

2020

# Apoptotic cell death promotes an immune evasion phenotype in tumor cells

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**APOPTOTIC CELL DEATH PROMOTES AN IMMUNE EVASION  
PHENOTYPE IN TUMOR CELLS**

by

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B.S., University of Vermont, 2017

Submitted in partial fulfillment of the  
requirements for the degree of  
Master of Science

2020

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## **ACKNOWLEDGMENTS**

I would like to acknowledge my advisor, Dr. Jamie McKnight, as well as my second reader and thesis advisor Dr. Raymond Birge. Additionally, I would like to thank my significant other, my family and my friends within the MAMS program for their continued support of my academic pursuits.

The information discussed in this thesis is a continuation of the work I had started in collaboration with the Birge Lab at the Rutgers New Jersey Medical School during two consecutive summer undergraduate research fellowships. I am happy to have had this opportunity to elaborate and expand these ideas.

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PHENOTYPE IN TUMOR CELLS**

**CONNOR B. DEVOE**

**ABSTRACT**

Cell death occurs by multiple pathways, though perhaps the most well-studied is apoptosis. Apoptosis describes a regulated, or programmed, form of cell death. While other forms of cell death, such as ischemic necrosis, occur via ATP-independent mechanisms, apoptosis is an ATP-dependent process that is reliant on intracellular enzymes known as “caspases”. Caspase-dependent cell death has been described as “death by a thousand cuts” as these proteases have a vast array of downstream targets which ultimately lead to chromatin condensation, extensive membrane blebbing and the formation of apoptotic bodies which are tagged for engulfment by professional and non-professional phagocytes.

While the machinery for cell death is expressed in all living cells, inhibitory proteins have been shown to exert control over apoptosis, particularly the B-cell lymphoma-2 (Bcl-2) protein and its family members. Bcl-2 itself is anti-apoptotic, alongside others such as Bcl-XL, Mcl-2 and Bcl-w. Others, such as BAK and BAX are pro-apoptotic and considered essential mediators of the mitochondrial outer membrane permeabilization characteristic of intrinsic apoptotic signaling. The highest level of control, however, appears to be exerted by the so-called “BH3-only” proteins, such as

BAD and BID, which disrupt Bcl-2-mediated antagonism of BAK/BAX and directly stimulate BAK/BAX activity. The balance of these proteins was described in the “Rheostat Model”, proposed in the 1990s by Stanley Korsmeyer and colleagues. The idea that oncogenic overexpression of anti-apoptotic proteins (i.e. the BCL-2 family) play an integral role in the survival and growth of tumors is a long-standing and well-supported hypothesis.

Compared to necrotic cell death, apoptosis is considered relatively “silent” to the immune system, as it does not generate a large-scale inflammatory response. Engulfment of apoptotic cells by phagocytic cells is also known as “efferocytosis”, and results in the release of anti-inflammatory cytokines. One molecule integral in mediating efferocytosis is phosphatidylserine (PS), a signaling lipid externalized on the cell membrane in apoptotic cells. PS interacts with PS receptors to mediate resolution of inflammation through triggering the release of anti-inflammatory cytokines and directly preventing pro-inflammatory cytokine release. Physiologically, this is a desirable process. Dysregulation of efferocytosis can lead to chronic inflammatory conditions, such as systemic lupus erythematosus.

Recently, however, evidence has emerged supporting a role of apoptosis in signaling proliferation of neighboring cells in the tumor microenvironment. This stems from an understanding of apoptotic signaling in healthy tissues, such as wound regeneration, and the analogy of tumors as “wounds that do not heal”. Work by Andreas Bergmann and colleagues has demonstrated this apoptosis-induced proliferation (AiP) in *Drosophila* and described how this could translate to higher organisms including

mammals. Cells neighboring a site of injury where apoptosis is occurring in massive amounts may undergo compensatory proliferation. In the tumor microenvironment, where apoptosis is occurring, efferocytosis and AiP may work in concert to suppress an appropriate immune response and promote proliferation of neighboring cells. AiP may be further implicated in tumorigenesis and recurrence after treatment with apoptosis-inducing chemotherapeutics. Sustained compensatory proliferation signaling may provide an explanation for this paradoxical phenomenon.

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## LIST OF ABBREVIATIONS

ADP .....	Adenosine triphosphate
AI .....	Apoptotic Index
AiP .....	Apoptotic-induced Proliferation
Apaf-1 .....	Apoptotic protease-activating factor 1
ATG .....	Autophagy-related protein
Bcl-2.....	B-cell Lymphoma 2
BH.....	Bcl-2 Homology
BU.....	Boston University
CAMs .....	Cancer-associated macrophages
Cdk5 .....	Cyclin-dependent kinase 5
CGD .....	Chronic granulomatous disease
COPD .....	Chronic obstructive pulmonary disease
CTLs .....	Cytotoxic T Lymphocytes
DAI .....	DNA-dependent activator of IRFs
DISC .....	Death-inducing signaling complex
DMT1.....	Divalent Metal Transporter 1
FADD.....	Fas associated via death receptor
GSH .....	Reduced Glutathione
Hh.....	Hedgehog mitogen
HOP .....	HSP70-90 Organizing Protein
ICAD.....	Inhibitor of Caspase-Activated Deoxyribonuclease

ICAM-1 .....	Intracellular adhesion molecule-1
ICE .....	IL-1B Converting Enzyme
IFN- $\beta$ .....	Interferon beta
IKK $\beta$ .....	Inhibitor of NF- $\kappa$ B kinase $\beta$
IRF .....	Interferon Regulatory Factor
ITIMs .....	Immunoreceptor Tyrosine-based Inhibitory Motifs
JNK.....	c-Jun N-terminal kinase
LAMP2A .....	Lysosome-associated membrane protein type 2A
LPS.....	Lipopolysaccharide
Mbc .....	Myoblast city
M-CSF .....	Macrophage colony-stimulating factor
MLKL.....	Mixed lineage kinase domain-like pseudokinase
MOMP .....	Mitochondrial outer membrane permeabilization
MPO .....	Myeloperoxidase
MPT .....	Mitochondrial permeability transition
NADPH.....	Nicotinamide adenine dinucleotide phosphate
NETs .....	Neutrophil extracellular traps
NF- $\kappa$ B .....	Nuclear factor kappa B
NK.....	Natural Killer cells
NPCs .....	Neuroprecursor cells
NSCLC .....	Non-small cell lung cancer
PAMPs .....	Pathogen-associated molecular patterns

PCNA .....	Proliferating cell nuclear antigen
PGE2 .....	Prostaglandin E2
PI3K .....	Phosphoinositide 3-kinase
PRRs .....	Pattern Recognition Receptors
PS .....	Phosphatidylserine
PSR .....	PS receptor
PUFAs .....	Polyunsaturated Fatty Acids
RHIMs.....	RIP Homotypic Interaction Motifs
RIP1,3 .....	Receptor-Interacting Proteins 1 & 3
ROS.....	Reactive Oxygen Species
SLE .....	Systemic Lupus Erythematosus
SREC .....	Scavenger receptor from endothelial cells
STAT.....	Signal Transducer and Activator of Transcription
TAMs .....	Tyro3, Axl and Mer receptor family
TLR.....	Toll-like receptor
TNF.....	Tumor Necrosis Factor
TNFR1 .....	Tumor Necrosis Factor Receptor 1
TRADD.....	TNFR-associated domain
TSP-1 .....	Thrombospondin-1
TUNEL .....	Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling
ULK1 .....	unc-51-like kinase 1
Upd.....	Unpaired, <i>Drosophila</i> Interleukin-6 analog

VEGF ..... Vascular endothelial growth factor

## INTRODUCTION

Cell death is an inevitable and necessary component of the cell's life cycle. An active area of research in many scientific disciplines, cell death has been shown to occur via a vast array of intra- and extracellular mechanisms. Depending on the type of death a cell is undergoing, cell-to-cell signaling and engulfment by phagocytic cells generates a plethora of consequences for cells immediately surrounding the dying cell, and perhaps even globally throughout the body. However, these consequences are not necessarily pathologic. Many common forms of cell death are highly regulated processes which work in concert with proliferation to homeostatically regulate the cellular environment. One such form of regulated cell death is apoptosis, which will be discussed at length in this thesis. Apoptosis differs from other forms of cell death in that it suppresses a larger immune response from happening; this is as if to say, "I am dying, but everything is OK. I am being cleared for the benefit of the cells around me."

Cancer is a disease with unquestionable global burden; in 2020, an estimated 16.9 million Americans have a history of cancer, with a projected 1.8 million new cases set to emerge in the coming year<sup>1</sup>. With over 600,000 cancer-related deaths projected for 2020, cancer remains the 2nd leading cause of death worldwide. Additionally, medical costs of cancer and cancer-related complications in 2015 were \$80.2 billion. Cancer as a disease is defined as an imbalance of cell proliferation relative to cell death. One theory suggests that dysregulation of regulated cell death (i.e. apoptosis) could lead to this unchecked proliferation and tumor formation, or "tumorigenesis"<sup>2</sup>. However, a deeper exploration of the mechanisms of cell death reveals a more sinister theory: apoptosis of tumor cells,

through suppression of an appropriate immune response, could be directly promoting neighboring tumor cell proliferation.

This thesis aims to explore the underlying mechanisms of cellular death, particularly apoptosis, as they relate to neighboring cell proliferation and tumorigenesis. In doing so, this thesis will propose an explanation for the seemingly paradoxical phenomenon of cell death driving new life, particularly of cancer cells.

## **Specific Aims**

The specific aims of this thesis are:

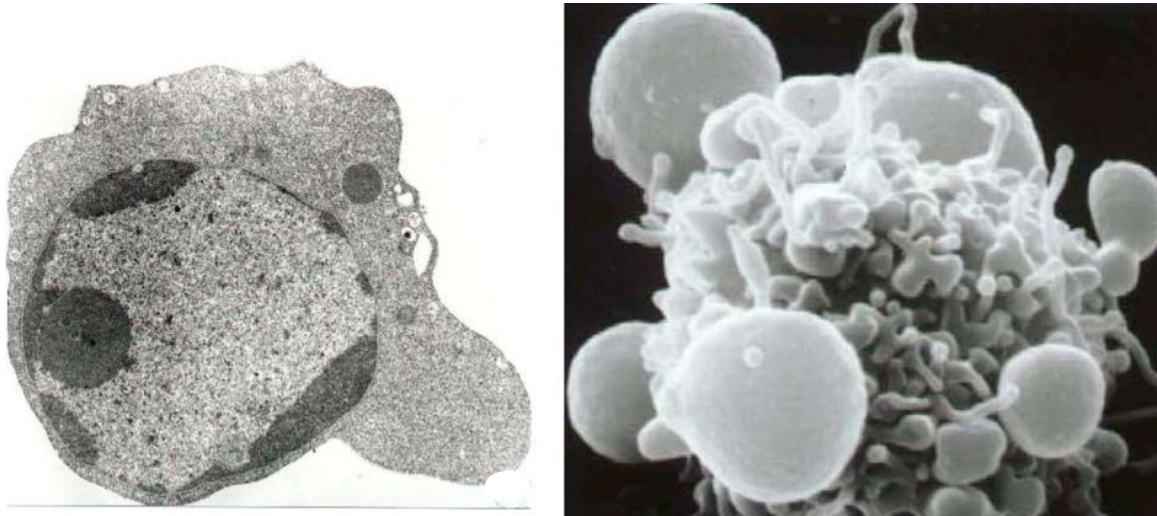
1. Exploring current models, including cellular, animal and clinical, for evaluating apoptosis-driven immune evasion in tumors.
2. Investigating potential correlation of apoptotic index with clinical outcomes and assessing any predictive ability of apoptotic index on clinical outcomes.

