“DEATH IS NOT THE END: THE ROLE OF REACTIVE OXYGEN SPECIES IN DRIVING APOPTOSIS-INDUCED PROLIFERATION“

A Dissertation Presented
By

Caitlin E. Fogarty

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The signatures of the Dissertation Defense Committee signify completion and approval as to style and content of the Dissertation

_____
Andreas Bergmann, Ph.D., Thesis Advisor

_____
Eric Baehrecke, Ph.D., Member of Committee

_____
Michael Brodsky, Ph.D., Member of Committee

_____
Hyung-Don Ryoo, Ph.D., Member of Committee

_____
Neal Silverman, Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

_____
Daryl Bosco, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

_____
Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

MD/PhD Program in Biomedical Sciences
June 2, 2015

Dedication

To my parents who have encouraged me to follow my passions and to persevere when faced by challenges; to my daughter who has encouraged me to do those things with a smile on my face and an appreciation for every day; and to my husband, who has supported me each and every step of the way.

Acknowledgements

I would like to thank my mentor, Andreas Bergmann, for his guidance, support, and trust throughout the course of my graduate studies. I truly value all of the opportunities afforded to me during my time in the lab, and I greatly appreciate all of his efforts challenging me to be the best scientist I can be.

I would like to thank my committee members for their thoughtful advice and feedback during the course of my thesis work. I am thankful for both the insightful critiques as well as the encouragement to “just try it out” when my hesitations and doubts held me back.

I would like to thank the members of the Bergmann Lab, for both their technical assistance in conducting this thesis work, and their friendship over the past several years. It can often feel like we are living in the lab, and I am glad to have had them as my lab family.

Finally, I would like to thank my colleagues in the MD/PhD program, my friends, and my family for making every day exciting and for supporting me unconditionally.

Abstract

Apoptosis-induced proliferation (AiP) is a compensatory mechanism to maintain tissue size and morphology following unexpected cell loss during normal development, and may also be a contributing factor to cancer growth and drug resistance. In apoptotic cells, caspase-initiated signaling cascades lead to the downstream production of mitogenic factors and the proliferation of neighboring surviving cells. In epithelial *Drosophila* tissues, the Caspase-9 homolog Dronc drives AiP via activation of Jun N-terminal kinase (JNK); however, the specific mechanisms of JNK activation remain unknown. Using a model of sustained AiP that produces a hyperplastic phenotype in *Drosophila* eye and head tissue, I have found that caspase-induced activation of JNK during AiP depends on extracellular reactive oxygen species (ROS) generated by the NADPH oxidase Duox. I found these ROS are produced early in the death-regeneration process by undifferentiated epithelial cells that have initiated the apoptotic cascade. I also found that reduction of these ROS by mis-expression of extracellular catalases was sufficient to reduce the frequency of overgrowth associated with our model of AiP. I further observed that extracellular ROS attract and activate *Drosophila* macrophages (hemocytes), which may in turn trigger JNK activity in epithelial cells by signaling through the TNF receptor Grindelwald. We propose that signaling back and forth between epithelial cells and hemocytes by extracellular ROS and Grindelwald drives compensatory proliferation within the epithelium, and that in cases of persistent signaling, such as in our sustained model of AiP, hemocytes play a tumor promoting role, driving overgrowth.

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List of Keywords

Apoptosis
Apoptosis-induced Proliferation
Cancer
Caspase
Catalase
Cell death
Chronic Inflammation
c-Jun Kinase (JNK) Signaling
Dcp-1
DrICE
Drosophila
Dronc
Duox
Hemocytes
Imaginal Discs
Non-apoptotic functions
Phoenix Rising
Reactive Oxygen Species
Regeneration
Regulatory Feedback Loop
TNF Signaling
Tumor Associated Macrophages
Wound Healing

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CHAPTER I:

INTRODUCTION

Portions of this chapter are adapted from:
Fogarty, CE and Bergmann, AB. “The Sound of Silence: Signaling by Apoptotic Cells.” Current Topics in Developmental Biology. 2015. Editor: H Steller.

Apoptosis: An Introduction

Apoptosis is a carefully choreographed process of cellular self-destruction, observed across the spectrum of metazoans from worms to flies to mammals (Kerr, Wyllie, & Currie, 1972; Ellis & Horvitz, 1986; Abrams et al., 1993). During development, programmed cell death shapes developing tissues by removing superfluous cells, sculpting out defined structures or regulating tissue size (Glucksmann, 1951; Saunders & Gasseling, 1962; Hinchliffe & Ede, 1973; Fernandez-Teran, Hinchliffe, & Ros, 2006; Suzanne & Steller, 2013). In adult organisms, apoptosis can be triggered in cells that are no longer functioning properly such as those injured by toxins or transformed by genetic aberrations. This removal is critical to maintaining tissue integrity and homeostasis, and it is the mechanism of removal that distinguishes apoptosis from other forms of cell death. Cells that are damaged, infected, or otherwise unwanted, are capable of initiating a tightly controlled cascade of events, which leads to the cessation of normal cellular activity, the degradation of major macromolecules including DNA, and ultimately the coordinated fragmentation of the cell so that it may be cleared via phagocytosis (Lockshin & Williams, 1965; Kerr et al., 1972; Schweichel & Merker, 1973; Schwartz et al., 1993; Enari et al., 1998; Sebbagh et al., 2001).

Apoptosis was initially distinguished from necrotic cell death based on the quiet nature of its cellular demise (Kerr et al., 1972). Unlike necrosis where cells spill their contents causing secondary tissue damage and infiltrating immune cells react with such fervor they induce significant inflammation, apoptosis is characterized by an unassuming departure, contained cellular contents, few immune cells, and no detectable inflammation.

This relatively benign process earned apoptotic cell death the moniker of “altruistic cell suicide,” and so for a time, the characterization of apoptosis as the silent cell death prevailed (Bar, 1996).

To better understand how apoptotic cells can die without causing further damage, it is helpful to first review the basics of apoptotic cell death. From worms to humans, there are a variety of ways to initiate the apoptotic cascade - some cascades are triggered by intrinsic developmentally regulated transcriptional programs, others by extrinsic death signals; some are triggered by active induction, others by neglect; some depend on the release of cytochrome C from the mitochondria, others can be driven by accumulation of pro-apoptotic factors (reviewed in (Steller, 1995; Danial & Korsmeyer, 2004; Domingos & Steller, 2007; Conradt, 2009; Xu et al., 2009; Bergmann, 2010; Czabotar et al., 2014)).

What all apoptotic deaths have in common, however, is the activation of caspases. These cysteine-dependent aspartate-directed proteases are the critical effectors of cell death (Miura et al., 1993; J. Yuan et al., 1993). Caspases are initially produced as zymogens, which are inactive until they are proteolytically cleaved. Autocatalytic activation of the initiator Caspase-9 most typically occurs via complex formation with the adaptor protein Apaf-1, along with cytochrome C and dATP (P. Li et al., 1997). Activated initiator caspases can cleave and activate effector caspases such as Caspase-3 and Caspase-7 (Zou et al., 1997; Brustugun et al., 1998). Activated effector caspases carry out the methodical process of executing cell death, directly activating other death enzymes such as nucleases and kinases, inactivating proteins required to sustain normal cellular processes, or indirectly disrupting normal physiological processes by

disassembling compartments such as the nucleus and the mitochondria (Gavrieli, Sherman, & Ben-Sasson, 1992; X. Liu et al., 1997; Enari et al., 1998; Susin et al., 1999; Coleman et al., 2001; L. Y. Li, Luo, & Wang, 2001; Sebbagh et al., 2001).

While only ten percent of specific caspase cleavage sites are conserved between worms and humans, there is incredible conservation of the biological pathways which are targeted by effector caspases (Crawford et al., 2012). Among these, there are a number of targets that do not seem to be involved in the actual disassembly of the dying cell, but instead many are released into the surrounding microenvironment. Over the past decade, interest in these apoptosis-derived signals has led to the discovery of critical communications between dying cells and their environment. While several signals act on nearby immune cells (regulating the clearance of apoptotic debris, preventing inflammation, and limiting fibrosis (Lauber et al., 2003; Miksa et al., 2007; Mueller et al., 2007; Gude et al., 2008; Truman et al., 2008; G. E. White et al., 2010; Gu et al., 2011; Tsai et al., 2014; Engel et al., 2015)), many apoptosis-derived signals act directly on neighboring surviving cells to maintain tissue integrity via growth control. The purpose of this thesis work is to investigate one of the mechanisms by which apoptotic cells in developing *Drosophila* epithelium signal to induce apoptosis-induced proliferation.

Drosophila as a model to study cell death

The fruit fly, *Drosophila melanogaster*, is an ideal system for studying the mechanisms of cell death and the various signals produced by apoptotic cells. In addition to a well-annotated and fully sequenced genome (M. D. Adams et al., 2000; Myers et al.,

2000; dos Santos et al., 2015), the *Drosophila* research community possesses a versatile genetic toolbox developed over the past century that allows for precise *in vivo* dissection of specific genetic pathways. Additionally, as a model organism, it is significant to note that 75% of human disease genes have functional equivalents in Drosophila (Reiter et al., 2001) and *Drosophila* are readily used to model a number of human diseases such as neurodegeneration and cancer (reviewed in (Bier, 2005)). However, one of *Drosophila's* greatest uses in biomedical research derives from the fact that the basic fundamental molecular pathways that guide normal development are highly conserved between flies and humans. Indeed, many of these critical growth and patterning programs were first discovered in flies (reviewed in (Rieder & Larschan, 2014)), and so by closely studying normal developmental processes in flies, we can be better prepared to understand what pathways are being perturbed in human disease.

Programmed cell death is one of these remarkably well-conserved pathways in *Drosophila,* and flies have allowed for the study of the *in vivo* genetic control that regulates initiation and execution of apoptosis (Bergmann, Agapite, & Steller, 1998; Xu et al., 2009). While the autonomously activated program of intrinsic cell death is conserved between flies and mammals, there are a few notable differences that will be discussed here (Figure 1.1).

First, in *Drosophila* the primary driving force initiating the intrinsic cell death program is the transcriptional upregulation of the pro-apoptotic genes *reaper*, *head involution defective (hid)*, and *grim* (K. White et al., 1994; Grether et al., 1995; P. Chen et al., 1996). The production of the respective pro-apoptotic proteins can precede

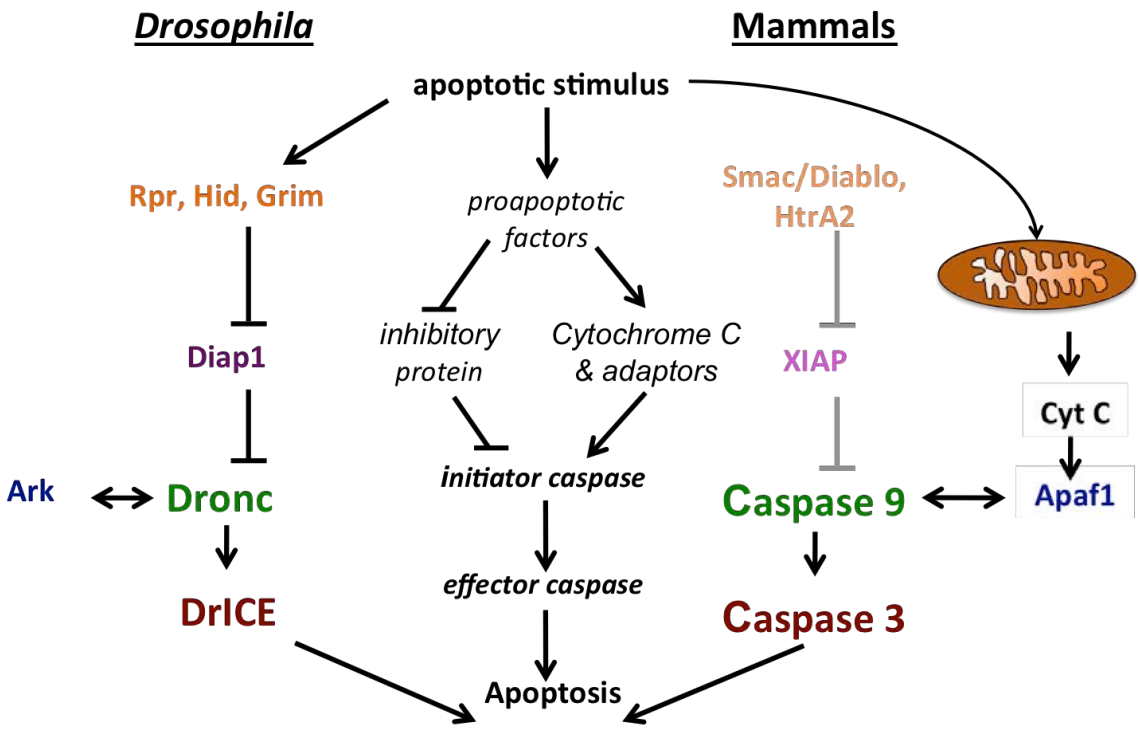

Figure 1.1
The Intrinsic Cell Death Pathway

Figure 1.1: The Intrinsic Cell Death Pathway.

The intrinsic cell death pathway is remarkably conserved between flies and mammals. The major components include the initiator and effector caspases, the apoptosome scaffold protein Ark/Apaf-1, inhibitors of apoptosis, and pro-apoptotic factors. Homologous proteins are denoted by shared colors.

execution of cell death by several hours, and Reaper, Hid, and Grim drive that execution by antagonizing the Drosophila Inhibitor of Apoptosis Protein 1 (DIAP1), a negative regulator of caspase activation (Goyal et al., 2000; Lisi, Mazzon, & White, 2000). In mammals, pro-apoptotic IAP antagonists exist, but their role is secondary and seemingly dispensable compared to the major driving force of the Bcl-2 family of proteins triggering the mitochondria-initiated cytochrome C dependent formation of the apoptosome (Zhivotovsky et al., 1998; Du et al., 2000; Srinivasula et al., 2000; Okada et al., 2002).

This brings us to a second striking difference between flies and mammals – while there is some evidence for cytochrome C involvement in executing certain forms of developmental cell death and facilitating non-apoptotic caspase function in *Drosophila* (Arama et al., 2006; Mendes et al., 2006), there is an apparent lack of cytochrome C requirement in initiating most *Drosophila* cell death (Varkey et al., 1999; Dorstyn et al., 2002). Whereas the mammalian apoptosome includes cytochrome C for full activation of Caspase-9, in *Drosophila* cytochrome C does not directly incorporate into the apoptosome for Dronc activation (Shi, 2002; Dorstyn & Kumar, 2006; S. Yuan et al., 2011). Instead, release of Dronc from DIAP1 inhibition, and complex formation with the scaffold protein Ark (Apaf-1 related killer) to form the apoptosome is sufficient for most cell death events in Drosophila, except for a possibly Ark-independent apoptosis in the embryo (Kanuka et al., 1999; Srivastava et al., 2007).

Finally, there are eleven caspases in humans, of which Caspases -2, -8, -9, and -10 function as initiators of apoptosis, while Caspases -3 and -7 are the major effector

caspases. The genome of *Drosophila melanogaster* encodes for seven caspase genes, three of these are involved in the initiation and execution of apoptosis: the initiator caspase Dronc (Dorstyn et al., 1999; Xu et al., 2005) and the effector caspases DrICE (Fraser & Evan, 1997; Fraser, McCarthy, & Evan, 1997) and Dcp-1 (Song, McCall, & Steller, 1997; Xu et al., 2006). The synthesis and activation of these three caspases are similar to that of their human homologs (Kumar, 2007).

Interestingly, recent studies have also shown that the roles of caspases in the fly go beyond traditional cell death and include a plethora of non-apoptotic functions. Caspases have been implicated in cell migration and morphogenesis (Geisbrecht & Montell, 2004; Suzanne et al., 2010; Kuranaga et al., 2011), cell differentiation and maturation (Arama, Agapite, & Steller, 2003; Arama et al., 2006), innate immunity (Leulier et al., 2000; Stoven et al., 2003), non-apoptotic alternative cell death (Yacobi-Sharon, Namdar, & Arama, 2013), and compensatory cell proliferation (Huh, Guo, & Hay, 2004; Kondo et al., 2006; Wells, Yoshida, & Johnston, 2006; Fan & Bergmann, 2008b).

Growth control signaling by apoptotic cells in Drosophila

Kerr, Wylie, and Currie initially characterized apoptosis as the kinetic counterpoint to mitosis, a process that actively contributes to tissue homeostasis and maintenance of cell populations (Kerr et al., 1972). Yet, since that time, apoptosis has often been regarded as a more passive, silent process (Bar, 1996). An emerging field of study focuses on the pathways utilized by apoptotic cells to produce instructive signals

influencing the growth state of neighboring survivor cells. Depending on the context, apoptotic cells are capable of producing pro-apoptotic, anti-apoptotic, mitogenic, and morphogenetic signals that act directly on the surrounding tissue, without an immunological intermediate (Figure 1.2). We will briefly review some of these signals as they have been characterized in *Drosophila.*

Throughout development there are several instances where entire populations of cells initiate apoptosis almost simultaneously. In certain contexts at least, this communal, or cohort, cell death is triggered by pro-apoptotic signals originating from the initial apoptotic cells (Perez-Garijo, Fuchs, & Steller, 2013). These authors found in developing *Drosophila* wing imaginal discs that induction of cell death in one tissue compartment results in additional ectopic apoptosis in distant compartments. This apoptosis-induced apoptosis (AiA) is dependent on the production and release of the *Drosophila* TNFα homolog, Eiger, from the initial population of dying cells.

Other recent studies have also found an apoptosis-induced death resistance (AiDR) program in *Drosophila*. Jaklevic et al. (2008) first noted that ionizing radiation of the developing wing disc generally increases the levels of *bantam*, a microRNA that stimulates cell proliferation and inhibits apoptosis by repressing the pro-apoptotic factor *hid* (Brennecke et al., 2003; Jaklevic et al., 2008). Additionally, *bantam* null animals were exceptionally susceptible to ionizing radiation, exhibiting increased apoptosis. However, follow-up studies by the same group revealed the surprising finding that when apoptosis is limited to a specific domain, the increase in *bantam*, and thus the anti-apoptotic effect, was non-autonomous (Bilak, Uyetake, & Su, 2014). These authors found

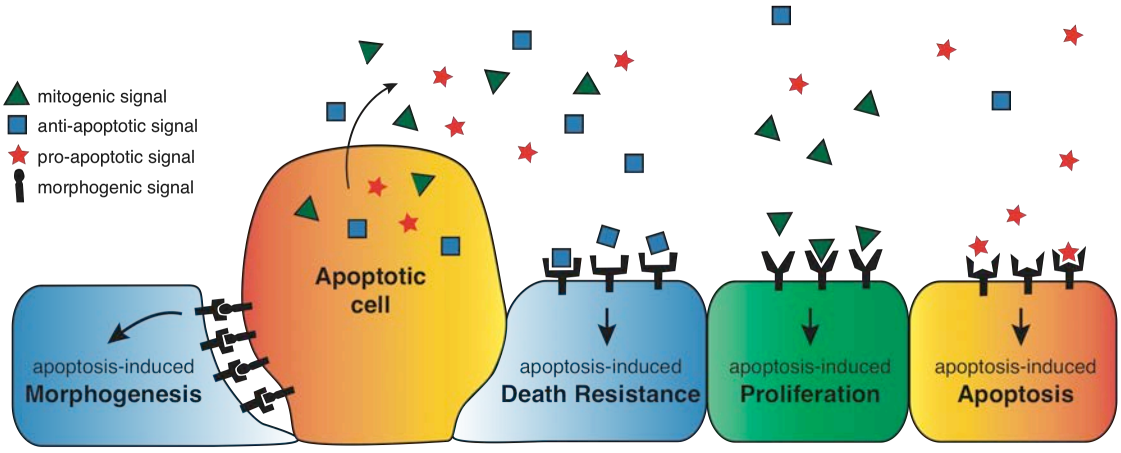

Figure 1.2
Apoptosis-derived signals regulate growth control

Figure 1.2: Apoptosis-derived signals regulate growth control.

Apoptotic cells can produce a variety of signals that can have an effect on the growth state of the surviving tissue. The outcome of this communication can depend on both the amounts of signal produced and the receptivity of the surviving cell to these different stimuli.

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(Fogarty & Bergmann, 2015)

that AiDR, which they termed “the Mahakali effect”, was mediated by the receptor tyrosine kinase Tie on the surviving cell, and the apoptosis-dependent production of the Pvf1 ligand. Interestingly, Pvf1 was required, but not sufficient, to induce AiDR, suggesting that other apoptosis-derived signals may also be required to stimulate this pro-survival effect.

This dissertation focuses on a third program - the phenomenon of apoptosis-induced proliferation (AiP). Generally, following significant cell death, compensatory proliferation can regenerate lost tissue via additional or accelerated cell divisions. In *Drosophila*, some of the first evidence for compensatory proliferation was uncovered by Haynie and Bryant when they demonstrated that up to sixty percent of cells in developing wing precursor tissue could be eliminated by radiation, and yet extra cell divisions within the surviving tissue resulted in a full-sized and normally-functioning adult wing (Haynie & Bryant, 1977). In 2004, it was reported that in the developing *Drosophila* epithelial tissues, induction of apoptotic cell death could induce non-autonomous proliferation in the surrounding cells (Huh et al., 2004; Perez-Garijo, Martin, & Morata, 2004; Ryoo, Gorenc, & Steller, 2004). Since that time, there has been a mounting interest in the concept that this proliferation, under certain contexts, is driven explicitly by mitogenic signals produced by the dying cell. There is still controversy today regarding which signals are actually produced by the dying cell, versus which mitogenic factors may be produced elsewhere, but in the past decade numerous studies have validated the concept in several model organisms from worms to mammals (Hwang et al., 2004; Vlaskalin, Wong, & Tsilfidis, 2004; Tseng et al., 2007; Chera et al., 2009; F. Li et al., 2010). Apoptosis-induced proliferation (AiP) therefore is a form of compensatory proliferation and is defined as the process by which apoptotic cells actively stimulate surviving cells to divide (Mollereau et al., 2013).

Taking these three programs together, it is important to keep in mind that dying cells may produce any combination of these signals depending on their pre-death state, the specific mode of apoptotic induction, the presence or absence of immune cells as signals are being released, or feedback from the surrounding immune cells and microenvironment. Moreover, signals produced by apoptotic cells will only have as much effect on neighboring cells, as those neighbors are receptive to detecting them. Balance in signaling events may also be important, whereby pro-growth and pro-death signals may simultaneously originate from dying cells, with the net effect being determined by the current state of the neighbor. While my work focuses on the specific mechanisms of AiP, it is important to remember that this physiological response is itself occurring in parallel or perhaps in direct competition with other stress responses.

Models of Apoptosis-induced Proliferation in Drosophila

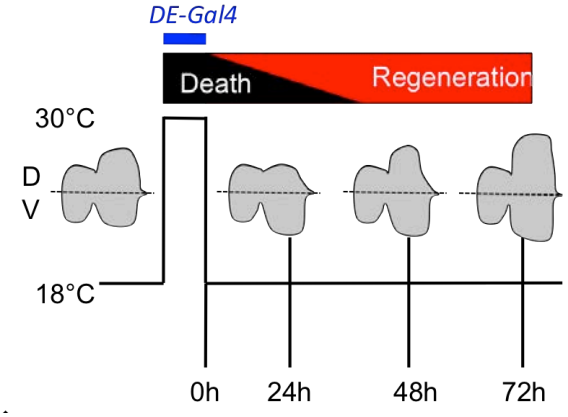
Over the past decade a number of research groups have developed genetic models to study AiP in *Drosophila*. *In vivo* studies of apoptotic cells and any apoptosis-derived signals during normal development are challenging due to the rapid induction and clearance of the apoptotic cell and the fleeting nature of its derived signals. However, in *Drosophila* it is possible to more closely examine the pathways driving AiP by capitalizing on the use of the effector caspase inhibitor P35 (Clem, Fechheimer, & Miller, 1991; Hay, Wolff, & Rubin, 1994). Therefore, these genetic constructs can be categorized as *p35*-independent (less tractable but more physiological) or *p35*-dependent

(more robust but artificial) models. The models used in this work are summarized below and in Figure 1.3.

