

ABSTRACT

Title of Thesis: EXPLORING THE ROLE OF NFκB
 HOMOLOGS IN AUTOPHAGIC CELL
 DEATH IN THE DROSOPHILA SALIVARY
 GLAND

Adrienne Lori Ivory, Master of Science, 2009

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The innate immune response is an ancient, highly conserved means of defense against pathogens. An important mediator of innate immunity is the NFκB (Nuclear Factor-Kappa B) family of transcription factors. Activation of immune-signaling pathways leads to the nuclear translocation of NFκB proteins which initiate the transcription of antimicrobial peptides (AMPs) that circulate and destroy microbes. In *Drosophila*, these AMPs are up-regulated during the destruction of larval salivary glands. Salivary gland cells are destroyed *via* autophagy during metamorphosis. This project sought to determine what, if any, role the NFκB transcription factors have in autophagic cell death. Using the *Drosophila* model, it was determined that a loss of AMP activity during metamorphosis results in a failure to completely degrade larval salivary glands, and this defect appears to be due to an inability to remove autophagic vacuoles. It is suggested that AMPs may serve to degrade the membranes of autophagic vacuoles.

EXPLORING THE ROLE OF NF κ B HOMOLOGS IN AUTOPHAGIC CELL
DEATH IN THE *DROSOPHILA* SALIVARY GLAND

By

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Chapter 1: Introduction & Background Information

1.1 Introduction

The innate immune response provides organisms with a broad-spectrum, rapid means of defense against invading pathogens. Features of the innate immune response in mammals, for example, include the barrier function of epithelial tissues, the production and secretion of antimicrobial peptides, and the uptake and destruction of microbes by professional phagocytes.

An important mediator of the innate immune response is the highly-conserved family of transcription factors known as Nuclear Factor-Kappa B (NF κ B). Members of this family of proteins have been identified throughout the Metazoans (Hoffmann & Reichhart, 2002). All NF κ B proteins contain a conserved N-terminal sequence known as a REL homology domain, and it is through this domain that they are regulated by the I κ B (Inhibitor of NF κ B) proteins. Under normal conditions (i.e., the absence of an immune stimulus) I κ B proteins bind to NF κ B proteins and sequester them in the cytoplasm. Immune challenge leads to signaling events that activate I κ B kinases which phosphorylate I κ B proteins, which are ultimately ubiquitinated and degraded, thereby releasing NF κ B factors to translocate to the nucleus and initiate the transcription of immune responsive genes. In mammals, NF κ B proteins regulate the transcription of genes involved in inflammation, as well as genes that coordinate the activation of the adaptive immune response (Ghosh & Hayden, 2008).

Intensive research into the complex regulation of NFκB proteins in mammalian immunity has led to the discovery of signaling pathways such as the Toll-like Receptor (TLR) and Tumor Necrosis Factor (TNF) pathways. The Toll-like Receptors (TLRs) are a family of transmembrane proteins that recognize and directly bind to pathogen-associated molecular patterns (PAMPS) from microbes ranging from bacteria to fungi to viruses (Arancibia et al., 2007). TNF is a cytokine produced by macrophages that have been stimulated by the LPS (lipopolysaccharide) of Gram-negative bacteria (Locksley et al., 2001). In both cases, activation of these pathways causes a signaling cascade which culminates in the nuclear translocation of NFκB proteins (Ghosh & Hayden, 2008).

Faulty regulation of NFκB proteins can lead to a number of human pathologies. For example, overexpression of NFκB proteins has been implicated in autoimmune diseases such as rheumatoid arthritis (Okamoto et al., 2008) and inflammatory bowel disease (Atreya et al., 2008). Due to the clinical significance of NFκB and associated signaling pathways, a thorough understanding of how this system functions and how its activity affects other aspects of host physiology is essential to developing safe and effective therapies for a number of important diseases.

There is ample evidence tying NFκB and associated pathways to the process of programmed cell death *via* apoptosis. However, little research has been conducted into the possible involvement of NFκB in an alternative form of cell death, specifically death *via* autophagy. The experiments described in this thesis were undertaken in an attempt to elucidate, using the *Drosophila* model, how the NFκB

transcription factor Relish and its antimicrobial peptide targets effect the destruction of native tissues by autophagy.

1.2 The *Drosophila* Humoral Immune Response

The humoral response in *Drosophila* is mediated largely by the fat body which, upon immune challenge, produces antimicrobial peptides which it then secretes into the hemolymph so that they can circulate throughout the fly and destroy invading pathogens (Bulet et al., 1999). Transcription of the AMP genes is coordinated by two distinct signaling pathways: Toll and Imd (Immune Deficiency), which are homologous to the mammalian Toll-Like Receptor and Tumor-Necrosis Factor pathways, respectively (Fig. 1). The Toll pathway serves to detect and initiate an immune response to fungal pathogens, viruses and Gram-positive bacteria, whereas the Imd pathway mediates the response to Gram-negative bacteria (Lemaitre, 1997).

Host recognition of Gram-positive bacteria or fungal pathogens initiates a serine protease cascade resulting in the activation of the cytokine, Spätzle (Kambris et al, 2006). Spätzle is the ligand for Toll, a transmembrane receptor with a cytoplasmic TIR (Toll/IL-1R) domain (Hashimoto et al., 1998)). Binding of Spätzle to the Toll receptor causes the assembly of a signaling complex which includes the cytoplasmic portion of Toll along with the death domain (DD) proteins dMyD88 (the *Drosophila* homolog of Myeloid Differentiation primary response protein) and Pelle, a