## PUBLISHED STUDIES

### 1. Apoptosis

#### 1.1 Characteristics of Apoptosis

Various mechanisms of cell death have been described, and the origins of cellular death have important and widespread consequences. Programmed cell death is a term initially coined by Richard Lockshin in early work characterizing silkworm development<sup>3</sup>. This study, as well as many others that followed, helped to explain predictable periods of cell death and their role in development. For example, one study indicates that triiodothyronine causes hormone-induced tail regression in tadpoles following upregulation of RNA and protein synthesis<sup>4</sup>. However, this tail regression can be suspended and led to survival of tails *in vitro* when RNA synthesis inhibitors were applied. Other examples of programmed cell death include apoptosis, a term initially coined in 1972 by Kerr, Wyllie and Currie, describes morphologically distinct form of programmed cell death which works synergistically with mitosis to homeostatically regulate cell proliferation<sup>5</sup>. Apoptotic cells undergo histologically organized processes prior to deletion. Pyknosis, or condensation and clumping of nuclear chromatin, and cell shrinking are two hallmarks of apoptotic cells<sup>6</sup>. Extensive blebbing and separation into fragments called “apoptotic bodies” enables phagocytosis via phagocytes; specifically “tingible body” macrophages, which contain nuclear debris from the engulfed apoptotic bodies<sup>7</sup>. These morphological changes observed in apoptotic body formation help illustrate the underlying molecular mechanisms of apoptosis (**Figure 1**).



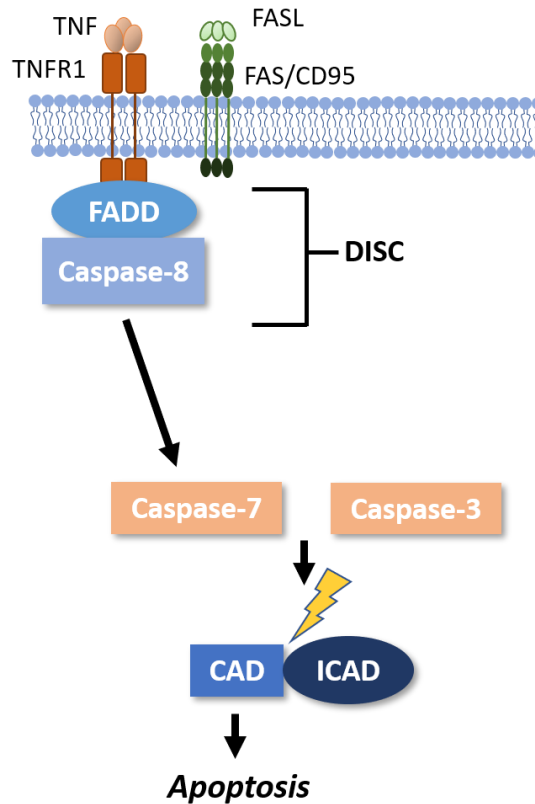
**Figure 1. Morphological Features of Apoptosis** Left) Transmission electron micrograph depicting hallmark morphological signs of apoptotic cells including membrane blebbing and chromatin condensation; Right) Scanning electron micrograph showing a cell with hallmark signs of apoptosis. *Images obtained from Purdue Cytometry CD-ROM Series, Vol. 4* <sup>8</sup>.

## 1.2 Mechanisms of Apoptosis

Since the initial characterization of apoptosis as a programmed form of cell death, three key pathways have been outlined. All three pathways appear to rely heavily on the family of aspartate-specific cysteine proteases, or caspases. The first caspase identified in the programmed cell death pathway was *Ced-3*, which was identified in the nematode *C. elegans*<sup>9</sup>. Through analysis of the genetic and protein structures of this molecule, Yuan et al. identified that it functioned similarly to the mammalian IL-1 $\beta$  converting enzyme (ICE) protein. The proposed role of this particular caspase (caspase 1, in humans) was in the proteolytic cleavage of inactive precursors to generate the mature form of the cytokine IL-1 $\beta$ <sup>10</sup>. Since then, other non-apoptotic roles for caspases have been proposed, such as immune system function, cell proliferation and cell structure remodeling<sup>11-13</sup>.

The first of three apoptotic pathways deemed the “extrinsic” or “death receptor” pathway, occurs through ligation of death receptors on the cell’s surface. These death receptors are members of the tumor necrosis factor (TNF) superfamily, and in addition to the primary ligand and its receptor, TNF- $\alpha$ /TNFR1, others such as the Fas ligand, FasL/FasR (CD95), can stimulate this pathway. Upon binding of the ligand, the proteins Fas associated via death receptor (FADD) forms a complex with caspase-8 (formerly known as FLICE). It should be noted that for the extrinsic pathway to occur, the death-inducing signaling complex (DISC) must first be formed and always includes FADD and caspase-8<sup>14</sup>. In this case, caspase-8 is acting as the “initiator” caspase, which cleaves the “effector” caspase-3 and -7. In fact, caspase-8 has been demonstrated as a “molecular switch” which regulates not only apoptosis via this pathway but is also involved in necroptosis and pyroptosis<sup>15</sup>. The “effector” or “executioner” caspase-3 and -7 have been shown to cause apoptosis by activation of the endonuclease CAD. In dividing cells, CAD is bound to its inhibitor ICAD, but the activated caspase-3 cleaves ICAD, freeing CAD to degrade chromosomal DNA (**Figure 2**) leading to the hallmark chromatin condensation observed by Kerr et al.<sup>5,16</sup>. It should be noted that there are a vast number of caspase substrates, leading to the phrase “death by a thousand cuts” often applied to caspase-mediated cell death. While the exact number and mechanism of caspase-substrates

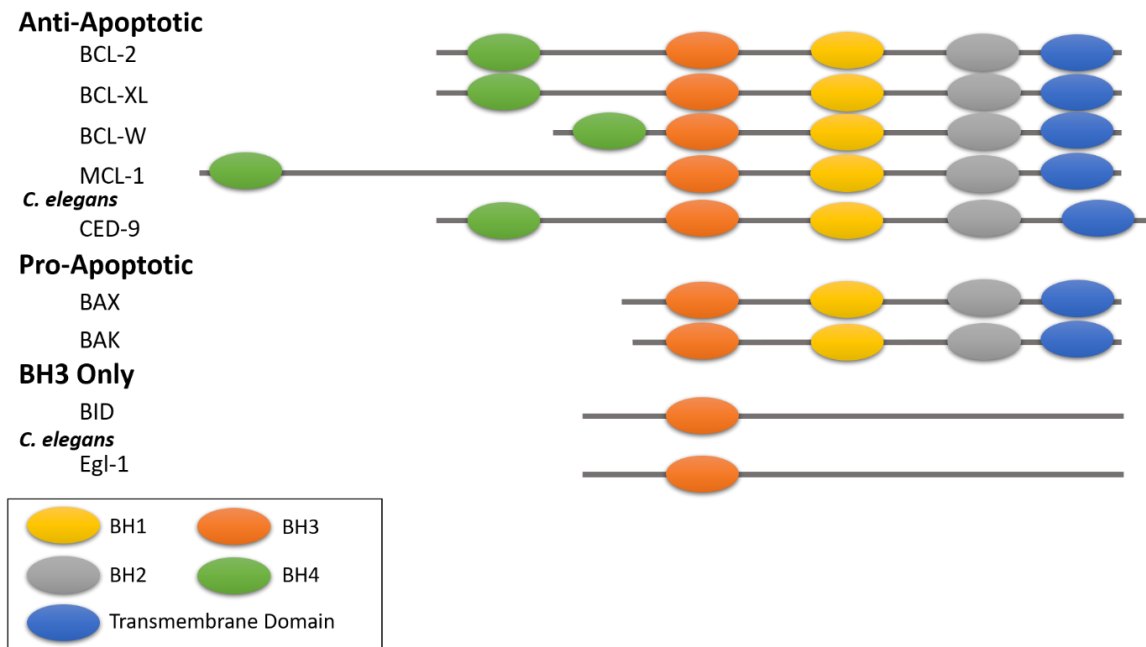
interactions is an active area of study, ICAD is integral to the chromatin condensation phenomenon.



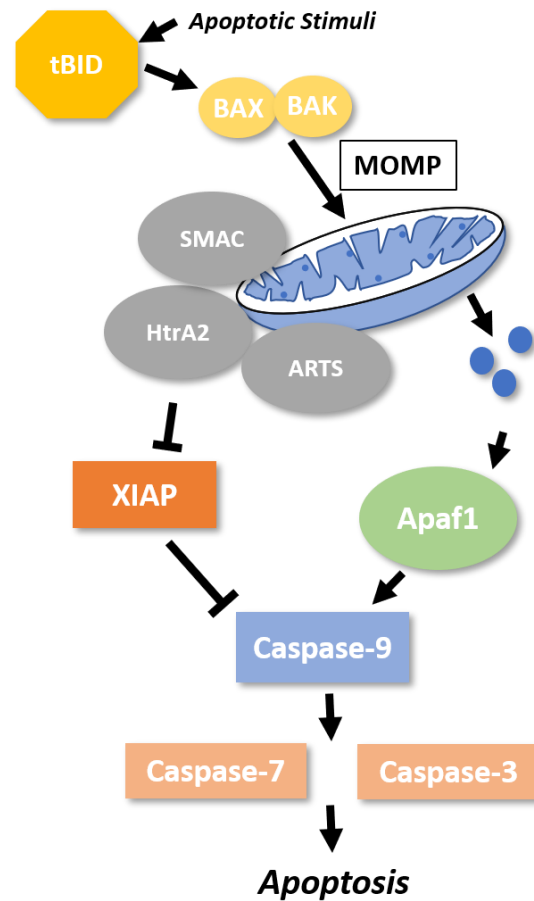
**Figure 2. Extrinsic Apoptosis.**

The second pathway is known as the “intrinsic”, “classical” or “mitochondrial” pathway and serves as the major route to cellular apoptosis. This pathway is managed by a checkpoint of pro-apoptotic and anti-apoptotic signaling factors. The anti-apoptotic factors are members of the B-cell lymphoma 2 (Bcl-2) protein family, specifically Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1<sup>17</sup>. The pro-apoptotic factors, BAK and BAX are considered essential mediators of the intrinsic pathway and are regulated by the anti-apoptotic Bcl-2 proteins. BID, described as a “BH3 domain only” due to its expression of only the BH3 domain homology of the Bcl-2 protein, is involved in overwhelming the anti-apoptotic

signals to lead to cell death<sup>18</sup> (**Figure 3**). Once activated, BID activates BAK and BAX to form oligomerized pores in mitochondria, inducing mitochondrial outer membrane permeabilization (MOMP). These pores in the mitochondrial membrane lead to the efflux of cytochrome c, which can complex with apoptotic protease-activating factor-1 (Apaf-1), a protein with homology to *Ced-4* in *C. elegans*, and caspase-9, forming an “apoptosome”<sup>19,20</sup>. In this intrinsic pathway, caspase-9 serves as the “initiator” caspase, and activation in an ATP-dependent manner triggers cleavage and activation of the effector caspase-3 and -7 (**Figure 4**)