Regenerative AiP – the "Genuine" model

In genuine models of AiP, tissues are first subjected to a limited injury or pro-apoptotic stimulus, and then they are allowed to recover and regenerate lost tissue through compensatory proliferation. These *p35*-independent models are useful for investigating the spatiotemporal dynamics of AiP and for monitoring responses in the surviving and regenerating cells (Smith-Bolton et al., 2009; Bergantinos, Corominas, & Serras, 2010; Herrera, Martin, & Morata, 2013; Fan et al., 2014). In this thesis work, I make use of a regenerative model in which *hid* expression is restricted to a 12-hour interval in the dorsal half of the eye imaginal disc by *tubulin-Gal80^{ts}*-controlled *dorsal eye-(DE)-Gal4* activity (*DE^{ts}>hid*). Following egg lay, growing larvae are kept at a restrictive temperature (18°C) until mid-2nd instar to allow for normal development. They are then shifted to the permissive temperature (29°C) to turn on the *DE-Gal4*, which drives *UAS-hid* and any other transgenes such as RNAi or mis-expression constructs. After 12 hours of death induction, the larvae are shifted back to the restrictive temperature, *tubulin-Gal80^{ts}* repression resumes, and physiological regeneration can proceed. Typically, cell death is greatest early in recovery but it takes 24 hours following the temperature down-shift for all caspase active cells to die and be cleared. During this time, c-Jun N-terminal kinase (JNK) activity increases in the surviving cells within the

A



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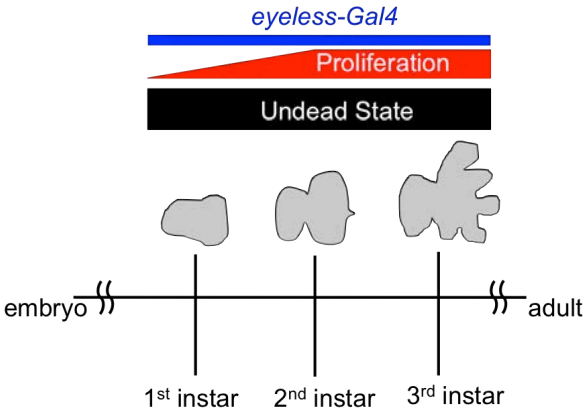

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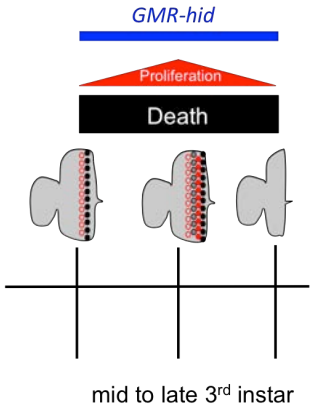


Figure 1.3

Genetic models of AiP

Figure 1.3: Genetic Models of AiP.

(A) Regenerative AiP - Spatiotemporally regulated induction of *hid* for 12 hours triggers limited cell death. From 0 to 72 hours recovery “genuine” AiP drives a regenerative response to compensate for the cell loss suffered in the dorsal half of the developing eye disc.

(B) Sustained AiP - Continuous co-expression of *hid* and *p35* under control of the *eyeless-Gal4* leads to a caspase-active, undead state; sustained mitogen production; and eventual overgrowth of the tissue. This produces an observable phenotype at both larval and adult stages.

(C) Post-mitotic AiP- In this model, the *GMR* promoter directly drives *hid* during later larval stages. Following the second mitotic wave (open red circles), cells typically exit the cell cycle. However, under *GMR-hid*, there is induction of cell death (solid black circles) which through effector caspase dependent mitogen production produces an additional mitotic wave (solid red circles). The balance, overall, still favors excessive death and tissue loss.

death domain, peaking 6-12hrs after recovery, and surviving proliferating cells contribute to complete regeneration by 72 hrs (Fan et al., 2014). Knockdown of genes important for the regenerative process, such as *basket* (the gene encoding JNK), can reduce the degree of regeneration by 72 hrs; however, as the RNAi expression is limited and normal gene expression can eventually resume, by the time animals eclose from the metamorphic pupal stages, adult eyes do not show any obvious defects or decrease in size. This has limited our use of the regenerative model for screening for new genes involved in AiP.

Sustained AiP – the "Undead" model

Through mis-expressing *p35* at the same time as triggering an apoptotic stimulus, we can uncouple the initiation of the apoptotic signaling cascade from the actual execution of cell death. This generates an "undead" state in which any apoptosis-derived signals are sustained. In the case of undead models of AiP, for example when death is induced and AiP sustained by *p35* in one compartment of the developing wing imaginal discs, mitogen production is sustained resulting in excessive proliferation and tissue overgrowth (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004). Importantly, proliferation occurs in both the posterior compartment where the undead cells exist, and in the genetically unaffected anterior region (Huh et al., 2004; Ryoo et al., 2004). In these original experiments, this strongly suggested the presence of a secreted and diffusible mitogen. Two mitogens identified at the time included Wingless (Wg, an homolog of Wnt) and Decapentaplegic (Dpp, homolog of TGFβ) (Perez-Garijo et al., 2004; Ryoo et al., 2004). Follow up work has also identified a role for Spitz (Spi, the EGF homolog in *Drosophila*) (Fan et al., 2014). In the undead model, these mitogens are produced in

response to JNK activity in the undead cells and are required for tissue overgrowth (Ryoo et al., 2004; Fan et al., 2014).

A common hypothesis in these studies had been that, following apoptosis induction, there is a bifurcation in the signaling cascade that ultimately results in both non-autonomous proliferation and autonomous execution of cell death. In this specific context, the bifurcation is at the level of the *Drosophila* initiator caspase, Dronc (Huh et al., 2004; Kondo et al., 2006; Wells et al., 2006; Fan et al., 2014). In genuine apoptotic cells, Dronc activates the effector caspases Drice and Dcp-1, as well as a currently unidentified target upstream of JNK activation, leading to compensatory proliferation (Fan et al., 2014). In undead cells, effector caspase activity is inhibited, and so Dronc continuously signals for compensatory proliferation, thus driving the tissue to hyper-proliferation[1]. Therefore, we see the undead model of AiP as both a tool to study the developmental pathways that can regulate regeneration, as well as a potentially fascinating tool to understand how AiP may play a role in the development of cancers.

In this work I make use of a sustained "undead" model of AiP, in which *hid* and *p35* are driven in the anterior proliferating tissue of the developing eye imaginal disc by an *eyeless-Gal4* construct (*ey>hid-p35*). This promoter turns on early in eye development and persists through late 3rd instar until differentiation programs initiate in the eye imaginal disc moving posterior to anterior (Hazelett et al., 1998; Hauck, Gehring, & Walldorf, 1999; Legent & Treisman, 2008). Overgrowth of the tissue in larval stages

[1] Since "apoptotic" cells in an undead model do not actually die, in this case of sustained AiP a more appropriate term may be caspase-induced proliferation (CiP), though I will continue to use AiP for the sake of simplicity.

leads to a variable overgrowth of the adult head capsule, duplications of bristles and sensory structures, and in extreme cases reduction of eye size. This clear adult phenotype allows for simple screening of RNAi constructs and dominant modifier mutants that can suppress this sustained AiP. For example heterozygous loss of *dronc*, mis-expression of a *basket* dominant-negative construct, or knockdown of endogenous JNK by *basket* RNAi are all sufficient to completely restore the wild-type adult head capsule.

AiP in post-mitotic tissues

Apoptosis-induced proliferation in *Drosophila* depends in part on the environmental context of the dying cell. Interestingly, the Dronc-dependent signaling cascade only applies to epithelial cells that have not begun terminal differentiation. Undifferentiated, actively proliferating tissue exists in the wing and the anterior of the eye imaginal disc. However, in the posterior eye disc differentiated photoreceptors induce AiP by a completely different mechanism (Fan & Bergmann, 2008a, 2008b). Here, apoptotic photoreceptor cells trigger a Drice and Dcp-1 dependent cascade that leads to release of Hedgehog (Hh). Hh secreted from these apical cells then stimulates the underlying unspecified progenitor cells to re-enter the cell cycle and proliferate. This effector caspase-dependent form of AiP can be seen when *hid* is expressed in the posterior eye disc under direct control of the *Glass Multimer Reporter (GMR)* promoter *(GMR-hid)* (Grether et al., 1995; Fan & Bergmann, 2008b). As death induction is so strong, *GMR-hid* produces an almost completely ablated adult eye, with the AiP phenotype only observable as a small wave of proliferating cells in the late 3rd instar eye imaginal disc, posterior to the morphogenetic furrow. This genetic model is frequently

used in our lab, and is currently being used to compare and contrast the mechanisms of Dronc- versus DrICE-dependent AiP, and will be briefly discussed as part of the future directions of this thesis work.

JNK activation in Dronc-dependent AiP

Since the first reports in 2004, many research groups have worked to better understand the molecular mechanisms and genetic control of AiP. One aspect that was evident early is that the stress-activated MAPK, JNK, is critically required for both regenerative and sustained AiP. Without JNK and the downstream activation of transcriptional targets proliferation does not occur. However, over a decade later the exact step-by-step mechanism by which Dronc activation leads to JNK activation is still unclear. To better understand the possible mechanisms of JNK activation, we will briefly review the stress-activated MAPK signaling cascade.

Mitogen-activated protein kinases include extracellular regulated kinase (ERK), p38, and c-Jun-N-terminal Kinase (JNK). The AP-1 transcription factors c-Jun and c-Fos are important for many developmental control programs and, when specifically phosphorylated by JNK, can activate genes important for survival or death depending on the context ((Derijard et al., 1994; Minden et al., 1994; Sluss et al., 1996; Kockel et al., 1997) and reviewed in (Kockel, Homsy, & Bohmann, 2001; Rios-Barrera & Riesgo-Escovar, 2013)). In *Drosophila,* this stress-induced MAPK (dJNK, encoded by the gene *basket*), is negatively regulated by a MAPK phosphatase (*puckered*) to keep basal levels of apoptosis-inducing JNK activity low (McEwen & Peifer, 2005). In order to be

activated, JNK is phosphorylated by either Hep/dMKK7 (*hemipterous*), or under certain pro-inflammatory conditions also by dMKK4 (*Mkk4*) (Boutros, Agaisse, & Perrimon, 2002; Geuking et al., 2009). Further upstream, a number of MAPKKKs, and signal cascade adaptor proteins contribute to the context dependent initiation of JNK signaling (reviewed in (Igaki, 2009; Rios-Barrera & Riesgo-Escovar, 2013)). These initiating signals can originate from within the cell, directly activating an upstream kinase, or from outside the cell activating one of the receptor/adaptors.

Therefore, the question in our system becomes: Is JNK specifically activated autonomously within the dying cell, or is JNK more generally activated by non-autonomous signals (receptor-mediated) that accumulate in areas with many dying cells? One clue uncovered by previous work in our lab using the *ey>hid-p35* model (Fan et al., 2014), is the fact that only a specific subset of upstream activating kinases are required for Dronc-dependent AiP (Figure 1.4). This could suggest that JNK activation is a specific targeted event following Dronc activation, and not a generalized stress response to apoptosis. Additionally, neither of the canonical ligand/receptor systems known to activate JNK are required for AiP, again suggesting an autonomous activation in caspase activated cells. However, not all evidence points toward an autonomous activation of JNK.

When investigating undead models of AiP, an individual cell can be caspase active, JNK active, producing mitogens, and responding to mitogens produced by neighboring undead cells. When compartments of the wing or eye disc are undead, and surrounding compartments are wild-type, these activities are still primarily restricted to

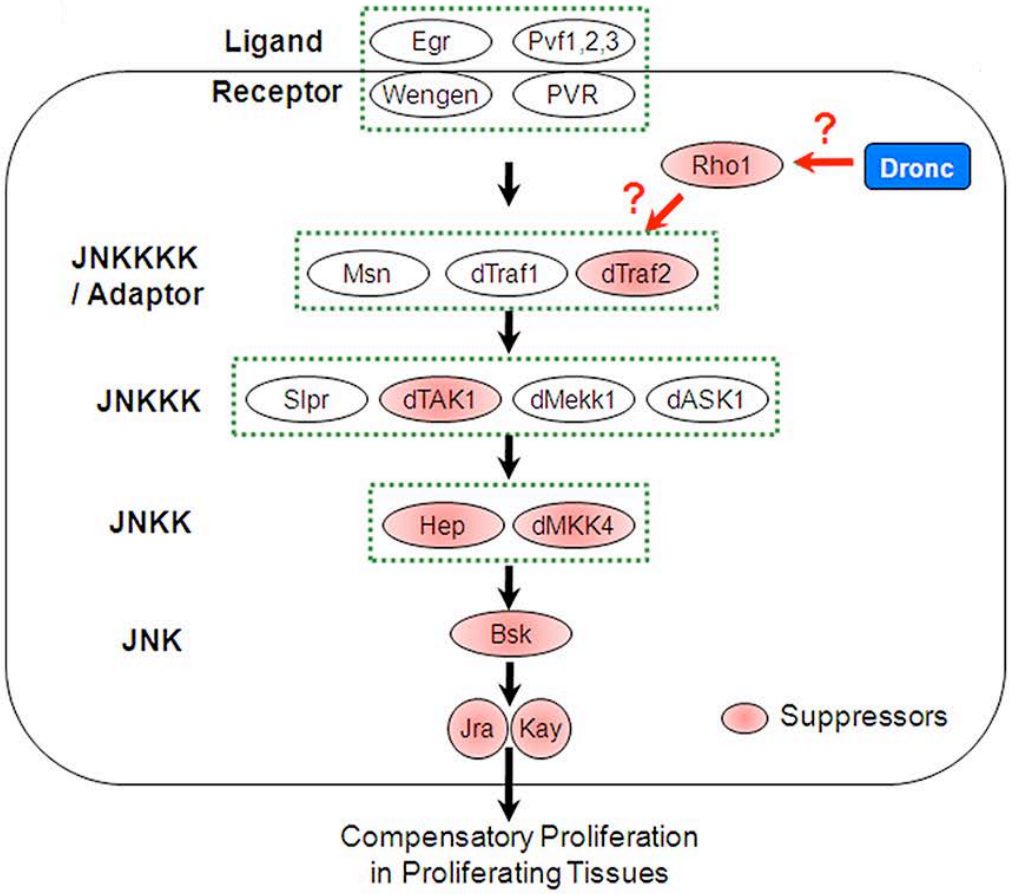


Figure 1.4:
Dronc-dependent AiP requires a specific subset of JNK activating kinases

Figure 1.4: Dronc-dependent AiP requires a specific subset of JNK activating kinases.

The JNK signaling pathway in *Drosophila* follows a typical MAPKKK/MAPKK/MAPK activation cascade. Of the several upstream activators, only a select subset (shown in red) are required for *ey>hid-p35* induced proliferation, as demonstrated by RNAi-mediated suppression.

Reproduced from:

Fan, Y., Wang, S., Hernandez, J., Yenigun, V. B., Hertlein, G., Fogarty, C. E., Lindblad, J. L., 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 Genetics. 10 (1) doi: 10.1371/journal.pgen.1004131

the undead region. This suggests that JNK activity and mitogen production in the context of AiP occurs autonomously in caspase active cells. However, in genuine models of AiP that do not rely on *p35* expression, depending on how long death is induced and how long the tissue is allowed to recover, JNK activity is detected both autonomously in dying cells and/or non-autonomously in neighboring proliferating cells (Bergantinos et al., 2010; Herrera et al., 2013; Fan et al., 2014).

This discrepancy has not only failed to provide resolution to our JNK activation question, but has also brought forth a controversy as to whether sustained AiP is mechanistically relevant to the study of physiological regenerative AiP. Perhaps instead sustained AiP is an artificial by-product of undead cells, and is not representative of the homeostatic pathways used in genuine AiP. This controversy has also been fueled by apparent differences in the requirement and the source of the Wg and Dpp mitogens in genuine versus undead models of AiP (Perez-Garijo et al., 2005; F. A. Martin, Perez-Garijo, & Morata, 2009; Perez-Garijo, Shlevkov, & Morata, 2009; Smith-Bolton et al., 2009). My thesis work has focused on further clarifying the mechanisms of Dronc-dependent activation of JNK, as well as attempting to provide some clarity to this controversy by investigating the commonalities between JNK activation in the genuine and undead models.

A role for reactive oxygen species in death and regeneration

Another mechanism by which JNK is activated under certain cellular stress conditions is via redox signaling. Reactive oxygen species, such as super oxide and

hydrogen peroxide, are frequently produced in living cells as byproducts of inefficient cellular respiration, and they can be dangerously destructive when they accumulate to high levels, non-specifically damaging lipids, proteins, and nucleic acids. However, ROS can also be produced by specialized enzymes such as the NADPH oxidase family of proteins, in response to specific triggers, for the purpose of tightly regulated growth control over cellular proliferation and differentiation (Geiszt & Leto, 2004; Owusu-Ansah & Banerjee, 2009; Schieber & Chandel, 2014). Additionally, hydrogen peroxide has been linked with malignant cell-associated proliferation via the upregulation of NADPH oxidases, and with the development of epithelial to mesenchymal transition type phenotypes through specific activation of JNK and production of Transforming Growth Factor beta (TGFβ) (Irani et al., 1997; Fukawa et al., 2012; Bauer, 2014).

In mammals, several components of the JNK cascade are known to be sensitive to redox signaling. The upstream MAPKKK, ASK1 can be negatively regulated by interaction with the redox sensitive protein thioredoxin. When ROS oxidize thioredoxin causing its dissociation, ASK1 is then able to oligomerize and activate via autophosphorylation (Saitoh et al., 1998; Tobiume, Saitoh, & Ichijo, 2002; Fujino et al., 2007). Additionally, the MAPK phosphatases, which negatively regulate JNK, can be inactivated via redox mechanisms, sensitizing the cell to JNK activating stimuli (Kamata et al., 2005). Unfortunately, in our model of AiP, neither ASK1 or the MAPK phosphatase Puckered appear to be involved in the specific activation of JNK downstream of Dronc; however, other ROS-dependent mechanisms of JNK activation exist.

Recent studies have demonstrated critical requirements for diffusible ROS during wound healing, regeneration, and compensatory proliferation. In certain contexts, these ROS function by activating JNK, though not necessarily via direct redox activation of the signaling cascade. Hydrogen peroxide, while categorized as “reactive”, is actually moderately stable, diffusible across cellular membranes especially with the help of aquaporins, and is utilized as a critical signaling molecule (Bienert, Schjoerring, & Jahn, 2006). In zebrafish models of wound healing and regeneration (following fin amputation), hydrogen peroxide recruits leukocytes for immediate wound healing, and sustained ROS production by NADPH oxidases leads to JNK-dependent regeneration of the lost tissue (Niethammer et al., 2009; Gauron et al., 2013). Similar ROS dependent regeneration is seen following tadpole tail amputations (Love et al., 2013). In *Drosophila,* ROS production following puncture of the embryonic epidermis also recruits immune cells (hemocytes) and contribute to transcriptionally regulated wound healing response pathways (Juarez et al., 2011; Razzell et al., 2013). Therefore, a possible role for redox signaling during AiP is the formation of an extracellular chemoattractant gradient for immune cells, which could both facilitate clearing of dying cells and contribute to the regenerative response.

In *Drosophila,* the cellular immune system consists of hemocytes, blood cells that serve a number of functions including phagocytosis, pathogen recognition and encapsulation, coagulation, clotting, melanization, and the production of antimicrobial peptides (reviewed in (Vlisidou & Wood, 2015)). There are three distinct populations of hemocytes in *Drosophila*: the vast bulk of circulating and tissue resident blood cells are

macrophage-like plasmatocytes (95%), with the remaining hemocytes being the crystal cells and lamellocytes, which have specialized roles in the *Drosophila* immune system (Tepass et al., 1994; Kurucz et al., 2007; Makhijani & Bruckner, 2012). With respect to epithelial biology, *Drosophila* hemocytes have indeed been implicated in wound healing responses downstream of ROS gradients, and hemocytes appear to play a role in epithelial growth control, as reports have highlighted both tumor suppressing and tumor promoting properties of hemocytes, depending on the context of the developing tumor (Pastor-Pareja, Wu, & Xu, 2008; Cordero et al., 2010; Razzell et al., 2013).

Preliminary in vivo evidence for reactive oxygen species in sustained AiP

Knowing that the JNK cascade is a stress-responsive pathway, early in my thesis work I wanted to determine what stresses undead cells might experience. Using a transcriptional reporter that detects cells responding to oxidative stress (*GstD-GFP*, (Sykiotis & Bohmann, 2008)), I found that undead *ey>hid-p35* eye imaginal discs had a moderate but significant increase in reporter activity over control *ey>p35* discs (Figure 1.5, 12.02% mean area of disc GFP+, 8-fold increase over control, $p<0.001$ by Student's t-Test, Holm-Sidak method)[2]. Based on these preliminary findings, and the published work in hydra, zebrafish, *Xenopus*, and *Drosophila* embryonic wound healing, I

[2] Interestingly, the reporter activity was predominantly upregulated in the peripodial membrane, and did not often co-label the undead caspase active cells. The significance of the reporter activity suggested to me that ROS were present in undead discs, but the absolute levels of activity suggested that either the total ROS burden was low, or the majority of cells did not detect the ROS "threat" as requiring an oxidative stress response. Evidence presented in Chapter II would seem to support the latter case, but I have not followed up specifically regarding the oxidative stress response in undead tissue.

