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The Role of NFκB Factor Relish in Developmentally Programmed Cell Death

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The Role of NFκB Factor *Relish* in Developmentally Programmed Cell Death

A Dissertation Presented

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Abstract

Several types of cell death including apoptosis, necroptosis and autophagic cell death play diverse roles in different biological processes. In addition to its essential roles in development and metabolism, programmed cell death is indispensable for host immunity. Interestingly, current research shows that these processes are connected but the nature and extent of the crosstalk between host defense and programmed cell death still remains an area of great interest.

The NFκB factor *Relish* is best characterized as a crucial component of *Drosophila* Imd pathway, which generates immune responses by producing antimicrobial peptides following Gram-negative bacterial infection. In this dissertation, I demonstrate a novel role of *Relish* in developmentally programmed cell death. During metamorphosis, *Drosophila* salivary glands are degraded by the collective actions of caspase-dependent and autophagic cell death. Here I show that *Relish* mutants displayed improper salivary gland degradation and the persistence of salivary gland cell fragments. Expression of *Relish* in salivary glands rescued this phenomenon. Among the upstream components of the Imd pathway, mutants in the bacterial peptidoglycan receptors, *PGRP-LC* and-*LE* also exhibited similar defects in gland degradation, but surprisingly none of the other Imd pathway components examined had any such effect. As both Relish and PGRPs are critical for host defense against bacterial infection, our next concern was the role of host microflora in salivary gland degradation. However, observation of normal salivary gland cell death in axenic flies ruled out possible involvement of microbiota. Robust genetic analyses proved that *Relish*-mediated cell death occurs in caspase-independent but autophagy-dependent manner. Moreover, expressions of either active version of *Relish* or *PGRP-LC* resulted in the premature gland degradation and induction of autophagy. Finally, I show that Relish controls autophagy by regulating the expression of *Atg1*, a core component of the autophagy pathway. Together these findings suggest the existence of a novel pathway, which connects immune response factors to developmentally programmed cell death.

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List of Abbreviation

AMP: Adenosine Monophosphate

AMPK: 5`-AMP activated Protein Kinase

AMPs: Antimicrobial Peptides

APF: Puparium Formation

ATG: Autophagy-Related Gene

BAK: Bcl-2 Homologous Antagonist/Killer

BAX: BCL2 associated X

BCL-2: B-cell Lymphoma 2

BECN1: Beclin1

BH3: Bcl-2 Homology 3

BIF1: Bax-Interacting Factor 1

BR-C: Broad complex

cGAS: cyclic GMP-AMP Synthase

ChIP: Chromatin-Immunoprecipitation

DAMP: Damage Associated Molecular Pattern

DAP: Diaminopimelic acid

DED: Death Effector Domain

DIABLO: Direct IAP Binding Protein with Low pI

DIF: Dorsal-related Immunity Factor

DISC: Death Inducing Signaling Complex

DREDD: Death-related ced-3/Nedd2-like protein

FADD: Fas Associated Death Domain

FIP200: FAK Family Kinase-Interacting Protein of 200 kDa

GNBP1: Gram-Negative Bacteria Binding Protein 1

GOPC: Golgi Associated PDZ and Coiled-Coil Motif Containing

HSV: Herpes Simplex Virus

IAP: Inhibitor of Apoptosis Proteins

IKK: IκB Kinase

IL: Interleukin

IMD: Immune Deficiency

ird5: Immune Response Deficient 5

LAMP2A: Lysosomal Associated Membrane Protein 2

LAP: LC3 Associated Phagocytosis

LC3: Light Chain 3

LIR: LC3-Interacting Region

LKB1: Liver Kinase B1

LPS: Lipopolysaccharide

MDP: Muramyl Dipeptide

MLKL: Mixed Lineage Kinase Domain-Like

MyD88: Myeloid Differentiation Primary Response 88

NDP52: Nuclear Domain 10 Protein 52

NFκB: Nuclear Factor kappa-light-chain-Enhancer of Activated B cells

NLR: NOD Like Receptors

NOD: Nucleotide Binding Oligomerization Domain

OPTN: Optineurin

PAMP: Pathogen Associated Molecular Pattern

PAS: Phagophore Assembly Site

PE: Phosphtidylethanolamine

PGN: Peptidoglycan

PGRPs: Peptidoglycan Binding Receptors

PI3K: Phostidylinositol 3-Kinase

PI3P: Phosphatidylinositol 3-Phosphate

PKA: Protein Kinase A

PRR: Pattern Recognition Receptors

RAG: Ras-related small GTPases

RBCC1**:** RB1-Inducible Coiled-Coil Protein 1

RelN: N-terminus of *Relish*

RHD: Rel Homology Domain

RHEB: Ras Homolog Enriched in Brain

RHIM: RIP Homotypic Interaction Motif

RIPK1: Receptor-Interacting Protein Kinase 1

RIPK2 or RIP2: Receptor Interacting Protein Kinase 2

ROS: Reactive Oxygen Species

SEM: Standard Error of The Mean

SMAC: Second Mitochondria-Derived Activator of Caspases

STING: Stimulator of Interferon Genes

TAB2: TAK1 Associated Binding 2

TAD: Transactivation Domain

TAK1: TGF-β Activated Kinase 1

TBK1: Tank Binding Kinase

TCT: Tracheal Cytotoxin

TLR: Toll Like Receptor

TNF: Tumor Necrosis Factor

TNFR1: Tumor Necrosis Factor Receptor 1

TOR: Target of Rapamycin

TRADD: TNFR-Associated Death Domain

TRAIL-R: TNF-Related Apoptosis-Inducing Ligand-Receptor

TSC: Tuberous Sclerosis complex

TUFM: Tu Elongation Factor

TUNEL: Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

UAS: Upstream Activation Sequence

UBD: Ubiquitin Binding Domain

ULK1/ULK2: Unc-51-Like Kinase ½

UVRAG: UV Radiation Resistance Associated Gene

VPS34: Vacuolar Protein Sorting-Associated Protein 34

VSVG: Vesicular stomatitis Indiana virus G protein

WIPI1: WD Repeat Domain, Phosphoinositide Interacting 1

XIAP: X-linked Inhibitor of Apoptosis Protein

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CHAPTER 1.

Introduction

Cell death is a natural and essential process that occurs from birth to death in all metazoans. Together with cell multiplication and survival, cell death is needed to ensure the proper development and homeostasis of organisms. Dysregulation in the balance among the pathways that facilitate cell death leads to abnormal conditions, ranging from different diseases to death. So, it is crucial to learn the intricacies of cell death and how this balance is maintained.

Cell death is induced by means of varied stimuli, such as nutrient deprivation; production of excess cells, or cellular damage or infection (Jacobson et al., 1997). Nutrient sensing is critical for survival of cells as starvation leads to developmental delays and potentially death in the case of prolonged nutrient deprivation. Cell death is also essential in the elimination of damaged cells (Krammer, 2000). Persistence of damaged cells may result in different abnormalities including uncontrolled growth. During the course of infection, cells with severe pathogen load are often eliminated by cell death to guarantee organism safety (Huang et al., 2015; Miao et al., 2010). Finally, during development, proper tissue and organ structure formation is dependent on various forms of programmed cell death (Zakeri et al., 1994) (Vaux and Korsmeyer, 1999).

Depending on the morphological characteristics, cell death is classified into different categories, including apoptosis, necrosis and autophagic cell death (Kroemer et al., 2009). However, recently it has also been classified as enzymatic and non-enzymatic, immunogenic or non-immunogenic, and programmed or accidental.

Part I. Apoptosis

The most prevalent form of cell death is termed ”apoptosis”. Apoptotic cell death was discovered in the early seventies (Kerr et al., 1972) .Subsequently, Bob Horvitz and colleagues used the nematode *Caenorhabtiditis elegans* to demonstrate that this form of cell death is genetically programmed (Horvitz, 1999). In animals, apoptosis is common during development (Meier et al., 2000). It is necessary for the maintenance of some cell populations and the removal of obsolete tissues (Kiess and Gallaher, 1998). Apoptosis is also necessary for the host defense as this form of cell death gets induced upon pathogen infection (Kepp et al., 2009; Zhang et al., 2012). Lastly cells that incur damage via cell division or by some mutagens are also eliminated through apoptosis (Brodsky et al., 2000).

Part I.A. Morphological Features

During apoptosis, cells undergo drastic morphological changes. The most stereotypic morphological phenotype associated with apoptosis is condensation of chromatin, also known as “pyknosis”. This is followed by karyorrhexis, or fragmentation of the chromatin. In addition to the nucleus, the golgi, endoplasmic reticulum and mitochondria also undergo fragmentation (Kerr et al., 1972; Kuwana et al., 2005; Lane et al., 2005; Williams et al., 1974; Wyllie, 1980). Another distinctive feature of apoptosis is blebbing of the plasma membrane and the packaging of cellular material into apoptotic bodies, which separate from the cells in a process called “budding” (Orlando et al., 2006). Subsequently, these apoptotic bodies are phagocytosed, which enables the cellular contents to be degraded without triggering inflammatory responses (Kurosaka et al., 2003).

Part I.B. Mechanism

The mechanism of apoptosis is quite complex, as it involves a series of events and numerous components, particularly the cysteinyl-aspartate specific proteases (caspases) (Thornberry and Lazebnik, 1998). Careful dissection of the factors and events involved in apoptosis revealed that both an intrinsic and extrinsic pathway are responsible for activation and execution of this process.

IB.a. Intrinsic Pathway

The intrinsic pathway is activated within a cell as a result of stressful conditions, such as hypoxia, infection, and radiation. These cytotoxic insults result in the activation of Bcl-2 homology (BH3) domain containing proteins, which inhibits the anti-apoptotic factors and promote the activation of the pro-apoptotic proteins, Bak and Bax. Subsequently, activated Bax and Bak oligomerize and cause pore formation in mitochondria (Newmeyer et al., 1994). The leakage of Cytochrome C proteins from mitochondria into the cytosol triggers the formation of the “apoptosome” complex, which consists of Cytochrome C, ATP and Apoptotic protease activating factor 1 (Apaf-1) (Chinnaiyan, 1999; Saelens et al., 2004; Youle and Strasser, 2008). The apoptosome then recruits and activates pro-caspase 9. Subsequently downstream caspases, -3, -6, and -7 are cleaved and activated. In addition to this particular complex formation, loss of mitochondrial permeability releases several other proteins, including SMAC/DIABLO, which aid in the apoptosis process by inhibiting the IAP (inhibitor of Apoptosis) proteins, such as XIAP. Under normal conditions, IAPs sequester and block the activation of caspases, -9, -3, and -7, this IAP inhibition enables caspase induction and promotes apoptosis (Duckett et al., 1996; van Loo et al., 2002).

IB.b. Extrinsic Pathway

The extrinsic pathway is activated upon interaction between certain death inducing ligands and receptors, such as TNFR1, Fas and TRAIL-R (Trauth et al., 1989; Yonehara et al., 1989). Upon ligand binding receptor oligomerization occurs, which leads to the formation of receptor specific signaling complexes such as complex II or the Death inducing signaling complex (DISC). This complex also includes the protein Fas Associated Death Domain (FADD) or TNFR-associated death domain (TRADD) which are essential for the recruitment of caspase-8 via its Death Effector Domain (DED) (Hsu et al., 1995; Kischkel et al., 1995; Sprick et al., 2000). The executioner caspases, -3 and -7 are cleaved and activated upon dimerization and subsequent activation of caspase-8. However, the activated caspase-8 can also stimulate the intrinsic pathway via cleavage and activation of the BH3 only protein Bid. Bid subsequently activates Bax and Bak, thereby engaging the intrinsic pathway (Li et al., 1998).

Part II Necrosis

Compared to apoptosis, necrosis has been considered an unregulated, accidental form of cell death. Necrotic cell death occurs following extreme trauma to the cells, such as rapid temperature change, osmotic shock, mechanical stress, hypoxia or infection.

Part II.A. Morphological Features

During necrosis, the cellular contents swell and plasma membrane deformities are observed early. This is followed by the loss of plasma membrane integrity and in the formation of pyknotic nuclei by chromatin condensation. Irregular degradation of chromatin follows and the cytoplasmic contents of the cells are released into the extracellular environment (Laster et al., 1988). This rupture of the plasma membrane causes the leakage of Damage Associated Molecular Pattern (DAMP) molecules, which are potent stimulants of the inflammatory pathways (Scaffidi et al., 2002).

Part II.B Necroptosis

Early discoveries and pathophysiological evidences suggested that necrosis is a disorderly cell death pathway, which can only occur after traumatic injury to the cells. However, recent scientific work proved that necrosis type of cell death could also be programmed and more recently multiple necrosis-regulating pathways are discovered (Degterev et al., 2005). This genetically programmed form of necrotic cell death is termed as “necroptosis”.

Part II.C Necroptosis: Mechanism

Necroptosis is generally induced when apoptosis is inhibited in the cells. Various death receptors such as TNFR1, Fas, TRAIL-R and Toll-like receptors including TLR3 and TLR4 are involved in necroptosis (He et al., 2011; Holler et al., 2000; Jouan-Lanhouet et al., 2012). Necroptosis is activated by proapoptotic ligands such as TNFα, FasL, and TRAIL in the absence of apoptosis or by different pathogen components such as bacterial lipopolysaccharide and viral nucleic acids (Upton et al., 2012). TNFα initiated necroptosis is the most well understood induction mechanism of this process. Stimulation of TNFR1 by TNFα leads to the formation of complex II, which consists of FADD and RIPK1.Caspase-8 recruitment to this complex and activation results in apoptosis. However, when apoptosis is inhibited, RIPK1 and the ser/thr kinase RIPK3 are stabilized and they interact through the common RIP homotypic interaction motif (RHIM) (He et al., 2009). RIPK1 and RIPK3 engagement forms a fibrillar structure, called a necrosome (Vanden Berghe et al., 2010). In the necrosome RIPK1 phosphorylates RIPK3, which recruits the protein mixed lineage kinase domain-like (MLKL) (Li et al., 2012). RIPK3 then phosphorylates MLKL and that leads to the oligomerization and translocation of MLKL to the plasma membrane(Sun et al., 2012). Here, MLKL damages the plasma membrane through a currently unknown mechanism. The current hypothesis is that MLKL upon localization to the plasma membrane induces ion influx either directly by pore formation or indirectly by influencing the ion channels (Dondelinger et al., 2014).

Part III Pyroptosis

Pyroptosis is another form of programed cell death that occurs exclusively in the context of infection. Pyroptosis was first detected in macrophages infected with *Shigella flexneri.* To date, it has only been observed in macrophages and dendritic cells (Zychlinsky et al., 1992). Although it is a caspase dependent cell death, pyroptosis is different from apoptosis in the distinct morphological features observed and the alternate mechanism involved.

Part III.A. Morphological Features

The most distinct feature of pyroptosis is the rupturing of the plasma membrane. During pyroptosis DNA damage occurs but unlike apoptosis pyknosis does not happen. Cytoplasmic swelling of pyroptotic cells causes formation of protrusions, which eventually rupture and enables the cellular contents to leak into the extracellular milieu (Fink and Cookson, 2006).

Part III.B. Mechanism

Pyroptosis is essential against intracellular infection. Different components of the pathogen such as bacterial LPS, toxin or cytosolic DNA induce the formation of a platform, called the Inflammasome. This multicomponent platform is necessary for the process inflammation, which is responsible for the production of the inflammatory cytokines, interleukin-1β (IL-1β) and interleukin-18 (IL-18) (Martinon et al., 2002). During this canonical inflammation process, caspase-1 is activated, which drives the cleavage and subsequent activation of cytokines (Bergsbaken and Cookson, 2007). However it has recently been found that caspase-1 also cleaves Gasdermin-D and this event is necessary for pyroptosis (Shi et al., 2015). Specifically, cleaved Gasdermin-D associates with the plasma membrane and possibly other membrane bound organelles, oligomerizes and induces membrane permealization, which ultimately leads to cell lysis and cell death (Ding et al., 2016; Liu et al., 2016).

Part IV Autophagy

Autophagy is a catabolic process, which encompasses sequestration and degradation of cytoplasmic contents of cells. The term autophagy originated from a Greek word, meaning, “self-eating”. Novikoff showed the first structural proof of an autophagic structure in 1959 in the epithelial cells of the kidneys. Shortly after, in 1962 Ashford and Potter confirmed the existence of vesicles that contain cytoplasmic material in rat hepatic cells (Ashford and Porter, 1962; Novikoff and Essner, 1962). Cell biologist Yoshinori Ohsumi identified many genes in the autophagy pathway by studying the process in *Saccharomyces cerevisiae,* which won him the Nobel Prize in 2016.

There are mainly three different main forms of autophagy, namely (1) chaperone-mediated autophagy (2) microautophagy and (3) macroautophagy. Chaperone-mediated autophagy involves shuttling of a particular set of proteins, containing the KFERQ amino acid sequence to lysosomes through channels made-up mostly of LAMP2A proteins (Terlecky and Dice, 1993). During Microautophagy small portions of cytoplasm are engulfed by lysosomal invaginations, followed by degradation within the lysosomes. This process and its components are still not defined properly (Ahlberg et al., 1982). The process macroautophagy involves encapsulation of cytoplasmic material into a double membrane structure, called autophagosomes. The ensuing fusion of autophagomes and lysosomes facilitates the degradation of the encapsulated cytoplasmic material. Macroautophagy is the most studied and well-documented autophagy pathway.

So, from now on, the term autophagy will denote macroautophagy unless stated otherwise.

Part IV.A. Autophagy Mechanism

Autophagy is induced in cells under a variety of conditions, including nutrient deprivation, hypoxia, and infection. It begins with the formation of vacuolar structures, called autophagosomes. The origin of this membranous structure is still controversial, however the smooth endoplasmic reticulum and in some instances the plasma membrane and mitochondria are hypothesized as the possible sources(Dunn, 1990; Hailey et al., 2010). The assembly of the autophagosome is initiated by the localization of multiple autophagic proteins to a particular site called phagophore assembly site (PAS). Also formed at the PAS is the isolation membrane, which sequesters the cytoplasmic content and generates the autophagosome. The outer membrane of autophagosomes later fuses with the lysosomes and lysosomal enzymes degrade both the inner membrane and the cytoplasmic contents (Mizushima and Komatsu, 2011).

Part IV.B. Autophagy Machinery

Much of what is currently known about the autophagy pathway and its components is from studies in yeast(Harding et al., 1995; Tsukada and Ohsumi, 1993). Most of the autophagy-related (Atg) genes that were identified in the yeast system later found to be conserved in higher species, as well. The autophagy pathway progresses via coordinated formation of different protein complexes, such as Atg1 complex, vacuolar protein sorting 34 (vps34) complex, and the atg5-atg12 complex.

Genetic studies place the formation of Atg1 complex as the most upstream event in the autophagic pathway. This particular complex consists of the serine/threonine kinase Atg1 (ULK1/ULK2 in mammals), Atg13, Atg101, FIP200 (RBCC1 in mammals and Atg17 in *Drosophila*). The kinase activity of Atg1 is necessary for the activation of this pathway and expression of Atg1 alone can induce autophagy in multiple systems(Berry and Baehrecke, 2007; Scott et al., 2007). Various studies suggest a dual role of Atg1 in phagophore assembly; Atg1 both recruits of other autophagy proteins and phosphorylates key autophagy components (Kamada et al., 2000; Scott et al., 2004).

An essential part of the autophagy induction is the establishment of the autophagosomal membrane. An important constituent of the autophagosomal membrane is the phospholipid phosphatidylinositol 3-phosphate (PI3P), which is generated by the action of phostidylinositol 3-kinase (PI3K) complex activity. The PI3K complex is comprised of the kinase Vps34, the serine/ threonine kinase Vps15 (p150 in mammals), Atg6 (Beclin1 in mammals), Atg14 and Vps38 (UVRAG in mammals). The nucleation and further elongation of the isolation membrane is dependent on these proteins. Furthermore different effectors such as Atg18 (WIPI1 in mammals) are also recruited to the autophagosomal membrane through interaction with PI3P and regulate the size of the vesicles (Efe et al., 2007; Kihara et al., 2001; Takahashi et al., 2007).

The ubiquitin like conjugation systems Atg12-Atg5-Atg16 and Atg8 (LC3 in mammals)-Phosphatidylethanolamine (Atg8-PE) play crucial roles in cargo selection and further membrane formation. Atg12 is activated by the E1 enzyme Atg7 and transferred to E2 enzyme Atg10. Finally Atg12 is conjugated to Atg5 via an irreversible covalent bond with an internal lysine residue in Atg5. The protein Atg16 binds this Atg12-Atg5 complex and further oligomerization results in the formation of a tetrameric complex, which is localized to the isolation membrane (Kuma et al., 2002; Shintani et al., 1999). By contrast, the Atg8 protein is cleaved by the cysteine protease Atg4 and this cleavage exposes the c-terminal glycine residue of the Atg8. The Atg7 enzyme activates the Atg8, which facilitates Atg8 transfer to Atg3. Finally, Atg8 is conjugated to the Phosphtidylethanolamine (PE) to form the Atg8-PE complex, which is observed both in isolation and in autophagosomal membranes. This lipid-conjugated form of Atg8 is widely used as the biomarker for autophagy induction (Hanada et al., 2007; Ichimura et al., 2000; Tanida et al., 1999).

