., Sinha, S.K.*, et al.* (2003). Eiger and its receptor, Wengen, comprise a TNF-like system in Drosophila. Oncogene *22*, 4860-4867.

Kelly, G.L., and Strasser, A. (2011). The essential role of evasion from cell death in cancer. Adv Cancer Res *111*, 39-96.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Khan, A.R., and James, M.N. (1998). Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. Protein Sci *7*, 815-836.

Khan, S.J., Abidi, S.N.F., Skinner, A., Tian, Y., and Smith-Bolton, R.K. (2017). The Drosophila Duox maturation factor is a key component of a positive feedback loop that sustains regeneration signaling. PLoS Genet *13*, e1006937.

Kim, B., Srivastava, S.K., and Kim, S.H. (2015). Caspase-9 as a therapeutic target for treating cancer. Expert Opin Ther Targets *19*, 113-127.

Kim, C.H., Paik, D., Rus, F., and Silverman, N. (2014). The caspase-8 homolog Dredd cleaves Imd and Relish but is not inhibited by p35. J Biol Chem *289*, 20092-20101.

Kim, J.Y., Jeong, H.S., Chung, T., Kim, M., Lee, J.H., Jung, W.H., and Koo, J.S. (2017). The value of phosphohistone H3 as a proliferation marker for evaluating invasive breast cancers: A comparative study with Ki67. Oncotarget *8*, 65064-65076.

Kim, M., Hernandez, L., and Annunziata, C.M. (2016). Caspase 8 expression may determine the survival of women with ovarian cancer. Cell Death Dis *7*, e2045.

Kim-Jo, C., Gatti, J.L., and Poirie, M. (2019). Drosophila Cellular Immunity Against Parasitoid Wasps: A Complex and Time-Dependent Process. Front Physiol *10*, 603.

Knapp, J.M., Chung, P., and Simpson, J.H. (2015). Generating customized transgene landing sites and multi-transgene arrays in Drosophila using phiC31 integrase. Genetics *199*, 919-934.

Koenig, U., Eckhart, L., and Tschachler, E. (2001). Evidence that caspase-13 is not a human but a bovine gene. Biochem Biophys Res Commun *285*, 1150-1154.

Kondo, S., Senoo-Matsuda, N., Hiromi, Y., and Miura, M. (2006). DRONC coordinates cell death and compensatory proliferation. Mol Cell Biol *26*, 7258-7268.

Kostura, M.J., Tocci, M.J., Limjuco, G., Chin, J., Cameron, P., Hillman, A.G., Chartrain, N.A., and Schmidt, J.A. (1989). Identification of a monocyte specific pre-interleukin 1 beta convertase activity. Proc Natl Acad Sci U S A *86*, 5227-5231.

Krelin, Y., Zhang, L., Kang, T.B., Appel, E., Kovalenko, A., and Wallach, D. (2008). Caspase-8 deficiency facilitates cellular transformation in vitro. Cell Death Differ *15*, 1350-1355.

Kumar, S. (2007). Caspase function in programmed cell death. Cell Death Differ *14*, 32-43.

Kumar, S., and Doumanis, J. (2000). The fly caspases. Cell Death Differ *7*, 1039-1044.

Kurada, P., and White, K. (1998). Ras promotes cell survival in Drosophila by downregulating hid expression. Cell *95*, 319-329.

Kuranaga, E. (2012). Beyond apoptosis: caspase regulatory mechanisms and functions in vivo. Genes Cells *17*, 83-97.

Kurokawa, M., and Kornbluth, S. (2009). Caspases and kinases in a death grip. Cell *138*, 838-854.

Kurucz, E., Zettervall, C.J., Sinka, R., Vilmos, P., Pivarcsi, A., Ekengren, S., Hegedus, Z., Ando, I., and Hultmark, D. (2003). Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in Drosophila. Proc Natl Acad Sci U S A *100*, 2622-2627.

La Marca, J.E., and Richardson, H.E. (2020). Two-Faced: Roles of JNK Signalling During Tumourigenesis in the Drosophila Model. Front Cell Dev Biol *8*, 42.

Lam, V.K., Tokusumi, T., Cerabona, D., and Schulz, R.A. (2010). Specific cell ablation in Drosophila using the toxic viral protein M2(H37A). Fly (Austin) *4*, 338-343.

Lambert, A.W., Pattabiraman, D.R., and Weinberg, R.A. (2017). Emerging Biological Principles of Metastasis. Cell *168*, 670-691.

Lamkanfi, M., Declercq, W., Kalai, M., Saelens, X., and Vandenabeele, P. (2002). Alice in caspase land. A phylogenetic analysis of caspases from worm to man. Cell Death Differ *9*, 358-361.

Landskron, G., De la Fuente, M., Thuwajit, P., Thuwajit, C., and Hermoso, M.A. (2014). Chronic inflammation and cytokines in the tumor microenvironment. J Immunol Res *2014*, 149185.

Lannan, E., Vandergaast, R., and Friesen, P.D. (2007). Baculovirus caspase inhibitors P49 and P35 block virus-induced apoptosis downstream of effector caspase DrICE activation in Drosophila melanogaster cells. J Virol *81*, 9319-9330.

Lee, G., Wang, Z., Sehgal, R., Chen, C.H., Kikuno, K., Hay, B., and Park, J.H. (2011a). Drosophila caspases involved in developmentally regulated programmed cell death of peptidergic neurons during early metamorphosis. J Comp Neurol *519*, 34-48.

Lee, H.H., Bellat, V., and Law, B. (2017). Chemotherapy induces adaptive drug resistance and metastatic potentials via phenotypic CXCR4-expressing cell state transition in ovarian cancer. PLoS One *12*, e0171044.