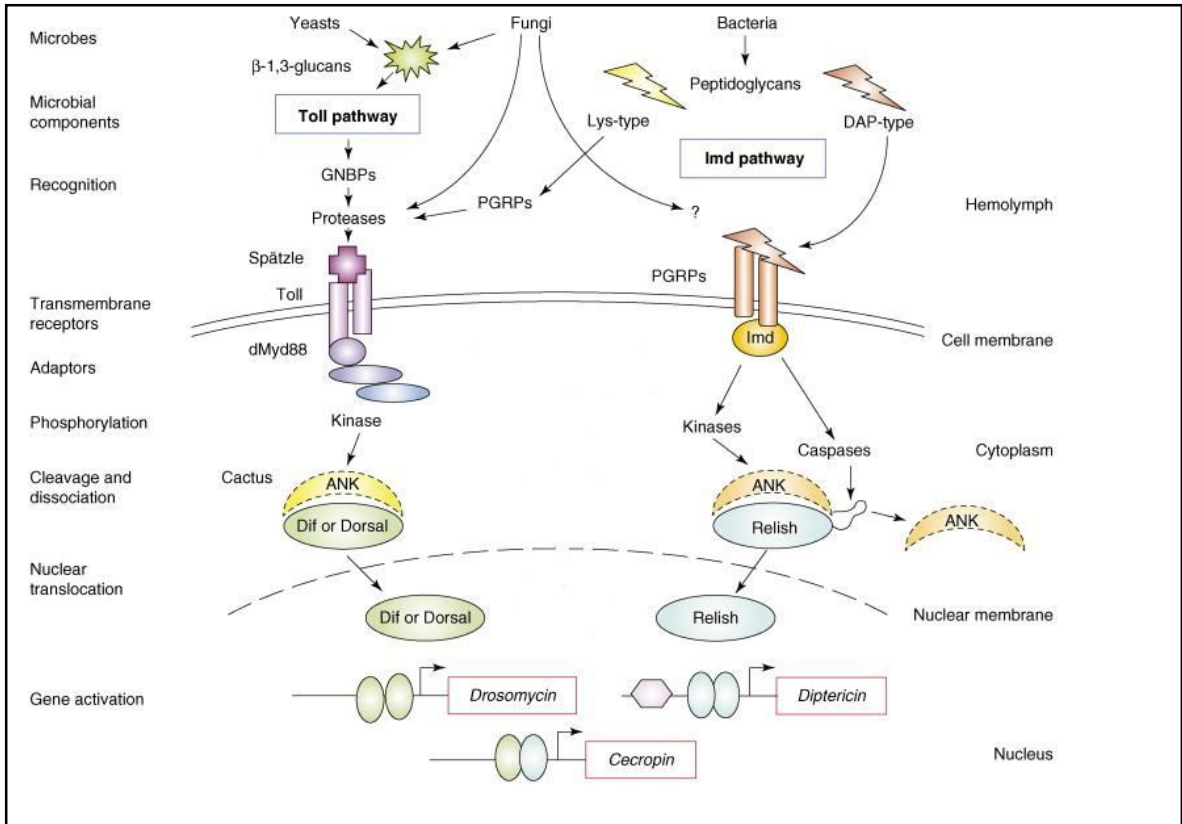


Fig. 1 The *Drosophila* Humoral Immune Response Is Regulated By NFκB Proteins. This figure illustrates the two pathways, Toll and Imd, that regulate the NFκB transcription factors in *Drosophila*. GNBPs= gram-negative bacterial-binding proteins; PGRPs= peptidoglycan recognition proteins; ANK= inhibitory ankyrin domain. (Figure adapted from Uvell & Engström, 2007)

serine/threonine kinase (Horng & Medzhitov, 2001; Tauszig-Delamasure et al., 2001; Sun et al., 2002; Charatsi et al., 2003; Hu et al., 2004; Sun et al., 2004; Moncrieffe et al., 2008). MyD88 is an adaptor protein which, like Toll, has a TIR domain. The assembly of the Toll signaling complex results in activation of Pelle's kinase activity, which in turn phosphorylates Cactus, a homolog of the mammalian IκB (Inhibitor of NFκB) that binds to the NFκB transcription factors Dorsal and Dif (Dorsal-related Immune Factor) to sequester them in the cytoplasm (Lemaitre et al., 1995; Nicolas et al., 1998; Meng et al., 1999). Once Cactus is degraded, Dorsal and/or Dif can translocate to the nucleus and initiate transcription of AMP genes with activity against Gram-positive bacteria and/or fungi. (Meng et al., 1999; Manfruelli et al., 1999).

The Imd pathway controls the humoral response to Gram-negative bacteria. Activation of the pathway is initiated when the transmembrane protein PGRP-LC, and in some cases the circulating form of the protein PGRP-LE, bind to and recognize Gram-negative peptidoglycan (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002; Kaneko et al., 2004; Kaneko et al., 2005; Kaneko et al., 2006). PGRP-LC activates Imd, a death domain protein similar to the mammalian RIP (Receptor Interacting Protein), by directly binding to a unique N-terminal domain (Georgel et al., 2001; Gottar et al., 2002, Choe et al., 2005). The exact mechanisms by which the Imd pathway ultimately leads to the transcription of AMP genes are not yet entirely clear, but many of the major components of the pathway have been identified. The active form of Imd forms a complex with the caspase Dredd (Death related ced-3/Nedd2-like protein) *via* the death adaptor protein dFADD (the *Drosophila* homolog

of the mammalian FADD, Fas-Associated protein with Death Domain) (Hu & Yang, 2000; Leulier et al., 2002; Zhou et al., 2005). The formation of this complex is dependent upon DIAP2 (the *Drosophila* homolog of IAP2, Inhibitor of Apoptosis Protein 2) (Leulier et al., 2006). The Imd/dFADD/Dredd complex activates the MAPKKK (Mitogen Activated Protein Kinase Kinase Kinase) homolog dTAK1, which activates the IKK (*Drosophila* I κ B Kinase) complex as well as the JNK pathway (Vidal et al., 2001; Zhou et al., 2005). Ultimately, the NF κ B protein Relish is activated through the cleavage of its I κ B-like inhibitory domain. This cleavage is downstream of the I κ B Kinase (IKK) complex and the caspase Dredd (Vidal et al., 2001). It has been suggested that Relish is phosphorylated by IKK and subsequently cleaved by Dredd (Stöven et al., 2003, Zhou et al., 2005). Once cleavage of Relish is achieved, the N-terminal portion of Relish translocates to the nucleus to initiate transcription of AMP genes with activity against Gram-negative bacteria (De Gregorio et al., 2002).

