Complement-Related Regulates Autophagy in Neighboring Cells

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COMPLEMENT-RELATED REGULATES AUTOPHAGY IN NEIGHBORING CELLS

A Dissertation Presented

By

LIN LIN

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COMPLEMENT-RELATED REGULATES AUTOPHAGY IN NEIGHBORING CELLS

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By
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This work was undertaken in the Graduate School of Biomedical Sciences Program in Cancer Biology
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This thesis is dedicated to my husband, Zhao, to my sons, Leo and Luke, to my parents; all of which were instrumental in helping me achieve this great success.
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Abstract

Autophagy is a conserved process that cells use to degrade their own cytoplasmic components by delivery to lysosomes. Autophagy ensures intracellular quality control and is associated with diseases such as cancer and immune disorders. The process of autophagy is controlled by core autophagy (Atg) genes that are conserved from yeast to mammal. Most Atg proteins and their regulators were identified through pioneering studies of the single cell yeast *Saccharomyces cerevisiae*, and little is known about factors that systematically coordinate autophagy within the tissues of multicellular animals. The goal of this thesis is to identify new autophagy regulators and provide a better understanding of the regulatory mechanisms within multicellular animals. My research determined Macroglobulin complement-related (Mcr), a *Drosophila* complement orthologue, can activate autophagy during developmental cell death. Unlike most known autophagy regulators, Mcr functions in a cell non-autonomous manner to trigger autophagy in neighboring cells. To my knowledge, this is the first identified autophagy factor that cell non-autonomously activates autophagy. Additionally, I found that Mcr, a secreted protein, instructs the autophagy machinery through the immune receptor Draper, suggesting a relationship between autophagy and the control of inflammation. Lastly, Mcr is dispensable for both nutrient deprivation-induced autophagy in the fat body and developmentally programmed autophagy in the dying midgut of *Drosophila*. Therefore, this study unveils a
mechanism in a multicellular organism by which autophagy is systematically controlled in distinct cell contexts.
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Copyright information

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CHAPTER I

Introduction

Part I: Autophagy

Autophagy is derived from the Greek word meaning “eating of self”. It is a general term for the process by which cytoplasmic material is delivered to lysosomes for degradation. In 1962, Ashford and Porter first reported the observation of degradation of mitochondria and other intra-cellular structures within lysosomes in rat liver cells after addition of glucagon (Ashford and Porter, 1962). In 1967, De Duve christened the process “autophagy”, and established that lysosomes are responsible for glucagon-induced autophagy (Deter and De Duve, 1967). In recent years, with the molecular understanding and appreciation of the physiological significance, a new era of autophagy research has begun.

There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (Blommaart et al., 1997; Dunn, 1994; Seglen and Bohley, 1992). During macroautophagy, an isolation membrane encloses a small portion of cytoplasmic material, including damaged organelles and unused proteins, to form a double-membraned structure called an “autophagosome” (Figure 1.1). The outer membrane of an autophagosome subsequently fuses with the membrane of lysosomes to become an “autolysosome”, in which the cytoplasmic material is degraded by lysosomal enzymes. In microautophagy, on the other hand, lysosome itself directly takes up cytosolic components through invagination of the lysosomal membrane (Castro-
Obregon, 2010). Both macroautophagy and microautophagy are able to engulf large cytosolic structures through both selective and non-selective mechanisms. In chaperone-mediated autophagy (CMA), targeted cytosolic proteins are unfolded and translocated across the lysosomal membrane with the assistance of chaperone proteins (such as Hsc-70) for degradation. CMA is significantly different from the other types of autophagy because it is extremely selective on the proteins that are degraded by this pathway, and it is also direct shuttle target proteins across the lysosomal membrane without the formation of additional vesicles (Bandyopadhyay et al., 2008; Cesen et al., 2012). Due to the increased interest in macroautophagy and its role in disease, this thesis focuses on the molecular control of macroautophagy (hereafter simply called autophagy) and its possible functions in programmed cell death.

**Core autophagy machinery**

The genes that regulate autophagy were first identified in yeast (Harding et al., 1996; Thumm et al., 1994b; Tsukada and Ohsumi, 1993a), although most of these factors are conserved in higher eukaryotes, including humans (Figure 1.1) (Mizushima and Komatsu, 2011b). To date, more than 30 autophagy-related (Atg) genes have been reported in yeast (Weidberg et al., 2010). Upon the induction of autophagy, Atg1/ULK1 kinase and its complex components Atg13, Atg17/FIP200, Atg29, and Atg31 translocate to the pre-autophagosomal structure (PAS) (Kamada et al., 2000; Matsuura et al., 1997; Weidberg et al., 2010). This leads to
recruitment of the autophagy-specific form of the phosphatidylinositol (PtdIns) 3-kinase (PI(3)K) complex, which includes Vps34, Vps15, Atg6/Beclin-1, and Atg14, to the PAS (Kihara et al., 2001; Simonsen and Tooze, 2009). The PI(3)K complex produces phosphatidylinositol-3-phosphate (PtdIns(3)P), which recruits effector proteins such as Atg18/WIPI1/2 to the PAS. Atg18 forms a complex with Atg2 that functions in autophagosome formation (Obara et al., 2008), and also controls the size of vesicles and phosphatidylinositol 3,5-bisphosphate (PtdIns (3, 5) P2) homeostasis in complex with other proteins (Efe et al., 2007). At the final step of autophagosome formation, elongation and closure of the isolation membrane requires two protein conjugation systems, the Atg12-Atg5-Atg16 complex16 and the Atg8/LC3-phosphatidylethanolamine (PE) complex (Ichimura et al., 2000). The ubiquitin-like Atg12 protein is conjugated with Atg5 by Atg7 (E1-like) and Atg10 (E2-like) enzymes, and then Atg12-Atg5 conjugate further to interact with Atg16 and function as a complex (Kuma et al., 2002; Shintani et al., 1999; Tanida et al., 1999). Atg8 is first processed by the protease Atg4, and is conjugated with PE by the Atg7 and Atg3 (E2-like) enzymes (Ichimura et al., 2000). Biochemical evidence supports a model in which the Atg12-Atg5 complex possesses an E3-like activity for efficient PE lipidation of Atg8 (Hanada et al., 2007).
Figure 1.1. Autophagy genetic regulatory pathway. After autophagy induction, the Atg1 complex (Atg1-Atg13-Atg17-Atg29-Atg31) translocates to the endoplasmic reticulum (ER), which is thought to be the major membrane source (other membrane sources may include mitochondria and the plasma). This leads to the recruitment of the autophagy-specific form of the phosphatidylinositol (PtdIns) 3-kinase (PI(3)K) complex, which includes Vps34, Vps15, Atg6/Beclin-1 and Atg14, to the ER. To form an autophagosome, elongation and closure of the isolation membrane requires two protein conjugation systems, the Atg12-Atg5-Atg16 complex and Atg8/LC3-phosphatidylethanolamine (PE) complex. See text for more details.
It is generally believed that all of the core machinery proteins are essential for autophagosome formation. However, several recent findings indicate that autophagy can proceed without some of the Atg proteins. Mouse embryonic fibroblast (MEF) cells from either Atg5\(^{-/-}\) or Atg7\(^{-/-}\) knockout mice formed autophagosomes and autolysosomes, and performed autophagy-regulated protein degradation (Nishida et al., 2009). However, lipidation of the microtubule-associated protein light chain 3 (LC3, the mammalian homolog of yeast Atg8) did not occur during this Atg5/Atg7-independent autophagy (Nishida et al., 2009). During the same year, Chu and colleagues reported that the parkinsonian neurotoxin MPP+ induces autophagy and mitochondrial degradation independent of Beclin-1 (Chu et al., 2007). In addition, Chang and colleagues showed that loss of either Atg7 or Atg3 function fails to influence the autophagy that participates in programmed reduction of cell size during Drosophila intestine cell death (Chang et al., 2013a). These studies indicate that autophagy can be controlled by different pathways in a cell context- and organism-specific manner.

In addition to their role in regulating autophagy activity, there is increasing evidence indicating that Atg proteins also have non-autophagic biological functions (Subramani and Malhotra, 2013). Atg6/Beclin-1 has been reported to function as a tumor suppressor, and Beclin-1\(^{+/-}\) tumors in mice possess elevated cell stress and p62 levels, altered NF-kB signaling, and genome instability (Mathew et al., 2009). Drosophila lacking Atg6 function exhibit blood cell tumors, but also possess defects in multiple vesicle trafficking pathways (Shravage et al.,
Therefore, it is possible that, in addition to autophagy, altered endocytosis and protein secretion may contribute to tumor development. Eukaryotic cells release proteins into the extracellular space by two main routes. One is the conventional secretion pathway for proteins that contain a signal for translocation into the ER, which is followed by their vesicular transport to Golgi membranes and subsequent export from the cell. The second is the unconventional secretion pathway for proteins that lack a secretion signal for entry into the ER-Golgi membrane pathway. Examples of proteins that use this secretory pathway include acyl-CoA-binding protein and the cytokines interleukin 1-β and interleukin-6. The mechanisms underlying the unconventional protein secretion pathway are poorly understood, but evidence indicates that these proteins are secreted by an autophagosome-like vesicular intermediate that requires Atg proteins such as Atg5, Atg7, and Atg12 (Dupont et al., 2011; Lock et al., 2014; Manjithaya et al., 2010). Unlike autophagy, these vesicles fuse with the plasma membrane, and bypass the final stages of autophagy.

Part II: Autophagy in cell death

Programmed cell death is a conserved and genetically regulated process that plays important roles throughout the lives of metazoans. Schweichel and Merker identified three types of programmed cell death based on morphology: apoptosis, autophagic cell death, and necrosis (Schweichel and Merker, 1973). In apoptosis,
dying cells usually present several morphology changes. These changes include cell blabbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Hacker, 2000). The dying cells eventually undergo phagocytosis by phagocytes, and being degraded in the lysosomes of phagocytes. In contrast, autophagic cell death is characterized by the presence of abundant autophagosomes in the dying cells and the lack of phagocyte participation. Dying cells degrade cytoplasmic components within their own lysosomes. While neither phagocytosis nor lysosomes are involved in necrosis (Schweichel and Merker, 1973). Dying cells become swollen; cell membranes become permeable and eventually release the cytoplasmic contents into surrounding tissue, lead to inflammation (Kerr et al., 1972; Majno and Joris, 1995; Trump et al., 1997).

Among the three types of programmed cell death, apoptosis is the most studied form. The genetic control and molecular mechanisms are relatively well understood. However, whether autophagy is the mechanism by which cells actually die (cell death by autophagy) or is simply present during cell death (cell death with autophagy) has been a subject of controversy because autophagy is well recognized as a cell survival mechanism (Baehrecke, 2005b; Levine and Yuan, 2005; Marino et al., 2014). During conditions of nutrient limitation, autophagy is used to generate amino acids and energy to maintain cell viability through the bulk degradation of cytoplasmic material. Accordingly, the presence of autophagy in dying cells has been proposed to be a stress response.
mechanism to prolong cell viability. Nevertheless, recent studies strongly support autophagy as a process that can promote programmed cell death. The contribution of autophagy to cell death has been studied most extensively in *Drosophila*. As described above, an increase in steroid hormone levels triggers the destruction of larval tissues during the transition from a larva to an adult (Jiang et al., 1997b). Destruction of the larval salivary gland requires both autophagy and caspase activities (Berry and Baehrecke, 2007b; Martin and Baehrecke, 2004b). Mutations in either multiple *Atg* genes or caspase genes, or overexpression of the caspase inhibitor p35, lead to incomplete degradation of larval salivary glands. However, combined inhibition of both autophagy and caspase activities increases suppression of salivary gland degradation. Further, *Atg1*-triggered autophagy in salivary glands is sufficient to induce premature cell death in a caspase independent manner (Berry and Baehrecke, 2007b). These data indicate that caspases and autophagy function additively in the degradation of *Drosophila* larval salivary glands. Yet how do caspases and autophagy intersect during this dying process is not clear. Both cleaved lamin and mCherry-Atg8a present ubiquitously within salivary gland cells at 14 hours after puparium formation. Indicates that caspases and autophagy function together within one cell but have different roles. Caspases function is required for DNA fragmentation in dying salivary glands. While autophagy may be necessary for self-degradation to make cells smaller since larval cells have endoreplication cell cycle that results in the production of gigantic cells. The number and size of cells may prohibit
engulfment and digestion by phagocytes. Autophagy also provides resources that are needed to construct the adult tissues. The large number of autophagosomes observed in dying cells may sever to recycle nutrients during metamorphosis when pupae do no feed.

There is no evidence of the presence of phagocytosis during Drosophila larval salivary glands degradation. No obvious phagocytes containing cell fragments were observed using TEM (Martin and Baehrecke, 2004b). Although salivary gland cells appear to use genes that are considered part of the core apoptotic machinery, including caspases, the morphology of these cells and cells undergoing apoptosis are distinct. In salivary glands, dynamic changes in vacuole structure immediately precede their demise, and such changes have not been reported in apoptotic cells (Kerr et al., 1972). Within one hour of salivary gland DNA degradation, large vacuoles appear to break into smaller vacuoles. As these large vacuoles fragment, smaller vacuoles accumulate near the plasma membrane, and autophagic vacuoles containing components of the cytoplasm, including mitochondria, are formed. Salivary gland cells then begin to fragment, and nuclei and components of the cytoplasm then disperse within the haemocoel. Although we cannot rule out the involvement of phagocytes in autophagic cell death, salivary gland cells proceed to late stages of degradation without the assistance of phagocytes. It has been suggested that phagocytes might play a role in the removal of cellular debris towards the end of autophagic cell death. Future studies should provide insights into the late stages of salivary glands
degradation.

In contrast to the *Drosophila* salivary gland, death of the larval midgut cells of the intestine is not disrupted by overexpression of p35 or by mutation of caspases, indicating that the canonical apoptosis pathway is not required for developmental midgut cell death (Denton et al., 2009). Interestingly, the impaired function of multiple *Atg* genes, including either *Atg1*, *Atg2* or *Atg18*, blocks larval midgut degradation (Denton et al., 2009). Additionally, caspase deficiency fails to enhance the *Atg* mutant phenotype in the midgut. These data indicate that autophagy, and not apoptosis pathway components, is essential for *Drosophila* midgut programmed cell death.