Lee, S., Rauch, J., and Kolch, W. (2020). Targeting MAPK Signaling in Cancer: Mechanisms of Drug Resistance and Sensitivity. Int J Mol Sci *21*.

Lee, T.V., Fan, Y., Wang, S., Srivastava, M., Broemer, M., Meier, P., and Bergmann, A. (2011b). Drosophila IAP1-mediated ubiquitylation controls activation of the initiator caspase DRONC independent of protein degradation. PLoS Genet *7*, e1002261.

Legrand, A.J., Konstantinou, M., Goode, E.F., and Meier, P. (2019). The Diversification of Cell Death and Immunity: Memento Mori. Mol Cell *76*, 232-242.

Leitao, A.B., and Sucena, E. (2015). Drosophila sessile hemocyte clusters are true hematopoietic tissues that regulate larval blood cell differentiation. Elife *4*.

Leulier, F., Ribeiro, P.S., Palmer, E., Tenev, T., Takahashi, K., Robertson, D., Zachariou, A., Pichaud, F., Ueda, R., and Meier, P. (2006). Systematic in vivo RNAi analysis of putative components of the Drosophila cell death machinery. Cell Death Differ *13*, 1663-1674.

Leulier, F., Rodriguez, A., Khush, R.S., Abrams, J.M., and Lemaitre, B. (2000). The Drosophila caspase Dredd is required to resist gram-negative bacterial infection. EMBO Rep *1*, 353-358.

Li, J., Brieher, W.M., Scimone, M.L., Kang, S.J., Zhu, H., Yin, H., von Andrian, U.H., Mitchison, T., and Yuan, J. (2007). Caspase-11 regulates cell migration by promoting Aip1-Cofilin-mediated actin depolymerization. Nat Cell Biol *9*, 276-286.

Li, J., Yin, H.L., and Yuan, J. (2008). Flightless-I regulates proinflammatory caspases by selectively modulating intracellular localization and caspase activity. J Cell Biol *181*, 321-333.

Li, J., and Yuan, J. (2008). Caspases in apoptosis and beyond. Oncogene *27*, 6194-6206.

Li, M., Sun, S., Priest, J., Bi, X., and Fan, Y. (2019). Characterization of TNF-induced cell death in Drosophila reveals caspase- and JNK-dependent necrosis and its role in tumor suppression. Cell Death Dis *10*, 613.

Lin, C.C., and Potter, C.J. (2016). Editing Transgenic DNA Components by Inducible Gene Replacement in Drosophila melanogaster. Genetics *203*, 1613-1628.

Liu, J., and Lin, A. (2005). Role of JNK activation in apoptosis: a double-edged sword. Cell Res *15*, 36-42.

Liu, X., He, Y., Li, F., Huang, Q., Kato, T.A., Hall, R.P., and Li, C.Y. (2015). Caspase-3 promotes genetic instability and carcinogenesis. Mol Cell *58*, 284-296.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell *86*, 147-157.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Lu, T.Y., MacDonald, J.M., Neukomm, L.J., Sheehan, A.E., Bradshaw, R., Logan, M.A., and Freeman, M.R. (2017). Axon degeneration induces glial responses through Draper-TRAF4-JNK signalling. Nat Commun *8*, 14355.

Luo, F.Y., Liu, Z.H., Hu, Q.H., Lin, G.Q., Tang, C.E., Zhang, W.X., and Zhuang, W. (2015). Association of BTBD7 with Metastasis and Poor Prognosis in Non-Small-Cell Lung Cancer Patients. J Cancer *6*, 477-481.

Luo, X., Puig, O., Hyun, J., Bohmann, D., and Jasper, H. (2007). Foxo and Fos regulate the decision between cell death and survival in response to UV irradiation. EMBO J *26*, 380-390.

Luster, A.D., Alon, R., and von Andrian, U.H. (2005). Immune cell migration in inflammation: present and future therapeutic targets. Nat Immunol *6*, 1182-1190.

Ma, X., Chen, Y., Zhang, S., Xu, W., Shao, Y., Yang, Y., Li, W., Li, M., and Xue, L. (2016). Rho1-Wnd signaling regulates loss-of-cell polarity-induced cell invasion in Drosophila. Oncogene *35*, 846-855.

MacCarthy-Morrogh, L., and Martin, P. (2020). The hallmarks of cancer are also the hallmarks of wound healing. Sci Signal *13*.

MacDonald, J.M., Beach, M.G., Porpiglia, E., Sheehan, A.E., Watts, R.J., and Freeman, M.R. (2006). The Drosophila cell corpse engulfment receptor Draper mediates glial clearance of severed axons. Neuron *50*, 869-881.

Mace, P.D., Shirley, S., and Day, C.L. (2010). Assembling the building blocks: structure and function of inhibitor of apoptosis proteins. Cell Death Differ *17*, 46-53.

MacKenzie, S.H., and Clark, A.C. (2008). Targeting cell death in tumors by activating caspases. Curr Cancer Drug Targets *8*, 98-109.

Makhijani, K., Alexander, B., Tanaka, T., Rulifson, E., and Bruckner, K. (2011). The peripheral nervous system supports blood cell homing and survival in the Drosophila larva. Development *138*, 5379-5391.

Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer-related inflammation. Nature *454*, 436-444.

Markus, R., Laurinyecz, B., Kurucz, E., Honti, V., Bajusz, I., Sipos, B., Somogyi, K., Kronhamn, J., Hultmark, D., and Ando, I. (2009). Sessile hemocytes as a hematopoietic compartment in Drosophila melanogaster. Proc Natl Acad Sci U S A *106*, 4805-4809.

Martin, F.A., Perez-Garijo, A., and Morata, G. (2009). Apoptosis in Drosophila: compensatory proliferation and undead cells. Int J Dev Biol *53*, 1341-1347.