There are seven known families of inducible AMPs in *Drosophila*, and all are regulated by one or both of the above mentioned pathways. Although the mode by which these peptides lead to the destruction of pathogens is not known for certain, *in vitro* studies of *Drosophila* AMPs (or their orthologs in other insects) have allowed for speculation as to their roles *in vivo*. For example, it has been demonstrated *in vitro* that Attacin, Cecropin, Defensin, Diptericin, and Drosomycin permeabilize the membranes of their targets, which leads to cell lysis (Carlsson et al., 1991; Gazit et al., 1994; Cociancich et al., 1993; Winans et al., 1999; Tian et al., 2008). Attacin and Diptericin target Gram-negative bacteria (Carlsson et al., 1991; Rabel et al., 2004;

Cudic et al., 1999; Winans et al., 1999), and Defensin acts upon Gram-positive bacteria (Cociancich et al., 1993; Dimarcq et al., 1994). Drosomycin exhibits activity against hyphae-forming fungal pathogens (Michaut et al., 1996; Tian et al., 2008), and Cecropin targets both Gram-positive and Gram-negative bacteria (Carlsson et al., 1991; Gazit et al., 1994; Rabel et al., 2004). Drosocin, which acts against Gram-negative bacteria, has also been studied *in vitro*, but the mode by which it contributes to pathogen destruction remains to be discovered. It has been determined that, unlike the other AMPs studied, it does not contribute to membrane permeabilization, but instead acts in an unknown manner on a stereospecific target (Bulet et al., 1993; Bulet et al., 1996). The only AMP that has not yet been studied *in vitro* is Metchnikowin. The peptide has, however, been isolated from *D. melanogaster* and characterized, and it has been shown that it is up-regulated in response to infection with Gram-positive bacteria as well as fungal pathogens (Levashina et al., 1995).

Both the Toll and Imd pathways can activate transcription of each AMP, and it has been suggested that the two pathways act synergistically to promote a more effective humoral immune response (Tanji et al., 2007; Pal et al., 2008). *Drosomycin* transcription is generally considered to be under the primary control of the Toll pathway (Meng et al., 1999; Manfrulli et al., 1999), and the *Diptericin* and *Drosocin* genes are predominantly controlled by the Imd pathway (De Gregorio et al., 2002). Both pathways contribute to the transcription of the remaining peptides (Hedengren et al., 1999; Rutschmann et al., 2000).

The fat body is not the only site of AMP production. It has been discovered that epithelial cells also produce AMPs, and this localized expression of AMP genes

is under the exclusive control of the Imd pathway (Ferrandon et al., 1998; Tzou et al., 2000; Onfelt Tingvall et al., 2001). A valuable tool for following real-time expression of AMP genes *in vivo* has been developed through the generation of transgenic *Drosophila* lines bearing reporter constructs in which the gene for the Green Fluorescent Protein (GFP) is fused to the promoter of each AMP gene (Tzou et al., 2000). There is no detectable GFP signal in the fat body of these reporter lines in the absence of immune challenge, but upon infection, the expression patterns of both GFP mRNA and protein correspond to the patterns of endogenous AMP expression patterns in wildtype flies. The accuracy of the reporter lines was further demonstrated by showing, through the use of HPLC and mass spectrometry, that wildtype levels of the AMPs Drosocin and Drosomycin are present in GFP-expressing epithelial tissues. Use of these reporter lines, however, is not without caveats. For example, tracking a real-time GFP signal is qualitative, and weaker signals may not be detected visually. Another possible problem is that the promoters of the AMP genes *Attacin* and *Defensin* have not been dissected, so there is no way of knowing for certain that the constructs used to generate these fly lines contained a sufficient portion of these promoters to serve as accurate reporters.

1.3 Autophagic Cell Death: An Alternative to Apoptosis

Autophagy is a highly conserved process which has been best characterized as part of the starvation response in yeast. In the absence of vital nutrients, cells degrade cytosolic components in order to recycle amino acids necessary for survival. Cell

components targeted for degradation are first isolated in a double-membrane bound organelle, the autophagosome. Assembly of this membrane is dependent upon a set of genes known as *atg* genes (Tsukada & Ohsumi, 1993). Once the autophagosome is complete, it fuses with a lysosome and its contents are processed and recycled by the cell (Fig. 2).

During development, autophagic vacuoles can serve to degrade native cells as a means of effecting cell death rather than survival. Studies of the destruction of *Drosophila* larval salivary glands during metamorphosis have provided numerous insights into how this form of cell death occurs. Dying larval salivary glands exhibit signs of autophagy as well as apoptosis. Destruction of salivary glands begins immediately following the pulse of the hormone ecdysone which occurs at approximately 10 to 12 hours after puparium formation (apf) (Fig. 3). This time point is accompanied by a rise in the transcription levels of *atg* genes as well as cell death genes including caspases and apoptosis-promoting genes such as *reaper*, *grim*, and *hid* (Gorski et al., 2003; Lee et al., 2003; Martin & Baehrecke, 2004). Dying salivary glands observed *via* light and electron microscopy exhibit signs of autophagy (Fig. 4). At 12 hours apf, numerous large autophagic vacuoles containing cellular components such as mitochondria appear in larval salivary glands. By 14 hours apf, vacuoles appear much smaller, and the cytoplasm soon begins to bleb and fragment. Within 15-16 hours apf, the larval salivary glands are completely destroyed. Throughout this process, phagocytic cells are conspicuously absent (Lee & Baehrecke, 2001; Martin & Baehrecke, 2004). Recent work by Berry & Baehrecke (2007) has conclusively shown that destruction of larval salivary glands is, in fact, dependent upon the