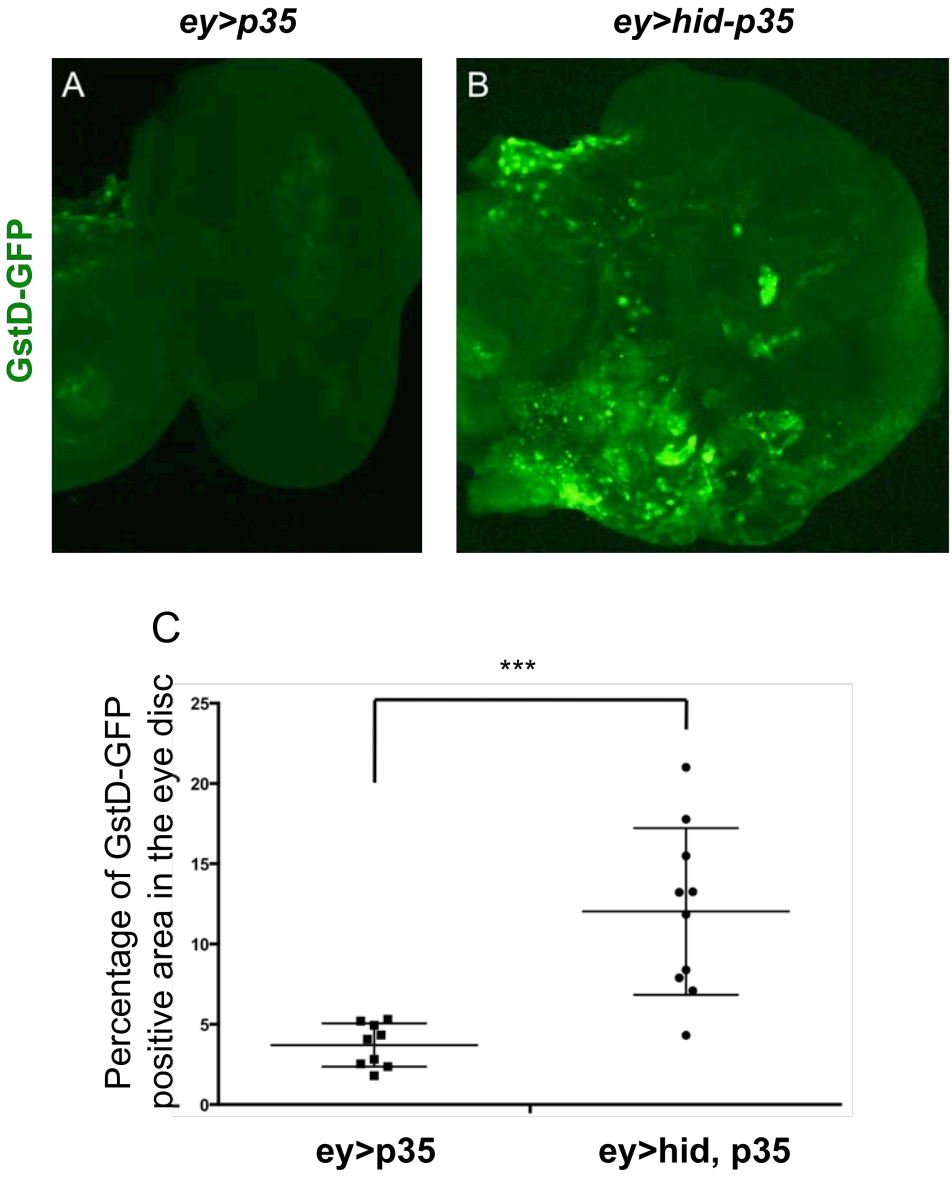


Figure 1.5
An Oxidative Stress Reporter Suggests the Presence of Oxidative Species During AiP

Figure 1.5: An Oxidative Stress Reporter Suggests the Presence of Oxidative Species During AiP

(A-C) The oxidative stress reporter construct *GstD-GFP,* which expresses GFP protein under the control of the glutathione-S-transferase D1 promoter, shows limited expression in control (*ey>p35*) eye discs, but dramatic induction in undead (*ey>hid-p35*) eye discs.

(A) Representative image of GstD-GFP expression in ey>p35 control discs. Note the faint signal between the anterior and posterior regions of the eye disc derives from adjacent intensely bright hemocytes, which are in a different plane. N=2, n=19

(B) Representative image of GstD-GFP expression in ey>hid-p35 undead discs. Most signal seen here is in the peripodial membrane overlaying the anterior eye disc and in some of the overgrown anterior eye disc proper. N>5, n>30

(C) These preliminary experiments were not co-labeled with anterior versus posterior eye markers, so quantification of GFP signal is over the entire eye disc, excluding the antennal disc, and is percentage of disc area positive for GFP over a threshold set based on calculated disc background. Error bars represent ±SEM, $p<0.001$ by Student's t-Test.

hypothesized that reactive oxygen species produced by dying or undead cells contribute to the activation of JNK during apoptosis-induced proliferation. I also aimed to test whether caspases, specifically the initiator caspase Dronc could trigger production of ROS in response to a sustained apoptotic stimulus. Finally, following observation of hemocytes adjacent to undead tissue in the *ey>hid-p35* model of sustained AiP, I hypothesized that ROS may recruit and activate hemocytes at the site of significant cell death. The work in Chapter II describes my investigations into the presence of ROS during the AiP and their requirement for proliferation during sustained AiP.

CHAPTER II:

EXTRACELLULAR REACTIVE OXYGEN SPECIES DRIVE APOPTOSIS-INDUCED PROLIFERATION VIA DROSOPHILA MACROPHAGES

A modified version of this work was accepted for publication:
Caitlin E. Fogarty, J.L. Lindblad, N. Diwanji, M. Tare, A. Amcheslavsky, K. Makhijani, K. Bruckner, Y. Fan, A. Bergmann. "Extracellular Reactive Oxygen Species drive Apoptosis-induced Proliferation via *Drosophila* Macrophages." Current Biology. (Fogarty et al., 2016)

CEF designed all experiments presented here and analyzed the data
JLL contributed raw data for Fig 2.10 E; 2.12 F
ND contributed raw data for Fig 2.10 C, D; and generated Fig 2.9;
MT assisted with Fig 2.3 D
AA contributed data for Fig 2.11 B
Christine Powers conducted the EM processing and imaging in Fig 2.8 C, D
Latisha Elijio assisted with collecting the data in Table 2.2

ABSTRACT

Apoptosis-induced proliferation (AiP) is a compensatory mechanism to maintain tissue size and morphology following unexpected cell loss during normal development, and may also be a contributing factor to cancer and drug resistance (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004; Fan & Bergmann, 2008a, 2008b; Huang et al., 2011; Fan et al., 2014; Kurtova et al., 2015). In apoptotic cells, caspase-initiated signaling cascades lead to the downstream production of mitogenic factors and the proliferation of neighboring surviving cells. In epithelial *Drosophila* tissues, the Caspase-9 homolog Dronc drives AiP via activation of Jun N-terminal kinase (JNK) (Ryoo et al., 2004; Kondo et al., 2006; Wells et al., 2006; Bergantinos et al., 2010; Herrera et al., 2013; Fan et al., 2014); however, the specific mechanisms of JNK activation remain unknown. Here, we show that caspase-induced activation of JNK during AiP depends on extracellular reactive oxygen species (ROS) generated by the NADPH oxidase Duox in epithelial cells. Extracellular ROS attract and activate *Drosophila* macrophages (hemocytes), which may in turn trigger JNK activity in epithelial cells by signaling through the TNF receptor Grindelwald. We propose that signaling back and forth between epithelial cells and hemocytes by extracellular ROS and Grindelwald drives compensatory proliferation within the epithelium. Furthermore, persistent signaling, such as in the case of an immortalized (‘undead’) model of AiP, promotes overgrowth indicating a possible tumor promoting role of macrophages.

INTRODUCTION

Apoptosis-induced proliferation (AiP) is a regenerative process that relies on JNK-dependent production of mitogens in *Drosophila* and other organisms including mammals (Bergmann & Steller, 2010). To facilitate screening for genes and mechanisms involved in AiP, we developed an AiP model in which we co-express the pro-apoptotic gene *hid* and the effector caspase inhibitor *p35*. *hid*/*p35*-expressing cells initiate the apoptotic process and also activate the initiator Caspase-9 homolog Dronc, but cannot execute apoptosis due to effector caspase inhibition by p35, thus producing "undead" cells (Fan et al., 2014). Because undead cells do not die, Dronc chronically signals for AiP and triggers hyperplastic overgrowth (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004; Kondo et al., 2006; Fan et al., 2014). For example, expression of *hid* and *p35* using *ey-Gal4* (*ey>hid-p35*) in the anterior compartment of eye imaginal discs causes overgrowth of adult heads compared to control (*ey>p35*) animals (Figure 2.1 A, B), often at the expense of the eye field in the posterior compartment (Fan et al., 2014). Disruption of key components of the AiP pathway with mutant alleles, RNAi, or other transgenes can lead to suppression of this overgrowth phenotype, restoring the wild-type head capsule size and architecture (Figure 2.1 C). This model provides the basis for phenotypic analysis and genetic screening.

When reactive oxygen species (ROS) accumulate indiscriminately within cells, they can be toxic leading to oxidative stress and possible cell death. However, at lower, controlled levels, ROS can have specific roles in growth control, proliferation and differentiation (Owusu-Ansah & Banerjee, 2009; Schieber & Chandel, 2014). Recent

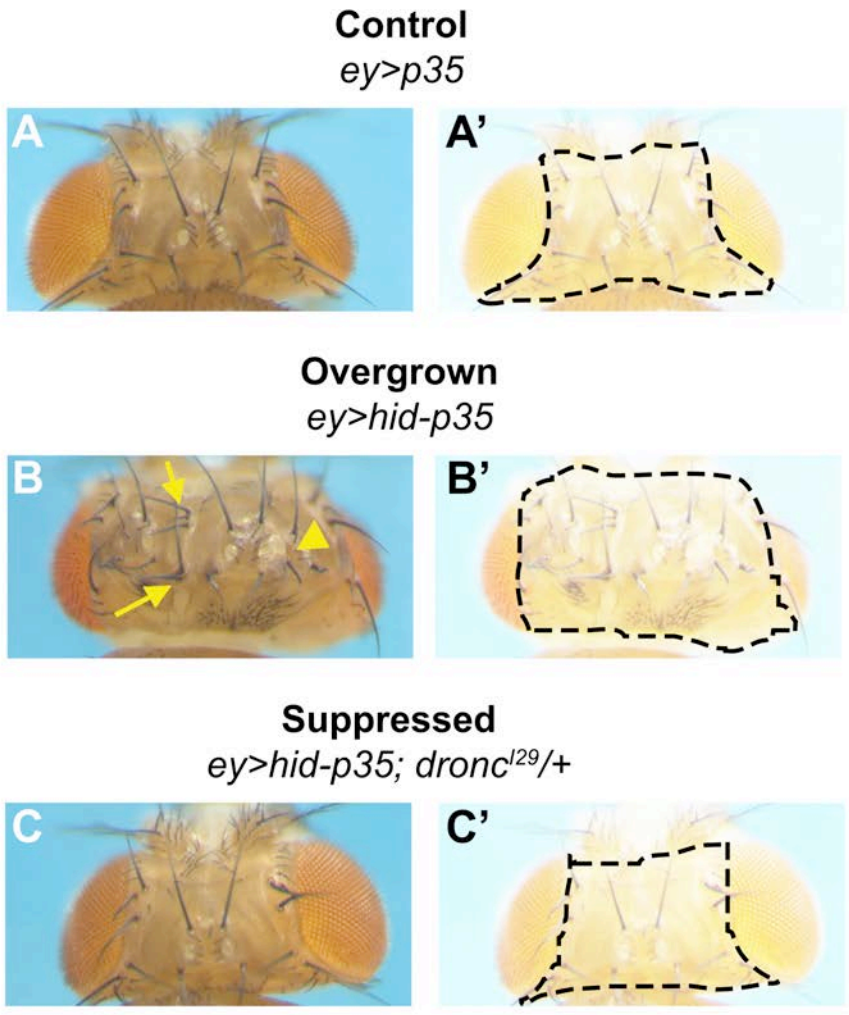


Figure 2.1
A Sustained Model of Apoptosis-induced Proliferation produces an Adult Overgrowth Phenotype

Figure 2.1: A Sustained Model of Apoptosis-induced Proliferation produces an Adult Overgrowth Phenotype

(A-C) The sustained ("undead") model of AiP yields a distinct adult phenotype that can be used in large scale genetic screens. Representative examples of adult heads are shown. Representative of N>20, n>1000 over time, where N= biological replicate genetic crosses, and n=total number of flies examined.

(A) Control animals express p35 under the tissue specific Gal4, but p35 alone does not cause an observable phenotype (*ey-Gal4, UAS-p35/+*). The bristles and ocelli appear in a stereotyped pattern and the head capsule is trapezoidal in shape (A').

(B) In experimental animals, the overgrowth phenotype is seen when Hid is co-expressed with p35,(*UAS-hid*; *ey-Gal4, UAS-p35/+;*). Defects include duplications of bristles (arrows) and ocelli (arrowhead), and expansion of the head capsule to a larger and more rectangular shape, often at the expense of eye tissue (B').

(C) In suppressed animals, the wild-type size, shape and pattern are restored, as seen here when Dronc activity is reduced with a mutant allele (*UAS-hid*; *ey-Gal4, UAS-p35/+; dronc^{I29}/+).*

studies have demonstrated critical requirements for ROS during wound healing and regeneration, and in certain contexts via activation of JNK (Niethammer et al., 2009) (Juarez et al., 2011; Gauron et al., 2013; Razzell et al., 2013; W. J. Lee & Miura, 2014; Muliyil & Narasimha, 2014).

RESULTS

Ectopic production of ROS in apoptosis-induced proliferation

In order to examine the role of ROS in AiP, we assessed *in vivo* ROS levels in *Drosophila* imaginal discs using the ROS-reactive dyes dihydroethidium (DHE) and the fluorescein based H_2-DCF-DA (Owusu-Ansah, Yavari, & Banerjee, 2008). These dyes generally detect superoxide and hydrogen peroxide, respectively; however, they may detect other ROS as well. For our purposes, we use these dyes as general reporters of ROS production. In undead eye imaginal discs, ROS are dramatically increased compared to control discs (Figure 2.2A, B, D, E). This increased ROS production in undead tissue is dependent on Dronc activity (Figure 2.2 C, F), consistent with the suppression of the adult head overgrowth phenotype by *dronc* mutations (Figure 2.1C). We also detected increased ROS in overgrown areas of undead wing imaginal discs (*ptc>hid-p35*, an analogous undead model previously published by (Rudrapatna, Bangi, & Cagan, 2013)) (Figure 2.3 A, B), suggesting that the production of ROS in response to caspase activation is not tissue-specific. These data together imply that ROS can be generated in developing epithelial tissues following initiator caspase activation, independent of cell death execution. Finally, in a genuine AiP model in which cells are

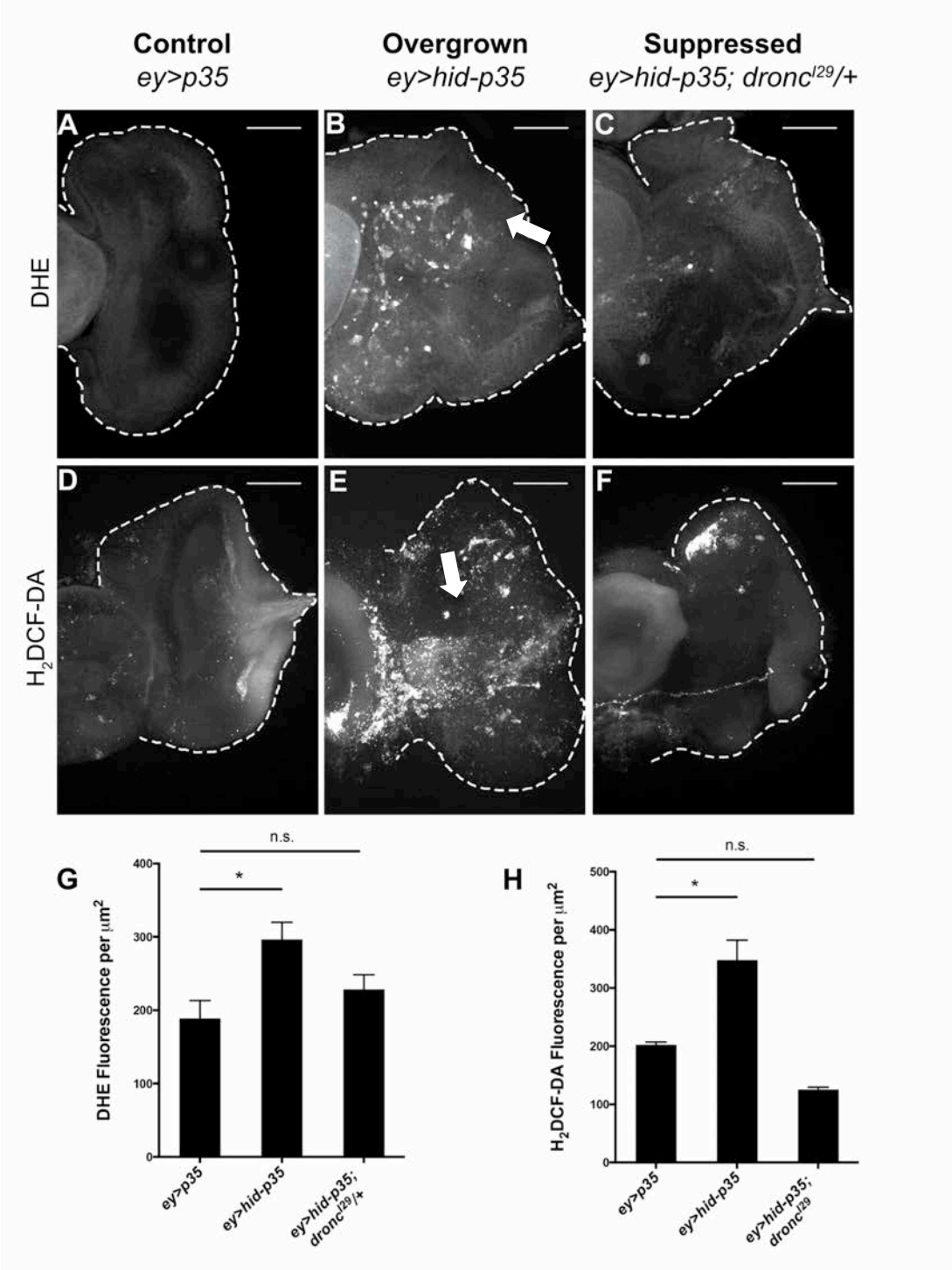


Figure 2.2
Reactive oxygen species are produced during Apoptosis-induced Proliferation by a Dronc-dependent mechanism.

Figure 2.2: Reactive oxygen species are produced during Apoptosis-induced Proliferation by a Dronc-dependent mechanism.

(A-H) ROS are produced in undead eye tissue but not control or suppressed eye tissue. Arrows in (B) and (E) indicate ROS in anterior undead tissue.

(A-C) ROS detection with dihydroethidium (DHE) primarily shows superoxide levels. Images are representative of N = 5, n = 3 to 5 per trial.

(D-F) ROS detection with the fluorescein based H_2-DCF-DA primarily indicates hydrogen peroxide levels, though may also represent other reactive species. N=3, n=10.

(G, H) Quantification reveals ROS levels are significantly higher in overgrown, undead discs (*ey>hid-p35*) than control (*ey>p35*) eye imaginal discs. while ROS levels are strongly suppressed in a $dronc^{I29}$ heterozygous background. Antibody labeling of the anterior versus posterior disc is not compatible with our live ROS dyes; therefore, quantification over the entire eye disc excluding the antennal disc is signal intensity per μm^2 ±SEM analyzed by one-way ANOVA, with Holm-Sidak test for multiple comparisons, * p= 0.031 (G) and * p=0.010 (H), n.s.= no statistically significant difference between control and suppressed states.

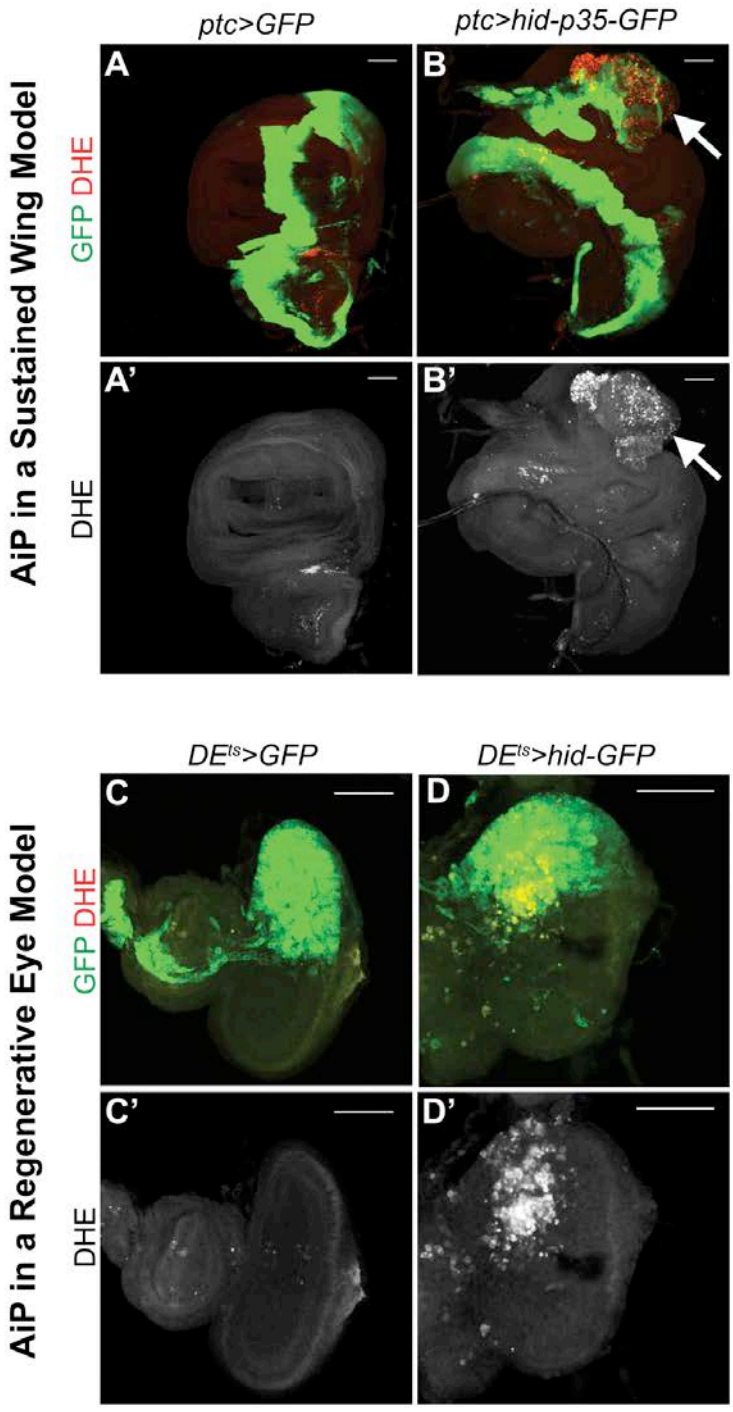


Figure 2.3
Ectopic production of ROS can be seen in additional models of Apoptosis-induced Proliferation.

Figure 2.3: Ectopic production of ROS can be seen in additional models of Apoptosis-induced Proliferation.

(A, B) ROS are detected in the overgrown area (marked by arrow) of an undead wing imaginal disc in an alternative sustained model of AiP. *UAS-hid; ptc-Gal4, UAS-GFP/UAS-p35;* +/+ N=2, n=9

(C, D) ROS are detected during AiP in the eye in a regenerative model (D), but not in control discs (C). In (D), ROS are produced within the specific cell death domains, marked by GFP. *UAS-hid; UAS-GFP, UAS-lacZ/+; DE-Gal4, tub-Gal80^{ts}*/+ N=4, n=12

allowed to complete apoptosis (Smith-Bolton et al., 2009; Bergantinos et al., 2010; Fan et al., 2014), ROS are specifically observed within the death domain (marked by GFP) following apoptosis induction (Fig. 2.3 C, D), suggesting that the ROS are a local response to or by dying cells. An added advantage of the genuine model is that we are able to observe ectopic ROS levels over time. We have noted that production of ROS is an early event in the death/regeneration process, with high levels detected immediately after apoptosis induction, and at least through 24 hours after apoptosis induction (Figure 2.4).