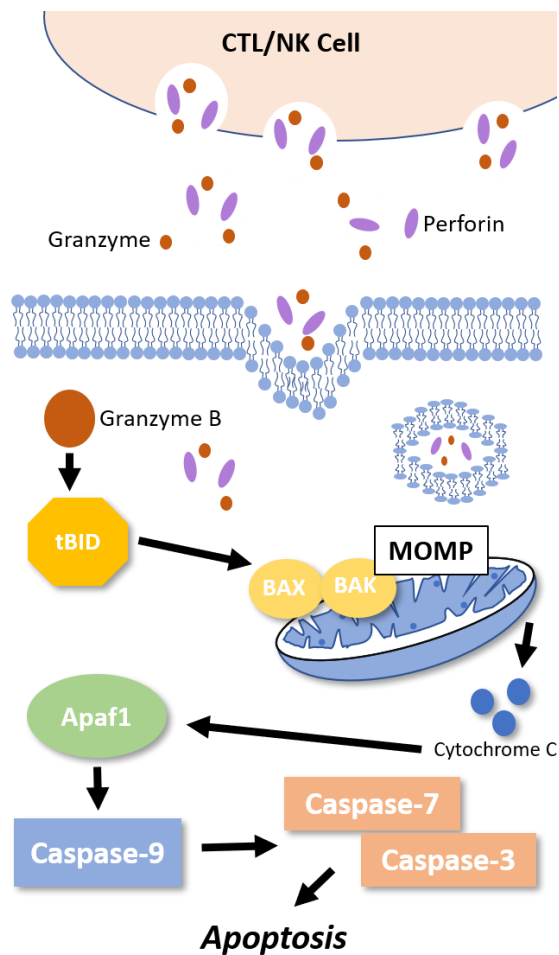
**Figure 3. Comparative Gene Constructs of BCL-2 Family Members.** Modified from Korsmeyer *et al.*, 2000<sup>18</sup>.



**Figure 4. Intrinsic Apoptosis.** Figure includes role of pro-apoptotic mitochondrial proteins SMAC (second mitochondrial-derived activator of caspase)<sup>21</sup>, HtrA2 (High-temperature requirement serine protease A2)<sup>22</sup>, and ARTS (apoptosis-related protein in the TGF-beta signaling pathway)<sup>23</sup> in promoting intrinsic apoptosis through inhibition of XIAP (X-linked inhibitor of apoptosis).

The third apoptotic pathway is regulated by perforins, pore-forming proteins, and serine proteases, termed granzymes, which are both released by granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. CTLs and NKs can also mediate the death receptor, extrinsic pathway of apoptosis, but they are crucial for mediating the aptly named “Perforin/Granzyme Pathway” of cell death. During immune surveillance, these immune cells utilize this pathway for unscheduled apoptosis of cells infected with viruses and other intracellular pathogens, as well as certain forms of cancer (i.e. B cell

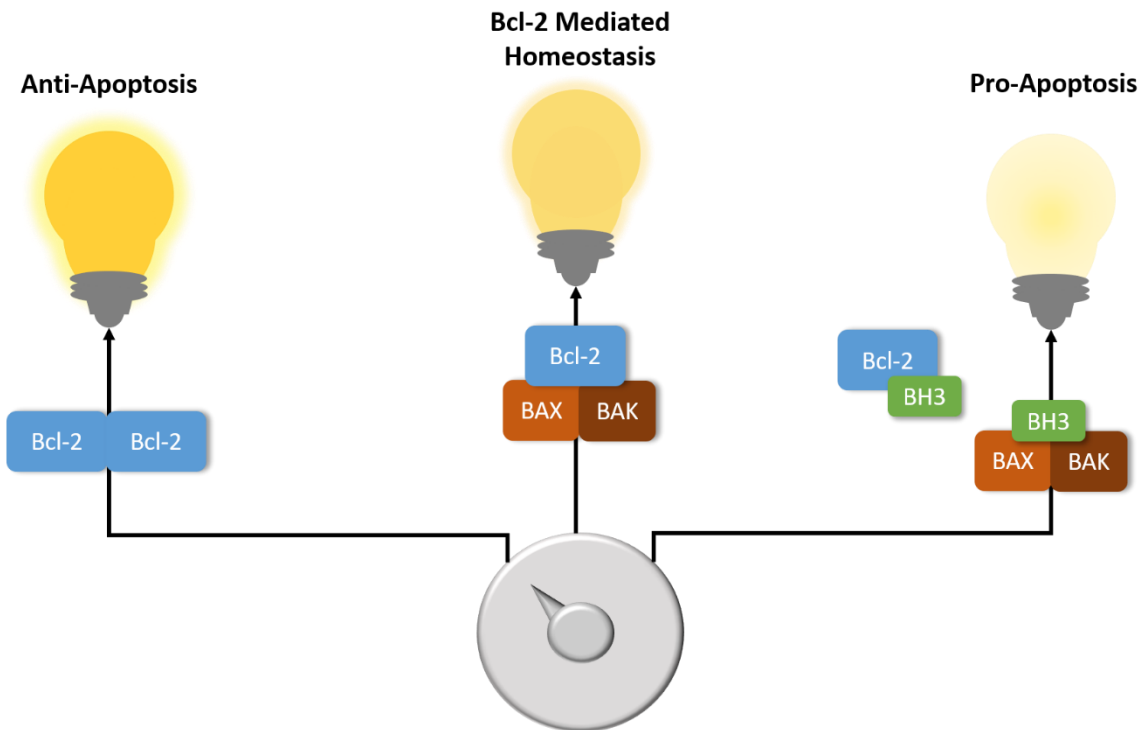
lymphoma)<sup>24</sup>. Some granzymes cause cell death in a caspase-independent manner, which may include direct cytotoxic DNA damage<sup>25</sup>; others, such as Granzyme B, cleave BID (tBID) to trigger mitochondrial outer membrane permeability (MOMP) and cytochrome c efflux or cleave effector caspase-3 and -7 directly to trigger apoptosis<sup>26</sup> (**Figure 5**).



**Figure 5. Perforin/Granzyme-Mediated Apoptosis.**

### 1.3 Homeostatic Regulation via Apoptosis

Apoptosis is a necessary component of developmental processes and those which continue through adulthood. In mammals, apoptosis is critical during embryogenesis. The maintenance of homeostasis, a balance between cell survival and apoptotic death has been proposed through the so-called “rheostat” model (**Figure 6**). This model, proposed in the 1990s by Stanley Korsmeyer, suggested that the interaction of Bcl-2/BAX proteins could indicate a cell’s “resistance” (high Bcl-2/BAX ratio) or “susceptibility” (low Bcl-2/BAX ratio) to apoptotic cell death<sup>27</sup>. The antagonism Bcl-2 demonstrates toward BAX was later shown to be disrupted by BH3-only homologs of the Bcl-2 family (i.e. BAD). BAD has been shown to free BAX from Bcl-2:BAX or Bcl-XL:BAX heterodimers, leading to restoration of apoptosis<sup>28</sup>. This model is supported by phenomenon seen *in vivo*, as in lymphoma where a Bcl-2:BAX ratio prevents apoptosis and sustained cell growth of these tumor cells, perhaps even in the presence of chemotherapeutic-induced apoptotic drugs (i.e. dexamethasone)<sup>29</sup>.



**Figure 6. Rheostat Model of Apoptotic Control.** Depicted as a lightbulb dimmer (an example of a rheostat), this model demonstrates how the presence of Bcl-2, BAX/BAK and BH3-only proteins determines the relative levels of apoptosis. Without BAX/BAK, Bcl-2 drives an anti-apoptotic phenotype and cells persist (unless there is a second mutation). Bcl-2-mediated antagonism in the presence of BAX/BAK helps preserve homeostatic levels of proliferation and death. In the presence of the BH3-only proteins, Bcl-2 is inhibited and BAX/BAK activated to drive a pro-apoptosis phenotype.

This interplay between the pro-apoptotic and anti-apoptotic members of the Bcl-2 family has since been expanded upon, and mRNA expression levels vary at critical time points of embryonic development. In the earliest stages of embryonic development, mRNA for BCL2L10 (also known as BCL-B or DIVA) is highest, although these levels wane with continued division<sup>30</sup>. BCL2L10 is thought to prevent apoptosis during these early stages, and this anti-apoptotic mRNA is inherited maternally. By day 3 however, when the number of cells reaches 6-8, the embryonic genome is activated, and high levels of the pro-apoptotic mRNA are observed. It is hypothesized that this provides an

increased sensitivity to apoptosis at this stage, given that these embryos produce higher levels of pro-apoptotic mRNA than mature MII oocytes and day 5/6 blastocysts<sup>30</sup>.

The anti-apoptotic gene *Mcl-2* is essential in embryonic development, as demonstrated by *Mcl-2*<sup>-/-</sup> mice. In these mice, blastocysts fail to implant, resulting in embryonic lethality. However, these cells do not show elevated levels of apoptotic activity, and the inner cells mass is capable of growing *in vitro*, suggesting the importance of *Mcl-2* in preimplantation development and adhesion<sup>31</sup>. Additionally, as levels of *Mcl-2* mRNA rise, *Bcl-XL* mRNA become elevated. Evidence from knockout studies suggests that these two genes work in concert with each other, with distinctive individual functions, particularly in neurogenesis. The two are temporally distinct, with *Mcl-2* importance in neuroprecursor cells (NPCs) and a prominent role for *Bcl-XL* in mature neurons, with both acting on BAX. Of note, in *Mcl-2* knockout mice, *Bcl-XL* can partially compensate for a loss of *Mcl-2*<sup>32</sup>. Additionally, individual knockout studies indicate that lack of anti-apoptotic effects of these genes do not lead to immediate cell death, but rather autophagic death of neurons with a lack of *Mcl-2*, or increased sensitivity to apoptosis in cells deficient in *Bcl-XL*<sup>33,34</sup>. Knockout of both genes, however, leads to a complete loss of the nervous system<sup>32</sup>. *Bcl-XL* also demonstrates protection from apoptosis in newly differentiated neurons in mice<sup>35</sup>.

