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The role of draper in phagocytic competency, corpse processing, and homeostasis

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THE ROLE OF DRAPER IN PHAGOCYTIC COMPETENCY, CORPSE PROCESSING, AND HOMEOSTASIS

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Boston University Graduate School of Arts and Sciences, 2015

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ABSTRACT

The clearance of apoptotic cells is an important process in animal development and homeostasis. Failure to dispose of dead cells leads to developmental defects as well as disease. The removal of dead cells within an organism is accomplished by the process of phagocytosis. Phagocytosis of apoptotic cells is the internalization of a dead cell by another cell. Once internalized, the apoptotic cell is subject to various processing events culminating in the complete degradation of the dead cell. Phagocytosis is carried out by specialized cells, known as professional phagocytes. However, phagocytosis can also be carried out by cells that are specialized for functions other than phagocytosis. These cells are known as nonprofessional phagocytes. Although the process of phagocytosis has been extensively studied, the mechanisms are poorly understood.

To better understand phagocytosis, this dissertation has focused on the Drosophila receptor Draper (Drpr). Drpr is a highly conserved transmembrane protein that has been shown to be crucial for proper phagocytosis. In this dissertation, I report novel roles for Drpr function. Specifically I show that in the ovary of Drosophila
*melanogaster*, the germline cells can be induced to die by starvation and their remnants are engulfed by surrounding epithelial follicle cells. During this process, the dying germline activates Drpr in the follicle cells. Drpr then activates c-Jun N-terminal kinase (JNK) leading to upregulation of Drpr as well as other engulfment genes. These results suggest that these nonprofessional phagocytes need to acquire a phagocytic phenotype to become phagocytic.

We also report that the absence of Drpr in glia leads to an accumulation of apoptotic neurons in the *Drosophila* brain. These dead cells persist throughout the lifespan of the organism and are associated with age-dependent neurodegeneration. Our data indicate that corpses persist because of defective phagosome maturation. Target of rapamycin complex 1 (TORC1) activation in glia is sufficient to rescue corpse accumulation and neurodegeneration. These results suggest that Drpr is important for phagocytic competency, corpse processing, and homeostasis.
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<table>
<thead>
<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>AMP</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>Atg</td>
<td>Autophagy-related gene</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAI-1</td>
<td>Brain-specific angiogenesis inhibitor 1</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>Bsk</td>
<td>Basket</td>
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<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>C. elegans</td>
<td><em>Caenorhabditis elegans</em></td>
</tr>
<tr>
<td>CA</td>
<td>Constitutively activated</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CED</td>
<td>Cell death abnormal</td>
</tr>
<tr>
<td>CRAC</td>
<td>Calcium-released-activated calcium channel</td>
</tr>
<tr>
<td>Crk</td>
<td>CT10 regulator of kinase</td>
</tr>
<tr>
<td>Crq</td>
<td>Croquemort</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole dichloride</td>
</tr>
<tr>
<td>dcp-1</td>
<td>Death Caspase-1</td>
</tr>
<tr>
<td>Diap-1</td>
<td><em>Drosophila</em> inhibitor of apoptosis-1</td>
</tr>
<tr>
<td>DmCaBP1</td>
<td><em>Drosophila melanogaster</em> Calcium Binding Protein 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant Negative</td>
</tr>
<tr>
<td>DOS</td>
<td>Daughter of sevenless</td>
</tr>
<tr>
<td>DRK</td>
<td>Downstream receptor kinase</td>
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<tr>
<td>Drpr</td>
<td>Draper</td>
</tr>
<tr>
<td>DYN</td>
<td>Dynamin</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibition motif</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>LAP</td>
<td>LC3-associated phagocytosis</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-associated protein light chain 3</td>
</tr>
<tr>
<td>LDL</td>
<td>oxidized low-density lipoprotein</td>
</tr>
<tr>
<td>Mbc</td>
<td>Myoblast city</td>
</tr>
<tr>
<td>MEGF</td>
<td>Multiple EGF-like-domains</td>
</tr>
<tr>
<td>MFG-E8</td>
<td>Milk fat globule EGF factor 8</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MJD</td>
<td>Machado Joseph Disease polyglutamine tract protein</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>PBT</td>
<td>Phosphate Buffered Saline buffer + 0.1% Triton X-100</td>
</tr>
<tr>
<td>Ph</td>
<td>Phase</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelial cells</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor interacting protein kinase</td>
</tr>
<tr>
<td>SIMU</td>
<td>Six microns under</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma</td>
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<tr>
<td>SSC</td>
<td>Saline Sodium Citrate</td>
</tr>
<tr>
<td>TAM</td>
<td>Tyro-3-Axl-Mer</td>
</tr>
<tr>
<td>TIM</td>
<td>T-cell immunoglobulin and mucin-domain containing molecule</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TORC1</td>
<td>Target of rapamycin complex 1</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TREM</td>
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Triggering receptor expressed on myeloid cells

Xkr8

Xk-related protein 8
CHAPTER ONE

Introduction

1.1 Programmed cell death

Programmed cell death (PCD) is the genetically encoded process of eliminating unwanted cells in multicellular organisms (Fuchs and Steller, 2011). PCD is crucial for development and tissue homeostasis. It is important in sculpting organs, regulating cell number, and removing infected or oncogenic cells among other things (Barres and Raff, 1999, Lindsten et al., 2000, Vousden and Prives, 2009). Failure in the proper execution of PCD can lead to autoimmunity, neurodegeneration, and cancer (Poon et al., 2014, Tower, 2015). There are many different types of PCD. The three that have been most extensively investigated are apoptosis, autophagic death, and regulated necrosis.

Apoptosis is the canonical form of PCD (Kerr et al., 1972). It is morphologically characterized by cell shrinkage, nuclear and chromatin condensation (pyknosis) and fragmentation (karyorrhexis), followed by membrane blebbing (Tower, 2015). The defining feature of apoptosis is that the process is executed by the activation of cysteine proteases known as caspases (Fuchs and Steller, 2011). Caspases are divided into initiator caspases and effector caspases (Feinstein-Rotkopf and Arama, 2009). Initiator caspases become activated through dimerization due to apoptotic signals. The apoptotic signals can come from the mitochondria (the intrinsic pathway) or from receptor signaling (the extrinsic pathway) (Feinstein-Rotkopf and Arama, 2009). Once dimerized, initiator caspases cleave effector caspases at specific aspartic residues triggering their activation.
Active effector caspases then cleave key intracellular components ultimately leading to cell death.

Autophagy is an intracellular degradation process important for both nutrient recycling during starvation and the clearance of damaged cellular components (Zhang and Baehrecke, 2015). Briefly, autophagy entails the formation of intracellular double membrane vesicles around cytoplasm or organelles. Once the vesicle has enclosed its content, the vesicle is targeted to the endocytic pathway. Vesicles in this pathway will fuse to endosomes and lysosomes culminating in the degradation of the cargo. Disproportionate levels of autophagy have been associated with various forms of cell death (Liu and Levine, 2015). Whether autophagy actively promotes death or is just a morphological characteristic of some forms of cell death still remains controversial (Shen et al., 2012). Autophagic death has been defined as cell death that can be suppressed by inhibition of autophagy by the Nomenclature Committee on Cell Death (Galluzzi et al., 2012). Using this definition then, there are only a few examples where this is the case (Shen et al., 2012). In *Drosophila melanogaster*, it has been shown that loss of function in genes that positively regulate autophagy severely delays the death of the larval midgut cells of the intestine. Conversely, blocking caspases has no effect on the removal of these cells (Denton et al., 2009). To date, this is the only instance in animals where autophagy has been shown to be important for cell death. Nevertheless, there are many instances where blocking autophagy delays death or disrupts the regular process (Marino et al., 2014). Therefore, although not necessarily causative, autophagy still plays an important role in some examples of programmed cell death.
Necrosis is a form of cell death characterized by a gain in cell volume, organellar swelling and disorganized dismantling of intracellular contents. The terminal step of this process is the rupture of the plasma membrane (Galluzzi et al., 2009). Necrosis was originally thought to be a passive process that occurred due to mechanical damage or other forms of injury. Recently, it has emerged that some forms of necrosis are regulated by cellular processes. Specifically, when caspases are inhibited two kinases, known as Receptor interacting protein kinase 1 and 3 (RIP1, RIP3), become engaged during extrinsic apoptotic signaling. Once active, they execute a sequence of events that ultimately leads to necrosis in cells (Cho et al., 2009). Regulated necrosis seems to have evolved as a defense against virus mediated inhibition of caspases. Regulated necrosis therefore allows the execution of cell death when the apoptotic machinery is inhibited. Death of these infected cells through regulated necrosis is important in preventing the spread of infection (Cho et al., 2009). A schematic of the 3 major forms of programmed cell death can be found in Figure 1.1.
Figure 1.1 Simple schematic of the three major forms of programmed death.

In red are the steps necessary for apoptosis to occur. These include caspase activation, nuclear shrinkage and membrane blebbing followed by nuclear fragmentation. The pathway in green corresponds to autophagy. Excessive derepression of autophagy proteins as well as their upregulation leads to high levels of autophagosome formation culminating in massive vacuolization, protein degradation, loss of organelles and consequently, death. In blue is the regulated necrosis pathway. In this pathway caspase inhibition coupled with extrinsic death signals lead to the activation of RIP kinases. Activation of RIP kinases triggers a cascade of events that cause cell swelling and organelle damage culminating in plasma membrane rupture and cell lysis. Adapted from (Tan et al., 2014).
1.2 Apoptosis in *Drosophila*

Apoptosis in the intrinsic pathway of mammals is initiated at the mitochondria (Fuchs and Steller, 2011). Apoptotic stimuli lead to the activation of Bcl-2 family members. These proteins lead to the formation of a pore on the mitochondria that releases molecules that suppress inhibitor of apoptosis proteins (IAPs), a family of proteins that bind to caspases and inhibit their function, and molecules that activate initiator caspases. In *Drosophila*, permeabilization of the mitochondria is not necessary for apoptosis to occur. Instead, apoptotic signaling typically activates a group of proteins known as IAP antagonist (Miura, 2012). *Drosophila* has four IAP antagonists; Reaper, Hid, Sickle and Grim. Once activated, these proteins will bind to and ubiquitinate IAP leading to its degradation. Degradation of IAP leads to de-repression of the initiator caspase, Dronc. Dronc then activates the effector caspases dcp-1 and Drice leading to the execution of apoptosis.

1.3 Phagocytosis

A common feature of programmed cell death in multicellular organisms is the formation of cellular corpses that need to be removed. The most widespread form of dead cell clearance is phagocytosis. Phagocytosis is a form of endocytosis that involves the internalization of a solid particle into a vesicle within a cell (Ravichandran and Lorenz, 2007). Unlike some other forms of endocytosis, the end goal of phagocytosis is the degradation of the particle within the cell that internalized it. This process can be carried out by cells that surround the cellular corpse. Additionally, specialized cells can be
recruited to the site of death to phagocytose the corpse. The cell that executes the phagocytic response is termed a phagocyte. The types of particles that normally get internalized are pathogens, dead cells or other forms of cellular debris. Failure to remove these particles can result in disease (Elliott and Ravichandran, 2010). Therefore, phagocytosis is a crucial process for organismal homeostasis. The main focus of this thesis will be on the removal of dead cells by phagocytes.

1.3.1 Types of phagocytes

When a cell dies, it needs to be removed from the site of death. To do so, the organism normally employs phagocytes to internalize the dead cell and subsequently destroy it. Phagocytes are any cells that are capable of phagocytosis. They can be divided into two different subtypes: professional and nonprofessional phagocytes (Hochreiter-Hufford and Ravichandran, 2013). Professional phagocytes are cells whose main role in an organism is to phagocytose dead cells. The three main phagocytic cell types in mammals are macrophages, immature dendritic cells, and neutrophils. Macrophages are responsible for most of the removal of dying cells (Hochreiter-Hufford and Ravichandran, 2013). Macrophages can be further subdivided into circulating macrophages and tissue-resident macrophages (Gordon and Taylor, 2005). Circulating macrophages are found in the blood stream. In response to cell death, macrophages will leave the blood to enter the tissue undergoing cell death. To enter the tissue, macrophages will permeate through capillary walls in a process known as diapedesis (Nourshargh and Alon, 2014). Once at the site of death, macrophages will actively phagocytose the dead
cells. Tissue resident macrophages are heterogeneous group of macrophages that populate and reside within the different organs in an animal. It has been shown that this subgroup has evolved specialized functions suited for the tissue in which they reside (Okabe and Medzhitov, 2014). Examples include the phagocytosis of synapses by microglia (the tissue resident macrophage of the brain) or bone reabsorption by osteoclasts (Murray and Wynn, 2011, Schafer et al., 2012).

Nonprofessional phagocytes are cells within an organism that are not specialized to phagocytose but can become phagocytic under certain conditions. Examples of nonprofessional phagocytes include epithelial cells, neuronal progenitors, and mesenchymal cells (Ichimura et al., 2008b, Jones et al., 2008a, Lu et al., 2011a). It is thought that nonprofessional phagocytes are not as efficient as professional phagocytes in proper cell clearance (Parnaik et al., 2000b). However, some animals such as *Caenorhabditis elegans* (*C.elegans*), are completely devoid of professional phagocytes, yet are efficient in removing dead cells (Mangahas and Zhou, 2005). Whether nonprofessional phagocytes have specialized phagocytic functions based on the tissue that they reside (akin to tissue-resident macrophages) is poorly understood. There are a few examples though nonprofessional phagocytes seem to have specialized phagocytic functions. One such example is the removal of less active synapses by astrocytes (Chung et al., 2013). Failure to phagocytose synapses results in aberrant connectivity within the brain. It remains to be seen how widespread the specialized phagocytic functions of nonprofessional phagocytes are.
1.3.2 Mechanisms of phagocytosis

Phagocytosis of dead cells can be subdivided into three steps (Figure 1.2) (Elliott and Ravichandran, 2010). The first step involves the recognition of the dead cell by the phagocyte via exposure of “eat me” signals, on the membrane of the dead cells. The phagocyte employs a complementary array of recognition receptors that bind directly, or indirectly through bridging molecules, to the “eat me” signal. In the second step, the binding of a recognition receptor to an “eat me” signal triggers a cascade of intracellular events that lead to cytoskeletal rearrangements. Specifically actin rearrangements allow the phagocyte to protrude membrane extensions around the dying cell, forming a phagocytic cup. This results in the internalization of the dead cell into a vesicle, known as a phagosome, within the phagocyte. In the third step, the phagosome undergoes successive fusion events to endosomes and lysosomes, culminating in the complete degradation of the apoptotic cell within the phagocyte (also known as corpse processing or phagosome maturation).
Figure 1.2 Schematic of the phagocytosis of dead cells.

Phagocytosis entails three steps: recognition, internalization, and corpse processing. Recognition is mediated by recognition receptors binding to “eat me signals on the surface of the dead cell. This triggers intracellular signaling that lead to cytoskeletal rearrangements resulting in the internalization of the dead cell into a vesicle within the phagocyte. The vesicle containing the dead cell then undergoes subsequent fusions to endosomes and lysosomes culminating in the complete degradation of the dead cell. Adapted from (Hochreiter-Hufford and Ravichandran, 2013).
To date, many different “eat me” signals have been identified. Examples of “eat me” signals include Calreticulin, Intracellular adhesion molecule 3 (ICAM-3) and oxidized low-density lipoprotein (LDL)-like moiety (Gardai et al., 2005, Ravichandran and Lorenz, 2007, Hochreiter-Hufford and Ravichandran, 2013). The best studied “eat me” signal is exposure of phosphatidyl serine (PS) (van den Eijnde et al., 1998). PS exposure has been shown to be widespread and conserved across organisms. In healthy cells PS distribution is maintained asymmetrically, with the bulk of it being found on the inner leaflet of the plasma membrane. A group of ATP-dependent translocases are responsible for this irregular distribution (Balasubramanian and Schroit, 2003). Upon the induction of death, PS gets externalized on the outer leaflet of the plasma membrane. Recent evidence has pointed to the existence of a phospholipid scramblase that actively externalizes PS during cell death (Suzuki et al., 2013). The protein has been identified as Xk-related protein 8 (Xkr-8) in mammals. Xkr-8 is a six transmembrane domain scramblase. Upon the induction of apoptosis, Xkr-8 gets cleaved by caspases at a specific residue resulting in its activation. Knockdown of Xkr-8 or disruption of the caspase cleavage site result in PS failing to externalize on the surface of dead cells. Apoptotic cells where PS is not externalized on the outer leaflet of the plasma membrane fail to get recognized by phagocytes. The Xkr-8 homolog in worms, Cell death abnormal protein 8 (CED-8), shows a high level of conservation with Xkr-8. CED-8 was also shown to be important for PS externalization (Suzuki et al., 2013).

Most of the work done on the process of phagocytosis has focused on recognition. Unlike “eat me” signals, there does not seem to be a universal recognition receptor
analogous to PS. Recognition receptors can be divided into 3 subtypes. The first type are those that bind to the “eat me” signal directly and have intracellular signaling capacities. Examples of such receptors include Brain-specific angiogenesis inhibitor 1 (BAI-1) which binds directly to PS and activates internalization through G-protein coupled signaling on the intracellular portion of the protein (Park et al., 2007). The second type are those that bind to the “eat me” signal indirectly through the use of bridging molecules. The Tyro-3-Axl-Mer (TAM) receptor family and integrins fall under this category. Specifically, TAM receptors and integrins bind indirectly to PS by binding to Protein S or Gas 6, in the case of TAM receptors, and Milk fat globule EGF factor 8 (MFG-E8) in the case of integrins (Nagata et al., 2010). Finally, there are those that bind to “eat me” signals directly but do not have intracellular signaling capabilities. For example, T-cell immunoglobulin and mucin-domain 4 (TIM-4) has been shown to bind directly to PS. However, it has a small transmembrane domain and fails to signal intracellularly (Park et al., 2009). It is thought to play the role of a tethering receptor, stabilizing the interaction between the apoptotic cell and the phagocyte which allow other recognition receptors to bind to the “eat me” signal and trigger an intracellular response.

Upon binding to an “eat me” signal, recognition receptors trigger a set of intracellular events that lead to cytoskeletal rearrangements. Genetic studies in C. elegans have identified two partially redundant signaling pathways that control internalization: the ced-1, 6, 7 and ced-2, 5, 12 pathways (Mangahas and Zhou, 2005, Pinto and Hengartner, 2012). Briefly, CED-1, a recognition receptor, activates CED-6 and Dynamin (DYN). At the same time integrins activate the CED 2-5-12 trimolecular
complex that has guanine nucleotide exchange activity. CED-6, Dynamin, and CED 2-5-12 then converge on CED-10, a Rac GTPase, leading to its activation. Once active, CED-10 triggers actin rearrangements that allow for internalization of the cell corpse. CED-7 also seems to work in conjunction with CED-1 and 6 but the mechanism is unclear. These two partially redundant pathways show a high degree of conversation from flies to mammals as shown in Figure 1.3.
The CED-1, 6, 7 and the CED-2, 5, 12 pathways converge on CED-10 to promote engulfment. Upon dead cell recognition, CED-1 recruits CED-6 and DYN. At the same time CED 2, 5, and 12 form a trimolecular complex which is thought to form in response to integrin signaling. Together these components activate CED-10 leading to actin rearrangements. The role of CED-7 is yet unknown but epistasis analysis suggest it works together with CED-1 and 6. The components of the pathway were first identified in *C. elegans* (black). The mammalian orthologs are listed in red. *Drosophila* orthologs are listed in purple. Modified from (Logan and Freeman, 2007).
A vesicle containing the dead cell that has been internalized (known as a phagosome), has to undergo subsequent fusions to endosomes and lysosomes to degrade the corpse within it. Phagosomes recruit Rab proteins; small GTPases that act as tethering factors to promote the fusion between intracellular organelles (Lu and Zhou, 2012). Specifically, work in *C. elegans* has shown that when a vesicle gets internalized, class III Phosphatidylinositol (PI) 3 Kinases, together with dynamin, change the membrane composition of the vesicle and recruit Rab5. Rab5 then recruits various other proteins that allow the fusion of the vesicle to an endosome. The process then continues with further lipid rearrangements on the vesicle as well as the recruitment of Rab7 to the vesicle. Rab7, as with Rab5 (which now has left the vesicle), recruits a set of proteins that allow the fusion of the vesicle to Lamp positive lysosomes. At this point, the lumen of the vesicle has significantly increased in acidification allowing for the activation of hydrolases and proteases that will degrade the corpse inside. Work in zebrafish has also shown that acidification of both the vesicle and the lysosome is important for proper fusion of these two organelles (Peri and Nusslein-Volhard, 2008). A schematic representation of phagosome maturation can be seen in Figure 1.4
Figure 1.4 Phagosome fusion to endosomes and lysosomes during corpse processing.

Fusion of endosomes and lysosomes to phagosomes requires the concerted effort of Rab proteins as well as lipid rearrangements on the vesicle. Subsequent fusions along the endocytic pathway lead to an increase acidity within the vesicle necessary for the proper processing of the corpse. Adapted from (Lu and Zhou, 2012).
Macroautophagy (referred to as autophagy) is an important process by which cells recycle organelles or proteins (Zhang and Baehrecke, 2015). This process serves as a quality control mechanism or in bouts of low nutrition, allowing the cell to survive by breaking down intracellular components into nutrients. During autophagy, organelles or portions of cytoplasm get internalized into double membrane vesicles known as autophagosomes. As with phagocytosis these vesicles get degraded by being shuttled into the endocytic pathway. Autophagy begins with inhibition of TORC1. TORC1 is a multi-protein complex that inhibits the canonical initiator of autophagy, Atg-1 (Zhang and Baehrecke, 2015). Inhibition of TORC1 disinhibits Atg-1, leading to the recruitment of various factors that form an isolation membrane termed the nascent phagosome. This nascent phagosome will then give rise to a full vesicle containing cytoplasmic space. An important component of this process is LC3 lipidation of the nascent autophagosome. During autophagy other Atg proteins lipidate LC3, the mammalian homolog of Atg-8, onto nascent membranes that begin to wrap around organelles or cytoplasm. This lipidation process has been shown to be important in internalizing the components into the autophagosome as well as targeting this vesicle to the endocytic pathway. In recent years it has been observed that these Atg proteins play a role in phagosome maturation during a process known as LC3-associated phagocytosis (LAP) (Florey and Overholtzer, 2012). In this type of phagocytosis, LC3 is lipidated onto vesicles containing dead cells. It is thought that lipidation of LC3 onto phagosomes is important for fusion to the lysosomes, however this remains poorly understood. LAP displays a large amount of variability as to which proteins are responsible for LC3 lipidation onto the phagosome. In
autophagy, LC3 get lipidated on nascent isolation membranes through two conjugation systems working together: the Atg5-12-16 system and the Atg3-7-4 system. In LAP, however, the requirement of these conjugation systems is variable for proper LC3 lipidation. For instance, macrophage dependent processing of dead cells requires both Atg5 and Atg7 (Martinez et al., 2011). In C.elegans, processing of dead Q-neuroblast does not require either of these proteins but is still dependent on LC3 (Li et al., 2012). Differences may be organism dependent or may be due to differences in professional versus nonprofessional phagocytes. A conserved feature of LAP is that it does not require activation of Atg1, the initiator of canonical autophagy. LAP is a newly described phenomenon and more work needs to be done to uncover how it participates in corpse processing as well as how widespread it is. A schematic representation of autophagy is shown in figure 1.5

Figure 1.5 Overview of the autophagic pathway

Autophagy starts when Tor becomes inhibited leading to the activation of Atg1 and then the subsequent steps of autophagy, culminating in cytoplasmic components becoming internalized into a vesicle. Adapted from (Zirin and Perrimon, 2010)
1.3.3 Phagocytosis in homeostasis and disease

The phagocytosis of dead cells is crucial for organismal homeostasis. Failure to properly dispose of dead cells can lead to disease. The main outcome of improper cell removal is the formation of secondarily necrotic cells. In secondary necrosis, dead cells that had died through non necrotic like mechanisms, acquire morphological features of necrotic cells. Rounding of the cell starts to occur, followed by cytoplasmic swelling, rupture of the plasma membrane and spilling of the intracellular content (Vanden Berghe et al., 2010). The spilling of the intracellular content is thought to be toxic to the surrounding cells.