Extracellular ROS are produced by Duox and are required for Apoptosis-induced Proliferation

To determine if there is a functional requirement for ROS in AiP, we mis-expressed ROS-reducing enzymes in the undead AiP model. However, mis-expression of cytosolic SOD and catalase transgenes did not significantly suppress *ey>hid-p35*-induced overgrowth (Figure 2.5 A, C). In contrast, mis-expression of two extracellular catalases, *immune-regulated catalase* (*IRC*) and a secreted human catalase (*hCatS)*, does suppress *ey>hid-p35*-induced overgrowth (Figure 2.5 B, C). Consistently, mis-expression of *hCatS* results in a strong reduction of ROS in undead eye discs (Figure 2.5 F, G). These observations suggest that extracellular ROS are required for AiP following induction of apoptosis.

Two enzymes known to generate extracellular ROS are the transmembrane NADPH oxidases Nox and Duox (Leto et al., 2009; Bae, Choi, & Lee, 2010). To examine if either of these enzymes are involved in ROS production during AiP, we

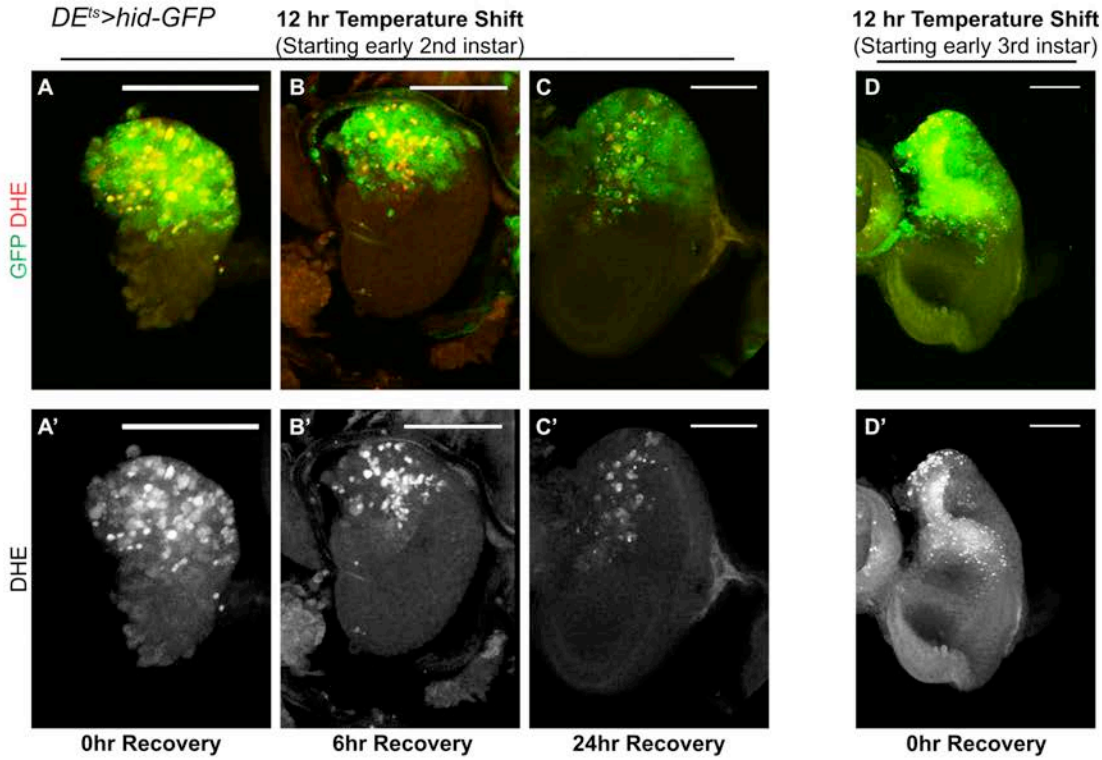
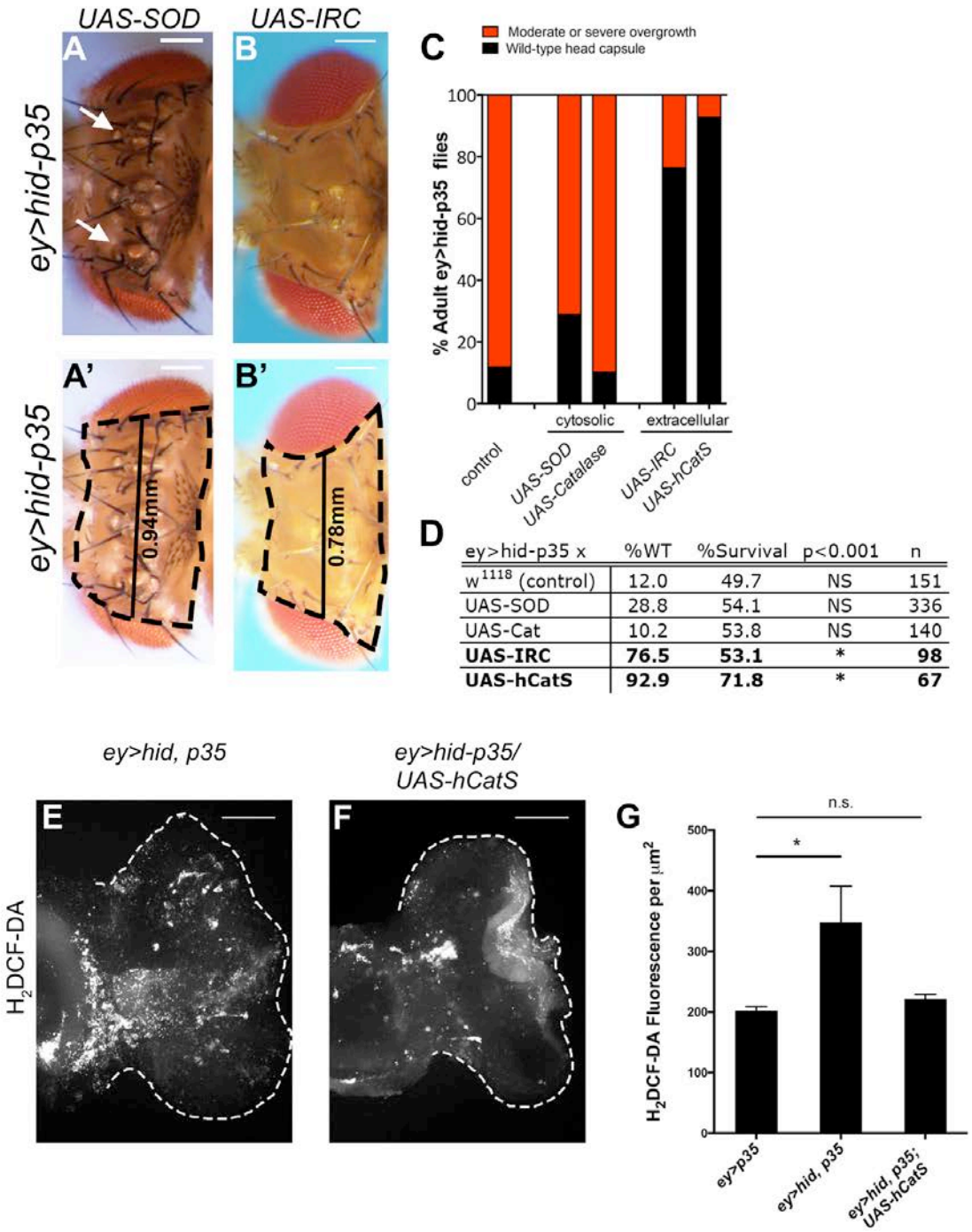


Figure 2.4
ROS production in a regenerative model of AiP

Figure 2.4: ROS production in a regenerative model of AiP.

(A-C) ROS (DHE; red in a-c, gray in a'-c') are detected in the death domain (labeled by GFP) of eye discs immediately after a 12-hour pulse of *hid* induction and persist throughout the death phase, up to 24 hours after *hid* induction.
UAS-hid; UAS-GFP, UAS-lacZ/+; DE-Gal4, tub-Gal80^{ts}/+ N=2, n=10

(D) The ROS production during regenerative AiP is independent of larval developmental timing. ROS are produced following *hid* induction during 2nd (A) or 3rd (D) instar of larval development. N=1, n=5



ey>hid-p35 x	%WT	%Survival	p<0.001	n
w^{1118} (control)	12.0	49.7	NS	151
UAS-SOD	28.8	54.1	NS	336
UAS-Cat	10.2	53.8	NS	140
UAS-IRC	**76.5**	**53.1**	*	**98**
UAS-hCatS	**92.9**	**71.8**	*	**67**

Figure 2.5
Extracellular ROS are required for AiP

Figure 2.5: Extracellular ROS are required for AiP.

(A-D) Reduction of extracellular ROS suppresses overgrowth. Representative examples of adult heads obtained from *ey>hid-p35* flies overexpressing the indicated reducing enzymes. When looking at qualitative indicators of overgrowth (presence of ectopic ocelli and bristles, arrows in A; expansion of mid-head capsule width, as in A'), expression of the extracellular reducing enzyme *IRC* (B) suppresses overgrowth, while the intracellular reducing enzyme SOD (A) has no effect. Scale bars = 200μm.

(C) Based on qualitative screening criteria, progeny are scored as wild type (black bars) or having the overgrowth phenotype (red bars).

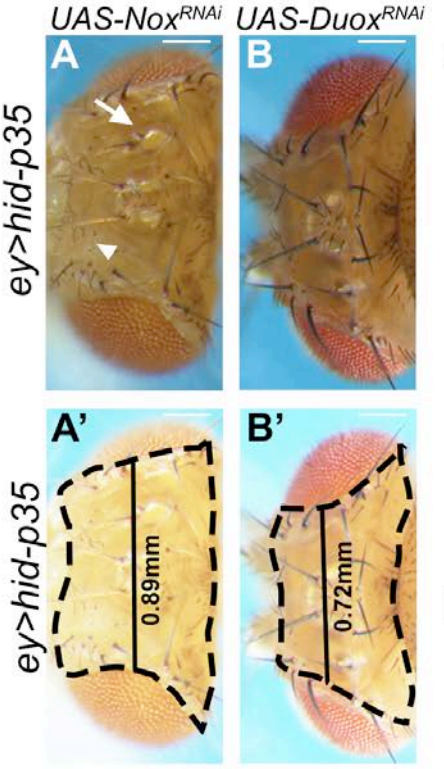
(D) Suppression is determined based on a shift in the percentage of wild-type versus phenotype that is significantly different based on a Pearson's chi-squared test for Df=1, χ^2 = 10.83 at p=.001. Strong suppressors may also increase the overall survival (% of expected animals that reached eclosion, % viability), whereas enhancers may decrease survival to adulthood (increased lethality).

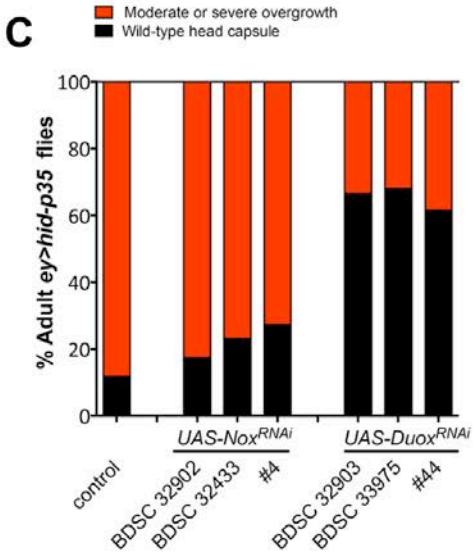
(E – G) ROS levels are strongly reduced by transgenic expression of human secreted catalase (*hCatS*). *UAS-hid; ey-Gal4, UAS-p35/UAS-hCats; +/+* . Quantifications of the ROS signal intensity per μm^2 ±SEM analyzed by one-way ANOVA, with Holm-Sidak test for multiple comparisons, *p=0.033.N=2, n=6

knocked down their expression by RNAi. Targeting *Nox* did not suppress the AiP overgrowth phenotype in *ey>hid-p35* animals (Figure 2.6 A, C). In contrast, RNAi against *Duox* produced a suppression of the AiP overgrowth phenotype (Figure 2.6 B, C). As *Duox* was autonomously inhibited in undead cells (using *ey-Gal4*), these data indicate that extracellular ROS originate from the same cells that have activated Dronc, consistent with the observation that ROS production requires Dronc. Combined, Duox activity in undead cells produces extracellular ROS, which is required for AiP-induced overgrowth.

Canonical mechanisms of Duox activation are not required for apoptosis-induced proliferation

Functionally, Duox is differentiated from Nox based on two domains: an extracellular peroxidase homology domain (PHD), and an intracellular calcium-binding regulatory (EF hand) domain. These EF hands can be activated by local increases in calcium. Under conditions of bacterial infection in the intestinal epithelium, activation of a phospholipase C complex (PLCβ and Gαq) triggers IP_3 dependent calcium release, activating Duox (Ha et al., 2009). Under conditions of embryonic wound healing, direct flux of calcium via the transient receptor potential cation channel TRPM is required for Duox activation (Razzell et al., 2013). We tested components of these two activation pathways with *ey>hid-p35*, but have not found them to be required in AiP (Table 2.1). We also attempted to reduce calcium levels using a calcium sponge, parvalbumin, with inconsistent results (Table 2.1). To determine whether any other transient receptor potential channels may be involved in AiP, we screened RNAi against each of these channels and identified two moderately strong suppressors, TRPP and TRPN (Table 2.2).





D

ey>hid-p35 x	%WT	%Survival	p<0.001	n
w^{1118} (control)	12.0	49.7	NS	151
UAS-Nox^{RNAi}	17.4	39.7	NS	81
UAS-Nox^{RNAi}	23.1	67.2	NS	97
UAS-Nox^{RNAi}	27.3	91.7	NS	70
UAS-Duox^{RNAi}	**68.2**	**57.9**	*	**120**
UAS-Duox^{RNAi}	**66.1**	**61.4**	*	**163**
UAS-Duox^{RNAi}	**61.5**	**76.5**	*	**94**

Figure 2.6
The extracellular ROS producing enzyme Duox is required for AiP

Figure 2.6: The extracellular ROS producing enzyme Duox is required for AiP.

(A-D) Reduction of extracellular ROS suppresses overgrowth. Representative examples of adult heads obtained from *ey>hid-p35* flies overexpressing the RNAi against the ROS producing enzymes Nox or Duox. When looking at qualitative indicators of overgrowth (presence of ectopic ocelli and bristles, arrow, arrowhead in A; expansion of mid-head capsule width, as in A'), knockdown of Duox (B) suppresses overgrowth, while knockdown of Nox (A) has no effect on overgrowth. Scale bars = 200μm

(C) Based on qualitative screening criteria, progeny are scored as wild type (black bars) or having the overgrowth phenotype (red bars).

(D) Suppression is determined based on a shift in the percentage of wild-type versus phenotype that is significantly different based on a Pearson's chi-squared test for Df=1, $\chi^2 = 10.83$ at p=.001. Strong suppressors may also increase the overall survival (% of expected animals that reached eclosion, % viability), whereas enhancers may decrease survival to adulthood (increased lethality).

Table 2.1: Targeted manipulation of DUOX activating pathways. Partial suppression is scored as greater than 50% wild-type animals, when viability was not reduced below 40%. Full suppression requires 90% and 50% respectively.

Gene	CG#	Stock	Percent WT	Percent Viability	p< 0.001	(n)
Control: *Canton*^{S}			22.61	66.80		117
UAS-IP$_3$-sponge^{M49}		(Usui-Aoki et al., 2005)	10.00	20.83	*lethal*	58
IP$_3$R RNAi	1063	VDRC 106982	0.00	24.00	*lethal*	105
G-α-q RNAi	17759	(Ha et al., 2009)	14.29	15.38	*lethal*	102
UAS-PV-Myc[1]		BDSC 25028	5.88	24.29	*lethal*	**87**
		BDSC 25029	**59.56**	**57.14**	*	**374**
		BDSC 25030	14.81	52.43	n.s.	**314**

[1]UAS-parvalbumin-myc is a reported calcium sponge (Harrisingh et al., 2007)

Table 2.2: RNAi Screen of Transient Receptor Potential Channels in *Drosophila.* Partial suppression is scored as greater than 50% wild-type animals, when viability was not reduced below 40%. Full suppression requires 90% and 50% respectively.

Gene	CG#	BDSC #	Percent WT	Percent Viability	p< 0.001	(n)
Control: w^{1118}			23.76	42.98		151
TRPM	44240	31672	60.00	9.09	*lethal*	60
		31291	25.00	5.48	*lethal*	77
		44503	71.43	11.29	*lethal*	69
		51713	45.45	21.15	*lethal*	63
TRP	7875	31649	47.90	55.35	*	334
		31650	70.93	40.19	*****	**300**
TRPV (nan)	5842	53312	73.53	43.93	*****	**557**
		31295	36.81	39.37	*lethal*	577
		31674	50.50	23.17	*lethal*	537
TRPV (iav)	4536	25865	55.95	74.01	*****	**395**
TRPML	8743	31294	40.91	21.02	*lethal*	380
		31673	74.42	36.91	*lethal*	638
TRPA	5751	31504	65.63	42.67	*****	**428**
		31384	27.38	30.43	*lethal*	360
TRPP (PKd2)	**6504**	**31296**	**52.50**	**47.24**	*****	**374**
		31675	**79.69**	**60.38**	*****	**340**
		51502	**83.10**	**58.20**	*****	**386**
TRPL	18345	26722	58.43	44.28	*****	**290**
TRPN (nompC)	**11020**	**51722**	**73.91**	**43.81**	*****	**302**
		31512	**80.43**	**58.97**	*****	**248**
pyx	17142	51836	66.67	40.91	**NS**	**31**
		31297	51.61	46.97	*****	**97**

Autonomous reduction of either of these calcium channels in *ey>hid-p35* animals resulted in significant ($p<0.001$) wild-type rescue consistently with multiple RNAi constructs. TRPP is also known as Polycystic Kidney Disease 2 (*Pkd2*) and has been studied for its role in sperm motility and phagocytosis of apoptotic cells by macrophages (Z. Gao, Ruden, & Lu, 2003; Watnick et al., 2003; Van Goethem et al., 2012).

Extracellular ROS may function upstream of JNK activity

Next, we attempted to examine the position of ROS function relative to JNK, which is a critical mediator of AiP (Ryoo et al., 2004; Bergantinos et al., 2010; Herrera et al., 2013; Fan et al., 2014). A standard tool used to detect JNK transcriptional activity is a gene trap reporter allele of the JNK transcriptional target *puckered.* The *puckered* gene encodes a phosphatase that negatively feeds back on JNK, but the puc^{E69} allele instead expresses the bacterial beta-galactosidase (*lacZ)* (Martin-Blanco et al., 1998). Using *puc-lacZ* as a marker of JNK activity, we know that *ey>hid-p35* results in a marked increase in JNK activity, especially in the anterior eye disc (Figure 2.7 A, B, F). We are able to distinguish proliferating “anterior” tissue from mature differentiated “posterior” tissue regardless of location, based on the pan-neuronal marker ELAV, expressed only in maturing neurons (Robinow & White, 1991; O'Neill et al., 1994). Often in overgrown discs, the ELAV+ area is reduced and distorted (Figure 2.7 B, B”).

We have previously demonstrated that suppression of AiP upstream of JNK can restore normal ELAV patterns by suppressing disc overgrowth and *puc-lacZ* activity (Fan et al., 2014). We have also previously shown that inclusion of the *puc-lacZ* allele alone, which results in a haplo-insufficient state of this negative JNK regulator, does not

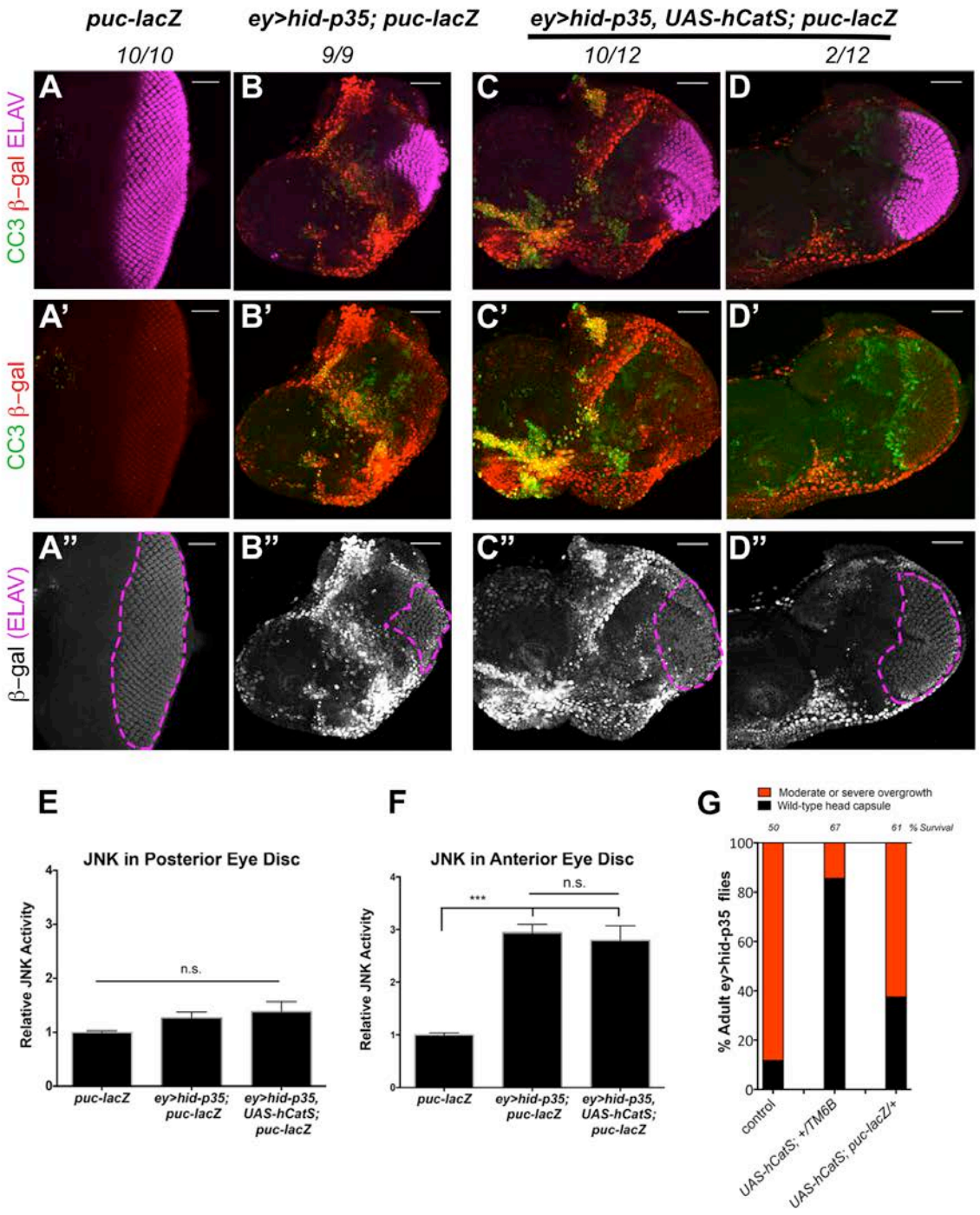


Figure 2.7
JNK activity is elevated in *ey>hid-p35* and may be affected by ROS levels

Figure 2.7: JNK activity is elevated in *ey>hid-p35* and may be affected by ROS levels

(A – D) Tissue level changes in JNK activity can be estimated using the puc^{E69} allele, which expresses a bacterial beta-galactosidase under the control of the endogenous *puckered* promoter (*puc-lacZ*). In wild-type 3rd instar imaginal discs, reporter activity is low, especially in the proliferating anterior region (A-A", red/gray: *puc-lacZ,* purple: ELAV, green: Cleaved Caspase 3). In *ey>hid-p35* discs, an increase in cleaved-caspase 3 staining is matched with a dramatic induction of the JNK reporter in the anterior (undead) region (B-B"). The increased *puc-lacZ* expression can be suppressed without affecting CC3 (suppression of AiP but not cell death) when extracellular ROS are reduced (D-D"), but more commonly reduction of ROS does not affect the *puc-lacZ* reporter (C-C"). N=2, n= as indicated. Scale bars = 50μm.