Part IV.C. Autophagy regulation

As discussed earlier, autophagy is regulated by different factors and conditions, including nutrients, energy, pathogens and hormones. Probably the best-characterized and most commonly found autophagy regulator is the availability of nutrients. The protein TOR (target of rapamycin) negatively regulates autophagy in the presence of nutrients (Chang and Neufeld, 2009). Recent discoveries showed that in response to amino acids, Ras-related small GTPases (Rag) cause TOR translocation to protein Ras homolog enriched in brain (Rheb) containing compartments. The direct interaction between GTP bound Rheb and TOR activates the TOR protein, which inhibits downstream Atg1 activity(Kim et al., 2008). However during starvation TOR is inhibited itself and autophagy is induced. In addition to the TOR pathway autophagy is also negatively regulated during nutrient rich condition by the Ras/cAMP Protein Kinase A (PKA) signaling pathway. Here, PKA inhibits autophagy by phosphorylating Atg1, which causes Atg1 detachment from the PAS (Budovskaya et al., 2004).

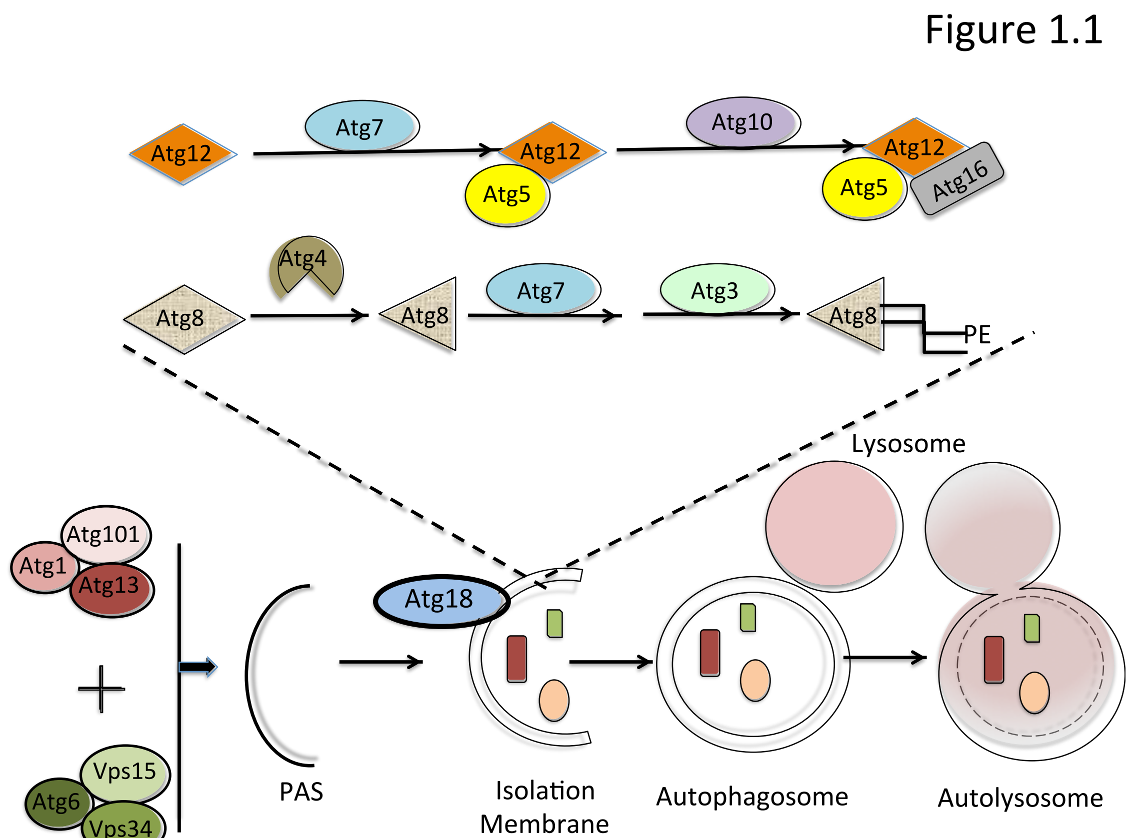


Figure 1.1 The Autophagy Core Pathway

The most upstream event of the autophagy pathway is the formation of Atg1 complex. This is followed by the generation of Vps34 complex. These complexes are formed at the PAS site and that leads to the formation of the isolation membrane. Further molecular events, including Atg12-Atg5-Atg16 conjugation cycle, Atg8 cleavage and lipidation result in the formation of autophagosome, which later fuses with lysosome and lysosomal enzymes to degrade the internal components of the autophagosome.

Under metabolic stress, the drastic change in the ratio of ATP and AMP activates the LKB1-AMPK pathway, which results in the activation of AMPK (5`-AMP activated Protein Kinase). Activated AMPK phosphorylates the Tuberous Sclerosis (TSC) complex. The phosphorylated TSC complex inhibits TOR activity and promotes autophagy (Inoki et al., 2003).

Part V. Autophagy and Cell Death

Autophagy is necessary for maintaining cellular homeostasis. Under nutrient deprived condition, the induction of autophagy recycles cellular material and eliminates damaged or unwanted organelles to protect the cells from early demise.

In addition to its role as a pro-survival mechanism, in some instances autophagy has also been shown to promote cell death. However, it is still a matter of debate whether autophagy induction actually kills cells or the presence of autophagic markers in the dying cells are marks of cells` last-ditch effort to survive. Nonetheless recent discoveries have shown that autophagy induction leads to cell death both in vitro and in vivo under certain conditions. In higher eukaryotes the double knockout of the key apoptotic genes Bax and Bak renders the cells deficient in mitochondrial apoptosis. Under these genetic conditions, further exposure to the DNA damaging agents forces the cells to undergo autophagic cell death. In this case, two key autophagy components ATG5 and Beclin-1 play important role as their knockdown protects cells (Shimizu et al., 2004). Similarly, upon inhibition of Caspase-8, L929 fibroblasts undergo autophagic cell death that is dependent on Beclin-1 and Atg7 (Yu et al., 2004a). Caspse-10 inhibition in multiple myeloma cell lines also induces autophagic cell death (Lamy et al., 2013). These examples pertinently show induction of autophagic cell death when apoptosis is blocked. However, in some cases autophagy can be induced even when active apoptosis tools are present. The overexpression of mutant Beclin-1 (lacks the Bcl-2 binding domain) can induce autophagic cell death, which can be blocked by knockdown of downstream autophagy gene Atg5. In another example, overexpression of the *Ras* oncogene induces *Beclin-1* expression that activates autophagy in ovarian surface epithelial cell line. Interestingly, Ras also induces the expression of Noxa, a BH-3 only protein. The expression of Noxa is necessary for autophagy induction as it blocks Bcl-2 mediated inhibition of Beclin-1 (Elgendy et al., 2011).

In vivo autophagic cell death is observed in several organisms, including *Drosophila melanogaster*, and *Dictyostelium discoideum.* The slime mold *Dictyostelium* has an interesting life cycle. It can exist in both unicellular and multicellular forms depending on the nutrient availability. In nutrient limiting condition, *Dictyostelium* switches to their multicellular form via adhesion and aggregation of unicellular amoebas. This particular multicellular fruiting body of *Dictyostelium* is comprised of a stalk and spores. The stalk cells undergo autophagic cell death during development. However the developmental autophagic cell death observed in *Dictyostelium* is difficult to compare to more evolved organisms, as *Dictyostelium* lacks the apoptosis machinery(Giusti et al., 2009).

In contrast to *Dictyostelium*, the fruit fly, *Drosophila melanogaster* has yielded significant insight into the model of autophagic cell death in vivo*.* In flies, autophagy occurs under different conditions and in different tissues. But, a clearly defined model of autophagic cell death is observed during metamorphosis under the influence of the molting hormone 20hydroxyecdysone (ecdysone). Specifically, two tissues that undergo ecdysone driven autophagic cell death during *Drosophila* metamorphosis are the larval salivary glands and midgut.

Part V.A. Autophagic Cell Death: *Drosophila* Salivary Gland

Drosophila larval salivary glands consist of two tube-shaped luminal organs that are joined by a common duct, which carries the glandular secretions to the larval mouth(Abrams et al., 2003). The salivary glands have been extensively studied as a model for developmental cell fate determination, due to its simple cellular architecture and lack of late mitotic cell division. The salivary glands develop from the ventral ectoderm and further increase in size through multiple rounds of endomitosis, which results in the formation of giant cells containing polytene chromosomes (Smith and Orr-Weaver, 1991). During *Drosophila* metamorphosis, when massive tissue remodeling occurs, salivary glands are also subjected to histolysis and eventually degraded completely (Jiang et al., 1997). The developmental degradation of salivary glands also makes these organs an ideal model to study different aspects of the cell death pathways.

The steroid hormone ecdysone plays many roles during *Drosophila* metamorphosis (Riddiford, 1993). Throughout metamorphosis, ecdysone promotes adult tissue formation and triggers context-specific cell death pathways. Two distinct ecdysone pulses are observed in metamorphosis. The first and relatively higher titer ecdysone pulse (referred as 0 hour ecdysone pulse) marks the transition from larval to prepupal stage, while the second pulse (referred as 12 hour ecdysone pulse) transforms the prepupa to pupa. The late 12-hour ecdysone pulse triggers the histolysis of salivary glands. Salivary gland destruction requires both the caspase-dependent and autophagic cell death pathways. Loss of only one pathway causes a mild defect in salivary gland degradation while blocking both pathways has a more profound phenotype (Berry and Baehrecke, 2007; Martin and Baehrecke, 2004). In *Drosophila* fatbody, autophagy is observed upon nutrient starvation, however autophagy is not induced in salivary glands in such condition. So, nutrient deprivation does not act as a trigger in case of salivary gland degradation. Relatively simple cellular structure, lack of interference from starvation induced autophagy signals, opportunities to study different aspects of multiple developmental cell death pathways, together make salivary gland degradation a very attractive model to study cell death.

Part V.B. Autophagic Cell Death: *Drosophila* Midgut

Distinct from the larval salivary gland, the *Drosophila* larval midgut is degraded as a result of the 0 hour ecdysone pulse. Two different ecdysone regulated transcription factors E93 and Broad complex (BR-C) promote the expression of several pro-apoptotic genes, including *reaper* and *hid.* At this time the effector caspase *Dronc* is also expressed and strong activity from other caspases such as Decay and Drice is also detected. During midgut programmed cell death, TUNEL positive cells are observed, which indicates the presence of DNA degradation and caspase activity. However inhibition of caspase activity does not block midgut degradation, suggesting dispensable roles of caspases in midgut cell death. Interestingly, many autophagy genes are also upregulated in the midgut before midgut destruction. Furthermore, mutation of the autophagy genes *Atg1, Atg2 or Atg18* impedes proper midgut degradation, which indicates that autophagic but not caspase dependent cell death is necessary for successful elimination of the larval midgut (Denton et al., 2009).

Part VI. Autophagy in Cancer

The role of autophagy in cancer is complex and rather controversial. During early stages of cancer development, autophagy suppresses cancer progression. However, as the cancer cells are metabolically stressed and the tumor microenvironment compromises cellular homeostasis, induction of autophagy can improve the survival of cancer cells.

Part VI .A. Autophagy: Promotion of Tumorigenesis

The hyper-proliferation of cancer cells creates a high demand for nutrients and oxygen, which induces metabolic stress and hypoxia in the tumor microenvironment. In this hostile environment, cancer cells are dependent on autophagy for their survival. Relative to non-malignant cell lines, various cancer cell lines survive longer under nutrient limiting conditions. This enables cancer cells to recover and regrow when favorable physiological conditions are established. Autophagy-triggered dormancy protects cancer cells from harsh cellular conditions, which enables recovery and proliferation when favorable conditions return. In fact, this is an underlying cause of cancer relapse. In solid tumors, poor vascularization results in defective oxygen delivery and hypoxic conditions. In the hypoxic regions of solid tumors, localized autophagy is observed. Inhibition of autophagy at these locations causes cell death and helps limit cancer progression (Degenhardt et al., 2006). In fact, deletion of *FIP200* (mammalian homolog of yeast *Atg17*) inhibits polyoma middle T antigen mediated breast cancer in mice (Wei et al., 2011).

Further deletion of Atg5 or Atg7 impairs RAS mediated tumorigenesis while knockdown of Atg5 in human pancreatic adenocarcinoma blocks tumor growth in a mouse xenograft model (Yang et al., 2011). Moreover, high levels of basal autophagy are observed in human melanomas with BRAF mutation, which suggests a positive role of autophagy in tumorigenesis (Corazzari et al., 2015).

Part VI .B. Autophagy: Suppression of Tumorigenesis

In contrast to the above observations, several studies suggest that autophagy can act as a tumor suppressor. Autophagy is necessary for maintaining cellular homeostasis and genomic integrity, which helps prevent malignant transformation of cells. The role of Beclin-1 (BECN1) in tumorigenesis presents the most prominent example of autophagy in tumor suppression. Monoallelic deletion of Beclin-1 is associated with human prostate, ovarian and breast cancers (Liang et al., 1999). In mice, heterozygous deletion of Beclin-1 leads to the formation of liver and lung tumors (Qu et al., 2003). Further, the autophagy regulator Bif-1 is also down regulated in different human cancers, including liver, gall bladder, prostate and gastric cancers. Consistently, mouse deficient of Bif-1 is more prone to tumor development(Takahashi et al., 2007). Mutations in other autophagy genes such as *Atg2B, Atg9, Atg5, Atg12* are observed in gastric and colorectal cancers (Kang et al., 2009). In mice, deficiencies of Atg5 and Atg7 result in the formation of benign liver tumors.

Part VII. Autophagy In Immunity

Autophagy has long been regarded as a survival mechanism for cells in nutrient limiting conditions. Autophagy is also important in the maintenance of cellular homeostasis and the clearance of damaged organelles and proteins. In addition, recent studies suggest that autophagy is essential for host immunity. In fact, autophagy has diverse roles in immunity, including regulation of inflammation and clearance of intracellular pathogens.

Part VII. A. Inflammation Regulation

Inflammation in response to foreign organisms is generally a protective mechanism of the hosts. Pathogen infection triggers the inflammatory cascade that recruits a diverse array of immune cells, which are essential for pathogen clearance and the regeneration of host tissues. Current advances in this field also suggest the necessity of autophagy in modulating inflammatory responses. A key feature of inflammation is the production of cytokines, such as the pro-inflammatory cytokine interleukin-1β (IL-1β). Production of Cytokines, including IL-1β, is dependent on the assembly and activation of the inflammasomes. The inflammasome is a multi-protein complex that is formed in response to either foreign Pathogen-associated molecular pattern (PAMP) or host Damage-associated molecular pattern (DAMP) molecules (Rathinam et al., 2012). These particles are recognized by cytosolic innate immune receptors called NOD-like receptors (NLRs). Activation of the NLRs initiates the formation of inflammasomes, subsequent activation of Caspase-1, and the production of inflammatory cytokines, including IL-1β and interleukin-18 (IL-18) (Brough and Rothwell, 2007; Thornberry et al., 1992).

A link between inflammasome activation and autophagy has been observed. For instance, the macrophages from *Atg16l1* knockout mice produce higher levels of processed IL-1β, compared to wild type controls, when exposed to bacterial LPS (Saitoh et al., 2008). This increased IL-1β production results from excessive activation of Caspase-1. Autophagy has also been proposed to eliminate cellular debris and damaged organelles, which may serve as endogenous inflammasome activators. These data suggest that inflammasome activation is dampened by autophagy (Zhou et al., 2011). Further, inhibition of autophagy leads to the accumulation of damaged mitochondria and the release of mitochondrial DNA and reactive oxygen species (ROS), which activates AIM2 and NLRP3-dependent inflammasomes(Nakahira et al., 2011). Upon Influenza virus infection the receptor interacting protein kinase 2 (RIPK2 or RIP2) activates Ulk1, a critical component of the autophagy pathway. Active Ulk1 stimulates mitophagy, which reduces inflammasome activation (Lupfer et al., 2013). Autophagy can also hinder inflammasome activation by selectively degrading assembled inflammasome components, as evidenced by the ubiquitin-mediated degradation of the Apoptosis-associated speck-like proteins (ASC). This degradation is mediated by p62, another component of the autophagy pathway.(Shi et al., 2012).

Part VII. B. Intracellular Pathogens

In addition to its role as a modulator of inflammasome activity in response to both foreign and self-molecules, autophagy can directly function in the elimination of the intracellular pathogens. Several mechanisms exist to achieve this autophagy-mediated removal. One such mechanism is “xenophagy.” Here, pathogens are sequestered inside autophagosomal compartments and subsequent autophagosomal and lysosomal fusion kills the invading microorganisms. Sometimes pathogens are engulfed inside single membrane phagosomal compartments, which are coated with LC3 proteins in a PI3K and LC3 conjugation dependent but ULK1 independent manner. This process is known as LC3 associated phagocytosis (LAP) and is essential for eliminating intracellular pathogens, such as *Burkholderia pseudomallei (Sanjuan et al., 2007).* By contrast, a process called “virophagy” degrades viral components. This process is different from xenophagy, as newly synthesized viral components are targeted for autophagy rather than complete virus particles, for example Sindbis viral capsid is degraded by autophagy in a p62 mediated but ubiquitin independent manner (Orvedahl et al., 2010).

Autophagy can be induced at different stages of host-pathogen interaction. At the early stages of infection; pattern recognition receptors (PRRs), such as TLRs and NLRs recognize PAMP molecules and initiate autophagy (Shi and Kehrl, 2010a). The autophagic pathway can be activated by macrophage-based phagocytosis of pathogens or triggered by pathogen-inflicted damages on the host cell.

The NLRs NOD1 and NOD2 interact with the bacterial component meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP) respectively, which results in the recruitment of the autophagy component ATG16L1 at the site of bacterial entry (Travassos et al., 2010b). Further, mitochondrial NLRX1 protein and its partner Tu Elongation Factor (TUFM) promote autophagy by associating with the Atg5-Atg12 complex (Lei et al., 2012).

During infection, the bacteria *Salmonella typhimurium* reside inside specific *Salmonella* containing vacuoles, which facilitate bacterial survival and multiplication. However, a small fraction of bacteria is found in the cytoplasm, likely as a result of bacteria released from damaged vacuoles. Cytoplasmic bacteria are rapidly ubiquitinated and recognized by the autophagic adaptor proteins p62, optineurin (OPTN) and NDP52. This NDP52 and OPTN mediated autophagy of *Salmonella* also requires Tank binding kinase (TBK1) *(Thurston et al., 2009)*.

Another example of antimicrobial autophagy is observed upon *Toxoplasma gondii* infection. It is an intracellular protozoan parasite, which causes the disease toxoplasmosis. An increased level of Beclin-1 promotes autophagy in *Toxoplasma gondii* infected macrophages and this autophagy induction is necessary to restrict the proliferation of this pathogen (Andrade et al., 2006).

Autophagy is also effective against different viruses. For instance, TLR7 mediated viral ligand recognition is autophagy-dependent. Here, autophagy is necessary for the induction of TLR7-directed cytokines following Sindbis or Vesicular stomatitis virus infection in plasmacytoid dendritic cells (Lee et al., 2007). Similarly, measles virus infection of epithelial cells induces autophagy, which relies on cell surface protein CD46 and scaffold protein GOPC (Joubert et al., 2009). Viral infection is also responsible for stress induced translational regulation, as evidenced by the activation of RNA dependent protein kinase (PKR) and eIF2α phosphorylation, which halts translation. Both of these events are necessary for autophagy induction following herpes simplex virus (HSV) infection (Talloczy et al., 2002). In Consistently, autophagy is blocked in PKR mutant neurons upon Sindbis virus infection. Also, Atg5 mutant mice die faster following Sindbis infection. However, it is to be noted also that some viruses such as, Dengue and Hepatitis B, promote autophagy to limit host immune responses and stimulate further viral proliferation.

Akin to mammals, autophagy serves as a defense mechanism in the fruit fly, *Drosophila melanogaster* as well. Excellent genetic tools, ease of use, and the conserved nature of many genes with the mammals, make *Drosophila* an excellent model to study antimicrobial autophagy. Knockdown of several autophagy genes in the *Drosophila* S2 cell line results in an increased vesicular stomatitis viral load. Similarly, when autophagy genes are silenced *in vivo,* heightened mortality and viral replication are observed upon infection with the same virus. The viral glycoprotein VSV-G acts like a PAMP and is sufficient to induce autophagy. The *Drosophila* Toll receptor Toll-7 recognizes VSV-G and triggers autophagy (Shelly et al., 2009).

Autophagy is also necessary against *Listeria monocytogenes* infection in flies. *Listeria* is a facultative, Gram-positive bacterium, which multiplies inside the cell. This bacterium causes serious food borne illness called listeriosis. Autophagy is important in restricting *Listeria* infection. In fact, knockdown of autophagy genes both in vitro and in vivo in flies causes increased death (Yano et al., 2008). The Peptidoglycan binding receptors (PGRPs), PGRP-LC and PGRP-LE are essential for fly defense against *Listeria* and autophagy induction in response to infection is particularly dependent on PGRP-LE. Both PGRP-LC and PGRP-LE were previously characterized components of the fly humoral immune system and they are responsible for the production of antimicrobial peptides (AMPs).

Part VIII. Drosophila Immune System

The fruit fly, *Drosophila melanogaster* thrives on rotten fruit and vegetables. So their natural habitat certainly consists of numerous bacteria and fungi. As the flies lack adaptive immunity, they possess a strong innate immune system to survive such a pathogen-infested environment. *Drosophila* innate immunity is divided into two categories, humoral and cellular immune responses.