The *Bcl-2* protein and family members are conserved across species and homologous proteins carry out similar functions in processes such as apoptosis (**Figure 7**). While the function of anti-apoptotic genes such as *BCL2L10*, *Mcl-2*, and *Bcl-XL* demonstrate clear importance in embryonic and nervous system development, pro-

apoptotic proteins are equally as crucial. In these processes, cells are over-produced, and pruning and plasticity are necessary and regulated by programmed cell death.

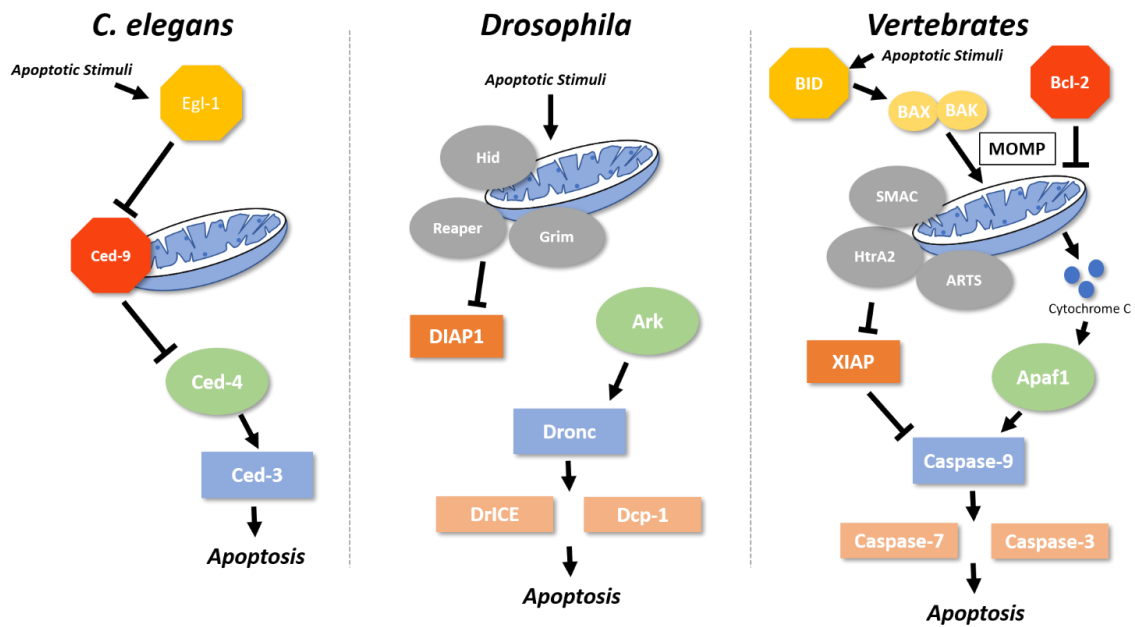
Particularly, in nervous system development, both the extrinsic and intrinsic pathway have been demonstrated in both NPCs and post-mitotic neurons. Mice deficient in the pro-apoptotic protein Apaf-1 demonstrate extensive cell growth in the periventricular proliferative zone and die during embryonic death<sup>36</sup>. This activation of the intrinsic pathway appears to be due to a lack of trophic factors in both NPCs and post-mitotic neurons<sup>37</sup>.

Many other homeostatic roles for apoptosis have been demonstrated. Wound healing, for example, involves the migration of many cells to the site of injury. Thus, the body requires a mechanism by which to clear these cells to ensure proper healing, and apoptosis provides a mechanism that does not potentiate further inflammation.

Additionally, key signaling pathways occur upon apoptosis of neutrophils, which have migrated to the site of injury and released their contents. Apoptotic neutrophils are taken up by macrophages via efferocytosis and the macrophages shift from a pro-inflammatory to anti-inflammatory phenotype<sup>38</sup>. Without this key transition, wounds become non-healing.

B and T lymphocyte maturation provides another instance of physiologic apoptosis. Programmed death of defective cells in the marrow or thymus is crucial to successful maturation B and T cells. Additionally, upregulation of anti-apoptotic Bcl-2 family member mRNA promotes cell survival of both pro-T and pro-B cells. Specifically, upregulation of *Mcl-2* mRNA promotes survival of these cells during development, and

at ablation of the gene results in rapid cell death<sup>39,40</sup>. The best example of this is seen in the upregulation of BAX and PUMA (both BH3-only proteins) by p53, a major cell cycle regulatory protein, following DNA damage<sup>41</sup>. Likewise, loss of *Bcl-XL* results in rapid death of immature (CD4<sup>+</sup>/CD8<sup>+</sup>) thymocytes and prevents appropriate maturation<sup>42</sup>.



**Figure 7. Cross-Species Conservation of Apoptotic Elements.** Modified from *Fuchs and Stellar, 2011*<sup>43</sup>.

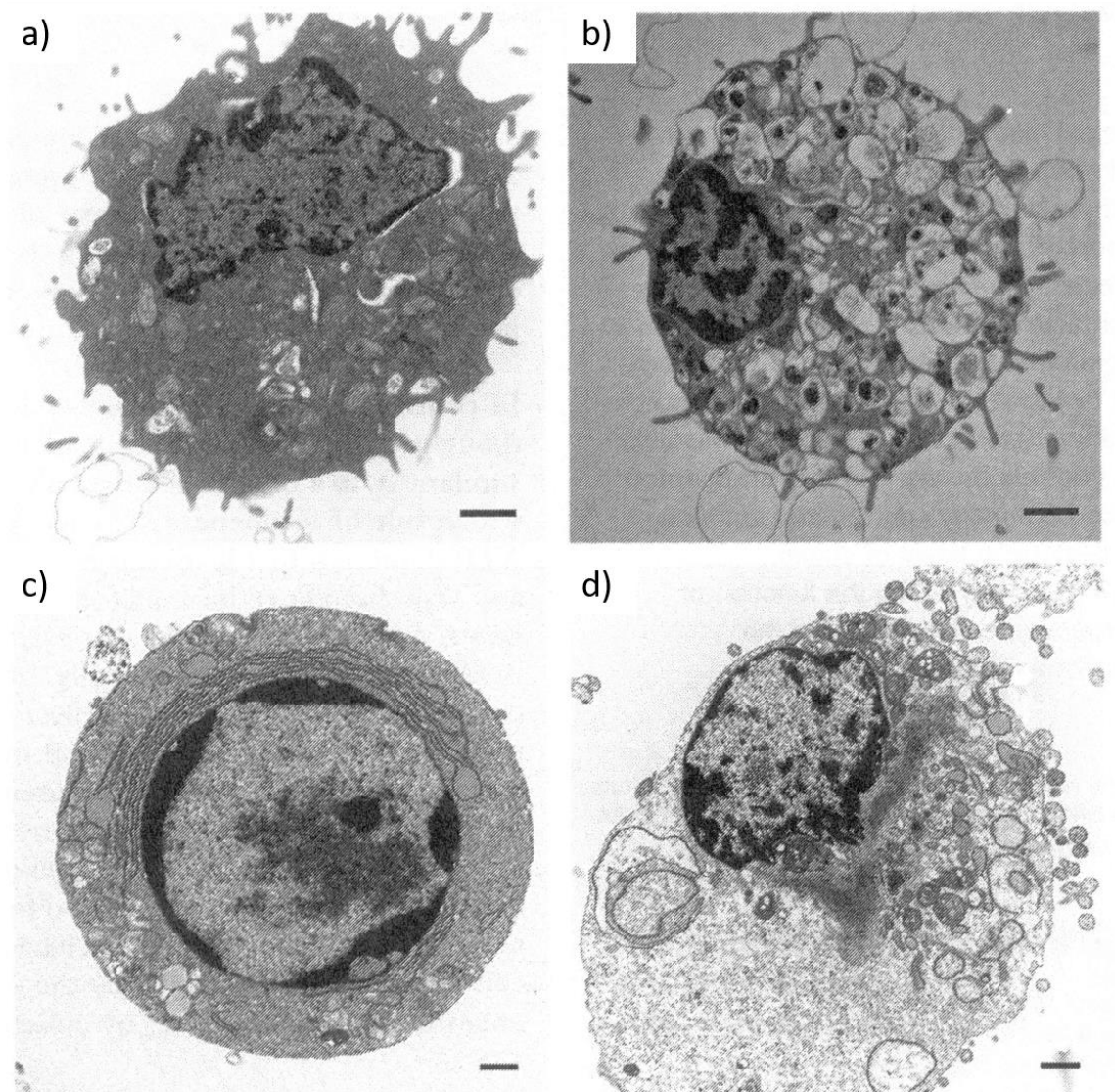
#### 1.4 Pathological Implications of Apoptosis

Abnormal apoptosis can lead to pathological presentation; too much apoptosis can lead to degenerative disease, while too little apoptosis can lead to unchecked proliferation and can lead to carcinogenesis.

## 2. Other Forms of Cell Death

### 2.1 Necrosis

In contrast to the programmed cell death in apoptosis, necrotic cell death is characterized by the uncontrolled release of cellular components following loss of integrity and eventual lysis of the cell membrane. Necrotic cell death is typically brought on by toxins, hypoxia, or traumatic damage to the cell, triggering membrane permeabilization, cellular swelling and rupture<sup>44</sup> (**Figure 8**). The process of cellular swelling, as opposed to the cellular shrinkage observed in apoptosis, is known as oncosis. This process is characterized by swelling, karyolysis, or dissolution of the nucleus, and vacuolation prior to cell lysis<sup>45</sup>. Considering necrosis as an antonym of apoptosis is convenient for the sake of comparison, although the two are not mutually exclusive. As apoptosis is an energy-dependent process, if a cell exhausts its stores of ATP, the cell may switch from apoptosis to necrosis. This is called secondary necrosis, characterized by swelling and lysis of previously apoptotic cells. Also, if oncosis is inhibited, cellular stresses may induce apoptosis of a previously necrotic cell<sup>45</sup>.