Martin, S.J., Henry, C.M., and Cullen, S.P. (2012). A perspective on mammalian caspases as positive and negative regulators of inflammation. Mol Cell *46*, 387-397.

Martinon, F., and Tschopp, J. (2004). Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. Cell *117*, 561-574.

Mayor, R., and Etienne-Manneville, S. (2016). The front and rear of collective cell migration. Nat Rev Mol Cell Biol *17*, 97-109.

McArthur, K., and Kile, B.T. (2018). Apoptotic Caspases: Multiple or Mistaken Identities? Trends Cell Biol *28*, 475-493.

McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K., and Davis, R.L. (2003). Spatiotemporal rescue of memory dysfunction in Drosophila. Science *302*, 1765-1768.

McIlwain, D.R., Berger, T., and Mak, T.W. (2013). Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol *5*, a008656.

Medzhitov, R. (2008). Origin and physiological roles of inflammation. Nature *454*, 428-435.

Meier, P., Silke, J., Leevers, S.J., and Evan, G.I. (2000). The Drosophila caspase DRONC is regulated by DIAP1. EMBO J *19*, 598-611.

Meinander, A., Runchel, C., Tenev, T., Chen, L., Kim, C.H., Ribeiro, P.S., Broemer, M., Leulier, F., Zvelebil, M., Silverman, N.*, et al.* (2012). Ubiquitylation of the initiator caspase DREDD is required for innate immune signalling. EMBO J *31*, 2770-2783.

Miles, W.O., Dyson, N.J., and Walker, J.A. (2011). Modeling tumor invasion and metastasis in Drosophila. Dis Model Mech *4*, 753-761.

Mirzoyan, Z., Sollazzo, M., Allocca, M., Valenza, A.M., Grifoni, D., and Bellosta, P. (2019). Drosophila melanogaster: A Model Organism to Study Cancer. Front Genet *10*, 51.

Miura, M. (2012). Apoptotic and nonapoptotic caspase functions in animal development. Cold Spring Harb Perspect Biol *4*.

Miura, M., Chen, X.D., Allen, M.R., Bi, Y., Gronthos, S., Seo, B.M., Lakhani, S., Flavell, R.A., Feng, X.H., Robey, P.G.*, et al.* (2004). A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells. J Clin Invest *114*, 1704-1713.

Mogi, M., and Togari, A. (2003). Activation of caspases is required for osteoblastic differentiation. J Biol Chem *278*, 47477-47482.

Mohammad, R.M., Muqbil, I., Lowe, L., Yedjou, C., Hsu, H.Y., Lin, L.T., Siegelin, M.D., Fimognari, C., Kumar, N.B., Dou, Q.P.*, et al.* (2015). Broad targeting of resistance to apoptosis in cancer. Semin Cancer Biol *35 Suppl*, S78-S103.

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

Moreno, E., Yan, M., and Basler, K. (2002). Evolution of TNF signaling mechanisms: JNK-dependent apoptosis triggered by Eiger, the Drosophila homolog of the TNF superfamily. Curr Biol *12*, 1263-1268.

Morin-Poulard, I., Vincent, A., and Crozatier, M. (2013). The Drosophila JAK-STAT pathway in blood cell formation and immunity. JAKSTAT *2*, e25700.

Mukherjee, A., and Williams, D.W. (2017). More alive than dead: non-apoptotic roles for caspases in neuronal development, plasticity and disease. Cell Death Differ *24*, 1411-1421.

Murphy, A.C., Weyhenmeyer, B., Schmid, J., Kilbride, S.M., Rehm, M., Huber, H.J., Senft, C., Weissenberger, J., Seifert, V., Dunst, M.*, et al.* (2013). Activation of executioner caspases is a predictor of progression-free survival in glioblastoma patients: a systems medicine approach. Cell Death Dis *4*, e629.

Muzzopappa, M., Murcia, L., and Milan, M. (2017). Feedback amplification loop drives malignant growth in epithelial tissues. Proc Natl Acad Sci U S A *114*, E7291-E7300.

Nagata, S. (2018). Apoptosis and Clearance of Apoptotic Cells. Annu Rev Immunol *36*, 489-517.

Nakajima, Y.I., and Kuranaga, E. (2017). Caspase-dependent non-apoptotic processes in development. Cell Death Differ *24*, 1422-1430.

Netea, M.G., Balkwill, F., Chonchol, M., Cominelli, F., Donath, M.Y., Giamarellos-Bourboulis, E.J., Golenbock, D., Gresnigt, M.S., Heneka, M.T., Hoffman, H.M., *et al.* (2017). A guiding map for inflammation. Nat Immunol *18*, 826-831.

Newton, K., and Dixit, V.M. (2012). Signaling in innate immunity and inflammation. Cold Spring Harb Perspect Biol *4*.

Nhan, T.Q., Liles, W.C., and Schwartz, S.M. (2006). Physiological functions of caspases beyond cell death. Am J Pathol *169*, 729-737.

Nikoloudaki, G., Brooks, S., Peidl, A.P., Tinney, D., and Hamilton, D.W. (2020). JNK Signaling as a Key Modulator of Soft Connective Tissue Physiology, Pathology, and Healing. Int J Mol Sci *21*.

Nimpf, S., and Keays, D.A. (2020). Why (and how) we should publish negative data. EMBO Rep *21*, e49775.

Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M.R., Carotenuto, A., De Feo, G., Caponigro, F., and Salomon, D.S. (2006). Epidermal growth factor receptor (EGFR) signaling in cancer. Gene *366*, 2-16.

Oda, K., Matsuoka, Y., Funahashi, A., and Kitano, H. (2005). A comprehensive pathway map of epidermal growth factor receptor signaling. Mol Syst Biol *1*, 2005 0010.

Olsson, M., and Zhivotovsky, B. (2011). Caspases and cancer. Cell Death Differ *18*, 1441-1449.