The most common consequence of secondary necrosis is inflammation and the onset of autoimmunity (Poon et al., 2014). Phagocytosis of apoptotic corpses leads to the production of anti-inflammatory molecules, such as transforming growth factor beta (TGF-β) and interleukin-10 (IL-10). Failure to remove these corpses prevents the release of these signals. Necrotic morphology, as a result of secondary necrosis, is recognized by the immune system as a sign of infection. The absence of anti-inflammatory signals, coupled with the formation of secondary necrotic cells triggers the release of pro-inflammatory molecules. Pro-inflammatory molecules, such as the release of reactive oxygen species and the production of death inducing ligands (e.g. tumor necrosis factor (TNF)), are thought to damage the surrounding tissue (Elliott and Ravichandran, 2010). In an attempt to clear the secondarily necrotic cell, organisms with an adaptive immune response will start producing antibodies that recognize components of the cell. Since those components are also present on healthy cells, the system starts eliciting an immune
response against the healthy cells as well. This leads to the onset of autoimmunity within the organism.

1.3.4 Phagocytosis in Drosophila melanogaster

Phagocytosis in Drosophila, as in mammals, is carried out by both professional and nonprofessional phagocytes. The professional phagocytes of Drosophila are a subgroup of blood cells known as plasmatocytes (Hartenstein, 2006). Like macrophages in mammals, they are found in the blood and have become specialized to phagocytose pathogens as well as dead cells. Unlike mammals, Drosophila do not have tissue resident macrophage equivalents. Many different cell types have been found to be nonprofessional phagocytes in Drosophila. These include glia cells, epidermal cells, and epithelial cells (Freeman et al., 2003, Ohsawa et al., 2011, Han et al., 2014). Nonprofessional phagocytes have been shown to be important not only in dead cell clearance but in synaptic pruning, cell-cell competition, and development (Awasaki et al., 2006, Li and Baker, 2007). Therefore the engulfment capabilities of nonprofessional phagocytes may be specialized depending on the tissue they reside, however this remains to be explored (Jenkins et al., 2013).

1.3.5 Draper dependent phagocytosis in Drosophila

One of the most important components of Drosophila phagocytosis is the CED-1 ortholog, Draper (Drpr). Drpr has been shown to play a central role in all forms of apoptotic cell clearance studied to date, either by professional phagocytes or
nonprofessional phagocytes (Freeman et al., 2003, Manaka et al., 2004, Fullard et al., 2009). In addition, Drpr is important for some forms of pathogen phagocytosis, synaptic pruning, removal of cellular debris, and cell elimination during cell competition (Awasaki et al., 2006, MacDonald et al., 2006, Li and Baker, 2007, Hashimoto et al., 2009). Drpr is a single pass transmembrane protein and plays a role as a recognition receptor (Freeman et al., 2003). The drpr gene encodes three isoforms, Drpr I, II, and III (Logan et al., 2012). These isoforms vary in the number of extracellular EGF-like repeats as well as sequences within the intracellular portion of the protein. One of these sequences is the immunoreceptor tyrosine-based activation motif (ITAM) present in Drpr I. ITAM motifs are important for signaling transduction of the immune cascade of many recognition receptors and are defined by the amino acid sequence YXXI/LX_{6-12}YXXI/L (Underhill and Goodridge, 2007). Drpr-I has been demonstrated to be required for proper engulfment. The ITAM motif within the Drpr-I isoform is partially responsible for its engulfment function. Specifically, data suggests that upon Drpr I activation, a member of the Src family of kinases, Src42A, phosphorylates Drpr I at the ITAM motif (Ziegenfuss et al., 2008). Phosphorylation of this sequence leads to the recruitment and activation of the non-receptor tyrosine kinase, Shark. The Drpr, Shark complex seems to be required for Rac-1 activation through an unknown mechanism (Ziegenfuss et al., 2012). Rac-1 activation leads to actin rearrangements important for the internalization of the apoptotic corpse. It has also recently been shown that the Drpr-Shark complex is important for the activation and nuclear localization of the transcription factor STAT92E (Doherty et al., 2014). It has been suggested that STAT92E directly upregulates Drpr by binding to the
Drpr upregulation seems to lead to more Drpr on the surface of the membrane. This amplifies the Drpr signaling response which is important for proper phagocytosis to occur.

The second isoform, Drpr II, differs because it lacks the ITAM motif and instead has an immunoreceptor tyrosine-based inhibition motif (ITIM). ITIM motifs have been shown to negatively regulate receptors that bear ITAMs through the recruitment of phosphatases (Underhill and Goodridge, 2007). Consequently, Drpr II has been shown to be required to terminate the phagocytic response (Logan et al., 2012). Specifically, it was shown that drpr II transcriptional induction occurs after Drpr I is upregulated in glia cells responding to axonal debrs. Drpr II recruits Corkscrew, a Src homology 2 (SH2) domain–containing protein tyrosine phosphatase. Corkscrew then dephosphorylates the ITAM motif of Drpr I preventing Shark from associating with Drpr I and restoring Drpr to basal levels. Overexpression of Drpr II or Corkscrew prevents Drpr dependent phagocytosis from occurring. Together, these experiments suggest that Drpr I is important for the execution of phagocytosis and Drpr II is important for terminating the response (Logan et al., 2012). The function of the Drpr III isoform remains unknown.

All 3 isoforms contain an amino acid sequence, known as the NPXY motif, on the intracellular portion of the protein. This NPXY motif has be shown to be important for the recruitment of the Drosophila homolog of CED-6, also called Ced-6 (Fujita et al., 2012). Ced-6 recruitment to Drpr has been shown to be an important mediator of phagocytosis in a variety of contexts. Ced-6 seems to be important for Rac-1 activation
like its homolog in worms (Ziegenfuss et al., 2012). Why the other Drpr isoforms have a recruitment site for Ced-6 is not clear. It could be, at least for Drpr II, Ced-6 recruitment to the other isoforms prevents its activation of Drpr I. This would further contribute to the termination of the phagocytic response which remains to be explored.

Drpr is thought to act as a recognition receptor. Phagocytosis of dead cells in *Drosophila* also involves the expression and recognition of “eat me” signals. Four “eat me” signals have been described in *Drosophila*. They are PS, Calreticulin, Drosophila melanogaster calcium-binding protein 1 (DmCaBP1), and Pretaporter (Kuraishi et al., 2007, Kuraishi et al., 2009b, Okada et al., 2012, Tung et al., 2013). All four are externalized on the surface of the dying cell. Loss of Calreticulin, DmCaBP1 or Pretaporter, as well as blocking PS, have been shown to lead to defects in phagocytosis. However, these experiments were conducted in professional phagocyte-mediated engulfment and it remains to be seen if such results extend to nonprofessional phagocytes. Interestingly, Calreticulin, *Drosophila melanogaster* Calcium Binding Protein 1 (DmCaBP1), and Pretaporter all reside in the endoplasmic reticulum (ER) of healthy cells. Previous studies in mammals have shown that under certain forms of cell death, Calreticulin gets targeted to the surface of the membrane by chaperones as a result of Calcium (Ca^{2+}) depletion in the ER (Peters and Raghavan, 2011). The above experiments were all conducted in plasmatocytes that were treated with cells that had undergone cycloheximide-induced apoptosis. Whether these proteins play a role in other forms of cell death is unclear.
Drpr has been shown to bind to 3 out of the 4 “eat me” signals characterized above. In particular, research has suggested that Drpr binds to PS, DmCaBP1, and Pretaporter (Kuraishi et al., 2009b, Okada et al., 2012, Tung et al., 2013). Why Drpr binds to three different ligands is unknown. The above experiments were all done in the same system under similar experimental conditions. This indicates that Drpr binds to all three ligands suggesting a potential synergy necessary for proper binding. Alternatively, it has been proposed that different dying cells express different patterns of “eat me” signals (Birge and Ucker, 2008). This theoretically would allow for a context specific phagocytic response. Therefore, Drpr may act as a pattern recognition receptor activating different downstream signaling cascades depending on the pattern present. Such function has normally been assigned to activation of a combination of recognition receptors but in *Drosophila* it may be more limited.

Ca$^{2+}$ has been show to play an important role in phagocytosis. Ca$^{2+}$ has been shown to be important for increasing the number of scavenger receptors, a type of recognition receptor, on the surface of a phagocyte (Beppu et al., 2001). It has also been shown to be important for microglia migration to the site of cell death (Sieger et al., 2012). In *Drosophila*, Ca$^{2+}$ signaling is also important for phagocytosis and this depends on Drpr (Cuttell et al., 2008). In phagocytes, data suggests that Drpr is in a complex with a junctophilin known as Undertaker. Junctophilins are proteins that connect the ER to the plasma membrane. Upon recognition of an apoptotic cell, Drpr, together with Ced-6, activates the ryanodine receptor on the ER through an unknown mechanism. Activation of the Ryanodine receptor triggers an influx of Ca$^{2+}$ from the ER to the cytosol. Due to
the apposition of the ER to the plasma membrane (due to Undertaker), calcium influx from the ER is sufficient to open Calcium-released-activated calcium (CRAC) channels. This allows a further influx of Ca\textsuperscript{2+} into the cell from the extracellular space. Blocking CRAC mediated Ca\textsuperscript{2+} influx into the cell prevent proper phagocytosis from occurring. However, how extracellular Ca\textsuperscript{2+} influx promote phagocytosis in Drosophila is unclear.

Drpr has also been shown to be involved in corpse processing. It was found that in Drosophila embryos, drpr null glia were still able to phagocytose dead neurons (Kurant et al., 2008). However, the dead neurons persist and accumulate inside of glia. This phenotype has only been reported in Drosophila embryos. In adults, drpr null phagocytes fail to internalize debris. Most studies on Drpr function have been carried out in adults, therefore understanding how Drpr plays a role in corpse processing has been difficult. Since debris fails to be internalized in adults defects in phagosome maturation cannot be observed because vesicle containing corpses are not present in the phagocyte. Recently it was found that Drpr is important for the upregulation of Downstream of receptor kinase (DRK), Daughter of sevenless (DOS), and the guanine nucleotide exchange factor Son of sevenless (SOS) in phagocytic glia (Lu et al., 2014). Knockdown of DRK, DOS, or SOS leads to a reduced internalization of debris. Reduced internalization of debris appears to be a result of incomplete activation of Rac-1. The authors also reported a decrease in LysoTracker (a marker of lysosomes) within glia, arguing that phagosome maturation may be disrupted. Since drpr mutant glia still internalize debris, albeit at a reduced levels, it is unclear whether the decrease in LysoTracker came from defects in processing or due
to a decreased number of the vesicles that needed to be processed. A schematic of Drpr and its signaling components can be found in Figure 1.5

Drpr function and structure is highly conserved in mammals. Drpr has three mammalian orthologs: Multiple EGF-like-domains 10 (MEGF10), MEGF11, and Jedi-1. All three are transmembrane receptors with phagocytic capabilities. MEGF10 and Jedi-1 have been shown to be important in nonprofessional phagocytosis by glia, both in the brain and in the PNS (Wu et al., 2009b, Chung et al., 2013). As with Drpr, MEGF10 and Jedi-1 get phosphorylated at their ITAM motif by Src family kinases. This phosphorylation allows the mammalian Shark ortholog, Syk, to bind to these receptors. Syk binding to either MEGF10 or Jedi-1 is important for proper phagocytosis (Scheib et al., 2012). Finally, the Ced-6 mammalian homolog, GULP, has been shown to bind Jedi-1 at its NPXY motif (Sullivan et al., 2014). This interaction is also important for the phagocytosis of dead cells.
Figure 1.6 Drpr, its ligands and signaling components.

Drpr is a single pass transmembrane protein that acts as a recognition receptor. Drpr I recognizes dead cells by binding to DmCABP1, PS or Pretaporter and as a response activates a multitude of components to ensure proper phagocytosis. Specifically, Drpr I gets phosphorylated by Src42a (red circle) which causes Shark to be recruited to Drpr I. Active Drpr I also recruits Ced-6. Drpr I, Ced-6, and Shark are thought to activate the DRK/DOS/SOS complex. Drpr I, Ced-6, and Shark together with the DRK/DOS/SOS complex activate Rac-1 leading to cytoskeletal reorganization and STAT92E mediated upregulation of Draper. Ced-6 is also recruited to Drpr I. Drpr I and Ced-6 have been shown to be important for the apposition of the ER to the plasma membrane through the junctophilin Undertaker. Apposition of the ER to the plasma membrane is important for ryanhodine receptor mediated Ca^{2+} influx from the ER which opens CRAC channels.
causing further increases in Ca²⁺ important for proper phagocytosis to occur. Once a corpse has been cleared, Drpr-II signals to terminate the response.

1.3.6 Additional important components for phagocytosis in Drosophila

Aside from Drpr, three other recognition receptors have been described in Drosophila. They are Croquemort (Crq), Six microns under (SIMU), and Integrins. All of these except SIMU have been shown to be important for professional phagocytosis, for both the engulfment of dead cells and bacteria (Franc et al., 1999, Cuttell et al., 2008, Nonaka et al., 2013). In addition, they have all been shown to be important for nonprofessional phagocytosis (Freeman et al., 2003, Kurant et al., 2008, Han et al., 2014) (Meehan et al., unpublished). The downstream signaling response executed by these receptors remains unclear. Crq has been shown to be important for preventing vesicles containing corpses to fuse with other corpse containing vesicles once internalized (homotypic fusion) (Han et al., 2014). Instead, Crq allows the phagosome to fuse with other components of the endocytic pathway (heterotypic fusion), allowing for proper processing of the dead cell. SIMU does not have a large intracellular domain, and the intracellular portion of SIMU is dispensable for its role in phagocytosis (Kurant et al., 2008). Therefore SIMU is thought to play the role of a tethering receptor. No downstream components have been identified for integrins in Drosophila. However, it is possible that integrins signal through CED-2, 5, 12 as they do in C.elegans.
The activation of recognition receptors leads to internalization of the dead cell through cytoskeletal rearrangements. As mentioned above, the ced-1, 6, 7 and ced-2, 5, 12 pathways work in a partially redundant manner to rearrange the cytoskeleton. In *Drosophila*, many of the components that make up the ced-1, 6, 7 and ced-2, 5, 12 pathways, as well as their interactions, are conserved. As mentioned in the previous section, Drpr and Ced-6 signal to Rac-1, just as CED-1 and CED-6 activate CED-10 in worms. However, unlike *Drosophila*, *C. elegans* does not have a Shark ortholog (Steele et al., 1999). The *Drosophila* orthologs of CED-2, 5, and 12 have also been implicated in phagocytosis. They are CT10 regulator of kinase (Crk), Myoblast city (Mbc), and Ced-12 respectively and have been implicated in both professional phagocytosis and nonprofessional phagocytosis (Van Goethem et al., 2012, Ziegenfuss et al., 2012). Like in *C. elegans*, it appears that the Crk/Mbc/Ced-12 pathway, together with the Drpr/Ced-6 pathway, converge on Rac-1 for proper phagocytosis. Unlike in *C. elegans* though, it seems there is a temporal dynamic to pathway activation. Specifically, the Drpr/Ced-6 pathway activates first, followed by the activation of the Crk/Mbc/Ced-12. This suggests that in *Drosophila* they may not be partially redundant but instead be required for different processes within the phagocytic response (Ziegenfuss et al., 2012).

The mechanisms of corpse processing remain understudied in *Drosophila*, as with other organisms. Outside of the known, shared components of endocytic trafficking, such as Rab5 and Rab7, only a few phagocytosis specific components have been identified. As previously mentioned, it appears that both Crq and Drpr have a role in corpse processing. A recent publication highlighted the role of another protein, Debris buster, in this process.
Like Crq, Debris buster is a member of the CD36 class of scavenger receptors. Interestingly, its role is not in recognition or internalization. Instead it seems that Debris buster is important for phagosome fusion to the lysosome downstream of Rab7 (Han et al., 2014).

1.4 The Drosophila ovary as a model for nonprofessional phagocytosis

The Drosophila female reproductive apparatus is composed of two ovaries. These ovaries continuously produce eggs that can be fertilized and give rise to new offspring. Each ovary is composed of 15-20 ovarioles. Ovarioles are chains of progressively developing egg chambers that mature as they are pushed towards the posterior of the ovary (Figure 1.6) (King, 1970, Spradling, 1993). Egg chambers originate from a specialized region at the anterior of the ovariole known as the germarium, which harbors germline and somatic stem cells. Egg chambers are 16-cell germline cysts, comprised of a single oocyte and 15 nurse cells that support the oocyte. Oogenesis is comprised of 14 stages. At the end of oogenesis, the nurse cells dump their cellular contents into the oocyte. At this point, the egg is now formed and can be fertilized. Each egg chamber is surrounded by a layer of somatically derived epithelial cells known as the follicle cells. The somatically derived follicle cells are responsible for various supportive functions throughout oogenesis such as yolk, vitelline membrane, and chorion synthesis (McCall, 2004, Wu et al., 2008, Pritchett et al., 2009, Jenkins et al., 2013).
Figure 1.7 Development and PCD in the *Drosophila* ovary.

A) Schematic of *Drosophila* ovariole. Egg chambers develop through 14 different stages of development. Towards the later portion of oogenesis the nurse cells dump their contents into the developing oocyte. The end of this process culminates in the formation of a fertilizable egg. B-F) In response to starvation, the germarium and mid-stage (stages 7-9) egg chambers undergo caspase dependent PCD. The surrounding follicle cells then phagocytose the germline debris (white arrow in f). NC, nurse cell. FC, follicle cell. O, oocyte. Adapted from (Bass et al., 2009).
The *Drosophila* ovary is a powerful model system for the study of cell death. Cell death occurs at distinct stages throughout oogenesis: PCD occurs in response to environmental stress such as starvation in the germarium and during mid-oogenesis (stages 7-9), and in response to developmental signals during late oogenesis (Giorgi and Deri, 1976, McCall, 2004, Pritchett et al., 2009) (Figure 1.6). The work done in this thesis will focus on death during stages 7-9 of mid-oogenesis.

During mid-oogenesis (stages 7-9), the germline undergoes PCD in response to a decrease in amino acids. The synthesis and uptake of yolk protein occurs during mid-oogenesis and is an energetically expensive process. Mid-oogenesis is therefore thought to be a checkpoint where nutritional conditions can be surveyed prior to investing further energy in to egg development (Giorgi and Deri, 1976, Jenkins et al., 2013). PCD during mid-oogenesis is apoptotic. Upon death, germline nuclei undergo karyorrhexis and pyknosis, two hallmarks of apoptosis. Moreover, PCD in the germline is caspase dependent. Specifically, egg chambers that lack the effector caspase dcp-1 fail to undergo cell death. Instead the germline acquires an undead phenotype, where the chromatin fails to fragment and condense (Jenkins et al., 2013).

The final step in the removal of the dying germline during mid-oogenesis is engulfment by the surrounding follicle cells (Figure 1.6). The follicle cells need to transition from being epithelial cells with developmental roles to functional phagocytes during PCD. Consequently, the *Drosophila* ovary provides a tractable system in which to
study nonprofessional phagocytosis. Prior to the work presented in this dissertation, the mechanisms of engulfment were completely uncharacterized in the Drosophila ovary.

1.5 The Drosophila brain

The adult Drosophila brain is composed of two cell types: neurons and glia (Ito and Awasaki, 2008). Neurons compose the majority of cells within the brain. As with mammals, neurons are important for the transmission and processing of information. The structure of neurons in Drosophila differs from mammals. Specifically, Drosophila neurons are unipolar in nature (Rolls, 2011). Unlike bipolar or multipolar neurons, unipolar neurons have only one neurite projecting from the cell body. The neurite then bifurcates giving rise to dendrites and axons. Partially due to this structure, the majority of neuronal cell bodies are localized on the surface of the brain (Figure 1.7). From the cell bodies, neurites project into the brain where they form synaptic connections with other neurons. The aggregation of synapses inside the brain gives rise to neuropiles. Different neuropiles form distinct anatomical and functional units under which the brain is organized (Ito et al., 2014).
Figure 1.8 Neuronal structure and cell body architecture in the *Drosophila* brain.

A) *Drosophila* neurons are unipolar in nature, and project their axons and dendrites into the brain. B-C) Cell bodies of neurons (white) are localized on the surface of the *Drosophila* brain. B is anterior view. C is posterior view. aimpr, anterior inferior medial protocerebrum. AL, antennal lobe. SOG, suboesophageal ganglion. vlpr, ventrolateral protocerebrum. LCBR, lateral cell body region. ca, mushroom body calyx. CC, coronal commissure. Adapted from (Ito and Awasaki, 2008).
Glia compose 10-15 percent of the cells within the adult *Drosophila* brain, as opposed to 85-90 percent in the human brain (Hartenstein, 2011). Glia have a wide range of functions, from ensuring proper neuronal communication to maintaining homeostasis. The adult *Drosophila* brain is composed of three main classes of glia based on their localization within the brain: surface, cortex, and neuropile glia (Stork et al., 2012). These can be further subdivided into morphologically distinct groups (Figure 1.8).

There are two types of surface associated glia: perineurial and subperineurial glia (Stork et al., 2012). As their name implies, they are localized on the surface of the brain, above the layer containing neuronal cell bodies. Together, they form the blood brain barrier (BBB) through cell-cell tight junctions. The BBB serves to isolate and protect the brain from pathogens and toxic substances.

There is only one type of cortex glia which is simply known as cortex glia (Stork et al., 2012). Cortex glia are found within the neuronal cell body layer. They project processes that wrap around individual neuronal cell bodies on the surface. One cortex glia can enwrap dozens of neurons at the same time. They are thought to provide structural, trophic, and metabolic support to the neurons.