**Figure 8. Morphological Features of Necrosis.** Comparison of morphological features of a) normal cell; b) autophagic cell (survival); c) apoptotic cell (dying); and d) necrotic cell. Note d) demonstrates the characteristics of necrotic cell death including cell membrane blebbing, karyolysis and vacuolation prior to cell lysis. Scale represents 1 $\mu$ m. *Image taken from Edinger & Thompson, 2004<sup>46</sup>.*

Programmed necrosis is also characterized by mitochondrial swelling and rupture, a process preceded by the phenomenon, “Mitochondrial Permeability Transition” (MPT)<sup>47,48</sup>. This process is triggered by pathological levels of calcium and ROS, although

the exact mechanisms underlying MPT are still under active investigation. Important in the formation of the pore leading to increased inner mitochondrial membrane permeability is the mitochondrial protein cyclophilin D (aka, peptidylprolyl isomerase F, PPIF). Loss of this protein in PPIF<sup>-/-</sup> mice shows protection from ischemia/reperfusion-induced cell death<sup>49</sup>. This phenomenon has been important to observe in medicine because blocking cyclophilin D activity via pharmacological application of cyclosporine A appears to be cardioprotective following ischemic injury<sup>50</sup>. This phenomenon has been duplicated experimentally, limiting reactive oxygen species (ROS) production, inflammation and mitochondrial decoupling following aortic cross-clamping related ischemic injury in rats<sup>51</sup>. Studies to show cardioprotective effects of cyclosporine A have shown mixed results, but it remains an optimistic therapeutic modulator of ischemic injury. Whereas MPT occurs on the inner mitochondrial membrane, Bcl-2 family members BAX/BAK have been shown to function as the outer mitochondrial pore contributing to MOMP (**Figure 4**), and could provide another possible therapeutic target<sup>52</sup>.

Necrosis occurs in two primary forms: Liquefactive and coagulative. These are based micro- and macroscopic changes to cells. Death by complete or partial dissolution of dead tissue into a liquid mass as a result of bacterial or fungal infections, or other inflammatory stressors indicates liquefactive (or colliquative) necrosis. In coagulative necrosis, cells maintain most of their normal architecture several days after death, but cellular swelling and microscopic changes indicate coagulative necrotic death<sup>53</sup>. For example, a lack of nuclei and hypertrophic appearance in cardiac myocytes following

acute infarct<sup>53,54</sup>. As highlighted by Adigun, Basit and Murrar<sup>53</sup>, distinctive clinical presentations have generated subsets of necrotic death. Caseous cell death, observed with bacterial tuberculosis infection, forms granulomas: eosinophilic centers surrounded by lymphocytes and activated macrophages leading to the cheese-like (caseous) appearance<sup>53,55</sup>. Fat necrosis, where adipocytes lyse to release digestive enzymes which break down lipids forming free fatty acids leading to anucleated adipocytes and calcium deposits<sup>53,56</sup>. Although not macroscopically difficult to distinguish, fibrinoid necrosis is another clinical subset used to describe the deposition of fibrin associated with vascular damage, as seen with immune-complex disease following a type III hypersensitivity reaction<sup>53,57,58</sup>. Similarly, gangrenous necrosis is clinically used to distinguish black skin with putrefaction in the limbs and is microscopically a combination of coagulative, due to ischemia, and liquefactive, with underlying bacterial infection, necrosis<sup>53</sup>.

## **2.2 Necroptosis**

As previously mentioned, apoptosis and necrosis are not entirely distinct, and there is speculation that they even exist as two ends of a spectrum of cell death. A regulated form of necrotic cell death, known as necroptosis, is one example of how these two processes are related. Like apoptosis, necroptosis relies on signaling from cell surface receptors, specifically death receptors such as tumor necrosis factor receptor 1 (TNFR1). This receptor is reliant on a necrosome, where receptor-interacting proteins 1 (RIP1) and 3 (RIP3) form an amyloid-like hetero-oligomeric signaling complex of protein aggregates<sup>59</sup>. Upon binding of the ligand, TNFR1 recruits a pro-survival complex

1 containing TNFR-associated death domain (TRADD), RIP1 and ubiquitin E2 ligases which polyubiquitinate RIP1. Following deubiquitination of RIP1 a decision is made; either RIP1 is deubiquitinated and complex IIa is formed, triggering caspase 8 and subsequent extrinsically-mediated apoptosis, or complex IIb is formed, caspase 8 is inhibited, and necroptosis is activated<sup>60</sup>. Recruitment and phosphorylation by RIP3 triggers formation of another signaling complex, the mixed lineage kinase domain-like (MLKL) pseudokinase, which, following oligomerization, induces loss of membrane integrity and necrotic death<sup>61</sup>. This signaling complex is known as the “necrosome”, and it is believed to poke holes in the cell membrane to cause necroptosis.

Despite its regulated, procedural activation, necroptosis is contrary to apoptosis in that it is a pro-inflammatory mechanism of cell death. This mechanism is believed to be due to, at least in part, nuclear factor-kappa B (NF- $\kappa$ B). In response to viral infection, endosomal toll-like receptors (TLR) 3, 7 and 8 are activated, and downstream signaling activates interferon regulatory factor (IRF) transcription, producing multiple components including interferon beta (IFN- $\beta$ ) and NF- $\kappa$ B<sup>62</sup>. The specific molecule responsible for activating transcription is the IFN-inducible protein DNA-dependent activator of IRFs (DAI)<sup>63</sup>. Shortly after discovery of this molecule, Rebsamen and colleagues observed that DAI recruits RIP1 and RIP3 through specific RIP homotypic interaction motifs (RHIMs) and activates NF- $\kappa$ B signaling, potentiating the pro-inflammatory consequences of necroptosis<sup>64</sup>.

### 2.3 NETosis

A third type of programmed cell death is characterized by the release of a “net”-like structure of chromatin- and histone-containing fibers known as neutrophil extracellular traps (NETs)<sup>65</sup>. Originally characterized in neutrophils, NET-like structures can also be extruded by other hematopoietically-derived cells including mast cells<sup>66</sup>, eosinophils<sup>67</sup>, and basophils<sup>68</sup>. Primarily a response to microbial pathogens, NETosis can be triggered by microbial signals including lipopolysaccharide (LPS) via TLRs<sup>69,70</sup>. Although the intracellular mechanisms which trigger NET extrusion are the subject of some debate, there is agreement that this mechanism is dependent on intracellular ROS. This has been demonstrated pharmacologically by the rescue of neutrophilic NET extrusion following both exogenous H<sub>2</sub>O<sub>2</sub> administration and via gene therapy in patients with chronic granulomatous disease (CGD) who otherwise cannot generate ROS or NETs<sup>71,72</sup>. The mechanism by which NETotic cell death occurs is also under debate. As highlighted by the 2018 Nomenclature Committee on Cell Death, it is thought that the Raf-MAPK-ERK2 pathway activates ROS generation, driving release of neutrophil-expressed elastase, ELANE, and myeloperoxidase (MPO). This simultaneous release promotes the MPO-dependent proteolytic activity of ELANE and drives cytoskeleton damage and chromatin decondensation, leading to plasma membrane rupture<sup>73</sup>. Interestingly, NET extrusion does not necessarily result in cellular lysis, and NETs have been implicated in human diseases such as diabetes and cancer<sup>70,74,75</sup>.

## 2.4 Ferroptosis

First proposed in 2012 by Dixon et al.<sup>76</sup>, *ferroptosis* represents a morphologically and microscopically distinct type of random cell death. This mechanism is apparently dependent upon the toxic accumulation of ROS. Morphologically, ferroptosis is characterized by small mitochondria, with condensed mitochondrial densities and either reduced or lost mitochondria cristae, as well as outer mitochondrial membrane rupture. It is suspected that these changes are due to high levels of lipid peroxidation and oxidative stress<sup>77</sup>. High lipid peroxidation can trigger aberrant iron metabolism, leading to reduction by the metalloredutase STEAP3 from  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , enabling transport by the divalent metal transporter 1 (DMT1). High intracellular iron levels leads to ROS production and accumulation by the Fenton reaction<sup>78</sup>. It would appear that this system is endogenously regulated by the reduced glutathione (GSH) dependent peroxidase GPX4, as knockdown and overexpression studies of GPX4 have been linked to direct control of ferroptotic activity<sup>79</sup>. More recently, the role of GPX4 in inhibiting ferroptosis has been linked to the prevention of phosphatidylethanolamine-containing polyunsaturated fatty acids (PUFAs), such as arachidonic and adrenic acids, which would otherwise act as death signals<sup>80</sup>.

## 2.5 Autophagy

Autophagy is the major homeostatic process by which cytosolic components of cells are degraded and recycled via the lysosome<sup>81</sup>. The initial step of autophagy is the formation of the phagophore via formation of the ULK1 (unc-51-like kinase 1) complex, and the class III phosphoinositide 3-kinase (PI3K) complex<sup>81</sup>. This complex contains

many components including VPS (vacuolar protein sorting) proteins VPS34 and VPS15, autophagy-related protein (ATG) 14L (ATG14-like), and BECLIN-1. Upon formation of these complexes, the phagophore elongates, utilizing the ATG5-ATG12-ATG16 complex and lipidated microtubule-associated protein light chain 3 (LC3II). The developed autophagosome, which contains the organelles and cytosolic proteins targeted for degradation, then fuses with the lysosome and undergoes degradation via lysosomal hydrolases<sup>82</sup>.

Of note, three major forms of autophagy have been identified, as summarized by Cuervo, 2004; macroautophagy, microautophagy and selective autophagy<sup>83</sup>. In macroautophagy, entire cellular cytosolic compartments are separated in double-membrane vesicles which fuse with lysosomes for degradation. Microautophagy is slightly more specific, as the organelles fuse directly with lysosomes, which occurs via the tagging of these organelles with surface proteins to trigger lysosomal fusion<sup>84</sup>. Finally, in selective autophagy (a.k.a. chaperone-mediated autophagy), heat shock protein HSC70 works with cochaperones such as HSP40 (a.k.a. DNABJ1) and HSP70-HSP90 organizing protein (HOP) to guide proteins to the lysosome-associated membrane protein type 2A (LAMP2A) for their selective degradation<sup>85</sup>. Autophagic mechanisms for promoting cell survival, such as aiding in the removal of toxic protein aggregates, are more deeply understood than roles in cell death. In recent years, efforts been made to clarify if autophagy directly causes cell death or simply accompanies and aids existing mechanisms of cell death<sup>86</sup>. It is most likely that autophagy is a survival mechanism, whereby cells delete cellular components to maintain a basal metabolic rate. These cells

will only die after periods of prolonged starvation, or if they have a viral infection. In the latter case, it is most likely immune-mediated where NK cells or CTLs would induce granzyme/perforin-mediated apoptosis of the infected cell.