(E, F) Quantification of relative JNK activity level in eye discs. JNK activity in the posterior differentiated region (ELAV+) remains relatively unchanged between control and experimental conditions (E). In the anterior region (undead) JNK reporter activity is significantly increased in experimental conditions over control (F). There is no significant difference in *puc-lacZ* reporter levels between *ey>hid-p35* alone and *ey>hid-p35, UAS-hCatS.* (One-way ANOVA, Holm-Sidak test for multiple comparisons, ***$p<0.0001$).

(G) Addition of the *puc-lacZ* transgene to *ey>hid-p35, UAS-hCatS* effectively negates the suppressive effect of the catalase (Chi-squared Df=1, χ^2 =10.83, p=0.001)

significantly affect (enhance) the overgrowth phenotype (Fan et al., 2014). Here, we observed that JNK activity can be strongly reduced in undead eye discs that mis-express *hCatS* suggesting ROS are upstream of JNK (Figure 2.7 D-D"). However, we do not see these suppressed discs at the frequency we would anticipate based on our previous results, and therefore we cannot show that there is a significant effect on JNK levels (Figure 2.7 C, D, F). Consistent with this observation, when undead animals expressing *UAS-hCatS* and *puc-lacZ* reach the adult stage, mis-expression of *hCatS* is no longer sufficient to suppress the adult head overgrowth phenotype (Figure 2.7 G). We hypothesize that ROS may be upstream of JNK, but that the combination of incomplete reduction of ROS by *hCatS* with the sensitized *puckered* haplo-insufficient state, could allow overgrowth to proceed. Future experiments to specifically dissect this relationship may be more informative, but with this limited finding, and because *puc-lacZ* and Cleaved-Caspase 3 often overlap (imperfectly) in undead discs (Ryoo et al., 2004; Herrera et al., 2013; Fan et al., 2014), we propose that extracellular ROS signal in an auto/paracrine manner to activate JNK in undead tissues.

ROS recruit and activate hemocytes for apoptosis-induced proliferation

Previous reports in fly and vertebrate models have demonstrated extracellular ROS can attract and activate innate immune cells to sites of tissue damage (Niethammer et al., 2009; Moreira et al., 2010; Evans & Wood, 2014). In our model of AiP we probed for immune cells adjacent to ROS-producing undead cells using cell-type specific antibodies against the three types of *Drosophila* immune cells, known as hemocytes. With the plasmatocyte specific anti-Nimrod (NimC) antibody (Kurucz et al., 2007), we

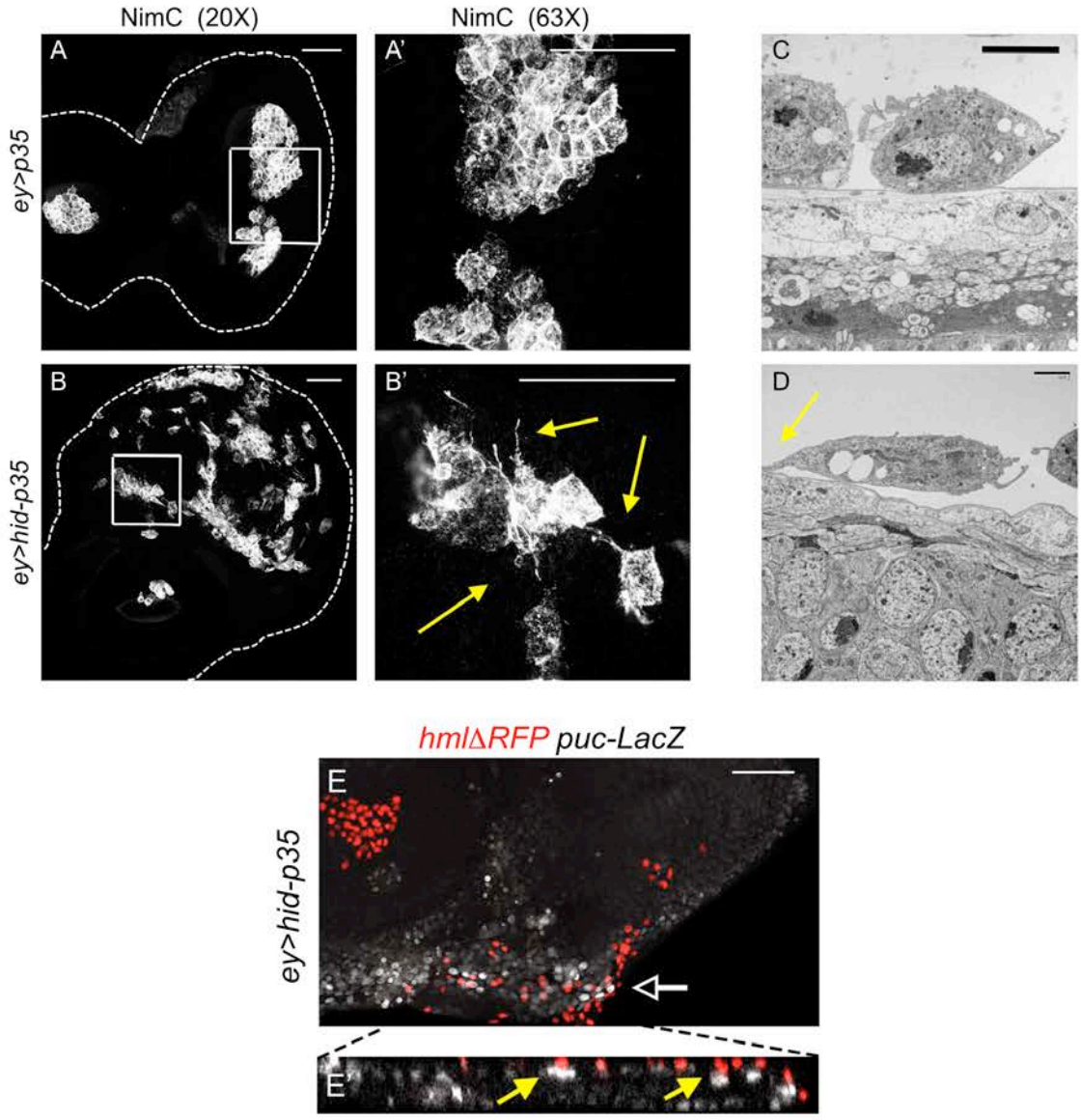


Figure 2.8:
Hemocytes are proximal to JNK active cells and the epithelial layer

Figure 2.8: Hemocytes are proximal to JNK active cells and the epithelial layer.

(A, B) Here plasmatocytes are labeled with anti-NimC (gray). Hemocytes attached to control discs clump as large aggregates (A, A'). Hemocytes attached to undead tissue project extensions (arrows in (B')), making extensive contacts with the epithelial layer of the imaginal disc. NOTE: Scale bars are all 50 μm. Representative images of N>3, n>15

(C, D) Transmission electron micrographs of hemocytes in contact with control and undead eye discs. In (D) the hemocyte has a more elongated "macrophage-like" morphology. NOTE: Scale bar in (C) is 5 μm, scale bar in (D) is 2 μm. N=2, n=10

(E) Hemocytes (visualized by nuclear *hml⊿RFP* marker, red) are often found directly adjacent to JNK-active epithelial cells (*puc-lacZ*, grey) in *ey>hid-p35* eye tissue. The white arrow in (E) indicates the location where the orthogonal (YZ) section was applied shown enlarged in (E'). Yellow arrows in (E') highlight examples where hemocytes are adjacent to JNK-active epithelial cells. The hemocyte cluster in the upper left is located in the antennal portion of the disc, which is not overgrown and therefore does not reveal JNK signaling. N=2, n=4

found hemocytes are adjacent to both control and undead eye discs (Figure 2.8 A, B). Most strikingly, although hemocytes are present at control eye discs, when they are adjacent to undead tissue, they change morphology to what is characterized as an activated macrophage-like morphology (Babcock et al., 2008; Kelsey et al., 2012). At control eye discs, rounded hemocytes group in large clusters (Figure 2.8 A, C) and are located around the border between anterior proliferating tissue and the posterior differentiating photoreceptors. In contrast, hemocytes on undead discs are often present as single cells, are less spherical and project membrane extensions (Figure 28 B, D). We see the same recruitment and these same morphological changes when we look at hemocytes recruited to undead wing discs (here identified by the pan-hemocyte antibody, anti-Hemese (H2) (Kurucz et al., 2003)) (Figure 2.9).

Interestingly, we have observed hemocytes with this macrophage-like morphology directly adjacent to the epithelial layer (Figure 2.8 D), and a number of hemocytes are directly adjacent to epithelial cells expressing the JNK marker *puc-lacZ* (Figure 2.8 E). Therefore, hemocytes may specifically interact with undead and JNK-activating cells.

Furthermore, the differentiation of the posterior eye tissue into mature photoreceptors, as marked by ELAV, is commonly disrupted in *ey>hid-p35* animals. Hemocytes are adjacent to the undead tissue that displaces this part of the posterior eye tissue as visualized by disrupted ELAV labeling (compare Figure 2.10B” and 2.10A”). Taken together, these changes in morphology and localization could suggest a functional role for hemocytes in driving proliferation.

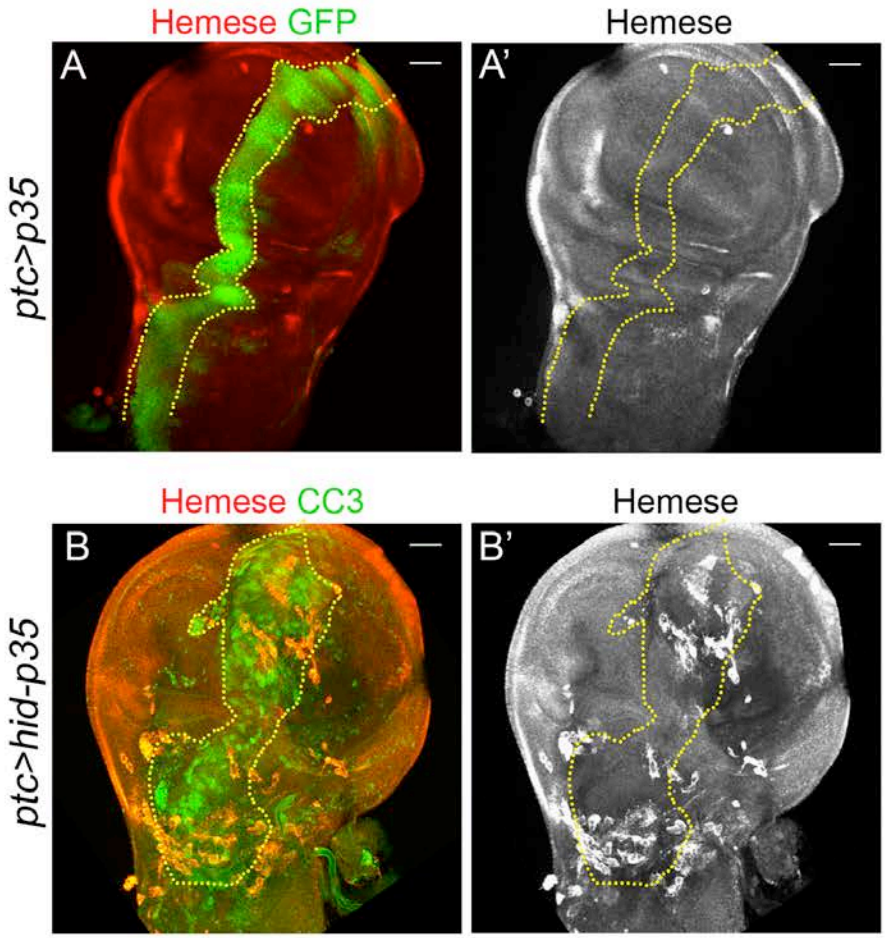


Figure 2.9:
Hemocytes are recruited to undead wing imaginal discs

Figure 2.9: Hemocytes are recruited to undead wing imaginal discs.

(A) In a *ptc>p35* control wing imaginal disc, expressing GFP in the *ptc* domain, and labeled with the pan-hemocyte marker Hemese (red in (A) and grey in (A')). Only a few hemocytes are detectable. N=2, n=8

(B) In *ptc>hid-p35* undead wing imaginal discs, hemocytes (red in (B) and grey in (B')) are recruited in large numbers. When overlying the undead cells in the *ptc* domain, these hemocytes also show similar alterations in morphology as observed in undead eye imaginal discs (B', within dashed lines). Cleaved Caspase 3 (CC3) labeling (B") is used here to mark the undead tissue, which should approximate the overgrown *ptc* domain. Most hemocytes attach to the undead portion of the wing tissue. N=3, n=10

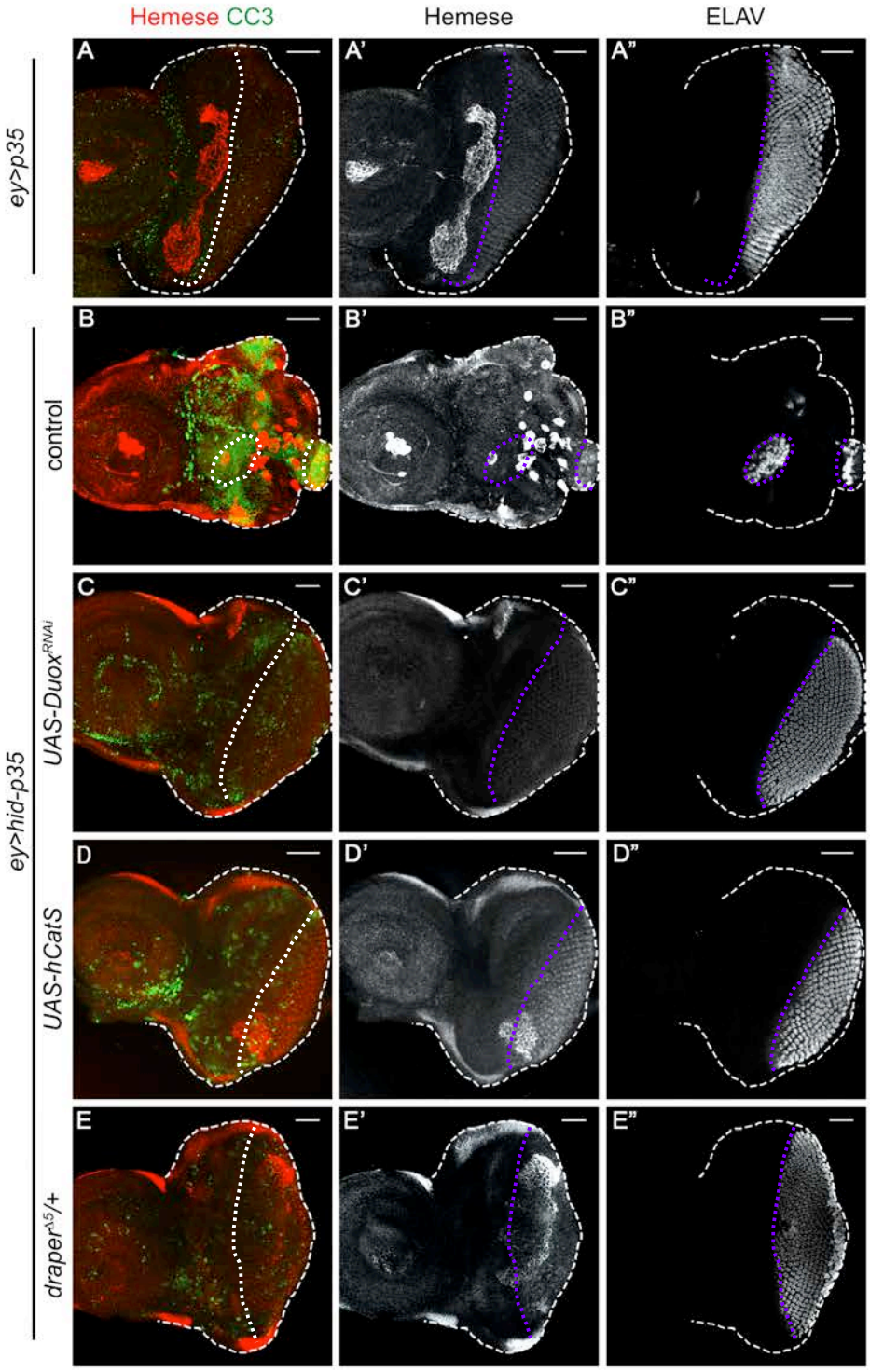


Figure 2.10:
ROS recruit and activate hemocytes for Apoptosis-induced Proliferation

Figure 2.10: ROS recruit and activate hemocytes for Apoptosis-induced Proliferation.

(A) In *ey>p35* control discs, hemocytes (anti-Hemese) are found in a clumped aggregate pattern (A') along the boundary between anterior proliferating and posterior differentiating eye tissue (visualized by ELAV labeling in A", boundary at purple dashed line in A"). They are also present as a cell aggregate in the antennal portion of the imaginal disc (left). Representative of N>5, n>50.

(B) Hemocytes adhere as single cells or small cell clusters on undead *ey>hid-p35* eye tissue with reduced spherical morphology. They are present in the overgrown anterior areas as marked by cleaved caspase 3 (CC3), which in this case extends into the "posterior" portion of the disc as visualized by the disrupted ELAV pattern (B", compare to A", mature tissue only within purple dashed lines). Representative of N>5, n>50.

(C,D) Hemocyte association with the eye disc is abolished or reduced to control levels upon loss of ROS by transgenic expression of $Duox^{RNAi}$ (C) and *hCatS* (D). Note that the ELAV pattern is normalized in these discs (C"-D") indicating suppression of abnormal growth. (C) N=2, n=12 (D) N=2, n=10

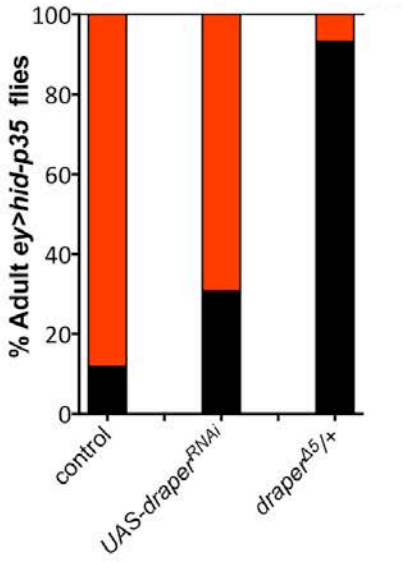
(E) Heterozygosity of *Draper* (*Drpr*) restores the morphology of hemocytes to the clumped cell aggregates observed in control discs (see (A)). This correlates with normalized ELAV pattern (E"). N=3, n=22

We further probed the relationship between hemocytes and overgrowth by examining the pattern and morphology of hemocytes when extracellular ROS levels were reduced. We found that upon reduction of ROS by expression of the extracellular catalase *hCatS* or *Duox* RNAi, hemocyte recruitment is strongly impaired and ELAV labeling is normalized (Figure 2.10 C,D). These results suggest that extracellular ROS may specifically attract hemocytes to undead tissue and that hemocytes play a role in regulating AiP.

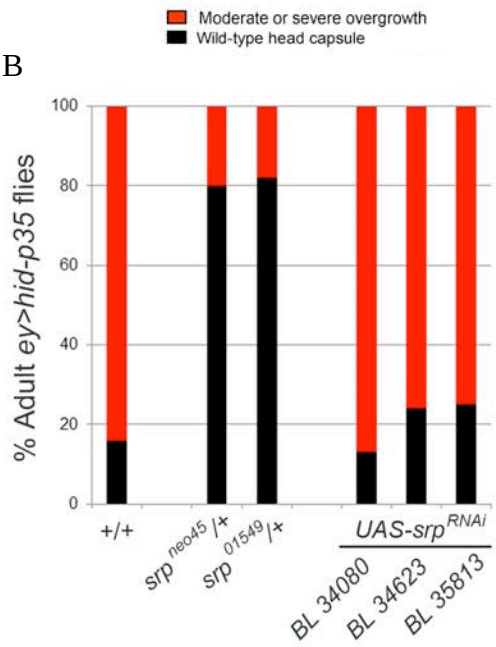
Next, we investigated whether hemocytes promote or restrict the overgrowth of undead tissue. To address this question, we analyzed *ey>hid-p35* animals that are mutant for *Draper* (*Drpr*), a cell surface receptor that integrates damage cues, is essential for phagocytic activity of hemocytes, and has recently been demonstrated to be involved in the regulating the "responsiveness" of hemocytes to ROS, specifically to hydrogen peroxide gradients. (Manaka et al., 2004; Cuttell et al., 2008; Hashimoto et al., 2009; Kuraishi et al., 2009; Doherty et al., 2014; Fullard & Baker, 2014; Evans et al., 2015). Examining at the disc level, we see the attachment and distribution of hemocytes to the undead eye discs mutant for *Drpr* is strongly impaired (Figure 2.10 E) and resembles control discs (Figure 2.10 A). As expected, the adult overgrowth phenotype of *ey>hid-p35* animals is dramatically suppressed when all cells are mutant for *Drpr* (Figure 2.11 A). Importantly, there is no suppression when *Draper* is only down-regulated in the disc epithelium by RNAi under *ey>Gal4* control. This supports the model that *Draper* functions other than autonomously in the epithelium to suppress overgrowth.

The next logical step to evaluate the requirement of hemocytes in AiP is to

A



B



C

ey>hid-p35 x	%WT	%Survival	p<0.001	n
w^{1118} *(control)*	13.6	76.0	NS	811
UAS-draper^{RNAi}	30.8	53.6	NS	149
draper $^{\Delta 5/+}$	**93.3**	**44.4**	***	**165**

ey>hid-p35 x	%WT	%Survival	p<0.001	n
w^{1118} *(control)*	15.9	66.0	NS	315
srp ^{neo45}	**80.0**	**75.0**	***	**53**
srp ^{01549}	**87.2**	**80.0**	***	**59**
UAS-srp^{RNAi}	13.5	70.0	NS	74
UAS-srp^{RNAi}	20.2	78.0	NS	146
UAS-srp^{RNAi}	27.2	72.0	NS	143

Figure 2.11:
Draper-Dependent Responsive Hemocytes are Required for Apoptosis-induced Proliferation

Figure 2.11: Draper-Dependent Responsive Hemocytes are Required for Apoptosis-induced Proliferation

(A) While specific loss of *draper* in the eye epithelium (by eye-specific RNAi) does not suppress overgrowth, whole animal loss (heterozygous mutant) of *draper* is sufficient to suppress overgrowth, suggesting a non-disc autonomous function of Draper in AiP.

(B) The *serpent (srp)* gene, which is critical for hemocyte development, is required for AiP. Loss of *srp* in the eye disc alone by RNAi has no effect on AiP, again suggesting a non-disc autonomous function of *srp.*

(C) Suppression is determined based on a shift in the percentage of wild-type versus phenotype that is significantly different based on a Pearson's chi-squared test for Df=1, $\chi^2 = 10.83$ at p=.001.

remove hemocytes from the system. This was particularly challenging, but we were able to demonstrate that animals heterozygously mutant for the GATA transcriptional regulator *serpent*, a key factor for hematopoiesis (Rehorn et al., 1996), also resulted in suppressed AiP (Figure 2.11 B). Combined, these results suggest that undead tissue produces extracellular ROS through activation of Duox, which triggers an inflammatory response by attracting and activating hemocytes via the "responsiveness receptor" Draper. Hemocytes in turn are required for the overgrowth of the undead epithelial tissue.