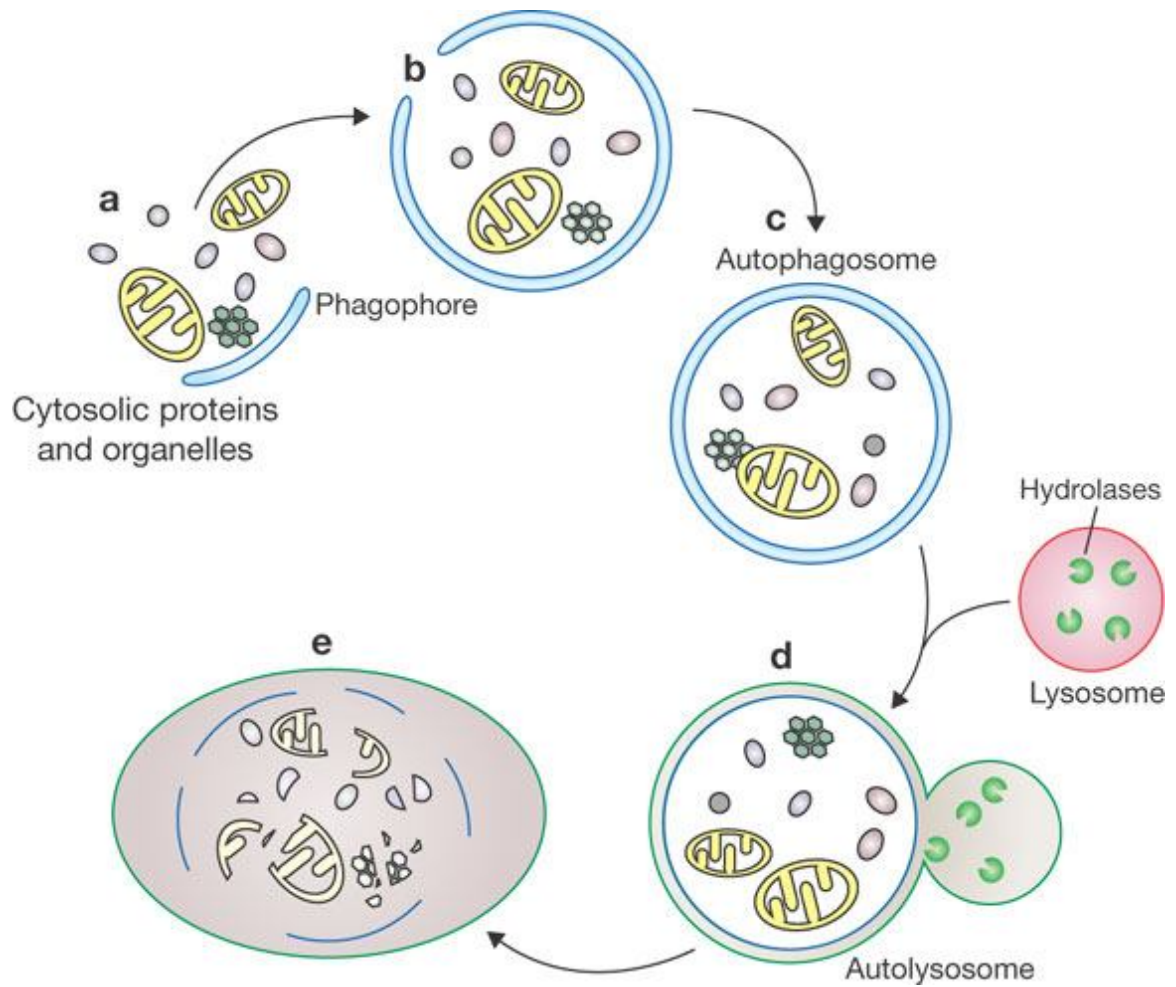


Fig. 2 Autophagy Allows Cells To Recycle Cytosolic Components In Response To Starvation. Starved cells can autonomously degrade non-vital native structures in order to harvest essential proteins. This is accomplished by sequestering cytosolic components in a double-membraned autophagosome which later fuses with a lysosome to form an autophagolysosome. In the mature autophagolysosome, the targeted structures are digested and their proteins recycled to promote cell survival. (Figure from Xie & Klionsky, 2007)

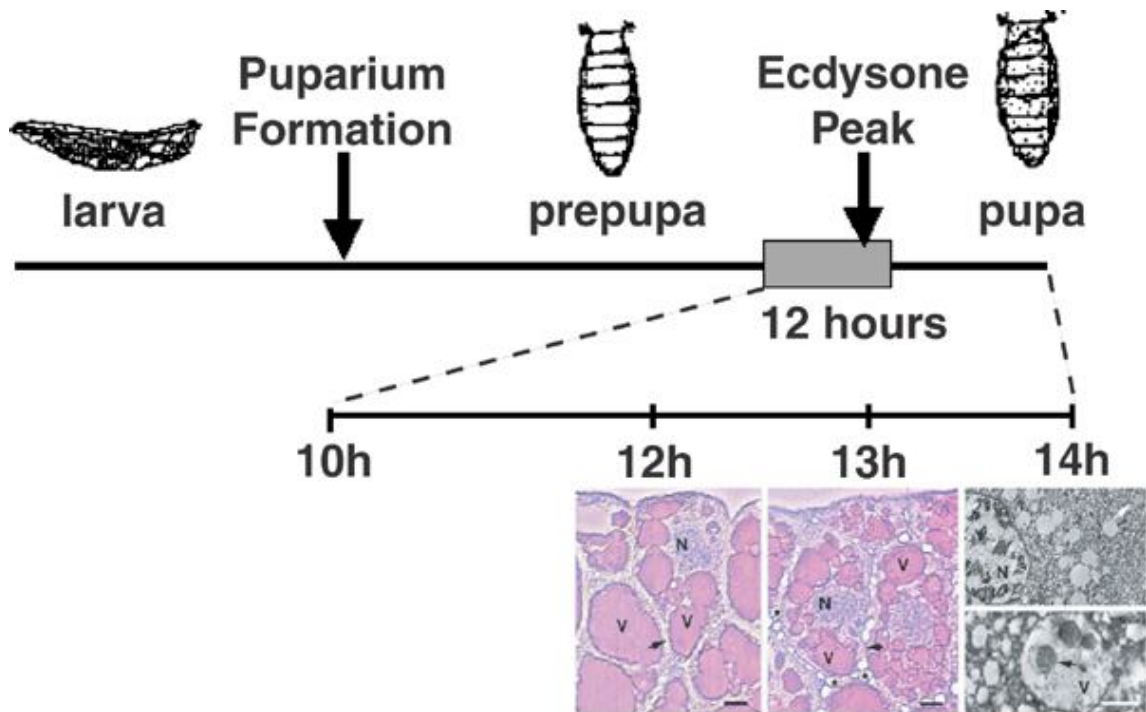


Fig. 3 *Drosophila* Larval Salivary Glands Are Destroyed By Autophagy.