Studies in mammalian systems also provide evidence in support of concurrent activation of autophagic and apoptotic pathways. In U937 monocyteid cells and L929 fibrosarcoma cells, knockdown of either *Beclin-1* or *Atg7*, two essential *Atg* genes, blocks non-apoptotic cell death induced by caspase-8 inhibition (Yu et al., 2004). In response to death stimulation, *Bax<sup>−/−</sup>Bak<sup>−/−</sup>* double knockout MEFs undergo non-apoptotic cell death. This cell death is associated with increased numbers of autophagosomes and autolysosomes, and can be reduced by knockdown of either *Atg5* or *Beclin-1* (Shimizu et al., 2004). In human ovarian surface epithelial cells, expression of oncogenic H-Ras<sup>V12</sup> leads to caspase-independent cell death with features of autophagy. This Ras-induced autophagy-dependent cell death was associated with upregulation of the BH3-
only protein Noxa and the autophagy regulator Beclin-1 (Elgendy et al., 2011). Furthermore, it has been reported that activation of autophagy by the autophagy-inducing peptide Tat-Beclin-1 can cause cell death with unique morphological features of autophagy. This type of cell death is blocked by either pharmacological or genetic inhibition of autophagy, but not by impairment of known regulators of either apoptosis or necroptosis (Liu et al., 2013b). In neonatal mice, neuron-specific deletion of Atg7 protects against cerebral hypoxia-ischemia-induced hippocampal neuron death (Koike et al., 2008), and in adult rats shRNA targeting Beclin-1 prevents neuronal death in the thalamus following focal cerebral infarction (Xing et al., 2012). Although such studies provide genetic data in support of autophagy function in the death of mammalian cells and tissues, additional analyses of autophagy in developmental cell death are needed in mammalian model systems.

Autophagy might be required for cell death, but little is known about how autophagy kills cells. One possibility is that autophagy causes a metabolic catastrophe by depleting mitochondria and metabolic substrates. Studies in Drosophila provide some support for this model, since high levels of autophagy that are induced by Atg1 expression are sufficient to induce cell death that is either dependent or independent of caspase function, depending on cell type (Berry and Baehrecke, 2007b; Chang et al., 2013a; Scott et al., 2007a). Another possible mechanism for autophagy-dependent cell death is the selective recruitment of cell survival factors to autophagosomes for degradation. Studies in
mammalian cells have shown that selective recruitment of cytoplasmic catalase to autophagosomes leads to accumulation of reactive oxygen species and cell death (Yu et al., 2004). Similarly, recruitment of the inhibitor of apoptosis Bruce to autophagosomes in the *Drosophila* ovary leads to the activation of caspases and cell death (Nezis et al., 2010). Finally, it has been proposed that autophagic membrane structures could serve as signaling scaffolds to enable activation of either apoptotic or programmed necrosis protein complexes. Limited data exist in support of each of these possible mechanisms, and more work is needed to determine how autophagy promotes cell death.

### Part III: Regulators of autophagy

Autophagy is a tightly regulated pathway that can be induced by a variety of stimuli, such as nutrient deprivation, hypoxia, reactive oxygen species, protein aggregates, and damaged organelles. The activation of autophagy by these stimuli involves multiple signaling pathways. For example, the mechanistic target of rapamycin (mTOR), a serine/threonine protein kinase, negatively regulates the activation of autophagy. In nutrient-rich conditions, mTOR interacts with Atg13 and phosphorylates it at several serine residues. The phosphorylation of Atg13 reduces both its affinity for Atg1/ULK1 and Atg1/ULK1 activity. Upon mTOR inhibition, for example by starvation, mTOR phosphorylation of Atg13 is reduced, enabling activation of Atg1/ULK1 kinase activity and autophagy (Kamada et al.,
Anti-apoptotic proteins, such as Bcl-2, Bcl-XL, and Mcl-1, have also been proposed as important negative regulators of autophagy. These proteins bind to the BH3 domain of Beclin-1 through their BH3-binding groove, and inhibit Beclin-1-dependent autophagy induction (Maiuri et al., 2007; Pattingre et al., 2005). Pro-apoptotic BH3-only proteins and pharmacological BH3 mimetics can induce autophagy by competitively disrupting the interaction between Beclin-1 and Bcl-2 or Bcl-XL (Maiuri et al., 2007). In addition, death-associated protein kinase (DAPK) can promote dissociation of Beclin-1 from Bcl-XL and induce autophagy by mediating phosphorylation of the BH3 domain of Beclin-1 (Zalckvar et al., 2009). However, it is important to note that the role of Bcl-2 in the regulation of autophagy is a subject of debate. For example, there is evidence indicating that the effect of prosurvival Bcl-2 family members on autophagy is instead an indirect consequence of their inhibition of the apoptosis mediators Bcl-2-associated X (Bax) and Bcl-2 homologous antagonist/killer (Bak). In the absence of Bax and Bak, antagonizing or altering the levels of prosurvival Bcl-2 family members has no detectable impact on autophagy (Lindqvist et al., 2014).

Inositol-1,4,5 trisphosphate (IP3) is a secondary messenger molecule that mediates calcium release from the ER by binding the IP3 receptor, which is an ER-localized calcium ion channel (Berridge et al., 2000). Increasing evidence shows that IP3 signaling pathway components, including the IP3 receptor, IP3
kinase 2, and calmodulin, are involved in regulating autophagy (Nelson et al., 2014b; Vicencio et al., 2009). An increase in the level of free cytosolic calcium also triggers autophagy. This process is mediated by calcium/calmodulin-dependent kinase kinase-β and AMP-activated protein kinase (AMPK) via mTOR inhibition (Hoyer-Hansen et al., 2007). In addition to inhibition of mTOR, AMPK directly interacts with and phosphorylates Atg1 to influence autophagy (Egan et al., 2011; Kim et al., 2011).

Steroid hormone has also been shown to regulate autophagy activity, and this has been best studied during development in *Drosophila melanogaster*. In *Drosophila*, pulses of the steroid 20-hydroxyecdysone (ecdysone) control the transitions through different developmental stages (Jiang et al., 1997b). At the end of the third larval instar stage, an ecdysone pulse triggers formation of the prepupa. The increase in ecdysone at this stage activates autophagy in the larval midgut, and this autophagy promotes intestine cell death (Lee et al., 2002a). Ten hours later, the subsequent increase in ecdysone triggers formation of the pupa, and activates autophagy that promotes programmed cell death of the salivary gland (Lee and Baehrecke, 2001). The molecular mechanisms underlying this process have been described extensively elsewhere (Berry and Baehrecke, 2007b; Lee et al., 2002b).

**Cell-Autonomous Regulation of Autophagy**
Autophagy appears to be influenced by systemic body-wide signals, but the proteins that control autophagy are largely thought to function within individual cells. In yeast, for example, nutrient deprivation induces a high level of autophagy, which provides a cell-autonomous source (by auto digestion of the cytosol) of energy and amino acids for the synthesis of proteins that are essential for survival. Nutrient deprivation is also shown to induce autophagy in both flies and mice (Kuma et al., 2004; Scott et al., 2004a). Animal starvation leads to decreased growth factors and insulin-like peptide levels, and it is assumed that this decrease in insulin is the systemic signal that leads to increased autophagy because of the well-known role for the class I PI3-Kinase and mTOR nutrient sensing pathways that are both downstream of insulin and inhibit autophagy (Arico et al., 2001; Scott et al., 2004a). In the Drosophila fat body, which is a nutrient storage organ, clonal knockdown of Atg5 inhibits starvation-induced autophagy only in cells with reduced autophagy gene function (Scott et al., 2004a). Similarly, clonal loss of Atg1 function in dying Drosophila midgut cells specifically inhibits autophagy in cells that possess reduced autophagy gene function (Figure 1.2) (Chang et al., 2013a). Draper, the Drosophila ortholog of the Caenorhabditis elegans engulfment receptor CED-1, is required for autophagy activation during larval salivary gland developmental cell death (McPhee et al., 2010b). Loss of draper prevents the induction of autophagy, and causes an incomplete larval salivary gland degradation phenotype. Interestingly, unlike its function in phagocytosis, Draper also regulates autophagy in a cell-autonomous
manner (McPhee et al., 2010b). Knockdown of *draper* does not prevent starvation-induced autophagy in the fat body, indicating that regulation of autophagy by Draper is also tissue specific. As Draper is an engulfment receptor, it is commonly accepted that there is a corresponding ligand; however, the ligand required for the regulation of autophagy by Draper remains unknown. If Draper does require a ligand for the regulation of autophagy, it is possible that this ligand functions in a non-cell autonomous manner. The most direct evidence for a cell non-autonomous signal that regulates autophagy comes from studies of pathogen-stimulated autophagy in host cells, where pathogen and host receptor interactions triggered an Atg-dependent phagocytic program (Joubert et al., 2009; Sanjuan et al., 2007a). Although extracellular metabolites have been shown to influence autophagy (Eng et al., 2010), little is known about how proteins from one cell activate autophagy in a different cell within an animal.
Figure 1.2. Cell-autonomous function of autophagy genes.

(A) Experiment design. A cell with Atg gene knockdown by RNAi co-expresses green fluorescent protein (GFP, green) and is surrounded by wild type cells (white). After autophagy induction, red autophagy reporter puncta are present only in wild type cells, and not in Atg gene RNAi knockdown cell. The size of the Atg gene RNAi knockdown cell is larger than wild type cell, because autophagy leads to cell size reduction.
(B) An image of the mid gut of the *Drosophila* larval intestine. Cells with Atg1 knock down are marked by GFP (green). mCherry tagged Atg8a protein (red), which localizes to autophagosomes and autolysosomes, serves as a reporter of autophagy. Two hours after puparium formation (APF), mCherry-Atg8a puncta are only present in the wild type mid gut cells but not in the Atg1 RNAi-expressing green cells. Scale bar represents 50 µm. Image courtesy of T.-K. Chang.
Part IV: Autophagy and disease

A. Autophagy and cancer

Role of autophagy in cancer development

Cancer was one of the first human disorders that was linked to a defect in autophagy (Levine and Kroemer, 2008; White and DiPaola, 2009). However, the role of autophagy in tumor progression is still an enigma. Inactivation of Fip200, the mouse homolog of yeast Atg17, in the polyoma middle T (PyMT) mouse mammary cancer model impairs tumor growth (Wei et al., 2011). Deletion of either Atg5 or Atg7 in the mouse liver causes hepatoma formation without progression to hepatocellular carcinoma (Takamura et al., 2011). Downregulation of essential autophagy proteins abrogates the tumorigenicity of oncogenic RAS-expressing human and mouse cancer cell lines (Guo et al., 2011; Guo et al., 2013). These findings suggest that autophagy can be tumor promoting in established cancers. It has been suggested that, through intracellular recycling, autophagy provides substrates that enable tumor cells to survive the metabolic stress in the tumor microenvironment and promotes tumor progression.

Paradoxically, other studies support a role for autophagy in tumor suppression. Mice with allelic loss of Beclin-1 have decreased autophagy and are more prone to the development of spontaneous tumors, including lymphomas, lung carcinomas, hepatocellular carcinomas, and mammary precancerous lesions (Qu et al., 2003; Yue et al., 2003). In addition, immortalized kidney and
mammary epithelial cells derived from Beclin-1 heterozygous-deficient mice showed increased tumorigenicity when transplanted into immunocompromised mice (Karantza-Wadsworth et al., 2007; Mathew et al., 2007). Atg5 is frequently downregulated in primary melanomas compared to benign nevi, and analyses of 158 patient biopsies showed that patients with low levels of Atg5 in their tumors had reduced progression-free survival (Liu et al., 2013a). The mechanisms by which autophagy functions in tumor suppression remain unclear, and it is possible that autophagy could directly restrict cell proliferation by inducing cell death. It is important to note that Laddha and colleges recently found that most Beclin-1 mutations are associated with mutations in the BRCA1 tumor suppressor gene (Laddha et al., 2014). Although the ability to draw strong conclusions may be limited by the current availability of cancer genome data, these data do raise doubts about the tumor suppressor function of Beclin-1.

Increasing evidence suggests that autophagy has a specific influence on tumor progression depending on context. Inactivation of either Atg5 or Atg7 impairs the initiation of KrasG12D-driven lung cancer, and deletion of the tumor suppressor p53 restores cancer progression in either Kras Atg5−/− or Kras Atg7−/− tumors (Rao et al., 2014). In a humanized genetically modified mouse model of Kras-triggered pancreatic ductal adenocarcinoma (PDAC), a small number of precancerous lesions developed into PDAC over time. If these mice also lacked either the Atg5 or Atg7 autophagy gene, they accumulated low-grade, premalignant pancreatic intraepithelial neoplastic lesions, and progression to
high-grade pancreatic intraepithelial neoplasias and PDAC was blocked. In contrast, in mice containing oncogenic Kras and lacking p53, loss of autophagy no longer blocked tumor progression and actually accelerated tumor onset (Rosenfeldt et al., 2013). However, decreased p53 function had no impact on the influence of autophagy inhibition on tumor growth in a different study of PDAC (Yang et al., 2014). The discrepancy between these studies may indicate that the timing of p53 impairment influences the impact of autophagy modulation on tumor growth. Furthermore, a recent study shows that the influence of autophagy on tissue overgrowth depends on the growth-inducing stimulus and cell type (Perez et al., 2015). These findings support the notion that autophagy may play distinct roles in cancer as both an inhibitor of initial oncogenesis and then as a facilitator of tumor progression, and that cell and tumor context influence how autophagy impacts tumor development. These studies have important implications for the use of modulators of autophagy for cancer therapy.