Onodera, T., Sakai, T., Hsu, J.C., Matsumoto, K., Chiorini, J.A., and Yamada, K.M. (2010). Btbd7 regulates epithelial cell dynamics and branching morphogenesis. Science *329*, 562-565.

Orme, M., and Meier, P. (2009). Inhibitor of apoptosis proteins in Drosophila: gatekeepers of death. Apoptosis *14*, 950-960.

Ouyang, Y., Petritsch, C., Wen, H., Jan, L., Jan, Y.N., and Lu, B. (2011). Dronc caspase exerts a non-apoptotic function to restrain phospho-Numb-induced ectopic neuroblast formation in Drosophila. Development *138*, 2185-2196.

Owen, K.L., Brockwell, N.K., and Parker, B.S. (2019). JAK-STAT Signaling: A Double-Edged Sword of Immune Regulation and Cancer Progression. Cancers (Basel) *11*.

Owusu-Ansah, E., Yavari, A., and Banerjee, U. (2008). A protocol for in vivo detection of reactive oxygen species. Protocol Exchange, [Preprint]. https://doi.org/10.1038/nprot.2008.1023.

Pagliarini, R.A., and Xu, T. (2003). A genetic screen in Drosophila for metastatic behavior. Science *302*, 1227-1231.

Pandya, P.H., Murray, M.E., Pollok, K.E., and Renbarger, J.L. (2016). The Immune System in Cancer Pathogenesis: Potential Therapeutic Approaches. J Immunol Res *2016*, 4273943.

Parisi, F., Stefanatos, R.K., Strathdee, K., Yu, Y., and Vidal, M. (2014). Transformed epithelia trigger non-tissue-autonomous tumor suppressor response by adipocytes via activation of Toll and Eiger/TNF signaling. Cell Rep *6*, 855-867.

Parrish, A.B., Freel, C.D., and Kornbluth, S. (2013). Cellular mechanisms controlling caspase activation and function. Cold Spring Harb Perspect Biol *5*.

Pastor-Pareja, J.C., Wu, M., and Xu, T. (2008). An innate immune response of blood cells to tumors and tissue damage in Drosophila. Dis Model Mech *1*, 144-154; discussion 153.

Perez, E., Lindblad, J.L., and Bergmann, A. (2017). Tumor-promoting function of apoptotic caspases by an amplification loop involving ROS, macrophages and JNK in Drosophila. Elife *6*.

Perez-Garijo, A. (2018). When dying is not the end: Apoptotic caspases as drivers of proliferation. Semin Cell Dev Biol *82*, 86-95.

Perez-Garijo, A., Fuchs, Y., and Steller, H. (2013). Apoptotic cells can induce non-autonomous apoptosis through the TNF pathway. Elife *2*, e01004.

Perez-Garijo, A., Martin, F.A., Struhl, G., and Morata, G. (2005). Dpp signaling and the induction of neoplastic tumors by caspase-inhibited apoptotic cells in Drosophila. Proc Natl Acad Sci U S A *102*, 17664-17669.

Perez-Garijo, A., and Steller, H. (2015). Spreading the word: non-autonomous effects of apoptosis during development, regeneration and disease. Development *142*, 3253-3262.

Pinal, N., Calleja, M., and Morata, G. (2019). Pro-apoptotic and pro-proliferation functions of the JNK pathway of Drosophila: roles in cell competition, tumorigenesis and regeneration. Open Biol *9*, 180256.

Pinal, N., Martin, M., Medina, I., and Morata, G. (2018). Short-term activation of the Jun N-terminal kinase pathway in apoptosis-deficient cells of Drosophila induces tumorigenesis. Nat Commun *9*, 1541.

Pingoud-Meier, C., Lang, D., Janss, A.J., Rorke, L.B., Phillips, P.C., Shalaby, T., and Grotzer, M.A. (2003). Loss of caspase-8 protein expression correlates with unfavorable survival outcome in childhood medulloblastoma. Clin Cancer Res *9*, 6401-6409.

Povea-Cabello, S., Oropesa-Avila, M., de la Cruz-Ojeda, P., Villanueva-Paz, M., de la Mata, M., Suarez-Rivero, J.M., Alvarez-Cordoba, M., Villalon-Garcia, I., Cotan, D., Ybot-Gonzalez, P.*, et al.* (2017). Dynamic Reorganization of the Cytoskeleton during Apoptosis: The Two Coffins Hypothesis. Int J Mol Sci *18*.

Prasad, M., Wang, X., He, L., Cai, D., and Montell, D.J. (2015). Border Cell Migration: A Model System for Live Imaging and Genetic Analysis of Collective Cell Movement. Methods Mol Biol *1328*, 89-97.

Pu, X., Storr, S.J., Zhang, Y., Rakha, E.A., Green, A.R., Ellis, I.O., and Martin, S.G. (2017). Caspase-3 and caspase-8 expression in breast cancer: caspase-3 is associated with survival. Apoptosis *22*, 357-368.

Puccini, J., Dorstyn, L., and Kumar, S. (2013). Caspase-2 as a tumour suppressor. Cell Death Differ *20*, 1133-1139.

Quesnelle, K.M., Boehm, A.L., and Grandis, J.R. (2007). STAT-mediated EGFR signaling in cancer. J Cell Biochem *102*, 311-319.

R Core Team (2020). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Rahmanian, N., Hosseinimehr, S.J., and Khalaj, A. (2016). The paradox role of caspase cascade in ionizing radiation therapy. J Biomed Sci *23*, 88.

Ramet, M., Lanot, R., Zachary, D., and Manfruelli, P. (2002). JNK signaling pathway is required for efficient wound healing in Drosophila. Dev Biol *241*, 145-156.