Destruction of larval salivary glands is a highly predictable process. Twelve hours after puparium formation (apf), salivary glands contain large autophagic vacuoles (V). By 14 hours apf, the vacuoles contain cellular components such as mitochondria. Within two hours, the glands are completely destroyed. (Figure from Baehrecke, 2003)

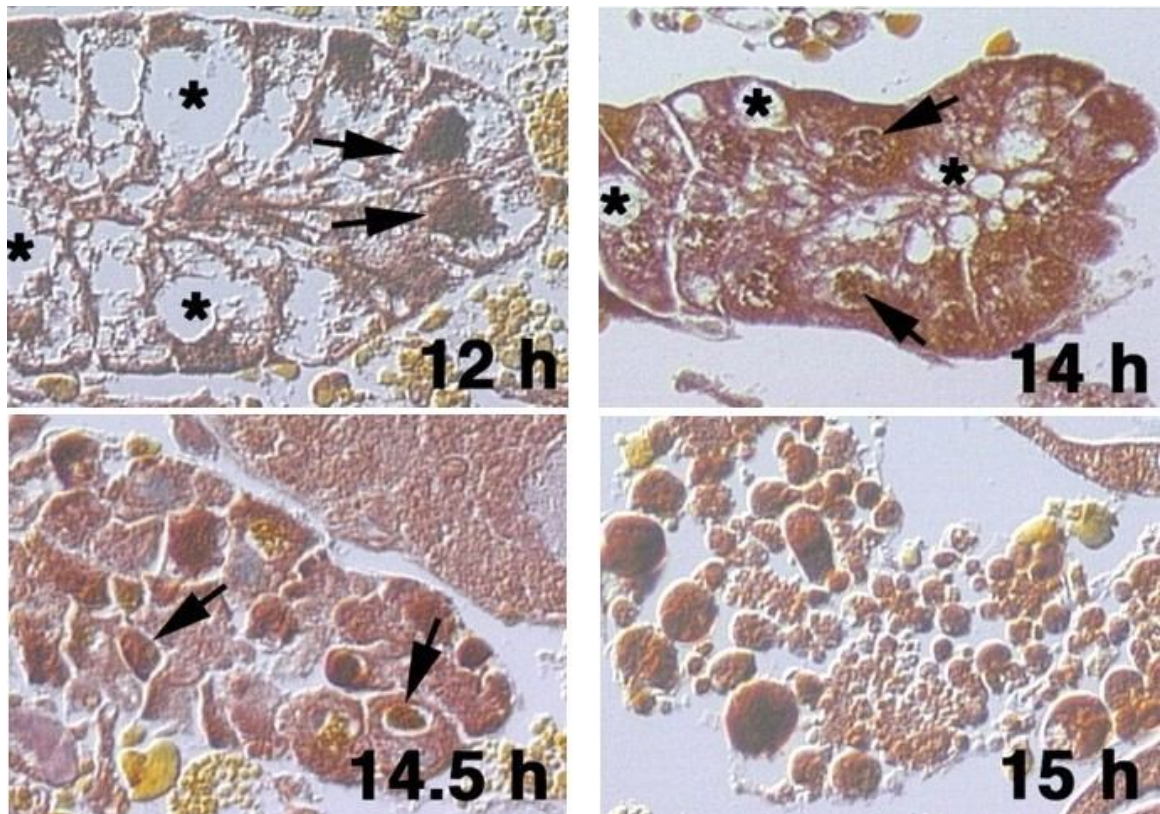


Fig 4 Salivary Gland Destruction Via Autophagy Occurs Between 12 And 16 Hours After Pupa Formation. Following the ecdysone peak at 12 hours apf, larval salivary glands exhibit a large number of autophagic vacuoles. By 14 hours apf, the vacuoles have begun to collapse and the glands have begun to condense. Within one hour, the remaining tissue has begun to disperse and disappear. Asterisks = vacuoles; arrows = nuclei. (Figure from Martin & Baehrecke, 2004)

function of *atg* genes, and that ectopic expression of *Atg1* actually leads to premature activation of salivary gland death.

Mutants in which the pathways controlling cell growth or death have been disrupted display varying degrees of persistent salivary glands at 24 hours apf. Persistent glands can be left completely intact, or may be partially degraded and exist only in fragments scattered throughout the fly's thorax and abdomen. Such fragments may be large or small, and typically appear to have progressed to the point in degradation at which the autophagic vacuoles have disappeared (Berry & Baehrecke, 2007; Martin et al., 2007; Dutta & Baehrecke, 2008). In a few cases, there have been reports of a phenotype in which persistent glands exist in fragments that contain abnormally large autophagic vacuoles. This form of persistent salivary gland has been observed in flies with mutations in various *atg* genes (Berry & Baehrecke, 2007) as well as in flies in which expression of *salvador* (a regulator of both cell death and proliferation) has been knocked down through RNAi (Dutta & Baehrecke, 2007).

1.4 NF κ B Activity During Autophagic Cell Death in the Salivary Gland

High-throughput studies of gene expression and protein content in dying salivary glands have revealed that various components of the Toll and Imd pathways, as well as antimicrobial peptides (AMPs) are upregulated during autophagic cell death. DNA microarrays revealed that, at the onset of salivary gland cell death, transcription levels of seven immunity genes (including *Cactus* and *Dif*) increased five-fold or more (Lee et al., 2003). A similar study using SAGE (serial analysis of gene expression) and qRT-PCR (quantitative real-time PCR) reported that transcription levels of *Cactus*, *Cecropin*, *Defensin*, and *Drosocin* increase

dramatically during salivary gland cell death (Gorski et al., 2003). Proteomic analysis of salivary glands have shown that a number of immune-related proteins including Cactus, Dif, Attacin, Defensin, Drosocin, and Metchnikowin are not present before cell death begins (i.e., at 6 hours apf) but are detectable in dying glands (i.e., at 13 hours apf) (Martin et al., 2007).

Only one study has been conducted to investigate the possible involvement of NFκB homologs in salivary gland cell death. Northern blots of RNA taken from salivary glands before and during autophagic cell death showed detectable levels of *Relish* RNA at 2 hours apf, and *Dif* and *cactus* RNA was detected at 12 hours apf. The authors examined flies with mutations in each of the NFκB transcription factors and did not find evidence of persistent salivary glands (Lehmann et al., 2002). In this study, persistence of salivary glands was determined by dissecting pupae and looking for remaining glands. Although this methodology would be adequate for identifying mutants in which glands remained completely intact, it is too crude a method to detect the more subtle phenotypes in which mere fragments persist. It is therefore possible that the only study which attempted to determine whether NFκB is involved in autophagic cell death overlooked important evidence in support of this possibility.

Chapter 2: Methods

2.1 Staging of *Drosophila* Pupae

Drosophila pupae from both wildtype (Canton-S and Oregon-R) and experimental lines were staged visually. White prepupae were collected from vials of fly lines maintained at room temperature on standard media. Prepupae were placed at 25°C until they reached the desired developmental stage, as determined by hours after puparium formation (apf). Development was considered to be progressing normally based on the occurrence of head eversion at 12 hours apf.