Part VIII. A. Cellular Immune System

Drosophila cellular immunity is dependent on mainly three processes; phagocytosis, melanization and encapsulation. Three distinct classes of blood cells (also called hemocytes), plasmatocytes, crystal cells and lamellocytes, respectively, accomplish these processes. The plasmatocytes are phagocytic cells, which recognize a range of particles, from foreign pathogens to apoptotic bodies. Crystal cells contain phenolperoxidase crystals and cause melanization upon infection. The third group of cells, termed lamellocytes, is only observed in the larvae and is responsible for the engulfment or encapsulation of large objects such as wasp eggs (Lanot et al., 2001; Scherfer et al., 2004; Tepass et al., 1994).

Part VIII .B. Humoral Immune Response

The humoral response utilizes soluble effectors, including turnadots and antimicrobial peptides to mount a response against invading microorganisms. Different signaling pathways are involved in the production of such effector molecules. Here I will discuss two of these pathways, the Toll and Imd.

Part VIII .B. a. Toll Pathway

The Toll pathway is critical for fly development, particularly in the dorso-ventral axis pattern formation(Nusslein-Volhard et al., 1980). However, in addition to its role in development, the Toll pathway plays a significant role in fly immunity. It is mostly activated by Gram-positive bacteria and fungi (Lemaitre et al., 1996). Unlike the mammalian membrane bound Toll Like Receptors (TLRs), Toll receptors in flies do not act like PRRs themselves, rather they are activated upon binding to the cleaved form of the cytokine Spätzle (Weber et al., 2003). Different upstream components such as PGRP-SA, GNBP1, and GNBP3 recognize bacterial and fungal structures and subsequent activation of a downstream signaling cascade results in the cleavage of Spätzle (Pili-Floury et al., 2004). The interaction between the Toll receptor and Spätzle initiates a signaling cascade that involves other pathway components, including Pelle, Tube, and MyD88, and culminates in the activation of NF-κB factors Dif and Dorsal (Gillespie and Wasserman, 1994). Activation of NF-κB drives the production of AMPs (Gross et al., 1996; Ip et al., 1993; Sun et al., 2002a; Tauszig et al., 2000).

Part VIII .B. b. Imd pathway

The Drosophila Imd pathway is activated upon infection by mostly Gram-negative bacteria. The diaminopimelic acid (DAP) type of cell wall peptidoglycan of Gram-negative bacteria is responsible for the activation of this pathway(Kaneko et al., 2004). Unlike Toll receptors, the PGRP receptors in the Imd pathway recognize the bacterial cell wall components directly. The membrane bound receptor PGRP-LC interacts with polymeric PGN, while the cytosolic PGRP-LE binds monomeric PGN, such as Tracheal cytotoxin (TCT) (Gottar et al., 2002; Takehana et al., 2004). Next, the IMD protein is recruited to the PGRPs. IMD then binds *Drosophila* homolog of Fas associated death domain (FADD) through homotypic interaction of the death domains. FADD then recruits the caspase Death-related ced-3/Nedd2-like protein (Dredd), which cleaves both Imd and the NFκB factor Relish (Hu and Yang, 2000). Cleaved Imd is then ubiquitinated, which leads to the recruitment of the TGF-β activated kinase 1 (Tak1) and Tak1 associated binding 2 protein (Tab2) (Georgel et al., 2001; Leulier et al., 2000). Tak1 then activates the downstream IKK complex, which consists of kinase immune response deficient 5 (id5) and Kenny. The activated IKK complex phosphorylates Relish. Upon phosphorylation, the N-terminal domain of the Relish translocates to the nucleus and induces transcription of several genes, including the AMP genes (Silverman et al., 2003; Silverman et al., 2000).

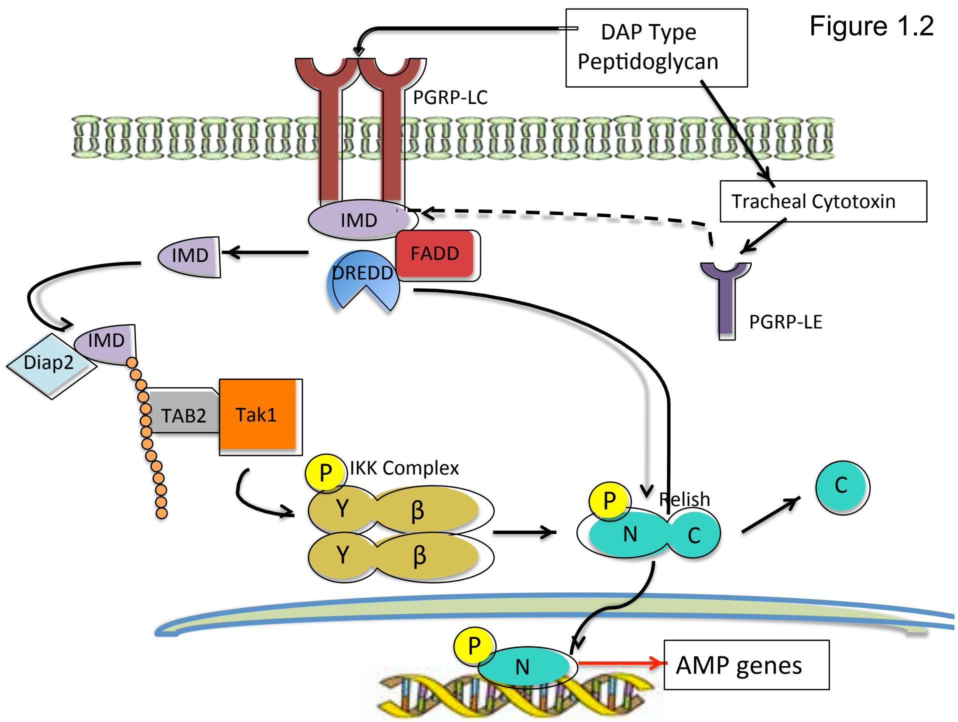


Figure 1.2 *Drosophila* IMD signaling Pathway

This pathway is responsible for the production of AMPs and is necessary for the protection against mostly Gram-negative bacterial infection. It is activated when either the membrane bound PGRP-LC or cytoplasmic PGRP-LE interacts with the polymeric / monomeric peptidoglycan of Gram-negative bacteria. This interaction results in the activation of the caspase Dredd and cleavage of the Imd protein, which is then ubiquitinated. Further downstream signaling events causes the cleavage of NF-κB protein Relish, which is translocated to the nucleus to activate the expression of several AMP genes.

Part IX. NFκB Factors

Nuclear factor kappa B (NFκB) is a highly conserved family of transcription factors that play crucial roles in different biological phenomena, including innate and adaptive immunity. This transcription factor was discovered by Ranjan Sen in David Baltimore`s lab in 1986 and initially thought to be only important for B-cell development (Sen and Baltimore, 1986). However it is now regarded as one of the most versatile transcription factors that are involved in multiple cell types and control a variety of gene expression. Even almost 30 years later and with thousands of publications worth of findings, it is still a matter of great interest to discover that how this particular factor regulates different sets of genes under variety of stimulus.

Part IX .A. NFκB in Mammalian System

There are five members in the mammalian NFκB family, p65 (RelA), p100/p52, p105/p50, c-Rel and RelB (Bonizzi and Karin, 2004). In unstimulated cells, they remain inactive. RelA, RelB and c-Rel are bound to inhibitory proteins IκB. The precursor proteins p100 and p105 possess inhibitory domains similar as IκB. Each family member possesses a Rel Homology Domain (RHD), which contains a DNA binding motif, a dimerization motif, and a region responsible for interacting with IκB proteins. Interaction with these inhibitory proteins prevents the dimerization and nuclear translocation of the NFκB members (Alkalay et al., 1995). Under distinct stimuli, the IKK kinase complex is activated, which leads to

the phosphorylation of IκB at specific serine residues (DiDonato et al., 1996). Then, the Skp1–Cullin–Roc1/Rbx1/Hrt-1–F-box (SCF or SCRF) type ubiquitin ligases mediate ubiquitination of these proteins and as a result these proteins are degraded by the proteasome and incase of precursor proteins, p105 and p100, undergo limited proteolysis (Suzuki et al., 1999; Verma et al., 1995).
Depending on the stimulus and the need of the cell, the NFκB proteins homo or heterodimerize and these complexes act either as activators or repressors. As the p50 and p52 do not possess a transactivation domain (TAD) like p65, RelB and c-Rel, they can only activate gene expression if they heterodimerize with any of the other three(Gilmore, 2006).

Part IX. B. NFκB in *Drosophila* System

The *Drosophila* genome encodes three NFκB factors, Dorsal, Dorsal-related Immune factor (DIF) and Relish. Among these three proteins, Dorsal and DIF share similarity with mammalian p65 while Relish functions as a NFκB precursor protein. However, it is to be noted that unlike mammalian precursor proteins, Relish is not processed via the proteasomes; rather the Caspase Dredd cleaves the inhibitory domain of Relish (Stoven et al., 2000). DIF and Dorsal are part of the Toll signaling pathway that is essential during both development and infection. Dorsal was the first identified NFκB factor in flies and it is important for dorso-ventral pattern formation during development (Anderson and Nusslein-Volhard, 1984). Another factor DIF is involved in the humoral immune response of the flies. In larval stage, both Dorsal and DIF function as immune pathway components while in adults the humoral immunity is mainly DIF dependent (Ip et al., 1993; Rutschmann et al., 2000). In unstimulated condition both Dorsal and DIF remain in the cytoplasm bound by the inhibitory IκB protein, Cactus (Manfruelli et al., 1999; Wu and Anderson, 1998). Phosphorylation of Cactus and subsequent ubiquitination by the gene *Slimb,* causes proteosomal degradation of Cactus, similar to the mammalian system (Belvin et al., 1995; Spencer et al., 1999). In the case of Relish, the caspase Dredd mediated cleavage uncouples the protein from its C-terminal domain, which contains inhibitory ankyrin repeats. The active N-terminus then translocates to the nucleus and activates downstream target genes, including AMPs (Belvin et al., 1995; Stoven et al., 2000).

Part IX. C. NFκB in Immunity

The prominence of NFκB in both innate and adaptive immune responses is well established. Interestingly NFκB is involved in multiple stages of the immune system. These proteins are crucial for the development of immune cells, including dendritic cells, neutrophils, B and T lymphocytes. In addition to this, development of both primary and secondary lymphoid organs is also dependent on NFκB factors. RelA and TNFR double knockout mice show severe defects in the formation of Peyer`s patches, the spleen and the lymph nodes (Alcamo et al., 2002). Different PRRs, such as TLRs utilize the NFκB mediated signaling pathway to produce downstream effectors, such as cytokines. For example, TLR4 recognize the bacterial lipopolysaccharide (LPS) and initiate NFκB dependent signaling pathways. The TLR4- NFκB mediated signaling pathway is dependent on either the adaptor protein MyD88 or TRIF. Similarly, cytoplasmic NOD receptors, NOD1 and NOD2 recognize meso-DAP (Diaminopimelic acid) type of peptidoglycan and muramyl dipeptide, respectively, and subsequently induce NFκB signaling pathway (Fitzgerald et al., 2001; Inohara et al., 1999; Yamamoto et al., 2003).

Thesis Objective

Cell death is induced under different biological conditions and is necessary for variety of purposes, including tissue development, survival and protection. The role of cell death in these individual biological pathways has been studied extensively. However, a growing body of evidence suggests that these phenomena are not exclusive and in many cases crosstalk between these pathways is essential for the benefit of the organism. Therefore, the involvement of previously characterized immune or developmental components in the context of cell death induction has become quite an exciting subject. In the following chapters, I intend to demonstrate a novel mechanism to decipher the interplay between the immune effectors, autophagic cell death and developmental cues, using *Drosophila* as a model system. I sincerely hope that my work would aid to understand these subjects better, and be used in the future to interpret the complex interaction of these biological processes.

CHAPTER 2.

The NF-κB Factor *Relish* Regulates *Atg1* Expression and Controls Autophagy

(Part of the following work is under review at *Cell Reports)*

Abstract

Macroautophagy and cell death both contribute to innate immunity, but little is known about how these processes integrate. *Drosophila* larval salivary glands require autophagy for developmentally programmed cell death, and innate immune signaling factors increase in these dying cells. Here we show that the NF-κB factor *Relish*, a component of the Imd immune pathway, is required for salivary gland degradation. Surprisingly, of the classic Imd pathway components, only *Relish* and the *PGRP* receptors were involved in salivary gland degradation. Significantly, *Relish* controls salivary gland degradation by regulating autophagy, but not caspases. In addition, expression of either *Relish* or *PGRP-LC* causes premature autophagy induction and subsequent gland degradation. *Relish* controls autophagy by regulating the expression of *Atg1*, a core component and activator of the autophagy pathway. Together these findings demonstrate that a novel NF-κB pathway regulates autophagy during developmentally programmed cell death.

Introduction

The NF-κB family of transcription factors is involved in diverse range of physiological processes, including cell division, cell death and most prominently innate and adaptive immunity (Bonizzi and Karin, 2004; Guttridge et al., 1999; Hayden and Ghosh, 2011). The mammalian NF-κB family consists of five members, RelA (p65), RelB, c-Rel, p50/p105 and p52/p100. These factors are critical for the production of cytokines, regulation of cell death and control of cell cycle progression in activated leukocytes and lymphocytes. Mutation in these factors leads to lethality, increased susceptibility to infection, and altered tissue development while constitutively active NF-κB leads to inflammatory diseases such as arthritis, inflammatory bowel disease and cancer (Li and Verma, 2002). The study of NF-κB factors and their proper regulation remains of great interest for many fields.

One powerful model to study the role of NF-κB factors in diverse areas of biology is the fruit fly *Drosophila melanogaster,* which encodes three NF-κB factors - *Dorsal*, *Dif* and *Relish*. *Dorsal* and *Dif* are similar to mammalian RelA and are activated following the cleavage of the cytokine Spätzle and its subsequent binding to and activation of the receptor Toll (Buchon et al., 2014). By contrast, *Relish* is an important component of the immune deficiency (Imd) pathway, which responds to the diaminopimelic acid (DAP) type peptidoglycan, from the cell wall of Gram-negative bacteria. Upon direct binding of DAP-type peptidoglycan to the Peptidoglycan Recognition Proteins-LC or -LE (PGRP-LC or -LE), a signaling cascade is triggered that results in the cleavage, activation and nuclear translocation of *Relish,* and transcription of antimicrobial peptide (AMP) genes (Choe et al., 2002; Hedengren et al., 1999). AMPs such as Diptericin, Cecropin, and Defensin are small cationic peptides with direct antimicrobial activity (Imler and Bulet, 2005).

Relish was characterized as an important component of the *Drosophila* immune system and is primarily responsible for the immune-induced expression of AMP genes. However, recent findings implicate *Relish* in several cell death paradigms. For example, *Relish* is required for the death of photoreceptor cells in a *Drosophila* model of light-dependent retinal degeneration (Chinchore et al., 2012). In another report, *Relish* was found to play a crucial role in elimination of "unfit" cells in a fly model of cell competition (Meyer et al., 2014). These findings suggest the involvement of *Relish* in caspase-dependent cell death pathways. Other reports have argued that Relish, through the production of antimicrobial peptides, can drive other types of cell death and neurodegeneration in the *Drosophila* CNS (Cao et al., 2013; Petersen et al., 2013). Moreover dying *Drosophila* larval salivary glands also show a marked increase in the expression of several NF-κB dependent antimicrobial peptide genes (Lee et al., 2003). The salivary gland is an excellent genetic model to study developmentally programmed cell death as steroid-induced salivary gland degradation requires both apoptotic caspases and autophagy (Berry and Baehrecke, 2007)

Autophagy is a catabolic process that sequesters cytoplasmic components inside a double membrane “autophagosome” structure followed by lysosomal fusion and content degradation. Although different types of autophagy have been characterized (Mizushima and Komatsu, 2011), here we will use the word “autophagy” to denote macro-autophagy. Autophagy serves different roles depending on cellular and environmental context (He and Klionsky, 2009). In times of starvation, autophagy promotes cell survival by recycling cellular contents. Dysregulation of autophagy has been implicated in different age-related disorders, including neurodegeneration (Qin et al., 2003; Yu et al., 2004b). Also, loss of autophagy contributes to genomic instability, tissue damage and in turn cancer (Karantza-Wadsworth et al., 2007; Mathew et al., 2007; White, 2015). Moreover, autophagy is involved in several immune pathways including inflammatory signaling, immune mediator secretion, antigen presentation and the elimination of cytosolic pathogens (Pengo et al., 2013; Saitoh et al., 2008; Schmid et al., 2007; Yano et al., 2008).

In *Drosophila*, autophagy and immune responses have been linked by several findings. The intracellular pathogen *L. monocytogenes* is controlled through the activation of autophagy following immune recognition by the cytosolic peptidoglycan receptor PGRP-LE (Yano et al., 2008). More recently, we found that complement-like factor Mcr also induces autophagy, via signaling through the scavenger receptor Draper, specifically in the salivary glands (Lin et al., 2017). Interestingly, we also observed that several AMP genes and other immune factors, which are controlled by NF-κB factors in the context of immunity, are upregulated in dying salivary glands (Lee et al., 2003). Together these findings suggested possible involvement of *Relish* and the Imd pathway in the control of autophagic cell death during salivary gland degradation.

Here we show that the *Drosophila* NF-κB family member *Relish* plays an essential role in salivary gland degradation. Surprisingly, apart from *Relish* and the two PGRP receptors involved in the Imd pathway, none of the other six components of the canonical Imd signaling pathway (*FADD, Dredd, IMD, Diap2, Tak1, IKKβ, IKKγ*) play any role in salivary gland cell death and degradation. The contribution of *Relish* to salivary gland degradation is caspase-independent, unlike that observed in either the *Relish*-dependent cell competition or retinal degeneration models. On the other hand, *Relish* was necessary and sufficient for activation of autophagy in the salivary gland. Our genetic and molecular data further indicate that Relish regulates autophagy by controlling the expression of *Atg1*, a key activator of autophagy. This study reveals a novel role for known immune pathway components, the NF-κB factor *Relish* and PGRP receptors, in the regulation of autophagy during programmed cell death.

Results

Relish is required for salivary gland cell death

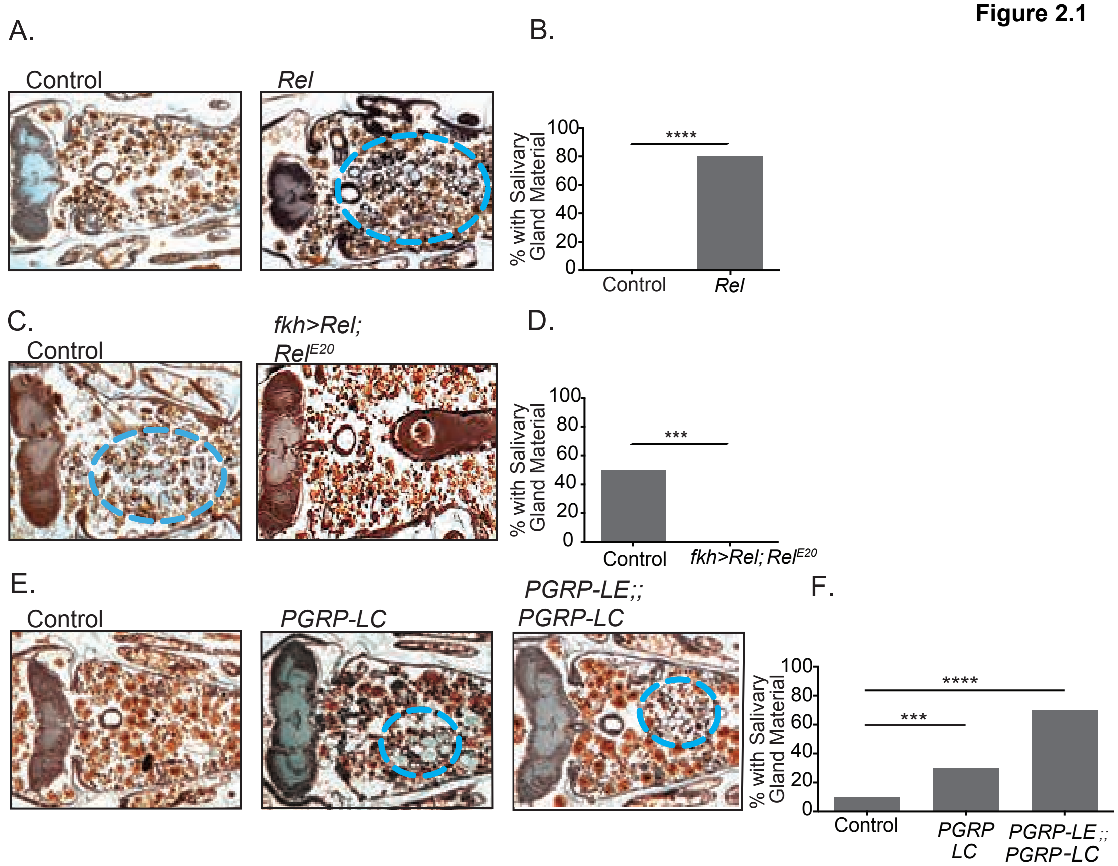
Genome wide transcriptome analyses of dying *Drosophila* salivary gland cells identified many genes that are either up regulated or down regulated during cell death (Lee et al., 2003). In addition to genes associated with apoptosis and autophagy, many NF-κB targets, including AMP genes, were upregulated in dying salivary glands. This prompted us to analyze whether loss of immunity genes would cause any change in salivary gland cell death.