Ray, M., and Lakhotia, S.C. (2019). Activated Ras/JNK driven Dilp8 in imaginal discs adversely affects organismal homeostasis during early pupal stage in Drosophila, a new checkpoint for development. Dev Dyn *248*, 1211-1231.

Razzell, W., Evans, I.R., Martin, P., and Wood, W. (2013). Calcium flashes orchestrate the wound inflammatory response through DUOX activation and hydrogen peroxide release. Curr Biol *23*, 424-429.

Recasens-Alvarez, C., Ferreira, A., and Milan, M. (2017). JAK/STAT controls organ size and fate specification by regulating morphogen production and signalling. Nat Commun *8*, 13815.

Riabinina, O., Luginbuhl, D., Marr, E., Liu, S., Wu, M.N., Luo, L., and Potter, C.J. (2015). Improved and expanded Q-system reagents for genetic manipulations. Nat Methods *12*, 219-222, 215 p following 222.

Riabinina, O., and Potter, C.J. (2016). The Q-System: A Versatile Expression System for Drosophila. Methods Mol Biol *1478*, 53-78.

Richardson, H., and Kumar, S. (2002). Death to flies: Drosophila as a model system to study programmed cell death. J Immunol Methods *265*, 21-38.

Richardson, H.E., and Portela, M. (2018). Modelling Cooperative Tumorigenesis in Drosophila. Biomed Res Int *2018*, 4258387.

Ridley, A.J. (2015). Rho GTPase signalling in cell migration. Curr Opin Cell Biol *36*, 103-112.

Riedl, S.J., and Shi, Y. (2004). Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol *5*, 897-907.

Riesgo-Escovar, J.R., Jenni, M., Fritz, A., and Hafen, E. (1996). The Drosophila Jun-N-terminal kinase is required for cell morphogenesis but not for DJun-dependent cell fate specification in the eye. Genes Dev *10*, 2759-2768.

Rodriguez, A., Oliver, H., Zou, H., Chen, P., Wang, X., and Abrams, J.M. (1999). Dark is a Drosophila homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. Nat Cell Biol *1*, 272-279.

Rohn, T.T., Cusack, S.M., Kessinger, S.R., and Oxford, J.T. (2004). Caspase activation independent of cell death is required for proper cell dispersal and correct morphology in PC12 cells. Exp Cell Res *295*, 215-225.

Rudrapatna, V.A., Bangi, E., and Cagan, R.L. (2013). Caspase signalling in the absence of apoptosis drives Jnk-dependent invasion. EMBO Rep *14*, 172-177.

Rudrapatna, V.A., Cagan, R.L., and Das, T.K. (2012). Drosophila cancer models. Dev Dyn *241*, 107-118.

Ryoo, H.D., and Bergmann, A. (2012). The role of apoptosis-induced proliferation for regeneration and cancer. Cold Spring Harb Perspect Biol *4*, a008797.

Ryoo, H.D., Gorenc, T., and Steller, H. (2004). Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. Dev Cell *7*, 491-501.

Safa, A.R. (2016). Resistance to Cell Death and Its Modulation in Cancer Stem Cells. Crit Rev Oncog *21*, 203-219.

Samadi, A.K., Bilsland, A., Georgakilas, A.G., Amedei, A., Amin, A., Bishayee, A., Azmi, A.S., Lokeshwar, B.L., Grue, B., Panis, C.*, et al.* (2015). A multi-targeted approach to suppress tumor-promoting inflammation. Semin Cancer Biol *35 Suppl*, S151-S184.

Santabarbara-Ruiz, P., Lopez-Santillan, M., Martinez-Rodriguez, I., Binagui-Casas, A., Perez, L., Milan, M., Corominas, M., and Serras, F. (2015). ROS-Induced JNK

and p38 Signaling Is Required for Unpaired Cytokine Activation during Drosophila Regeneration. PLoS Genet *11*, e1005595.

Santarpia, L., Lippman, S.M., and El-Naggar, A.K. (2012). Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. Expert Opin Ther Targets *16*, 103-119.

Scaltriti, M., and Baselga, J. (2006). The epidermal growth factor receptor pathway: a model for targeted therapy. Clin Cancer Res *12*, 5268-5272.

Scarpa, E., and Mayor, R. (2016). Collective cell migration in development. J Cell Biol *212*, 143-155.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B.*, et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods *9*, 676-682.

Sepp, K.J., and Auld, V.J. (2003). RhoA and Rac1 GTPases mediate the dynamic rearrangement of actin in peripheral glia. Development *130*, 1825-1835.

Sever, R., and Brugge, J.S. (2015). Signal transduction in cancer. Cold Spring Harb Perspect Med *5*.

Shalapour, S., and Karin, M. (2015). Immunity, inflammation, and cancer: an eternal fight between good and evil. J Clin Invest *125*, 3347-3355.

Shalini, S., Dorstyn, L., Dawar, S., and Kumar, S. (2015). Old, new and emerging functions of caspases. Cell Death Differ *22*, 526-539.

Shaukat, Z., Liu, D., and Gregory, S. (2015). Sterile inflammation in Drosophila. Mediators Inflamm *2015*, 369286.

Shaw, T.J., and Martin, P. (2009). Wound repair at a glance. J Cell Sci *122*, 3209-3213.

Shen, H.M., and Liu, Z.G. (2006). JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. Free Radic Biol Med *40*, 928-939.

Shia, A.K., Glittenberg, M., Thompson, G., Weber, A.N., Reichhart, J.M., and Ligoxygakis, P. (2009). Toll-dependent antimicrobial responses in Drosophila larval fat body require Spatzle secreted by haemocytes. J Cell Sci *122*, 4505-4515.

Shin, M., Cha, N., Koranteng, F., Cho, B., and Shim, J. (2020). Subpopulation of Macrophage-Like Plasmatocytes Attenuates Systemic Growth via JAK/STAT in the Drosophila Fat Body. Front Immunol *11*, 63.