**Autophagy in response to anticancer agents**

Autophagy has been reported to play contradictory roles in tumor initiation and progression. Therefore, both repression and stimulation of autophagy should be considered as therapeutic approaches depending on the many factors that contribute to cancer. Therapeutic induction of autophagy-associated cell death can be accomplished by modulation of regulators of autophagy. mTOR is a key regulator of cell growth and an autophagy repressor, and inhibition of this kinase
leads to the activation of Atg1/ Ulk1 and stimulation of autophagy. Treatment with the mTOR inhibitor rapamycin led to a reduction in carcinogen-induced lung tumors in a murine model (Granville et al., 2007). In addition, rapamycin treatment showed antitumor effects in the MCF7 and MDA-MB-231 breast cancer cell lines in xenografted tumors, and it was suggested that this was because of inhibition of angiogenesis (Seront et al., 2013). Furthermore, continuous long-term rapamycin treatment in APC^{Min/+} mice, which have enhanced AKT-mTOR signaling, was shown to markedly inhibit intestinal neoplasia (Koehl et al., 2010). Recently, combining autophagy activation by mTOR inhibition with radiation was shown to lead to enhanced therapeutic effects in cancer cells and xenografts (Nam et al., 2013). Another strategy that has been implemented is the induction of autophagy by inhibition of mTOR while impairing lysosome function by treatment with chloroquine (Yang et al., 2011). This leads to the accumulation of what is presumably toxic autophagic cargo without the lysosomal capacity to degrade this material and thus results in cell death.

As described earlier, autophagy can also be induced by the protein kinase AMPK. Metformin is an inhibitor of the mitochondrial electron transport chain complex I that leads to decreased ATP production and increased levels of AMP. This causes AMPK activation and autophagy induction. Metformin has been reported to prevent tobacco carcinogen-induced lung tumorigenesis in a rodent cancer model (Memmott et al., 2010), and to improve tumor oxygenation and
hence the radiotherapy response (Zannella et al., 2013). Furthermore, the combination of chemotherapy and metformin is more harmful to breast cancer cells than treatment with chemotherapy or metformin alone (Liu et al., 2012). Significantly, the first clinical trial that combined autophagy inhibition and chemotherapy demonstrated that patients harboring the BRAF$^{V600E}$ mutation who were treated with the RAF inhibitor vemurafenib and chloroquine had decreased brain tumor growth (Levy et al., 2014). Although the contribution of autophagy to these cancer therapeutic strategies is not completely clear, the results of this clinical trial illustrate how targeting autophagy could lead to new cancer therapies.

Growing evidence indicates that autophagy not only preserves cellular homeostasis in conditions of endogenous stress, but also plays an important role in controlling intracellular pathogens. Thus, autophagy represents one of the most primitive innate immune responses. From the immunological point of view, cancer can only develop when premalignant cells escape immunosurveillance by either losing their antigenic properties or by actively suppressing the antitumor immune response (Schreiber et al., 2011). Autophagy is often thought to be suppressed in tumor cells during early oncogenesis, such as upon allelic loss of Beclin-1. Indeed, autophagy-deficient tumors fail to elicit an anticancer immune response upon exposure to chemotherapy (Michaud et al., 2011). Many preclinical studies of the role of autophagy in killing tumor cells may have missed important non-autonomous influences on the immune system, since previous work has mostly been conducted either in vitro or in animal models with defective
immune systems. Therefore, when considering modulation of autophagy for cancer therapy, it is important to consider the role of autophagy in the immune and anticancer responses.

**B. Autophagy in immunity and inflammation**

**Autophagy tackles microbes**

Autophagy was originally considered to be a non-selective bulk degradation process, but it is now clear that autophagosomes can selectively degrade substrates (Kraft et al., 2010). In addition to endogenous substrates, autophagy degrades intracellular pathogens in a selective form of autophagy, named xenophagy. Cellular xenophagy is an innate component of immune responses (Alexander and Leib, 2008), though the precise membrane dynamics and specificity determinants of xenophagy are not fully understood.

Several lines of evidence suggest that autophagy proteins function in the process of phagolysosomal maturation during antigen presentation and microbial invasion. Autophagy proteins are required for the fusion of phagosomes that contain Toll-like receptor (TLR)-ligand-enveloped particles with lysosomes in macrophages (Sanjuan et al., 2007a). The self-ligand and cell-surface receptor SLAM functions as a microbial sensor that recruits the Beclin-1- class III PI(3)K complex to phagosomes containing Gram-negative bacteria, facilitating
phagolysosomal fusion and activation of the antibacterial NADPH oxidase (NOX2) complex (Berger et al., 2010). Furthermore, the engagement of TLR or Fcγ receptors during phagocytosis recruits LC3 (and Atg12) to the phagosome through NOX2-dependent generation of reactive oxygen species (ROS) (Huang et al., 2009). Another autophagosome-independent function of autophagy proteins in pathogen destruction has been described in interferon-γ (IFN-γ)-treated macrophages infected with the parasite *Toxoplasma gondii*. The parasite-derived membrane undergoes destruction through a mechanism that involves Atg5-dependent recruitment of the immunity-related GTPase proteins to the parasitophorous vacuole (Khaminets et al., 2010; Zhao et al., 2008), leading to the death of the parasite in the infected cell (Zhao et al., 2009; Zhao et al., 2008). Together, these studies suggest that autophagy proteins have diverse roles in membrane dynamics to benefit the host in the removal of invading pathogens. Thus, autophagy can be targeted as a therapy strategy against infectious diseases.

**Autophagy and inflammatory disease**

The role of autophagy in inflammatory diseases was initially established through genome-wide association studies (GWASs) (Wellcome Trust Case Control, 2007). Polymorphisms in autophagy-associated genes, such as *Atg16L* and *IRGM*, are linked to Crohn’s disease (Wellcome Trust Case Control, 2007). In addition to single nucleotide polymorphisms, *IRGM* is an example of a gene
dosage correlation with a predisposition to Crohn’s disease in human populations (Wellcome Trust Case Control et al., 2010). One of the common \textit{IRGM} polymorphisms in Crohn’s disease leads to an escape from the negative regulation of IRGM expression by a microRNA (miRNA) (Brest et al., 2011). Moreover, a link has been reported between Crohn’s disease and the autophagy-targeting factor SMURF1 (Jostins et al., 2012). Furthermore, polymorphisms in \textit{ULK1} have also been linked with Crohn’s disease (Henckaerts et al., 2011).

In addition, polymorphisms in autophagy-associated genes have been associated with autoimmune disorders. \textit{IRGM} polymorphisms may be a risk factor in systemic lupus erythematosus (SLE) (Ramos et al., 2011). A new human autophagy locus, which was first identified in \textit{C. elegans} screens and which was shown in mice to be required for autophagosomal maturation (Zhao et al., 2013), has been linked to the complex Vici syndrome that includes immunodeficiency (Cullup et al., 2013). Thus, autophagy shows clinical relevance and could be a target for inflammatory disorders treatments.

\textbf{Part V: Complement system}

The complement system was discovered many years ago as a component of normal plasma that augments the opsonization of bacteria by antibodies and allows antibodies to kill some bacteria. This activity was said to 'complement' the antibacterial activity of antibody, hence called the complement system. Although
first discovered as an effector arm of the antibody response, complement can be activated in the absence of antibodies. It is a part of the innate immune system that enhances the ability of antibodies and phagocytic cells to clear microbes and damaged cells, induce a series of inflammatory responses, and attack pathogens.

The complement system consists of a number of small proteins found in the blood and normally circulating as inactive precursors (pro-proteins). Many of complement proteins are proteases that are themselves activated by proteolytic cleavage. Such enzymes are called zymogens. When stimulated by one of several triggers, a protease in the system undergoes self-cleavage and becomes active, then cleaves its substrate, another complement zymogen, to its active enzymatic form. This in turn cleaves and activates the next zymogen in the complement pathway. In this way, the activation of a small number of complement proteins at the beginning of the pathway is amplified, resulting in the generation of large complement response. This eventually leads to the stimulation of phagocytes to clear foreign and damaged material.

There are three distinct pathways through which complement can be activated on pathogen surface. These pathways depend on different molecules for their initiation, but they converge to generate the same set of effector molecules. C1q is the initiating protein of the classical complement pathway. When C1q binds to and coats (opsonizes) dead cells, pathogens, or debris, it
triggers a protease cascade, leading to the deposition of the downstream complement protein C3 (Gasque, 2004). Opsonization with activated C3 fragments (C3b and iC3b) leads to cell or debris elimination in two different ways. Deposited C3 can directly activate C3 receptors on macrophages, therefore triggering elimination by phagocytosis, or activated C3 can trigger the terminal activation of the complement cascade, leading to cell lysis through the formation of a lytic membrane attack complex.

Other than the function in innate immune system, recent work suggests more diverse roles for complement proteins. Mice deficient in complement protein C1q or the downstream complement protein C3 exhibit large sustained defects in CNS synapse elimination (Stevens et al., 2007a). In a mouse model of glaucoma, C1q, which is normally downregulated in the adult CNS, becomes upregulated and synaptically relocalized in the adult retina early in the disease (Stevens et al., 2007a). Furthermore, in the mouse model of Alzheimer’s disease (AD), inhibition of C1q, C3 or the microglial complement receptor CR3, reduces the number of phagocytic microglia as well as the extent of early synapse loss (Hong et al., 2016b). These findings indicate that unwanted synapses are tagged by complement for elimination and suggest that complement-mediated synapse elimination may become aberrantly reactivated in neurodegenerative disease.

In addition, the mosquito immune factor Thioester-containing protein 1 (TEP1), which determines mosquito resistance to a wide range of pathogens,
including malaria parasites, has been shown to promote mosquito male fertility. During spermatogenesis TEP1 binds to and removes damaged cells through the same complement-like cascade that kills malaria parasites in the mosquito midgut (Pompon and Levashina, 2015b). Therefore, complement may possess diverse biological functions within animals.

**Part VI: Some outstanding questions**

Like cell growth and division, programmed cell death plays a fundamental role in tissue and organism homeostasis. When approaching the term of programmed cell death, apoptosis is often the only form of cell death being considered. Yet, autophagic cell death has been shown to play important roles in the development of insects, plants and potentially mammals (discussed in part II). Moreover, autophagic cell death has also be related with plant innate immune response (Liu et al., 2005). Therefore, the potential prominence of this type of cell death could be greatly under-estimated.

Most of the core autophagy machinery proteins are conserved from yeast to higher eukaryotes, including humans. Evidence indicates that at least some of the mechanisms that regulate autophagic cell death are conserved between evolutionarily distant species (Lam and Golstein, 2008). Furthermore, the presence of autophagic cell death has been observed during development in higher metazoans such as mammals (Clarke, 1990). Thus, a better
understanding of the regulatory mechanisms of autophagic cell death in multicellular model organisms may help us understand the mechanism of autophagic cell death in humans. As the apoptosis machinery proteins are usually disrupted in diseases, such as cancer, autophagic cell death may serve as an alternative strategy which we can use to kill tumor cells. Therefore, further studies that focus on understanding how does autophagy promote cell death and the function of autophagic cell death would significantly benefit the treatment of diseases.

Other than its function in promoting cell death, autophagy is well recognized as a cell survival mechanism. During nutrition deprivation situation, cells can induce autophagy to survive the starvation condition. However, the mechanisms that distinguish these two functions of autophagy are not clear. Since the outcomes of these two functions of autophagy are completely different, the question is: how do cells determine to go one way while not the other? Could these cells be "primed" for their fate when autophagy gets induced so they go down one of the two paths? If these cells are primed, how do they do this and how do they maintain this “primed” property?
CHAPTER II

Macroglobulin complement-related regulates autophagy in neighboring cells

Preface

The work presented in this chapter was a collaborative effort: L.L., F.S.L.M.R., C.K., W.W. and E.H.B. designed the experiments. All experiments were performed by L.L., except Fig. 2.10 and Fig. 2.11 by F.S.L.M.R., Fig. 2.2B and 2.2C by C.K., M.L. provided transgenic flies, R.H.G.B. provided recombinant proteins, L.L., C.K. and E.H.B. wrote the manuscript and all authors commented on it.
Summary

Autophagy degrades cytoplasmic components and is important for development and human health. Although autophagy is known to be influenced by systemic intercellular signals, the proteins that control autophagy are largely thought to function within individual cells. Here we report that *Drosophila* Macroglobulin complement-related (Mcr), a complement orthologue, plays an essential role during developmental cell death and inflammation by influencing autophagy in neighboring cells. This function of Mcr involves the immune receptor Draper, suggesting a relationship between autophagy and the control of inflammation. Interestingly, Mcr function in epithelial cells is required for macrophage autophagy and migration to epithelial wounds, a Draper-dependent process. This study reveals, unexpectedly, that complement-related from one cell regulates autophagy in neighboring cells via an ancient immune signaling program.
Introduction

Autophagy is a conserved process that cells use to degrade their own cytoplasmic components by delivery to lysosomes (Mizushima and Komatsu, 2011a). Autophagy ensures intracellular quality control and is associated with diseases such as cancer, immune disorders and neurodegeneration (Mizushima et al., 2008). Most autophagy studies have focused on cell survival under nutrient limiting conditions, but it has also been associated with cell death (Baehrecke, 2005a). The *Drosophila* larval salivary gland undergoes steroid-triggered cell death during development and is an excellent model to study autophagy in dying cells. Both autophagy (*Atg*) genes and caspases are required for larval salivary gland degradation (Berry and Baehrecke, 2007a), but how autophagy is regulated during cell death is poorly understood.

Most *Atg* proteins and their regulators were identified through pioneering studies of the single cell yeast *Saccharomyces cerevisiae* (Harding et al., 1995; Thumm et al., 1994a; Tsukada and Ohsumi, 1993b), and little is known about systemic factors that signal between different cells to control autophagy within the bodies of multicellular animals.

The complement system is best known as a regulator of inflammation and immune clearance of pathogens (Janeway et al., 2001). Complement proteins exist as inactive protease zymogens that become activated to opsonize pathogens to facilitate clearance by engulfment, and these factors are conserved
from invertebrate organisms to humans (Williams and Baxter, 2014). Recent work suggests more diverse roles for complement, including roles in male fertility in mosquitoes (Pompon and Levashina, 2015a) and in synapse pruning and a model of Alzheimer’s disease in mice (Hong et al., 2016a; Stevens et al., 2007b). Therefore, complement may possess diverse biological functions within animals.