## **2.4 Pyroptosis**

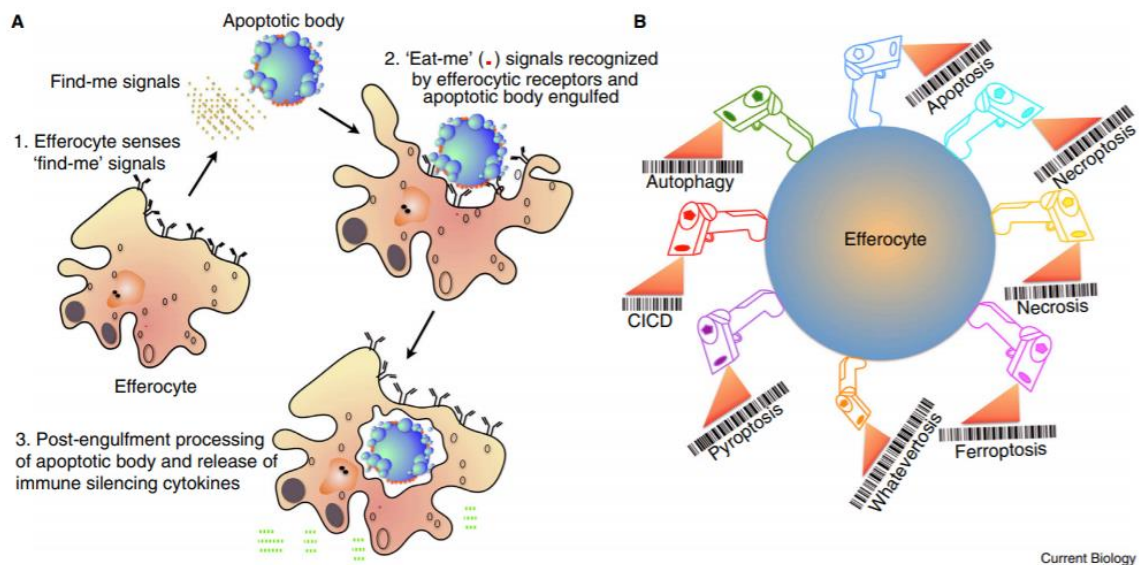
Pyroptosis is a programmed form of necrosis. Initially, pyroptosis was described as caspase-1 mediated cell death, due to the caspase-1-dependent formation of ion pores in the plasma membrane which leads to pathological efflux of ions and cellular lysis<sup>87</sup>. Caspase-1, as initially mentioned, is also known as ICE – interleukin (IL) converting enzyme and is responsible for production of IL-1 $\beta$ <sup>10</sup>. It has since been discovered that the mechanism of activation for pyroptosis is two-fold: First, a signaling cascade from pattern recognition receptors (PRRs) pathogen-associated microbial patterns (PAMPs) triggers formation of the inflammasome. This activates the cleavage of caspase-1 from the zymogen procaspase-1. Activated caspase-1 can then trigger the activation of IL-1 $\beta$  from pro-IL-1 $\beta$ , leading to activation of gasdermin D (GSDMD), a substrate representative of a large family of pore-forming proteins. The second pathway is triggered specifically by LPS, which causes activation of caspase-4/5/11 (via cleavage from procaspase-4/5/11), which activate GSDMD and pore formation<sup>88</sup>.

## **3. Physiological and Pathological Fates of Apoptosis**

### **3.1 Efferocytosis**

As important as the balance between mitosis and apoptosis is in maintaining homeostasis is the clearance of apoptotic bodies by surrounding cells, a process known as

*efferocytosis*. This term, coined by Henson et al.<sup>89</sup>, comes from the Greek “to bury”, and has been researched in greater depth in recent years. Professional phagocytic cells, such as macrophages (**Figure 9**) and dendritic cells, are believed to be the primary mediators of efferocytosis. However, neighboring non-professional or “amateur” phagocytic cells, such as epithelial/endothelial cells, fibroblasts and microglial cells have demonstrated capacity for efferocytosis, though with varying levels of efficiency<sup>90,91</sup>.



**Figure 9. Efferocytosis of Apoptotic Bodies via Efferocytes.** A) Uptake of intact apoptotic bodies by macrophages, dendritic cells or other efferocytes. Apoptotic bodies displaying cell-surface signals are engulfed via membrane extensions. B) Recent evidence suggests that these signaling molecules act as barcodes, and that efferocytes can modify the cytokines released following efferocytosis to potentiate cell-death outcomes. *Image obtained from Kumar & Birge, 2016*<sup>92</sup>.

Various genes have been implicated in efferocytosis, starting in the model organism *C. elegans*. In 2001, Zhou et al. demonstrated the role of the *ced-1* gene and protein product as a transmembrane scavenger receptor which mediates engulfment with neighboring cells<sup>93</sup>. Since then, a pathway has been elucidated between *ced-1*, *ced-7*, an analogue of

the ABC-1 cassette transporter<sup>94</sup>, and *ced-6*, which contains a phosphotyrosine domain and is integral in mediating engulfment signaling<sup>95,96</sup>. This pathway works in conjunction with a pathway involving *ced-2*, *ced-5*, *ced-12* and *ced-10*, a conserved pathway in *Drosophila* and mammals which triggers signaling of engulfment of the dying cells<sup>97</sup>. Additionally, *ced-8* encodes a lipid scramblase, which is important in the externalization of phosphatidylserine (PS), an integral “eat-me” signaling lipid<sup>98</sup>.

These intracellular signaling pathways are highly conserved across species, including *Drosophila* and mammals, with each *ced* gene possessing a counterpart homolog/analogue (**Table 1**). *Ced-1* corresponds to the CD91/scavenger receptor from endothelial cells (SREC)-like protein, while *ced-7* corresponds with the ABC1 transporter (Eato in *Drosophila*), and *ced-6* with the GULP adaptor protein, which binds the CED-1 or CD91/LRP (low density lipoprotein receptor-related protein) phosphotyrosine domain<sup>99–101</sup>. CED-8 has been shown to function similarly to the Xk-related protein 8 (Xkr8), a mammalian scramblase promoting externalization of PS<sup>98</sup>. Additionally, the *ced-2*, *ced-5*, *ced-12* pathway appears to function identically to the CrkII, DOCK180 (mammals), ELMO1 pathway in mammals. *Ced-5* is analogous to the *Drosophila* homolog myoblast city (Mbc), and its function in apoptotic clearance in *Drosophila* development is essential<sup>102</sup>. *Ced-10* demonstrates GTPase activity equivalent to Rac1 in mammals<sup>97,103</sup>.

**Table 1. Conservation of Engulfment Signaling Molecules from *C. elegans*, *Drosophila* and Mammals.**

<i>C. elegans</i>	<i>Drosophila</i>	Mammals	Role/Function
CED-1	Draper	CD19/LRP	Surface receptor; Recognizes dying cells
CED-2	CG1587	CrkII	Contains SH2 and two SH3 domains; physically interacts with CED5/Mbc/DOCK180
CED-3	DRONC	Caspase 9	Effector Caspase and part of the “apoptosome”
CED-4	Ark	Apaf1	Key signaling molecule which, in mammals, complexes with cytochrome c and caspase 9 to form “apoptosome”
CED-5	Mbc	DOCK180	Activator of Rac/CED12 GTPase activity
CED-6	Dmel\Ced-6	GULP	PTB domain-containing adaptor protein; trafficking of PS receptor (PtdSer)
CED-7	Eato	ABC1	Transmembrane Scavenger receptor; Aids in PS externalization
CED-8		Xkr8	Phospholipid scramblase
CED-9	Drob-1	Bcl2	“Brake” on apoptotic signaling
CED-10	Rac-2	Rac1	GTPase; common point of signal transduction across engulfment pathways
CED-12	Dmel\Ced-12	ELMO1	Cytoskeletal rearrangement for engulfment

### 3.2 Resolution of Inflammation

As previously mentioned, the externalization of PS is a key step in efferocytosis which ultimately dictates engulfment of the dying cell. While the *C. elegans* model has provided an excellent framework of the proteins involved in triggering efferocytosis, PS/PS receptor (PSR) signaling is a complexed and nuanced phenomenon which is best studied in higher metazoans. A myriad of PSRs have been identified, as well as their complementary ligands. These receptors include stabilin-1 and stabilin-2, the TAM (Tyro, Axl, Mer) family of receptors, the integrins  $\alpha_v\beta_{3/5}$ , the T cell immunoglobulin mucin 1 (TIM-1, as well as TIM-3 and TIM-4), the CD300 family (CD300b and CD300a), as well as BAI1<sup>104</sup>. These receptors interact with PS differently: BAI1 is a transmembrane protein which interfaces directly with PS, triggering signaling via ELMO and DOCK180, whereas Mer or  $\alpha_v\beta_{3/5}$  integrins are tyrosine kinases which require

ligands (GAS6 or protein S for TAMs, milk fat globule-EGF factor 8 protein (MFG-E8) for integrins) to interface with PS and trigger signaling<sup>105,106</sup>. The increasing numbers of PS-PSR interactions identified in recent years make clear the underlying complexity of PS signaling in the clearance of apoptotic cells. As Kumar et al.<sup>107</sup> point out in their review of efferocytosis, multiple PS receptors are often activated simultaneously leading to cooperative and synergistic downstream signaling. Thus, it is sensible to consider a “phagocytic synapse”, analogous to the immunological synapse in T cell signaling, when discussing PS signaling.

### **3.3 Consequences for Immune Response (Signaling Factors)**

Being the prominent “eat me” signal, PS-PSR signaling has an important role to play in programmed cell death, specifically, in preventing potentially harmful immune responses. Improper clearance of apoptotic cells can be both the cause and effect of chronic inflammatory diseases. As discussed, if cells fail to complete apoptosis (i.e. due to ATP depletion), cells can enter secondary necrosis and lyse. Secondary necrosis has been implicated in autoimmune diseases, such as systemic lupus erythematosus (SLE). Interestingly, apoptotic remnants in the germinal follicular centers, possibly presented via tingible body macrophages, can result in autoimmune antibody production, triggering SLE<sup>108</sup>. Additionally, damage to skin cells by UV light can trigger accumulation of autoantigens, and cause flares in SLE patients and potentially immune complex formation, undoubtedly contributing to chronic inflammation in these patients<sup>109,110</sup>. Autoantibody production has also been linked directly to impaired efferocytosis<sup>111,112</sup>.

Ensuring proper management of “eat me” signals is therefore necessary to maintain homeostasis and prevent the etiology and pathogenesis of chronic disease. Two prominent classes of PSRs have been shown to directly inhibit the production of pro-inflammatory cytokines. The TAM receptor Axl, in conjunction with its ligands GAS6 and Protein S, suppresses TLR signaling and prevents expression of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IFN $\alpha$ <sup>113,114</sup>. Similarly, Mertk receptor signaling results in a blockade of NF- $\kappa$ B and pro-inflammatory cytokine release by dendritic cells<sup>115</sup>. The TIM family of PSRs possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs), and, in addition to serving as tethering receptors in the “phagocytic synapse” and activating T cell responses, have been implicated in suppressing pro-inflammatory signals<sup>116,117</sup>. Genetic ablation studies have implicated the role of these suppressive signals in preventing autoimmune and chronic inflammatory conditions. Selective ablation of Axl or GAS6 leads to impaired recovery of neurons after pharmacologically-induced damage<sup>118–120</sup>. However, exogenous GAS6 was sufficient to provide therapeutic rescue following pharmacologically-induced damage<sup>121</sup>. Additionally, a genome-wide analysis of over 9,000 patients has implicated a link between polymorphisms of Mertk and development of multiple sclerosis<sup>122</sup>. Conversely, increasing efferocytotic clearance of apoptotic cells, such as through increased BAI1 expression, has been shown to reduce inflammation in chronic inflammatory disease<sup>123</sup>.