Neuropile glia within the brain are composed of two cell types: ensheathing glia and astrocyte-like glia (Stork et al., 2012). Ensheathing glia are associated with the neuropile and work to ensure structural as well as functional connectivity between neurons. Specifically, ensheathing glia cover and wrap around individual neuropiles. They are important in providing the structural divisions between neuropiles. Astrocyte-
like glia project into the neuropile. Like their mammalian counterparts, they associate with synapses themselves and are important for proper communication between neurons by regulating various aspects of neurotransmitter release.

Glia are the main phagocytes within the *Drosophila* brain. Like the follicle cells in the *Drosophila* ovary, glia are nonprofessional phagocytes. They are important for the removal of apoptotic corpses during development, synaptic pruning, and clearance of injured cells (Awasaki et al., 2006, MacDonald et al., 2006, Kurant et al., 2008). Three of the five differentiated glia subtypes have been shown to play a role in phagocytosis: ensheathing, cortex, and astrocyte glia. In adults, ensheathing glia and cortex glia seem to be the main phagocytes (Doherty et al., 2009). Ensheathing glia are responsible for the removal of damaged axons within the adult neuropile. In a model of axonal injury, ensheathing glia permeate into the neuropile that they ensheathe and phagocytose the axonal debris (Doherty et al., 2009). Cortex glia are responsible for the removal of the cell body during apoptosis or injury (Doherty et al., 2009, Tasdemir-Yilmaz and Freeman, 2014). Unlike ensheathing glia, cortex glia seem to be important for phagocytosis during development and adulthood. Astrocyte glia are important for the pruning of axons during development (Tasdemir-Yilmaz and Freeman, 2014). A role for astrocytes in phagocytosis during adulthood has not been elucidated. It could be that astrocytes, like their mammalian counterparts, are important in the removal of dendrites in adult brains. There is some evidence that glia phagocytose synapses in the olfactory lobe of adult *Drosophila*, however further studies are needed to confirm this.
Figure 1.9 Schematic representation of glia morphology and organization

Circle represents brain from the surface (the outer edge of the circle) going into the neuropile (white portion of the circle). Perineurial and subperineurila glia form the BBB. Cortex glia wrap around neuronal cell bodies. Ensheathing glia wrap around neuropiles and astrocytes associate with synapses. Glia are in red. Cortex glia cell body in white. Neurons in black. Figure adapted from (Coutinho-Budd and Freeman, 2013).
1.6 Thesis rationale

Professional phagocytes are specified to become phagocytic at differentiation (Gordon and Taylor, 2005). Nonprofessional phagocytes are not fully phagocytic at differentiation. Depending on the cell type, nonprofessional phagocytes are engaged in processes that do not involve phagocytosis. Instead, under the right conditions, nonprofessional phagocytes need to upregulate various proteins in order to become competent to phagocytose. The mechanisms underlying the transition to competent phagocytes are not well understood. In order to better understand how nonprofessional phagocytes achieve a phagocytic phenotype, I have used the ovary of *Drosophila melanogaster* as a model system to investigate the mechanisms underlying this transition.

The *Drosophila* ovary provides a powerful model system to study the mechanisms of nonprofessional phagocytosis. In response to conditions of low amino acid availability, the germline undergoes PCD. In response to death of the germline, the epithelial layer of cells surrounding the germline, known as follicle cells, become competent to phagocytose. The dying germline is engulfed by surrounding epithelial follicle cells. Therefore, the ovary is a naturally inducible *in vivo* model of cell death and nonprofessional engulfment. Furthermore, *Drosophila* has powerful genetic tools and a simplified biology which make it an excellent model organism to study nonprofessional phagocytosis.

Whether cellular corpses affect homeostasis directly is not understood. Failure to remove dying cells from tissue results in the onset of inflammation and the development of autoimmunity. These processes negatively affect tissue homeostasis. The
consequences of impaired cell clearance aside from inflammation are not known. In addition, certain organs, such as the brain, prevent cells of the adaptive immune system from entering (Erickson et al., 2012). Therefore, defects in phagocytosis in organs that prevent the entry of the adaptive immune system do not elicit an adaptive immune response. Consequentially, autoimmunity does not develop. It is thought that the direct consequence of uncleared corpses is the spilling of the intracellular contents due to secondary necrosis. The release of these contents to the extracellular space is thought to be toxic to the surrounding cells; however this has not been studied. Furthermore, it remains controversial whether the intracellular content of cells that have undergone apoptosis is toxic. It has been suggested that caspases, along with other components of PCD, inactivate substances that are toxic to the organism (Martin et al., 2012). In summary, whether uncleared corpses have a negative effect on surrounding tissue is unknown.

The components of programmed cell death are conserved across species. The fact that phagocytosis is conserved across organisms goes counter to the proposal that caspases are sufficient to inactivate toxic intracellular components and therefore cellular corpses are not detrimental to the surrounding cells. Many of these organisms do not possess an adaptive immune system and have a limited inflammatory response. This suggests that dead cells themselves have a detrimental effect on the surrounding tissue. Therefore, the clearance of dead cells is necessary to maintain organism homeostasis. To test whether the persistence of dead cells directly affects homeostasis I have used the
brain of *Drosophila melanogaster* as a model to study whether corpse accumulation damages is toxic to tissue

*Drosophila* does not have an adaptive immune system (Kurata, 2010). The brain of *Drosophila*, like the mammalian brain, is composed of neurons and glia (Freeman and Doherty, 2006). Glia form a blood brain barrier that restricts the entry of macrophages. Furthermore, none of the glia in the Drosophila brain, unlike microglia in the mammalian brain, are derived from white blood cells. This indicates that the *Drosophila* brain does not exhibit adaptive immunity and, because nonprofessional phagocytes have limited inflammatory repertoires, shows little inflammation. The genetic tools available to *Drosophila*, as well as the tractability of this system, make the brain a powerful model to study how improper clearance of dead cells affects homeostasis.

Corpse processing is arguably the least studied component of phagocytosis. Many of the components important for corpse processing are shared among many other intracellular degradation systems (Lu and Zhou, 2012). How a cell is able to distinguish vesicles containing apoptotic cells as opposed to other endocytosed material remains poorly understood. Recent findings have demonstrated that some apoptotic recognition receptors are not only important for internalization of corpses but are also important for phagosome maturation (Lu and Zhou, 2012). This dual role may allow the cell to distinguish apoptotic versus necrotic cells or pathogens and execute the appropriate response by coupling recognition to a specific set of processing signals (Blander and Medzhitov, 2006). The signaling mechanisms responsible for coupling recognition to phagosome maturation are poorly characterized. Drpr has been shown to important for
recognition and processing. The signaling mechanisms that Drpr uses to couple recognition to corpse processing are not known. We therefore have used the *Drosophila* brain to uncover the signaling mechanisms that Drpr uses to ensure proper corpse processing. Since glia are the main phagocytes within the *Drosophila* brain, we have disrupted Drpr in these cell types and uncovered new roles of Drpr in corpse processing. Our findings have led to further understanding of the signaling mechanisms that Drpr uses to ensure proper phagosome maturation.
CHAPTER TWO

Materials and methods

(Portions of this chapter were previously published in Etchegaray, Timmons et al. 2012)

2.1 Drosophila stocks and husbandry

Unless otherwise indicated, flies were obtained from the Bloomington Stock Center and raised at 25°C on standard cornmeal/molasses food. A “cleaned up” version of \textit{drpr}^{\Delta5} (Freeman et al., 2003) was provided by Estee Kurant. \textit{FRT2A} was recombined onto the \textit{drpr}^{\Delta5} chromosome and a recombinant was isolated and confirmed by PCR. The \textit{drpr} \textit{FRT} homozygous mutant showed the same ovary phenotype in trans to a deficiency (\textit{Df(3L)BSC181}), and this line was used for all further studies. \textit{PWIZ-drpr^{RNAi} #7b} (MacDonald et al., 2006) and \textit{UAS-Drpr-I} (McPhee et al., 2010) were provided by Mary Logan and Marc Freeman. The reporter for JNK activity was \textit{puc-lacZ}^{A251.1F3}, \textit{ry/TM3} (Martin-Blanco et al., 1998). \textit{G89} (\textit{G00089}) and \textit{G71} (\textit{G00071}) are germline-specific GFP gene traps from FlyTrap (Morin et al., 2001). \textit{dcp-I^prev1}, \textit{UASp-Diap1}, \textit{UASp-t-dcp-I} and \textit{UASp-fl-dcp-I} were generated in our laboratory (Laundrie et al., 2003, Peterson et al., 2003, Hou et al., 2008). The cortex and astrocyte glial subtype GAL4 drivers (NP2222 and NP3233) were obtained from the \textit{Drosophila} Genomics Resource Center. Germline clones were generated using the \textit{ovo^D} method as described (Chou and Perrimon, 1996).

Fly crosses were performed at 25°C, unless otherwise indicated. The \textit{GR1-GAL4 G89 > UAS-bsk^{DN}} cross was performed at 18°C and adults were incubated for several days at 25°C before analysis. Because of lethality or experimental paradigm, some
crosses required use of tubulin-GAL80ts. Crosses using GAL80ts were performed at 18°C and adults were switched to 29°C to inactivate GAL80ts. For the expression of UAS-hepCA with GAL80ts, flies were raised at 18°C and the adult progeny were conditioned with yeast paste at 18°C. To inactivate GAL80ts, flies were transferred to 29°C overnight. For the expression of all death effectors in neurons, flies were raised at 18°C. Upon eclosion, flies were moved to 29°C until death. GAL80ts drpr RNAi crosses used to determine at what point in development corpses accumulate were kept at 18°C until respective times in development and then transferred to 29°C where the progeny eclosed.

2.2 Drosophila binary expression systems

To control the spatiotemporal expression of transgenes in Drosophila, binary expression systems have been developed (Rodriguez et al., 2012). These systems are comprised of two separable components: an exogenous transcription factor under the control of endogenous enhancer/promoter sequences and exogenous DNA promoter sequences that control the expression of any transgene inserted downstream of these sequences. Upon the activation of enhancer or promoter sequences, the exogenous transcription factor gets upregulated. The transcription factor then binds to the exogenous DNA sequences leading to the expression of the transgene placed downstream. This essentially allows the expression of the transgene to be controlled by endogenous enhancer/promoter sequences. Enhancer and promoter sequences have tissue and developmental patterns of activation. Therefore binary systems induce transgene expression in a spatiotemporal manner. The components of binary systems are separable,
meaning that different transcriptional factors can be coupled to different exogenous DNA sequences. This has led to the creation of expression libraries that can be mixed and matched with each other, allowing for great versatility in gene control. Additional components have been added to these systems. Specifically, proteins that repress the interaction between the transcription factor and exogenous DNA sequences have been developed. The stability or expression of these proteins can be controlled by temperature or chemical means therefore adding a third layer of spatial temporal control to the system. So far, three systems have been developed. In this thesis, we have done experiments using two of them: the UAS-GAL4 system and the Q-system (Figure 2.1).

The UAS-GAL4 system is a binary transgene expression system created for *Drosophila* that uses an expression system discovered in yeast (Brand and Perrimon, 1993). In this system, an enhancer or promoter controls the expression of GAL4. Upon the activation of the enhancer or promoter, GAL4 becomes upregulated. GAL4 is a transcription factor that recognizes and binds to the UAS DNA sequences. The binding of GAL4 leads to the expression of any transgene downstream of UAS. Additionally, a repressor element known as GAL80 has been added to this system. GAL80 binds to GAL4 preventing it from activating the UAS sequences (McGuire et al., 2003). GAL80, like GAL4, is under the control of endogenous enhancer or promoter sequences; however, these sequences may be different than those controlling GAL4. Therefore, the expression of GAL80 restricts the expression of the UAS controlled transgene to cells and/or developmental time points where these enhancer/promoter sequences are not active. A temperature sensitive version of GAL80 has also been created under the control
of the alpha tubulin promoter. This form of GAL80 is inactivated at temperatures at or above 29°C. This allows for further control of the expression of the transgene.

Another binary system analogous to the UAS-GAL4 system is the Q system (Potter et al., 2010). This system has taken expression components from Neurospora and works almost identically to UAS-GAL4. In this system, QF is comparable to GAL4 in that it binds to and activates sequences that act like UAS known as QUAS. The Q system also has an element that represses QF-QUAS known as QS. Unlike GAL80, removal of QS repression is mediated by feeding flies quinic acid. These two systems do not interact with each other, therefore using them together is a powerful way to manipulate two different transgenes in two separate spatiotemporal manners.
Figure 2.1 The UAS-GAL4 system and the Q system of binary transgene expression

These UAS-GAL4 system is illustrated on the left and the Q system is on the right. X equals reporter but can also be substituted for other transgenes such as a gene, RNAi, etc. Figure adapted from (Potter and Luo, 2011).

2.3 Drosophila ovary dissections

Flies were dissected in Grace’s media and egg chambers were fixed in Graces media fix (3 parts Graces media, 2 parts heptane, 1 part 16% paraformaldehyde) for 20 minutes. Ovaries were then washed 3 times in Phosphate Buffered Saline buffer + 0.1% Triton X-100 (PBT), 15 minutes each while rotating, and then samples were placed in VectaShield with DAPI (Vector Labs) overnight at 4°C and subsequently mounted on
slides. For antibody staining, after 3 washes in PBT, ovaries were blocked for 1 hour in PBT + 0.5% BSA + 5% Normal Goat serum (PBANG) and then placed in antibody diluted in PBANG overnight at 4°C. Ovaries were then washed 3 times in PBT + 0.5% BSA and then placed in secondary antibody diluted in PBANG for 1 hour. Brains were then washed again 3x in PBT + 0.5% BSA. Samples were placed in VectaShield with DAPI (Vector Labs) overnight at 4°C and subsequently mounted on slides. Primary antibodies used during ovary dissections were: α-β-gal (1:400, Promega or 1:200, Developmental Studies Hybridoma Bank (DSHB)), α-Dlg (1:1000, DSHB), α-Drpr (1:500, Marc Freeman), and α-DCAD2 (1:10, DSHB). Secondary antibodies were used at 1:200: goat-α-rabbit Alexa Fluor 488 (Invitrogen), goat-α-mouse Cy3, goat-α-rabbit Cy3, and goat-α-rat Dylight 649 (Jackson Immunoresearch). Egg chambers were imaged on an Olympus FV10i confocal microscope. Images were processed in ImageJ and Adobe Photoshop.

2.4 Drosophila brain dissections

Flies were dissected in 4% paraformaldehyde diluted in PBT and fixed for 50 minutes. Brains were washed 3 times in PBT, 15 minutes each while rotating, and then samples were placed in VectaShield with DAPI (Vector Labs) overnight at 4°C and subsequently mounted on slides. For antibody staining, after 3 washes in PBT, brains were blocked for 1 hour in PBANG and then placed in antibody diluted in PBANG for 4 days. Brains were washed 3x in PBT + 0.5% BSA and then placed in secondary antibody diluted in PBANG for 4 hours. Brains were then washed again 3 times in PBT + 0.5%
BSA. Samples were placed in VectaShield with DAPI (Vector Labs) overnight at 4°C and subsequently mounted onto slides. Primary antibodies were used at the following dilutions: 1:100 rabbit cleaved *Drosophila* Dcp-1 (Cell Signaling Technology) and α-Dlg (1:200 DSHB). Secondary antibody Cy3 anti-rabbit IgG (Jackson ImmunoResearch) was used at 1:200. Brains were imaged on an Olympus FV10i confocal microscope. All z-projection images consisted of thirty 1μm stacks starting from the surface of the brain from the first point where the antennal lobe becomes visible. Images were processed in Fiji and Adobe Photoshop.

### 2.5 Ovary engulfment quantifications

To quantify engulfment, we used ImageJ to measure the intensity of GFP in the germline compared to the GFP intensity of the entire egg chamber (germline + follicle cells) for each phase (Ph) of death (Figure 2.2). As a second method, we quantified the area of unengulfed germline compared to the area of the entire egg chamber. The mean ratio for wild-type (WT) Ph0 egg chambers was normalized to 100%. Data for WT were combined from *G71/+* and *GRL-GAL4 G89/TM6B* control lines. n≥ 3 egg chambers were analyzed for each phase and genotype, except *dMekk1* Ph2 (n=2). P-values were determined using a student two-tailed t-test.
Figure 2.2 Methods of quantification of engulfment.

(A, A’) Using ImageJ, the area of the unengulfed germline (A) and the area of the total egg chamber (A’) were measured based on the outline of the FC membranes as shown for a phase 2 egg chamber. The ratio of the area of the unengulfed germline compared with the area of the entire egg chamber was calculated, and referred to as the percentage unengulfed germline. (B, B’) Using the same outline as in A, A’ based on FC membranes, the intensity of the GFP in the unengulfed germline (B) and the intensity of the GFP in the entire egg chamber (B’) were measured by ImageJ. Again, a ratio was taken and referred to as percentage unengulfed germline.

2.6 TUNEL staining

The DeadEnd Fluorometric TUNEL system (Promega) was used on male brains. Briefly, after fixing and washing as for antibody staining, tissue was incubated for 10 minutes in 20μL of equilibration buffer from the kit while rotating. The equilibration buffer was
removed and rTdT incubation buffer was added (45μL Equilibration buffer, 5μL Nucleotide mix, 1μL rTdT enzyme). Samples were then incubated for 3 hours in a 37°C water bath. The reaction was stopped by adding 300μL of 2X saline sodium citrate (SSC) solution for 1 minute while rotating. The solution was removed and replaced with another 300μL of 2x SSC, rotating for 15 minutes. Tissue was washed 3 times in PBT while rotating for a total of 30 minutes. Samples were then placed in VectaShield with DAPI. To quantify the number of TUNEL-positive cells, we used the 3D object counter plugin in FIJI. Unpaired t-test was used for all statistical analysis in chapter 4.

2.7 Sectioning

Histology was conducted by the Feany lab. Briefly, flies were processed for paraffin embedding and sectioned at a thickness of 4μm. Sections from the entire brain were placed on a single glass slide and stained with hematoxylin and eosin. For quantifications, vacuoles 4μm or greater in size were counted. Researchers were blinded during quantifications. Unpaired t-test was used for all statistical analysis.

2.8 Lifespan analysis

Flies were raised at 18°C until eclosion. Once flies eclosed, they were put at 29°C to remove GAL80 mediated repression. Vials at 29°C were checked every day and the number of flies that remained alive were counted. Survival plots were made in Microsoft Excel by taking the number of starting flies minus the number of flies that died, divided by starting flies times 100 for each day.
2.9 PCR of the *drpr* gene

PCR was used to confirm the *drprΔ5 FRT* recombinant. Two whole files of either *w^{1118}, drprΔ5*, or *drprΔ5 FRT* were homogenized in 49μL of Sodium Chloride-Tris-EDTA (STE) buffer. Squished flies in buffer were incubated with 1μL of proteinase K (1:50) at 56°C for 5 hours. We then incubated solution at 95°C to inactivate proteinase K. We then added 1μL of this solution to 19μL of Phusion PCR master mix (New England Biolabs): 4μL 5x Phusion HF buffer, 0.4μL 10mM dNTPs, 1μL *drpr* forward primer (GCA CGG TGT AAA CTG GTT TTC), 1μL *drpr* reverse primer (GCT GTA CAG AAT GGG AGG ATC), 0.6 μL DMSO, 0.2μL Phusion DNA polymerase, and 12.8μL of autoclaved dH2O. Samples were then subjected to PCR and run on 1.2% agarose gel with 1μL of ethidium bromide for a concentration of 1:150. We observed the 2 kilobases (kb) product expected for *w^{1118} based on the primer sequences. Both *drprΔ5* and *drprΔ5 FRT* showed a 500 base pair product (Figure 2.3) resulting from the P-element excision previously reported (Freeman et al., 2003).
Figure 2.3 PCR on the *drpr* gene for *w*1118, *drpr*Δ5, or *drpr*Δ5 FRT

PCR products for *w*1118 (Lane 1, arrow), *drpr*Δ5 (Lane 2, 4, 6) and *drpr*Δ5 FRT (Lane 3, 5, 7). As expected *w*1118 shows a 2000 bp product while *drpr*Δ5 and *drpr*Δ5 FRT show a 500bp product resulting from the same *P*-element excision.
2.10 Cloning of dcp-1 into a QUAS vector