The complement related \textit{mcr} gene was identified as a factor that is required for phagocytosis of the fungal pathogen \textit{Candida albicans} in \textit{Drosophila} (Stroschein-Stevenson et al., 2006b). Here we show that \textit{mcr} is necessary for autophagy but not caspase activity during \textit{Drosophila} salivary gland degradation during development. Unlike most known regulators of autophagy, \textit{mcr} functions in a cell non-autonomous manner to regulate autophagy in neighboring cells within the dying salivary gland. Interestingly, \textit{mcr} appears to function upstream of the conserved immune receptor Draper, a factor that functions in a cell autonomous manner to regulate autophagy in dying salivary gland cells. Surprisingly, \textit{mcr} does not influence either nutrient deprivation-induced autophagy in the fat body or developmentally programmed autophagy in the dying midgut of \textit{Drosophila}. Rather, \textit{mcr} is required for autophagy in embryonic macrophages where Draper is known to be required for an inflammatory response to epithelial wounds. Remarkably, this requirement for \textit{mcr} is in the embryonic epidermis, indicating that this complement related molecule also functions in a novel cell non-autonomous manner to regulate autophagy and migration to wounds. Moreover, the addition of recombinant Mcr protein to an
embryonic macrophage-derived cell line is sufficient to induce autophagy that depends on *draper* and multiple *Atg* genes. These studies reveal an unexpected role for complement in the regulation of autophagy in neighboring cells that depends on an ancient immune signaling program.

**Results**

*mcr* functions in a caspase-independent manner during salivary gland cell death

The immune receptor Draper is required for autophagy during salivary gland degradation where it functions upstream of *Atg* genes (McPhee et al., 2010a). This suggests that the Draper receptor, which mediates phagocyte recognition of dying cells (Freeman et al., 2003; MacDonald et al., 2006), may sense an extracellular signal to control autophagy within dying cells. To test this model, we investigated the role of the Draper extracellular domain. We found that the extracellular domain of Draper was required for salivary gland degradation (Figures 2.1A and 2.1B). Significantly, loss of the only reported Draper ligand, Pretaporter (Prtp) (Kuraishi et al., 2009), that is expressed in salivary glands did not affect salivary gland degradation (Figures 2.2A-2.2C), suggesting an unknown extracellular factor activates Draper-dependent autophagy.
Figure 2.1. The Draper-I extracellular domain and Mcr are required for salivary gland cell degradation.
(A) Wild-type Canton-S animals ($n = 20$), *draper* null mutant animals ($n = 22$), animals that express salivary gland-specific expression of Draper-I lacking the extracellular domain ($n = 8$), and *draper* null mutants with salivary gland-specific expression of Draper-I lacking the extracellular domain ($n = 26$) analyzed by histology for the presence of salivary gland material (yellow circles) 24h after puparium formation. The images on the bottom emphasize salivary gland cellular fragments with other tissues removed.

(B) Quantification of data from (A). Statistical significance: Chi-square test.

(C) Western blot analyses of Mcr and Tubulin 6h, 12h, and 14h after puparium formation in salivary gland extracts from control and *mcr* knockdown animals.

(D) Quantification of data from (C). All samples are normalized to Tubulin and plotted relative to their respective 6h samples. Error bars, mean ± SEM; $n=3$.

(E) Control ($n = 27$) and salivary gland-specific *mcr* knockdown animals ($n = 24$) analyzed by histology for the presence of salivary gland material (yellow circles) at 24h after puparium formation.

(F) Quantification of data from (E). Statistical significance: Chi-square test.
Mcr, a conserved complement-related protein (Williams and Baxter, 2014), is expressed in salivary glands and increases following the rise in steroid that triggers cell death 12 hours after puparium formation (Figures 2.1C and 2.1D). This prompted us to consider if mcr is required for larval salivary gland degradation. We screened for persistence of salivary gland material 8 hours after this tissue is normally degraded (Jiang et al., 1997a; Lee and Baehrecke, 2001), at 24 hours after puparium formation. We expressed an upstream activating sequence (UAS)-promoted double-stranded inverse repeat construct designed to target mcr (UAS-mcrIR) with the salivary gland-specific fkh-Gal4 driver, which depleted Mcr protein levels in salivary glands (Figures 2.1C and 2.1D). Significantly more mcrIR-expressing animals possessed persistent salivary gland cell fragments compared to control animals (Figures 2.1E and 2.1F). Targeting a different region of mcr also impaired salivary gland degradation compared to controls (Figures 2.2D and 2.2E).
Figure 2.2. Pretaporter is not, but mcr is, required for salivary gland cell degradation.

(A) Western blot analyses of Prtp and Tubulin protein levels in salivary glands isolated from wild-type (Canton-S) animals 6h, 12h, and 14h after puparium formation.

(B) Control animals lacking one allele of \( prtp^{\Delta 2} \) \( (n = 10) \) and \( prtp \) null mutants \( (n \)
(B) Quantification of data from (A). Statistical significance: Chi-square test.

(C) Quantification of data from (B). Statistical significance: Chi-square test.

(D) A mcr-RNAi line that targets a different sequence exhibits the same phenotype by histological analyses 24h after puparium formation as Figure 2.1E and F. Control ($n = 26$), mcr knockdown ($n = 26$), salivary gland material (yellow circles). Bottom images: salivary gland cellular fragments without other tissues.

(E) Quantification of data from (D). Statistical significance: Chi-square test.
Mcr binds to the surface of the fungus *Candida albicans* to promote phagocytosis and clearance (Stroschein-Stevenson et al., 2006b). Therefore, Mcr could also function in phagocytic blood cells to mediate salivary gland degradation. However, driving UAS-\textsuperscript{IR} mcr expression in blood cells did not lead to a defect in salivary gland clearance (Figures 2.3A and 2.3B). Salivary gland destruction requires cell growth arrest and an increase in the steroid hormone 20-hydroxyecdysone (Berry and Baehrecke, 2007a; Jiang et al., 1997a). Experiments with a cell growth reporter indicated that the salivary gland degradation defect in mcr knockdown animals was not due to a failure in cell growth arrest (Figures 2.3C). In addition, reduced mcr function failed to alter the steroid response factors EcR and BR-C (Figures 2.3D-G). These data indicate that mcr is required for larval salivary gland clearance, does not alter either cell growth or steroid signaling, and functions tissue-autonomously in salivary glands during degradation.
Figure 2.3. Decreased mcr function alters neither cell growth nor hormone signaling.
(A) Control ($n = 24$) and blood cell-specific knockdown of $mcr$ ($n = 27$) analyzed by histology for the presence of salivary gland material at 24h after puparium formation.

(B) Quantification of data from (A). Statistical significance: Chi-square test.

(C) Salivary gland (sg) tGPH analyses in feeding larvae and 14h after puparium formation in control (feeding, $n = 17$, 14h, $n = 14$) and salivary gland-specific knockdown of $mcr$ (feeding, $n = 15$, 14h, $n = 27$) animals. Scale bars, 50 µm.

(D) EcR and Tubulin protein levels in salivary gland extracts isolated from control and salivary gland-specific $mcr$ knockdown animals at 6h, 12h, and 14h after puparium formation.

(E) Quantification of data from (D). All samples are normalized to Tubulin and plotted relative to their respective 6h samples. Error bars, mean ± SEM; $n=3$. Statistical significance: Student’s t-test.

(F) BR-C and Tubulin protein levels in salivary gland extracts isolated from control and salivary gland-specific $mcr$ knockdown animals at 6h, 12h, and 14h after puparium formation.

(G) Quantification of data from (F). All samples are normalized to Tubulin and plotted relative to their respective 6h samples. Error bars, mean ± SEM; $n=3$. Statistical significance: Student’s t-test.
Caspases and autophagy function additively in the clearance of larval salivary glands (Berry and Baehrecke, 2007a). Inhibition of caspases leads to the persistence of condensed cellular fragments, while blocking autophagy results in the persistence of vacuolated cell fragments. Simultaneous blockade of both caspases and autophagy leads to robust morphological preservation of large salivary gland fragments. We found that while salivary gland-specific expression of the caspase inhibitor p35 led to the persistence of condensed cell fragments, \( mcr^{IR} \) expression led to the presence of vacuolated cell fragments (Figures 2.4A and 2.4B), similar to autophagy mutant phenotypes. In addition, simultaneous expression of both p35 and \( mcr^{IR} \) in salivary glands led to persistence of multicell salivary gland tissue fragments (Figures 2.4A and 2.4B), indicating that Mcr functions additively with caspases. Furthermore, similar to autophagy mutants, \( mcr \) knockdown in salivary glands did not influence the degradation of the caspase substrate nuclear Lamin (Martin and Baehrecke, 2004a) (Figures 2.4C-E). Combined, these results indicate that Mcr does not influence caspases, and suggests that Mcr signals in the autophagy branch of salivary gland degradative pathways.
Figure 2.4. *mcr* functions in an additive manner with caspases during salivary gland degradation.

(A) Animals with salivary gland-specific p35 expression (*n* = 16), *mcr* knockdown (*n* = 24), and p35 expression plus *mcr* knockdown (*n* = 25) analyzed by histology for the presence of salivary gland material at 24h after puparium formation. Yellow circles, cell fragments; red circle, salivary gland fragments.

(B) Quantification of data from (A). Statistical significance: Chi-square test.
(C and D) Salivary glands dissected 6h (C) and 14h after puparium formation (D) from control animals and those with salivary gland-specific mcr knockdown, stained with anti-cleaved Lamin antibody (green) and Hoechst (blue). Scale bars, 50 µm.

(E) Quantification of data from (C and D). Error bars, mean ± SEM; n = 12 (control 6h), n = 10 (control 14h), n = 10 (mcrIR 6h), n = 14 (mcrIR 6h). Statistical significance: Student's t-test.
*mcr* is necessary for autophagy in neighboring cells during salivary gland cell death

We next tested whether an *Atg* gene mutant enhances the *mcr*IR salivary gland persistence phenotype. Animals with either knockdown of *mcr*, *Atg13*−/− mutant animals, or knockdown of *mcr* in *Atg13*−/− mutant animals all possess similar salivary gland cell fragment phenotypes (Figures 2.5A and 2.5B), consistent with *mcr* functioning in the autophagy pathway. Atg1 mis-expression is sufficient to induce autophagy in salivary glands and other tissues (Berry and Baehrecke, 2007a; Chang et al., 2013a; Scott et al., 2007b), and suppressed the salivary gland clearance defect caused by reduced *mcr* function (Figures 2.5C and 2.5D). These data indicate that Mcr regulates autophagy and functions upstream of Atg1.

We then asked whether *mcr* influences autophagy markers. The cargo receptor Ref(2)P (p62 in mammals) is degraded by autophagy (Nezis et al., 2008), and we analyzed if *mcr* loss influences Ref(2)P levels during salivary gland degradation. Unlike wild-type animals, which have decreased Ref(2)P levels as salivary glands activate autophagy prior to cell death, *mcr* knockdown in salivary glands resulted in Ref(2)P accumulation (Figures 2.5E and 2.5F). We expressed *mcr*IR in all salivary gland cells and analyzed mCherry-Atg8a autophagy reporter activity (Denton et al., 2012). Consistent with a role for Mcr in autophagy, we found that salivary glands that express *mcr*IR possessed
significantly fewer mCherry-Atg8a puncta compared to controls (Figures 2.5G and 2.5H). These data indicate that Mcr is required for autophagy in salivary gland cells.
Figure 2.5. Mcr regulates autophagy during salivary gland cell degradation.
(A) Atg13 null mutants ($n = 14$), salivary gland-specific mcr knockdown ($n = 21$), and Atg13 null mutants with salivary gland-specific mcr knockdown ($n = 11$), analyzed by histology for the presence of salivary gland material (yellow circles) at 24h after puparium formation.

(B) Quantification of data from (A). Statistical significance: Chi-square test.

(C) Animals with salivary gland-specific mcr knockdown ($n = 24$) and those with salivary gland-specific mcr knockdown expressing Atg1 ($n = 24$) analyzed by histology for salivary gland material (yellow circle) at 24h after puparium formation.

(D) Quantification of data from (C). Statistical significance: Chi-square test.

(E) Western blot analysis of Ref(2)P and Tubulin 6h, 12h, and 14h after puparium formation in salivary gland extracts from control and salivary gland-specific mcr knockdown animals.

(F) Quantification of data from (E). Ref(2)P in control and mcr$^{IR}$ samples normalized to Tubulin and plotted relative to their respective 6h sample levels. Error bars, mean ± SEM; $n=3$. Statistical significance: Student’s t-test.

(G) mCherry-Atg8a puncta in control salivary glands ($n = 18$) and salivary gland with tissue-specific mcr knockdown ($n = 24$). Scale bars, 50 µm.

(H) Quantification of data from (G). Error bars, mean ± SEM. Statistical significance: Student’s t-test.
Although extracellular signaling molecules have been suggested to regulate autophagy, genetic evidence in support of such an extracellular signal is limited. Given that Mcr appears to be a secreted protein (Stroschein-Stevenson et al., 2006b), it could regulate autophagy activity in neighboring cells in a non-autonomous manner. To test this hypothesis, we produced mosaic salivary glands with mcr mutant cell clones and compared mCherry-Atg8a autophagy reporter puncta formation between wild-type control (that express GFP) and mcr mutant cells (lacking GFP). To our surprise, unlike Atg gene mutants and other known autophagy regulators, including Draper, which function in a cell autonomous manner, mCherry-Atg8a puncta were present in both mcr mutant cells and neighboring control cells (Figures 2.6A and 2.6B). Similar results were obtained with mcr knockdown (Figure 2.7A). Consistent with these data, we detected Flag-tagged Mcr protein multiple cell distances away from cells where it was expressed in a pattern that is similar to endogenous Mcr (Figure 2.6C), and similar immune reactivity was not detected in salivary glands lacking Flag-tagged Mcr (Figure 2.7B). These data support a model whereby secreted Mcr functions in a cell non-autonomous manner to regulate autophagy in salivary gland cell neighbors.