We found that loss of the NF-κB factor *Relish* impaired salivary gland degradation. Salivary gland cell death is activated by a rise in steroid hormone 12 hours after puparium formation, and by 16 hours after puparium formation this tissue is largely degraded. We used Rel^{E23} as control animals, because Rel^{E23} is an exact excision of the same P-element used to create the Rel^{E20} *allele* (via imprecise excision) and thus serves as the best control strain (Hendengren et al. 1999). Like wild type animals, control animals possessed no remnants of salivary glands 24 hours after puparium formation (APF) (Fig 2.1 A, B), 8 hours after this tissue is destroyed. By contrast, a null allele of *Relish* (Rel^{E20}) exhibited persistent salivary gland cell fragments at 24h after puparium formation in ~80% of the cases examined (Fig 2.1A,B & Fig 2.2A, middle, displaying the salivary gland cell fragments without other tissue). In addition, ectopic expression of *Relish* in the salivary glands, using the GAL4 UAS system, rescued the salivary gland degradation defect observed in *Relish* mutant animals (Fig 2.1C,D).

Together these results indicate that *Relish* is required for complete salivary gland degradation and clearance.

Relish is an essential component of *Drosophila* Imd pathway. Therefore, we next sought to determine if other components of the Imd pathway are also involved in salivary gland degradation. Surprisingly, only *Relish* or *PGRP-LC* mutants, or *PGRP-LC, -LE* double mutants displayed a significant defect in salivary gland degradation; none of the other Imd pathway components effected salivary gland destruction and clearance (Fig 2.1E,F, and Fig 2.2B)

Figure 2.1


A.
Control
Rel
B.
% with Salivary Gland Material
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80
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0
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Control
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C.
Control
fkh>Rel; RelE20
D.
% with Salivary Gland Material
***
Control
fkh>Rel; RelE20
E.
Control
PGRP-LC
PGRP-LE;; PGRP-LC
F.
% with Salivary Gland Material
****
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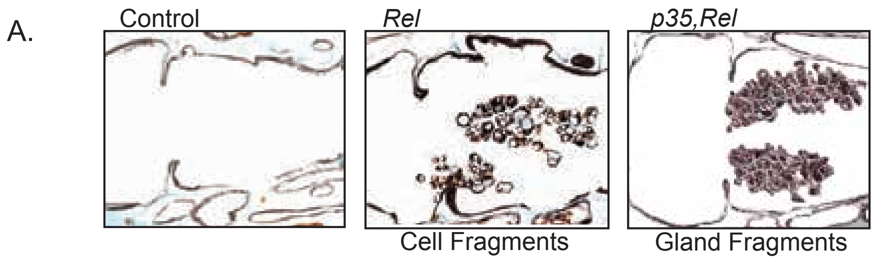
Control
PGRP LC
PGRP-LE;; PGRP-LC

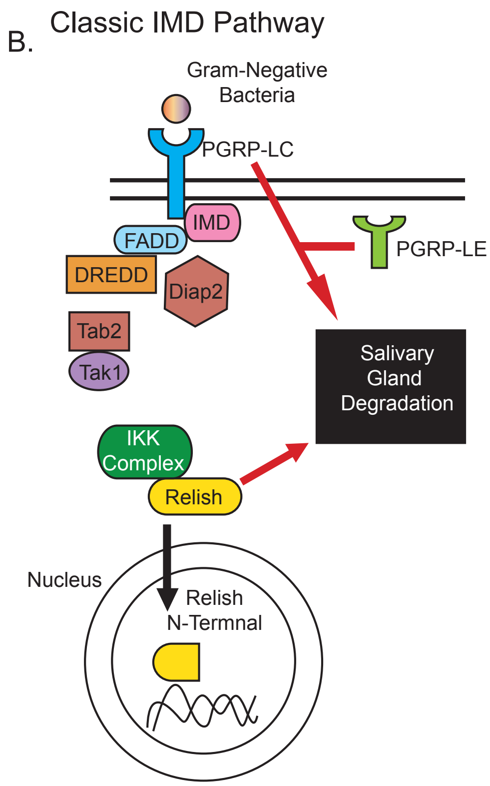
Figure 2.1. The *Drosophila* NF-κB factor *Relish* is essential for salivary gland degradation

(A) Representative histological sections of control (*Rel* ^{E23}) and *Relish* mutant (*Rel*^{E20}) flies 24 h APF. Salivary gland fragments observed in the *Relish* mutants are highlighted within dotted blue circle. (B) Quantitation of histology from 20 independent samples for each strain as in (A), statistical analysis by chi-square test. (C) Representative histological sections of samples of control *Relish* mutants (*fkh-Gal4*/+;; *Rel*^{E20}, left), and *Relish* mutants with salivary gland specific expression of transgenic *Relish*, (*fkh-Gal4*/+; *UAS-Relish/*+; *Rel*^{E20}*,* right) analyzed 24 h APF. Salivary gland fragments are highlighted within dotted blue circle. (D) Quantitation of histology from 20 independent samples for each strain as in (C), statistical analysis by chi-square test. (E) Representative histological sections of control (*PGRP-LE*^{112}/+*;; PGRP-LC*$^{\Delta E}$ /+, left) and *PGRP-LC* mutants (*PGRP-LC*$^{\Delta E}$, middle) and *PGRP-LC* and *PGRP-LE* double mutants (*PGRP-LE*^{112}*;; PGRP-LC*$^{\Delta E}$, right) flies 24 h APF. Salivary gland fragments observed in *PGRP* mutants are highlighted within dotted blue circle. (F) Quantitation of histology from 20 independent samples for each strain as in (E), statistical analysis by chi-square test. **** P<0.0001, *** P<0.001.

Major transcriptional targets of Relish during the immune response include the AMP genes. Previous transcriptomic analyses of the dying salivary glands showed upregulation of several AMP genes, including *Cecropin-C* and *Attacin-A*. The expression profile of these two AMP genes in the salivary glands was determined both at 0 hour (puparium formation) and 14 hours after puparium formation (when salivary gland degradation occurs) by qRT-PCR. Wild type and Imd pathway mutants were compared (Fig 2.2D,E). While *PGRP* double mutants exhibited dramatic effects on salivary gland degradation, they showed robust expression of *Attacin-A* but reduced *Cecropin-C.* By contrast, *Relish* mutants, which also effected salivary gland degradation, showed the opposite with low *Attacin-A* and elevated *Cecropin-C* expression. Moreover, mutants that do not affect salivary gland degradation, such as *Dredd* and *TAK1*, also showed reduced *Attacin-A,* but not *Cecropin-C.* All together, this pattern of AMP gene expression did not correlate with the salivary gland phenotypes (or lack thereof) exhibited by these mutants, and suggests AMPs are not involved in this process.

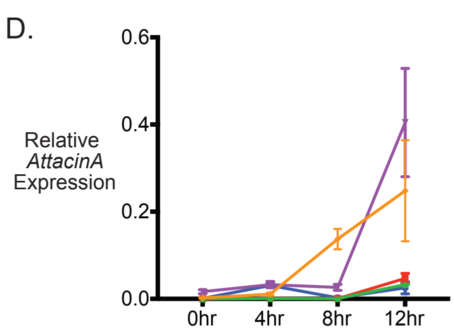
Figure 2.2





C.

IMD PATHWAY COMPONENTS	SALIVARY GLAND DEGRADATION PHENOTYPE
PGRP-LC	YES
PGRP-LE	YES (Partial)
IMD	NO
FADD	NO
DREDD	NO
DIAP2	NO
TAB2	NO
TAK1	NO
IKK COMPLEX	NO
RELISH	YES



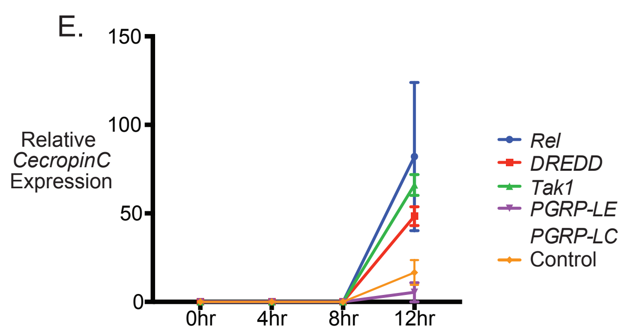


Figure 2.2 Role of Imd pathway Components in Salivary Gland Degradation and AMP expression profile in different Imd Pathway mutants

(A) Representative histological sections of control (*Rel*^{E23}, left) Relish mutant (RelE20, middle), and Relish mutants expressing p35 (*p35, Rel*^{E20}, right) 24 h APF. All tissues except the salivary gland cellular fragments and gland fragments were removed in Photoshop. (B) Diagram of core components of the Imd pathway and (C) a table indicating their role in salivary gland degradation. (D, E) Salivary gland expression of the antimicrobial peptides Attacin A and Cecropin C in wild type (Canton-S) and several Imd pathway mutants at different time points, as indicated, APF.

Previous reports demonstrated that ectopic expression of AMPs could drive neurodegeneration (Cao et al., 2013). Therefore, we tested whether ectopic expression of AMPs is sufficient to induce premature salivary gland degradation. However, ectopic expression of several AMPs in the salivary gland did not result in any discernible effect, further indicating that AMPs do not function in salivary gland degradation (Fig.2.3A).

We next considered the possibility that the endogenous microflora could provide a stimulus through the PGRPs, which are activated by the bacterial cell wall (Kaneko et al., 2004; Leulier et al., 2003), to activate *Relish* and contribute to salivary gland degradation. However, axenic flies, which were negative for bacterial 16S sequences and devoid of any colony forming microbes (Fig 2.3B,C), showed normal salivary gland degradation (Fig 2.3D), excluding a role for the microflora in salivary gland degradation. Combined these data suggest that PGRP receptors and Relish function in a novel pathway to regulate cell death.

Figure 2.3

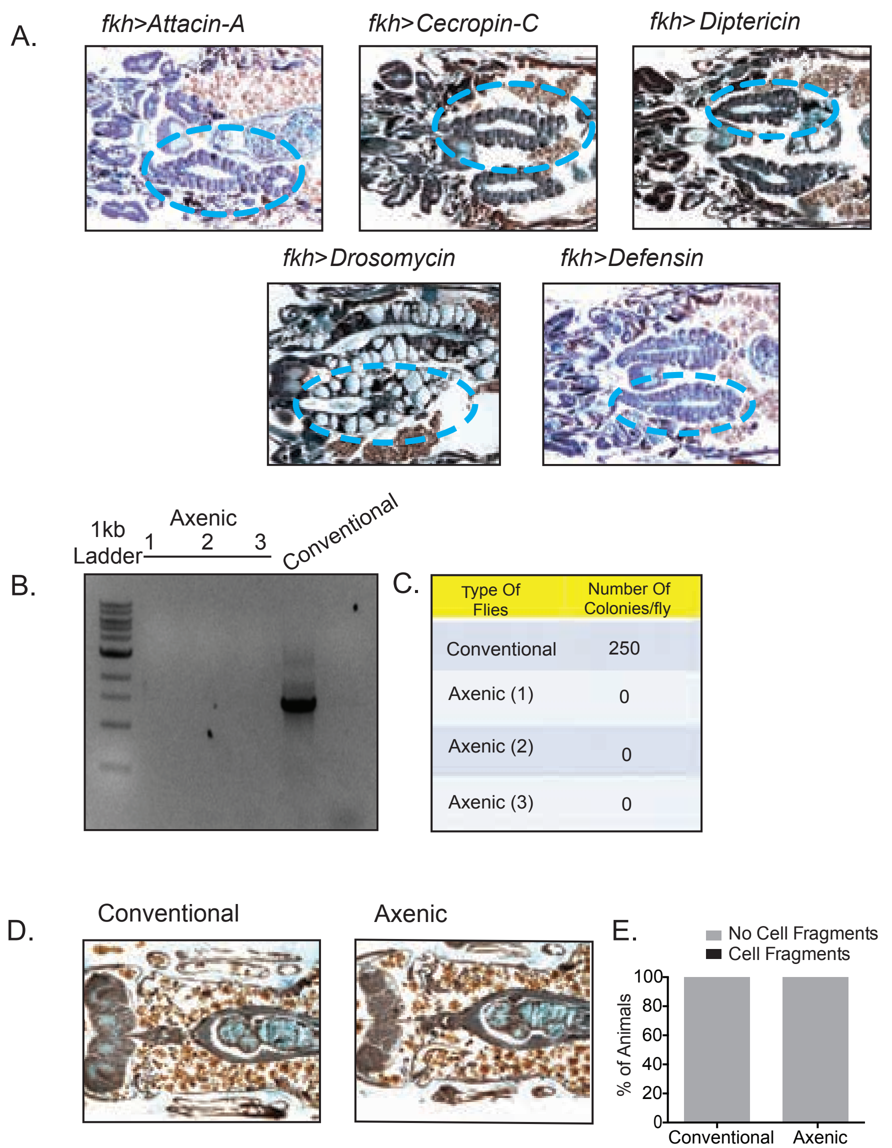


Figure 2.3. AMPs and Microflora are not required for Salivary Gland Degradation.

(A) Representative histological sections from animals ectopically expressing several AMP genes in the salivary gland (w; UAS-AMP/+; fkh-Gal4/+) 6 h APF. 12 independent samples for each AMP gene were similarly analyzed without any premature gland degradation. (B) 16S rDNA gene PCR from three axenic and one conventional fly samples. (C) Bacterial loads from three axenic strains and one conventional strain. (D) Representative histological sections of conventionally reared (w1118, left) and axenic (w1118, right) fly 24 h APF. 10 and 11 independent samples respectively from each condition were histologically analyzed and no salivary gland cell fragments were observed.(E) Quantitation of histology from samples for each genotype as in (D)

***Relish* contributes to autophagic, but not caspase-dependent cell death**

Caspases and autophagy are both necessary for complete salivary gland degradation(Berry and Baehrecke, 2007). To determine if *Relish* contributes to the caspase-dependent pathway, p35, a potent baculoviral inhibitor of effector caspases, was expressed in the salivary glands of wild type or Rel^{E20} mutant animals. As expected, p35 expression in the salivary glands of wild type animals resulted in the accumulation of <u>cell</u> fragments in ~60% of animals analyzed, with more intact tissue, known as <u>gland</u> fragments, in the other ~40%. These gland fragments are indicative of a more severe failure in salivary gland degradation. When p35 was expressed in the *Relish* mutants, <u>gland</u> fragments were observed in over 80% of animals (Fig 2.4 A, B & Fig 2.2A, right, displaying the salivary gland fragments without other tissue). The enhanced severity of this phenotype suggests that *Relish* mediated cell death and caspase-dependent apoptotic pathways are distinct, working in tandem contributing to salivary gland degradation.

Cleaved caspase-3 is used as a marker of caspase activity (Fan and Bergmann, 2010). The accumulation of cleaved caspase-3 in salivary glands was examined by immunofluorescence, and *Relish* had no effect on the appearance of this apoptotic marker (Fig. 2.4C). Together, these results indicate that caspase-dependent and *Relish*-mediated cell death pathways function in parallel, converging on the degradation and clearance of the larval salivary gland.

Figure 2.4

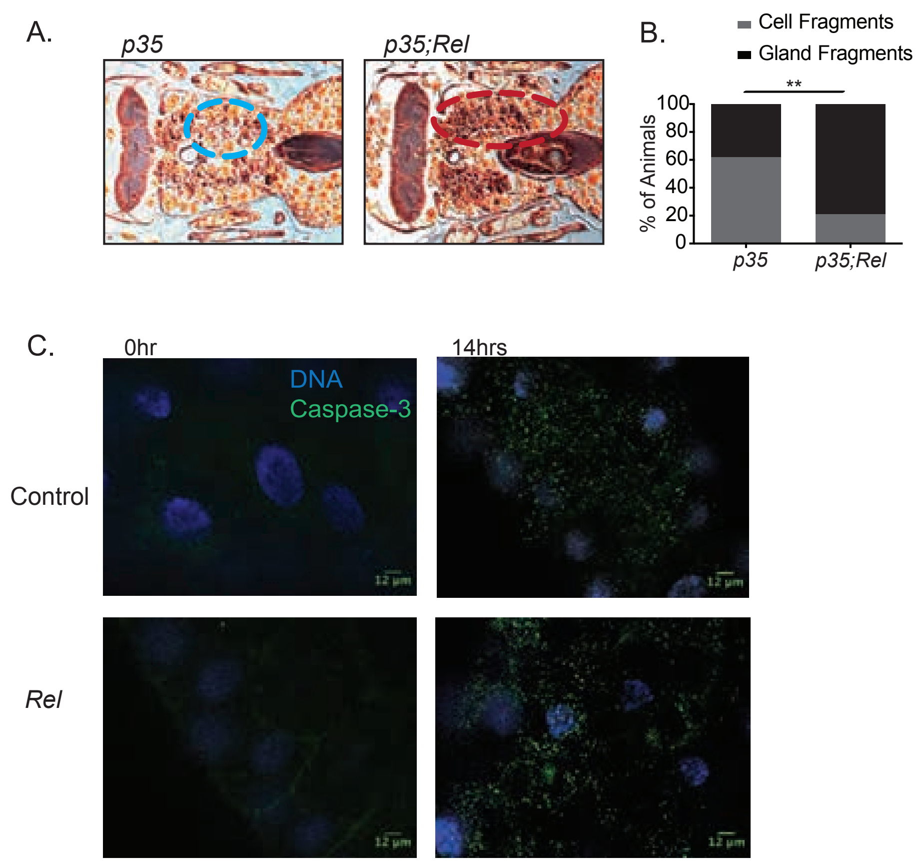


Figure 2.4. *Relish* controls salivary gland degradation independent of caspase activity

(A) Representative histological sections of animals with salivary gland specific expression of *p35* (*fkh-gal4/+;* UAS-*p35/+; Rel*^{E20}*/+,* left), *Relish* mutants with salivary gland specific expression of *p35* (*fkh-gal4/+;* UAS-*p35/+; Rel*^{E20}*,* right) 24 h APF. Salivary gland cell fragments are within dotted blue and gland fragments are within dotted red circle. (B) Quantitation of histology from 21 and 24 independent samples respectively for each strain as in (A), statistical analysis by chi-square test comparing gland fragments in these two genotypes. ** P<0.01. (C) Cleaved caspase-3 antibody staining (green) and DAPI (blue) in salivary glands of control (*Rel*^{E23}) and mutant (*Rel*^{E20}) animals at 0 h and 14 h APF, representative images from at least five independent samples analyzed in two independent trials.

We next examined the relationship between *Relish* and autophagy. Decreased *Atg18* function results in persistence of salivary gland cell fragments (Berry and Baehrecke, 2007), a phenotype that is similar to *Relish* mutants. In *Atg18, Relish* double mutants, salivary gland cell fragments were present 24 hours after puparium formation, similar to that observed in either single mutant (Fig. 2.5A,B), suggesting *Relish* functions through autophagy to regulate salivary gland destruction. To further examine the connection between *Relish* and autophagy, mCherry-Atg8a puncta were visualized 14 hours after puparium formation. Control animals (*Relish*^{E23}*)* showed distinct puncta in salivary gland cells, while the amount of Atg8a puncta were significantly decreased in the salivary glands of *Relish* mutant animals (Fig 2.5 C, D).

In *Drosophila*, the expression of *Atg1* induces premature autophagy in multiple *Drosophila* tissues (Berry and Baehrecke, 2007; Chang et al., 2013; Scott et al., 2007). We expressed *Atg1* in salivary glands to test if this is sufficient to suppress the *Relish* phenotype. Indeed, *Atg1* expression in the salivary glands of *Relish* mutants suppressed the salivary gland degradation defect observed in

Relish mutants (Fig. 2.5 E,F). Taken together, these data indicate *Relish* is required for autophagy during salivary gland degradation.

However, there is a caveat in the cleaved Atg8 assay. This assay cannot distinguish between the induction of autophagy or block in autophagosomal fusion with lysosomes, as both the processes would cause accumulation of cleaved Atg8 puncta. To ensure proper autophagic flux, another assay involving the adaptor protein Ref(2)P was used. This protein is a fly homolog of mammalian p62. Ref(2)P functions as an adaptor, which couples ubiquitinated components and Atg8 by its ubiquitin binding domain (UBD) and LC3-interacting region (LIR) respectively. During autophagosomal fusion with lysosomes, Ref(2)P is degraded and thus presence of accumulated Ref(2)P serves as an indication of block in autophagy pathway. Immunofluorescence experiments in fly salivary glands showed that compared to controls, Relish mutants demonstrate significantly higher levels of Ref(2)P puncta in salivary gland cells(Fig 2.5 G, H), supporting earlier findings that indeed Relish functions through autophagy.