Shinoda, N., Hanawa, N., Chihara, T., Koto, A., and Miura, M. (2019). Dronc-independent basal executioner caspase activity sustains Drosophila imaginal tissue growth. Proc Natl Acad Sci U S A *116*, 20539-20544.

Shlevkov, E., and Morata, G. (2012). A dp53/JNK-dependant feedback amplification loop is essential for the apoptotic response to stress in Drosophila. Cell Death Differ *19*, 451-460.

Siegrist, S.E., Haque, N.S., Chen, C.H., Hay, B.A., and Hariharan, I.K. (2010). Inactivation of both Foxo and reaper promotes long-term adult neurogenesis in Drosophila. Curr Biol *20*, 643-648.

Sild, M., Chatelain, R.P., and Ruthazer, E.S. (2013). Improved method for the quantification of motility in glia and other morphologically complex cells. Neural Plast *2013*, 853727.

Smith-Bolton, R.K., Worley, M.I., Kanda, H., and Hariharan, I.K. (2009). Regenerative growth in Drosophila imaginal discs is regulated by Wingless and Myc. Dev Cell *16*, 797-809.

Sofia Vala, I., Martins, L.R., Imaizumi, N., Nunes, R.J., Rino, J., Kuonen, F., Carvalho, L.M., Ruegg, C., Grillo, I.M., Barata, J.T.*, et al.* (2010). Low doses of ionizing radiation promote tumor growth and metastasis by enhancing angiogenesis. PLoS One *5*, e11222.

Srivastava, A., Pastor-Pareja, J.C., Igaki, T., Pagliarini, R., and Xu, T. (2007). Basement membrane remodeling is essential for Drosophila disc eversion and tumor invasion. Proc Natl Acad Sci U S A *104*, 2721-2726.

Stec, W., Vidal, O., and Zeidler, M.P. (2013). Drosophila SOCS36E negatively regulates JAK/STAT pathway signaling via two separable mechanisms. Mol Biol Cell *24*, 3000-3009.

Stupack, D.G., Teitz, T., Potter, M.D., Mikolon, D., Houghton, P.J., Kidd, V.J., Lahti, J.M., and Cheresh, D.A. (2006). Potentiation of neuroblastoma metastasis by loss of caspase-8. Nature *439*, 95-99.

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

Su, T.T. (2020). Non-apoptotic roles of apoptotic proteases: new tricks for an old dog. Open Biol *10*, 200130.

Sun, Y., Han, Y., Wang, X., Wang, W., Wang, X., Wen, M., Xia, J., Xing, H., Li, X., and Zhang, Z. (2016). Correlation of EGFR Del 19 with Fn14/JAK/STAT signaling molecules in non-small cell lung cancer. Oncol Rep *36*, 1030-1040.

Swarup, S., and Verheyen, E.M. (2012). Wnt/Wingless signaling in Drosophila. Cold Spring Harb Perspect Biol *4*.

Szymczyk, K.H., Freeman, T.A., Adams, C.S., Srinivas, V., and Steinbeck, M.J. (2006). Active caspase-3 is required for osteoclast differentiation. J Cell Physiol *209*, 836-844.

Tang, D., Kang, R., Berghe, T.V., Vandenabeele, P., and Kroemer, G. (2019). The molecular machinery of regulated cell death. Cell Res *29*, 347-364.

Tang, H.L., Tang, H.M., Fung, M.C., and Hardwick, J.M. (2015). In vivo CaspaseTracker biosensor system for detecting anastasis and non-apoptotic caspase activity. Sci Rep *5*, 9015.

Tao, Y.M., Huang, J.L., Zeng, S., Zhang, S., Fan, X.G., Wang, Z.M., Yang, H.X., Yuan, X.H., Wang, P., Wu, F.*, et al.* (2013). BTB/POZ domain-containing protein 7: epithelial-mesenchymal transition promoter and prognostic biomarker of hepatocellular carcinoma. Hepatology *57*, 2326-2337.

Tassetto, M., Kunitomi, M., and Andino, R. (2017). Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in Drosophila. Cell *169*, 314-325 e313.

Teitz, T., Inoue, M., Valentine, M.B., Zhu, K., Rehg, J.E., Zhao, W., Finkelstein, D., Wang, Y.D., Johnson, M.D., Calabrese, C.*, et al.* (2013). Th-MYCN mice with caspase-8 deficiency develop advanced neuroblastoma with bone marrow metastasis. Cancer Res *73*, 4086-4097.

Thomas, S.J., Snowden, J.A., Zeidler, M.P., and Danson, S.J. (2015). The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours. Br J Cancer *113*, 365-371.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*, 1456-1462.

Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J.*, et al.* (1992). A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature *356*, 768-774.

Tolwinski, N.S. (2017). Introduction: Drosophila-A Model System for Developmental Biology. J Dev Biol *5*.

Torres, V.A., Mielgo, A., Barbero, S., Hsiao, R., Wilkins, J.A., and Stupack, D.G. (2010). Rab5 mediates caspase-8-promoted cell motility and metastasis. Mol Biol Cell *21*, 369-376.

Tournier, C. (2013). The 2 Faces of JNK Signaling in Cancer. Genes Cancer *4*, 397-400.

Tremblay, M., Fugere, V., Tsui, J., Schohl, A., Tavakoli, A., Travencolo, B.A., Costa Lda, F., and Ruthazer, E.S. (2009). Regulation of radial glial motility by visual experience. J Neurosci *29*, 14066-14076.

Trengove, M.C., and Ward, A.C. (2013). SOCS proteins in development and disease. Am J Clin Exp Immunol *2*, 1-29.

Trinchieri, G. (2012). Cancer and inflammation: an old intuition with rapidly evolving new concepts. Annu Rev Immunol *30*, 677-706.