To further examine the cell non-autonomous function of Mcr, we used the temperature-sensitive Gal80 system to express mcr\textsuperscript{IR} in a subset of salivary gland cells. By varying the temperature shift length we produced salivary glands with varying numbers of wild-type and mcr\textsuperscript{IR}-expressing cells (Figure 2.6D). In
contrast to controls that lack both mCherry-Atg8a reporter puncta and Mcr protein on the cell cortex (Figures 2.7C and 2.7D), following temperature shift, we observed mCherry-Atg8a autophagy reporter puncta at 14 hours after puparium formation, and the number of puncta was associated with the number of control cells lacking GFP (Figures 2.6E – 2.6G). Under these conditions, Mcr protein was detected on the cortex of salivary gland cells (Figure 2.7E). Taken together, these data provide further support for the model that Mcr can function in a cell non-autonomous manner to regulate autophagy in neighboring cells.
Figure 2.6. Mcr regulates autophagy in a cell non-autonomous manner in salivary glands.

(A) Salivary glands dissected 14h after puparium formation containing a loss-of-function \( mcr^{EY07421} \) mutant cell clone (lacking GFP), imaged for mCherry-Atg8a (red), GFP (green) and Hoechst (blue). Scale bars, 50 μm.

(B) Quantification of data from (A). Error bars, mean ± SEM; \( n = 24 \) (control), \( n = 19 \) (mcr \(^{-} \)). Statistical significance: Student’s t-test.

(C) Salivary glands expressing Mcr-flag specifically in GFP-marked cells at wandering third instar larval stage were dissected and stained with antibodies against Flag (left) and Mcr (right). Scale bars, 20 μm.

(D) Strategy for Gal80 flip-in induction of salivary glands with variable numbers of \( mcr^{IR} \)-expressing salivary gland cells.

(E and F) Salivary glands dissected 14h after puparium formation and imaged for mCherry-Atg8a puncta (red) after varying the numbers of \( mcr^{IR} \)-expressing cells (green, GFP-positive). Following temperature shift, Gal80 is expressed in a subset of cells, repressing Gal4 activation of UAS-\( mcr^{IR} \). There are more mCherry-Atg8a puncta in salivary glands with many wild-type cells (lacking green GFP) (E), compared to glands with one or two wild-type cells (arrow head, F). Scale bars, 50 μm.
(G) Quantification of data from (E) and (F). Error bars, mean ± SEM; \( n = 12 \)

(many WT cells), \( n = 12 \) (few WT cells). Statistical significance: Student’s t-test.
Figure 2.7. Gal80 flip-in assay control experiments to induce mcrIR-expressing salivary gland cells, and controls for Flag and Mcr localization.
(A) Salivary glands expressing mCherry-Atg8a in all cells, and \textit{mcr}^{IR} specifically in GFP-marked cells at 14h after puparium formation, imaged for mCherry-Atg8a puncta (red), GFP (green) and Hoechst (blue). \textit{n} = 20. Scale bars, 50 µm.

(B) Wandering larval (WL) salivary glands were dissected from wild-type animals (Canton-S) and stained with anti-Flag (left) and anti-Mcr (right) antibodies. Scale bars, 20 µm.

(C) The \textit{tub}^{P}_{>\text{stop}}\text{Gal80} flip-in system functions to restrict Gal4 expression. In flies without temperature shift, \textit{mcr}^{IR} and GFP are expressed in all salivary gland cells and there are no mCherry-Atg8a puncta at 14h after puparium formation. Nuclei are stained with Hoechst (blue). \textit{n} = 16. Scale bars, 50 µm.

(D and E) Wandering larval (WL) salivary glands were dissected from animals either without (D, \textit{n} = 18) or with (E, \textit{n} = 22) temperature shift, and stained with anti-Mcr antibody (red) and Hoechst (blue). Scale bars, 50 µm.
*mcr* regulates autophagy via immune receptor signaling in both salivary glands and during macrophage inflammatory response to wounds

Draper functions upstream of Atg proteins (McPhee et al., 2010a). Surprisingly, the complement molecule C1q was recently shown to bind Megf10, the most similar protein to Draper in mice (Iram et al., 2016). We therefore explored the possibility that Mcr might act upstream of Draper, potentially as a Draper ligand to activate salivary gland degradation and autophagy. We first expressed *mcr*IR in the salivary glands of *draper* null animals, and found there was no clear difference in the cell fragment phenotypes of either *draper* null mutant animals, expression of *mcr*IR in salivary glands, or combined *draper* mutant and *mcr*IR knockdown animals (Figures 2.8A and 2.8B), indicating that *mcr* functions in the same pathway as *draper*. Moreover, animals with combined loss of one allele of both *mcr*EY07421 and *draper*Δ5 possessed a significant defect in salivary gland clearance compared to control animals with allelic loss of either *mcr*EY07421 or *draper*Δ5 at 24 hours after puparium formation (Figures 2.8C and 2.8D). Significantly, expression of Atg1 in the salivary glands of animals with combined loss of one allele of both *mcr*EY07421 and *draper*Δ5 suppressed the defect in salivary gland clearance at 24 hours after puparium formation (Figures 2.8C and 2.8D). In addition, *mcr*EY07421 *draper*Δ5 trans-heterozygous animals possessed significantly fewer mCherry-Atg8a puncta in their salivary gland cells at 14 hours after puparium formation compared to control animals (Figures 2.8E and 2.8F).

These data support a strong genetic relationship between Mcr and Draper. If Mcr
is upstream of Draper, activation of signaling events downstream of Draper should bypass the need for Mcr. The protein kinase Src42A phosphorylates Draper and is required for downstream clearance of dying salivary glands (McPhee et al., 2010a; Ziegenfuss et al., 2008b). We found that expression of constitutively active Src42A (Src42A\textsuperscript{CA}) was sufficient to suppress the \textit{mcr}^{JR} knockdown salivary gland clearance defect (Figures 2.8G and 2.8H). Taken together, these data argue strongly that Mcr and Draper are in the same genetic pathway, and that Mcr functions upstream of activation of the Draper receptor.
Figure 2.8. Mcr functions with Draper to activate autophagy and salivary gland degradation.

(A) draper null mutants (n = 20), salivary gland-specific mcr knockdown (n = 32), and draper mutants with salivary gland-specific mcr knockdown (n = 24) analyzed by histology for the presence of salivary gland material (yellow circles) at 24h after puparium formation.

(B) Quantification of data from (A). Statistical significance: Chi-square test.

(C) Animals lacking one allele of mcr\textsuperscript{EY07421} (n = 23), one allele of draper\textsuperscript{Δ5} (n = 27), one allele of both mcr\textsuperscript{EY07421} and draper\textsuperscript{Δ5} (n = 22), and one allele of both mcr\textsuperscript{EY07421} and draper\textsuperscript{Δ5} with expression of Atg1 in salivary glands (n = 18) analyzed by histology for the presence of salivary gland material (yellow circles) at 24h after puparium formation.

(D) Quantification of data from (C). Statistical significance: Chi-square test.

(E) mCherry-Atg8a puncta in salivary glands of wild type (WT) animals, animals lacking one allele of draper\textsuperscript{Δ5}, one allele of mcr\textsuperscript{EY07421}, and one allele of both mcr\textsuperscript{EY07421} and draper\textsuperscript{Δ5}. Scale bars, 50 µm.

(F) Quantification of data from (E). Error bars, mean ± SEM; n ≥ 18. Statistical significance: Student’s t-test.

(G) Animals with either salivary gland-specific knockdown of mcr (n=31) or salivary gland-specific knockdown of mcr with expression of constitutively active
src42A \( (n=12) \) analyzed by histology for the presence of salivary gland material (yellow circles) at 24h after puparium formation.

(H) Quantification of data from (G). Statistical significance: Chi-square test.
Draper is required for activation of autophagy in salivary glands, but not for starvation-induced autophagy in the larval fat body (McPhee et al., 2010a). This prompted us to investigate whether mcr is required for autophagy in other cell contexts. Reduced mcr function in either the larval fat body of starved animals (Scott et al., 2004b) or the developing larval intestinal midgut (Chang et al., 2013a) failed to influence autophagy (Figures 2.9A – 2.9D). In addition, starved larvae did not exhibit a significant change in Mcr levels in the fat body (Figures 2.9E and 2.9F).
Figure 2.9. *mcr* does not influence autophagy in either the fat body or the midgut.
(A) Fat body expressing mCherry-Atg8a in all cells, and mcrIR specifically in GFP-marked clone cells. Third instar larvae were starved for 4h and fat bodies were dissected and imaged for mCherry-Atg8a (red) and GFP (green). Representative images are shown. n = 11. Scale bars, 50 µm.

(B) mCherry-Atg8a was expressed in the fat body of control and those with fat body-specific mcr knockdown. Third instar larvae were starved for 4h and fat bodies were dissected and imaged for mCherry-Atg8a (red). Representative images are shown. Scale bars, 50 µm.

(C) Quantification of data from (B). Atg8a puncta were quantified using Zeiss Automeasure software. Error bars, mean ± SEM; control (n = 11), mcrIR (n = 17). Statistical significance: Student's t-test.

(D) Midgut expressing mCherry-Atg8a in all cells, and mcrIR specifically in GFP-marked clone cells. Midguts were dissected from animals at puparium formation (0h) and imaged for mCherry-Atg8a (red) and GFP (green). Representative images are shown. n = 12. Scale bars, 50 µm.

(E) Mcr and Tubulin levels in fatbodies isolated from feeding and starved 2nd instar larvae.

(F) Quantification of data from (E). All samples are normalized to Tubulin. Error bars, mean ± SEM; n=3. Statistical significance: Student’s t-test.
We next explored whether mcr is required for macrophage migration to epithelial wounds in the Drosophila embryo where both Draper and Src42A are required (Evans et al., 2015; Weavers et al., 2016). We knocked down mcr specifically in epithelial cells (Figure 2.10A) and analyzed macrophage recruitment to laser-induced epithelial wounds. Remarkably, reduced mcr function in the epithelial cells resulted in the recruitment of significantly fewer macrophages to wounds (Figures 2.11A and 2.11B), indicating that Mcr mediates an efficient inflammatory response to damage. Importantly, reduced mcr function failed to impact either macrophage number (Figure 2.10B), the calcium wave associated with wounding (Figures 2.11C and 2.11D), or wound closure rate (Figure 2.10C). These results prompted us to examine if embryonic macrophages possess mCherry-Atg8a autophagy reporter puncta. Not only did these cells possess autophagy reporter puncta, but both mcr and draper mutant embryonic macrophages possessed significantly fewer mCherry-Atg8a puncta (Figure 2.11E). Moreover, embryos with knockdown of mcr specifically in epithelial cells possessed significantly fewer mCherry-Atg8a puncta in their macrophages than controls (Figures 2.11F and 2.11G). These data indicate that Mcr mediates the inflammatory response of macrophages to wounds, and that this cell migration is associated with Mcr regulation of autophagy.
Figure 2.10. Knockdown mcr in epithelial cells alters neither macrophage number nor wound closure in embryos.

(A) Analyze of mcr knockdown efficiency in epithelial cells. Control and mcrIR stage 15 embryos were immunostained for Mcr, showing a significant reduction in overall levels of Mcr following RNAi knockdown. Scale bar, 20 µm.
(B) Macrophage numbers are unaffected in epithelial-driven $mcr^{IR}$ animals. $(n \geq 24)$.

(C) Mcr has no effect on wound closure at stage 15. Control $(n = 10, \text{black circles})$ and $mcr^{EY07421} (n = 7, \text{red squares})$ wound perimeter was measured every 10 min for 1 h and normalized to the 5 min post-wound perimeter. Second order polynomial fit, preferred model one curve fits both sets of data.
A Control  \( mcr^{IR} \)

\[ \text{Actin} \]

Macrophages

C Control  \( mcr^{EY07421} \)

\[ \text{UAS:GCaMP6m} \]

D  \( mcr^{EY07421}/+ \)  \( mcr^{EY07421} \) null

Wave height (\( \mu m \))

\[ \text{Wound height (\( \mu m \))} \]

E

\[ \text{Alpha puncta per macrophage area} \]

\( mcr, \text{GFP} \)  \( mcr, \text{FPR} \)  \( sph, \text{GFP} \)  \( sph, \text{FPR} \)  \( mcr, \text{GFP} \)  \( mcr, \text{FPR} \)  \( sph, \text{GFP} \)  \( sph, \text{FPR} \)

F Control  \( mcr^{IR} \)

\[ \text{Macrophages} \]

\[ \text{mCherry-Alg8a} \]

G

\[ \text{Number of puncta per \( \mu m^2 \)} \]

\( \text{Control} \)  \( mcr^{IR} \)

\( p < 0.001 \)
Figure 2.11. Mcr is required in a cell non-autonomous manner for macrophage recruitment to wounds.

(A) Epithelial-driven $mcr^{IR}$ reduces recruitment of stage 15 embryonic macrophages to laser-induced wounds compared to control. Z-projection of macrophage recruitment to laser-induced wounds 60 minutes post-wounding. Macrophages (green) can be seen around the wound edge of the epithelium (red). Scale bar, 20 µm.

(B) Number of macrophages at the wound edge per µm of wound perimeter normalized to control ($n \geq 22$). Error bars, SD; Statistical significance: unpaired t-test with Welsh correction.

(C) Wound-induced calcium wave is not affected in $mcr$ mutants. Still images of calcium wave (green) around the wound edge of the embryonic epithelium (magenta) immediately after wounding. Scale bar 20 µm.

(D) Wound height versus height of calcium wave was calculated for both heterozygous embryos (black circles) and homozygous $mcr$ mutant embryos (red squares).

(E) mCherry-Atg8a puncta in stage 15 embryonic macrophages are reduced in $mcr$ and draper mutants. Number of mCherry-Atg8a puncta per macrophage cell normalized to cell area ($n \geq 49$ macrophages and $n \geq 4$ embryos per treatment). Error bars, 95% CI; Statistical significance: Kolmogorov-Smirnov test.
(F) Epithelial-driven \( mci^{IR} \) reduces mCherry-Atg8a puncta in stage 15 embryonic macrophages. Single Z-slice of macrophages (green) and mCherry-Atg8a puncta (red) in control and epithelial-driven \( mci^{IR} \). Scale bar, 10 µm.