Figure 2.5

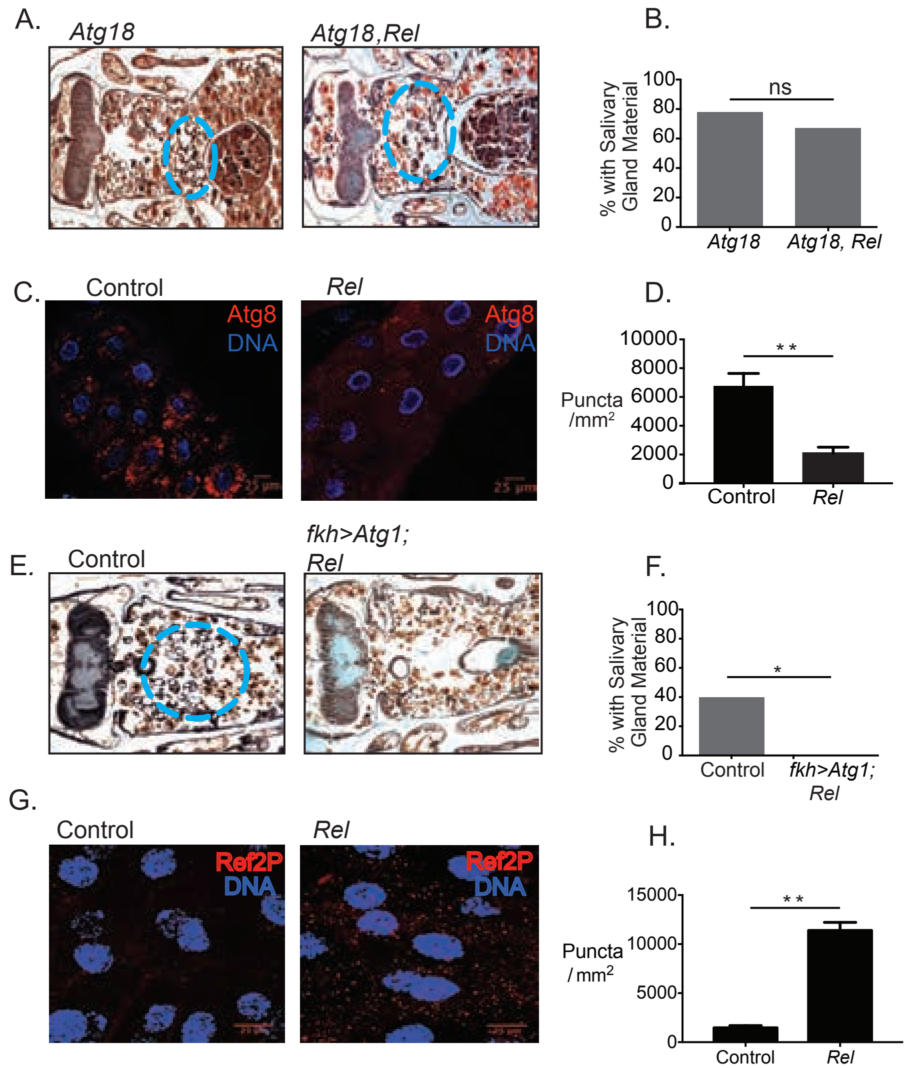


Figure 2.5. *Relish* mediated salivary gland degradation is autophagy dependent

(A) Representative histological sections of *Atg18* mutants, (left, *Atg18*^{KG03090}/Df (3L)$^{\text{Exel6112}}$) or *Relish, Atg18* double mutants (right, If/CyO; *Atg18*$^{\text{KG03090}}$, *Rel*^{E20}/Df (3L) Exel6112, *Rel*^{E20}) 24 h APF. Salivary gland fragments are within blue dotted circle. (B) Quantitation of histology from 9 and 12 independent samples for each genotype as in (A) respectively, statistical significance by chi-square test. (C) Representative images of mCherry-Atg8a expressed in the salivary glands of control animals (w; *pmCherry-Atg8a*; *Rel*^{E20}/TM6b) or *Relish* null mutants (w; *pmCherry-Atg8a*; *Rel*^{E20}). Salivary Glands were dissected and visualized 14 h APF. (D) Quantitation of data from (c) N=5, data presented as mean ± SEM and statistical analysis by unpaired two-tailed t-test with Welch`s correction, ** P<0.01, ns not significant. (E) Representative histological sections of *Relish* mutants (UAS-*Atg1*^{6A}; *Rel*^{E20}, left), and *Relish* mutants with transgenic salivary gland specific *Atg1* expression, (*fkh-Gal4*/+; UAS-*Atg1*^{6A} /+; *Rel*^{E20}*,* right) analyzed 24 h APF. Salivary gland fragments are highlighted within dotted blue circle. (F) Quantitation of histology from 20 independent samples for each strain as in (E), statistical analysis by chi-square test; * P<0.05. (G) Representative immunofluorescence images of Ref2P in the salivary glands of control animals (*Rel*^{E20}/TM6b) or *Relish* null mutants (*Rel*^{E20}). Salivary Glands were dissected and visualized 14 h APF. (H) Quantitation of data from (G) N=18, data presented as mean ± SEM and statistical analysis by unpaired two-tailed t-test with Welch`s correction, ** P<0.01

Expression of the *Relish* N-terminus or *PGRP-LC* Causes Premature Gland Degradation

Relish and *PGRP-LC* are crucial components of the Imd pathway and expression of these factors can activate Imd pathway even without an immune challenge (DiAngelo et al., 2009; Gottar et al., 2002; Wiklund et al., 2009). Our data also suggest that *Relish* and *PGRP-LC* positively regulate salivary gland autophagic cell death pathway. Therefore, we hypothesized that expression of active versions of these factors will cause early gland degradation. To test this hypothesis, we expressed either full length *Relish*, the N-terminus of *Relish* (*RelN,* an active form), *PGRP-LCx*, *Dredd* or *imd* in salivary glands and analyzed by histology, before salivary glands normally degrade. Salivary gland expression of either *RelN* or *PGRP-LC* caused premature gland degradation, but no such phenotype was observed with similar expression of full-length *Relish, imd* or *Dredd* (Fig. 2.6A-J). In particular, gland specific expression of *RelN* or *PGRP-LC* caused a marked loss of lumen structure and a severe reduction of gland size.

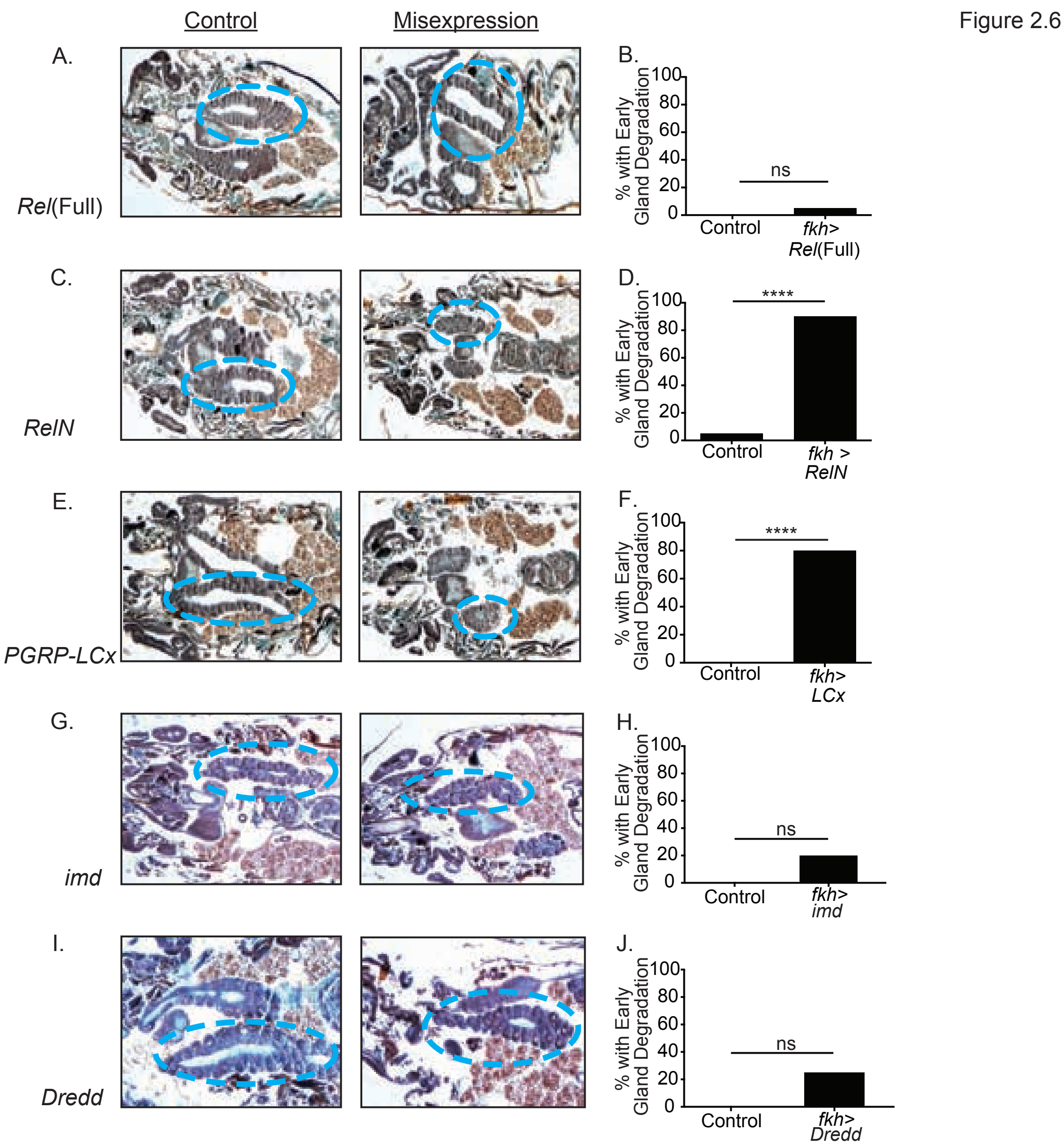
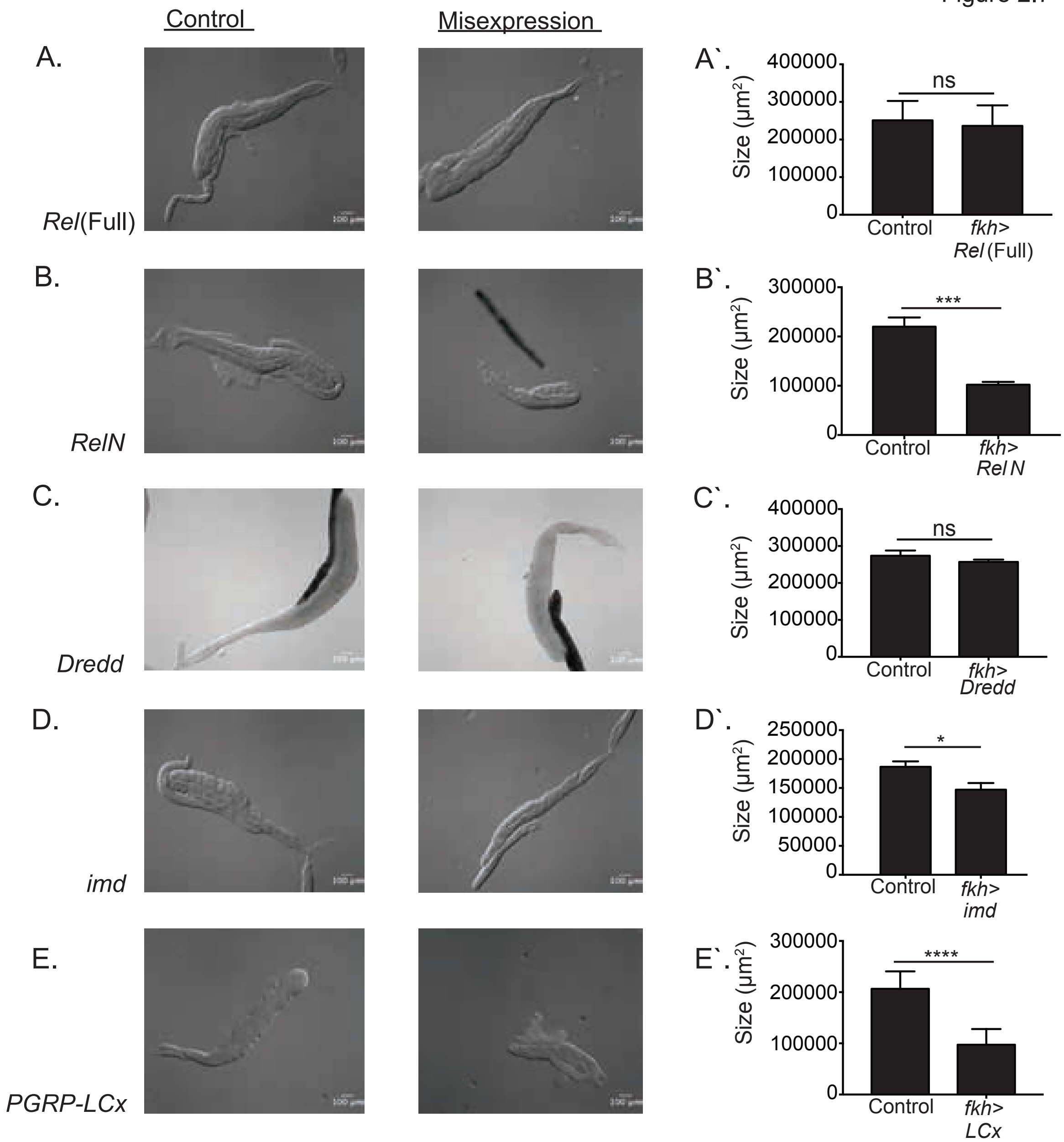


Figure 2.6. *Relish-N* or *PGRP-LC* misexpression causes premature gland degradation

(A, C, E, G, I) Representative histological sections from of control animals (left, w; UAS-*Relish* full-length, UAS-*RelN*, w; UAS-*PGRP-LCx*, w; UAS-*imd*, w;; UAS-*Dredd* respectively,) or animals expressing those genes ectopically in salivary glands (right, w; UAS-*Relish* full-length /+; fkh-Gal4/+, UAS-*RelN*/+;; *fkh-Gal4*/+, w; UAS-*PGRP-LCx*/+;f*kh-Gal4*/+, w;UAS-*imd*/+; *fkh-Gal4*/+, w;;UAS-*Dredd/fkh-Gal4*). Salivary glands are highlighted within blue dotted circles. (B, D, F, H, J) Quantitation of histological sections from 20 independent samples for each strain as in (A, C, E, G, I), statistical significance by chi-square test. **** P<0.0001, ns not significant.

Similarly, expression of these genes in 3rd instar salivary glands also caused severe gland size reduction (Fig. 2.7). *imd* expression caused a mild degree of gland size reduction in the 3rd instar, while *Relish* full-length and *Dredd* had no effect. These data demonstrate that expression of active *Relish* and *PGRP-LCx* in salivary glands is sufficient to trigger a pathway of gland degradation.

Figure 2.7


Control
Misexpression
A.
Rel(Full)
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100000
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Size (μm²)
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Control
fkh>
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C.
Dredd
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300000
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Size (μm²)
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Size (μm²)
*
Control
fkh>
imd
E.
PGRP-LCx
E`.
300000
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100000
0
Size (μm²)
****
Control
fkh>
LCx

Figure 2.7. Ectopic expression of RelN or PGRP-LC Reduces Salivary Gland Size

(A-E) DIC images of whole salivary glands and (A`-E`) quantitation of salivary gland size from animals ectopically expressing various Imd pathway components. (A) Left, control (pmCherry Atg8a/UAS-Relish full length, N=6), and right, misexpression of full length Relish (pmCherry Atg8a/ UAS-Relish full length; fkh-Gal4/+, N=4). (B) Left, control (UAS-RelN/+; pmCherry Atg8a/+, N=8), and right, misexpression of RelN (UAS-RelN/+; pmCherry Atg8a/+; fkh-Gal4/+, N=10). (C) Left, control (pmCherry Atg8a/+; UAS-Dredd/+, N=11), and right misexpression of Dredd, (pmCherry Atg8a/+; fkh-Gal4/UAS-Dredd, N=13). (D) Left, control (pmCherry Atg8a/UAS-imd, N=7), and right, misexpression of imd (pmCherry Atg8a/ UAS-imd; fkh-Gal4/+, N=7). (E) Left, control (pmCherry Atg8a/UAS-PGRP-LCx, N=16), and right, misexpression of PGRP-LCx (pmCherry Atg8a/ UAS-PGRP-LCx; fkh-Gal4/+, N=29). Statistical analysis was performed by unpaired two-tailed t-test with Welch`s correction. **** P<0.0001, *** P<0.001, * P<0.05, ns not significant.

Premature Gland Degradation due to *Relish* and *PGRP* expression is Autophagy-Dependent

We next tested if the early salivary gland degradation induced by *RelN* expression was dependent either on caspases or autophagy. *RelN* was expressed in salivary glands along with *p35* or in a homozygous *Atg18* mutant background. Inhibition of caspases by expression of *p35* did not suppress the early gland degradation caused by *RelN* expression (Fig 2.8A,B). By contrast, when *RelishN* was expressed in *Atg18* mutant animals, a complete suppression of early gland degradation was observed (Fig. 2.8C & D).

Drosophila midgut cells undergo dramatic size reduction due to autophagy induction during pupation (Chang et al., 2013). Similarly, expression of either *RelN* or *PGRP-LCx* in salivary gland cell clones also caused significant cell-autonomous reduction and autophagy, as assayed by mCherry-Atg8a puncta formation (Fig 2.8E-H).

Figure 2.8

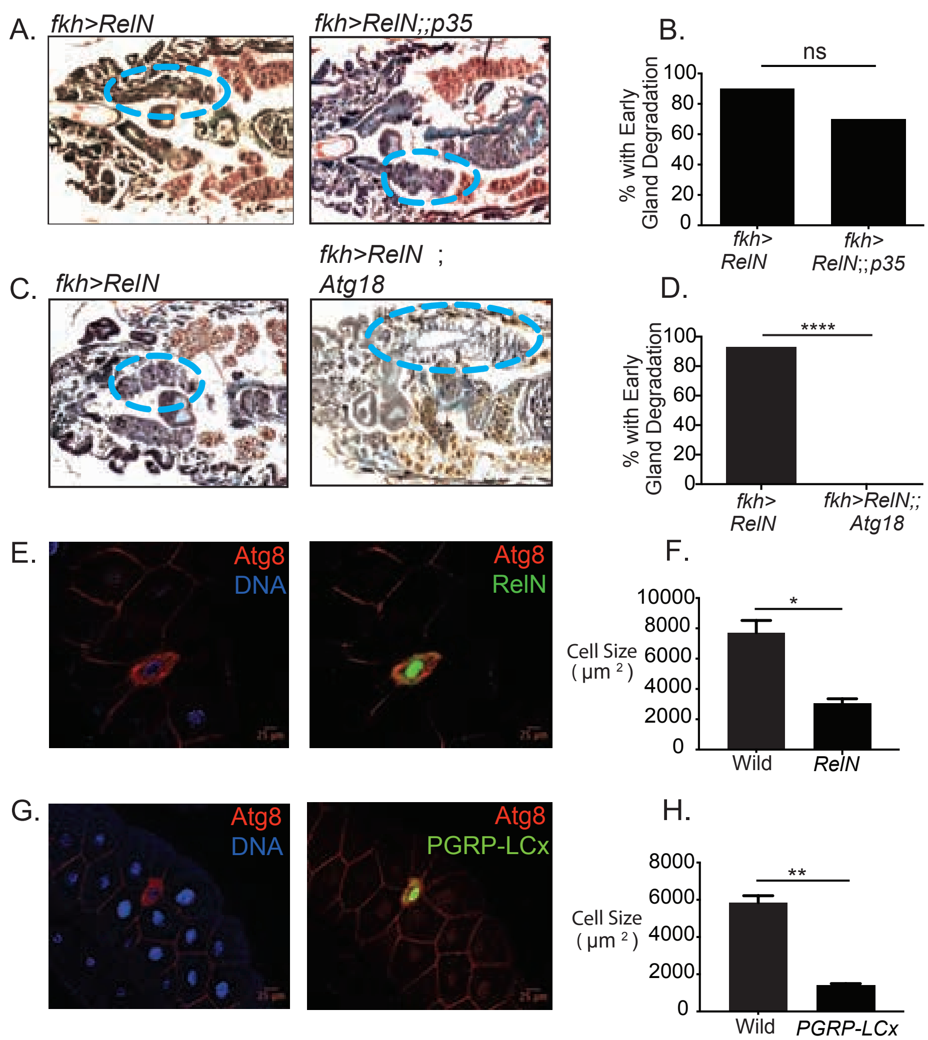

Figure 2.8. ***Relish-N*** **and** ***PGRP-LC*** **Mediated Early Gland Degradation is Autophagy dependent**

(A) Representative histological sections of animals expressing *RelN* (left, UAS-*RelN*/+;; *fkh-Gal4*/+) and animals expressing *RelN* and *p35* together (UAS-*RelN*/+;; *fkh-Gal4*/UAS-*p35*), in salivary glands. Salivary glands are highlighted within blue dotted circles. (B) Quantitation of histology from 20 samples for each genotype as in (A), statistical significance by chi-square test. (C) Representative histological sections of animals expressing *RelN* in salivary glands of wild type flies (left, UAS-*RelN*/+;; *fkh-Gal4*/+) and in *Atg18* mutant flies (right, UAS-*RelN*/*fkh-Gal4*;; *Atg18*^{KG03090}*/Df (3L) Exel6112*) . Salivary glands are highlighted within blue dotted circles. (D) Quantitation of 14 and 10 independent samples respectively from each genotype as in (C), statistical significance by chi-square test. **** P<0.0001, ns not significant. (E) Representative images of dissected salivary glands from wandering larvae. All cells express *mCherryAtg8*, while RelN is expressed in GFP marked clone cells (*hsflp/*UAS-*RelN; pmCherryAtg8/CyO*; *act<FRT, cd2, FRT>Gal4*; UAS-*GFP*/+). Quantitation of the cell size of RelN expressing cells compared to neighboring wild type cells is shown in (F), N=3, data presented as mean ± SEM and statistical analysis by unpaired two-tailed t-test with Welch`s correction. (G) Representative images of dissected salivary glands from wandering larvae. All cells express *mCherryAtg8*, while *PGRP-LCx* is expressed in GFP marked clone cells (*hsflp/w)*; *pmCherryAtg8/*UAS-*PGRP-LCx*; *act<FRT, cd2, FRT>Gal4*; UAS-*GFP*/+). Quantitation of the cell size of *PGRP-LCx* expressing cells and wild type cells is shown in (H), N=3, data presented as mean ± SEM and statistical analysis by unpaired two-tailed t-test with Welch`s correction., ** P<0.01, * P<0.05.