Tsai, C.R., Wang, Y., and Galko, M.J. (2018). Crawling wounded: molecular genetic insights into wound healing from Drosophila larvae. Int J Dev Biol *62*, 479-489.

Tsai, Y.C., and Sun, Y.H. (2004). Long-range effect of upd, a ligand for Jak/STAT pathway, on cell cycle in Drosophila eye development. Genesis *39*, 141-153.

Ugur, B., Chen, K., and Bellen, H.J. (2016). Drosophila tools and assays for the study of human diseases. Dis Model Mech *9*, 235-244.

Uhlirova, M., and Bohmann, D. (2006). JNK- and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in Drosophila. EMBO J *25*, 5294-5304.

Unsain, N., and Barker, P.A. (2015). New Views on the Misconstrued: Executioner Caspases and Their Diverse Non-apoptotic Roles. Neuron *88*, 461-474.

Vaishnav, M., MacFarlane, M., and Dickens, M. (2011). Disassembly of the JIP1/JNK molecular scaffold by caspase-3-mediated cleavage of JIP1 during apoptosis. Exp Cell Res *317*, 1028-1039.

Vlisidou, I., and Wood, W. (2015). Drosophila blood cells and their role in immune responses. FEBS J *282*, 1368-1382.

Voll, R.E., Herrmann, M., Roth, E.A., Stach, C., Kalden, J.R., and Girkontaite, I. (1997). Immunosuppressive effects of apoptotic cells. Nature *390*, 350-351.

Wagner, E.F., and Nebreda, A.R. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer *9*, 537-549.

Waldman, A.D., Fritz, J.M., and Lenardo, M.J. (2020). A guide to cancer immunotherapy: from T cell basic science to clinical practice. Nat Rev Immunol.

Wang, C., and Youle, R.J. (2009). The role of mitochondria in apoptosis*. Annu Rev Genet *43*, 95-118.

Wang, J., Li, D., Cang, H., and Guo, B. (2019). Crosstalk between cancer and immune cells: Role of tumor-associated macrophages in the tumor microenvironment. Cancer Med *8*, 4709-4721.

Wang, L., Kounatidis, I., and Ligoxygakis, P. (2014). Drosophila as a model to study the role of blood cells in inflammation, innate immunity and cancer. Front Cell Infect Microbiol *3*, 113.

Wang, S.L., Hawkins, C.J., Yoo, S.J., Muller, H.A., and Hay, B.A. (1999). The Drosophila caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. Cell *98*, 453-463.

Weaver, B.P., Weaver, Y.M., Omi, S., Yuan, W., Ewbank, J.J., and Han, M. (2020). Non-Canonical Caspase Activity Antagonizes p38 MAPK Stress-Priming Function to Support Development. Dev Cell *53*, 358-369 e356.

Wei, S., Xie, Z., Filenova, E., and Brew, K. (2003). Drosophila TIMP is a potent inhibitor of MMPs and TACE: similarities in structure and function to TIMP-3. Biochemistry *42*, 12200-12207.

Wells, B.S., Yoshida, E., and Johnston, L.A. (2006). Compensatory proliferation in Drosophila imaginal discs requires Dronc-dependent p53 activity. Curr Biol *16*, 1606-1615.

Wen, W., Wu, J., Liu, L., Tian, Y., Buettner, R., Hsieh, M.Y., Horne, D., Dellinger, T.H., Han, E.S., Jove, R.*, et al.* (2015). Synergistic anti-tumor effect of combined inhibition of EGFR and JAK/STAT3 pathways in human ovarian cancer. Mol Cancer *14*, 100.

Wen, X., Lin, Z.Q., Liu, B., and Wei, Y.Q. (2012). Caspase-mediated programmed cell death pathways as potential therapeutic targets in cancer. Cell Prolif *45*, 217-224.

Wilke, C.O. (2020). ggridges: Ridgeline Plots in ‘ggplot2’. R package version 052 https://CRANR-projectorg/package=ggridges.

Williams, M.J. (2007). Drosophila hemopoiesis and cellular immunity. J Immunol *178*, 4711-4716.

Willsey, H.R., Zheng, X., Carlos Pastor-Pareja, J., Willsey, A.J., Beachy, P.A., and Xu, T. (2016). Localized JNK signaling regulates organ size during development. Elife *5*.

Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D.A., Agapite, J., Steller, H., and Meier, P. (2002). The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. Nat Cell Biol *4*, 445-450.

Winfree, L.M., Speese, S.D., and Logan, M.A. (2017). Protein phosphatase 4 coordinates glial membrane recruitment and phagocytic clearance of degenerating axons in Drosophila. Cell Death Dis *8*, e2623.

Wong, R.S. (2011). Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res *30*, 87.

Woodcock, K.J., Kierdorf, K., Pouchelon, C.A., Vivancos, V., Dionne, M.S., and Geissmann, F. (2015). Macrophage-derived upd3 cytokine causes impaired glucose homeostasis and reduced lifespan in Drosophila fed a lipid-rich diet. Immunity *42*, 133-144.

Worley, M.I., Alexander, L.A., and Hariharan, I.K. (2018). CtBP impedes JNK- and Upd/STAT-driven cell fate misspecifications in regenerating Drosophila imaginal discs. Elife *7*.

Wu, J.S., and Luo, L. (2006). A protocol for dissecting Drosophila melanogaster brains for live imaging or immunostaining. Nat Protoc *1*, 2110-2115.

Wu, M., Pastor-Pareja, J.C., and Xu, T. (2010). Interaction between Ras(V12) and scribbled clones induces tumour growth and invasion. Nature *463*, 545-548.

Wu, Q., Wu, W., Fu, B., Shi, L., Wang, X., and Kuca, K. (2019). JNK signaling in cancer cell survival. Med Res Rev *39*, 2082-2104.