(G) Number of mCherry-Atg8a puncta per cell normalized to cell area \( (n \geq 226 \) macrophages, \( n \geq 10 \) embryos). Error bars, SD; Statistical significance: Mann-Whitney test.
To determine whether application of Mcr to individual cells is sufficient to induce Atg8a puncta and activate autophagy, we used *Drosophila* embryonic macrophage-derived S2R$^+$ cells that possess a stable GFP-Atg8a autophagy reporter construct that is sensitive to nutrient deprivation (Anding and Baehrecke, 2015). Significantly, the addition of recombinant Mcr protein into culture medium was sufficient to induce similar numbers of GFP-Atg8a puncta as cells cultured under nutrient limiting conditions (Figures 2.12A and 2.12B). By contrast, addition of recombinant mosquito TEP1, a known orthologue of complement C3, did not induce GFP-Atg8a puncta (Figures 2.12A and 2.12B). The effect of Mcr appears to represent activation of autophagy, since the addition of both Mcr and the lysosome inhibitor Bafilomycin resulted in increased abundance of Atg8a-II compared to addition of either Mcr or Bafilomycin alone (Figures 2.12C and 2.12D).

To determine if the formation of GFP-Atg8a puncta in S2R$^+$ cells by addition of Mcr is dependent on *Atg* genes, we used double stranded (ds)RNA to decrease the function of multiple *Atg* genes. We found that knockdown of either *Atg1*, *Atg3* or *Atg5* suppressed the formation of GFP-Atg8a puncta in S2R$^+$ cells after addition of Mcr (Figures 2.12E and 2.12F). Finally, consistent with the Draper receptor receiving the signal to activate autophagy, we found that two distinct dsRNAs that target *draper* also inhibited Mcr induction of GFP-Atg8a puncta in S2R$^+$ cells (Figures 2.12E and 2.12F). Taken together, these results
indicate that Mcr functions in a cell non-autonomous manner through Draper to activate autophagy in multiple cell contexts.
Figure 2.12. Mcr is sufficient for induction of autophagy in S2 cells.
(A) S2R\(^+\) cells stably expressing GFP-Atg8a were either untreated (no treatment), cultured with serum free-medium (starvation), treated with 50 µg (110nM) TEP I protein (TEP I) or treated with 50 µg (82nM) Mcr protein (Mcr). GFP-Atg8a puncta were assessed at 20h. Representative images from three independent experiments are shown. Scale bars, 10 µm.

(B) Quantification of data from (A). Error bars, mean ± SEM; Statistical significance: Student's t-test.

(C) Western immuno-blots of Atg8a and Actin in extracts of S2R\(^+\) cells stably expressing GFP-Atg8a and treated with either 82nM Mcr, 100nM bafilomycin A1, or both.

(D) Quantification of data from (C). All samples are normalized to Actin and plotted relative to no treatment samples. Error bars, mean ± SEM.

(E) S2R\(^+\) cells with stable expression of GFP-Atg8a were treated with dsRNAs against either Luciferase, *Atg1, Atg3, Atg5* or *draper* and then treated with 50 µg (82nM) Mcr protein. GFP-Atg8a puncta were assessed at 20h. Representative images from three independent experiments are shown. Scale bars, 10 µm.

(F) Quantification of data from (E). Error bars, mean ± SEM; Statistical significance: Student's t-test.
Discussion

Much is known about the regulation of autophagy, including that a conserved group of core Atg proteins function within individual cells to control this process (Mizushima and Komatsu, 2011a). Although body-wide signals are thought to control autophagy, particularly during nutrient restriction, direct genetic evidence in support of systemic activators of autophagy is limited. Our findings reveal a novel function for the complement related protein Mcr in the control of autophagy in neighboring cells. Mcr appears to signal through the immune receptor Draper to regulate autophagy during programmed cell death and the inflammatory response of macrophages to wounds, but not during nutrient deprivation-induced autophagy in the fat body. Therefore, this study highlights a mechanism by which autophagy is controlled in distinct cell contexts within an animal.

The mechanisms that underlie the cell-specific roles of autophagy must vary. Although it is logical that the core Atg proteins function in diverse cell types to control this conserved catabolic process, multiple mechanisms likely account for differences in autophagy. For example, different signaling and regulatory pathways (Chang et al., 2013a; McPhee et al., 2010a; Nelson et al., 2014a; Tracy et al., 2016), recruitment of distinct autophagic cargoes (Stolz et al., 2014), different rates of autophagy (Mizushima and Komatsu, 2011a), and other regulatory mechanisms may account for cell context-specific autophagy programs. Here we describe an autophagic program that is controlled by Mcr and
Draper, proteins that have been implicated in inflammation (MacDonald et al., 2006; Stroschein-Stevenson et al., 2006b). Significantly we show that mcr is neither required for nutrient deprivation-induced autophagy, nor for developmentally programmed autophagy in dying intestine cells. By contrast, mcr is required for autophagy in both developmentally programmed cell death of salivary glands and inflammation associated with embryonic epithelial wound healing. The identification of such specific regulators of autophagy in distinct cell contexts may be important, as this concept is at the foundation of precise modulation of autophagy for therapeutic purposes.

The relationship between cells that die by apoptosis and inflammation has been extensively studied (Ravichandran and Lorenz, 2007). Dying cells release pro-inflammatory factors, such as cytokines, and present eat me signals that facilitate inflammatory macrophage removal of dying cells. Draper, the Drosophila orthologue of the C. elegans CED-1, is a well-known engulfment receptor that functions in the recognition of apoptotic cells (MacDonald et al., 2006; Zhou et al., 2001). By contrast, dying Drosophila salivary gland cells do not appear to be eaten by phagocytes (Martin and Baehrecke, 2004a; McPhee et al., 2010a), and do not require Pretaporter, the only known ligand of Draper (Kuraishi et al., 2009), for salivary gland clearance. Rather, dying salivary glands use autophagy, at least in part, to facilitate self-degradation (Berry and Baehrecke, 2007a). Therefore, it is particularly interesting that the inflammatory proteins Mcr and Draper function to mediate inter-and intra-cellular activation of autophagy
during salivary gland degradation. Mcr appears to signal from one cell to another via the immune receptor Draper that activates a cell autonomous autophagy program.

Autophagy is also known to influence inflammation and the immune response. The impacts of autophagy on inflammation can be multifaceted, and can include modulation of pro-inflammatory signaling as well as influencing secretion of immune mediators (Deretic et al., 2013). Furthermore, autophagy influences infection by clearance of pathogens by either xenophagy (Levine et al., 2011) or LC3-associated phagocytosis (Sanjuan et al., 2007b). By contrast, this study implicates autophagy in the regulation of inflammatory response to sterile wounds in the Drosophila embryo. Remarkably, Mcr from the wounded epidermis is required to activate autophagy in and migration of macrophages to the injury. Therefore, this study highlights the possibility that the program to control autophagy in the dying salivary gland has similarities to the inflammatory response during wound healing. It is possible that these seemingly different cell types use a common program to control autophagy without similar cellular consequences. Alternatively, autophagy could have common purposes in salivary glands and macrophages that could be important for efficient wound healing and regeneration of tissues. Although dying salivary gland cells are clearly not migratory, numerous tissue changes are occurring in the forming adult tissues at this stage in development, and it is possible that autophagy may contribute to this tissue formation by providing metabolic substrates. In addition,
it is possible that during this developmental period with extensive tissue remodeling, including that within the intestine, autophagy in the salivary gland somehow helps to prevent infection.

The relationship between Mcr and Draper is likely an ancient mechanism for activation of the Draper immune receptor, as C1q has recently been shown to activate Megf10 in mammals (Iram et al., 2016). Complement has been most studied in the context of pathogen clearance, but recent studies also highlight the importance of complement in other contexts (Kolev et al., 2014), including roles in microglial synapse pruning and in a mouse model of Alzheimer’s disease (Hong et al., 2016a; Stevens et al., 2007b). This study highlights the potential role of autophagy in complement-associated processes. Since autophagy machinery has been associated with both neurodegeneration (Mizushima et al., 2008) and immune disorders, for example through the function of non-canonical LC3-associated phagocytosis (Martinez et al., 2016b), it will be interesting to determine if complement and autophagy are associated in human diseases.

**Materials and methods**

*Drosophila strains*

Fly crosses and experiments were performed at 25°C unless noted otherwise. We used Canton-S as the wild-type control. For loss of function studies, we used
mcr^{EY07421} (Hall et al., 2014), draper^{Δ5} (MacDonald et al., 2006), Atg13^{Δ74} (Chang and Neufeld, 2009), pttp^{Δ2} (Kuraishi et al., 2009). We used the following Vienna Drosophila RNAi Center (VDRC) stocks: UAS-mcr^{IR} VDRC Transformant ID (TID) 100197, UAS-mcr^{IR} VDRC TID 2785. The sequences used for VDRC knockdown strains are available for each TID at http://stockcenter.vdrc.at/control/main. For mis-expression studies, we used UAS-p35 (Hay et al., 1994), UAS-Atg1^{GS10797} (Scott et al., 2007b), UAS-src42a^{ca} (Tateno et al., 2000), UAS-Draper-I-NSS::TMD/ICD. For clonal mis-expression and RNAi studies we used yw hsFlp; +; Act>CD2>Gal4 (“>” is FRT site), UAS-GFP (nls) (Bloomington Drosophila stock center), yw hsFlp; pmCherry-Atg8a; Act>CD2>Gal4, UAS-GFP (nls) and Sp/Cyo; Tub^{P}>stop>Gal80/ Tb (Bohm et al., 2010). mCherry-Atg8a was used as a marker for autophagy (Denton et al., 2012), and tGPH was used as an activity reporter of phosphatidylinositol-3,4,5-P3 (Britton et al., 2002). To obtain flies containing Flag-tagged Mcr, we inserted the entire coding region of Mcr cDNA isolated from clone LD23292 (Drosophila Genomics Resource Center, Bloomington, IN, USA) into pTWF vector from Drosophila Gateway vectors and we generated transgenic flies following standard procedures (Rubin and Spradling, 1982). Six fly lines carrying the transgene on the second or third chromosome were established, and one with it on the third chromosome was used.
**Protein Extracts and Western Blotting**

Protein extraction and western blotting were performed as described previously (Dutta and Baehrecke, 2008). We used guinea pig anti-Mcr (1:500, Robert Ward), mouse anti-ecdysone receptor (1:500, Developmental Studies Hybridoma Bank), mouse anti-Broad Complex (1:100, Developmental Studies Hybridoma Bank), rat anti-Ref(2)P (1:5000, H. Stenmark), rabbit anti-Atg8 (1:2000, Gabor Juhasz), rat anti-pretaporter (1:500, Yoshinobu Nakanishi), mouse anti-actin (1:100, Developmental Studies Hybridoma Bank) and mouse anti-β-Tubulin (1:50, Developmental Studies Hybridoma Bank) primary antibodies. Three independent biological repeats were performed.

**Histology**

Histology was performed as described previously (Muro et al., 2006).

**Immunolabeling and microscopy**

For immunohistochemistry, salivary glands were dissected from animals staged relative to puparium formation at 25°C, fixed in 4% paraformaldehyde overnight at 4°C, blocked in PBS, 1% BSA and 0.1% Tween-20 (PBSBT) for 2 hours at room temperature, and incubated with primary antibodies overnight at 4°C. We used rabbit anti-cleaved-Lamin (Asp 230) (1:500, Cell Signaling Technology),
guinea pig anti-Mcr (1:200, Robert Ward) antibodies, and mouse anti-Flag (1:200, Sigma). Following incubation with primary antibodies, salivary glands were washed for 4 X 30 min in PBSBT, incubated with appropriate secondary antibodies for 2 hours at room temperature, and washed for 1 hour in PBSBT. Salivary glands were mounted in Vectashield (Vector Laboratories) and examined using a Zeiss Axiophot II microscope. For mCherry-Atg8a and tGPH analyzes, salivary glands were dissected from animals staged relative to puparium formation at 25°C, fixed in 4% paraformaldehyde containing 2 µM Hoechst stain for 15 min at room temperature, washed in PBS, and mounted in PBS. mCherry-Atg8a puncta were quantified using Zeiss Automeasure software.

Embryos were fixed and immunostained as previously described (Evans et al., 2015), using rat anti-cherry (1:500, Cappel), purified mouse anti-Fascin (1:200 clone sn7C, Developmental Studies Hybridoma Bank) and guinea pig anti-Mcr (1:200, Robert Ward) as primary antibodies with goat anti-rat-CF594 (Sigma), goat anti-mouse-FITC (Jackson Immunoresearch Laboratories) and goat anti-guineapig-AlexaFluor647 (Molecular Probes) used as secondary antibodies, respectively.

**Induction of cell clones**

To induce RNAi-expressing cell clones in *Drosophila* tissues, we obtained an overnight egg lays at 25°C, and temperature shifted embryos at 37°C for 30 min.
To induce $mcr^{EY07421}$ null mutant cell clones, we crossed $yw$ $hsFlp$; FRT40A, *ubiquitin* (*ubi*)-GFP (nls); pmCherry-Atg8a virgins to $w$; FRT40A, $mcr^{EY07421}$ males. We obtained 6 hour egg lays at 25 °C, and following the egg lay, temperature shifted embryos at 37 °C for 1 hour. To induce Gal80 flip-in clones, we crossed $yw$ $hsFlp$; $mcr^{IR}$; *Tub>P>stop>Gal80* virgins to $w$; pmCherry-Atg8a; *fkh*-Gal4, UAS-GFP (nls) males. We obtained an overnight egg lay at 25 °C, and following the egg lay, temperature shifted embryos at 37 °C for 1 hour.

**Starvation of larvae**

We either allowed early third instar larvae to remain in the food (fed) or transferred them from food to 20% sucrose (starved) in PBS for 4 hours.