Equally as important in suppressing harmful inflammatory responses following apoptosis is the release of so-called “calming” agents, such as TGF- $\beta$ , IL-10, adenosine diphosphate (ADP), prostaglandin E2 (PGE<sub>2</sub>) and thrombospondin-1 (TSP-1). This

process of tempering the inflammatory response following apoptotic activity is termed “catabasis”<sup>124</sup>. In addition to these signaling factors, a host of endogenous mediators work in concert to mediate this resolution of inflammation, known as resolvins, protectins and lipoxins<sup>125</sup>. These molecules appear to operate on a switch, with chemical signals influencing which lipid mediators are produced. For example, PGE<sub>2</sub> specifically switches production of damaging, pro-inflammatory leukotrienes and prostaglandins to anti-inflammatory lipoxins, such as lipoxin A<sub>4</sub><sup>126</sup>. Additionally, PGE<sub>2</sub> appears to upregulate production of pro-resolution factors such as E-series and D-series resolvins and protectins<sup>125</sup>. Other factors, such as the local secretion of vascular endothelial growth factor (VEGF) by Mertk and Mfge8 expressing macrophages, have shown to be critical in repairing injury following myocardial infarction<sup>127</sup>. Faulty resolution of inflammation is hypothesized to result in chronic inflammatory conditions, such as chronic obstructive pulmonary disease (COPD)<sup>128</sup>.

One major complementary pathway which assists in appropriate efferocytosis and catabasis is alternative macrophage activation to a so-called, anti-inflammatory “M2” phenotype. This appears to be mediated, at least in part, by the secretion of TGF- $\beta$ <sup>129</sup>. Physiologically, a balance of M1 (classically activated) and M2 macrophages exists. A shift in this balance is seen during human development and pregnancy (M2 polarization), and during pathogenesis (M1 polarization) such as in atherosclerosis or obesity<sup>130</sup>. A polarization toward M2 phenotype is favorable following apoptosis, as seen following ischemic/reperfusion liver injury after surgery which is mediated by D1 resolvins<sup>131</sup>. In 2014, Das et al. demonstrated the importance of microRNA-21 (miR-21) in polarizing

macrophages toward an anti-inflammatory M2 phenotype in wound healing<sup>132</sup>. In a recent study, the same group demonstrated that a modified collagen gel dressing improved wound healing through increased production of anti-inflammatory IL-10, IL-4 and pro-angiogenic VEGF due to increased macrophage recruitment and upregulation of miR-21<sup>133</sup>. In summary, efferocytosis of PS-expressing cells drives macrophages to express an M2, anti-inflammatory phenotype.

#### **4. Consequences of Pathological and Chronic Apoptosis**

##### **4.1 Compensatory Apoptosis-Induced Proliferation**

Externalization of PS and PS/PSR signaling are just one downstream consequence of caspase activation via apoptosis. Compensatory proliferation, a mechanism of replenishing lost tissue due to damage or injury, is another such consequence. Although regenerative capacity varies greatly across species, it is apparent that compensatory proliferation is necessary to repair tissue following injury, and following apoptosis. Early work in *Drosophila* characterized a unique form of compensatory proliferation, reliant on apoptotic machinery and specifically the initiator caspase Dronc. Since then, the term “apoptotic-induced proliferation” (AiP) has been used to describe compensatory proliferation coupled with apoptotic activity<sup>134</sup>.

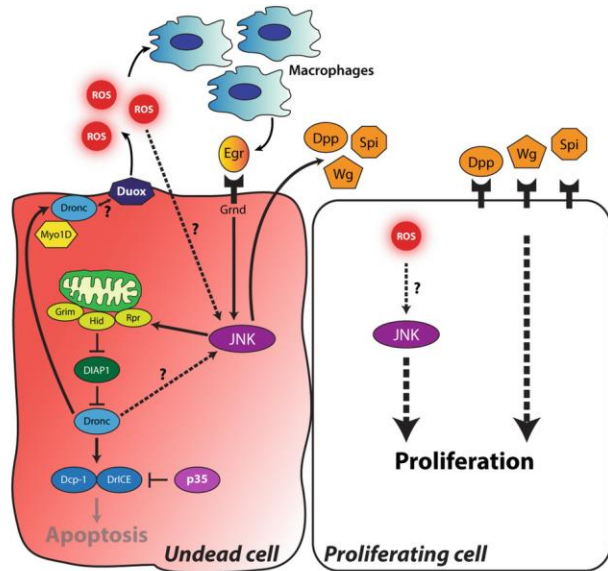
Andreas Bergmann and colleagues at the University of Massachusetts Medical School have proposed a three-part model for AiP in *Drosophila melanogaster*: “Undead”, “genuine” and “post-mitotic”<sup>135</sup> (**Figure 10**). The first model (**Figure 10A**), also known as the “overgrowth” model, occurs when apoptotic cells exist in an *immortalized* state, primarily due to the expression of the caspase inhibitor p35. In this “undead” cell, the

transporter Myo1D carries Dronc (the initiator caspase in *Drosophila*) to the membrane, inducing the NADPH oxidase Duox to generate ROS<sup>136</sup>. Macrophages attracted to the “undead” cell release the TNF ligand *Eiger*, which activates the JNK stress-signaling pathway; this in-turn leads to expression of *hid* and *reaper*, creating an amplification loop<sup>137,138</sup>. Additionally, JNK signaling causes the release of proliferation factors, such as *Dpp*, *Wg*, and *Spi*<sup>139,140</sup>. The combination of the amplification loop perpetuating Dronc signaling and the release of these proliferative factors provides a convincing model for unchecked proliferation in the “undead” model of AiP.

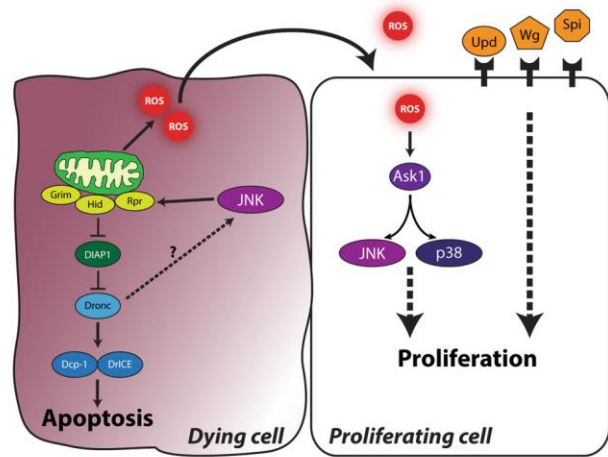
The second model, also known as the “regenerative” model (**Figure 10B**), occurs independent of p35 and is more conservative. Spatial and temporal restrictions are placed on dying cells in order to prevent excessive apoptosis. This model relies on JAK/STAT and p53 signaling in addition to the JNK signaling observed in the “undead” model of AiP. ROS generation appears to occur intracellularly, propagating the signal to neighboring cells where the JNK pathway is activated; p38 signaling through Akt and the redox-sensitive Ask1 factor induce expression of *Unpaired (Upd)*, which is an IL-6 paralog<sup>141</sup>. Unlike the “undead” model, the role of proliferative signaling factors is undefined in the “genuine” model, and this may provide an explanation for the context-dependent nature of AiP. The third model, “post-mitotic” AiP (**Figure 10C**), is best demonstrated in the developing retina of *Drosophila*, as dying photoreceptor neurons produce and secrete the mitogen *Hedgehog (Hh)* in a DrICE/Dcp1-dependent manner<sup>142</sup>, promoting proliferation of neighboring, undifferentiated (yet post-mitotic) cells. Contrary

to the “undead” and “genuine” models of AiP, this model relies on the Hippo signaling pathway, rather than JNK<sup>143</sup>.

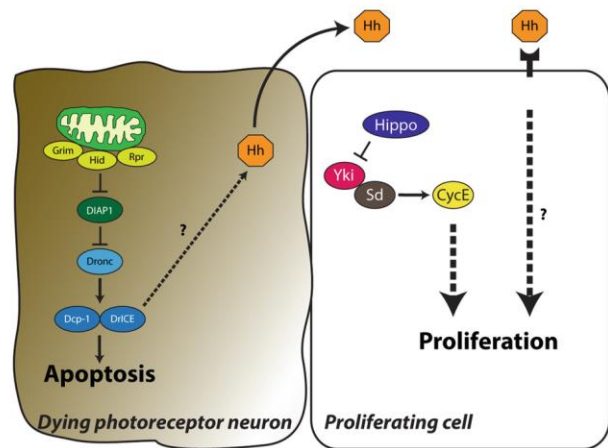
A. "Undead" model



B. "Genuine" model



C. "Post-mitotic" model



**Figure 10. Apoptotic-induced Proliferation (3-Part model).** Proposed 3-part model of apoptotic-induced cellular proliferation in *Drosophila*. **A:** The “undead” model is in an *immortalized* cell, which possess the caspase inhibitor p35. Production of ROS recruits macrophages to secrete cytokines (i.e. TNF) which activate the JNK pathway and induces an amplification loop which initiates unchecked proliferation. **B:** The “genuine” or “regenerative” model, in which intracellular ROS production promotes cell proliferation but temporal and spatial restrictions provide a check on this proliferation. **C:** The “proliferative” or “post-mitotic” model in which proliferation is triggered by the secreted Hedgehog mitogen (Hh) and the pathway does not involve JNK signaling. This pathway is observed in the developing retina in *Drosophila*. Figure taken from Diwanji and Bergmann<sup>135</sup>.

#### 4.2 Apoptotic Index (including prognostic research)

The fundamental principles of chemotherapy are to kill rapidly dividing cells as a means of “targeting” tumor cells. Given the lack of a concurrent inflammatory response, death by apoptosis would be the preferred mechanism for chemotherapeutic agents. With that in mind, why would a high number of apoptotic cells in the tumor microenvironment not be indicative of a good prognosis for cancer patients? This can be quantified, via the number of apoptotic cells per 100 intact neoplastic nuclei<sup>113</sup>, otherwise known as the “apoptotic index” (AI). Existing theories, and a simplistic perspective, would lend credence to the idea that high levels of apoptosis (high AI) could overwhelm the rapid division and prevent tumor proliferation. However, as this thesis has discussed, apoptosis is a complex phenomenon influencing many other factors and involving prolific cell-to-cell communication. It is possible that, in certain cancers, a high AI could create an overall immunosuppressive tumor micro-environment, promoting immune escape, tumorigenesis and unchecked proliferation.