Additionally, when expressed throughout the 3rd instar salivary gland, *RelN* or *PGRP-LC* triggered Atg8a puncta formation, similar expression of *imd* triggered only mild puncta formation, while *Relish* full-length or *Dredd* expression did not cause this phenotype (Fig. 2.9). Taken together, these data indicate that premature gland degradation caused by *RelN* and *PGRP-LC* mis-expression is due to premature activation of autophagy.

Figure 2.9

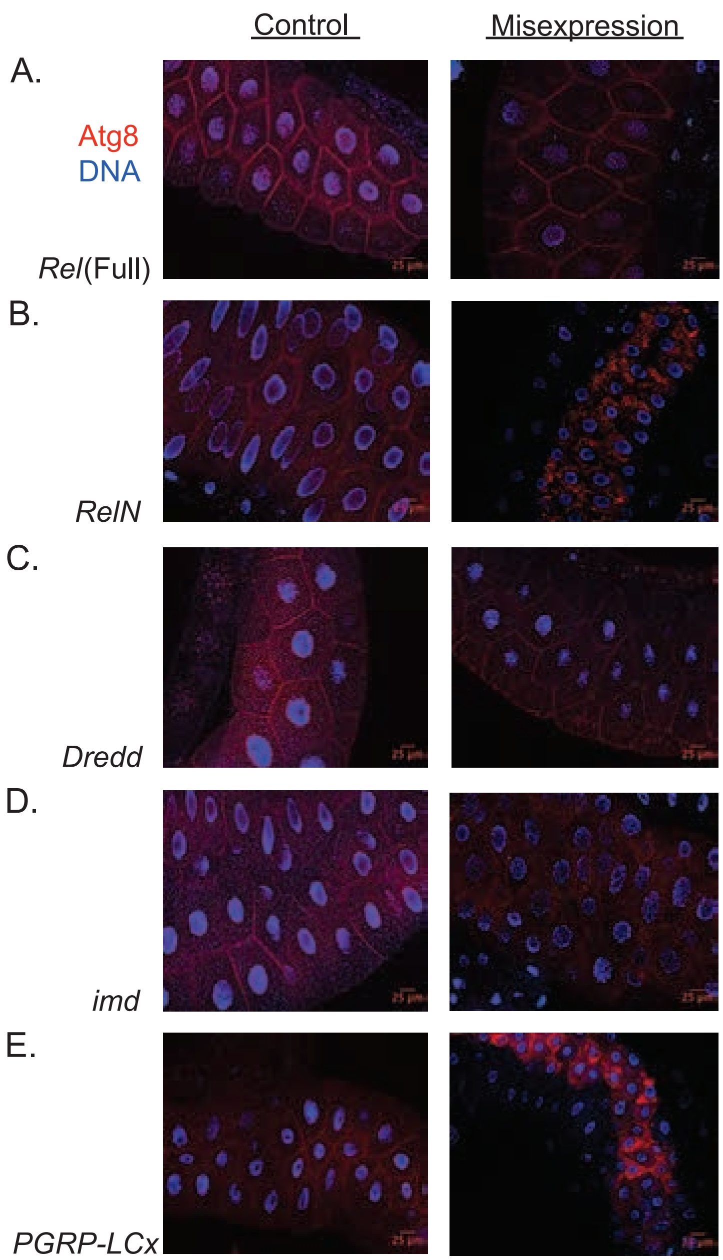

Figure 2.9. *Misexpression of RelN or PGRP-LC induces autophagy in Larval Salivary Glands*

Fluorescent microscopy of larval salivary glands from the same genotypes as

Shown in Figure 2.7 visualizing mCherry-Atg8 puncta formation. Representative images, replication for each genotype as indicated in previous legend.

Relish **and** ***PGRP-LCx*** **Function Upstream of the Autophagy Pathway**

To begin to map the pathway by which *Relish* and *PGRP-LC* control autophagy in salivary glands, we next determined the epistatic relationship between these two classic immune signaling components. PGRP-LCx, -LE, Relish triple mutant does not show any increase in phenotype than the single mutants (Fig.2.10)*,* suggesting they act in the same pathway. Furthermore, *PGRP-LCx*-induced early gland degradation was suppressed in *Relish* mutants (Fig.2.11 A, B), indicating that *PGRP-LCx* acts upstream of *Relish,* as observed in the immune signaling context.

The above data indicates that *Relish* affects the autophagy pathway upstream of both *Atg8* and *Atg18*. To further map the interaction between *Relish* and the autophagy pathway, *RelN* misexpression was combined with knockdowns or mutations of two genes upstream in the autophagy pathway, *Atg1* or *Atg13* (Fig. 2.10 C-F). In particular, *RelN* -induced salivary gland degradation was suppressed by loss of *Atg1*, but not by *Atg13*. These results suggest Relish acts upstream of *Atg1* but downstream or parallel of *Atg13*.

Figure 2.10

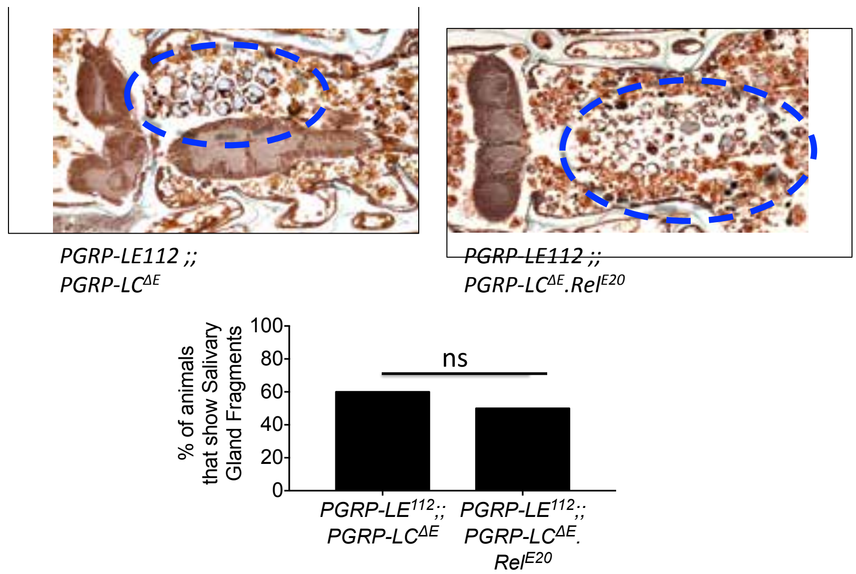

Figure 2.10 PGRPs and Relish Function In The Same Pathway In The Context Of Salivary Gland Degradation

(A) Representative histological sections of *PGRP* mutants (left, $LE^{112};;LC^{\Delta E}$) and PGRP, *Relish* triple mutants ($LE^{112};;LC^{\Delta E}.Rel^{E20}$) flies 24 h APF. Salivary gland fragments observed in the mutants are highlighted within dotted blue circle. (B) Quantitation of histology from 20 independent samples for each strain as in (A), statistical analysis by chi-square test. ns not significant

Since *Relish* is best characterized as a transcription factor that can drive gene expression when activated or mis-expressed, we investigated whether *Relish* influences *Atg1* expression. It has been reported that *Atg1* expression is sufficient to drive autophagy in the salivary gland (Berry and Baehrecke, 2007). The level of *Atg1* expression in the salivary glands of both control (Rel^{E23}) and *Relish* mutant animals (Rel^{E20}) was determined at both 0 hour and 14 hours after puparium formation. We found that expression of *Atg1* gene is significantly reduced at both time points in *Relish* mutants compared to controls (Fig. 2.10 G). On the other hand, *RelN* expression in salivary glands significantly increased *Atg1* transcription. These data indicate that *Relish* controls autophagy through the regulation *Atg1* expression (Fig. 2.10 H).

Figure 2.11

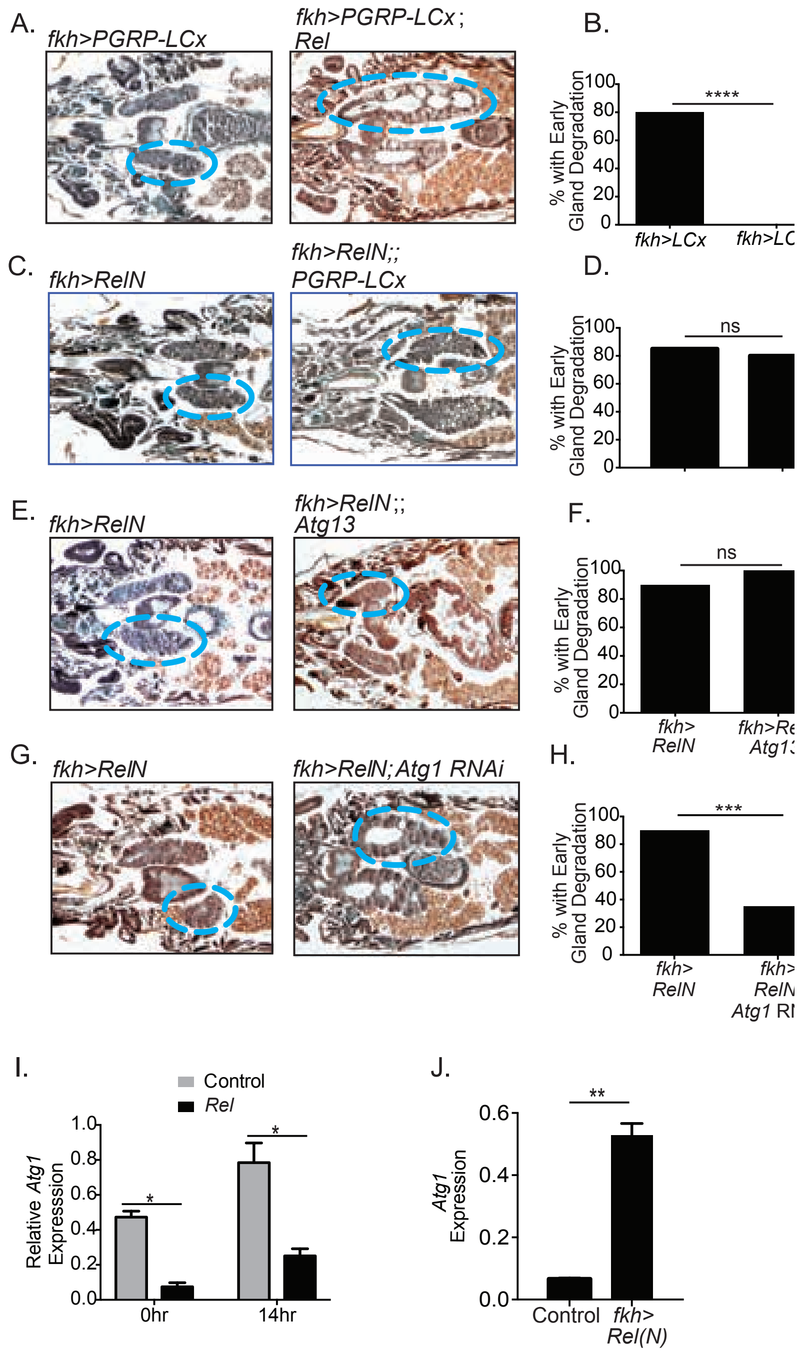
A.
fkh>PGRP-LCx
fkh>PGRP-LCx ; Rel
B.
% with Early Gland Degradation
****
fkh>LCx
fkh>LC
C.
fkh>RelN
fkh>RelN;; PGRP-LCx
D.
ns
E.
fkh>RelN
fkh>RelN;; Atg13
F.
ns
fkh> RelN
G.
fkh>RelN
fkh>RelN; Atg1 RNAi
H.
***
fkh> RelN
I.
Control
Rel
Relative Atg1 Expresssion
0hr
14hr
J.
Atg1 Expression
**
Control
fkh> Rel(N)

Figure 2.11. ***Relish* controls autophagy through *Atg1* expression**

(A) Representative histological sections of animals expressing *PGRP-LCx* in salivary glands of wild type flies (left, w; UAS-*PGRP-LCx*/+; *fkh-Gal4*/+) and *Relish* mutant flies (right, *fkh-Gal4*/+; UAS-*PGRP-LCx*/+; Rel^{E20}) . (B) Quantitation of 20 independent samples from each genotype as in (A), statistical significance by chi-square test. (C) Representative histological sections of animals expressing *RelN* in salivary glands of wild type flies (left, UAS-*RelN*/+;; *fkh-Gal4*/+) and in *PGRP-LCx* mutant flies (right, UAS-*RelN*/*fkh-gal4*;; $PGRP\text{-}LCx^{\Delta E}$ *fkh-gal4, Atg13*^{74}) 6 h APF (D) Quantitation of 20 independent samples respectively from each genotype as in (C), statistical significance by Chi-square test. (E) Representative histological sections of animals expressing *RelN* in salivary glands of wild type flies (left, UAS-*RelN*/+;; *fkh-Gal4*/+) and in *Atg13* mutant flies (right, UAS-*RelN*/+;; *fkh-gal4, Atg13*^{74}) (F) Quantitation of 10 and 11 independent samples respectively from each genotype as in (E), statistical significance by Chi-square test. (G) Representative histological sections of animals expressing *RelN* (left, UAS-*RelN*/+;; *fkh-Gal4*/+) and animals expressing *RelN* and *Atg1* RNAi together (UAS-*RelN*/+; UAS-*Atg1* RNAi/+; *fkh-Gal4*/+), in salivary glands. (H) Quantitation of histology from 20 samples for each genotype as in (G), statistical significance by chi-square test. For A, C, E & G, salivary glands are highlighted within blue dotted circles. (I) *Atg1* gene expression levels in salivary glands of control (Rel^{E23}) and Rel^{E20} animals, at 0 h and 14 h APF, measured by qRT-PCR, N=3, data presented as mean ± SEM, and statistical analysis by unpaired two-tailed t-test with Welch`s correction. (J) *Atg1* gene expression in salivary glands of control (UAS-*RelN*) and *RelN* expressing animals (UAS-*RelN*/+;; *fkh-Gal4/+)*, 6 h APF quantified by qRT-PCR, N=3, data presented as mean ± SEM) and statistical analysis by unpaired two-tailed t-test with Welch`s correction. **** P<0.0001, *** P<0.001, ** P<0.01, * P<0.05, ns not significant.

Bioinformatic analysis of Atg1 promoter using different transcription factor binding prediction software (like Consite, Jasper, Twine) revealed four putative NF-κB binding sites at -1706, -1229, -723, and -26bp respectively (Fig.2.11A). We performed a Chromatin-Immunoprecipitation (ChIP) assay to check whether Relish induces *Atg1* expression by directly binding to the promoter region. Diptericin promoter was used as a positive control and Diedel and Atg6 promoters as well as Atg1 downstream region (9284bp, no putative NF-κB site) were used as negative controls. Our data shows that indeed Relish N-terminal binds to the *Atg1* promoter region (Fig 2.11B), particularly to the region closest to the transcription start site.

Figure 2.12

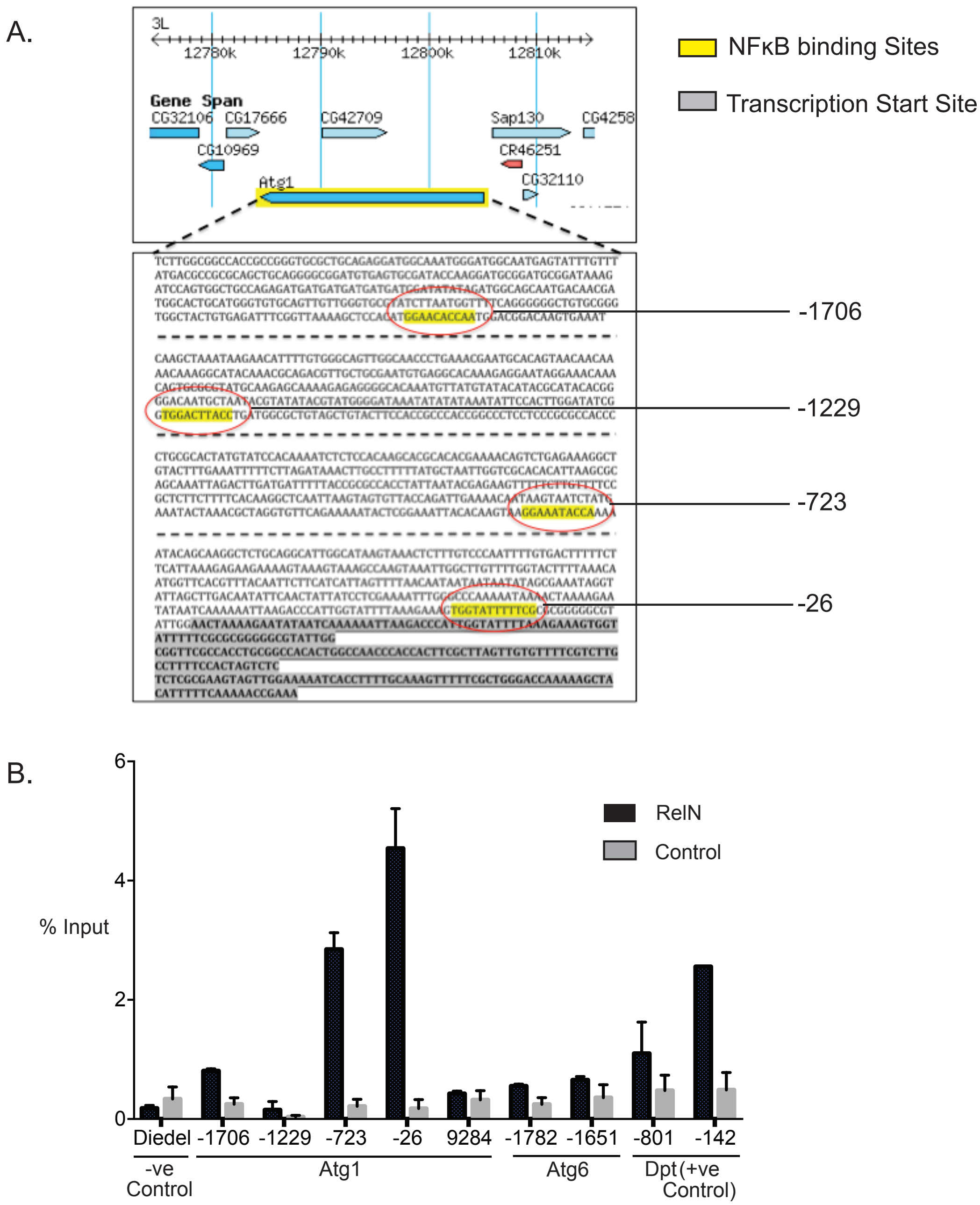

Figure 2.12. Relish directly binds the promoter region of *Atg1* to induce *Atg1* expression

(A) DNA sequence of the *Atg1* promoter region. The putative NFκB sites

(-1706bp, -1229bp, -723bp, -26bp) are highlighted in yellow and the transcription initiation site is highlighted in grey. (B) The recruitment of Relish N-terminal (UAS-FLAG-*RelN*/+; tub-Gal80^{ts}/+; *fkh-Gal4*/+) to the promoters of *Diedel, Atg1, Atg6, Diptericin* in salivary glands. *Diedel* and *Diptericin* were used as negative and positive controls respectively. All values are represented as percent fraction of total input DNA. Data was calculated against the driver only control (w; tub-Gal80^{ts}; *fkh-Gal4*) and is representative of two independent experiments.

Starvation induced Autophagy and Midgut Degradation are *Relish* Independent

Nutrient deprivation triggers autophagy as autophagy inhibitor TOR is inhibited in this condition. To check whether *Relish* also influences this kind of autophagy pathway we starved the animals and observed the fatbody for autophagy induction. The *Relish* mutants did not show any discrepancy in mcherry-Atg8 puncta formation (Fig.2.12A, B) compared to the control animals suggesting that Relish is not involved in starvation-induced autophagy.

Larval midgut is also degraded during metamorphosis and that degradation is autophagy dependent. So, we decided to check whether this developmental autophagy is also influenced by Relish. However, we found autophagy induction occur normally in the Relish mutant animals (Fig.2.12C, D), indicating that Relish is not involved in autophagy mediated midgut histolysis.