*E.coli* carrying the QUAS plasmid were bought from Addgene. *E.coli* carrying the plasmid were grown in Lysogeny broth (LB) agar + Ampicillin (AMP) plates overnight. Toothpicks were then used to swab 4 colonies on the QUAS* E.coli* plates. These 4 toothpicks were placed in liquid 200ml of LB + AMP (AMP at concentration of 50μl/ml). 2 toothpicks were used to swab *E.coli* carrying a full length NB40p dcp-1 cDNA plasmid (Song et al., 1997) from a stock at -80°C. These toothpicks were also placed in LB + AMP. The following day, Qiagen minipreps were done to isolate the plasmid. Specifically, 1.5 ml were taken from each of the tubes containing the *E.coli* in solution. The tubes containing the 1.5 ml were centrifuged at 4°C at 16,000 rpm for 30 seconds. Supernatant from these tubes was then discarded and pellet was conserved. To the pellet was added 100μL of cold solution 1 from miniprep kit and vortexed to resuspend the pellet. After 200μL fresh solution 2 were added and mixed gently. Solution 1 was not removed. 150μL of solution 3 were added, vortexed, and left on ice for 5 minutes. Tubes were then centrifuged for 5 minutes at 4°C. The supernatant was then transferred to a new tube where 2 volumes of 100% ethanol were added for 2 minutes. Tubes were then centrifuged for 5 minutes at 4°C. Supernatant was removed and 70% ethanol was added to the tube. Tubes were then air dried for 15-20 minutes. Pellet was re-dissolved in 50μL 1x TE + 20μg/ml RNAse and stored at -20°C. To these purified plasmids a restriction digest diagnostic was done to make sure plasmids were correct. Specifically dcp-1 plasmid was digested with XhoI and XbaI, or XhoI by itself. For the restriction digests, the following components were added: 2μL dcp-1 plasmid from miniprep, 1μL buffer
(New England Biolabs (NE) buffer 2), 1μL of the restriction enzyme, and 10μL of autoclaved H₂O (Note, solution was 2x for the XhoI w/XbaI treatment) Treatments were then placed in 37°C water baths for 2 hours. After, samples were run on 1% agarose with 1μL of ethidium bromide (1:150). As control we ran an uncut plasmid. The same approach was done for QUASt plasmid except NotI was the only restriction enzyme used with NE buffer 3 instead. After confirming that the plasmids were right, dcp-1 was cut again with XbaI and XhoI and the band corresponding to the dcp-1 gene was gel extracted. DNA was then purified using a QIAquick gel extraction kit as per manufacturer’s instructions. We determined that we had a concentration of 8ng/μL using a NanoDrop spectrophotometer. QUASt plasmid was also digested with XbaI and XhoI. The restriction digest was done for 3 hours and transferred to a 65°C water bath to inactivate the enzymes. A ligation reaction was set up as follows: 2μL of digested QUASt, 4μL of digested and gel purified dcp-1, 1μL of T4 DNA ligase, 1μL of 10x T4 DNA ligase buffer, 2μL of dH₂O. As a control, QUASt was incubated alone with 4μL of extra dH₂O instead of purified dcp-1. Tubes with ligation reaction were then placed at 18°C overnight. The next day, JM109 *E.coli* competent cells (Promega) were thawed on ice. 5μL of vector ligation (control) and vector ligation + insert (QUASt +dcp-1) were added to 100μL of cells separately. Tubes were left on ice for 10 minutes. Cells were then heat shocked for 45-50 seconds in a water bath at 42°C. Tubes were then placed on ice for 2 minutes. After, 900 μL of LB was added to each transformation reaction and tubes were incubated for 60 minutes at 37°C while shaking. We then plated 100 μL of transformation reaction on LB+AMP plates overnight. We then took and grew the 4
colonies in LB+AMP solution for one day. A restriction enzyme diagnostic test was then performed using XhoI and XbaI to test the colonies for the right plasmid. Out of the 4 colonies, 1 and 2 showed the right digest. To further confirm that these colonies had the right insert as opposed to a UAS insert, we did another diagnostic test with PstI and XbaI with Acc65 (the two reactions use NE buffer 3). PstI mediated digest of a QUAST plasmid should yield a 152 bp band and another that is similar in size to the the full length plasmid. A PstI mediated digest of UAS should lead to two similar size bands. A XbaI/Acc65 cut of QUAST should yield a band the same size as the full length linear size of the plasmid. A UAS digest with XbaI/Acc65 should yield a band the size of the dcp-1 insert and a band the size of the UAS plasmid. Based on the band products of the digest, we obtained the QUAST plasmid with the dcp-1 insert. PCR of this plasmid was done to further confirm that it is QUAST and not UASp using the same specifications for the drpr PCR protocol except using the primers specified by Addgene for QUAST detection. Our findings confirmed that the right plasmid was obtained. We then sent this plasmid for sequencing. Once confirmed by sequencing, we sent the plasmid to an injection service and obtained transgenic animals in return. We then crossed these flies to M2ET-QF, a QF insertion in 49E6 near the Posterior sex combs gene. This QF line has been shown to drive expression in the Drosophila eye (Potter et al., 2010). Cell death induced in the eye produces a rough eye phenotype (Wolff and Ready, 1991). Since dcp-1 is an effector caspase, we screened for rough eye phenotype to obtain lines where dcp-1 could be expressed by QF drivers. Out of the flies screened, we found approximately 10 lines that
had a rough eye phenotype indicating these flies had QUAS-dcp-1 that could be driven by QF.
CHAPTER THREE

Draper acts through the JNK pathway to control synchronous engulfment of dying germline cells by follicular epithelial cells

(Portions of this chapter were previously published in Etchegaray, Timmons et al. 2012)

3.1 Introduction

Programmed cell death (PCD) is a critical process in metazoan development and is a major factor in many diseases. In the human body, billions of cells die daily and are removed by phagocytic cells (Elliott and Ravichandran, Birge and Ucker, 2008, Erwig and Henson, 2008, Lleo et al., 2008, Fullard et al., 2009, Kinchen, 2010). A lack of phagocytosis of apoptotic cells can lead to secondary necrosis, culminating in an inflammatory response, or in severe examples, auto-immune diseases such as lupus. Thus, phagocytosis of apoptotic cells is essential for organism homeostasis.

Phagocytosis of apoptotic cells (also called efferocytosis) occurs by the actions of both professional phagocytes, such as macrophages, and nonprofessional phagocytes. In C. elegans, which lack macrophages, efferocytosis is carried out by neighboring cells (Mangahas and Zhou, 2005). In mammals, significant effort has been placed on understanding the mechanisms of engulfment by macrophages, but much less is known about engulfment in nonprofessional phagocytes. Other cell types known to be involved in efferocytosis include epithelial cells, mesenchymal cells, granulosa cells, glia, and neuronal progenitors (Inoue et al., 2000, Wiegand et al., 2001, Ichimura et al., 2008, Jones et al., 2008, Monks et al., 2008, Lu et al., 2011). Some nonprofessional phagocytes clear apoptotic cells less efficiently than professional phagocytes (Parnaik et al., 2000),
but it is not known if this is a general phenomenon. Whether nonprofessional phagocytes can be “activated” to increase their engulfing ability is also unknown.

Genetic analysis in *C. elegans* has defined two parallel pathways required for engulfment of apoptotic cells (Mangahas and Zhou, 2005, Hurwitz et al., 2009). One pathway involves *ced-2, -5* and -12 and activates CED-10, a Rac GTPase. A second pathway utilizes CED-1, a putative apoptotic cell receptor. Other genes acting in this pathway are *ced-7, ced-6* and *dyn-1*, and this pathway may also converge on CED-10 (Kinchen, 2005). Extensive study of engulfment in mammals has revealed a high level of complexity (Kinchen and Ravichandran, 2007). As in worms, the CED-1,6,7 and CED-2,5,12 pathways play prominent roles in engulfment in mammals. There are many putative apoptotic cell receptors, suggesting they may function redundantly or be specific for certain cell types.

*Drosophila* appears to use a smaller number of engulfment components than mammals, but unlike *C. elegans*, flies utilize both professional and nonprofessional phagocytes. Engulfment has been studied in four cell types in *Drosophila*: macrophages, embryonic epidermal cells, glia, and epithelial imaginal disc cells (Fullard et al., 2009, Kinchen, 2010). The *ced-1* ortholog, *drpr (drpr)*, is important in several of these contexts. *drpr* plays a role in the engulfment of neurons, severed axons, bacteria, and imaginal disc cells (MacDonald et al., 2006, Li and Baker, 2007, Cuttell et al., 2008). The *ced-5* and *ced-10* orthologs, *mbc* and *Rac1*, have partial effects in imaginal discs, suggesting that this pathway plays a more minor role in engulfment than the CED-1,6,7 pathway in flies (Li and Baker, 2007).
Activation of the stress-activated MAP kinases, c-Jun N-terminal kinase (JNK) and p38, is an early event following recognition of apoptotic cells by macrophages and epithelial cells in mammals, but a requirement for these pathways has not been demonstrated (Patel et al., 2006, Patel et al., 2010). The Drosophila JNK pathway is well-characterized for its roles in development, innate immunity, and apoptosis (Stronach, 2005, Igaki, 2009). Recently the JNK pathway was shown to be required for the removal of imaginal disc cells succumbing to cell competition (Ohsawa et al., 2011), suggesting that JNK may play a conserved role in promoting engulfment.

The Drosophila ovary is an established model for many aspects of cell biology, but efferocytosis is not well-characterized. Cell death of developing egg chambers can be induced during mid-oogenesis (Stage (St) 7-9) by the removal of protein from the diet (Pritchett et al., 2009). Dying mid-stage egg chambers display a reproducible series of morphological events, with the germline nurse cells (NCs) showing the first signs of degeneration (Giorgi and Deri, 1976, Nezis et al., 2000, Peterson et al., 2003). Fly ovaries have few circulating cells such as macrophages (King, 1970), indicating that germline remnants are removed largely by nonprofessional phagocytes. Indeed, the epithelial follicle cells (FCs), which surround developing germline cysts, engulf the NC remnants (Giorgi and Deri, 1976), but the engulfment mechanisms are unknown.

Here we show that the Drosophila ovary provides a powerful in vivo system for the study of engulfment in nonprofessional phagocytes. Unlike the redundant pathways described in other systems, we find that the Drpr pathway alone is essential for corpse clearance by FCs. We find that Drpr activates the JNK pathway, which is also required
for engulfment, and that JNK activity feeds back to promote an increase in Drpr protein in engulfing cells. Remarkably, activation of JNK can restore engulfment in the absence of Drpr. Our findings indicate that the Drpr and JNK pathways are critical activators of engulfment in nonprofessional phagocytes.

3.2 *Drosophila* follicle cells coordinately engulf dying germline cells

To visualize engulfment in egg chambers, we obtained strains expressing GFP restricted to germline cytoplasm, so that uptake by surrounding FCs could be detected. Flies were starved to induce PCD, and egg chambers were labeled with an antibody against Discs large (α-Dlg), a scaffolding protein that labels FC membranes. As previously described (Pritchett et al., 2009), the first signs of egg chamber degeneration were observed in NC nuclei which displayed condensed chromatin, progressing to nuclear fragmentation (Figure 3.1). FCs initially did not show any uptake of NC cytoplasm (Figure 3.1B, “Phase (Ph) 1”), but as NC chromatin condensed, germline GFP was engulfed by surrounding FCs which synchronously enlarged (Figure 3.1C-D, “Ph2-3”). The germline appeared to be taken up through macropinocytosis, consistent with previous studies using electron microscopy (Giorgi and Deri, 1976, Tanner et al., 2011). As PCD proceeded, FCs grew and the germline shrunk as it was engulfed by FCs (Figure 3.1E, “Ph4”). At late phases, the germline region was reduced to a small sliver and then completely engulfed by enlarged FCs (Figure 3.1F, “Ph5”). Quantification of engulfment by measuring GFP intensity or germline area showed a decrease beginning in Ph2 (Figure
3.1G, methods). The area measurement showed a more dramatic decrease, presumably because GFP fluorescence declined during engulfment.

### 3.3 The receptor Drpr is required for engulfment by follicle cells

The engulfment receptor Drpr and related molecules are established components of the phagocytosis machinery (Kinchen and Ravichandran, 2007). To determine whether Drpr was involved in the phagocytosis of NCs, we stained ovaries of starved flies with antibodies against Drpr (Figure 3.2A-C). Healthy egg chambers showed low levels of Drpr staining around FC membranes (Figure 3.2A). Once NC nuclei began to condense and FCs started to enlarge, Drpr became enriched on the apical membrane of engulfing FCs, bordering the germline (Figure 3.2B). Drpr staining intensity peaked at Ph5 (Figure 3.2C). In addition to plasma membrane staining, Drpr was detected in punctate speckles within FC cytoplasm. These data suggest that Drpr is up-regulated throughout engulfment, and the puncta suggest that Drpr becomes clustered and internalized during phagosome formation.

To determine whether Drpr was required for engulfment, we examined ovaries from flies carrying the $drpr^{Δ5}$ mutation, a null allele (Freeman et al., 2003). In the absence of starvation, $drpr^{Δ5}$ mutant egg chambers developed normally through mid-oogenesis (Figure 3.2E). However, FCs of degenerating egg chambers from starved $drpr^{Δ5}$ flies showed significant defects in engulfment and failed to enlarge as NC chromatin condensed (Figure 3.2E'). $drpr^{Δ5}$ FCs also showed little engulfment, with minimal uptake of NC nuclear fragments and germline GFP. As death progressed, $drpr^{Δ5}$
FCs died (Table 3.1) without clearing most NC debris (Figure 3.2E’’, 2I). These results suggest that Drpr is crucial for proper engulfment of the dying germline. Quantification of egg chambers revealed that drpr<sup>Δ5</sup> mutants have more than a six-fold increase in the number of Ph5 egg chambers compared to controls, indicating a pronounced defect in corpse removal (Figure 3.3).

Since the drpr<sup>Δ5</sup> mutation affects both the NCs and FCs, we assessed whether it was required specifically in the engulfing FCs. We performed a FC-specific knockdown of drpr by expressing UAS-drpr<sup>dsRNA</sup> with the FC-specific GR1-GAL4 driver (Methods). As seen in Figure 3.2F’-F’’ and 3.2I, FC-specific knockdown of drpr prevented FC enlargement and NC debris uptake, similar to drpr<sup>Δ5</sup>. Additionally, we rescued the drpr<sup>Δ5</sup> defect by over-expressing drpr<sup>+</sup> in the FCs (Figure 3.2G, J). In these egg chambers, FCs enlarged and took up germline debris, similar to WT (Figure 3.2G’-G’’). Furthermore, the rescued drpr<sup>Δ5</sup> FCs did not show premature death. These results show that drpr is specifically required in FCs for proper engulfment of NCs during starvation-induced PCD. We also made drpr<sup>Δ5</sup> germline clones (data not shown) to see whether drpr was required in the germline. The majority of egg chambers in these clones had proper engulfment; however there were some that had somewhat aberrant engulfment, likely because the protocol for creating germline clones (Laundrie et al., 2003) leads to some unmarked FC clones.

To further analyze the role of drpr in engulfment we over-expressed drpr in FCs of WT egg chambers (Figure 3.2H). Interestingly, we found that drpr over-expression in the FCs was sufficient to induce germline PCD in the egg chambers of unstarved flies.
NCs condensed and fragmented in St8 and St9 egg chambers, but instead of engulfing, the FCs thinned out during the early phases of PCD (Figure 3.2H’). By Ph4, the FCs engulfed normally, culminating in the removal of the germline (Figure 3.2H”). These findings indicate that over-expression of *drpr* is sufficient to induce NC death, but that there is a delay between the onset of death and engulfment compared to WT. The delay could be because egg chambers from well-fed flies lack “eat me” signals or starvation-induced signals for engulfment, or that *drpr*-induced death has delayed exposure of “eat me” signals.

### 3.4 Follicle cells require a subset of known engulfment genes

We next determined whether other engulfment genes were required in FCs. The kinase Shark is known to act downstream of Drpr in glia (Ziegenfuss et al., 2008), and Rac-1 is a conserved GTPase required for cytoskeletal rearrangements that occur during engulfment (Kinchen et al., 2005, Li and Baker, 2007). As seen in Figure 3.4, FCs failed to enlarge in both *shark*dsRNA and *Rac-1*DN (dominant negative) degenerating egg chambers. By Ph4-5 of cell death, NC and FC debris lingered in both *shark*dsRNA and *Rac-1*DN egg chambers (Fig 3.4B’’, 3.4C’’), and FCs died prematurely (Table 3.1), similar to *drpr* mutants. Thus, *shark* and *Rac-1* are required in FCs for proper engulfment during starvation-induced PCD. Whereas *drpr* and *shark*-deficient FCs resembled WT when flies were well-fed, egg chambers of *Rac-1*DN flies displayed FC death even without starvation, indicating that *Rac-1* is required for another aspect of FC function. However, *Rac-1*DN FCs from starved flies usually did not die until later phases, suggesting that FC
death is not responsible for the engulfment defects. We also investigated mutants of \textit{simu}
and \textit{prêt-a-porter} (Kurant et al., 2008, Kuraishi et al., 2009), but neither displayed defects
in engulfment, suggesting that only a subset of known engulfment genes are required in
FCs.

3.5 JNK signaling is required for engulfment by the follicle cells

The increase in Drpr levels in engulfing FCs suggested that signaling pathways
are activated in FCs to increase their competence for engulfment. The JNK pathway has
been shown to be activated in engulfing mammalian macrophages and nonprofessional
phagocytes (Patel et al., 2006, Patel et al., 2010), making it an excellent candidate
pathway to be involved in germline engulfment. To visualize JNK activity, egg chambers
carrying a \textit{lacZ} enhancer trap in the JNK target gene \textit{puckered} (Martin-Blanco et al.,
1998) were analyzed with \textit{α-β-gal}. Egg chambers were co-labeled with \textit{α-Drpr} to
visualize the timing of JNK activity relative to Drpr induction. Healthy egg chambers had
no \textit{puc-lacZ} staining and minimal Drpr staining in mid-stage egg chambers (Figure
3.5A). Ph1 dying egg chambers displayed activation of \textit{puc-lacZ} in a few FCs and had
increased Drpr staining, particularly in posterior FCs (Figure 3.5B). Antibody staining
indicated that the increase in Drpr levels preceded \textit{puc-lacZ} activation. As the egg
chambers progressed through death, \textit{puc-lacZ} and Drpr staining became widespread and
robust in FCs (Figure 3.5C-D).

The increase in JNK activity in engulfing FCs suggested that JNK might be
required for engulfment. To investigate a role for JNK (called \textit{basket} or \textit{bsk} in flies), egg
chambers over-expressing dominant negative $bsk$ (Adachi-Yamada et al., 1999) in the FCs and germline-specific GFP were examined. Healthy egg chambers appeared WT (Figure 3.5E) and Ph1-2 dying egg chambers began the process of engulfment, including the enlargement of the FCs and uptake of germline GFP (Figure 3.5E’). However, there were engulfment defects seen in Ph3-5 dying egg chambers. FCs failed to enlarge, no longer stained for Dlg, and the FC nuclei became pyknotic, indicating that the FCs were dying (Table 3.1). Furthermore, NC nuclei and germline GFP were not taken up by the FCs (Figure 3.5E’’-4E’’’). Ph3-5 egg chambers in WT had < 50% of unengulfed remaining germline, while $bsk^{DN}$ mutants had 100% remaining germline (Figure 3.5I). Interestingly, the NC nuclei failed to fragment properly, making it difficult to assess the specific phases of degeneration (Figure 3.6). Thus, JNK signaling is required in the FCs for fragmentation of NC nuclei and engulfment of dying NCs. Similar engulfment defects were seen by knocking down $bsk$ in the FCs with dsRNA, including the lack of FC enlargement, pyknotic FCs, and lingering germline material (Figure 3.5F-5F’’’, 3.5I, Table 3.1), resembling $drpr$ (Figure 3.2). In unstarved flies expressing $bsk^{DN}$ or $bsk^{dsRNA}$ in the FCs, there were few degenerating egg chambers, indicating that the death of the FCs was due to engulfment defects and not a general requirement for $bsk$.

3.6 Upstream components of the JNK signaling pathway are required for engulfment

In *Drosophila*, the JNK pathway is simpler compared to mammals, with only one JNK ($bsk$) and two JNK kinases (JNKKs) (Stronach, 2005). However, upstream of the
JNKKs, there is increased complexity with six putative JNKK kinases, activated by multiple upstream pathways. One upstream regulator is Rac-1, which we have demonstrated to be required for engulfment by FCs. To determine if other upstream activators of the JNK signaling pathway were required, we expressed dsRNA against several components of the JNK pathway specifically in the FCs of starved flies, including the JNKK *hemipterous* (*hep*), the JNKKKs *slipper* (*slpr*) and *dMekk1*, and the JNKKKK *misshapen* (*msn*). We found that FCs in dMekk1 dsRNA egg chambers failed to enlarge and died prematurely, similar to bsk (Figure 3.5E-G,I-J, Table 3.1). In the absence of starvation, mid-stage egg chambers appeared normal, indicating that the FC phenotype was due to defective engulfment and not a general requirement for dMekk1. The death of the NCs and subsequent engulfment by the FCs occurred normally with expression of *hep, slpr,* or *msn* dsRNA (data not shown). However, these knockdowns may not be strong enough to produce a phenotype. We additionally analyzed mutants of *eiger*, which encodes the fly TNF ortholog and is a ligand known to activate the JNK pathway (Igaki et al., 2002, Moreno et al., 2002). Like *bsk* and *dMekk1*, *eiger* mutant FCs failed to enlarge and take up NC debris, and died prematurely (Figure 3.5H,J, Table 3.1). Importantly, excessive FC death was observed only in late phases of degeneration (Table 3.1) after engulfment defects were apparent in the mutants (Figure 3.5I,J), indicating that engulfment defects were not a consequence of FC death. Furthermore, many mid-stage egg chambers remained healthy (Ph0) under starvation conditions, and these did not show FC death (Table 3.1). Our findings demonstrate the requirement for JNK signaling in
the FCs and the involvement of the upstream signaling components, *eiger, Rac-1, and dMekk1*.

### 3.7 JNK acts downstream of Drpr to promote engulfment

Because both *drpr* and JNK pathway mutants displayed defects in engulfment by FCs, we wished to determine if they acted in the same pathway, and if so, which gene acted upstream. To determine if over-expression of *drpr* was sufficient to activate JNK, we examined expression of *puc-lacZ* in ovaries from well-fed flies over-expressing *drpr* in FCs (Figure 3.7A). *puc-lacZ* was induced in these egg chambers before they showed any signs of degeneration (Figure 3.7A’). *puc-lacZ* was first detected in anterior FCs, but then gradually spread to all FCs, and increased as the germline began to die (Figure 3.7A’’). Interestingly, *puc-lacZ* was not detected until St8 of oogenesis, although *drpr* over-expression was observed by St6 with the *GR1-GAL4* driver (Chapter Two). These findings indicate that Drpr is sufficient to activate JNK, but only at mid-oogenesis. To confirm that Drpr acts upstream of JNK, we examined *puc-lacZ* expression in starved *drpr*\(^{\Delta5}\) mutants (Figure 3.7B). Consistent with the over-expression results, *puc-lacZ* was not induced in Ph1-2 (Figure 3.7B’-B’’), but surprisingly was detected in some FCs of late phase degenerating egg chambers (Figure 3.7B’’’). These observations suggest that Drpr activates JNK in engulfing FCs, but another pathway can activate JNK in the late phases of germline PCD.

To determine if JNK activity promotes the increase in Drpr levels during engulfment, we examined egg chambers from starved flies expressing *bsk^{DN}* in the FCs.
Drpr staining was observed on FC membranes in early dying egg chambers, but it declined as the FCs died (not shown). We additionally over-expressed constitutively active hep (Adachi-Yamada et al., 1999), which encodes a JNKK. Activation of the JNK pathway led to an increase in Drpr in the FCs of healthy egg chambers in the absence of starvation (Figure 3.7C). Furthermore, the hep\(^{CA}\)-expressing FCs from unstarved flies displayed a hyper-engulfment phenotype in which they invaded and surrounded apparently healthy NCs (Figure 3.7C-C’’), culminating with FCs engulfing intact NCs and inducing their death (Figure 3.7C’’). These results indicate that JNK activity is sufficient for the up-regulation of Drpr observed during engulfment.