**Live imaging of *Drosophila* blood cells**

For live imaging experiments, stage 15 embryos were collected from overnight apple juice agar plates and mounted on slides in a minimal volume of 10S Voltalef oil (VWR), following dechorionation in bleach for 1 min and extensive washing in water. All imaging was carried out at room temperature. For quantification of wound responses, epithelial wounds were induced using a nitrogen-pumped Micropoint ablation laser tuned to 435 nm (Andor Technologies), as previously described (Evans et al., 2015). Embryos were then
imaged at 60 min post-wounding using a 40X oil immersion objective lens on a PerkinElmer UltraView spinning disc microscope. To quantify blood cell responses to wounds, the number of blood cells in contact with/inside the wound edge was determined from z-stack images. Wound size was determined from red channel images in ImageJ. The number of macrophages was then divided by the wound perimeter and the value normalized to the appropriate control.

**S2R+ cell culture and RNAi**

*Drosophila* S2R+ cells (Anding and Baehrecke, 2015) were cultured in Schneider's medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% Glut-MAX (Gibco), and 0.2% Penicillin-Streptomycin (Pen-Strep). S2R+ cells were plated into 96-well plates at a density of 30,000 cells per well in a culture volume of 150 µl per well. dsRNA was added to a final concentration of 13 µg/ml, and the cells were incubated for four days at 25 °C to allow for depletion of the corresponding gene product.

**Recombinant Protein Expression and Purification**

Recombinant proteins were produced using the Bac-to-Bac™ baculovirus expression system (ThermoFisher). The purification of *A. gambiae* TEP1 was performed as previously described (Baxter et al., 2007). The entire Mcr
ectodomain (sMcr) including the native signal peptide (aa 1–1725 of DGRC cDNA clone LD23292) was subcloned into pFastbac1 with a C-terminal 6×His tag. Protein was secreted by *T. ni* cells cultured in ESF-921 media (Expression Systems LLC) and harvested at 72 hpi. Following concentration and diafiltration in 0.2 M NaCl, 20 mM Tris pH 7.8, sMcr-6xHis was purified on Co-Talon™ (Clontech) and eluted with a gradient on 0-250 mM imidazole. The crude eluate was purified to homogeneity by anion exchange chromatography on MonoQ 10/10 (GE Healthcare) with 20 mM Tris pH 8.5, 80-600 mM NaCl, and size-exclusion chromatography Superdex200 16/60 (GE Healthcare) with 20 mM Hepes pH 7.5, 150 mM NaCl. The purified protein was concentrated to >1 mg/ml with 20% w/w glycerol, aliquots flash-frozen in liquid nitrogen and stored at -80 °C until use.

**Statistical analyses**

All experiments were performed independently at least three times. For GFP-Atg8a puncta quantification, at least 15 random images were chosen and the number of cells with GFP-positive puncta were counted. An average of 80 cells were examined for each group, and P values were calculated using a two-tailed unpaired t-test. For animal studies, sample sizes were determined empirically based on previous studies to ensure appropriate statistical power. No animals
were excluded from statistical analyses, the experiments were not randomized, and the investigators were not blinded.

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Chapter III

Discussion

Part I: Functions of Mcr

Mcr is a member of the widely-conserved thioester-containing protein (TEP) family. Phylogenetically, TEPs from insects share sequence similarities with both the vertebrate complement factors C3/C4/C5 and the $\alpha_2$-macroglobulin family of protease inhibitors (Nonaka and Yoshizaki, 2004). In vertebrates, complement factors contain a highly reactive intrachain thioester bond that mediates covalent binding to pathogen surfaces, targeting them for phagocytosis or lysis. $\alpha_2$M, the most abundant serum protein in human blood, acts as an antiprotease that inactivates a broad spectrum of proteases. Upon proteolytic cleavage of a ‘bait’ region, $\alpha_2$M undergoes a conformational change and covalently binds the attacking protease to the exposed thioester (Borth, 1992). The $\alpha_2$M-protease complex is subsequently cleared by receptor-mediated endocytosis. Insect TEPs were first studied in Drosophila melanogaster and Anopheles gambiae and are involved in the response to pathogens and parasites (Levashina et al., 2001). Drosophila has six TEP family members, four of which (Tep1-4) are predicted secreted proteins expressed in hemocytes and thought to play roles in innate immunity (Bou Aoun et al., 2011; Lagueux et al., 2000). Tep5 appears to be a pseudogene in Drosophila melanogaster (Bou Aoun et al., 2011). Mcr (Tep6) is a diverged TEP family member with a mutated thioester motif and a
transmembrane domain not found in other TEPs, suggesting that the function of Mcr may be distinct from those of other TEPs.

**Regulation of innate immunity**

Animals have evolved multiple immune systems to eliminate pathogenic viruses. The innate immune response plays a crucial antiviral role in the early stage of infection. Immune responses are initiated based on the recognition of viral surface components by a group of recognition receptors. This recognition activates the complement system and intracellular antiviral signaling cascades, leading to the phagocytosis of viruses and infected cells. In a RNAi screen for the cellular components that are required for phagocytosis of a fungal pathogen, *Candida albicans*, Stroschein-Stevenson et al. showed that Mcr is secreted by *Drosophila* S2 cells and binds tightly to *C. albicans* in the absence of S2 cells. The defect in phagocytosis caused by mcr RNAi can be reversed through the addition of conditioned media from normal S2 cells, suggesting that secreted Mcr may be the active Mcr that is required for efficient phagocytosis of *C. albicans* (Stroschein-Stevenson et al., 2006a). Unlike aTep1 or complement in mammals (Fujita et al., 2004; Gasque, 2004; Levashina et al., 2001), there is no evidence of proteolytic processing of Mcr, suggesting that the full-length protein is the active form. Moreover, Mcr binding exhibits specific recognition for *C. albicans*. This is consistent with the roles of the other four closely related *Drosophila* TEPs, which each individual member showing specificity for certain classes of
pathogens. Thus, this family of five closely related proteins collectively functions to promote the phagocytosis of a diverse set of pathogens. This study resembles the opsonizing effect of Mcr. However, it was done in vitro in tissue culture cells. Although Drosophila S2 cell line is believed to be derived from embryonic plasmatocytes and shares many properties with plasmatocytes, it is worth to address the function of Mcr in regulation of innate immune response in vivo. Furthermore, it is not clear how Mcr recognizes and binds to C. albicans. Mcr lacks the critical cysteine residue in the thioester motif. Therefore, unlike the other TEPs proteins, Mcr cannot form covalent bond with its target at the thioester motif. Since there is no evidence of proteolytic processing of Mcr, the full-length protein is the active form. It is possible that Mcr bonds to microbial surfaces receptor via formation of covalent bond at the N terminal alpha 2-macroglobulin domain. Unbiased screens for the genes that are required for specific pathogen recognition should give a broad view of the mechanisms by which Mcr activates innate immune response.

Maintenance of septate junction structure

Polarized epithelia play crucial roles as barriers to the outside world and provide distinct compartments for organs to carry out essential functions in all metazoans. These functions require a physiologically tight epithelium to provide a barrier to the flow of small molecules between the apical and basal sides of the epithelium. This paracellular barrier is established and maintained by tight
junctions (TJs) in the epithelia of vertebrate organisms, and by septate junctions (SJs) in invertebrate organism. TJs and SJs play crucial roles in organizing basic epithelial functions during development. Accordingly, disruption of SJs in embryonic epithelia and glia results in embryonic lethality, with characteristic defects in the epidermal cuticle (Lamb et al., 1998), dorsal closure (Baumgartner et al., 1996; Fehon et al., 1994; Woods and Bryant, 1991), and embryonic paralysis due to a disrupted blood brain barrier (Baumgartner et al., 1996). Mcr was identified as a new SJ-associated protein in screens for new SJs components (Batz et al., 2014; Hall et al., 2014). It localizes to the lateral membranes of epithelial cells, where its distribution overlaps with known SJ components. mcr mutant epithelial tissues have defective SJ organization and function. And it is required in a cell-autonomous fashion for the correct localization of other SJ components, suggesting that membrane-bound rather than secreted Mcr isoforms are involved in SJ formation. Finally, mcr is essential for SJ-dependent tracheal tube size control and epithelial barrier function. These results, along with previous identified function in innate immunity regulation, suggest that Mcr plays dual roles in epithelial barrier formation and innate immunity.

**Regulation of autophagy**

Autophagy is related to normal development and many human diseases. Therefore, it is important to understand the mechanisms that regulate autophagy.
The mechanism that regulates cell survival function of autophagy is relatively well studied, however, the regulatory mechanisms of autophagic cell death is less clear. To identify genes that may regulate autophagy in cell-specific contexts, the former Baehrecke lab members performed proteomic analysis from serial time points during salivary gland degradation. Several factors that have been implicated in the engulfment of apoptotic cells are induced in dying salivary glands, whereas there are no detectable changes in these genes after laval starvation. Although many engulfment factors are pleiotropic through their regulation of the cytoskeleton and vesicular transport, the identification of phagocytosis factor mcr is intriguing, as salivary gland degradation is thought to be largely independent of phagocytes.

In this thesis, I have demonstrated that mcr regulates autophagy during salivary gland developmental cell death but not during nutrient deprivation in *Drosophila melanogaster*. Unlike most known regulators of autophagy, mcr functions in a cell non-autonomous manner to regulate autophagy in neighboring cells within the dying salivary gland. This is the first known autophagy regulator that cell non-autonomously regulates autophagy. Interestingly, mcr appears to function upstream of the conserved immune receptor Draper, a factor that functions in a cell autonomous manner to regulate autophagy in dying salivary gland cells. Moreover, mcr is required for autophagy in embryonic macrophages where Draper is known to be required for an inflammatory response to epithelial wounds. These studies reveal an unexpected role for complement in the
regulation of autophagic cell death in neighboring cells that depends on an ancient immune signaling program. My results, along with previous work (Batz et al., 2014; Hall et al., 2014; Stroschein-Stevenson et al., 2006a), suggest that Mcr plays multiple roles in autophagic cell death regulation, innate immunity and epithelial barrier formation. These finds call attention to a potential link between autophagy, pathogen defense mechanisms and the structure of occluding cell-cell junctions in epithelia.

**Mcr may be involved in LAP**

Among the core autophagy machinery proteins, Atg8 is the most commonly monitored, and its lipidated form, Atg8a-II, is present on both autophagosomes and autolysosomes during canonical autophagy (Klionsky et al., 2012). Thus, we can use fluorescence-labeled Atg8a as a reporter for autophagy activity. However, LC3, mammalian homologue of Atg8, has been identified as a regulator of LC3-associated phagocytosis (LAP) (Sanjuan et al., 2007a). LAP is a process triggered following phagocytosis of particles that engage cell-surface receptors such as toll-like receptor (TLR), resulting in recruitment of some, but not all, members of the autophagics machinery to stimulus-containing phagosomes, facilitating rapid phagosome maturation, degradation of engulfed pathogens, and modulation of immune response (Florey et al., 2011; Henault et al., 2012; Huang et al., 2009; Kim et al., 2013; Martinez et al., 2011; Sanjuan et al., 2007a). Although some of the autophagy machinery are recruited to the TLR-
engaged phagosome, it has been demonstrated that LAP and autophagy are functionally and mechanistically distinct processes (Dishaw et al., 2005; Florey et al., 2011; Henault et al., 2012; Kim et al., 2013; Martinez et al., 2011). While there is no direct evidence, it is possible that LAP also happens in *Drosophila* to eliminate pathogens. Therefore, fluorescence-labeled Atg8a could serve as a reporter for the activity of both autophagy and LAP. The formation of GFP-Atg8a puncta after addition of recombinant Mcr protein into S2 cell culture medium could be because of the induction of either LAP or autophagy. Interestingly, knockdown of CG12772, the *Drosophila* homolog of mammalian LAP regulator *Rubicon*, suppressed the formation of GFP-Atg8a puncta in S2R*+* cells after addition of Mcr (Figure 3.1). This result suggests that Mcr may also regulate LAP in *Drosophila*. Yet, further analysis is required to elucidate the role and mechanism of Mcr in the regulation of LAP.
Figure 3.1. CG12772 dsRNAs suppress formation of GFP-Atg8a puncta in S2R\textsuperscript{+} cells after addition of Mcr.

(A) S2R\textsuperscript{+} cells with stable expression of GFP-Atg8a were treated with dsRNAs against either Luciferase or CG 12772 and then treated with 50 µg (82nM) Mcr protein. GFP-Atg8a puncta were assessed at 20h. Representative images from three independent experiments are shown. Scale bars, 10 µm.

(B) Quantification of data from (A). Error bars, mean ± SEM; Statistical significance: Student's t-test.
Part II: Differences in autophagy programs in different *Drosophila* tissues

The larval midgut

During *Drosophila* development, successive pulses of the steroid hormone ecdysone trigger differentiation and morphogenesis of imaginal discs to give rise to adult tissues, and programmed cell death of larval cells to eliminate obsolete tissues (Thummel, 1996). An increase in ecdysone titer at the end of third larval instar stage triggers puparium formation and marks the onset of metamorphosis. The larval midgut cells undergo programmed cell death in response to this ecdysone titer. Future adult midgut cells use the dying larval midgut as a substrate and form epithelium that surround larval midgut. Dying larval midguts express the *rpr*, *hid*, *ark*, *dronc*, and *crq* apoptosis genes, however, inhibiting or reducing the activities of these core apoptosis machinery have no significant effect on larval midgut cell death (Denton et al., 2009). Moreover, due to the presence of adult epithelium, professional phagocytes of the blood cell lineage have no direct access to the dying larval midgut, therefore these larval midgut cells must degrade themselves. By contrast, midgut degradation was severely delayed by inhibition of autophagy genes, and the dying larval midgut cells possess vacuoles that contain cellular organelles, indicating that these cells die by autophagy (Denton et al., 2009). Therefore, despite the high levels of caspase activity in the *Drosophila* larval midgut during programmed cell death, caspases
do not have a significant function in midgut degradation. Instead, autophagy is the sole known cellular process that is essential for the proper removal of the midgut.