Figure 2.13

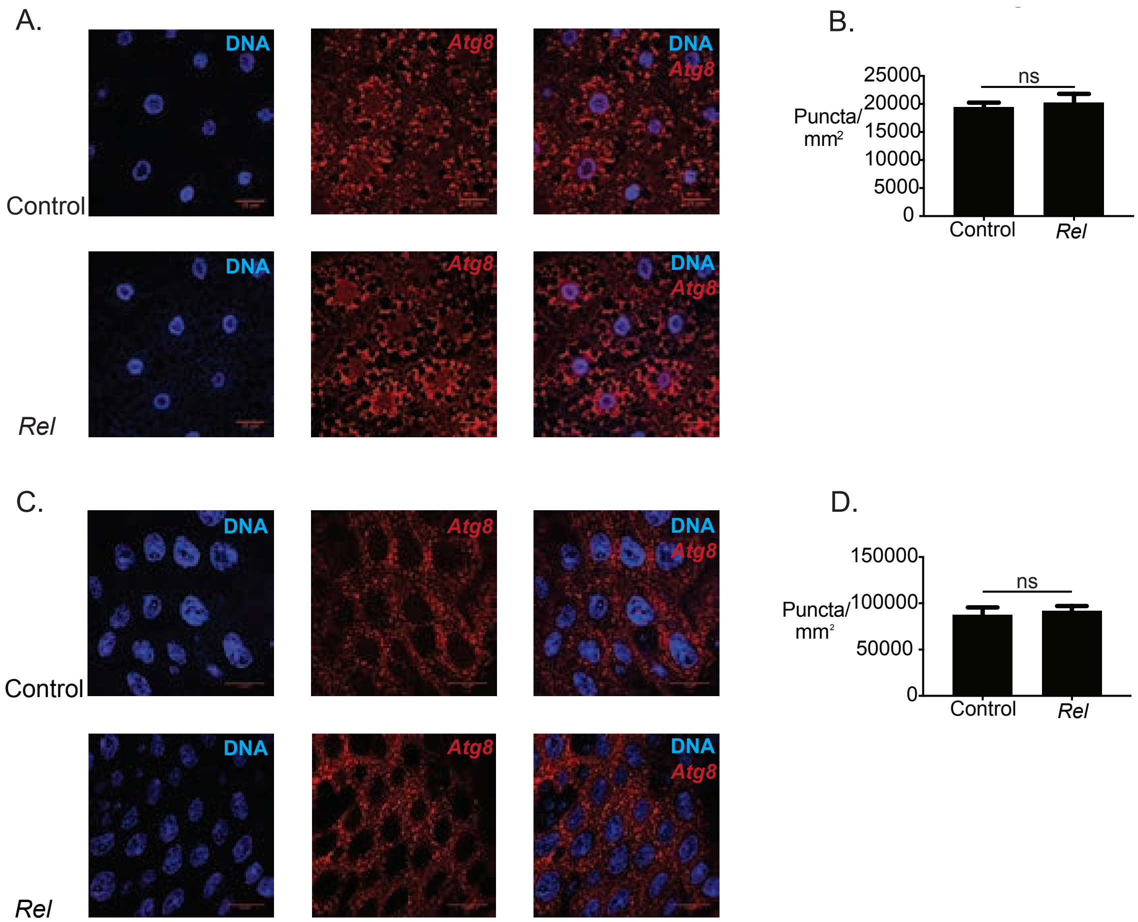

Figure 2.13. *Relish* is not involved in Starvation induced Autophagy or Midgut degradation.

(A) Representative images of mCherry-Atg8a expressed in the fat bodies of control animals (w; *pmCherry-Atg8a*; *Rel*^{E20}/TM6b) or *Relish* null mutants (w; *pmCherry-Atg8a*; *Rel*^{E20}). Fat bodies were dissected and visualized 4 h after starvation. (B) Quantitation of data from (A) N=10, data presented as mean ± SEM and statistical analysis by unpaired two-tailed t-test with Welch`s correction, ns not significant. (C) Representative images of mCherry-Atg8a expressed in the midguts of control animals (w; *pmCherry-Atg8a*;*Rel*^{E20}/TM6b) or *Relish* null mutants (w; *pmCherry-Atg8a*; *Rel*^{E20}).

(D) Quantitation of data from (C) (control, N=8 and Relish mutants, N=9), data presented as mean ± SEM and statistical analysis by unpaired two-tailed t-test with Welch`s correction, ns not significant.

RelN Influenced Eye deformities are also Autophagy Dependent

In a previous report *RelN* expression in the eyes causes eye deformities. The authors attributed this phenotype as a result of toxicity. However, as we demonstrated earlier that the expression of the active version of *Relish* results in the premature gland degradation, we thought the eye phenotype could be the result of that. *RelN* mediated eye deformities are suppressed by the knockdown of *Atg1* and *Atg2*, but not *Atg13*, indicating that *Relish* mediated eye deformities are also caused by autophagy (Fig.2.13 A-D). It has already been reported that autophagy gene knockdown in *Drosophila* eye alone does not change eye morphology(Chen et al., 2012; Nandi et al., 2014).

Figure 2.14

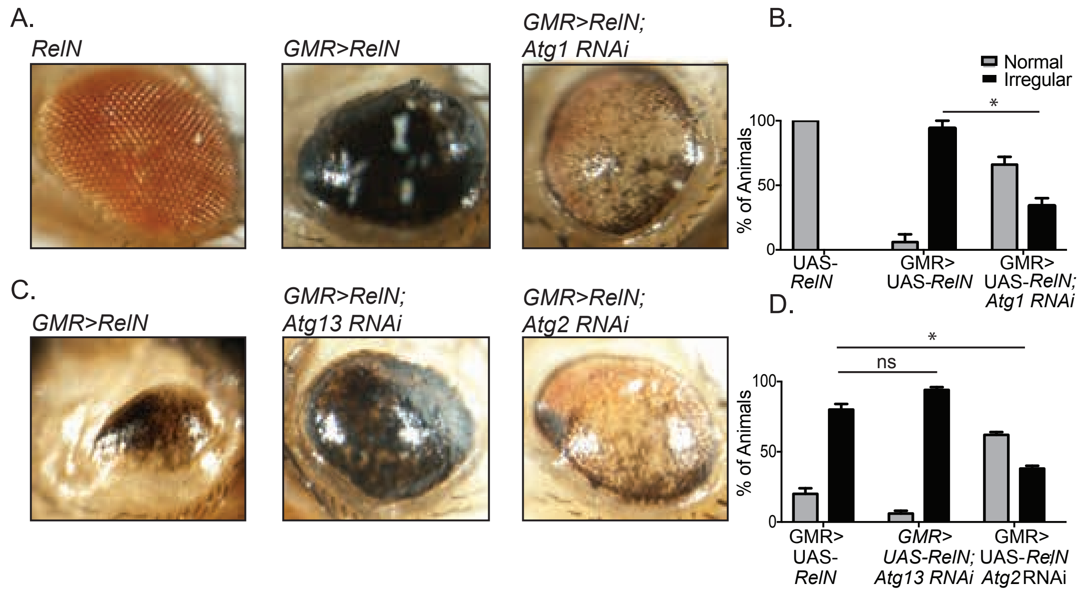

Figure 2.14. *Relish-N* mediated Eye Deformities are Autophagy Dependent. (A) Representative images of the eyes of Drosophila, control (left), expressing *RelN* (UAS-*RelN/+; GMR-Gal4/+,* middle), and expressing *RelN* and *Atg1* RNAi together (UAS-*RelN/+; GMR-Gal4/* UAS-*Atg1* RNAi*,* right). (B) Quantitation of data from (A) from each genotypes (N=25). (C) Representative images of the eyes of Drosophila, expressing *RelN* (UAS-*RelN/+; GMR-Gal4/+,* left), expressing *RelN* and *Atg13* RNAi together (UAS-*RelN/+; GMR-Gal4/* UAS-*Atg13* RNAi*,* middle) and expressing *RelN* and *Atg2* RNAi together (UAS-*RelN/+; GMR-Gal4/* UAS-*Atg2* RNAi, right). (D) Quantitation of data from three independent biological experiments from (C) from each genotypes (N=25). Data presented as mean ± SEM) and statistical analysis by unpaired two-tailed t-test with Welch`s correction. * P<0.05, ns not significant.

Discussion

Different aspects of autophagy have been extensively studied over the years, particularly during nutrient deprivation, and the role of metabolites in the regulation of autophagy is well established. Autophagy is critical for genomic stability and alleviation of oxidative stress, and in turn the prevention of tumorigenesis(White, 2015). In recent years autophagy has become an attractive target for cancer therapy (Thorburn et al., 2014). Moreover, it has also been observed that autophagy plays important roles in different immune defenses, especially against intracellular pathogens.

Our findings suggest that the NF-κB factor *Relish,* an important component of the fly immune system, plays a significant role in steroid hormone triggered autophagy in the salivary glands of *Drosophila*. *Relish* positively regulates autophagy as evidenced by the inhibition of autophagy in salivary glands of *Relish* mutant flies. Ectopic expression of active *Relish* induces autophagy and causes premature gland degradation. Furthermore, we present a novel mechanism by which *Relish* regulates autophagy. *Relish* drives the expression of *Atg1*, which is both necessary and sufficient for autophagy induction and programmed cell death of salivary glands (Berry and Baehrecke, 2007; Scott et al., 2007).

As *Relish* is the key transcription factor regulating *Drosophila* immunity via the Imd pathway, we examined all other components of this pathway to determine whether they also contribute to salivary gland degradation. However, apart from the bacterial sensing receptors, *PGRP-LC* and *–LE* and *Relish*, none of the other Imd pathway components affected salivary gland degradation. *PGRP-LC* functions upstream of *Relish* during gland degradation, similar to that observed in the immune signaling context. These results are surprising and suggest several possibilities including a direct interaction between Relish and PGRP-LC/LE or the existence of a novel pathway that connects PGRP-LC and/or PGRP-LE to Relish without the involvement of other traditional Imd pathway components. We also report here that the *PGRP-LC* and *PGRP-LE*, which encode receptors known to directly bind bacterial peptidoglycan, function in the context of salivary gland degradation even though microbial triggers are not involved in this biological process, as salivary gland degradation occurs normally in axenic flies. It has been reported that *PGRP-LE,* but not *Relish,* is crucial to mount an autophagic response against cytosolic *Listeria monocytogenes* infection (Yano et al., 2008). So it seems unlikely that the autophagy activation upon *Listeria* infection is regulated in the same way as the *Relish*-controlled autophagy pathway in salivary gland degradation. Thus the Imd pathway can trigger autophagy in different contexts involving *PGRP* receptors and/or *Relish* but the mechanism involved likely differ depending on the context.

Recently, *Relish* has been implicated in several cell death related processes. For example, *Relish* was found to be essential for the light-dependent death of photoreceptor cells in *norpA* mutant flies (Chinchore et al., 2012). Another report suggests that Relish is crucial in removing unfit cells in a *Drosophila* wing-disc model of cell competition (Meyer et al., 2014). *Relish* has also been linked to neurodegeneration in *Drosophila,* where *Relish*-dependent expression of AMPs was shown to cause increased neuronal damage (Cao et al., 2013). Several of these studies either speculated or showed that *Relish* influences caspase dependent cell death. Yet in salivary gland degradation *Relish* does not affect caspase-dependent processes, and instead controls the activation of autophagy and cell death. To the best of our knowledge, no previous reports have implicated *Relish* in the regulation of autophagy.

It has been reported that some autophagy components play important roles in both immunity and tumorigenesis, such as *ATG6/BECN1* that acts downstream of cGAS-STING signaling pathway as well as downstream of TLR4 signaling upon cytosolic DNA and LPS exposure respectively (Cadwell, 2016). However it has been reported that deletion of *BECN1* also results in the generation of liver and lung tumors as well as lymphomas in mice (Qu et al., 2003). So, clearly these results demonstrate that some autophagy components play dual role in both immunity and cellular homeostasis. Interestingly, our findings also demonstrate a dual role of the PGRP receptors and Relish in both immune responses and regulation of developmentally controlled cell death. The lack of any microbial involvement linking PGRP receptors and Relish to the death of salivary gland cells indicates the possibility that these factors are regulated by developmental cues in this context. We have previously demonstrated that a rise in steroid hormone induces *PGRP-LC* and *Relish* expression (Rus et al., 2013).

In fact, we also observed increased expression of both *PGRP-LC* and *Relish* in dying salivary glands (Fig. 2.14A,B)

Figure 2.15

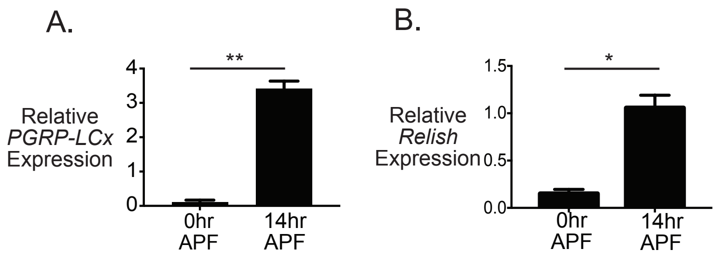

Figure 2.15. *PGRP-LCx* and *Relish* are Upregulated during Salivary gland Degradation

(A, B) Analysis of *PGRP-LCx* and *Relish* expression at 0 h and 14 h APF by qRT-PCR. N=3, statistical analysis was performed by unpaired two-tailed t-test with Welch`s correction. ** P<0.01, * P<0.05.

Elevated levels of *PGRP-LC* are sufficient to activate the classical Imd pathway (Choe et al., 2002). Together these findings suggest that in the presence of high levels of steroid, *PGRP-LC* is upregulated within the salivary glands, to a level that triggers a non-classical *PGRP-LC* to *Relish* pathway, which in turn induces *Atg1* expression. *Atg1* expression *per se* is sufficient to activate autophagy in the salivary gland (Berry and Baehrecke, 2007). Although in physiological context *Atg13* is required for proper gland degradation and *Atg1* activation, we have demonstrated that in case of active relish misexpression, *Atg13* is not required. This could be due to high level of *Atg1* expression through active relish and this bypasses the need for *Atg13*. We speculate that in normal condition both relish mediated *Atg1* expression and subsequent complex formations by *Atg13* are necessary for autophagy induction in salivary glands. Hence, even in the absence of any microbial stimulus, steroid hormone signaling, through elevated expression of *PGRP-LC* and *Relish,* could contribute to the activation of programmed cell death by transcriptionally regulating the *Atg1* and autophagy. Alternatively, in this context PGRPs could be activated by a yet-to-be identified developmentally regulated ligand, to activate Relish and autophagy. Future studies will be necessary to discriminate between these possibilities.

CHAPTER 3.

Experimental Procedures

Fly Strains

All strains have been previously described including *Relish*^{E23}, a precise P-element excision allele, and *Relish*^{E20}*,* a congenic imprecise deletion allele (Hedengren et al., 1999), *PGRP-LC*$^{\Delta E}$ and the *PGRP-LE*^{112}*;; PGRP-LC*$^{\Delta E}$ double mutant (Gottar et al., 2002; Takehana et al., 2004), UAS-*Relish Full* length (BL-9459)(Hedengren et al., 1999), UAS-*RelN* (Wiklund et al., 2009) , UAS-*imd* (Georgel et al., 2001), UAS-*Dredd* (Leulier et al., 2002)*,Tak1*^{2} (Vidal et al., 2001)*,* UAS-*AMP*s(Cao et al., 2013), UAS-*PGRP-LCx (Kaneko et al., 2006)*, hsflp; +, act<FRT, cd2, FRT> Gal4,*UAS-GFP*, *pmcherry-Atg8a (Denton et al., 2012)*, UAS-*Atg1*^{6A} *(Mohseni et al., 2009),* UAS-*Atg1* RNAi (VDRC-16133), UAS-*p35 (Hay et al., 1994) Atg13*^{74} *(Chang and Neufeld, 2009)*, *Atg18a*^{KG03090} (BL-13945), Df(3L)Exel6112 (BL-7591).

Axenic Fly Preparation

The fly embryos were collected on grape juice agar plates and later washed sequentially with 2.7% sodium hypochlorite solution, 70% Ethanol, sterile PBS and transferred to vials containing autoclaved fly food with an antibiotic cocktail of tetracycline, ampicillin, rifamycin (50 μg/ml, 500 μg/ml, or 200 μg/ml respectively). 16S ribosomal DNA PCR was performed to determine the axenic status of the flies, and whole fly lysates, from 10 animals, were serially diluted and plated on LB agar plates to quantify culturable microbes.

Immunostaining and Microscopy

Salivary glands were dissected in cold PBS solution and then fixed in 4% paraformaldehyde for overnight at 4°C. The glands were washed with PBST (PBS with 0.1% tween-20) and then with PBSBT (PBS with 0.1% tween-20 and 1% BSA). Next, they were incubated in PBSBT at room temperature for 2 hours and kept in primary antibody (rabbit anti-cleaved caspase-3, 1:400, Cell-Signaling, #9664) overnight at 4°C. The glands were washed with PBSBT, incubated with secondary antibody for 2 hours at room temperature and washed again with PBSBT for 1 hour. Finally, the glands were mounted in Vectashield (Vector Laboratories). For mcherry-Atg8 analysis, salivary glands were dissected in cold PBS and fixed with 2% paraformaldehyde for 1 hour in room temperature. The glands were then mounted in 50% glycerol containing 2 μM Hoechst stain. Imaging was performed using Zeiss AxioImager microscope and mecherry-Atg8 puncta analysis and cell size measurement were performed with ImageJ software.

Quantitative RT-PCR analysis

Salivary glands were dissected in cold PBS and RNA isolated as described (Andreas and Thummel, 1994). 500ng of total RNA was treated with DNaseI (Invitrogen) and used as template in an iscript cDNA synthesis reaction (Bio-Rad), followed by qRT-PCR reaction using SYBR green supermix (Bio-Rad).

Histology

Salivary gland histology was performed as described previously (Muro et al. 2006).

Induction of Cell Clones

Misexpressing cell clones were induced in *Drosophila* salivary glands as described previously (McPhee et al. 2010).

Starvation Assay

Starvation of larvae was done as described previously (McPhee et al. 2010)

Chromatin-Immunoprecipitation Assay

Wandering larvae were kept at 29ºC for 3 hours to induce *Relish N-terminal* expression and ~100 pairs of salivary glands were dissected from these larvae. The glands were washed with cold PBS and then suspended in 1ml of PBS solution. The glands were cross-linked using 1% formaldehyde; at room temperature for 10 min. Glycine was added to quench the cross-linking at a concentration of 125mM. Then, the glands were washed with ice-cold TBS and resuspended with 500 μl of sonication buffer (50mM Hepes-pH7.8, 150mM NaCl, 1mM EDTA, 1% Triton-X 100, 0.1% sodium Deoxycholate, 1% SDS, Protease Inhibitor Cocktail). The glands were ground with pestle and then freeze-thawed. Finally, the solution was sonicated using the diagenode-bioruptor sonicator (20min sonication, 30sec On and 30sec Off cycle, setting-high). The chromatin was co-immunoprecipitated overnight using Dynabeads (thermofisher, catalog no-10003D) conjugated with anti-FLAG antibody (Sigma, catalog no-F1804), reverse-crosslinked and purified. Quantitative RT-PCR was performed using primers designed to sites of interest.

CHAPTER 4.

Discussion

Autophagy, which initially was thought to be cells` last resort for survival and only a recycling mechanism, now found out to be involved in numerous other biological processes, including development and immunity. Its role in cancer and other disorders made it an attractive target for future drug therapy. Therefore the mechanisms that regulate autophagy are of great interest. Here, in my dissertation I have described a novel transcriptional regulation of autophagy pathway, mediated by the NFκB transcription factor Relish.

Role Of NFκB in regulation of Autophagy

NFκB is a versatile transcription factor, which influences a large number of biological pathways. The fruit fly *Drosophila melanogaster* has three NFκB factors, Dorsal, DIF and Relish. Dorsal is essential for the dorso-ventral pattern formation during development. Stimulation of the Toll pathway during development results in the nuclear localization of Dorsal, which then activates several genes, including *snail* and *twist (Jiang et al., 1991).* However, later the role of Dorsal in expression of several AMP genes was also discovered. The second NFκB factor DIF is also a component of the Toll pathway. DIF is also necessary for the induction of proper humoral immune response as it also controls the expression of several AMP genes. It has been found that Dorsal/ DIF controls AMP expression during larval stage in a redundant manner. However, in adults DIF is the principal NFκB factor, which is involved in Toll pathway dependent AMP production (Ip et al., 1993). The last NFκB factor, Relish, was characterized as a component of the Imd pathway, which is essential for generation of proper humoral immune response against Gram-negative bacteria (Hedengren et al., 1999).

Developmentally programmed cell death is observed in *Drosophila* during metamorphosis. At this stage major tissue remodeling, which include both old tissue histolysis and generation of new tissues, occurs. *Drosophila* salivary glands and midgut are degraded at this time under the influence of major cell death pathways. As apoptosis and autophagic cell death both take place in the salivary glands to ensure its complete destruction, this particular tissue is an extremely useful model to study different aspects of both cell death pathways. Interestingly, a high level of AMPs, whose expression is controlled by Relish, is observed in dying salivary glands (Lee et al., 2003). As the salivary glands are degraded by both apoptosis and autophagy, I was curious whether Relish plays any role to control these pathways.