To further examine the relationship between JNK and Drpr, we carried out epistasis analysis. First, we expressed hep\(^{CA}\) in a drpr\(^{Δ5}\) mutant background (Figure 3.7D). Over-expression of hep\(^{CA}\) in FCs led to the formation of many abnormal egg chambers, but close examination showed that hep\(^{CA}\) could restore engulfment in drpr\(^{Δ5}\) mutant egg chambers, seen by enlargement of FCs and uptake of germline GFP (Figure 3.7D’-D’’, H). Furthermore, expression of hep\(^{CA}\) still led to hyper-engulfment of the germline in a drpr mutant background (Figure 3.7D’’’, H). These findings support our previous conclusion that JNK acts downstream of Drpr, but also reveal that, remarkably, drpr is not required for engulfment in the presence of activated JNK. We also conducted epistasis analysis with bsk\(^ {DN}\) and UAS-drpr, but found that this combination was lethal even at low temperatures.
3.8 Caspase activity in the dying germline is necessary but not sufficient for engulfment

We next determined if engulfment by FCs was dependent on caspase activity in the dying germline. Inhibition of caspases in the germline leads to a striking “undead” egg chamber phenotype where NC nuclei fail to condense, but FCs disappear (Laundrie et al., 2003). To determine if engulfment was initiated when caspases were inhibited in the germline, we starved flies and examined egg chambers that were starting to show morphological abnormalities, but still had most of their FCs (Figure 3.8A-D). We first examined egg chambers from dcp-1prev1 homozygous mutants (where both FC and NC lack dcp-1), and found that engulfment was largely inhibited with only occasionally enlarging FCs (Figure 3.8A). dcp-1 is the only caspase mutant found to completely disrupt mid-stage NC death, and only one allele has been described. Therefore, we generated additional alleles of dcp-1 to confirm that the partial phenotype was not due to residual activity in the dcp-1prev1 allele. Two new EMS-induced alleles were generated: dcp-12 (P92L) and dcp-13 (W243Stop). The new alleles showed ovary phenotypes that were largely indistinguishable from the dcp-1prev1 allele, with very little engulfment by FCs (Figure 3.8B-C). Some engulfment by FCs was seen in all dcp-1 alleles, so we examined whether inhibition of additional caspases would show a stronger phenotype. Egg chambers over-expressing the caspase inhibitor Diap1 in the germline also showed a partial inhibition of engulfment (Figure 3.8D). 30% (n=138) of dcp-12 undead egg chambers showed some FC enlargement, and similarly, 36% (n=64) of Diap1-over-expressing undead egg chambers showed some FC enlargement. These findings indicate that dcp-1 is the major caspase
mediating engulfment signals in the ovary. To determine if dcp-1 was sufficient to induce engulfment, we over-expressed dcp-1 in the germline. Although engulfment by FCs was observed, it was delayed compared to WT (Figure 3.8E-F), suggesting that dcp-1 does not induce engulfment directly, but that subsequent events in the dying NCs can induce engulfment.

To further investigate the role of caspases in activating engulfment in the surrounding FCs, we examined Drpr and JNK induction. Surprisingly, we saw induction of both Drpr and JNK in dcp-1 mutants and in egg chambers over-expressing diap-1 in the germline (Figure 3.8A’-D’,G-G’, starved flies). This indicates that dcp-1 acts in a pathway independent of JNK and Drpr induction (Figure 3.9). Consistent with these findings, we found that germline over-expression of dcp-1 in the absence of starvation led to Drpr and JNK induction (Figure 3.8E’-F’, H-I’), but it was delayed compared to WT, based on NC chromatin morphology. These findings indicate that JNK and Drpr are activated independently of caspase activity in NCs, and their activation alone is not sufficient to drive engulfment. This suggests that at least two pathways are required for engulfment: one caspase-dependent and one acting through JNK and Drpr.

3.9 Discussion

How nonprofessional phagocytes respond to dying cells and modulate their phagocytic capabilities is unclear. Here we have used the Drosophila melanogaster ovary as a model to study engulfment by nonprofessional phagocytes. In this system, the germline can be induced to undergo PCD upon starvation. Following the initiation of PCD, a layer of
epithelial FCs synchronously engulfs the dying germline. We have shown that the engulfment genes *drpr*, *shark*, and *Rac-1* are required for engulfment by FCs. We have also found that the JNK pathway is specifically activated during engulfment and is required for proper engulfment by FCs. Our analysis suggests that *drpr* and JNK are involved in a circuit, where the dying germline activates Drpr, which activates JNK, and JNK signaling leads to an increase in Drpr and likely other engulfment genes (Figure 3.9). Surprisingly, activation of JNK is sufficient to rescue *drpr* engulfment defects, indicating that other pathways can carry out engulfment in the absence of *drpr*. A likely candidate pathway is CED-2,5,12, which can promote engulfment in the absence of *ced-1* in *C. elegans*.

In other systems, such as *C. elegans* and mammalian macrophages, there is redundancy among engulfment pathways (Kinchen and Ravichandran, 2007, Kinchen, 2010). In *Drosophila* embryos lacking *drpr*, unprocessed apoptotic particles are detected within glia (Kurant et al., 2008), suggesting that other pathways can facilitate engulfment of corpses. However, in FCs, *drpr* is essential for corpse removal. This may be because FCs die if they are engulfment-defective, and there may not be time to activate redundant pathways prior to FC death. It is important to note that *drpr* (and JNK pathway) mutant FCs survive in healthy egg chambers under starvation conditions; it is only during terminal phases of egg chamber degeneration that they die (Table 3.1). Why do the FCs die if they are engulfment-defective? Perhaps they have a metabolic requirement, and starve if they cannot obtain nutrients from the germline. Another possibility is that WT FCs are programmed to die after completing engulfment, and this PCD may be activated
prematurely if engulfment is defective. Mammalian macrophages eliminate themselves after engulfment of specific pathogens or following efferocytosis in ABC transporter mutants (Navarre and Zychlinsky, 2000; Yvan-Charvet et al., 2010). Alternatively, FCs may die because of death “by confusion,” where disruption of the proper signaling network culminates in PCD. We attempted to block FC death by expression of caspase inhibitors p35 and Diap1, but FC death was still observed in control, \( \text{drpr}^{\Delta 5} \) and \( \text{bsk}^{\text{DN}} \)-expressing egg chambers, indicating that FCs die via a caspase-independent pathway.

In mammals, JNK is activated in engulfing professional and nonprofessional phagocytes (Patel et al., 2006, Patel et al., 2010), although it remains to be determined whether JNK is required for engulfment. Recently in \( \text{Drosophila} \), JNK has been found to be required for the removal of imaginal disc cells succumbing to cell competition (Ohsawa et al., 2011). These findings suggest that JNK may play a conserved role in engulfment. To our knowledge, a role for JNK in engulfment has not been explored in \( \text{C. elegans} \) and no transcription factor has been shown to activate engulfment genes. This is surprising since levels of CED-1 increase in engulfing cells (Zhou et al., 2001).

How does JNK become activated during engulfment? It may occur via Shark, a kinase that has been shown to interact with both Drpr and JNK in \( \text{Drosophila} \) (Fernandez et al., 2000, Tran and Berg, 2003, Ziegenfuss et al., 2008). Another candidate is Rac-1, which can act upstream of JNK and may act downstream of Drpr (Kinchen et al., 2005). Interestingly, JNK activity is sufficient to restore engulfment in \( \text{drpr} \) null egg chambers, suggesting that Drpr’s primary role is to activate JNK. Thus, functions attributed to Drpr such as actin reorganization, calcium signaling, the formation of junctional complexes,
and autophagy (Cuttell et al., 2008, McPhee and Baehrecke 2010), may depend on JNK activity. Indeed, JNK has been shown to induce autophagy genes in \textit{Drosophila} (Wu et al., 2009a).

Remarkably, \textit{drpr} or \textit{hep}\textsuperscript{CA} over-expression in FCs promoted death of egg chambers even when flies were not starved. To our knowledge, this is the first time that over-expression of an engulfment gene has been shown to induce non-autonomous cell death. In other systems, engulfment can promote the death of cells that are weakened, perhaps on the brink of death. For example, mutations in engulfment genes can lead to the survival of cells fated to die in \textit{C. elegans ced-3} hypomorphs (Reddien et al., 2001; Hoeppner et al., 2001), and to the survival of “loser” cells in \textit{Drosophila} imaginal discs (Li and Baker, 2007). In mammals, neuronal exposure to amyloid A\textit{β} peptide or LPS leads to cell death, which can be inhibited by blocking phagocytosis (Neher et al., 2011, Neniskyte et al., 2011). Interestingly, treated neurons transiently expose phosphatidylserine, perhaps to announce their vulnerability. Our findings differ from these scenarios in that the egg chambers are healthy. However, mid-stage egg chambers are more susceptible to death stimuli than egg chambers at other stages of oogenesis (McCall, 2004). Over-expression of Drpr in early oogenesis did not lead to egg chamber death, but death was observed later in mid-oogenesis. Thus, it may be that Drpr is not sufficient to kill the germline until mid-oogenesis, when it is more vulnerable. The factors that contribute to this vulnerability are unknown.

Over-expression of \textit{drpr} in FCs led to death of the underlying NCs before there was any engulfment by the FCs, suggesting that \textit{drpr} produces a death signal that is sent to the
germline. Over-expression of the JNKK hep^{CA} led to destruction of egg chambers earlier in oogenesis than over-expression of drpr, suggesting that JNK did not require the vulnerability at mid-oogenesis. Furthermore, hep^{CA}-expressing FCs engulfed intact NCs ("hyper-engulfment"), rather than inducing death first. This phenotype resembles the process of entosis, where living cells are engulfed by their neighbors (Overholtzer et al., 2007).

Germline PCD in mid-oogenesis requires caspases, and our results indicate that caspase activity is required to stimulate FCs to engulf the germline. Surprisingly, germline caspase activity was not necessary or sufficient to activate JNK or induce Drpr in the FCs. This suggests that a caspase-dependent pathway, distinct from the pathway(s) that activate Drpr-JNK, is required for engulfment in mid-oogenesis. The caspase-dependent signal and the responding pathway in the FCs remain to be elucidated. Another open question is how Drpr, and thereby JNK, become activated in response to the dying germline. The complexity of cell surface modifications that occur during apoptosis will make this a challenge to determine. Drpr and JNK may become activated directly in the FCs in response to starvation, however this scenario seems less likely than activation by the dying germline for two reasons. First, many egg chambers do not die immediately upon starvation, and activation of Drpr and JNK was observed only in egg chambers that had begun to die. Second, germline death triggered by over-expression of dcp-1 could lead to Drpr and JNK activation in FCs in the absence of starvation. The activation of JNK and Drpr illustrate ways in which nonprofessional phagocytes change in response to
apoptotic cells. Future work will reveal the network of pathways activated in nonprofessional phagocytes to enhance apoptotic cell clearance.
Figure 3.1 Progression of cell death and engulfment.

Egg chambers from starved flies were labeled with DAPI to label DNA (cyan, top panels), and α-Dlg (magenta). Egg chambers express a germline-specific GFP gene trap (G89, green). Lower panels are enlargements of boxed regions in middle panels. State of chromatin condensation and fragmentation is used to characterize the different “phases” of egg chamber degeneration. Egg chambers in Figs. 3.3 are from St8 to early St9 of oogenesis. (A) Healthy (Ph0) egg chamber shows dispersed chromatin in NC nuclei, and FC nuclei surround the egg chamber. Scale bar=50μm. (B-F) Progression of cell death. NC nuclei become highly condensed and fragmented (arrows). Middle and lower panels show that FC membranes enlarge and engulf germline GFP (arrowheads). (B) Ph1, NC
chromatin is disordered. (C) Ph2, NC chromatin is condensed but individual nuclear regions are still apparent. (D) Ph3, NC chromatin becomes highly condensed into individual balls. (E) Ph4, NC chromatin is fragmented and widely dispersed. (F) Ph5, few NC nuclear fragments remain. (G) Quantification of engulfment from control G71/+ and GR1-GAL4 G89/TM6B flies shows decrease in germline GFP and area as death progresses. Error bars = SEM.

Credit: Images acquired by Adam Klein.
Figure 3.2 Drpr is required in follicle cells for proper engulfment of nurse cells. (A-C) WT (w^{118}) egg chambers labeled with DAPI (cyan), α-Drpr (yellow), and α-Dlg (magenta) (from starved flies). (A) Healthy egg chamber. Scale bar=50µm. (B) Ph3 dying egg chamber. (C) Ph5 egg chamber. Drpr staining intensity increases in the FCs (arrowheads in B) as engulfment proceeds in dying egg chambers. Arrow in B merge shows internalization of a NC nuclear fragment, and arrow and inset in C indicate Drpr puncta within FCs. (D-H) Egg chambers from starved flies expressing germline specific GFP (G71, green) stained with DAPI (DNA, cyan), and α-Dlg (magenta). Egg chambers are Ph0, 3 and 5 (left to right). (D-D’’) Control G71/+ egg chambers show normal death and engulfment. (E-E’’) drpr^{AS} flies show normal healthy egg chambers (E) but are defective in engulfment (E’) and show premature FC death (arrowheads) and lingering germline debris (arrows) (E’’). (F-F’’) Expression of drpr dsRNA in the FCs with GRI-GAL4 shows the same phenotype. (G-G’’) Expression of drpr^{+} in the FCs of drpr^{AS} egg chambers rescues engulfment defects. FCs enlarge and take up NC debris (arrows). (H-H’’’) Over-expression of drpr in the FCs of otherwise WT egg chambers induces NC death (flies NOT starved). FCs first thin out (arrowheads) (H’) but engulfment eventually begins and proceeds normally (H’’’). (I) Quantification of unengulfed germline (WT data from Figure 1G). Degree and pattern of chromatin condensation were used as the primary criteria for assigning phases of death in mutant egg chambers. For Ph5 egg chambers, a reduced number of NC nuclear fragments and/or >50% pyknotic FC nuclei were additional criteria. Ph5 drpr egg chambers show >100% unengulfed germline because of FC death. * p<0.05, ** p<0.005, *** p<0.001. (J) Percentages of egg
chambers that show no engulfment, partial engulfment (less than WT), complete
engulfment (similar to WT), and hyper-engulfment (engulfment before NC chromatin
condensation) for drprΔ5 (n=34) and UAS-drpr; drprΔ5 GRI-GAL4 (n=192).
Figure 3.3 *Drosophila* egg chambers respond rapidly to starvation and all phases are present at all times of starvation.

The mean number of degenerating egg chambers at each phase (± S.E.M.) per 100 ovarioles is shown. Three different control genotypes were combined and averaged: *w*¹¹¹⁸, UAS-*mCD8::GFP*/GR1-GAL4 and *drpr*Δ⁵/+. At least five replicates with a minimum of 44 total flies for each time point were analyzed. All flies were 3-6 days old after 2 days of conditioning and were starved on apple juice agar plates as described in Chapter 2. A two-tailed t-test was performed comparing the number of egg chambers in each phase at 4, 8 and 18 hours with the number observed in the absence of starvation (0 hours). *P<0.05.

Credit: Tracy Meehan
Table 3.1 Percentage of pyknotic follicle cells in egg chambers from starved flies.

<table>
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<th>Phase</th>
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1 Pyknotic follicle cells were counted and compared to the total number of follicle cells in a central confocal slice. n≥ 3 egg chambers were analyzed for each phase and genotype, except dMekk<sup>1</sup> phase 2 (n=2).

2 Data for WT were combined from G71/+ and GRI-GAL4-G89/TM6B control lines.

3 Homozygous mutants.

4 Driven by the follicle cell-specific GRI-GAL4 line.
Figure 3.4 Shark and Rac-1 are required in follicle cells for proper engulfment.

Egg chambers from starved flies express G71 GFP in the germline and are stained with α-Dlg (magenta), and DAPI (cyan). (A) GRI-GAL4 alone shows normal progression of mid-oogenesis PCD. Scale bar=50µm. (B) UAS-shark<sup>dsRNA</sup>/+; GRI-GAL4/+ shows defective clearance of NCs (arrow). FC membranes do not enlarge and GFP is largely absent in the FCs (arrowheads). FCs are pyknotic (arrowheads) as in drpr<sup>A5</sup> mutants. (C) UAS-Rac-1<sup>DN</sup>/+; GRI-GAL4/+ shows same phenotype. (D) Quantification of the unengulfed area.
Figure 3.5 The JNK pathway is activated and required in follicle cells during engulfment.

(A-D) Healthy and progressively dying egg chambers from starved flies are stained with DAPI (cyan), α-Drpr (yellow), and α-βgal (red). Egg chambers carry a lacZ enhancer trap in puc. (A) Healthy egg chambers express minimal Drpr and no puc-lacZ. Scale
bar=50µm. (B) Ph1 dying egg chambers begin to express Drpr and puc-lacZ in the FCs (arrowheads). (C-D) Drpr and puc-lacZ are expressed robustly in actively engulfing FCs in Ph4-5 dying egg chambers. (E-G) Healthy and progressively dying egg chambers from starved JNK pathway mutants stained with DAPI (cyan) and α-Dlg (magenta).

Arrowheads indicate the pyknotic nuclei of dying FCs or FCs that have failed to enlarge. (E-E’’) Egg chambers from UAS-bskDN/+; GRL-GAL4 G89/+ flies express bskDN in the FCs and germline GFP (green). Healthy (E, Ph0) and dying (E’, Ph1) egg chambers look normal but later phase egg chambers (E’’-E’’’, Ph4,5) display defects in engulfment. (F-G’’) Egg chambers expressing bskdsRNA or dMekk1dsRNA in FCs show a similar phenotype. Egg chambers in F-F’’’ are Ph0,1,3,5, and egg chambers in G-G’’’ are Ph0,3,4,5. (H-H’’, Ph0,1,2,5) eiger3 egg chambers show little FC enlargement and premature death of the FCs. (I,J) Quantification of the unengulfed area.

Credit: Allison Timmons
Figure 3.6 Engulfment mutants have defects in the breakdown of NC nuclei in late phases of death.
(A-D) Using ImageJ, the diameter of the largest remaining NC nucleus was measured in phase 3-5 dying egg chambers from a central confocal slice. \( n>3 \) for each phase of death in every genotype. Control egg chambers display a gradual decrease from phase 3 to phase 5 in the average diameter of the largest remaining NC nuclei. All of the engulfment-defective mutants show a larger NC diameter size than controls, and all except \( drpr^{A5} \) and \( hsk \) dsRNA show statistically significant differences. *P<0.005.
Figure 3.7 Drpr and JNK regulate each other during engulfment.

(A-A’’) Egg chambers over-expressing drpr in FCs show activation of puc-lacZ (red, arrowheads; flies NOT starved). Egg chambers stained with DAPI (cyan) and α-DCAD2 to label FC membranes (blue). Scale bar=50µm. (B-B’’) drprΔ5 egg chambers from starved flies do not express puc-lacZ (red, arrowhead) until late phases of death. Egg chambers are stained as in (A) and express G71 GFP (green). (C-C’’) FCs that over-express hepCA (tubulin-GAL80ts/+; UAS-hepCA/GRL G89) induce Drpr expression (red) in the absence of starvation. Arrowheads indicate FCs and arrows indicate dying NCs. (C’’) Lower magnification image shows widespread induction of Drpr and few late stage egg chambers. Scale bar=50µm. (D-D’’) Over-expression of hepCA in drprΔ5 background (tubulin-GAL80ts/+; UAS-hepCA/G71; drprΔ5 GRI-GAL4/drprΔ5 flies incubated at 29°C, starved) suppresses the drpr phenotype. (D) Healthy egg chamber appears normal. (D’-D’’). FCs enlarge (arrowheads) and engulf germline GFP. (D’’) Some egg chambers show a hyper-engulfment phenotype, where FCs (arrowheads) engulf intact NCs (arrows). (E-G) Enlargements of FCs from Figure 2D’ (E), Figure 2E’ (F), and Figure 5D’ (G) show that control and UAS-hepCA; drprΔ5 FCs engulf GFP (arrowheads) while drprΔ5 FCs do not (F, arrow). (H) Quantification of engulfment as in Figure 2J for drprΔ5 (n=128) and UAS-hepCA; drprΔ5 (n=745). Egg chambers from flies incubated at 29°C.
Figure 3.8 Caspase activity is required for proper engulfment but is not required for upregulation of Drpr or JNK activity.

(A-F) Caspase mutant egg chambers stained with DAPI (cyan), α-Drpr (yellow), and α-Dlg (magenta). (A’-F’) Drpr staining only (white). (A-C) Egg chambers from three homozygous alleles of dcp-1 (dcp-1prev1, dcp-1^2, and dcp-1^3) show Drpr up-regulation in the FCs when starved. However, most FCs fail to enlarge and display thinning out of their membranes (arrowheads). (D-D’) Egg chambers over-expressing diap1 in the germline (NGT/UASp-diap1; nanos-GAL4/+; starved flies) show Drpr up-regulation. FCs fail to enlarge and display thinning out of their membranes (arrowhead). (E-F) Egg chambers from unstarved flies over-expressing full-length dcp-1 (nanos-Gal4-tubulin (NGT)/UASp-fl-dcp-1; nanos-GAL4/+) in the germline show germline death but delays in engulfment and Drpr induction (compare E’ and F’ to Figure 2B,C). (G-I) Caspase mutant egg chambers stained with α-β-gal to detect puc-lacZ (red) and DAPI (cyan). (G-G’) Egg chambers from starved flies over-expressing diap1 in the germline (NGT/UASp-
diap1; nanos-GAL4/puc-lacZ) show an induction in puc-lacZ. G’ is likely a later phase egg chamber than G because of the FC loss. (H-I’) Germline over-expression of truncated dcp-1 (nanos-GAL4 UASp-tdcp-1/ puc-lacZ) in the absence of starvation leads to death of the germline with delayed puc-lacZ expression. The same egg chamber is shown in H,H’, and I,I’ with the red channel only shown in H’-I.’ I-I’ is a later phase egg chamber than H-H’ based on nuclear morphology.
Figure 3.9 Model for Drpr-JNK circuit in engulfing FCs.
Drpr (black rectangles) recognizes an unknown ligand(s) (purple circle) on germline cells, leading to its initial activation in FCs. Drpr becomes phosphorylated by Src kinase, and physically interacts with Shark. Shark activates Rac1 and leads to cytoskeletal changes and activation of Basket (JNK). The phosphorylation of Basket leads to the activation of the transcription factor AP-1, which translocates to the nucleus and activates its downstream target, puckered. Additionally, AP-1 is proposed to transcriptionally activate drpr, leading to enrichment of Drpr on the membrane and other engulfment genes. Our data suggest that the caspase dcp-1 activates an independent pathway that contributes to engulfment.

Credit: Allison Timmons
CHAPTER FOUR

Defective phagocytic corpse processing results in neurodegeneration and can be rescued by TORC1 activation

4.1 Introduction

Phagocytosis is a crucial component of development and organismal homeostasis. Failure in the phagocytic process can result in a wide range of disease states such as autoimmunity and cancer (Poon et al., 2014). In the mammalian central nervous system (CNS), microglia and astrocytes have been shown to be the most active cell types in executing the phagocytic response. These two glia subtypes are responsible for the removal of dead cells and bacteria, and the refinement of circuits within the CNS (Chung et al., 2013, Sierra et al., 2013). In recent years, a strong link has been established between defective phagocytosis by glia and neurodegenerative diseases. Phagocytic glia have been observed in nearly all neurological diseases (Fu et al., 2014). Furthermore, genome wide association studies have found a correlation between various alleles of genes involved in glia engulfment and a risk of developing neurodegenerative disorders (Kauwe et al., 2014). The mechanisms underlying phagocytosis are still not well understood and how defects in the clearance of apoptotic cells contribute to neurodegeneration remains understudied.