JNK is activated through the TNF receptor Grindelwald

One possible mechanism by which hemocytes could drive AiP is via release of cytokines, in turn activating one or more signaling receptors on the disc epithelial cells, to induce JNK activity and promote proliferation. The *Drosophila* TNF, Eiger (Egr), is a well-established JNK-activating cytokine (Igaki et al., 2002; Moreno, Yan, & Basler, 2002; Kauppila et al., 2003), which we see aggregated in the area of hemocytes on overgrown undead discs, but not control discs (Figure 2.12 A-D"). We have previously shown by *ey-Gal4*-driven RNAi that Eiger is not required disc-autonomously in epithelial cells for AiP (Fan et al., 2014); however, whole animals homozygous mutant for *Egr*, do show a non-autonomous requirement for *Egr* (Figure 2.12 E).

Alternatively, when considering candidate signaling receptors,Wengen, PVR, and Grindelwald are known to activate JNK in epithelial cells (Kanda et al., 2002; Kauppila et al., 2003; Ishimaru et al., 2004; Macias et al., 2004; Wood, Faria, & Jacinto, 2006; Bond & Foley, 2009; Igaki, 2009; Andersen et al., 2015). Again, we showed previously that Wengen and PVR are not required in undead tissue during AiP (Fan et al., 2014).

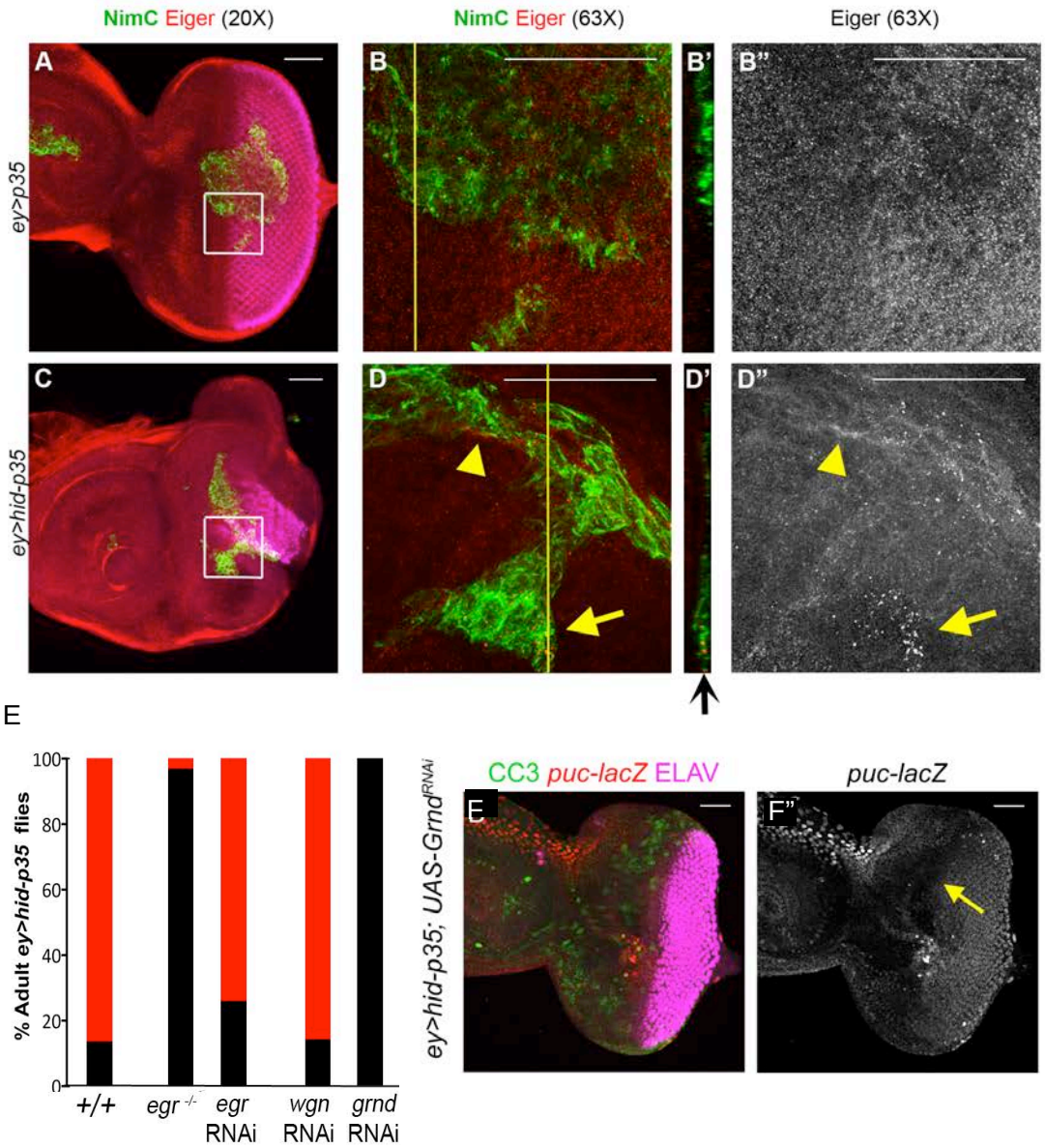


Figure 2.12:

Eiger drives AiP through the TNF receptor Grindelwald

Figure 2.12: Eiger drives AiP through the TNF receptor Grindelwald.

(A - D) Anti-Eiger labeling (red) of control (ey>p35) eye discs (A, B) and undead (ey>hid- p35) eye discs (C, D) with attached hemocytes (NimC, green). The boxed area in (A) is magnified in (B), box in (C) is magnified in (D). The yellow lines in (B) and (D) mark the orthogonal (YZ) sections shown in (B') and (D').

(A, B) Diffuse Eiger staining is seen in the disc epithelium but not in hemocytes (B"). (C, D) Increased Eiger labeling can be seen in hemocytes in the overgrown region of an undead disc (arrows in D - D") as well as in epithelial cells close to hemocytes (arrowheads in D, D").

(E) Loss of Eiger in the eye disc alone (RNAi) cannot suppress AiP, but a whole animal *Egr* null condition does suppress. Eye-specific (*ey-Gal4*) knockdown of *Grindelwald* (*Grnd*), but not *Wengen (Wgn)*, strongly suppresses the overgrowth of the adult head cuticle of *ey>hid-p35* animals.

(F) *Grnd* RNAi suppresses JNK activity (*puc-lacZ*) in the anterior eye disc (see Figure 2.7 for comparison). The suppression by *Grnd* RNAi correlates with normalization of the ELAV pattern (magenta) in *ey>hid-p35* tissue. Cleaved caspase 3 (CC3, green) labeling is present in *Grnd* RNAi discs suggesting that it is downstream of caspase activation and does not affect apoptosis. N=1, n =10

However, we also showed that JNK activation in AiP specifically requires the upstream factors Traf2 and Tak1 (Fan et al., 2014). Grindelwald (Grnd) is a TNF receptor that triggers JNK activation via Traf2 and Tak1 (Andersen et al., 2015). Consistently, two independent RNAi constructs targeting *Grnd* in the undead epithelium strongly suppressed overgrowth of *ey>hid-p35* animals (Figure 2.12 E). Additionally, upon *Grnd* knockdown in the undead tissue, the ectopic JNK activity as reported by the *puc-lacZ* construct is lost (Figure 2.12 F). These results provide evidence that Egr-mediated, Grnd-dependent activation of JNK may serve as an intermediary of hemocyte/epithelial crosstalk, which is required for overgrowth of undead epithelial tissue.

DISCUSSION

The role of ROS, specifically of H_2O_2, as a regulated form of redox signaling in damage detection and damage response is becoming increasingly clear (Schieber & Chandel, 2014). Here, we have shown in *Drosophila* that extracellular ROS generated by the NADPH oxidase Duox drive compensatory proliferation following *hid*-induced activation of the initiator caspase Dronc in developing epithelial tissues. We find that at least one consequence of ROS production is the recruitment and/or activation of hemocytes to the epithelial tissue. This work also helps resolve a controversial issue, namely which epithelial cells activate JNK, either apoptotic cells or neighboring surviving cells(Ryoo et al., 2004; Bergantinos et al., 2010; Herrera et al., 2013; Fan et al., 2014). Because our data indicate that hemocytes trigger JNK activation in epithelial cells, the location of hemocytes on the imaginal discs determines which epithelial cells receive

the signal for JNK activation. Nevertheless, we do not exclude the possibility that there is also an autonomous manner of JNK activation in undead cells.

The role of hemocytes in driving proliferation is less clear and likely context-dependent. In *Drosophila* embryos, hemocytes are required for epidermal wound healing, but this is a non-proliferative process (Razzell et al., 2013). With respect to tumor models in *Drosophila*, much of the research to date has focused on the tumor suppressive role of hemocytes and the innate immune response (Pastor-Pareja et al., 2008; Hauling et al., 2014; Parisi et al., 2014). In contrast, in our undead model of AiP, we find that hemocytes have a growth- and tumor-promoting role. Therefore, the state of the damaged tissue and the signals produced by the epithelium may have differential effects on hemocyte response. These considerations are reminiscent of mammalian systems, where many solid tumors are known to host alternatively activated (M2) tumor-associated macrophages, which promote tumor growth and are associated with a poor prognosis (reviewed in (Biswas, Allavena, & Mantovani, 2013)).

As tumors are considered “wounds that do not heal” (Dvorak, 1986), we see the undead model of AiP as a tool to probe the dynamic interactions and intercellular signaling events that occur in the chronic wound microenvironment. Future studies will investigate the specific mechanisms of hemocyte-induced growth and the tumor promoting role of inflammation in *Drosophila* as well as roles of additional tissue types, such as the fat body, on modulating tumorous growth.

EXPERIMENTAL PROCEDURES

Flystocks

The following mutants and transgenic stocks were used: *dronc*^{I29}; *draper*$^{\Delta 5}$; *ey-Gal4*; *ptc-Gal4*; *UAS-p35*; *UAS-hid*; *puc-lacZ. UAS*-based overexpression and RNAi stocks of the following genes were obtained from the stock centers (VDRC, Bloomington and NIG): *SOD1, catalase, Nox, Duox, Grnd*. The following stocks were a kind gift from Won Jae Lee: *UAS-IRC, UAS-hCatS, UAS-Duox*^{RNAi} #44, and *UAS-Nox*^{RNAi} #4. All stocks were maintained at 18°C in a humidity controlled dark environment.

Genetic Screening and Statistical Analysis

The exact genotype of *ey>p35* is *+/+; ey-Gal4 UAS-p35/CyOAct-GFP; +/+* (control parent). The exact genotype of *ey>hid-p35* is *UAS- hid/UAS-hid; ey-Gal4 UAS-p35/CyO,tub-Gal80;+/+* (experimental parent). Screens for suppressors of the AiP phenotype were conducted as follows: 6-10 virgin females of the control or experimental backgrounds were incubated with 3-6 males carrying the RNAi or genetic construct of interest (this equals one cross). All crosses were incubated on standard low yeast brown food (6.5 g/L Agar; 23.5 g/L Yeast; 60 g/L Cornmeal; 60 ml/L Molasses; 4ml/L Acid Mix; 0.13% Tegosept), and maintained in a controlled 22°C incubator. Parental adults were transferred to new vials every three days to prevent crowding of the progeny, as crowding tended to increase lethality among the experimental animals. F1 progeny were collected daily for up to 7 days from first eclosure.

A cross between an experimental parent female and a homozygous RNAi male should yield progeny where 50% of animals contain the construct of interest but no *ey-Gal4* driver (balancer progeny) and 50% contain the desired combination construct of interest and the *ey-Gal4* driver (experimental progeny). Animals expressing the balancer marker (curly wings) were counted to determine expected population size (50 balancer flies = expected 100 total progeny). The head capsules of experimental progeny were scored for the AiP phenotype based on a standardized protocol within our lab, taking into consideration (major criteria) number of ocelli, number of bristles, gross integrity and morphology of the cuticle, as well as (minor criteria) number of antennae, morphology and size of the eyes. Experimental progeny were scored as wild-type (WT), mild overgrowth, moderate overgrowth, and severe overgrowth. For the purpose of simply identifying suppressors of AiP, screening results are presented as the percentage of animals with WT+mild versus percentage of moderate+severe phenotype, with 100% set as the total number of eclosed non-balancer animals. However, many animals with severe overgrowth do not eclose, therefore the percent survival or viability is also reported, which is the number of eclosed experimental progeny as a percentage of a the total number of expected experimental progeny based on Mendelian ratios, calculated from the number of eclosed balancer progeny.

Constructs screen positive as suppressors of AiP when they result in at least 50% wild-type animals, but only when viability is not reduced below 40%. Strong or full suppression requires 90% WT and 50% viability. If viability is less than 40% the construct of interest is deemed “lethal” and the scored results are inconclusive. If viability

is greater than 40%, the scored results are analyzed for significance using a Pearson's chi-squared test (degrees of freedom=1, $\chi^2 = 10.83$ at p=0.001, expected values for phenotypes derived from replicate controls).

Imaging of adult head capsules

Adult flies were collected, anesthetized with carbon dioxide, placed in a 1.5mL eppendorf tube and placed at -80°C for 24hrs. Flies were then stored at -20°C until imaging. Head capsule images were taken using a Zeiss Stereo Discovery.V12 microscope equipped with an AxioCam ICC1 camera, and processed using the Zen Blue software's extended depth of field wavelets algorithm.

ROS in vivo staining

Imaginal discs and adjacent structures were dissected from 3rd instar larvae (2nd instar for *DE^{ts}>hid*) in fresh Schneider's medium for DHE staining and in fresh PBS for H_2-DCF-DA staining, according to the protocol by Owusu-Ansah et al. (Owusu-Ansah et al., 2008). Care was taken to avoid severing the eye imaginal discs from the brain lobes prior to staining, else traumatic injury of the developing photoreceptor axons resulted in excessive signal. Samples were incubated in their respective dyes for 5 minutes, washed, dissected from remaining structures in PBS, mounted in Vectashield mounting media, and imaged immediately on a Zeiss LSM700 confocal microscope, using the same presets of laser and gain settings developed during early trials with the respective reagents. Samples were discarded if they were not imaged within 30 minutes of the final

wash. Unless noted all image scale bars represent 50μm. Quantifications of ROS, specifically in the eye region of the eye-antennal disc, are reported as signal per area, determined using ImageJ. Images were not altered prior to quantification, but representative images printed here have increased brightness, and adjustment of the gray scale levels to better visualize ROS signal with respect to background. Statistical analysis was performed using one-way ANOVA with the Holm-Sidak test for multiple comparisons, $\alpha=0.05$.

Immunohistochemistry

Imaginal discs were dissected from late 3rd instar larvae, fixed, and stained using standard protocols (Fogarty & Bergmann, 2014). Antibodies to the following primary antigens were used: anti-cleaved Caspase- 3 (Cell Signaling), β-GAL (Promega), ELAV (DHSB). Anti-Hemese (H2), Nimrod (P1a,P1b), Atilla (L1), and C1 were a kind gift from István Andó (Kurucz et al., 2007). Secondary antibodies were donkey Fab fragments from Jackson ImmunoResearch. Images were taken with a Zeiss LSM700 confocal microscope and processed using ImageJ. Unless noted all image scale bars represent 50μm. Unless otherwise noted, images are maximum intensity projections of contiguous optical sections (z-stack) from the basal edge of the disc proper to the apical edge of the disc proper, thus excluding the peripodial membrane for clarity. In images containing hemocyte staining, additional optical sections were taken extending from the basal edge of the eye disc through the population of hemocytes to their most distal signal. Samples being directly compared were imaged during single sessions using identical

laser and gain settings. Most samples were labeled with three distinct fluorophores (FITC, CY3, CY5), as well as a nuclear marker; therefore, great care was taken to limit the collection of emission spectra such that bleed-through signal was not included in these IF images.

Electron microscopy

Eye imaginal discs and brain lobes were dissected from late 3rd instar larvae in ice cold PBS, washed once and then fixed in 2.5% gluteraldehyde in 0.1M Na cacodylate buffer, pH 7.4 for 45 minutes at room temperature, then washed three times for 15 minutes each in the cacodylate buffer. Eye discs were then fully dissociated from remaining tissue and placed in fresh cacodylate buffer at 4°C for no more than 24 hrs. Samples were then sent to the UMass EM Core Facility (Christine Powers). After post-fixation in aqueous 2% osmium tetroxide at room temperature for 1hr, and processing for SPI-pon/Araldite embedding, sections were studied in a Philips CM10 transmission electron microscope (TEM).

Chapter III:

DISCUSSION AND CONCLUSIONS

The section on “Implications of AiP” in this chapter is adapted from:
Fogarty, CE and Bergmann, AB. “The Sound of Silence: Signaling by Apoptotic Cells.” Current Topics in Developmental Biology. 2015. Editor: H Steller.

DISCUSSION

The hyperplastic overgrowth of a sustained model of AiP depends on the production of extracellular ROS, functional hemocytes, and JNK activation by Eiger/Grindelwald.

Apoptosis-induced proliferation is a mechanism of caspase-activated compensatory proliferation. In *Drosophila,* AiP in developing epithelial tissues is dependent on Dronc mediated activation of JNK. In this dissertation, I have uncovered a role for reactive oxygen species, specifically produced by apoptotic cells, to facilitate this activation of JNK. ROS have been implicated in triggering compensatory proliferation previously, following trauma or wounding; but in those cases, generation of ROS was attributed to general oxidase activity following loss of cellular integrity (Gauron et al., 2013). Here, I have demonstrated a clear and specific caspase-initiated production of ROS during regenerative and sustained models of AiP. In the course of this work, I have also found that these specifically generated ROS are critical for the hyperplastic overgrowth associated with the sustained AiP model in *Drosophila.* Extracellular ROS are produced by the NADPH oxidase family member Duox, and reduction of these extracellular ROS by secreted catalases suppresses the frequency of overgrowth in adult *ey>hid-p35* flies.

I also investigated possible mechanisms by which Dronc may lead to Duox activity, including via IP_3-mediated calcium signaling and via the transmembrane transient receptor potential channel TRPM; however, neither of these established mechanisms of Duox activation appear to be required for Dronc-dependent AiP. Other TRP channels, including TRPN and TRPP, may be involved in mediating calcium levels

during AiP, and are worth investigating further during future work. Until this point, it has been difficult to probe the role of calcium in sustained AiP, as traditional *in vivo* calcium sensors are optimized to detect the fleeting pulses of calcium associated with neuronal signaling (Akerboom et al., 2012). Excitingly, a newly reported *in vivo* transcriptional reporter of intracellular calcium (TRIC) may facilitate the study of subtle changes in steady-state calcium levels within undead tissue (X. J. Gao et al., 2015).

Finally, I have demonstrated that extracellular ROS likely function upstream of JNK activity, and that these extracellular ROS also recruit and activate hemocytes. Currently we propose that hemocyte activation is the critical step required for proliferation during sustained AiP, and that one or more factors derived from hemocytes such as Eiger signal back to the undead epithelium. This signal may be received via the TNF receptor Grindelwald, a newly reported JNK signaling pathway component that specifically activates the subset of kinases known to be required for AiP (Andersen et al., 2015). RNAi mediated knockdown of *grindelwald* in undead tissue strongly suppresses the sustained AiP overgrowth phenotype. A proposed model of sustained AiP based on these findings is presented in Figure 3.1.

The role of ROS in regenerative AiP remains undefined

While there is a clear role for ROS in the sustained model of AiP, I have not yet been able to demonstrate the same requirement for ROS in the regenerative model of AiP. Preliminary results in our lab show that knockdown of Duox by RNAi, or mis-expression of the secreted human catalase (*hCatS*), at the same time as the induction of

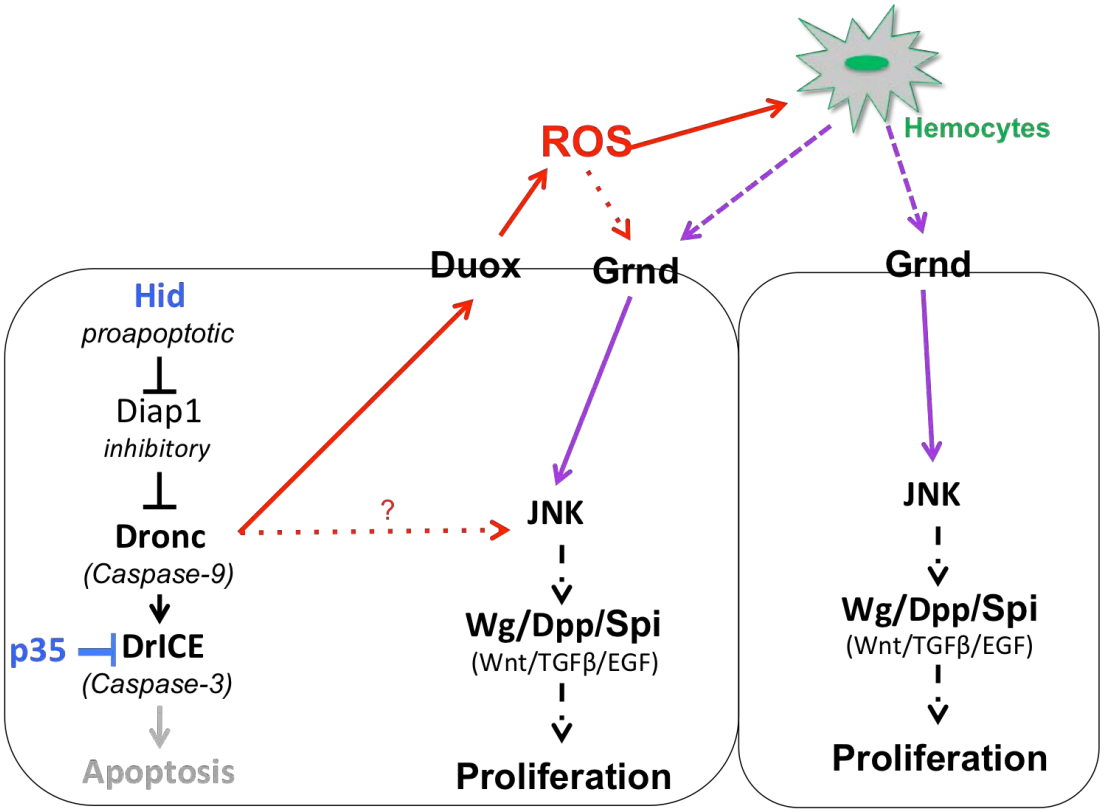


Figure 3.1
Model of ROS induced AiP

Figure 3.1: Model of ROS induced AiP.