2.2 AMP Expression Profiling Using GFP-AMP Reporter Lines

Reporter lines for each individual AMP have been developed by fusing the Green Fluorescent Protein (GFP) to the promoters of each AMP (Tzou et al., 2000). These transgenic flies have been used successfully to track the expression of individual AMPs in various epithelial tissues (Tzou et al., 2000). In order to visualize induction of AMP expression during salivary gland cell death, 6 to 27 pupae from each of these transgenic lines were staged at 25°C and photographed at 12, 14, and 16 hours apf using a Discovery V.8 stereoscope (Zeiss). Approximate location of salivary glands was estimated based on familiarity with pupal anatomy as the result of extensive histological examination of dying salivary glands.

2.3 Histology of Salivary Glands

In order to visualize the morphology of larval salivary glands, *Drosophila* pupae were prepared for histological analysis according to established methods (Martin & Baehrecke, 2004; Muro et al., 2006; Berry & Baehrecke, 2007; Martin et al., 2007; Dutta & Baehrecke, 2008). At the appropriate developmental time points, pupae (n>10) were fixed at 4°C in a solution of 1% glutaraldehyde, 4% formaldehyde, 5% glacial acetic acid, and 80% ethanol. Once fixed, pupae were then dehydrated in a series of ethanol dilutions, cleared in xylenes, and embedded in paraffin. Paraffin-embedded specimens were sectioned to 7µm using a rotary microtome. Sectioned pupae were then stained with Weigert's Hematoxylin and Pollack's Trichrome and viewed and photographed using an Axiophot II microscope (Zeiss).

Salivary gland phenotypes were assessed by examining the thorax of each specimen to determine whether or not glands were present. If salivary glands were present, they were evaluated based on their relative size, the presence or absence of autophagic vacuoles, and whether the glands were intact or had begun to fragment and disperse throughout the thorax and/or abdomen.

2.4 AMP Expression Profiling Using Quantitative Real-Time PCR

Quantitative Real-Time PCR (qRT-PCR) was used to profile AMP expression during salivary gland cell death in wildtype (Oregon-R) and *Relish* mutant flies. Salivary glands (>20) were dissected from pupae at 6 and 13.5 hours apf, and RNA was extracted using an RNA STAT-60 kit (Isotex Diagnostics). For each sample,

RNA was used to generate cDNA through a RT-PCR reaction using Superscript II (Invitrogen). The resulting cDNA was measured through Real-Time PCR using primers for each of the seven families of AMPs in an ABI7300. Rp49 was used as a control in each experiment. Reactions were run in triplicate and experiments were repeated three times. Paired t-tests were used to determine the statistical significance of the induction of each AMP at 13.5 hours apf relative to 6 hours apf. Standard deviations of the three repetitions of each experiment for each AMP were also determined. Statistical analyses were performed using Microsoft Excel.

2.5 Driving Constitutive Expression of a Single Antimicrobial Peptide in the Salivary Glands of *Relish* Mutants

In order to determine whether a single antimicrobial peptide was sufficient to restore salivary gland destruction in *Relish* mutants, flies were generated that carried the salivary gland-specific enhancer construct *forkhead (fkh) GAL4*, and the GAL4 target sequence as the promoter of the *Cecropin* gene, in a *Relish^{E20}* background. To generate such flies, stocks were obtained carrying the *UAS-Cecropin* transgene (Tzou et al., 2002) and standard genetic crosses were used to create flies containing this construct in a *Relish^{E20}* background. Standard crosses were also used to generate a fly line carrying the *fkhGAL4* enhancer construct (Henderson & Andrew, 2000) in a *Relish^{E20}* background. Once these two lines were established, they were crossed to obtain *fkhGAL4; UAS-Cecropin; Rel^{E20}* progeny. These flies would have expressed the *Cecropin* gene in salivary glands even in the absence of a functional Relish

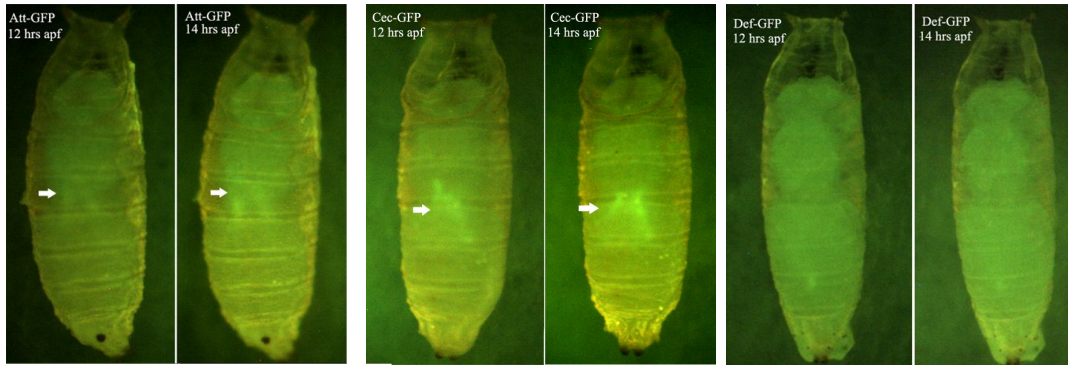
transcription factor. Pupae from this cross were staged to 24 hours apf and prepared for histological analysis as described above. Sections were examined *via* light microscopy to determine whether or not they displayed persistent salivary gland fragments. *fkhGAL4; Rel^{E20}* pupae were used as a control.

Chapter 3: Results

3.1 GFP Reporter Lines Show that AMP Genes are Transcribed in Dying Salivary Glands

Reporter lines for each individual AMP gene have been developed by fusing the Green Fluorescent Protein (GFP) gene to the promoters of each AMP gene, and these flies have been used to visualize AMP expression patterns in epithelia (Tzou et al., 2000). In order to follow expression of AMPs in salivary glands undergoing autophagic cell death, pupae from each AMP-GFP line were staged to 12 hours apf and examined every hour through 17 hours apf.