Wu, S., Liu, Y., Zheng, Y., Dong, J., and Pan, D. (2008). The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. Dev Cell *14*, 388-398.

Wu, Y., and Zhou, B.P. (2009). Inflammation: a driving force speeds cancer metastasis. Cell Cycle *8*, 3267-3273.

Xiao, T.S. (2017). Innate immunity and inflammation. Cell Mol Immunol *14*, 1-3.

Xu, D., Li, Y., Arcaro, M., Lackey, M., and Bergmann, A. (2005). The CARD-carrying caspase Dronc is essential for most, but not all, developmental cell death in Drosophila. Development *132*, 2125-2134.

Xu, D., Wang, Y., Willecke, R., Chen, Z., Ding, T., and Bergmann, A. (2006). The effector caspases drICE and dcp-1 have partially overlapping functions in the apoptotic pathway in Drosophila. Cell Death Differ *13*, 1697-1706.

Xu, D., Woodfield, S.E., Lee, T.V., Fan, Y., Antonio, C., and Bergmann, A. (2009). Genetic control of programmed cell death (apoptosis) in Drosophila. Fly (Austin) *3*, 78-90.

Xu, D.C., Arthurton, L., and Baena-Lopez, L.A. (2018a). Learning on the Fly: The Interplay between Caspases and Cancer. Biomed Res Int *2018*, 5473180.

Xu, D.C., Yamada, K.M., and Baena-Lopez, L.A. (2020). Widespread non-apoptotic activation of *Drosophila* Caspase-2/9 limits JNK signaling, macrophage proliferation, and growth of wound-like tumors. bioRxiv, [Preprint]. https://doi.org/10.1101/2020.1107.1127.223404.

Xu, Y., So, C., Lam, H.M., Fung, M.C., and Tsang, S.Y. (2018b). Apoptosis Reversal Promotes Cancer Stem Cell-Like Cell Formation. Neoplasia *20*, 295-303.

Yamanaka, N., Rewitz, K.F., and O'Connor, M.B. (2013). Ecdysone control of developmental transitions: lessons from Drosophila research. Annu Rev Entomol *58*, 497-516.

Yan, X.X., Najbauer, J., Woo, C.C., Dashtipour, K., Ribak, C.E., and Leon, M. (2001). Expression of active caspase-3 in mitotic and postmitotic cells of the rat forebrain. J Comp Neurol *433*, 4-22.

Yang, C., and Novack, D.V. (2013). Anti-cancer IAP antagonists promote bone metastasis: a cautionary tale. J Bone Miner Metab *31*, 496-506.

Yang, H., Kronhamn, J., Ekstrom, J.O., Korkut, G.G., and Hultmark, D. (2015). JAK/STAT signaling in Drosophila muscles controls the cellular immune response against parasitoid infection. EMBO Rep *16*, 1664-1672.

Yang, L., Wang, T., Zhang, J., and Wang, X. (2017). BTBD7 silencing inhibited epithelial- mesenchymal transition (EMT) via regulating Slug expression in human salivary adenoid cystic carcinoma. Cancer Biomark *20*, 461-468.

Yang, X., Zhong, D.N., Qin, H., Wu, P.R., Wei, K.L., Chen, G., He, R.Q., and Zhong, J.C. (2018). Caspase-3 over-expression is associated with poor overall survival and clinicopathological parameters in breast cancer: a meta-analysis of 3091 cases. Oncotarget *9*, 8629-8641.

Yi, C.H., and Yuan, J. (2009). The Jekyll and Hyde functions of caspases. Dev Cell *16*, 21-34.

Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., Wang, S.L., Feldman, R.M., Clem, R.J., Muller, H.A., and Hay, B.A. (2002). Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. Nat Cell Biol *4*, 416-424.

Yoshimura, A., Naka, T., and Kubo, M. (2007). SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol *7*, 454-465.

Yu, X., Wang, L., Acehan, D., Wang, X., and Akey, C.W. (2006). Three-dimensional structure of a double apoptosome formed by the Drosophila Apaf-1 related killer. J Mol Biol *355*, 577-589.

Yuan, S., Yu, X., Topf, M., Dorstyn, L., Kumar, S., Ludtke, S.J., and Akey, C.W. (2011). Structure of the Drosophila apoptosome at 6.9 a resolution. Structure *19*, 128-140.

Zeke, A., Misheva, M., Remenyi, A., and Bogoyevitch, M.A. (2016). JNK Signaling: Regulation and Functions Based on Complex Protein-Protein Partnerships. Microbiol Mol Biol Rev *80*, 793-835.

Zhang, Q., Zhu, B., and Li, Y. (2017). Resolution of Cancer-Promoting Inflammation: A New Approach for Anticancer Therapy. Front Immunol *8*, 71.

Zhao, R., Kaakati, R., Lee, A.K., Liu, X., Li, F., and Li, C.Y. (2018). Novel roles of apoptotic caspases in tumor repopulation, epigenetic reprogramming, carcinogenesis, and beyond. Cancer Metastasis Rev *37*, 227-236.

Zhou, M., Liu, X., Li, Z., Huang, Q., Li, F., and Li, C.Y. (2018). Caspase-3 regulates the migration, invasion and metastasis of colon cancer cells. Int J Cancer *143*, 921-930.

Zhou, Q., Krebs, J.F., Snipas, S.J., Price, A., Alnemri, E.S., Tomaselli, K.J., and Salvesen, G.S. (1998). Interaction of the baculovirus anti-apoptotic protein p35 with caspases. Specificity, kinetics, and characterization of the caspase/p35 complex. Biochemistry *37*, 10757-10765.

Zou, H., Henzel, W.J., Liu, X., Lutschg, A., and Wang, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell *90*, 405-413.