My data shows that *Relish* controls salivary gland degradation. Further investigation also revealed that Relish mediated salivary gland degradation is autophagy dependent. This is the first in vivo data showing the involvement of *Relish* in regulation of autophagy. Furthermore the PGRPs, which are primarily characterized as necessary components of humoral immune response, also found to be acting in the same pathway. Although mammalian NOD receptors also control autophagy, they mostly respond to microbial components, not developmental cues (Travassos et al., 2010a). In this regard this pathway is quite unique and more research is needed to properly delineate the pathway.

Relish null mutant animals show incomplete salivary gland degradation, as salivary gland cell fragments are observed in the histological sections of the *Relish* mutants compared to the control animals. Expression of wild type *Relish* gene in the salivary glands in Relish mutant background rescues the phenotype, further proving the necessity of *Relish* in salivary gland histolysis. Surprisingly the other components of the Imd pathway, except the receptors *PGRP-LC* and-*LE*, did not show any such phenotype. Furthermore the *Relish* mediated salivary gland cell death appears to be caspase independent rather it relies on autophagy. Expression of both the active versions of *Relish* and *PGRP-LC* results in the premature gland degradation and autophagy induction, proving that *Relish* and *PGRP-LC* are necessary and sufficient to induce autophagy. Interestingly, my data also indicates that *Relish* controls autophagy by regulating the expression of *Atg1*, as evidenced by the comparatively lower expression of *Atg1* in *Relish* mutants and induction of *Atg1* expression in salivary glands when *Relish* N-terminal was misexpressed. Bioinformatic analyses and ChIP assay further demonstrated direct interaction of *Relish* N-terminal to the putative NFκB sites at the *Atg1* promoter region.

Different pathways, including the TOR network, regulate autophagy. As mentioned earlier, TOR pathway monitors the nutrient status of the cells and in a nutrient rich condition TOR is activated and that leads to cell growth. TOR kinase causes protein synthesis by phosphorylating downstream protein S6 kinase (RPS6-p70-protein kinase). Phosphorylation of the ribosomal S6 protein results in protein synthesis. TOR also inhibits the translational repressor Thor/4E-BP (Eukaryotic Translation Initiation Factor 4E binding), by phosphorylating it (Gingras et al., 2001). So, together with inhibition of autophagy and promotion of cell growth, TOR activity ensures proper development of tissues and organs (Noda and Ohsumi, 1998). As both nutrients and growth factors such as IGF (Insulin like Growth Factor) influence TOR activity I thought whether Relish activates autophagy by influencing these particular signaling cascades. However, we found that starvation-induced autophagy occurs normally in *Relish* mutants. This suggests that *Relish* mediated autophagy is separate from the one controlled by TOR/IIS axis.

As I have demonstrated that PGRP-LC and Relish also act in the same pathway in the context of cell death, the next question was, what is activating PGRP-LC and initiating subsequent Relish activation? Microbes were ruled out as possible candidates as axenic flies show normal gland degradation. Interestingly it has been observed that the molting hormone 20-hydroxyecdysone (20E), which is essential for fly development and metamorphosis, induces *PGRP-LC* expression.

Ecdysone Pathway

Ecdysone is secreted from the prothoracic ring glands into the hemolymph and processed by several enzymes to produce the molting hormone 20-hydroxyecdysone (20E). Two different nuclear receptors, ecdysone receptor (EcR) (homolog of vertebrate farnesoid receptor or liver X receptor) and ultraspiracle (USP) (homolog of retinoid X receptor), form a heterodimer complex, which recognizes this particular form of ecdysone (Koelle et al., 1991). EcR has three different isoforms; -A, -B1 and-B2 while USP has only one. Three isoforms of EcR has similar DNA and ligand binding domains but they differ at the amino termini(Talbot et al., 1993). Differential expression isoforms led to tissue specific responses of 20E during development, as evidenced by the observations that EcR-A mutants display improper salivary gland degradation and abnormality in leg development while metamorphosis is blocked in EcR-B mutants, which also exhibit defects in tanning of pupa and neuronal network formation (Bender et al., 1997). The EcR-B isoforms are strong activators of gene expression due to their AF-1 domains. During each stages of the developmental cycle, there is a pulse of 20E that propels the animal towards next phase of development. Upon 20E binding, the EcR and USP complex initiate different gene expression, which are categorized into early and late response genes, depending on their time of expression. The early response class of genes, that are expressed during larval to prepupal transition, includes *Broad complex (Br-C), E74, E75,* which are transcription factors themselves. In addition to these early genes, a second 20E pulse that results in the prepupal to pupal transition also activates the helix-turn-helix factor *E93.* These transcription factors are responsible for the expression of late response genes that includes pro-apoptotic genes *reaper* and *hid (Baehrecke and Thummel, 1995; Woodard et al., 1994)*.

In an earlier publication from our lab, we observed that 20E alone could drive the expression of various Imd pathway genes such as *PGRP-LC* in *Drosophila* cell lines (Rus et al., 2013). I also noticed during metamorphosis, when the 20E titer is particularly high, *PGRP-LC* and *Relish* expression is also upregulated in the salivary glands. This suggests that 20E or ecdysone might be the stimulus that drives *PGRP-LC* expression, which leads to Relish cleavage and subsequent autophagy induction. However as the ecdysone controls both apoptosis and autophagy during salivary gland degradation and is also involved in plethora of developmental pathways, it is rather difficult to ascertain the possible role of ecdysone in controlling autophagy through *PGRP-LC* and *Relish*. However identification and selective mutation of ecdysone responsive elements in *PGRP-LC* and possibly *Relish* promoters might be useful to address this question.

Relish mediated cell death is tissue specific and also varies depending on the stimuli. For example, in a previous report *Relish* is necessary for the light dependent cell death of photoreceptor cells. In another, during cell competition unfit cells are killed through the involvement of both *Relish* and Toll pathway components (Chinchore et al., 2012). However in both cases the cell death appears to be caspase dependent. In our model, we have shown that *Relish* controls another form of cell death, called autophagy, in the salivary glands of Drosophila. Interestingly, autophagy mediated cell death is also observed during the degradation of midgut and that event is also ecdysone dependent. However we did not find any link between autophagy and *Relish* during midgut histolysis, suggesting Relish mediated autophagy induction is tissue specific. These observations also imply that *Relish* can influence both caspase dependent and autophagic form of cell death in different scenarios. In addition, according to our hypothesis ecdysone dependent activation of *Relish* is important, however high titer of ecdysone in midgut did not show any Relish dependency to induce autophagy. So, probably differential expression of ecdysone receptors in different tissues might be driving *Relish* activation or there are other tissue specific stimuli that results in the activation of *Relish* and subsequent autophagy induction.

Another interesting aspect of my research is the involvement of PGRP receptors in developmentally programmed salivary gland degradation. Both the membrane bound receptor *PGRP-LC* and cytosolic receptor *PGRP-LE* is found to be necessary for proper gland degradation. Ectopic expression of *PGRP-LC* causes premature gland degradation and autophagy, further justifying that PGRP receptors are both necessary and sufficient to induce autophagic cell death.

In *Drosophila* PGRP receptors were known as part of the Imd pathway, which is activated upon Gram-negative bacterial infection. However *PGRP-LE* was also found to be necessary for autophagy induction upon intracellular pathogen

Listeria monocytogenes infection. Interestingly, even in that case the classical Imd pathway did not seem to be involved. This leaves us with several questions, are *PGRP-LE* mediated autophagy upon Listeria infection and *PGRP-LC* and –*LE* mediated autophagy in salivary gland completely different? It's quite possible that in different situations and under different stimulation the receptors behave differently and engage different pathways to accomplish the same thing, induction of autophagy. However, as we already discussed that same components can behave differently in different tissues, such as *Relish* controlling either Caspase dependent or autophagic cell death, there is a possibility that those pathways might utilize similar factors. In both cases classical Imd pathway did not seem to be involved so it would be really interesting to identify components that link those two pathways together.

Mammalian PRRs and Autophagy

In mammalian system pattern recognition receptors (PRR) play necessary roles in activating autophagy. The TLR4 recognizes bacterial lipopolysaccharide and that leads to the activation of ubiquitin ligase TRAF6, which ubiquitinates Beclin1 resulting in its dissociation from Bcl2, thus promoting autophagy. Interestingly, stimulation of TLR4 pathway also leads to the activation of NFκB, which causes *A20* transcription. A20 negatively regulates autophagy by de-ubiquitinating Beclin1 (Shi and Kehrl, 2010b). Cytosolic NOD receptors influence autophagy by interacting with Atg16L1 and localizing it to the plasma membrane, particularly to the entry site of microorganisms. By contrast the receptors NLRC4 and NLRP4 inhibit autophagy by sequestering Beclin-1. The NACHT domain of these two proteins interacts with Beclin-1 ECD domain and this interaction is necessary for the inhibition of autophagy (Jounai et al., 2011). Autophagy is also activated through the IKK complex utilizing different mechanisms. The canonical IKK complex activates autophagy through AMPK, which phosphorylates ULK1, upstream component of the autophagy pathway. Surprisingly, this mode of IKK mediated autophagy initiation is NFκB independent (Comb et al., 2011).

Hormonal Involvement in mammalian Innate Immunity

Hormonal control of PRR expression and immune function became an exciting field as it indicates possible immunomodulatory roles of hormones. Hormones, particularly the glucocorticoids play important role in regulation of both innate and adaptive immune pathways. It has been observed that glucocorticoids dampen cytokine production, as evidenced by the enhanced production of IL-12, TNF, IL-1β, and IL-6 in conditional glucocorticoid receptor mutant mice. These mice also show higher mortality compared to wild type controls (Bhattacharyya et al., 2007). The inhibition of cytokine production by this type of hormones is accomplished by different ways, such as inhibiting of NFκB activity either by direct sequestration or coding of other NFκB inhibitors, like IκB and glucocorticoid-induced leucine zipper protein (GILZ). However, prolonged signaling through TLRs in pDCs (Plasmacytoid Dendritic Cells) circumvents this particular mode of inhibition (Guiducci et al., 2010; Scheinman et al., 1995). Surprisingly in other studies it has been demonstrated that glucocorticoids can also act as immunoenhancers. Low dose of corticosterone activates inflammatory gene expression in macrophages, which were primed with LPS and IFN-γ. Pro-inflammatory cytokine level is increased in rats upon glucocorticoid treatment, however that effect decreases following LPS exposure. In respiratory epithelial cell line glucocorticoid treatment enhances TLR2 expression. Furthermore, NACHT, LRR and PYD domains-containing protein 3 (NLRP3) expression is also upregulated in glucocorticoid treated primary and immortalized macrophages (Dhabhar and McEwen, 1999; van de Garde et al., 2014).

This curious observation that glucocorticoid performs both immunostimulatory and immunosuppressive actions in cells led to the hypothesis that immunomodulatory role of glucocorticoids is dose-dependent. At low concentration this hormone exposure results in the expression of PRRs potentiating cytokine expression, however high concentration diminishes that response (Sapolsky et al., 2000).

This hypothesis might prove useful in explaining the ecdysone mediated cell death through *PGRP-LC* and *Relish* in salivary glands. Comparatively higher dose of ecdysone at 0 hour during larval to prepupal transition does not induce autophagy in the salivary gland. By contrast, lower surge of the same hormone drives salivary gland degradation at 14 hour, during prepupal to pupal transition.

However, ecdysone mediated salivary gland degradation is dependent on PGRP-LC and Relish. So, it is possible that comparatively low dose of ecdysone in the glands causes *PGRP-LC* upregulation, Relish cleavage and subsequent autophagy induction. While, higher dose of ecdysone at 0 hour dampens *PGRP-LC-Relish* expression.

Questions and Future Directions: -

In our model (Fig 4.2) we proposed a novel manner of autophagy regulation through the regulation of key autophagy gene expression by immune receptors and NF-κB factor. However some interesting questions remained.

1. The cleavage of relish in the context of salivary gland degradation was not explored properly. We have established the role of relish as a transcription factor and demonstrated that active cleaved relish is sufficient to induce autophagy, however the caspase Dredd, which cleaves relish, was not involved in gland degradation. This led us to postulate several possibilities, like relish could be cleaved by some hitherto unknown mechanism. In that case relish nuclear translocation in salivary glands and genetic screen for known drosophila proteases in terms of salivary gland degradation defect might be useful to understand the relish cleavage mechanism.

2. RIP Homology Interaction Motif (RHIM) is a conserved protein domain that was discovered in mammalian Receptor-Interacting-Proteins (RIP) (Sun et al., 2002b). This domain is necessary for the association of RIP1 and RIP3 and

further signal transduction. Later it was discovered in other proteins as well, such as the adaptor TIR-domain-containing adapter-inducing interferon-β (TRIF) and cytosolic DNA sensor DAI/ZBP1 (DNA-dependent activator of IRFs/Z-DNA binding protein-1) (Kaiser and Offermann, 2005; Rebsamen et al., 2009). In *Drosophila* RHIM like motifs were discovered in the PGRP receptors, -LC and –LE. This motif is essential for signal transduction and mutation in these motifs blocks IMD pathway activation in response to PGN stimulation (Kaneko et al., 2006). Recently we also demonstrated that similar as mammalian RHIM domains, drosophila RHIM like motifs also form amyloid structure and Drosophila RHIM could replace mammalian RHIM, suggesting the conserved nature of this domain (Kleino et al., 2017). Interestingly, it has also been shown that the death domain containing protein IMD and NFkB factor Relish both have putative RHIM domain

[Putative Relish RHIM Domain-

N-Terminal-

...PYQNQLLNNGGICQLGATNLINSTGVSFGVANVTSFGNMYMDHQYFVP...

C-terminal] (VSFG residues serve as the core of the RHIM domain)

We were interested whether this domain is necessary for the salivary gland degradation as well, since the canonical IMD pathway was not involved. Surprisingly the PGRP-LCx RHIM mutant failed to induce salivary gland degradation upon misexpression (Figure 4.1), suggesting possible role of the RHIM domain in salivary gland degradation. This led us to speculate the possible interaction between RHIM containing proteins, such as PGRP-LC, LE and Relish might be important for autophagy induction in salivary glands. Possible experiments that would prove this hypothesis include (a) interaction of Relish in PGRP receptors in both *Drosophila* Cells and in heterologous expression system. (b) RHIM domain mutation in endogenous Relish and PGRP receptors and check that whether these mutations affect salivary gland degradation.

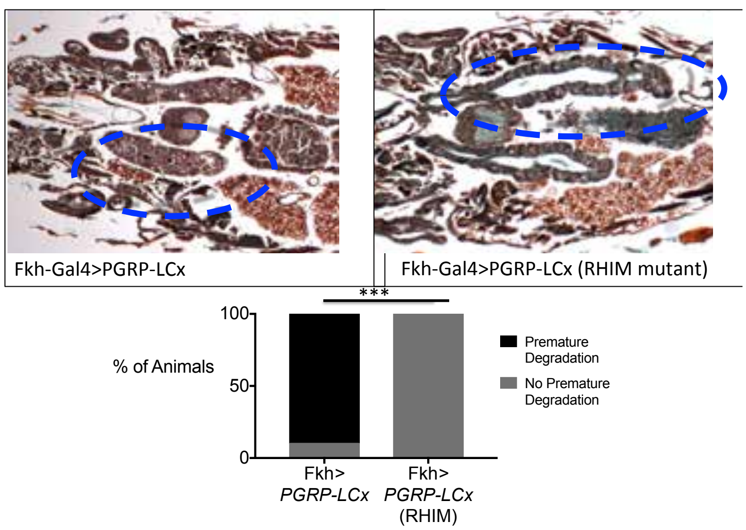

Figure 4.1

Figure 4.1 *PGRP-LCx* RHIM Domain Is Essential For Salivary Gland Degradation

(A) Representative histological sections of animals expressing *PGRP-LCx* in salivary glands of wild type flies (left, w; UAS-*PGRP-LCx*/+; *fkh-Gal4*/+) and *PGRP-LCx* RHIM mutant flies (right, *w;* UAS-*PGRP-LCx*^{RHIM}/+; *fkh-Gal4*/+) (B) Quantitation of 20 independent samples from each genotype as in (A), statistical significance by chi-square test. *** P<0.001

3. Further exploration of the role of the hormone ecdysone in LC-Relish mediated gland degradation is necessary. We have shown we have demonstrated that *PGRP-LCx* and *Relish* expression are upregulated in dying salivary gland and this upregulation occurs at the same time of ecdysone pulse. However the role of ecdysone specifically in this event was not characterized properly. Inhibition of ecdysone signaling in salivary glands either by expressing dominant negative ecdysone receptor or knockdown of ecdysone induced transcription factors and subsequent change in PGRP-LC and Relish expression would be informative. Further, identification of specific ecdysone responsive elements in either *PGRP-LC* or *Relish* promoter region and selective mutation of those elements would also be helpful to elucidate the role of ecdysone in mediating the gland degradation through the expression of *PGRP-LC* and *Relish*.

4. As the canonical IMD pathway is not involved in salivary gland degradation, it is still unclear if there is any external stimulus that involves LC-Relish mediated gland degradation. Currently we are unaware about the any other ligands of PGRP receptors except microbial components. However that does not rule out the existence of such factors. To test whether PGRP receptors require any such ligands for gland degradation, expression of *PGRP* receptors without ligand-binding domain and subsequent monitoring of salivary gland degradation would be worthwhile.

5. It would be interesting to investigate the role of these particular factors, PGRP receptors and Relish in induction of autophagy in other tissues or in different

biological context. For example, we demonstrated that active relish expression results in the eye deformities, which can be suppressed by knockdown of key autophagy genes. Similarly, it has been reported earlier that *PGRP-LE* is involved in autophagy induction in response to *Listeria* infection (Yano et al., 2008). Further experiments to elucidate the role of these components in the context of intracellular pathogens as well as in other tissues of *Drosophila* in response to developmental cues or otherwise, would be of significance.

Although earlier reports demonstrated the role of several PRRs in autophagy induction, none of the findings suggested transcriptional regulation through NF-κB. Hormonal control of cell death and immune responses is well known, however here we showed that hormones can also regulate cell death through previously characterized immune response factors, and possibly this mechanism is dose-dependent. As, all three pathways, immunomodulatory function of hormones, Regulation of autophagic cell death, crosstalk between immune effectors and autophagy components, are of clinical significance, further study would be extremely beneficial for the advancement of drug development.

Figure 4.2

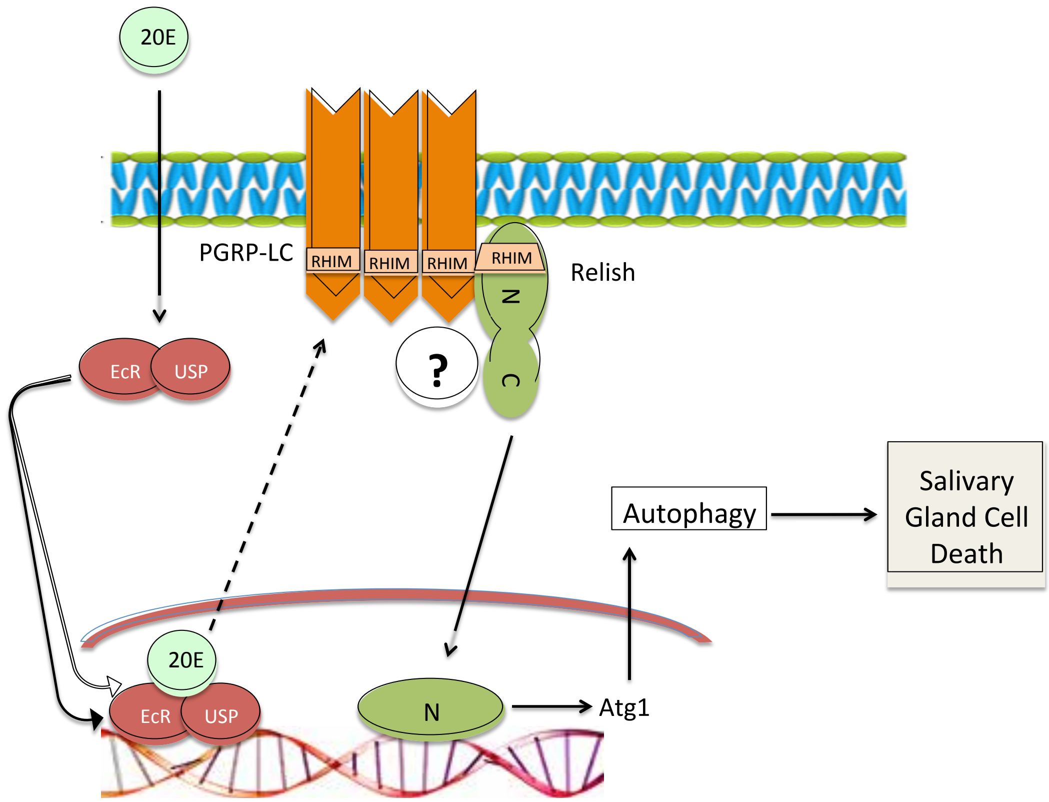

Figure 4.2 *Relish* Mediated autophagy Induction

Our Proposed model shows that external stimuli, possibly the hormone ecdysone induce the expression of PGRP-LC receptor. *PGRP-LC* and Relish interact via the RHIM domain and this interaction causes Relish cleavage and translocation to the nucleus where Relish activates the expression of the key autophagy gene, *Atg1*.

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