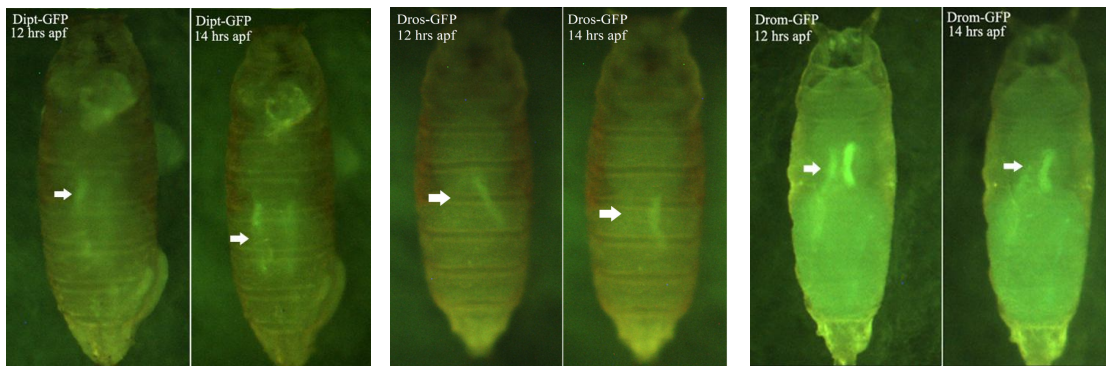
In the majority of pupae observed from *Cecropin-GFP* (100%, n=13), *Diptericin-GFP* (100%, n=7), *Drosocin-GFP* (92.5%, n=27), and *Metchnikowin-GFP* (83.3%, n=6) lines, fluorescent signals were localized to the area around the larval salivary glands (Fig 5). The signals intensified between 12 and 14 hours apf, and faded as salivary glands were destroyed. This same pattern of expression was observed in 50% (n=6) of *Attacin-GFP* pupae. *Drosomycin-GFP* (95.5%, n=22) levels appeared high at all time points and throughout the pupae, with a more intense signal located in the salivary glands. A GFP signal was not detectable in any *Defensin-GFP* (n=19) pupae at any time point.



Attacin (50%, n=6)

Cecropin (100%, n=13)

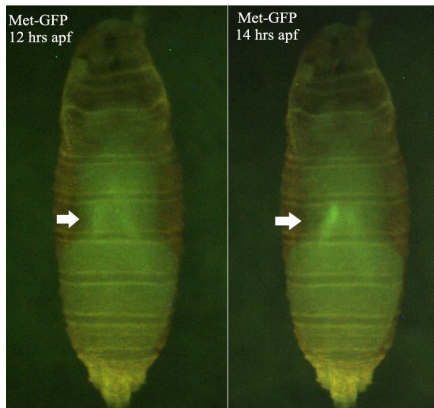
Defensin (100%, n=19)



Diptericin (100%, n=7)

Drosocin (92.5%, n=27)

Drosomyacin (95.5%, n=22)



Metchnikowin (83.3%, n=6)

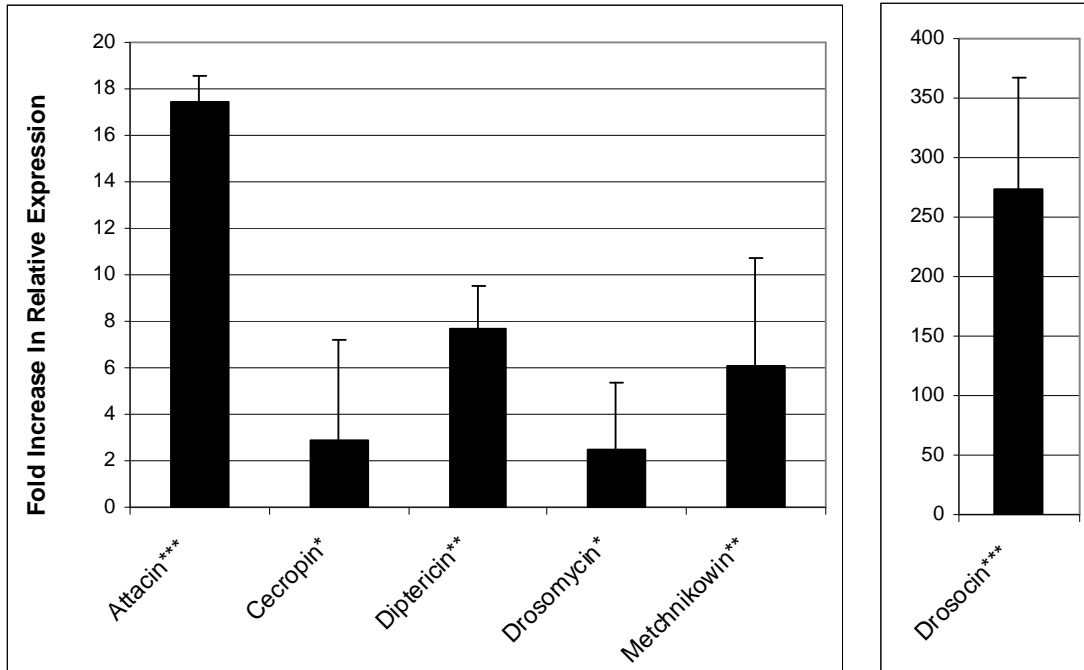
Fig. 5: Localization of Antimicrobial Peptide Gene Expression During Autophagic Programmed Cell Death in *Drosophila* Pupae. Transgenic pupae containing GFP reporter constructs for each antimicrobial peptide were observed *via* fluorescent microscopy during metamorphosis. GFP signals appeared to localize to the area containing larval salivary glands during the time when these organs are typically destroyed by autophagic programmed cell death. For each GFP line, 6-27 pupae were observed beginning at 12 hours apf. Images shown are representative of observed results. Att = Attacin; Cec = Cecropin; Def = Defensin; Dipt = Diptericin; Dros = Drosocin; Drom = Drosomycin; Met = Metchnikowin.

Based on the patterns of GFP expression during salivary gland death, it appears that the majority of AMP genes are transcribed in this tissue during its destruction *via* autophagy. The absence of a detectable signal in *Defensin*-GFP pupae may indicate that this particular AMP gene is not induced during salivary gland cell death, or it may simply be expressed at levels too low to be detected using this visual approach. Another possibility is that the reporter construct did not contain a sufficient portion of the *Defensin* promoter.

3.2 qRT-PCR Confirms that AMP Genes are Up-Regulated at the Onset of Autophagic Cell Death in Salivary Glands

Once it was confirmed that AMP genes are transcribed in dying salivary glands, qRT-PCR was used to obtain a more precise, quantitative characterization of this trend. RNA was isolated from wildtype salivary glands before and during the time period when autophagic cell death of this tissue occurs (i.e., at 6 and 13.5 hours apf). Equal volumes of RNA from each sample were subjected to RT-PCR, and the cDNA products were used to determine the relative expression levels of each AMP *via* qRT-PCR. Each reaction was performed in triplicate, and each experiment was repeated three times using Rp49 as a control.