A schematic summary of the AiP events presented in this work: *hid*-activated Dronc in undead epithelial cells triggers generation of extracellular ROS, which attract and activate hemocytes. Hemocytes may signal via Eiger to stimulate Grindelwald-dependent activation of JNK in epithelial cells, which promotes production of growth-promoting factors such as Wg, Dpp and Spi. Depending on the location of hemocytes on the imaginal disc, either the undead or the neighboring surviving epithelial cells receive this signal for JNK activation. Solid arrows indicate confirmed signaling events, though one or more intermediates may be excluded (e.g. As far as we can tell, Dronc does not directly act on Duox, but is directly upstream of it and its ROS production). Dotted lines and question marks indicate remaining uncertainties and possible accessory signaling paths.

hid, is not sufficient to suppress regeneration at 72 hrs recovery (Lindblad, Bergmann Lab, unpublished)[3]. However, recalling that in this model genetic manipulation is limited to the death phase (see Figure 1.3), I have not yet established whether either of these conditions is actually capable of suppressing ROS for the entirety of the death and regeneration phases. In fact preliminary results indicated that ROS are still strongly produced during the recovery phase in the genuine model, despite the expression of extracellular catalase (Fogarty, Bergmann Lab, unpublished). The limited duration of catalase expression in the death phase may not be enough to prevent ongoing ROS production through the critical window 6-12hrs after recovery, when JNK is typically activated.

There are a few possible ways to overcome this limitation of the regenerative model. First, to test the involvement of Duox or Nox in regeneration, we could employ exogenous chemical inhibitors of these oxidases. The studies that elucidated a role for NADPH oxidases in zebrafish fin tip regeneration utilized the chemical inhibitor VAS2870, a pan-NOX family inhibitor (Gauron et al., 2013). A great advantage of zebrafish as a model organism is the ease with which chemical inhibitor screens can be completed simply by adding the drug of interest to the water in which the fish grow and develop (Peterson et al., 2000). In *Drosophila*, similar chemical inhibitor studies are a bit more difficult, with drugs being delivered through the gastrointestinal system of growing larvae. However, one possible way to test Duox/Nox chemical inhibitors in our

[3] Ongoing work is being conducted by a number of individuals in our lab investigating aspects of both the regenerative and sustained AiP models. Unpublished findings included in this chapter are attributed by last name to the appropriate person.

Drosophila models of AiP would be through *ex vivo* disc culture. Imaginal discs can be kept in *ex vivo* culture for up to two weeks under the correct conditions (Davis & Shearn, 1977). While removing discs from their supporting environment substantially reduces the benefits of having this *in vivo* model, here an *ex vivo* method may provide a quick answer to the requirement of oxidases in regenerative AiP.

Second, to simply assess the requirement for ROS in regenerative AiP, we could generate a constitutively expressing secreted catalase, for example directly under *eyeless*-promoter control, which would be independent from the temperature restriction imposed on the Gal4/UAS based constructs that we employed. However, this single reagent will not be sufficient to fully tease apart the spatiotemporal requirements for other potential mediators of AiP.

The next major advancement in understanding AiP, both regenerative and sustained, will come with the development of a second tissue specific binary genetic system to use in parallel with our current models, such as the development of an *eyeless-lexA* or *eyeless-QF* (Lai & Lee, 2006; Potter et al., 2010). Just as the use of *p35* has allowed us to uncouple cell death initiation from execution, by independently manipulating the dying cells versus the surviving cells or the epithelium versus the surrounding tissues, we will be able to uncouple the apoptosis-derived signals from the microenvironment and system-wide responses. Separate binary systems in the genuine model could be especially useful to reduce target protein levels (such as Duox) prior to death induction, thereby eliminating contribution from pre-existing, stable protein. Additionally, separate binary systems in the post-mitotic AiP model could allow for a

more precise dissection of whether Dronc in any way contributes or attempts to contribute to AiP in the posterior eye.

LIMITATIONS

One of the most significant contributions my work makes to the field of AiP is in providing a shift in focus, from what was previously thought of as a localized, cell-autonomous process, to a more expansive and dynamic process engaging the microenvironment and potentially beyond. However, by rapidly expanding outside of the adjacent epithelial cells, we have encountered a number of limitations in our work.

Possible confounders: Tissue growth versus animal viability in the undead model

In our genetic screens for modifiers of AiP, we are looking for genes that affect the gross phenotype of the adult head capsule, which we are using as a proxy for the target tissue – the developing eye imaginal disc. Genes identified by the screen are then more thoroughly characterized at the tissue and cellular level. However, we now understand AiP can be a non-autonomous process involving the apoptotic cell, neighboring epithelia, local immune cells, and diffusible factors such as ROS. These components are not totally isolated from the rest of the developing organism, and signals from the apoptotic cells or from the responding intermediaries such as hemocytes may be influencing other developmental programs throughout the fly larva, not just the eye discs.

We have long observed that flies with the AiP overgrowth phenotype do not eclose at expected Mendelian ratios (increased lethality compared to control). This

increased death is most likely a “side effect” due to upstream signals from the undead cells diffusing outward disrupting global developmental programs. With some screen constructs we can see an enhancement of this lethality side effect, which at times can skew results if not properly taken into account. For example, a cross which results in 7 WT animals and 3 overgrown animals would appear to have 70% WT and might be interpreted as a suppressor of AiP. Yet, if we compare these numbers to the expected population size based on a balancer population of 200, we can quickly see this construct simply has enhanced lethality (5% survival to eclosion) and is not a suppressor of AiP. For this reason, we set a threshold of survival (viability) greater than 40% for each individual cross in order to consider statistical significance of WT versus phenotype. Yet, as a “side effect”, the lethality is not specifically a part of the AiP phenotypic spectrum; therefore, after passing the survival threshold, construct results were only analyzed based on the WT as a percentage of total eclosed. We believe this is the most informative analysis of the data, while remaining honest to the possible “side effects.”

Similarly, we have also noted that strong suppression of AiP during the screen, such as with loss of JNK, does not automatically restore the viability of all animals (eg *ey>hid-p35; UAS-Bsk RNAi* has 100% WT, only 50% viability). If lethality and the AiP phenotype were truly two distinct outcomes, it would follow then, that in a genetic screen, certain constructs might better suppress the AiP pathway without improving viability, others might suppress the “death side effects” increasing survival but not have any effect on the local tissue overgrowth, and yet other constructs may fortuitously affect both. In fact this is what we see – with constructs such as Nox RNAi improving viability

(up to 90% survival), yet without any improvement in AiP phenotype (27.3% WT) – compared to Duox RNAi, which partially suppresses AiP (68% WT) with no major survival benefit (57.9% survival). These results can be interpreted as follows: in the undead state both Nox and Duox act downstream of *hid* expression, but Nox contributes to non-AiP related death while Duox is responsible for AiP mediated proliferation.

A final point to consider includes the non-specific effects of individual screen constructs. While conducting our screens, we attempted to test at least two if not three independent constructs per gene of interest, ideally testing RNAi knockdown first, and then confirming with mutant alleles. However, when there were discrepancies between constructs, we erred on the side of caution and did not highlight those as suppressors of AiP (e.g. TRPA from Table 2.2, one construct showed partial suppression, but the other showed strong lethality – until another construct shows suppression we are not classifying this as a suppressor). Conversely, all of the genes presented as suppressors in this work have been confirmed by either multiple RNAi constructs or with mutants.

Dronc as a death enzyme or a life-promoter?

Dronc has been well characterized in its cell death role over the past two decades – from cataloging its requirement in different types of developmental cell death, to probing the biochemical mechanisms of Dronc activation, to defining cleavage substrate specificities (Dorstyn et al., 1999; Xu et al., 2005; Kondo et al., 2006; Dorstyn & Kumar, 2008; Snipas et al., 2008). Given that in both cell death and AiP, Dronc functions downstream of Hid and requires the apoptosome scaffold protein Ark, it is easy to

assume Dronc triggers both pathways in a similar manner, namely cleaving a single specific downstream target based on its established biochemical signature. However, there is a significant problem with this assumption: Dronc mutants do not equally suppress cell death and AiP.

In the above Dronc studies, and countless others, it has been well established that *dronc* mutant alleles are recessive with respect to WT *dronc* alleles in cell death; a single copy of functional *dronc* is sufficient to execute a cell death program (Xu et al., 2005). Mechanistically, this makes sense, since as a death enzyme even very small amounts of active caspase can trigger the self-perpetuating and self-expanding cascade of apoptosis by acting on and activating the effector caspases Drice and DCP-1 (Dorstyn & Kumar, 2008; Snipas et al., 2008). However, in AiP we clearly see dramatic suppression of ROS and the tissue overgrowth with loss of just one copy of *dronc* (see Figures 2.1 and 2.2), meaning here Dronc functions as a dominant modifier of AiP.

There are not many examples in the literature of recessive genes functioning dominantly in alternative contexts, except for several examples here in the context of AiP. In fact we see two other traditionally recessive mutants also acting as dominant modifiers in our AiP screen – the apoptosome scaffold gene *ark* and the hemocyte phagocytosis receptor *draper* are both able to suppress AiP when they are whole animal heterozygous for null allele mutants (see Fan et al., 2014 and Figure 2.11)[4]. These

[4] What is important to note here is that in the AiP screen whole animal homozygous mutants for *dronc* and *draper* are lethal with only a few animals reaching pupal stages or eclosion; which is consistent with their critical roles in cell death and development, but limits our ability to comment on the dose effect of these genes on AiP. One commonly used *Drosophila* genetic technique not employed in this dissertation, but previously

findings could be consistent with a high threshold dependent process. We know that not every apoptotic cell death results in AiP. Perhaps, proliferation is only triggered when caspase activity reaches such a profound level that it crosses a threshold, something that might not be attainable in a haplo-insufficient state. Interestingly, there is some evidence from the mammalian literature to support this threshold model. In human melanoma samples and cell lines, high Caspase-3 activity is associated with apoptosis, but also with malignant, proliferative, and metastatic behaviors in non-apoptotic cancer cells (Y. R. Liu et al., 2013; Donato et al., 2014). When Caspase-3 activity is blocked with a low level of the inhibitor z-DEVD-fmk, the degree of invasion and malignant behavior is reduced, but high levels of inhibitor are required to suppress apoptosis (Y. R. Liu et al., 2013). While these experiments are looking at effector caspase function, and while they did not specifically test for proliferation after inhibitor in the Liu study, these results are consistent with non-apoptotic functions of caspases (including AiP) requiring greater quantities of active enzyme to overcome higher signaling thresholds. Future studies of Dronc-dependent AiP could utilize caspase inhibitors or catalytically inactivated mutants to further investigate this possibility.

ROS, JNK, and a feedback loop

One of the complexities of Dronc-dependent AiP not yet addressed in this work is that of the *hid*/Dp53/JNK feedback loop. Initial work by Wells et al., (2006) identified a

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employed in the study of AiP, is the use of clone-generating recombinases such as in the MARCM (T. Lee & Luo, 1999) or Ey-Gal4/UAS-FLP (Stowers & Schwarz, 1999) systems. These systems allow for the study of nearly homozygous mutant eye tissue in an otherwise viable animal and could help characterize the threshold effect in AiP.

role for the *Drosophila* p53 (*dp53*) in sustaining Dronc activity during AiP via a positive feedback response on the pro-apoptotic genes *hid* and *reaper*. Different isoforms of Dp53 can transcriptionally upregulate *hid* and *rpr* depending on the apoptotic context, and it is currently understood that the shorter isoform more potently induces *hid* during AiP (Brodsky et al., 2000; Wells et al., 2006; Fan et al., 2010; Dichtel-Danjoy et al., 2013). Subsequent work by Shlevkov and Morata (2012) demonstrated that JNK activity is both upstream of Dp53 mediated *hid* induction, and downstream of *hid* mediated Dronc activity. By isolating each segment of the loop, they found that mis-expression of *dp53* or the constitutively active JNKK, hep^{ACT}, in *dronc* mutant clones was sufficient to induce *rpr* or *hid* expression; whereas, mis-expression of *hid* only led to activation of Dp53 or JNK when *dronc* was wild-type (Shlevkov & Morata, 2012). The sustaining stimulus from the *hid*/Dp53/JNK loop provides a potential mechanism by which whole tissues might regulate whether or not certain cell death stimuli warrant a compensatory proliferative response. In the context of our undead model, when the loop is activated by *UAS-hid* expression, the AiP process is amplified through JNK-mediated upregulation of the endogenous *hid* locus. However, this sustaining loop also complicates our analysis of triggering events during AiP with respect to ROS production, hemocyte signaling, and JNK activation.

Specifically, in order to demonstrate that ROS are upstream of JNK, we showed overexpression of an extracellular catalase can suppress AiP, and in examining individual imaginal discs we found when AiP is suppressed, then activation of JNK is reduced (see Figure 2.7). However, our use of *puc-lacZ,* a reporter inserted into the endogenous locus

of a negative regulator, inadvertently counteracted that catalase-driven suppression in most animals, leaving our results inconclusive. Interestingly, this very flaw had been questioned before, and we had erroneously thought it adequately addressed.

The original concern derived from the fact that *puckered* encodes a dual-specificity phosphatase that provides significant negative regulation of the JNK cascade (Martin-Blanco et al., 1998). By disrupting one of the endogenous alleles with the *lac-Z* gene trap, we begin with a haplo-insufficient state of this negative regulator, thus creating some baseline of active JNK, free from repression, independent of any positive AiP stimulus. The original concern was that "JNK activity" measured in overgrown discs, as represented by beta-galactosidase levels detected via IF, was a non-specific artifact of JNK/puckered dysregulation and could not be trusted as a readout for AiP. However, at that time I had shown similar "JNK activity" results could be detected in undead discs using *TRE-dsRed* (compare Figure 3.2 C-C" to Figure 2.7 B-B") – a different reporter and an exogenous transgene, simply comprised of AP-1 binding sites upstream of a fluorophore, with no possible effects on the endogenous signaling pathways (Sykiotis & Bohmann, 2008; Fan et al., 2014). This, combined with a previous lab member's determination that there was no obvious difference in adult phenotype between *ey>hid-p35* control and *ey>hid-p35;puc-lacZ* animals, suggested the puckered haplo-insufficiency did not meaningfully increase baseline JNK activity within an AiP model and was not a major concern for our experimental design.

However, what we neglected to formally test was the effect of *puckered* haplo-insufficiency on a partially suppressed AiP construct. In *ey>hid-p35; UAS-hCatS*, there is

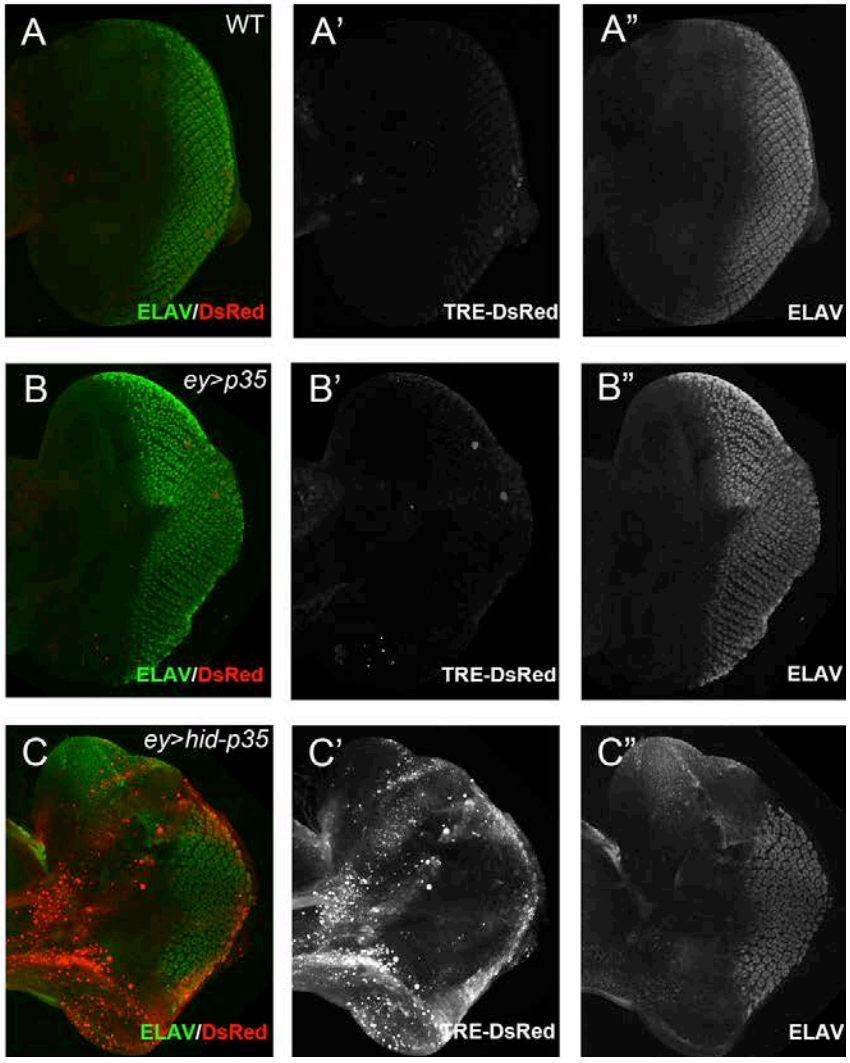

Figure 3.2
An Independent Exogenous Reporter shows JNK activity in AiP

Figure 3.2: An Independent Exogenous Reporter shows JNK activity in AiP

The JNK activity reporter construct *TRE-dsRed* is present in all cells but is only induced in 'undead' *ey>hid-p35* cells. Discs are labeled with dsRed (JNK marker, red in A–C; gray in A′–C′) and ELAV (photoreceptor neurons, green in A–C; gray in A″–C″).

(A) Wild-type (wt) animals show no reporter activity, normal disc morphology. N=2, n=4

(B) *ey>p35* control animals show little reporter activity, mild disruption to maturing posterior eye disc patterning (ELAV) but overall normal disc morphology. N=2, n=6

(C) *ey>hid-p35* eye imaginal discs exhibit strong expression of the ds-Red reporter, indicating high JNK activity acting on downstream AP-1 sites, primarily in the overgrown anterior eye disc. Compare to puc-lacZ activity in Figure 2.7 C. N=3, n=15

Reproduced from:

Fan, Y., Wang, S., Hernandez, J., Yenigun, V. B., Hertlein, G., Fogarty, C. E., Lindblad, J. L., 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 Genetics. 10 (1) doi: 10.1371/journal.pgen.1004131

presumably active Hid, active Dronc, active ROS production, and then sufficient catalase activity to reduce the ROS, dampening any JNK-stimulating signal and preventing AiP. However, if a JNK negative regulator is removed from this condition, we have active Hid, active Dronc, active ROS production, active reduction of most ROS, but perhaps enough ROS persisting to trigger activation of a sensitized JNK. Then, in the context of the feedback loop, this mildly-stimulated but sensitized JNK could sufficiently upregulate endogenous Hid, which can lead to more ROS, strengthening of the JNK-activating stimulus, driving the AiP cascade over a threshold to proliferation.

While we could employ the *TRE-dsRed* reporter to repeat any experiments which relied on *puc-lacZ,* more pressingly, we must clearly determine whether ROS are exclusively upstream of JNK, whether they contribute to the feedback loop, or whether some artifact has led to an erroneous interpretation of the epistasis involved. The most direct way will be to isolate each segment of the loop, similar to the experiments of Shlevkov and Morata (2012). Fortunately, there are solidly established tools for probing these interactions. First, if ROS are truly downstream of Dronc and upstream of JNK, then mis-expression of *pro-Dronc* in a *hid* null background should lead to the production of extracellular ROS (suppressible by $Duox^{RNAi}$ or catalase), and the activation of JNK (suppressible by $Grnd^{RNAi}$). Additionally, mis-expression of hep^{ACT} in a *hid* null background should activate JNK, but not produce extracellular ROS (unaffected by $Duox^{RNAi}$, catalase, or $Grnd^{RNAi}$). However, it is possible that ROS is also produced downstream of JNK activity, as has been previously reported in other systems (Sakurai et

al., 2008; Kanda et al., 2011). In this case, in the *hep*^{ACT} condition, JNK may produce ROS that may or may not be suppressible by extracellular catalase expression.

Yet another layer of complexity is added when we consider the role of Grindelwald in this feedback loop model. The newly reported TNF receptor integrates both extracellular stimuli (from the TNF, Eiger) as well as autonomous signals (from the apical polarity complex) (Andersen et al., 2015). While we do show a requirement for Eiger in AiP, it is not completely resolved which of these input pathways is important for Dronc-dependent AiP. Andersen et al., demonstrated that in a *Ras*^{V12}*/scrib*$^{-/-}$ tumor model, Grindelwald facilitates invasive behavior through Egr-dependent *Matrix metalloprotease-1* (*Mmp1*) expression. We see some production of MMP1 in undead eye discs, but I found that inhibiting MMP1 activity via mis-expression of *UAS-TIMP* (tissue inhibitor of metalloproteases) in *ey>hid-p35* animals does not restore wild-type phenotypes (0% WT, 18% viability), suggesting this specific function of Grindelwald is not responsible for AiP. Andersen et al., also showed that mis-expression of a form of Grindelwald possessing only the intracellular domain is sufficient to activate JNK and induce apoptosis. However, in our system activation of JNK leads to proliferation. These seemingly contradictory findings may suggest an important role for opposing modulatory domains or even yet to be identified accessory proteins.

Alternatively, there may be successive cycles of AiP signals, with different subsets each cycle activating JNK towards apoptosis or proliferation. Assuming ROS and hemocytes are upstream of extracellular stimulation of Grindelwald, I hypothesized that reduction of Grindelwald or JNK by RNAi should suppress proliferation, but should not

suppress “upstream” ROS production or changes in hemocyte morphology and distribution. However, preliminary observational results showed knockdown of Grindelwald or JNK was associated with loss of ROS signal and with hemocytes having a distribution and morphology resembling control (Figure 3.3 and Lindblad, Bergmann Lab, unpublished). This suggests Grindelwald signaling is required for the detectable ROS in undead discs, perhaps placing ROS downstream of the feedback loop’s secondary stimulus from endogenous *hid,* as opposed to our primary stimulus from exogenous *UAS-hid.* A proposed model accounting for ROS/JNK/*hid* feedback is presented in Figure 3.3.

More confounders: A limited sampling of reactive species

Finally, three of the largest limitations of our current studies could be masking even greater complexity within the signaling cascades driving AiP. First, we limited our investigations to reactive oxygen; we have not yet even begun to explore the possible role of other reactive small molecules such as nitric oxide and reactive nitrogen species, which have both recently been highlighted as more critical to cellular signaling than previously thought (Bonafe, Guarnieri, & Muscari, 2015; Weidinger & Kozlov, 2015).