Phagocytosis of dead cells can be subdivided into three steps (Elliott and Ravichandran, 2010). The first step involves the recognition of the dead cell by the phagocyte via exposure of “eat me” signals on the membrane of the dead cell. The phagocyte then employs a complementary array of recognition receptors that bind
directly, or indirectly through bridging molecules, to the “eat me” signal. In the second step, the binding of a recognition receptor to an “eat me” signal triggers a cascade of intracellular events that lead to cytoskeletal rearrangements allowing the phagocyte to protrude membrane extensions around the dying cell, forming a phagocytic cup. This results in the internalization of the dead cell into a vesicle, known as a phagosome, within the phagocyte. In the third step, the phagosome undergoes successive fusion events to endosomes and lysosomes, culminating in the complete degradation of the apoptotic cell within the phagocyte (known as corpse processing or phagosome maturation).

Little is known about the pathways responsible for proper corpse processing. In particular, how a cell is able to distinguish vesicles containing apoptotic cells as opposed to other endocytosed material remains poorly understood. Recent findings have demonstrated that some apoptotic recognition receptors are not only important for internalization of corpses but are also important for phagosome maturation (Lu and Zhou, 2012). This dual role may allow the cell to distinguish apoptotic versus necrotic cells or pathogens and execute the appropriate response by coupling recognition to a specific set of processing signals (Blander and Medzhitov, 2006). Given this dual role, disrupting the function of these receptors may have a disproportional impact on proper phagocytosis and homeostasis. In fact, one of the strongest known risk factors for Alzheimer’s disease is a rare variant of the gene, TREM2 (Guerreiro et al., 2013, Jonsson et al., 2013). TREM2 has recently been shown to fall under this category of dual role phagocytic recognition receptors (Cantoni et al., 2015). Better understanding of the signaling
mechanisms employed by this class of receptors may be crucial in elucidating how defective phagocytosis by glia affects brain homeostasis.

Phagocytosis across species is well conserved. Work in *C.elegans* has identified two partially redundant pathways, known as the CED-1/6/7 and CED-2/5/12 pathways, which show a high degree of conservation from invertebrates to mammals (Hochreiter-Hufford and Ravichandran, 2013). Of the proteins important for this pathway, it has been shown that CED-1, as well as its orthologs in mammals, MEGF10 and JEDI (Scheib et al., 2012), act as recognition receptors. CED-1 has also been shown to be important in phagosome maturation. Specifically it has been shown that CED-1 leads to the recruitment of dynamin, a large GTPase involved in processing nascent phagosomes (Yu et al., 2008). Whether MEGF10 or JEDI are important for corpse processing remains to be determined.

*Drosophila melanogaster* has emerged as a powerful model for understanding mechanisms underlying glia-dependent phagocytosis as well as neurodegenerative diseases (Shulman et al., 2003, Fullard et al., 2009). Drpr, the *Drosophila* ortholog of CED-1, has been shown to be an important recognition receptor for glia-dependent phagocytosis (Freeman et al., 2003). Similar to CED-1, Drpr carries out a dual function and participates in corpse processing as well as recognition (Kurant et al., 2008). However, the signaling mechanisms important for Drpr-dependent corpse processing are unknown. The dual function of Drpr in phagocytosis, as well as its high conservation across species, led us to investigate whether defects in *drpr*-dependent glia phagocytosis could cause disruptions in brain homeostasis. We found that the absence of Drpr in glia
cells led to a massive accumulation of neuronal corpses in adult *Drosophila* brains and eventually neurodegeneration. Our findings indicate that these phenotypes arise from ineffective corpse processing rather than defects in apoptotic cell recognition. Moreover, these phenotypes were rescued by inactivating autophagy through TORC1 activation, pointing to a novel role for TORC1 in regulating phagosome maturation. Our data suggest a new function for *drpr* in corpse processing that is TORC1-dependent and crucial for brain homeostasis.

### 4.2 Apoptotic corpses persist in *drpr*<sup>−/−</sup> brains

To determine the impact of defective phagocytosis on brain homeostasis, we examined the brains of *drpr*<sup>−/−</sup> flies for persistent cellular corpses. Specifically, we looked for the presence of pyknotic nuclei, highly condensed chromatin suggestive of dead cells (Savill et al., 2002). We dissected brains from 1-day-old *drpr*<sup>−/−</sup> flies, stained them with DAPI, and surveyed the surface of the brain, where most cell bodies are located (Ito et al., 2014) (Figure 4.1A). The nuclei of control (w<sup>1118</sup>) brains appeared normal, with diffuse uncondensed DNA and a single bright condensed speckle corresponding to heterochromatin (Frost et al., 2014) (Figure 4.1B). As with control brains, the majority of nuclei in *drpr*<sup>−/−</sup> brains had normal chromatin morphology (Figure 4.1C). However a large number of the nuclei had a highly condensed appearance that was uniform throughout the nucleus, indicating pyknosis (Figure 4.1C). These pyknotic nuclei were found throughout the surface of the central brain as well as the surface of the optic lobes (Figure 4.1C, data not shown). To confirm that the large number of pyknotic nuclei seen in *drpr*<sup>−/−</sup> brains
were dead cells, we performed TUNEL labeling, which detects fragmented DNA, as well as antibody staining for cleaved dcp-1, an effector caspase (Song et al., 1997). Control flies displayed one to two TUNEL-positive cells per brain at 1 day of age (Figure 4.1D’, F). *drpr*−/− brains displayed a much higher number of TUNEL-positive nuclei, with an average of 663 dead cells per brain (Figure 4.1E’, F). Furthermore, TUNEL labeling in *drpr*−/− brains highly colocalized with pyknotic nuclei as detected with DAPI staining (Figure 4.1E”). *drpr*−/− brains were also highly positive for cleaved dcp-1 compared to control (Figure 4.2A’, B’). As with TUNEL staining, the brain regions with high levels of cleaved dcp-1 corresponded to the regions of pyknotic nuclei (Figure 4.2B”). These results indicate that the pyknotic nuclei correspond to apoptotic corpses. These experiments were performed on 1-day-old flies, so we were interested in determining the fate of these corpses as flies aged. We compared TUNEL labeling on flies at 1, 20, and 40 days of age. To our surprise, the number of TUNEL-positive corpses did not change with time (Figure 4.2C). This suggests that corpses remain in the brain throughout the lifespan of the organism.

The above analyses were conducted in *drpr* null flies. The defect in corpse clearance could be due to an absence of *drpr* in glia, neurons, or macrophages outside of the brain. To determine where *drpr* was required to prevent the persistence of apoptotic cells, we knocked down *drpr* in specific cell types using the GAL4-UAS system (Brand and Perrimon, 1993). We found that pyknotic nuclei formed in the brains of flies expressing *drpr* RNAi in glia using repo-GAL4 (Figure 4.3 A-B), but did not form when expressed in neurons or macrophages when using elav-GAL4 or croquemort-GAL4.
large number of pyknotic nuclei that formed using \textit{drpr} RNAi \textit{repo-GAL4} were also TUNEL-positive indicating that they were apoptotic corpses (Figure 4.3D', D'', E). As with mammals, \textit{Drosophila} contain different glia subtypes in the brain with different function. These include astrocyte, ensheathing, cortex, perineurial, and subperineurial glia (Awasaki et al., 2008). To determine which glia subtype acted as phagocytes to remove apoptotic corpses, we knocked down \textit{drpr} in the individual glia subtypes with different glia-specific GAL4 drivers. We found that expressing \textit{drpr} RNAi in astrocyte and cortex glia led to persistent pyknotic nuclei (Figure 4.3G, H). Knocking down \textit{drpr} in the other glia subtypes had no effect.

The \textit{drpr} gene encodes three different isoforms with distinct functions. The Drpr I isoform positively regulates phagocytosis while Drpr II negatively regulates engulfment (Logan et al., 2012). The function of Drpr III is unknown (Logan et al., 2012). The RNAi line we used above knocks down all three isoforms. To narrow down which isoform is responsible for the corpse phenotype we separately knocked down all three isoforms and found that the absence of the first isoform, but not the second or the third, was sufficient for the phenotype (Figure 4.3F). Taken together, these data indicate that \textit{drpr} (isoform I) is required in astrocyte and cortex glia to prevent the accumulation of apoptotic corpses in the brain. Furthermore, once these corpses form, they likely remain in the brain for the lifespan of the organism.
4.3 The apoptotic corpses are neurons that died during late larval and early pupal development

Our results indicate that pyknotic nuclei correspond to apoptotic cells that have not been cleared. However, the cellular identity of these corpses remained elusive, as they did not label with antibodies against neuronal or glial proteins which are typically lost early during cell death. The *Drosophila* brain is predominantly composed of neurons, with glia approximately constituting only 10% of the cells (Edwards and Meinertzhagen, 2010). The high number of corpses led us to believe that they were likely neurons. To determine whether the corpses were neurons, we marked neuronal membranes with myristoylated-GFP using *elav-GAL4* in a *drpr* / background. In control brains, myristoylated-GFP surrounded neurons in a membrane like fashion (Figure 4.4A-D). In the *drpr* / background, GFP collapsed around corpses (Figure 4.4E-H), suggesting that they were neuronal in origin. To further confirm that the corpses were neurons, we expressed a fluorescently tagged version of Histone 2A, His2A-RFP, in neurons (by using *elav-GAL4*) in a *drpr* / background. We found that corpses were positive for His2A-RFP (Figure 4.5A-B”), indicating that they were indeed apoptotic neurons. As a control, we expressed His2A-RFP in glia in *drpr* / brains (by using *repo-GAL4*) and found that corpses largely did not colocalize with His2A-RFP (Figure 4.5C-D”). These findings indicate that the corpses are dead neurons.

*Drosophila* has five discrete stages of development: embryo, 1st, 2nd, and 3rd instar larva, and pupa. To determine the specific time point in development that these corpses formed, we used a temperature sensitive form of GAL80 (a repressor of the UAS-GAL4
system (McGuire et al., 2003)) to induce *drpr* RNAi expression in glia at different time points during development. Specifically, larvae were shifted at different developmental time points from 18°C, a temperature at which GAL80 actively represses expression of the UAS driven transgene, to 29°C, a temperature at which GAL80 degrades and therefore the UAS driven transgene can become active. We observed that pyknotic nuclei were present in the central brain of adult flies when they had been shifted to 29°C at the 2nd instar phase of larval development (Figure 4.4I). Corpses were mostly absent when flies were shifted to 29°C at the beginning of the 3rd instar phase of larval development (Figure 4.4J). Corpses in the optic lobe, however, were still present at this time (Figure 4.4K). Conversely, when flies were shifted to 29°C towards the end of larval development, corpses were absent from the optic lobe (Figure 4.4L). These data suggest that the corpses that are observed in the central brain and optic lobe of adult *drpr* / flies form during the beginning and late portions of the 3rd instar larval phase respectively. Surprisingly, our findings also indicate that dying neurons arising later in development are efficiently cleared without Drpr.

**4.4 Knockdown of *drpr* in glia leads to age-dependent neurodegeneration and glial death**

We next wanted to determine if the persistence of neuronal apoptotic corpses in *drpr* mutant flies would disrupt brain homeostasis, potentially leading to neurodegeneration. Thus we sectioned *drpr* / brains at 1 and 40 days of age and examined them for the presence of vacuoles, a hallmark of neurodegeneration in
Drosophila (Hegde et al., 2014). We found that $drp^{−/−}$ brains exhibited high levels of vacuolization at 40 days when compared to control but not at 1 day of age. We then repeated this experiment in flies that had $drp$ specifically knocked down in glia. At 1 day of age, brains expressing $drp$ RNAi or GFP RNAi control in glia cells did not show high levels of vacuolization (Figure 4.6A, B, E). However, at 40 days of age, brains expressing $drp$ RNAi in glia, but not GFP RNAi, showed significant levels of vacuolization (Figure 4.6C-E). Consistent with our results, neurodegeneration in $drp^{−/−}$ brains was recently reported (Draper et al., 2014). These experiments show that $drp$ is required in glia for brain homeostasis and the prevention of age-dependent neurodegeneration.

Our previous work has shown that knocking down $drp$ in another model of engulfment, the Drosophila ovary, leads to premature death of the phagocytes (Etchegaray et al., 2012). In mammals, mutations in other phagocytosis components have also been shown to cause death of the phagocyte (Yvan-Charvet et al., 2010). We were interested to see if knockdown of $drp$ in glia also led to glial death, which could be a cause of the neurodegeneration. We used His2A-RFP to mark glia with repo-GAL4 and stained for TUNEL to visualize cell death. Control brains of flies expressing His2A-RFP in glia showed little to no TUNEL-positive glia (Figure 4.7A-A”, C). In contrast, brains of flies that expressed His2A-RFP and $drp$ RNAi showed some overlap between TUNEL and RFP indicating glia death (Figure 4.7B-B”, C). Furthermore, it appeared that glia that were TUNEL-positive were actively dying, as opposed to the majority of corpses which were neurons that died previously and persisted in the brain. This conclusion stems from the finding that His2A-RFP, TUNEL-positive glia were heterogeneous in size, with
many displaying very small nuclei (Figure 4.7B’, B” white arrows). This suggests that unlike most corpses, dying glia cells disappear. Moreover, unlike the majority of TUNEL-positive cells which were neurons, dying glia cells decreased in numbers with time (Figure 4.7C) suggesting that they were cleared. These results suggest that the absence of drpr in glia cells leads to autonomous cell death.

4.5 Uncleared apoptotic cells arise from a corpse processing defect in drpr-deficient glia

*drpr<sup>-/-</sup>* brains contain large numbers of persisting corpses as well as actively dying glia. The age-dependent neurodegeneration observed in these brains could be caused by either or both of these defects. To better understand the phenotype, we conducted a small screen to determine whether we could replicate the *drpr* phenotype. We knocked down other known interactors of *drpr*, as well as other *Drosophila* engulfment genes. To our surprise, none of the candidates we tested, including *shark*, *ced-6*, and *ced-12*, displayed persistent apoptotic cells (data not shown). However, Drpr has been shown to be important for two distinct steps in phagocytosis: the recognition and the processing of the corpse (Kurant et al., 2008). The engulfment genes that we analyzed have been shown to be primarily involved in the recognition and/or cytoskeletal reorganization important for the internalization of the dead cell into the phagocyte (Doherty et al., 2009, Ziegenfuss et al., 2012). Therefore, we reasoned that the *drpr<sup>-/-</sup>* phenotype may be due specifically to the corpse processing function of *drpr*. 
To assess whether *drpr*-deficient glia have defects in phagosome maturation, we co-expressed fluorescently-tagged phagosome maturation markers in glia cells that also had *drpr* knocked down. Failure of vesicles containing corpses to undergo fusion with organelles in the endocytic pathway has been shown to result in the accumulation of markers associated with corpse processing (Kinchen and Ravichandran, 2010). To this end, we expressed Rab7 and lysosomal-associated membrane protein 1 (LAMP1) GFP fusions, markers that are associated with corpse fusion to late endosome and lysosomes respectively (Lu and Zhou, 2012). We found that when expressed alone, Rab7-GFP had a membraneous appearance and somewhat uniform distribution throughout the surface of the brain (Figure 4.8A’,A”). However, knocking down *drpr* in glia led to a high accumulation of Rab7-GFP (Figure 4.8B’, B”). Specifically, Rab7-GFP highly accumulated and completely surrounded corpses but not healthy cells in these brains (Figure 4.8C’-C””). LAMP1-GFP had similar dynamics, where it appeared membrane-like and uniform in control brains (Figure 4.8D’, D””) but appeared to accumulate around corpses in brains where *drpr* RNAi was expressed in glia (Figure 4.8E’, E””). As with Rab7-GFP, LAMP1-GFP completely surrounded corpses but not healthy cells in these brains (Figure 4.8F’-F””). Unlike Rab7-GFP which seemed to “wrap” around corpses, LAMP1-GFP had a punctate appearance. The presence of both of these markers around corpses suggests that *drpr*-deficient glia have defects in phagosome maturation.
4.6 Activating TORC1 in glia cells suppresses corpse formation and rescues neurodegeneration

Our findings suggest that cellular corpses may result from defective phagosome processing in *drpr*-deficient glia. It has previously been shown that null mutants of *transient receptor potential mucolipin (Trpml)*, a cation channel responsible for vesicle fusion, show high levels of late apoptotic cells and neurodegeneration (Venkatachalam et al., 2008). Defects in *Trpml*-dependent vesicle fusion can be rescued by TORC1 activation (Wong et al., 2012). Furthermore, it has been shown that glia-specific knockdown of *raptor*, a component of TORC1, leads to defective phagocytosis in a model of axonal injury (Doherty et al., 2014). Therefore, TORC1 may be involved in apoptotic corpse processing during phagocytosis. Since our data indicate that corpse persistence in *drpr*−/- flies is a result of flawed phagosome maturation, we explored whether activation of TORC1 could modulate the *drpr* phenotype. Thus, we overexpressed Rheb, an activator of TORC1, in glia cells that also expressed *drpr* RNAi. Compared to expressing *drpr* RNAi by itself, expressing both Rheb and *drpr* RNAi led to a large reduction in the number of corpses forming in the brain. Flies mutant for *raptor* were shown to have lower levels of Drpr expression, suggesting that the function of TORC1 could be to upregulate Drpr (Doherty et al., 2014). To circumvent this potential issue, we expressed Rheb in glia cells in a *drpr* null background. *repo-GAL4, drpr-/-* brains, like *drpr*−/- alone, displayed a large accumulation of apoptotic cells on the surface of the brain (Figure 4.9A’, A”, D). However, overexpressing Rheb in glia cells of *drpr*
null flies strongly suppressed the phenotype close to wild-type levels (Figure 4.9B’, B’’, D).

TORC1 activates a variety of different pathways important for cell growth, motility, proliferation, and protein translation (Betz and Hall, 2013). Of particular interest, TORC1 has been shown to negatively regulate the initiation of autophagy. Proteins involved in autophagy have been shown to be important for vesicle processing, yet in most studies the initiation complex is not required (Martinez et al., 2011, Li et al., 2012). We therefore inferred that inhibition of autophagy initiation may be necessary to allow autophagy proteins to localize to vesicles containing corpses as opposed to vesicles containing non-specific cytoplasmic material. We found that the formation of corpses in \( drpr^{+/−} \) brains was strongly suppressed by knocking down \( Atg1 \), an initiator of autophagy that is inhibited by TORC1 (Figure 4.9C’, C”’, D). These results suggest that TORC1 activation, through autophagy inhibition, suppresses the corpse processing defect seen in \( drpr^{+/−} \) brains. Furthermore, TORC1 suppression of corpse formation persisted throughout the lifespan of the organism (Figure 4.9D).

Two other phenotypes that we observed in \( drpr \) mutants were glial death and neurodegeneration. To determine whether glial death could be rescued by TORC1 activation, we overexpressed \( Rheb \) and His2A-RFP in glia cells in a \( drpr^{+/−} \) background. We found that the number of glia dying was similar in 1 day old \( drpr^{+/−} \) brains and \( drpr^{+/−} \) brains over-expressing \( Rheb \) in glia (Figure 4.10A-A”, C). These results were not due to Rheb’s expression being dampened by co-expression of His2A-RFP since the suppression of corpse formation was similar to expressing Rheb by itself (compare Figure
4.9B’ to Figure 4.10A’). These results suggest that the glia death is most likely due to a defect in the recognition role that Drpr plays in phagocytosis, and not due to a corpse processing defect.

To determine whether the corpse processing role of drpr underlies age-dependent neurodegeneration, we overexpressed Rheb in glia cells of drpr⁻/⁻ flies and sectioned their brains. 40 day old drpr⁻/⁻ brains exhibited high levels of vacuolization (Figure 4.9E, G). 40 day old drpr⁻/⁻ brains overexpressing Rheb in glia showed a decrease in the amount of vacuolization (Figure 4.9F, G). This suggests that TORC1 activation in glia can suppress the age-dependent neurodegeneration phenotype in drpr null brains. This finding, together with the finding that expressing Rheb in glia cells rescues corpse formation but not glia death, suggests that the persistence of corpses causes neurodegeneration. Therefore, defects in corpse processing, but not recognition, result in age-dependent neurodegeneration through the persistence of corpses in adult brains. These defects can be rescued by activating TORC1 in glia, which we show plays a novel role in phagosome maturation.

4.6 Discussion

How defects in the clearance of dead cells contribute to neurodegeneration remains elusive. Our work has shown that the accumulation of apoptotic cells in the brain, due to defects in glia mediated corpse processing, leads to neurodegeneration. We also showed that corpse accumulation, as well as neurodegeneration, can be suppressed by TORC1-mediated inhibition of autophagy. To our knowledge, it has not been
previously shown that deficits in clearance of apoptotic cells by glia directly cause neurodegeneration in vivo.

Corpse accumulation in *drpr*/* brains results from defects in phagosome maturation. This is evidenced by the accumulation of glia-derived Rab7-GFP and LAMP1-GFP around neuronal pyknotic nuclei. We showed that the defect in phagosome maturation can be rescued by activating TORC1. Activating TORC1 in glia cells of *drpr*/* flies suppresses corpse formation but not glia death. Since glia are still actively dying in these brains it is unlikely that adult phagocytic defects associated with *drpr*/* are suppressed through TORC1 activation. However TORC1 activation suppresses age-dependent neurodegeneration. These findings indicate that processing defects in development, as opposed to phagocytic defects in adulthood, lead to persistent corpses. Our findings indicate that persistent corpses are responsible for age-dependent neurodegeneration in adulthood.

We found that corpses in adult *drpr*/* brains formed during development. Corpse numbers remained constant even though the neurodegeneration phenotype indicated that neurons were continuing to die in *drpr*/* brains. Why corpse accumulation does not increase in adults, even though neurodegeneration is occurring, is unclear. It may be because *drpr* has distinct roles at different stages of development. During embryogenesis, *drpr*-deficient glia can still internalize dead cells but are unable to process them (Kurant et al., 2008). Conversely in adults, axotomy experiments showed that *drpr*-deficient glia fail to internalize debris, suggesting that after eclosion Drpr plays a stronger role in
recognition (MacDonald et al., 2006). We propose that the critical role of Drpr during larval development is in corpse processing.

*Drosophila* development is characterized by high levels of apoptosis. Thus, persistent corpses could arise from high levels of cell death overwhelming *drpr*-deficient glia. The increased intake of multiple apoptotic corpses would require efficient processing, and an inability to do so would lead to persistent corpses. The levels of cell death occurring in *drpr*−/− brains during adulthood may be low enough to allow other recognition receptors and processing systems to ensure the removal of the corpses. This point could have relevance in situations of high cell death such as in disease or trauma. Specifically, during these conditions there could be an increased need for very efficient processing. Individuals with defective alleles in genes for corpse processing, such as in *TREM2*, could be highly vulnerable to the persistence of corpses and further exacerbations of neurodegeneration.