Examining Bergmann's model of AiP in *Drosophila*, the "undead" model could provide a model for the tumor microenvironment. In this model, the abundance of p35, the caspase inhibitor, results in the complexing of DRONC with Myo1D and DUOX to produce ROS and locally recruit macrophages<sup>135</sup>. High pro-inflammatory cytokines are thus produced by the recruited macrophages, such as TNF- $\alpha$ . The presence of ROS, activated macrophages and continued cytokine release leads to activation of JNK signaling pathways, promoting an amplification loop and unchecked proliferation with apoptosis prevented by p35. Cyclin dependent kinase 5 (Cdk5), a downstream target of p35, has been shown to be dysregulated in cancer and associated with poor prognosis. Thought to be upregulated in response to DNA damage, Cdk5 triggers STAT3 signaling pathways and upregulates pro-survival factors. Inhibiting Cdk5 is associated with decreased tumorigenesis, proliferation and metastasis further underscoring its importance<sup>145</sup>. The role of Cdk5/p35 signaling in human carcinomas could provide evidence of the "undead" model of AiP as a mechanism of high AI resulting in tumorigenesis and potentiating poor prognosis.

In 1999, Tanaka et al.<sup>146</sup> described a study of 236 patients undergoing surgery for previously untreated non-small cell lung cancer (NSCLC) and analyzed both AI and proliferative index (PI). After stratifying the patients across lowest AI (AI <5.0), lower AI ( $5.0 \leq \text{AI} < 11.0$ ), higher AI ( $11.0 \leq \text{AI} < 25.0$ ) and highest AI ( $25.0 \leq \text{AI}$ ), the group determined 5-year survival rate. The group found that the lower, higher and highest AI groups also had higher PI (48.0%, 54.3% and 50.7%, respectively), and the lower and higher groups had poorer prognosis than the lowest AI group (PI=32.3%). They

concluded that AI is suitable as an independent prognostic factor in NSCLC. The study used the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) labelling technique<sup>147</sup>. PI is measured by the presence of the antigen Ki-67, a marker of proliferation.

Studies emerging after Tanaka et al. shared mixed results, but several studies have supported the idea that AI could provide prognostic value. One group correlated AI with proliferating cell nuclear antigen (PCNA) and several other biomarkers in NSCLC patients, and found that individuals with high PCNA and high AI have a particularly poor prognosis<sup>148</sup>. Several other studies have corroborated this idea in other cancers, including non-Hodgkins lymphoma<sup>144</sup>, endometrial adenocarcinoma<sup>149</sup>, synovial sarcoma<sup>150</sup>, prostate carcinoma<sup>151</sup>, squamous cell lesions of the oral cavity<sup>152</sup>, breast cancer<sup>153</sup> and bladder cancer<sup>154</sup>.

### **4.3 Immune Evasion**

Long understood is the concept of tumors as “wounds that do not heal”<sup>155</sup>. As such, the tumor micro-environment is a rich inflammatory environment with competing signaling molecules dictating tumorigenesis. In support of this analogy, the tumor micro-environment is rich in macrophages, termed cancer associated macrophages (CAMs), which express M2-like phenotype. This appears to be due to high levels of IL-10, IL-4, IL-13 and glucocorticoids<sup>156,157</sup>. This might be due to high levels of efferocytosis of apoptotic cells, and thus indicates a hijacking of a physiologically beneficial pathway. In addition, the tumor micro-environment contains high levels of macrophage colony-

stimulating factor (M-CSF) and TGF $\beta$ , which potentially play a role in polarizing developing monocytes into M2-like macrophages<sup>158</sup>.

The M2-like phenotype would theoretically be ideal in this environment; however, the immunosuppressive secretions of CAMs are likely diminishing an appropriate immune response to the tumor. In fact, one study has demonstrated that intracellular adhesion molecule-1 (ICAM-1) is involved in mediating macrophage polarization. ICAM-1 deficient mice demonstrate increased M2 macrophage infiltration and accelerated liver metastasis of colon carcinoma; however, when efferocytosis is inhibited, these cells show diminished metastatic capacity and lacked M2 macrophage polarization<sup>159</sup>.

TNF and IL-6 are also elevated in the tumor micro-environment and lead to NF- $\kappa$ B activation via the classical, inhibitor-of-NF- $\kappa$ B kinase- $\beta$  (IKK $\beta$ ) pathway. This activation has been implicated as an essential promoter of inflammation-associated carcinomas<sup>160</sup>. Not only does the inflammatory tumor micro-environment provide immune escape via M2-like macrophages, but also through increased tumorigenesis and cell growth. The activation of NF- $\kappa$ B promotes expression of anti-apoptotic proteins such as Bcl-XL, BFL (another Bcl-2 family member), and growth arrest and DNA-damage-inducible 45 $\beta$  (GADD45 $\beta$ )<sup>160</sup>.

Another means of immune evasion occurs via the upregulation of checkpoint inhibitors, such as PD-L1, CD80 and other B7 family members. These immunological regulators provide tumors with T cell tolerance; for example, CD80/CTLA-4 and CD80/CD28 activity induce T cell anergy<sup>161,162</sup>, whereas PD-L1/PD-1 activity potentiates

T cell exhaustion<sup>163</sup>. In specific cancer types, such as epidermal growth factor receptor (EGFR)-driven NSCLC, upregulation of PD-L1 has been directly linked to potentiating immune escape<sup>164</sup>. Oncogenes provide one potential means of upregulating these checkpoint inhibitors, such as the myelocytoma oncogene (MYC) driving increased expression of CD47 and PD-L1<sup>165</sup>. Other studies have implicated exogenous pharmaceutical agents, such as resveratrol and piceatannol, in upregulating PD-L1 expression in colorectal and breast cancer cells via NF- $\kappa$ B signaling<sup>166</sup>.

#### **4.2 Treatment Implications**

The multiplicity of effects of apoptosis in the tumor microenvironment have profound implications for treatment. Those cells in the tumor microenvironment which are sensitive to chemotherapies and do undergo apoptosis may stimulate proliferation of neighboring tumor cells through cytokine release and AiP. As suggested by Fogarty & Bergmann<sup>167</sup>, there could exist a link between chemotherapeutics and the release of PGE<sub>2</sub>, release of VEGF and subsequent angiogenesis, and/or death of vascular endothelial cells in the tumor microenvironment. All of these are possible mechanisms by which AiP could be induced, and if following administration of chemotherapies, could result in prolific and chemo-resistant tumors.

However, high levels of apoptosis in the tumor microenvironment may reveal novel, effective targets for cancer therapy. If tumors have adopted PS-PSR recognition and other as a means of immune suppression, inhibiting this interaction would prevent efferocytosis and the release of anti-inflammatory cytokines. Similarly, for cancers over-expressing checkpoint inhibitors such as members of the B7 family (i.e. PD-L1, CD80), it

should be possible to block this pathway to prevent T cell exhaustion/anergy. These therapies would drive an appropriate immune response to the tumors and enable the body to clear the cells effectively.

## **DISCUSSION**

Cancer is defined by an imbalance of cellular proliferation relative to cell death, but that does not inherently mean tumor cells are not dying. Imagine a solid tumor as a sphere of cells; while the innermost cells may lack proper vascularization and die by ischemic necrosis, those in the circumferential perimeter act as a shield from immune interaction. These cells are immune-susceptible, as they are exposed to interact with professional phagocytes and T cells. However, by co-opting an apoptotic phenotype and undergoing efferocytotic engulfment, these cells generate immunosuppressive signals in the immediate environment. This tricks the immune system into believing that it has appropriately cleared the apoptotic cells, remaining blind to the growing tumor underneath.

This theory is further complicated by current methods of treatment.

Chemotherapies which induce even greater levels of apoptosis may outweigh proliferation to the point of suppressing tumor growth and diminishing tumor size. However, while the intention of triggering apoptotic death may be to spare patients from a full immune response, this may be worsening the situation. One mechanism for this would be through AiP; as high levels of apoptosis may send signals to the surrounding environment to “re-generate” lost tissue, particularly once chemo- or radiotherapy has

ceased. This phenomenon could be creating opportunities for tumor recurrence and/or chemoresistance.

By discussing the complexities of the interplay between regulated cell death (i.e. apoptosis, necroptosis) and tumor cell growth and development, this thesis offers an introductory glance into how this phenomenon could manifest.

## LIST OF JOURNAL ABBREVIATIONS

Am J Pathol.	American Journal of Pathology
Arthritis Rheum.	Arthritis & Rheumatology
Br J Cancer	British Journal of Cancer
Brain Res.	Brain Research
Cell Bio Int.	Cell Biology International
Cell Death Differ.	Cell Death and Differentiation
Cell Mol Life Sci.	Cellular and Molecular Life Science
Cell Tissue Res.	Cell Tissue Research
Curr Biol.	Current Biology
Curr Med Chem.	Current Medicinal Chemistry
Curr Opin Immunol.	Current Opinion in Immunology
Dev Biol.	Developmental Biology
Dev. Cell	Developmental Cell
EMBO J.	EMBO Journal
EMBO Rep.	EMBO Reports
Eur J Cancer Prev.	European Journal of Cancer Prevention
Exp Cell Res.	Experimental Cell Research
Front Immunol.	Frontiers in Immunology
Genes Dev.	Genes & Development
Immunol Rev.	Immunologic Research
Int J Cancer	International Journal of Cancer

Int J Oncol.	International Journal of Oncology
J Am Acad Dermatol.	Journal of the American Academy of Dermatology
J Cell Bio.	Journal of Cell Biology
J Exp Med.	Journal of Experimental Medicine
J Immunol.	Journal of Immunology
J Innate Immun.	Journal of Innate Immunity
J Insect Physiol.	Journal of Insect Physiology
J Microsc Ultrastruct	Journal of Microscopy and Ultrastructure
J Neurosci.	Journal of Neuroscience
J Pathol	Journal of Pathology
J Vasc Surg	Journal of Vascular Surgery
JIOH	Journal of International Oral Health
Mol Cell Biochem	Molecular and Cellular Biochemistry
Mol Cell Neurosci.	Molecular and Cellular Neuroscience
Mol Cell.	Molecular Cell
Nat Chem Biol.	Nature Chemical Biology
Nat Med.	Nature Medicine
Nat Rev Immunol.	Nature Reviews Immunology
NEJM	New England Journal of Medicine
PLoS Biol.	PLoS Biology
PLoS Genet.	PLoS Genetics
PNAS	Proceedings of the National Academy of Sciences of the United States of America

Radiol Res Pract.	Radiology Research and Practice
Respir Res.	Respiratory Research
Sci Rep.	Scientific Reports
Sci Signal.	Science Signaling
Semin Cancer Biol.	Seminars in Cancer Biology
Trends Neurosci.	Trends in Neuroscience

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## CIRRICULUM VITAE