The larval salivary gland

The death of the *Drosophila* larval salivary glands differs from larval midgut. The larval salivary glands do not initiate programmed cell death at the time of puparium formation. Rather, 12 hours after puparium formation, there is another steroid pulse which triggers larval salivary glands to undergo programmed cell death (Berry and Baehrecke, 2007b). Presumably, the salivary glands do not degrade at the stage when midgut is triggered to die because salivary glands are still synthesizing and secreting polypeptide glue. In addition, unlike adult midgut which uses larval midgut as a substrate, larval salivary gland appears to be dispensable for adult salivary gland formation, and larval salivary gland cells die in a synchronous way. Finally, high level of autophagy occurs during larval salivary gland degradation, and genetic inhibition of autophagy results in incomplete gland degradation (Berry and Baehrecke, 2007b; Lee and Baehrecke, 2001). However, unlike the larval midgut, in addition to autophagy, caspases are also necessary for salivary gland destruction. These two processes appear to function in parallel, inhibit either one of these two processes leads to partial degradation of the larval salivary glands (Berry and Baehrecke, 2007b). These differences indicate that larval midgut and salivary glands utilize similar, yet
distinct, genetic mechanism during steroid regulation of programmed cell death. In fact, previous work from the Baehrecke lab has shown that both engulfment receptor Draper and microRNA machinery are new autophagy activity regulators. Genetic knockdown of either Draper or miR-14 in salivary glands result in defects in larval salivary gland destruction (McPhee et al., 2010b; Nelson et al., 2014b). However, these genes have no effect on larval midgut programmed cell death. Clonally knockdown of these genes in midgut does not affect either cell size reduction or autophagy level (McPhee et al., 2010b; Nelson et al., 2014b). In contrast, the larval salivary gland degradation requires all of the core autophagy pathway proteins, whereas the larval midgut possess a unique Atg8 lipidation-independent form of autophagy. This form of autophagy does not need Atg7 or Atg3, instead, requires Uba1, the E1 enzyme used in ubiquitylation (Chang et al., 2013b). In this thesis, I also identified a new autophagy regulator, Mcr, which specifically regulates autophagy in salivary glands programmed degradation but not in the larval midgut. Further, Mcr cell non-autonomously regulates autophagy during salivary gland degradation. The rationale of this regulation mechanism is to ensure salivary gland cells die in a synchronous way, also explained the reason that larval salivary glands do not initiate programmed cell death at the time of puparium formation. Together, these findings strengthen the argument that Drosophila larval midgut and salivary gland use different genetic mechanisms to regulate autophagy and tissue destruction. Future analyses are needed to clarity the mechanisms that regulate autophagy in different cells within
an animal.

It seems that hemocytes are not involved in larval salivary gland degradation. First, there is no evidence of the presence of phagocytosis during Drosophila larval salivary glands degradation. No obvious phagocytes containing cell fragments were observed using TEM (Martin and Baehrecke, 2004b). Second, specifically knock down mcr or draper in hemocytes doesn’t affect Drosophila larval salivary gland clearance (Lin et al., 2017; McPhee et al., 2010b). However, we cannot rule out the involvement of hemocytes in autophagic cell death. Future studies with Drosophila that lacking functional hemocytes should provide insights into the role of hemocytes in the regulation of salivary gland cell death (Defaye et al., 2009).

**The larval fat body**

Drosophila fat body is a primary nutrient-responsive tissue that emulates the functions of the liver and adipose tissue of vertebrates (Colombani et al., 2003). The fat body has the capacity for both starvation-induced and developmentally programmed autophagy. In response to starvation, fat body cells recover nutrients through autophagy. 3 hours in a protein-free starvation diet resulted in a striking increase in the size and abundance of lysosomes in larval fat body (Scott et al., 2004a). Double membrane-bound vesicles containing undigested cytoplasmic material were often observed adjacent to these enlarged lysosomes, typical of autophagosomes just prior to lysosomal fusion (Scott et al.,
Interestingly, TOR/PI3K signaling is necessary and sufficient to suppress starvation-induced autophagy. Both TOR mutants or loss-of-function mutations in Rheb, an upstream activator of TOR, lead to induction of autophagy regardless of nutrient conditions. In contrast, mutations in other growth regulators such as Myc and CDK4 do not induce autophagy (Scott et al., 2004a).

On the other hand, developmental autophagy is initiated in fat bodies at the last larval stage (L3), which is at least 6 hours before puparium formation (Rusten et al., 2004). This induction of autophagy in larval fat body is triggered by a low but rising titer of ecdysone. Overexpression of either one of the dominant-negative versions of the ecdysone receptor, EcRF645A or EcRW650A lead to a strong reduction of both the number and size of GFP-LC3/lysotracker-positive structures relative to the control (Rusten et al., 2004). Surprisingly, elevation of PI3K signaling during the period of programmed autophagy prevented the biogenesis of autolysosomes. And a strong reduction and ultimately loss of PI3K signaling was observed in the fat body during the induction of programmed autophagy (Rusten et al., 2004), suggesting that ecdysone signaling has the ability to promote autophagy through the downregulation of PI3K signaling. However, other than the PI3K signaling, the mechanism that regulates programmed autophagy in fat body is less clear. Neither Draper nor Mcr or the microRNA machinery are necessary in the induction of autophagy in fat body. Future studies are needed to clarity the mechanisms that regulate autophagy in different cells within an animal.
Part III: Conclusions and Future directions

Autophagy is an extremely fascinating cellular process. It plays essential roles in regulating development, homeostasis, cell survival, and cell death. The ability of autophagy to influence various cellular situations offers the potential for its manipulation in the treatment of different diseases. Due to its significance, tremendous effort has been made to dissect this pathway in the past, yet still little is known about the regulation of autophagy in different cell contexts. In this thesis, I have demonstrated that the complement factor Mcr can serve as a regulator that is being secreted by one cell and activate autophagy in a different cell within an animal. This is the first known autophagy regulator that cell non-autonomously regulates autophagy. Interestingly, mcr is only necessary for autophagy but not for caspases activity during Drosophila salivary gland degradation. And it does not influence either nutrient deprivation-induced autophagy in the fat body or developmentally programmed autophagy in the dying larval midgut, suggesting that Mcr can serve as a regulator to distinguish between the uses of autophagy during different developmental situations and cell contexts. Surprisingly, mcr is also required for autophagy in embryonic macrophages for an inflammatory response to epithelial wounds. This requirement is in the embryonic epidermis, indicating that this complement related molecule also functions in a cell non-autonomous manner to regulate macrophages autophagy and migration to wounds. Moreover, the functions of Mcr in salivary gland degradation and macrophages migration to wounds are all
involve the immune receptor Draper; and *mcr* appears to function upstream of *draper*. These studies reveal an unexpected role for complement in the regulation of autophagy in neighboring cells that depends on an ancient immune signaling program. It is worth to look at the mammalian homolog of *mcr*, complement C3, whether it influences autophagy and uses the same manner as Mcr during cell death in mammalian system.

Other than its function in regulating programmed cell death and cell survival under nutrient deprivation condition, autophagy is also known to influence inflammation and the immune response. The fact that Mcr functions through Draper to regulate both larval salivary gland destruction and macrophages response to wounds highlights the possibility that the program to control autophagy in the dying salivary gland has similarities to the inflammatory response during wound healing. It is possible that these seemingly different cell types use a common program to control autophagy without similar cellular consequences. Alternatively, autophagy could have common purposes in salivary glands and macrophages that could be important for efficient wound healing and regeneration of tissues. Although dying salivary gland cells are clearly not migratory, numerous tissue changes are occurring in the forming adult tissues at this stage in development, and it is possible that autophagy may contribute to this tissue formation by providing metabolic substrates. In addition, it is possible that during this developmental period with extensive tissue remodeling, including that within the intestine, autophagy in the salivary gland
somehow helps to prevent infection. Future studies that try to understand these possibilities will open the doors to manipulate autophagy pathway for the treatment of human diseases.

**Appendices**

Autophagy is an important metabolic process that has different outcomes under different circumstances. For example, autophagy can function either as a tumor suppressor or promote tumor growth depending on the cell context. Therefore, it is important to understand the function and regulation of autophagy in a variety of cell contexts. The research presented in this dissertation contributes to our understanding on contexts specific regulation of autophagy, but it also raises more questions that need to be addressed. Mcr and Draper are two context-specific regulators of autophagy, and understanding the details of how they regulate and function to control autophagy is important.

**Physical interaction between Mcr and Draper**

In this thesis, I have shown that mcr and draper function within the same pathway, and mcr appears to function upstream of draper to regulate autophagy during programmed cell death and the inflammatory response of macrophages to wounds. However, I do not have direct evidence to show that Mcr directly interacts with Draper. I have attempted several rounds of co-immunoprecipitation (co-IP) experiments in *Drosophila* S2 cells under different conditions, but I was not able to co-IP either Draper or Mcr (Figure 3.2).
**Figure 3.2. Experiments to co-IP Mcr and Draper in S2 cells.** Transfection of constructs for over-expression (OE) of either Mcr, HA-tagged Draper, or HA-tagged Draper and Mcr were used to attempt co-IP of Mcr and Draper.
With the help of my collaborators, we next expressed the full-length extracellular domains of Mcr and Draper using the baculovirus system, and purified these proteins. Then we conducted interaction experiments at an alkaline pH 8.5 to minimize self-association of both proteins. Using a monoclonal antibody against Mcr, we performed a co-IP experiment of recombinant Mcr and Draper. Draper did not co-IP with Mcr (Figure 3.3). To test further whether Mcr interacts directly with Draper, we performed analytical ultracentrifugation experiments of Draper and Mcr at 4.5 µM and 1.65 µM, respectively. Draper sedimented as a single peak at $s = 4.0$ S and Mcr sedimented as a single peak at $s = 8.9$ S. A mixture of Draper and Mcr was well-fit by a superposition of the two individual proteins; no evidence of a distinct complex was observed (Figure 3.4). These results suggest the full-length extracellular domains of Draper and Mcr do not form a high affinity complex ($K_D \geq 15$ µM).
Figure 3.3. Purification and co-IP of recombinant Mcr and Draper. (A)
Coomassie staining and α-6xHis Western blotting of purified protein. (B)
Immunoprecipitation of Mcr with α-Mcr mAb 4C7F10, α-6xHis Western blotting.
Draper does not co-IP with Mcr.
Figure 3.4. Sedimentation velocity analytical ultracentrifugation of Draper and Mcr. (A) Draper (orange) sediments at $s = 4.0$ S ($f/f_0 = 1.97$) whereas Mcr (purple) sediments at $s = 8.9$ S ($f/f_0 = 1.41$); the mixture of both proteins (grey) is a sum of the individual $c(s)$ distributions. (B–D) Fit of $c(s)$ model to experimental data for Draper (B), Mcr (C), and Draper + Mcr (D).
Obviously, these negative results are uninformative, as the interaction of Mcr with Draper may be too transient to survive the rigors of immunoprecipitation, may not be direct, or may need the presence of a co-factor. Continue study is necessary to understand the exact mechanism by which Mcr functions through Draper to regulate autophagy.

**How does Draper initiate autophagy?**

Although it has been shown that Draper is required for the activation of autophagy during programmed cell death. How Draper functions to regulate autophagy specifically in a cell death context remains unclear. It has been reported that as an engulfment receptor, Draper signals through an evolutionarily conserved Src/Shark family kinase cascade to drive cytoskeletal rearrangements and target engulfment through Rac1 (Ziegenfuss et al., 2008a). In fact, Src is required for downstream clearance of dying salivary glands (McPhee et al., 2010b; Ziegenfuss et al., 2008b), and expression of constitutively active Src protein can suppress the mcr knockdown salivary gland clearance defect. These data argue that Draper may function through Src to phosphorylate Atg proteins or other unknown factors to active autophagy, but this awaits further exploration. On the other hand, Draper is also activated in response to axonal injury, which in turn promoted JNK signaling through TRAF4 and MSN to alter glial transcription via dAP-1 and STAT92E (Lu et al., 2017). TNF receptor associated factor 4 (TRAF4) is a novel Draper binding partner that is required for reporter activation
and phagocytosis of axonal debris. It is worth to exploring the roles for TRAF4, dAP-1 and STAT in *Drosophila* larval salivary gland degradation. This could provide further important molecular insight into Draper regulation of transcription changes in these physiological contexts.

**Is LAP involved in salivary gland degradation during development?**

LAP is a form of non-canonical autophagy, in which phagosomes containing engulfed particles, including dying cells and pathogens. Recruitment of some of the autophagy pathway elements facilitate the maturation of phagosomes and digestion of their contents. It has been shown that mice lacking any of several components of the LAP pathway show increased serum levels of inflammatory cytokines and autoantibodies, glomerular immune complex deposition, and evidence of kidney damage (Martinez et al., 2016a). However, whether LAP is present in *Drosophila* is not clear. The fact that knockdown of CG12772, the *Drosophila* homolog of mammalian LAP regulator *Rubicon*, suppressed the formation of GFP-Atg8a puncta in S2R⁺ cells after addition of Mcr (Figure 3.1) suggests that Mcr may also regulates LAP in *Drosophila*. Since Mcr is required for the degradation of larval salivary gland, I decided to check whether LAP is also involved in *Drosophila* larval salivary glands degradation. Surprisingly, specific express UAS-CG12772IR46141 in salivary gland does not affect larval salivary gland degradation at 24h after puparium formation (Figure 3.5). In
addition, targeting another two different regions of *CG12772* also do not affect salivary gland destruction (data not shown). This result indicates that LAP may not be involved in salivary gland degradation. However, I cannot rule out the possibility that the RNAi lines that I used to knockdown *CG12772* are not strong enough, there is still *CG12772* protein to activate LAP in dying salivary glands. The knockdown efficiency needs to be validated in the future. It is also worth noting that Ref2p/p62 levels are influenced by *mcr* in salivary glands, indicating that this cargo receptor is involved in recruitment of cargos into autophagosomes in salivary glands. Since Ref2p/p62 is not involved in LAP, these data further suggest that LAP does not occur in dying salivary glands. It is also important to generate *CG12772* mutants with CRISPR/Cas9 system and check larval salivary gland clearance phenotype in mutant animals, but this will dependent on the viability of such mutant animals.
Figure 3.5. CG12772 is not required for salivary gland cell degradation.

Control (n = 20) and salivary gland-specific CG12772 knockdown animals (n = 21) analyzed by histology for the presence of salivary gland material at 24h after puparium formation.


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