How corpse processing defects cause neurodegeneration remains to be elucidated. It is unlikely that the corpses themselves cause death to the surrounding cells. This stems from the finding that the onset of neurodegeneration is age-dependent even though the corpses are present from earlier in development. It is therefore more likely that the presence of corpses interferes with normal neuronal or glia development/function and this then leads to neurodegeneration. Recent work has shown that phagocytosis is important in regulating the number of neural precursor cells in mammalian development as well as in adults (Lu et al., 2011, Cunningham et al., 2013). It may be that defective corpse processing in *drpr*−/− brains results in aberrant neural stem cell production. Furthermore,
in mice it was found that the MeCP2 mutation that causes Rett Syndrome, an autism spectrum disorder, led to defective phagocytosis in glia (Derecki et al., 2012). Although not a neurodegenerative disease, patients with Rett syndrome do display increased cell loss in the cerebellum (Oldfors et al., 1990). Therefore, corpse processing defects observed in drpr/− could lead to developmental defects which then give rise to age dependent neurodegeneration.

Previous work has indirectly implicated TORC1 in phagocytosis. Raptor, a component of TORC1, as well as the phosphoinositol 3-kinase (PI3K) signaling pathway, an activator of TORC1, have been shown to be important for phagocytosis in Drosophila (Doherty et al., 2014). Furthermore, in both mammals and C. elegans, disrupting PI3K signaling leads to defects in the corpse processing of bacteria and apoptotic cells respectively (Fratti et al., 2001, Kinchen et al., 2008). Our work, in conjunction with these previous findings, suggests that TORC1 acts downstream of PI3K to regulate phagosome maturation.

We found that the suppression of corpses was rescued by knocking down Atg1 in glia. This suggests that TORC1 promotes corpse processing by inhibiting autophagy initiation. At first glance this seems contrary to data accumulated both in C. elegans and mammals. Studies have shown that autophagy proteins localize to vesicles containing corpses, allowing for proper processing of dead cells, in a process known as LC3-associated phagocytosis (LAP) (Florey et al., 2011, Martinez et al., 2011, Li et al., 2012). LAP does not appear to require Atg1 or the autophagy initiation process (Florey et al., 2011, Martinez et al., 2011, Li et al., 2012). Phagocytosis has been shown to be an energy
intensive process (Han and Ravichandran, 2011). Autophagy initiation may need to be suppressed to prevent the formation of autophagosomes and autolysosomes (which form under conditions of high energy demand). This would then free up other autophagy proteins and allow their recruitment to vesicles containing corpses. Within the endocytosis pathway, some proteins, such as Rab5 and Rab7, have been shown to be important for many membrane trafficking events. TORC1 mediated inhibition of autophagy may also be important in preventing these proteins from localizing to autophagosomes (Figure 4.11). This would allow their proper localization to vesicles containing corpses. Autophagy and phagocytosis are essential processes in most multicellular organisms. Autophagy inhibition may have evolved across species as a key mechanism to promote the proper localization of trafficking proteins during phagocytosis.
Figure 4.1 Drpr is required for corpse clearance in the adult brain.

A) Brain stained with DAPI (blue) to label nuclei (arrows) and anti-Discs Large (magenta) to label neuropil. White box represents the area of the brain that is shown in subsequent images. All images are z projections unless otherwise noted. B-C) DAPI staining shows normal nuclear morphology in w^{1118} (B) but pyknotic nuclei amassing on the surface of drpr^{-/-} brains (w; drpr^{Δ5}/ drpr^{Δ5}) (C). Insets represent zooms of white boxes in B and C. D-E”) TUNEL labeling shows that control brains have few TUNEL-positive cells (D’, D”) whereas drpr null brains contain a large number of positive cells (E, E’’). F) Quantification of TUNEL positive cells in the central brain of 1 day old control and drpr null brains. (n ≥ 6, ***P ≤ 0.001). Data presented are mean ± SEM.
Figure 4.2 The absence of *drpr* leads to persistence of apoptotic cells in the brain over the lifespan of the fly.

A-B’’’*w*[^118] brains are mostly negative for anti-cleaved dcp-1 caspase staining (A, A’’’), while *drpr*’’ brains are positive for anti-cleaved dcp-1 (B’’) that overlaps with pyknotic nuclei (B’’’). Insets show zooms of boxes in A’’’ and B’’’. C) Quantification of TUNEL-positive cells in the central brain of 1, 20, and 40 day old *drpr* null brains. (n ≥ 8)
Figure 4.3 Knockdown of drpr in glia leads to corpse accumulation.

A-B) DAPI staining on control (repo-GAL4) brain shows typical DNA morphology (A) while DAPI staining of drpr RNAi driven by repo-GAL4 in glia (UAS-drpr-RNAi/+; repoGAL4 /+) (B), shows pyknotic nuclei on the surface of the brain. C-D) Control brains are mostly TUNEL-negative (C’) whereas drpr RNAi driven in glia results in a large number of TUNEL-positive cells (D’). E) Quantification of TUNEL-positive cells in the central brain of 1 day old drpr RNAi driven in glia and control brains. (n ≥ 6, ***P ≤ 0.001). F) DAPI staining of a brain in which drpr I RNAi is driven by repo-GAL4 (UAS-drpr-I-RNAi/repo-GAL4) shows pyknotic nuclei on the surface. G-H) DAPI staining of drpr RNAi being driven by promoters specific to cortex glia (UAS-drpr-RNAi/+; NP2222-GAL4/) (G) or astrocyte glia (UAS-drpr-RNAi/NP3233-GAL4) (H) shows pyknotic nuclei accumulating on the surface of the brain. Insets represent zooms of white boxes in A, B, F, G, and H.
Figure 4.4 Neuronal corpses form during development in *drpr* deficient brains.

A) DAPI stain on brains that express myristoylated-GFP under *elav-GAL4 (elav-GAL4/+; UAS-IVS-myristoylated::GFP/+)*, shows GFP surrounding neurons (A). B-D) Zoom of white box in (A), shows myristoylated-GFP (C) surrounding neurons (B) in panel (D) E) DAPI stain on *drpr* null brains that also express myristoylated-GFP under *elav-GAL4 (elav-GAL4/+; UAS-IVS-myristoylated::GFP/+; drprΔ5/drpΔ5)*, shows GFP collapsed around pyknotic nuclei (E). F-H) Zoom of white box in (E), shows myristoylated-GFP (G) surrounds pyknotic nuclei (G, arrowheads) in panel (H). I-J)
Corpses persist in the adult central brain of tub-GAL80ts/+; UAS-drpr-RNAi/+; repo-GAL4/+ flies when 2nd instar larva are shifted to 29°C (I) and largely disappear when flies are shifted to 29°C during the beginning of the 3rd instar larval stage (J). K-L)

Corpses persist in the adult optic lobes of tub-GAL80ts/+; UAS-drpr-RNAi/+; repo-GAL4/+ flies when larvae are shifted to 29°C during the beginning of the 3rd instar larval stage (K) and largely disappear if flies are shifted to 29°C during at the end of the 3rd instar larval stage (L). Below the images are schematics for the temperature shift protocol used for I-J. Briefly, flies were raised at 18°C, shifted to 29°C at different time points during development, and raised at 29°C until eclosion. E- embryo, 1- 1st instar larva, 2-2nd instar larva, 3- 3rd instar larva, P- Pupa, and A- adult. Images from A-H are all single slice images.
Figure 4.5 Pyknotic nuclei largely correspond to neurons.

A-A’’) Pyknotic nuclei labeled with DAPI (A) and His2A-RFP (A’) driven specifically in neurons with the elav-GAL4 promoter (elav-GAL4/+; UAS-His2A-RFP, drpr^{A5}/drpr^{A5}) colocalize with one another (A’’). B-B’’) Zooms of white boxes in A-A’’. Pyknotic nuclei in outlined area (B) colocalize with His2A-RFP in the same outlined area (B’-B’’), although His-RFP is less intense than in some surrounding areas. C-C’’) Pyknotic nuclei (C) do not colocalize with His2A-RFP (C’-C’’) when driven specifically in glia with repo-GAL4 (UAS-His2A-RFP, drpr^{A5}/repo-GAL4, drpr^{A5}). D-D’’) Zooms of white boxes in C-C’’. Outline of area with pyknotic nuclei (D) is devoid of His2A-RFP (D’-D’’), indicating that the majority of corpses are not glia.
Figure 4.6 Knockdown of drpr in glia leads to age-dependent neurodegeneration.

A-D) Brain slices of UAS-GFP RNAi/+; repo-GAL4/+ (A) and UAS-drpr-RNAi/+; repo-GAL4/+ (B) show little vacuolization at 1 day of age. However, at 40 days of age, drpr RNAi brains (D) show significant levels of vacuolization (black arrows) while GFP RNAi brains (C) do not. E) Quantification of the number of vacuoles per brains for GFP RNAi and drpr RNAi brains at 1 day and 40 days respectively (n=6, *P ≤ 0.05)
DNA  His2A-RFP  TUNEL  MERGE

A  A'  A''  A'''

B  B'  B''  B'''

C

Number of TUNEL positive glia per brain

1 day  10 days

- His2A-RFP
- His2A-RFP drpr RNAi

***  *  ***
Figure 4.7 *drpr*-deficient glia actively undergo cell death.

A-B”’) Glia-specific His2A-RFP does not colocalize with TUNEL in control brains (*repo-GAL4/UAS-His2A-RFP*) (A-A”), whereas His2A-RFP in glia expressing *drpr* RNAi (*UAS-drpr-RNAi/+; repo-GAL4/UAS-His2A-RFP*) shows some overlap with TUNEL staining (B-B”). Inset represents zoom of white box. C) Quantification of the number of His2A-RFP positive cells that colabel with TUNEL for control and *drpr* RNAi at 1 and 10 days respectively. (n ≥ 6 *P* ≤ 0.05, ***P* ≤ 0.001)
Figure 4.8 Corpses become arrested during late phagosome maturation.

A-A’’) Rab7-GFP driven in glia cells has a membrane like appearance in control (UAS-Rab7::GFP/+; repo-GAL4/+, A’-A’’). B-C’’) Rab7-GFP highly accumulates around pyknotic nuclei (B’, B’’) in brains where drpr has been knocked down in glia (UAS-Rab7::GFP/ UAS-drpr-RNAi; repo-GAL4/+). C-C’’ are zoomed images of areas outlined by white rectangles in B-B’’. C’’’ is a Y-Z representation of white line in C’’. Rab7-GFP completely surrounds corpses but does not surround nuclei with normal nuclear morphology (C’’, C’’’). D-D’’) LAMP-GFP has a speckled, as well as membranous appearance in control brains (UAS-LAMP::GFP/+; repo-GAL4/+ (D’, D’’). E-F’’) In brains where drpr has been knocked down in glia (UAS-LAMP::GFP/ UAS-drpr-RNAi; repo-GAL4/+), LAMP-GFP speckles highly accumulate around pyknotic nuclei (E’,E’’). F-F’ are zooms of area outlined by white rectangles in E-E’’. F’’’ is a Y-Z representation of white line in E’’. LAMP-GFP speckles completely surrounds corpses but not nuclei with normal morphology (F’’, F’’’).
Figure 4.9 Activation of TORC1, through Atg1 inactivation, suppresses corpse formation and partially rescues neurodegeneration in drpr null mutant brains.

A-B’’) The accumulation of TUNEL-positive cells (A’, A’’) seen in drpr null brains is suppressed by expressing Rheb specifically in glia cells (UAS-Rheb; drprΔ5 / repo-GAL4, drprΔ5) (B’, B’’). C-C’’) Knocking down Atg1 in glia of drpr null flies (UAS-Atg1 RNAi; drprΔ5 / repoGAL4, drprΔ5) phenocopies the TUNEL suppression (C’, C’’) seen by overexpressing Rheb (B’, B’’). D) Quantification of TUNEL-positive corpses in drpr null brains with repo-GAL4, repo-GAL4 UAS-Rheb, or UAS-Atg1 RNAi at 1, 20, and 40 days respectively (n > 5, **P < 0.01, ***P < 0.001) (D) E-F) Brain slices of drpr null flies
expressing Rheb in glia cells show a decrease in the number of vacuoles at 40 days (F) when compared to drpr by itself (E). H) Quantification of the number of vacuoles per brain for drpr^Δ5 / repo-GAL4, drpr^Δ5 brains and UAS-Rheb; drpr^Δ5 / repo-GAL4, drpr^Δ5 brains at 40 days of age (n=6, p <0.06).
Figure 4.10 Activation of TORC1 does not rescue glia death in \textit{drpr} null flies.

\textbf{A-A''} Glia-driven histone-RFP colocalizes with TUNEL staining in brains of \textit{drpr} null flies even when \textit{Rheb} is overexpressed in glia (\textit{UAS-Rheb; UAS-His2A-RFP, drpr^{Δ5}/repo-GAL4, drpr^{Δ5}}) (A-A’’). Inset represents zoom of white box. \textbf{C} Quantification of the number of histone-RFP positive cells that colabel with TUNEL for \textit{drpr}^{Δ5}/\textit{repoGAL4}, \textit{drpr^{Δ5}} and \textit{UAS-Rheb/+; drpr^{Δ5}/repo-GAL4, drpr^{Δ5}} at 1 day of age. (n \geq 4)
Figure 4.11 Model illustrating how TOR prevents mislocalization of components important for phagocytic processing.

Activation of TOR suppresses autophagy therefore preventing components important for corpse processing from localizing to the autophagosome.
CHAPTER FIVE

Tools for screening in Drosophila

5.1 Introduction

There are still many unknowns in PCD and phagocytosis. To further the understanding of these processes, I have developed reagents to conduct high throughput screens. One of the powers of conducting research in Drosophila is the ability to conduct large genetic screens. However, such screens although possible are many times laborious in nature. There are many reasons for this. One of the reasons that most significantly can contribute to this problem is the assay under which the screen is conducted. A common way to circumvent this problem is to make the assay simpler in nature or if situations allow, turn the screen into a selection. In particular, selections which revolve around examining the viability of the fly are of great utility. In these selections, the researcher just needs to see whether fly viability under testing conditions increased or decreased after a particular gene or gene mutation was introduced. To this end, I developed reagents that could be used to conduct large genetic selections in the brain. Specifically, I screened through various death effector lines to examine which ones induce death of neurons in a reproducible and reliable manner when expressed pan-neuronally. Once efficient death effectors were found, I tested to see what area of the fly brain is required for viability. To do so, I expressed the efficient death effectors in various areas of the brain using brain area specific drivers. I found that Diap-1 RNAi, dcp-1, MJD, and Ricin are all efficient death effectors. Furthermore, I found that when Diap-1 RNAi is expressed in the ellipsoid body it leads to organismal death. Finally, since most flies use the UAS-GAL4
system for transgene expression, creating a transgenic fly that expresses the death effectors under the control of a different transgene expression system would be of particular use. This is because such a system would prevent cross talk between the system used to drive expression of your gene of interest (UAS GAL4 system) and the system used to drive the death effector. To this end, I created a transgenic fly where dcp-1 is under the control of a different transgene expression system. By examining delays or exacerbations in organismal survival, these reagents can be used to uncover novel processes in cell death and phagocytosis in an expedient way.

5.2 Diap-1 RNAi, MJD, dcp-1 and Ricin are efficient death effectors when expressed under a pan-neuronal driver

The brain of Drosophila is crucial for organismal viability. Inducing massive amounts of cell death in the brain leads to a decrease in organismal survival (Lessing and Bonini, 2009). To develop a system that allows high throughput selection by looking at survival rates, we focused on inducing cell death in neurons. To this end we selected candidate death effectors under the control of UAS and expressed them in neurons using the pan neuronal driver elav-GAL4. Death effectors were selected based on literature searches for their ability to induce cell death. Two types of death effectors were chosen for this assay: those that induce apoptosis and those that induce non-canonical forms of cell death. A list of death effectors tested and their modes of cell death can be seen in Table 5.1. Since we wanted to induce cell death specifically in the adult, we avoided
lethality during development by crossing in a temperature sensitive version of GAL80, a repressor of UAS-GAL4 expression. Flies were raised at 18°C. Upon eclosion flies were moved to 29°C, a temperature where GAL80 breaks down and the UAS-GAL4 system is de-repressed. The percent survival of flies per day after gene expression was quantified. From death effectors tested, we found that expression of Diap-1 RNAi, dcp1, and SCA3 induced organismal death compared to control (Figure 5.1). Ricin also induced organismal death compared to control, however, Ricin also blocks protein synthesis (Spooner and Lord, 2015). We were not able to determine whether organismal death was caused because of neuronal cell death or defects in synaptic transmission. We therefore excluded Ricin from further analysis.

### 5.3 PCD in the ellipsoid body leads to organismal death

The *elav-GAL4* driver is pan-neuronal. Different neurons may have different sensitivities to death effectors that are expressed by *elav-GAL4*. Furthermore, different areas of the brain may be more important for the organisms viability that others. In our system, it is therefore unknown what neurons are dying and in what area of the brain is the expression of these death effectors leading to organismal death. To narrow down which neurons were dying and what areas are important for organismal viability, we crossed region-specific GAL4 drivers to *Diap-1* RNAi. As with experiments using *elav-GAL4*, temperature sensitive GAL80 was used as well. Experiments were then carried out as described above. We tested 29 different GAL4 drivers with different expression patterns in the brain. Out of the lines tested, only two led to organismal death (Figure 5.2,
5.3). Both lines express specifically in the ellipsoid body, the brain area responsible for movement in the fly. The two GAL4 lines with ellipsoid body expression were 50020 and 48692. The GAL4 driver in these two lines is inserted in or near the genes shot (for 50020) and spineless (for 48629). GAL4 is presumed to be under the control of DNA sequences responsible for the expression of these genes. Interestingly organismal death was faster when Diap-1 RNAi was driven by these two drivers than when driven by elav-GAL4. Furthermore, the fact that two different GAL4 lines expressing in the same brain area led to organismal death, suggest that expression of the death effector in the ellipsoid body is responsible for death of the organism as opposed to potential off-target expression patterns of these drivers.

Inactivating Diap-1 leads to caspase activation. Although the main role of caspases is to induce cell death, caspases have been found to have other functions outside of PCD (Fuchs and Steller, 2011). To make sure that organismal death in flies expressing Diap-1 RNAi in the ellipsoid body is due to neuronal death as opposed to another effect of caspase overactivation, we decided to measure neuronal viability. To measure neuronal viability, we determined whether there was a decrease in fluorescence. We decided to use loss of fluorescence as opposed to other cell death markers because many cell death markers use caspase activity as a readout for death. Since we are overactivating caspases by knocking down Diap-1, cell death markers could indicate high caspase activity in the absence of death. Furthermore, phagocytosis is a very efficient process. Therefore, observing high levels of cell death by using cell death markers when phagocytosis is intact can be difficult (Hochreiter-Hufford and Ravichandran, 2013). To
this end, we expressed a membrane fluorescent reporter, mCD8::RFP, and a nuclear fluorescent reporter, Redstinger, under the control of the ellipsoid GAL4 drivers. We then also expressed Diap-1 RNAi and analyzed whether expressing Diap-1 RNAi led to a loss of fluorescence. Loss of fluorescence of both membrane and nuclear reporters is indicative of death. Flies were raised at 18°C and were transferred to 29°C at eclosion. We then examined fluorescence after five days at 29°C. We found that expressing Diap-1 RNAi led to a strong decrease in membrane fluorescence as well as a decrease in the number of cells expressing a nuclear reporter when driven by 48692 (Figure 5.4, Figure 5.5). We did not find a decrease in fluorescence when Diap-1 RNAi was driven by 50020. These results indicate that Diap-1 RNAi leads to cell death when driven in ellipsoid body neurons with the 48692 promoter but not with 50020.

5.4 Cloning dcp-1 into the Q system

The UAS-GAL4 system of transgene expression offers several advantages. Tissue specific expression of exogenous transgenes is one of them. However, a problem with UAS-GAL4 system is the inability to induce different transgenes in different cell types at the same time. Having two GAL4s and two UAS driven genes leads to expression of both transgenes in both GAL4 expressing cell types. To circumvent this problem, researchers have developed the Q system (Potter et al., 2010). Like the UAS-GAL4 system the Q system is a binary transgene expression system. This system does not cross talk with the UAS-GAL4 system. In other words, GAL4 cannot activate QUAS sequences, nor can QF activate UAS sequences. Coupling both of these system allows for expression of two
different transgenes in two different cell types if QF and GAL4 have different expression patterns. In our system, this could allow for expression of death effectors in a brain area of choice and the expression of another transgene in surrounding neurons or glia cells. Other transgenes could express RNAi or overexpress proteins for example. This system can be used to conduct high throughput selection by combining our death effector expression system developed above and manipulating either surrounding neurons or glia. Organismal viability can then be compared after cell type specific manipulations.

To develop this system, we decided to clone dcp-1 under the control of QUAS. We decided to clone dcp-1 instead of Diap-1 RNAi because the Diap-1 RNAi transgene under the control of QUAS sequences is being developed by Drosophila RNAi Screening Center and will be made publicly available. To this end, we used a previously created dcp-1 plasmid and by using restriction enzymes we isolated the dcp-1 sequences. We then obtained a QUAS plasmid from Addgene and by using restriction enzymes as well as ligation systems, we cloned dcp-1 into QUAS vector and generated transgenic flies. These flies carried independent insertions throughout the genome. We then crossed these flies to M2ET-QF, a QF insertion 49E6 near the Posterior sex combs gene. This QF line has been shown to drive expression in the Drosophila eye (Potter et al., 2010). Cell death induced in the eye produces a rough eye phenotype (Wolff and Ready, 1991). Since dcp-1 is an effector caspase we screened for rough eye phenotype to obtain lines where dcp-1 could be sufficiently expressed by QF drivers. Out of the flies screened, we found approximately 10 lines that had a rough eye phenotype indicating these flies had levels of QUAS-dcp-1 sufficient to kill cells when driven by QF.
5.5 Discussion

Screening is a laborious process. To develop a high throughput way to identify novel regulators of cell death and phagocytosis, we created reagents to perform selections. Specifically, we analyzed various death effectors for their ability to induce neuronal death by measuring organismal survival. We also analyzed areas of the brain required for adult viability by inducing death effectors in these areas. We found that Diap-1 RNAi, dcp-1 and MJD are all efficient death effectors when expressed pan-neuronally, as measured by their ability to induce organismal death. We also found that expressing Diap-1 RNAi in the ellipsoid body, the movement center of the Drosophila brain, leads to organismal death. This death correlated with loss of neurons suggesting that decreases in organismal viability were due to neuronal death. We also cloned dcp-1 into the Q system to allow, in conjunction with UAS-GAL4, for the manipulation of two different cell types by expressing two different transgenes. These reagents will allow for rapid identification of novel genes in cell death and phagocytosis.

An interesting finding was the inability of many known death effectors to induce death when expressed pan-neuronally. Furthermore, expression of death effectors that did induce organismal death did so after a variable number of days, even though GAL80 degradation and transgene expression should be on by six to twelve hours after flies had been placed at 29°C. The reason why neurons were resistant to cell death is unclear. It has been shown in other organisms that once neurons mature, they become highly resistant to cell death. Specifically, mature neurons seem to upregulate anti-apoptotic
proteins such as XIAP (the mammalian homologue of Diap-1) and downregulate the expression of initiator and effector caspases, such as Apaf-1 and caspase 3 (the mammalian homologue of dcp-1) (Kole et al., 2013). It could be that Drosophila neurons have co-opted a similar mechanism. Therefore, the expression of a single death effector may be insufficient to induce neuronal death. Instead expression of multiple death effectors may be required to induce PCD in neurons.