Second, and perhaps more importantly, our experimental design assumed that all ROS in our system are acting in a common pathway generating a common response; but we know from other published studies that ROS can acts as a chemoattractant or as a direct redox activator of signal pathways depending on the ROS identity, location, and concentration (Tobiume et al., 2002; Niethammer et al., 2009; Evans et al., 2015). In our initial studies, I used multiple ROS-detecting reagents. DHE specifically detects

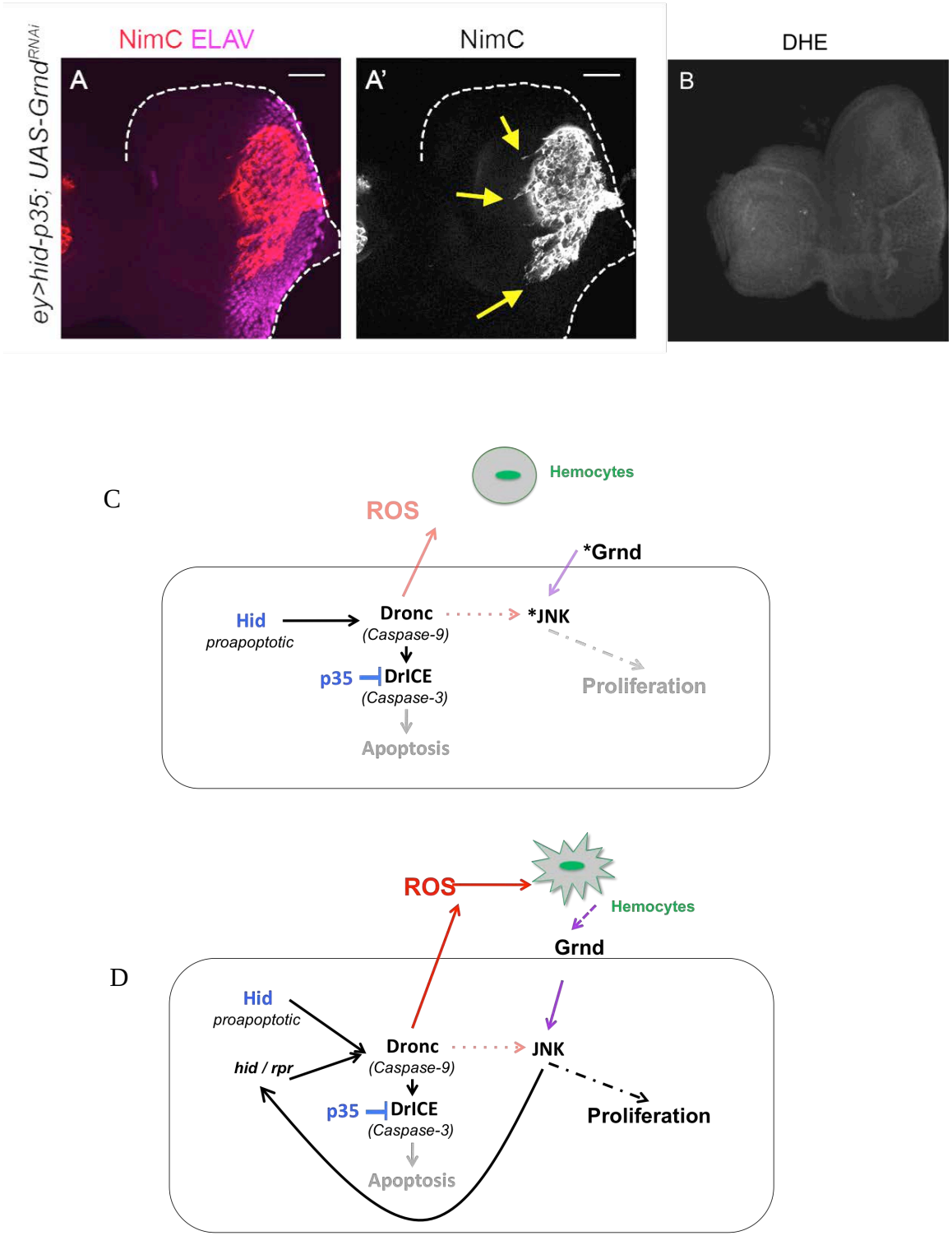

Figure 3.3
A Proposed Feedback Loop through ROS, Grindelwald, and JNK

Figure 3.3: A Proposed Feedback Loop through ROS, Grindelwald, and JNK.

(A, A') In *ey>hid- p35; Grnd*^{RNAi} eye discs, hemocytes exhibit a mostly control type distribution and morphology. However, occasional projections from the large central cluster were observed on select discs. Hemocytes are marked with anti-NimC (red, gray), with ELAV highlighting the normalized photoreceptor field (purple). N=1, n =5

(B) ROS production is limited in *ey>hid- p35; Grnd*^{RNAi} eye discs, as marked by DHE (gray). N=1, n=3

(C) Without an intact feedback loop, for example if Grnd or JNK are inactivated, ectopic *hid* expression is not sufficient to sustain Dronc activation and establish a stable ROS gradient. Any transient ROS would dissipate; hemocytes would then remain in a quiescent state.

(D) With an intact feedback loop including Grnd and JNK, ectopic *hid* expression activates Dronc, which activates JNK-mediated feedback on the endogenous *reaper/hid* locus. Here, sustained Dronc activity may cross the threshold to cause the build up of a pool of ROS that can recruit hemocytes and trigger JNK-mediated proliferation.

superoxide, while H_2DCF-DA detects many types of ROS including hydrogen peroxide(Owusu-Ansah et al., 2008). Both reagents yielded a positive signal during AiP that could be suppressed in a Dronc mutant state. Due to technical limitations that prevented us from combining the fluorescein based H_2DCF-DA (green) with some of our GFP-labeled (green) mutants, and due to the greater consistency in signal detection with DHE, I elected to use the DHE reagent as a proxy for detecting all ROS in the rest of my experiments. However, we know based on genetic evidence and previously published reports that H_2O_2 is likely the most critical molecule, and we know it is not directly measured by DHE. Therefore, while the ROS detected by DHE in my experiments are real, they may not truly represent the initial driving force behind the AiP phenotype. Specifically, the loss of DHE signal seen in Figure 3.3 B above does not provide any information as to the level of H_2O_2 produced. It is possible that Dronc induces H_2O_2 upstream of JNK, which then as part of the feedback loop induces superoxide as detected by DHE. When JNK signaling is blocked, only the later would be suppressed. Additional testing with H_2O_2 specific detection reagents will help clarify this point.

Third and finally, based on the results of our genetic screen we concluded that extracellular ROS were the critical signaling molecule in AiP. We showed a requirement for the enzyme Duox, which produces extracellular species, and we only saw suppression of AiP when expressing extracellular reducing enzymes but not intracellular enzymes such as superoxide dismutase and catalase. However, this again is a simplified view of potential sources of ROS and redox control. The major intracellular anti-oxidant enzymes present in *Drosophila* include superoxide dismutase, catalase, and thioredoxin reductase

(Missirlis, Phillips, & Jackle, 2001). Superoxide dismutase exists as both SOD1 and SOD2; the cytosolic and mitochondrial forms respectively (Y. M. Lee, Misra, & Ayala, 1981)). We only utilized a *UAS-SOD* construct that expresses in the cytosol (Watson et al., 2008), but did not test for ROS in the mitochondria. For Catalase, there is only one major gene in *Drosophila* (except for a second testes specific catalase), but the enzymatic activity of this endogenous catalase can be found both freely in the cytosol as well as in its traditional subcellular localization of the peroxisomes (Beard & Holtzman, 1987). The *UAS-Catalase* construct has commonly been used to generally influence the redox state of the intracellular cytoplasmic compartment (Missirlis et al., 2001), but we did not specifically quantify whether our construct was primarily expressing in one compartment or the other. We did not test expression of thioredoxin reductase in our system at all.

On the other side of the cellular membrane, we used two different constructs encoding extracellular reducing enzymes, *UAS-IRC* and *UAS-hCatS, (Ha et al., 2005),* to test the requirement of extracellular ROS. While we did see an effect with over-expression of the *Drosophila* enzyme IRC, a catalase typically only expressed by the fat body, we saw a more dramatic suppression of AiP with expression of the human transgenic secreted catalase. It could be that the redox signaling in AiP is so highly regulated that over-expression of native *Drosophila* reductases is not sufficient to overcome the complex regulatory network, but by introducing a completely novel reductase into the system without endogenous regulation we artificially enhanced the suppression. Perhaps a human cytosolic catalase transgene will suppress AiP just as well.

Therefore, a crucial next step will be to systematically, and precisely, tease apart the exact nature of the reactive species, the subcellular or extracellular compartments, and regulators of redox signaling both in and out of the epithelial cell during AiP. This should start with the generation of a positive control for our experiments. Specifically, in the ey>p35 control, we should induce ROS from various cellular locations, by applying exogenous hydrogen peroxide, known free radical generators such as paraquat or arsenic, chemical inhibitors of the redox enzymes, or through the targeted genetic disruption of mitochondrial oxidative pathways. We would use our ROS detecting reagents to confirm the production of ROS, and then characterize disc morphology and adult phenotype to establish if and from where ROS are sufficient to induce AiP. Then we could more directly test the UAS-driven reductases to first confirm their functionality as cytosolic reductases, and then to better test whether intracellular ROS play any role in driving AiP. We could also explore the use of other existing mutants and transgenic constructs related to redox biology such as reductases targeted to cellular compartments where they are typically absent (Kwong et al., 2000).

FUTURE DIRECTIONS

Further definition of the role of hemocytes in AiP

Based on results presented in Chapter II, I propose that hemocytes contribute to AiP downstream of ROS, but upstream of JNK. However, one of the most significant questions that remains unclear is: what are the mechanisms which directly connect hemocytes with the upstream and downstream signals in AiP? This can be generally

broken into three smaller, though still quite open, questions – 1) How are hemocytes recruited and ‘activated’ by epithelially derived ROS?, 2) What signals are produced by hemocytes to drive proliferation?, and 3) Do hemocytes also produce signals that may inhibit proliferation?

We attempted to answer the first of these questions indirectly by reducing hemocyte functionality with a mutant of the phagocytic receptor and damage cue sensor, *draper* (Manaka et al., 2004; Doherty et al., 2014; Fullard & Baker, 2014). Recent work has shown *draper* to be critical in regulating the migratory response of hemocytes to damage sites based on an extracellular ROS signal (Evans et al., 2015). Whole-animal heterozygous-loss of *draper* dramatically suppressed ey>hid-p35 overgrowth. However, this is complicated by the fact that Draper itself can be upstream of JNK in non-professional engulfing cells including epithelial cells (Etchegaray et al., 2012). . Reducing Draper in the whole animal may have reduced overall JNK activity independent of its role in hemocytes. Therefore, as stated above, the most important next step in probing the precise relationships in AiP is to establish a second binary transcriptional system in the sustained AiP model. Once established, we could conduct targeted knockdown only in hemocytes of Draper, its downstream adaptors, and targets to tease apart their functions outside the proliferating tissue.

Next, we must determine what growth promoting and growth inhibiting signals are derived from hemocytes in the undead tissue. We have demonstrated a requirement for Egr in AiP, and by immunofluorescence have detected Egr antigens colocalizing with AiP associated hemocytes, which when combined with the epithelial requirement for the

Egr receptor Grindelwald, is highly suggestive of direct AiP-derived Egr signaling. However, previous studies have demonstrated hemocytes are capable of producing several additional cytokines, including the Toll ligand Spätzle, in both invasive tumor models where the hemocytes suppress tumor growth (Parisi et al., 2014), as well as in models where hemocytes promote tumor growth (Cordero et al., 2010). Once the second binary system is established we could specifically evaluate each of the previously identified cytokines for positive or inhibitory roles in AiP.

In addition to looking at tumor models, another approach for identifying signals modulating AiP would be to draw parallels to other ROS mediated immune responses in the fly. Duox is best characterized as a source of bacteriocidal ROS during intestinal infection. However, in this scenario, the ROS can also still attract hemocytes to help fight infection and repair epithelial damage. Interestingly, in this condition, hemocytes are a source of Dpp, which triggers intestinal stem cell proliferation (conference proceedings (Jasper, 2015)). Therefore, we need to test if eye-disc associated hemocytes directly produce any of the established AiP mitogens (Wg, Dpp, Spi). Several studies have shown the presence of ectopic Wg and Dpp in the disc, and under certain genetic conditions demonstrated their requirement for proliferation; however, in other experimental designs, specific loss of these mitogens in dying cells did not affect the proliferative response (Perez-Garijo et al., 2005; F. A. Martin et al., 2009; Perez-Garijo et al., 2009; Smith-Bolton et al., 2009). If Wg and Dpp are produced by both dying cells and recruited hemocytes, that may help resolve the controversy regarding their requirement.

Can disc-associated hemocytes model tumor-associated macrophages of mammalian systems?

A key component of the apoptotic program is the efficient and controlled clearance of the dying cells by phagocytes, such as macrophages. In addition to the traditional “find me” and “eat me” signals, certain apoptosis-derived signals serve the additional function of modulating the immune response to cell death. For example, shortly following initiation of the apoptotic program, phosphatidylserine (PS) begins to accumulate on the outer leaflet of the dying cell’s membrane (Fadok et al., 1992; S. J. Martin et al., 1995). This exposure is critically required for recognition and engulfment of the dying cell by the phagocyte (Fadok et al., 2001). Effector caspase activation promotes exposure of PS on the outer leaflet through both the inactivation of flippases and the activation of scramblases (S. J. Martin et al., 1996; Bratton et al., 1997; Mandal et al., 2005; Segawa et al., 2014; Suzuki, Imanishi, & Nagata, 2014). Interestingly, PS exposure on the apoptotic cell also dictates what type of immunological response should occur. Upon recognizing the apoptotic “eat me” signal, macrophages actively induce production of anti-inflammatory cytokines including TGFβ and prostaglandins[5], and suppress production of typical pro-inflammatory cytokines such as TNFα and IL-1β (Fadok et al., 1998). These immunosuppressive programs are dependent on the activation of the PS receptor on the engulfing cell, and they prevent the further recruitment of other,

[5] Hopefully, it has not escaped your notice these are the some of the same cytokines that can be produced by dying cells during AiP. It may be interesting to investigate in the future whether the controversy regarding requirement and source of mitogen production in AiP can be explained by a dual production model, with Dpp produced in both the JNK active epithelium as well as the overlying hemocytes.

potentially more damaging, immune cells such as neutrophils (Fadok et al., 2000; Huynh, Fadok, & Henson, 2002).

These anti-inflammatory programs are important for wound healing and tissue remodeling; however, they can also allow for developing tumors to evade immunological suppression. When tissue associated macrophages turn on anti-inflammatory programs, they are alternatively activated (M2 polarization) (D. O. Adams & Hamilton, 1984; Stein et al., 1992; Mills et al., 2000). When this happens in the context of a solid tumor, they are considered to be alternatively activated Tumor Associated Macrophages (TAMs). Higher numbers of TAMs in cancer are associated with a worse prognosis (Bingle, Brown, & Lewis, 2002). Current research in the field of TAMs aims to understand the mechanisms that regulate M1 versus M2 polarization, and whether it is possible to redirect TAMs to execute anti-tumor functions or selectively remove tumor promoting TAMs (Biswas et al., 2013).

Based on the requirement for proliferation-inducing hemocytes in our hyperplastic model of AiP, and based on the evidence the *Drosophila* hemocytes can be either tumor suppressing or tumor promoting, the undead model may be a genetically tractable system to help us better understand the interaction of factors and contexts that determine immune cell response to cancer. Again, this will be best accomplished with the establishment of alternative binary expression systems (i.e. hemocyte specific – *lexA*); but in the interim, I encountered a curious finding.

As described earlier, in the undead model *eyeless-Gal4* drives expression of *UAS-hid* and *UAS-p35* in the developing eye tissue. I considered that adding in a second,

hemocyte-specific *Gal4* might either kill hemocytes or reduce hemocyte functionality, incapable of participating in AiP. Addition of an *hmlΔ-Gal4* had no effect on the *ey>hid-p35* phenotype (11.5% WT, 27.5% Viability), but surprisingly addition of *crq-Gal4* did suppress the frequency of overgrowth of *ey>hid-p35* animals (75.0% WT, 41.3% Viability). In both cases, circulating hemocyte numbers appear slightly reduced compared to *ey>hid-p35* alone, though there were still a majority of hemocytes present (~ 20% reduction, p=0.07 for n=5 of each genotype). However, a difference between these two hemocyte specific drivers is that *crq-Gal4* begins driving expression in early embryonic hemocytes, the blood cells that make up the tissue resident hemocyte populations in the larva, whereas *hmlΔ-Gal4* is limited in embryonic hemocytes and instead strongly expresses in the circulating larval hemocyte and lymph gland populations (Franc et al., 1996; Goto, Kadowaki, & Kitagawa, 2003; Sinenko & Mathey-Prevot, 2004; Olofsson & Page, 2005).

Therefore, it is worth considering the possibility that there are subsets of plasmatocytes, uniquely primed by their lineage or trophic support, which may contribute to AiP. For example, the sessile hemocytes that form hematopoietic pockets along the body wall receive trophic signals from sensory neurons, input that is critical for priming these cells for appropriate immune responses in the adult (Makhijani et al., 2011).

A next step would be to determine if there is anything unique about the disc-associated hemocytes that distinguish them from the general circulating population. If sub-populations do exist, the undead model may be an excellent tool to investigate the conflicting roles of hemocytes in tumor promotion (Cordero et al., 2010; Kelsey et al.,

2012) versus tumor suppression in *Drosophila* (Pastor-Pareja et al., 2008; Hauling et al., 2014; Parisi et al., 2014), with implications for TAMs and mammalian tumor progression.

The local versus systemic damage response: How might distant signals contribute to AiP?

One of the most frustrating aspects of the sustained model of AiP is the dramatic variability in phenotype severity. The degree of head capsule overgrowth, the degree of eye size reduction, and especially the structural integrity of larval eye imaginal discs greatly depends on a number of environmental factors, including: subtle shifts in temperature, crowding of the food, and excessive yeast in the food. Preliminary results even indicate that concurrent bacterial infection with *Serratia* spp. during larval development is correlated with a suppression of the adult AiP phenotype (Amcheslavsky, Bergmann Lab, unpublished). This variability, and sensitivity to environmental factors strongly suggests AiP is not purely a local, autonomous process. In a regenerative model of AiP in *Drosophila*, Herrera et al. (2013) found that localized death induction leads to an early, widespread proliferative response, which after 48 hrs subsides to a limited local response. Systemic factors may mediate this early, generalized response while local factors dominate once cell death ceases and regeneration is established. Hemocytes, fat body, and soluble circulating enzymes in the hemolymph are all capable of contributing to systemic responses against infection and even tumor growth (Nam et al., 2012; W. J. Lee & Miura, 2014; Parisi et al., 2014). Future work in the sustained AiP model,

especially under independent binary control, will likely reveal a complex and interconnected signaling network that promotes coordinated systemic and local responses, which are integrated into a final variable phenotype of AiP.

IMPLICATIONS OF AIP

How might regenerative pathways be misused in cancer?

In the regenerative and sustained AiP models, we induce cell death essentially through a genetic wound to the tissue. We can view these two models then as tools to probe the dynamic interactions and intercellular signaling that occur in the acute versus chronic wound microenvironment. As chronic wound healing can be associated with substantial disease including cancer (Wu et al., 2014), emerging studies are investigating the ways in which physiological regenerative pathways such as AiP may be hijacked in malignant transformation and growth.

Li et al., (2010) first characterized the regenerative AiP pathway “Phoenix Rising” through *in vitro* co-culture assays, designed to test the effect of a large number of irradiated dying cells on a smaller population of fluorescently labeled non-irradiated surviving cells. Excitingly, in these assays, dying cells stimulated proliferation of the fluorescently labeled cells via Caspase-3 dependent activation of calcium-insensitive phospholipase A_2 ($iPLA_2$). This enzyme had previously been identified as an effector caspase cleavage target important for the release of phospholipid derived “find me” signals (Atsumi et al., 1998; Lauber et al., 2003), but in this model of regeneration, the

important signal produced by $iPLA_2$ is prostaglandin E_2. PGE_2 is known to promote stem and progenitor cell proliferation (Hagedorn et al., 2014).

Importantly, the Phoenix Rising pathway can be co-opted in cancer and can contribute to tumor repopulation following radiation and chemotherapy (Huang et al., 2011). These findings were validated in breast cancer, melanoma, and pancreatic ductal adenocarcinoma cell lines (Donato et al., 2014; Cheng et al., 2015; Kurtova et al., 2015). Additionally, this Caspase-3/$iPLA_2$/PGE_2 AiP signaling cascade can be activated in dying tumor cells to promote growth of surviving tumor cells, but also extends to dying vascular endothelial cells that promote tumor growth. One treatment considered for many solid tumors is anti-angiogenic therapy to limit oxygen to the developing tumor mass (Sitohy, Nagy, & Dvorak, 2012). However, if dying vascular endothelial cells are also capable of activating the Caspase-3/$iPLA_2$/PGE_2 AiP cascade, such as in one study of glioma, also targeting this AiP pathway may be critically important for successful therapy (Mao et al., 2013). Continued work in other cancer cell lines (such as the Panc1 line derived from pancreatic ductal carcinoma), has revealed other cascades that also trigger AiP. There is a role for Caspase 7-PKCδ-Akt/p38 MAPK stimulated mitogen production (Cheng et al., 2015), as well as evidence of Caspase-3/Sonic Hedgehog (sHH) modulating AiP in a paracrine manner (Ma et al., 2013; Ma et al., 2015).

These Phoenix Rising studies focus on the mechanisms by which established tumors maintain bulk and repopulate following chemotherapy. A separate area to consider would be the role of AiP in tumor initiation. In mammalian systems, chronic inflammatory states, often characterized by repeated cycles of cell death and wound

healing, can lead to the development of fibrotic disease or certain cancers. For example, in an injury-based model of liver carcinogenesis in mice, persistent cell death of hepatocytes drives a Kupffer cell-mediated, EGFR-dependent compensatory proliferation and eventual transformation (Lanaya et al., 2014). Decreasing apoptosis in the parenchyma is sufficient to prevent the development of tumors, both in number and size (Wree et al., 2015). In comparison, in the case of acute kidney injury, apoptotic cells induce compensatory proliferation of the renal tubular epithelium via release of ATP (Nakagawa et al., 2014). However, re-entry into the cell cycle increases the susceptibility of these renal tubular epithelial cells to any remaining toxic insults, creating potential amplification of injury and compensatory proliferation through "proliferation-associated death" (B. C. Chen et al., 2014). Yet, here chronic injury progresses to tubulointerstitial fibrosis, a debilitating chronic kidney disease, but not to cancer. Better understanding of the differences between these two AiP-induced hyperproliferative diseases may allow us to better prevent either disease from progressing.

The findings in mice and human cell lines, taken with the *in vivo* work in *Drosophila*, would suggest that AiP is likely a commonly used mechanism to promote regeneration, whether normal or cancerous. Historically, cancer has been called a wound that will not heal (Dvorak, 1986). It appears probable that some of the very pathways designed to promote wound healing, are the ones promoting tumor growth. A goal I would have for continued work in *Drosophila* models of AiP, would be the establishment of an intermediate AiP condition that allowed exploration of the sequential steps between acute regeneration and sustained hyperplasia, and then a transforming AiP condition that

allowed similar exploration of the steps between hyperplasia and dysplasia. Most notably, even with the diverse signals already catalogued, it is very likely that we have only begun to uncover the many paths to AiP, which will vary based on the dying cell, the surviving cell, the developmental context, and the surrounding microenvironment.

CONCLUSIONS

In this dissertation, I have detailed the important role of initiator caspase-induced production of reactive oxygen species for the activation of JNK during AiP, and for the recruitment of potentially tumor-promoting hemocytes. As a final note on the significance of this work, it is surprising to me that the initiator caspase-dependent mechanism of AiP has only been substantially investigated in *Drosophila.* Reviews of the current literature in other model organisms only highlight effector caspase-dependent mechanisms of AiP (Bergmann & Steller, 2010; Ryoo & Bergmann, 2012). It is possible that a lack of evidence of initiator caspase-dependent AiP in other model systems derives from *Drosophila* having a uniquely evolved mechanism of Dronc-dependent AiP, a fortuitous back-up for developing cells when Drice-dependent AiP is not sufficient or appropriate. However, I think it more likely that the versatile genetic toolkit including the ability to specifically block effector caspase activity with *p35,* along with the over 100 years of collective understanding of *Drosophila* genetics and development, allowed for a fortuitous discovery of what is likely a conserved mechanism for wound healing and regeneration. Continued work investigating the exact mechanisms of Dronc-dependent AiP in *Drosophila,* including further clarification of the interactions among ROS,

hemocytes, and epithelial JNK, will lead the way to determine which pathways should be evaluated in other model systems.

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