The finding that expressing death effectors in the ellipsoid body leads to cell death is interesting. Our main hypothesis is that motor function is important for viability. Blocking movement may impair many functions important for homeostasis and proper feeding. However, it could be that these drivers expressed Diap-1 RNAi at higher levels than the other ones tested. This is unlikely, since the lab that developed these drivers tested their expression and could not find striking differences (flylight.org, unpublished data). Finally, we observed that the 50020 GAL4 driver did not seem to induce neuronal death but did induce decreases in organismal viability. The reasons for this is unclear. It could be that the 50020 GAL4 driver is weaker than the 48698 driver but target a neuronal population more important for viability within the ellipsoid body than 48698. Therefore, the addition of the UAS-RFP transgene construct to UAS Diap-1 RNAi would decrease the amount of GAL4 driving each construct. In this situation, the 50020 GAL4, being a weaker line than 48698, may not express sufficient GAL4 to drive both RFP and Diap-1 RNAi to a level where it can induce cell death. Future experiments need to be done to address this question.
These reagents can be used to uncover novel processes in cell death and phagocytosis. Specifically, they can be used to uncover genes that negatively or positively regulate PCD by examining whether organismal death became delayed or exacerbated. Furthermore, genes can be specifically expressed in glia cells by using the UAS-GAL4 system while at the same time inducing death of the neurons using the Q system. Delays or exacerbations in organismal viability can uncover genes involved in the role of glia in PCD, which includes the process of phagocytosis.
### Table 5.1 Death effectors tested

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<thead>
<tr>
<th>Apoptotic death effectors</th>
<th>Reference</th>
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<tr>
<td>Reaper (IAP inhibitor)</td>
<td>(Mohseni et al., 2009)</td>
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<tr>
<td>Hid (IAP inhibitor)</td>
<td>(Bergmann et al., 2002)</td>
</tr>
<tr>
<td>Grim (IAP inhibitor)</td>
<td>(Wing et al., 2002)</td>
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<tr>
<td>Diap1-RNAi (IAP RNAi)</td>
<td>(Orme and Meier, 2009)</td>
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<td>Diap2-RNAi (IAP RNAi)</td>
<td>(Orme and Meier, 2009)</td>
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<tr>
<td>Drice (effector caspase)</td>
<td>(Keller et al., 2011)</td>
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<tr>
<td>dcp-1(effector caspase)</td>
<td>(Song et al., 1997)</td>
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<tr>
<td><strong>Non-canonical death effectors</strong></td>
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<tr>
<td>Diptheria Toxin (inhibitor of RNA translation)</td>
<td>(Andrews et al., 2014)</td>
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<tr>
<td>Ricin (inhibitor of RNA translation)</td>
<td>(Neuburger et al., 2006)</td>
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<tr>
<td>Pros-β2 DN (proteosome inhibitor)</td>
<td>(Scuderi and Letsou, 2005)</td>
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<tr>
<td>Machado Joseph disease polyglutamine tract protein (MJD) (induces glutamine toxicity)</td>
<td>(Warrick et al., 1998)</td>
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Expressing Diap-1 RNAi (blue), dcp-1 (green), and MJD (purple) pan-neuronally with elav-GAL4 leads to organismal death faster than control. Days after gene expression has been induced is time when flies were transferred from 18°C to 29°C. Percent survival was taken as the number of starting flies minus the number of flies that died divided by starting flies time 100.

Figure 5.1 Diap-1 RNAi, dcp-1 and MJD induce death upon neuronal expression
Figure 5.2 Expression of Diap-1 RNAi in the ellipsoid body with the 50020 GAL4 driver leads to organismal death faster than control

Expression of Diap-1 RNAi (blue) in the ellipsoid body, using the 50020 GAL4 driver, leads to organismal death faster than control (driver by itself, green). Days after gene expression has been induced is time when flies were transferred from 18°C to 29°C. Percent survival was taken as the number of starting flies minus the number of flies that died divided by starting flies time 100.
Figure 5.3 Expression of Diap-1 RNAi in the ellipsoid body with the 48698 GAL4 driver leads to organismal death faster than control

Expression of Diap-1 RNAi (blue) in the ellipsoid body, using the 48698 GAL4 driver, leads to organismal death faster than control (driver by itself, green). Days after gene expression has been induced is time when flies were transferred from 18°C to 29°C. Percent survival was taken as the number of starting flies minus the number of flies that died divided by starting flies time 100.
Figure 5.4 Expression of Diap-1 RNAi in the ellipsoid body using the 48698 GAL4 driver leads to loss of neurons

A) Expression of membrane bound RFP (mCD8::RFP) in neurons using 48698 GAL4 shows high expression and outlines the ellipsoid body. B) Expression of Diap-1 RNAi together with mCD8::RFP leads to a pronounced decrease in fluorescence as well as the disappearance of the ellipsoid body.
Figure 5.5 Expression of Diap-1 RNAi in the ellipsoid body using the 48698 GAL4 driver leads to neuronal death

A) Expression of nuclear Redstinger in neurons using 48698 GAL4 shows high expression and indicates the number of cell bodies in the ellipsoid body. B) Expression of Diap-1 RNAi together with Redstinger leads to a pronounced decrease in fluorescence as well as a decrease in the number of cells within the ellipsoid body.
CHAPTER SIX

Discussion and future perspectives

The work covered in this dissertation shows a function for Drpr in phagocytic competency, corpse processing and brain homeostasis. However, many open questions remain. In this chapter, I will briefly summarize the findings outlined above and discuss the potential consequences of these findings in the larger context of phagocytosis. I will also suggest future research directions that may be taken to further understand Drpr’s role in these processes.

6.1 Summary of findings: Drpr acts through the JNK pathway to control synchronous engulfment of dying germline cells by follicular epithelial cells

In conditions of low nutrient availability, developing egg chambers in the ovary of Drosophila undergo PCD. Specifically, the germline becomes apoptotic at stages 7-9 of oogenesis. In response to PCD, the surrounding follicle cells become phagocytic and synchronously begin to engulf the dying germline. During phagocytosis of the germline, follicle cells upregulate the transmembrane receptor Drpr. Our work shows that Drpr is required for proper phagocytosis of the germline by the follicle cells. Disruption of the drpr gene in follicle cells lead to the persistence of germline cell debris and the premature death of these cells. Likewise, overexpression of Drpr led to death of the germline in
abundant nutrient conditions and hyperengulfment when flies were deprived of amino acids. We show that Drpr is required for JNK activation as overexpressing Drpr leads to ectopic JNK activation and mutations in \textit{drpr} lead to a suppression of JNK activation. Furthermore, we were able to rescue the \textit{drpr} null phenotype by overactivating JNK in the follicle cells. Finally, we show that caspase activity is not required for Drpr activation since blocking caspases still leads to Drpr upregulation and JNK activation. Together, these results suggest that Drpr through JNK activation is crucial for the nonprofessional phagocyte mediated clearance of the germline.

\textbf{6.2 Open questions and future directions}

Our work shows that Drpr activates JNK which is crucial for proper phagocytosis to occur. JNK is important in the activation of transcription factors (Igaki, 2009) and work in the lab has shown that in our model, JNK mediates its effects through the activation of the \textit{Drosophila} homolog of AP-1 (Meehan et al., unpublished). The activation of JNK could be a mechanism by which nonprofessional phagocytes acquire the phagocytic phenotype necessary to remove cellular debris. Indeed, work done in mammals has shown that JNK is activated during macrophage-mediated engulfment (Patel et al., 2006) suggesting that JNK is required for phagocytic competency in multiple species. However, since JNK is also important in professional phagocytes it may not be specific for nonprofessional phagocytosis. Instead it may just be a broader requirement for phagocytic capabilities. Future experiments are needed to address whether JNK specifically mediates the transition to phagocytic competency in nonprofessional
phagocytes. Work should be done to identify what genes get upregulated or downregulated by JNK mediated signaling and compared to JNK activation in other *Drosophila* models of professional phagocytosis. In particular, an emphasis should be given on genes that become downregulated.

During engulfment, follicle cells transition from cells with supportive roles necessary for oogenesis to phagocytes. To become fully phagocytic, the genes involved in these supportive roles most likely need to be turned off. Therefore, the mechanisms to downregulate these genes are the most likely to be specific for nonprofessional phagocytosis. Current studies in the lab are being focused on transcriptional profiling of phagocytic follicle cells. To complement this approach, future work could use modENCODE data to see what genes are highly expressed in the ovary. Then by using this information, a screen can be conducted where genes that are highly expressed in the ovary can be overexpressed, by using the GAL4-UAS system, to prevent them from being downregulated during phagocytosis. Defects in phagocytosis would be indicative of a necessity to turn these genes off in order for the proper transition to a phagocytic state to occur. Since all glia in *Drosophila* are nonprofessional phagocytes, a similar approach could be done in the brain. These experiments may converge on some conserved factor present in nonprofessional phagocytes that needs to be turned off.

Most of the work done on nonprofessional phagocytes has focused on factors conserved throughout phagocytosis. Little work has been done on how nonprofessional phagocytosis is different from professional phagocytosis. In our model of phagocytosis, the follicle cells engulf the germline synchronously, something that is not seen in most
instances of phagocytosis. The mechanisms of how this is achieved are poorly understood. Interestingly, follicle cells in the stage where germline death occurs are heterogeneous (Klusza and Deng, 2011). They consist of four populations: main body follicle cells, anterior follicle cells, posterior follicle cells, and polar cells. Some of these cells can be manipulated independently with specific GAL4 drivers. Future studies should look at disrupting phagocytosis in the different cell populations and examine whether synchrony or phagocytosis in general is disrupted. This would allow for a better understanding of this coordinated, nonprofessional, phagocytic response that follicle cells engage in.

6.3 Summary of findings: Defective phagocytic corpse processing results in neurodegeneration and can be rescued by TORC1 activation

The clearance of apoptotic cells is crucial for organismal homeostasis (Lu et al., 2011). How the persistence of apoptotic cells contributes to tissue dysfunction is poorly understood. Our work reveals that disrupting drpr expression in glia leads to the massive accumulation of neuronal apoptotic corpses on the surface of the Drosophila brain. These corpses arise in development and stay in the brain throughout the lifespan of the organism. The persistence of corpses causes age-dependent neurodegeneration in Drosophila. We also show that mutations in drpr lead to glia death and, unlike neuronal cells, glia corpses do become cleared. Persistence of corpses seems to be caused from defects in phagosome maturation as observed by the high levels of corpse processing proteins that associate with these dead cells. Finally, we show that inhibiting autophagy
by activating TORC1 is sufficient to suppress corpse accumulation and age dependent neurodegeneration. These results suggest that Drpr activates TORC1 in order for proper phagosome maturation to occur.

6.4 Open questions and future directions

Defects in the phagocytosis of apoptotic cells has been shown to lead to inflammation, and/or autoimmune disease, both of which negatively impact tissue homeostasis (Poon et al., 2014). How dead cells affect tissue in the absence of inflammation and autoimmunity is not well understood. Our studies show that the absence of Drpr in glia leads to the persistence of apoptotic corpses. The accumulation of apoptotic corpses then leads to age-dependent neurodegeneration. *Drosophila* have a limited inflammatory repertoire and do not permit the innate immune system to permeate into the brain. Furthermore, *Drosophila* does not have an adaptive immune system. These data coupled with our findings suggests that the persistence of corpses leads to age-dependent neurodegeneration in the absence of inflammation and autoimmunity.

The mechanism by which corpses cause age dependent neurodegeneration are not known. Uncleared apoptotic cells undergo secondary necrosis (Vanden Berghe et al., 2010). It is thought that the leakage of intracellular contents during secondary necrosis is toxic to the surrounding cells, nevertheless this remains controversial. Some studies have suggested that caspases dispose of most of the intracellular contents that could be toxic (Martin et al., 2012). Therefore, the content that is spilled from secondarily necrotic cells has a minimal impact on homeostasis. Our studies are in agreement with these findings.
Corpses form in development and are highly apparent in the $drpr^{-/}$ brain upon eclosion. However, the onset of neurodegeneration occurs late in the lifespan of the organism. Furthermore, the neurodegeneration phenotype observed is less severe than other *Drosophila* neurodegeneration models, such as that of spinocerebellar ataxia (Fernandez-Funez et al., 2000). This suggests that corpses do not lead to acute toxicity within the brain. Whether low levels of toxicity are contributing to the phenotype remains a possibility. Future studies should test whether caspases make secondary necrotic cells innocuous by inhibiting caspase activity in a $drpr^{-/}$ background. Specifically, *elav-GAL4* can be used to drive Diap-1 in neurons in the absence of Drpr. Neurodegeneration can then be examined through sectioning to see if caspase activation exacerbates the $drpr$ deficient phenotype. Alternatively GAL80$^{ts}$ could be used as well to stop Diap-1 overexpression after eclosion to prevent any confounding factors.

We hypothesize that the $drpr^{-/}$ neurodegeneration phenotype results from defects in development that then give rise to neurodegeneration as the fly ages. Developmental defects have been shown to cause neuronal loss in other situations. For example, patients with Rett’s syndrome, a disease which is thought to be partially caused by defects in phagocytosis, experience loss of neurons in the cerebellum (Derecki et al., 2012). To test this hypothesis, we could use the GAL80$^{ts}$ system to turn off $drpr$ only in development or adulthood. We could then examine whether the brain undergoes neurodegeneration in either of these conditions.

Our studies show a novel role of TORC1 in Drpr-mediated corpse processing. Specifically, we showed that TORC1 mediated inhibition of the initiation of autophagy is
sufficient to rescue corpse accumulation. These results suggests that autophagy may need to be turned off in order for the proper maturation of corpse containing vesicles to occur. Phagocytosis is an energy expensive process (Han and Ravichandran, 2011). Autophagy has been shown to be active during high metabolic processes (Galluzzi et al., 2015). Activating autophagy during phagocytosis may sequester the intracellular components required for corpse processing to the autophagosome. This would prevent proper phagosome maturation from occurring and therefore lead to the accumulation of dead cells within the phagocyte. This is an attractive hypothesis since autophagy and phagocytosis are conserved from worms to mammals. Our work provides the first evidence of how phagocytosis may circumvent autophagy from occurring in order to prevent unwanted consequences of autophagosome formation. Furthermore, this mechanism, by preventing autophagosome formation, may allow the recruitment of autophagy specific proteins important in processing to the phagosome. This would allow for efficient phagosome maturation.

To study the validity of these claims, we propose to examine corpse formation in larval phases in a \( drpr^- \) fly. Our data show that corpses form during 3\textsuperscript{rd} instar larva, therefore in the absence of Drpr, autophagy should go unregulated. This can be tested by crossing in genetic reporters of autophagy and examine their expression/localization in \( drpr \) deficient glia. We can then overexpress Rheb in similar conditions and examine whether it rescues any defect observed in autophagy expression/localization. In our studies, we propose that inhibiting autophagy initiation may allow corpse processing autophagy proteins to localize to phagosomes and assist with maturation. To test this, we
can knockdown autophagy proteins involved with this process (such as LC3) to examine whether it is sufficient to prevent TORC1 mediated suppression of corpse accumulation in \textit{drpr\textsuperscript{-/-}}.

In our studies, we found that the absence of Drpr in glia, led to death in these cells. We also observed the same result in follicle cells of the \textit{Drosophila} ovary. Both instances seem to be caspase independent: dying glia cells are not caspase positive and blocking caspases in follicle cells does not prevent their death. Therefore, their death is likely non-apoptotic. The absence of Drpr in glia cells leads to a high level of lysosomal activity in dying cells (Figure A.1). It may be that lysosomes become upregulated during phagocytosis. In the absence of Drpr, the lysosomes cannot fuse with phagosomes because there isn’t any internalization (as in adulthood) or the processing aspect is disrupted. Lingering lysosomes may then leak into the cytoplasm causing cell death. This type of death is observed in many forms of lysosomal storage disorders (Wong et al., 2012). Patients with Gaucher’s disease, a lysosomal disorder, are unable to break down glycolipids due to the absence of glucocerebrosidase (Aflaki et al., 2014). In Gaucher’s disease, the main cell type with disease pathology are macrophages and it is thought that the disease occurs because of not being able to properly process phagocytosed erythrocytes. Therefore the death of nonprofessional phagocytes within our system could be lysosomal in nature. Future studies need to be done in order to address this question. Specifically, blocking lysosomal biogenesis by knocking down TFEB, the transcription factor important for lysosomal processes (Settembre et al., 2013), may be sufficient to rescue the death of phagocytes. These results, coupled with the observed
neurodegeneration as well as potential fertility defects, posit the exciting possibility that defects in phagocytosis lead to a cell-type specific form of lysosomal processing disorder.
APPENDIX A

Death of glia cells

When \textit{drpr} signaling is disrupted we find an increase in the persistence of both apoptotic germline and neuronal corpses. Interestingly, we find that disrupting \textit{drpr} also leads to premature death of the follicle cells (Figure 3.2), as well as glia death (Figure A.1). Glia death is characterized by an increase in the lysosomal marker LAMP when analyzed through antibody staining or genetic methods (Figure A.1). Furthermore, the autophagy marker Atg8a also clusters around dying glia cells when expressed under the control of a glia promoter (A.1). These data suggest that machinery in the endocytic pathway, as well as in the autophagic pathway, is dysregulated in glia cells that are undergoing cell death due to the absence of Draper. Whether lysosomes are somewhat contributing to death is unknown.
Figure A.1 Lysosomes and autophagosomes cluster around dying glia cells.

A-B) Lamp-GFP (B) expressed in glia cells clusters around large pyknotic nuclei (A, B arrow). C-E) Large nuclei (C arrow) are immunoreactive for lamp antibody (D, E arrow). F-G) Atg8a mCherry (F) expressed in glia cells clusters around large pyknotic nuclei (F, G arrow)
APPENDIX B

Screens for corpses and suppressors of corpses

The following RNAi constructs were driven specifically in glia cells and screened for the appearance of corpses (Table A.1). Mutations of certain genes were also tested. No RNAi line or mutation tested was able to replicate the appearance of corpses seen in \textit{drpr}^{-/-} and \textit{drpr} RNAi brains. Furthermore, expressing \textit{drpr} in neurons (Table A.1, entry 37) does not rescue the \textit{drpr}^{-/-} phenotype. We also screened a number of autophagy related genes for their ability to suppress corpses (Table A.2). Knocking down \textit{Atg1}, \textit{12}, and \textit{16} as well as overexpressing Rheb in glia cells was sufficient to suppress the corpse phenotype seen in \textit{drpr} RNAi brains. All constructs were expressed in glia cells.

Table A.1 Screen for corpses

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Table A.2 Screen for suppressor of corpses

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2008  B.S., Biology, Specialization in Neuroscience. Boston University, Boston MA.

RESEARCH EXPERIENCE

2009-present  Ph.D. Student, Dr. Kim McCall, Boston University, Boston, MA. Studying the mechanisms of engulfment in Drosophila melanogaster.

2008-2009  Ph.D. Student, Dr. Susan Tsunoda, Boston University, Boston, MA. Investigated the role of the Retinal degeneration B protein in localizing signaling components to the rhabdomere of fly photoreceptors.

2007-2008  Undergraduate Researcher, Dr. Susan Tsunoda, Boston University, Boston, MA. Conducted an assay to detect Drosophila melanogaster mutants that are deficient in light induced Gq-alpha translocation in the fly photoreceptor.

Summer 2006  Undergraduate Researcher, Dr. Elizabeth Langley, Universidad Autonoma de Mexico, Mexico D.F., Mexico. Examined estrogen receptor activation and nuclear translocation in rhesus monkey cell culture

AWARDS AND HONORS

2015  Brenton R. Lutz award for Excellence in Neurobiology, Boston University, Boston, MA
2014  Keystone Symposia Scholarship for Cell Death Signaling in Cancer and the Immune System, São Paulo, Brazil (Travel Award)

2014  Selected Attendee, GYSS @ one-north, Global Young Scientist Symposium, Singapore

2011- 2014  NIH F31 Fellowship GM099425
“Elucidating the effectors of cell clearance in immunoprivileged organs”

2011  Selected Attendee, Cold Spring Harbor Course on the Neurobiology of Drosophila

2010-2011  NIH Diversity Supplement Award, Boston University, Boston, MA (to Kim McCall)

2009  NIH Diversity Supplement Award, Boston University, Boston, MA (to Susan Tsunoda)

2008-2009  Teaching Fellowship Award, Boston University, Boston, MA

PUBLICATIONS

1. Etchegaray, J.I., Tran, J., Elguero, J., Sinatra, V., Feany, M., and McCall, K. Glia corpse processing defects result in neurodegeneration and can be rescued by activating TORC1, submitted


ORAL PRESENTATIONS

2015  Invited speaker, “Blocking autophagy rescues processing defects in draper null flies”, Biogen, Cambridge, MA

2014  Departmental Seminar, “Death without the curtains: Glia cells undergo autophagic death in the absence of Draper”, Boston University, Boston, MA
2013 **Departmental Seminar,** “In the face of death: the response and contribution of glia to neuronal death in *Drosophila melanogaster*”, Boston University, Boston, MA

2012 **Departmental Seminar,** “In search of protection: establishing *Drosophila* as a potential model to study the regulation of the immune response during neurodegeneration,” Boston University, Boston, MA

2012 **Platform speaker,** “JNK and Draper regulate the engulfment of nurse cells by follicle cells during starvation-induced mid-oogenesis cell death in the *Drosophila* ovary,” 53rd *Drosophila* Research Conference, Chicago, IL

2011 **Departmental Seminar,** “Elucidating the effectors of engulfment in the *Drosophila* ovary”, Boston University, Boston, MA

**POSTER PRESENTATIONS**


2012  

2012  

2011  
Genome Science Institute Research Symposium, Boston University Medical School, Boston, MA. “Basket and Draper regulate the engulfment of nurse cells by follicle cells during starvation induced mid-oogenesis cell death.” A.K. Timmons, **J.I. Etchegaray**, T.L. Pritchett, E. Welch, and K. McCall.

2011  

2007  
Boston University Undergraduate Research Symposium, Boston, MA. “Development of an *in vivo* assay to test for Gq-alpha GFP translocation in *Drosophila* photoreceptors.” **J.I. Etchegaray** and Susan Tsunoda.

**TEACHING EXPERIENCE (BOSTON UNIVERSITY)**

2009  
Lab instructor, BI108 Introductory Biology lab

2008, 2009  
Lab instructor, BI211 Human Physiology lab

**OUTREACH ACTIVITIES AND PROFESSIONAL DEVELOPMENT**

2009-2015  
Resident Assistant, Boston University, Boston MA

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Mentor, to Vincent Sinatra, undergraduate researcher, recipient of a UROP grant.

2012-2014  
Mentor, to Jennifer Tran, undergraduate researcher, recipient of two UROP grants.