Analysis of qRT-PCR data showed that all seven AMPs are up-regulated during salivary gland cell death (Fig. 6). The most dramatically up-regulated AMP genes were *Attacin* and *Drosocin*, with averages of 17.44- and 274.13- fold increases



*p<0.10 p**<0.05 p***<0.0005

Fig. 6: Antimicrobial Peptide Genes are Up-regulated During Salivary Gland Destruction. Quantitative Real-Time PCR was used to measure expression levels of each AMP in salivary glands during autophagic cell death. Data are represented as the fold-change in mRNA levels in wildtype salivary glands at 13.5 hours apf (i.e., during cell death) relative to wildtype (Oregon-R) glands at 6 hours apf (i.e., before the onset of cell death). Results depicted are means calculated from three independent experiments. Each reaction was performed in triplicate, and Rp49 was used as a control. Error bars show standard deviation.

($p < 0.0005$) in transcription levels relative to expression levels at 6 hours apf, respectively.

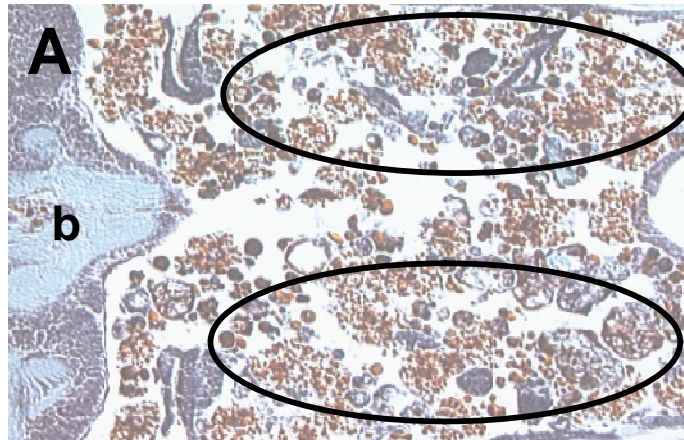
Taken together, the results from both the observation of GFP reporter lines and the qRT-PCR results clearly indicate that dying salivary glands exhibit a dramatic increase in the transcription of AMP genes. These results confirm prior reports that mRNA levels of *Cecropin* and *Drosocin* increase upon the onset of salivary gland destruction (Gorski et al., 2003) and that Attacin, Drosocin, and Metchnikowin peptides are present in dying salivary glands (Martin et al., 2007). This is the first demonstration, however, that the *Diptericin* and *Drosomyacin* genes are also up-regulated during salivary gland cell death.

3.3 *Relish* Null Mutants Display Incomplete Destruction of Larval Salivary Glands

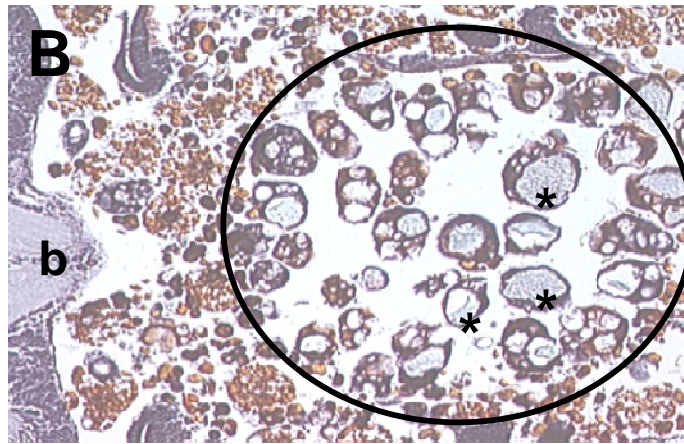
The intriguing results demonstrating the up-regulation of AMP genes in dying salivary glands prompted an investigation into whether these peptides are necessary for autophagic cell death. Another question raised was whether, if AMPs do function in autophagic cell death, they are still regulated by the immune-response pathways or by another independent signaling pathway. It was reasoned that if AMPs are utilized in the destruction of salivary gland cells during metamorphosis, and if they are still regulated by the Toll and/or Imd pathways in this context, then flies expressing null alleles for the NF κ B transcription factors should be unable to effectively eliminate this tissue.

In wildtype flies, salivary gland degradation is largely complete by 16 hours apf, and pupae show virtually no salivary gland remains by 24 hours apf (Lee & Baehrecke, 2001). If pupae from fly lines with the null alleles *dorsal*¹ (Isoda et al., 1992), *Dif*^d (Rutschmann et al., 2000) or *Relish*^{E20} (Hedengren et al., 1999) have persistent salivary glands beyond that time point, this would indicate that these genes or the target genes controlled by their respective pathways are required for destruction of salivary glands.

dorsal, *Dif*, and *Relish* mutant pupae, as well as wildtype (Canton-S) pupae, staged to 24 hours apf were fixed and embedded in paraffin wax, sectioned using a microtome, and stained for examination *via* light microscopy. *Dif* (n=20) and *dorsal* (n=2; analysis was difficult due to a pupal-lethal phenotype) null mutants did not exhibit signs of persistent salivary glands at 24 hours apf. *Relish* null mutants, however, consistently displayed remnants of partially degraded salivary gland cells (55%, n=20) (Fig. 7). The remaining fragments contained what appear to be abnormally large autophagic vacuoles. This seems to indicate that *Relish* and/or the AMPs it regulates are required for the complete destruction of salivary glands. It is important to note that development appeared to progress normally in *Relish* mutants. Head eversion occurred 12 hours apf, just as in wildtype pupae (data not shown). It is unlikely, therefore, that the *Relish* phenotype is due to any developmental delays. The fact that *Dif* and *dorsal* mutants did not have persistent salivary glands indicates that the Toll pathway is not involved in this process, or they may be acting redundantly. An alternative explanation is that these transcription factors overlap in function and therefore only one is necessary for efficient salivary gland cell death.



Wildtype 24 hrs apf



***Relish* mutant 24 hrs apf**

Fig. 7: *Relish* Mutants are Defective in Autophagic Cell Death of Salivary Glands. NF κ B mutant lines were screened for persistent salivary glands at 24 hours apf, a point at which glands are completely destroyed. (A) Salivary glands are absent at 24 hours apf in wildtype (Canton-S) pupae. (B) *Relish* null mutants contain remnants of salivary glands at 24 hours apf, and these remnants contain what appeared to be large, aberrant autophagic vacuoles. Images are representative of 20 samples from each line. b= brain; asterisk= autophagic vacuoles

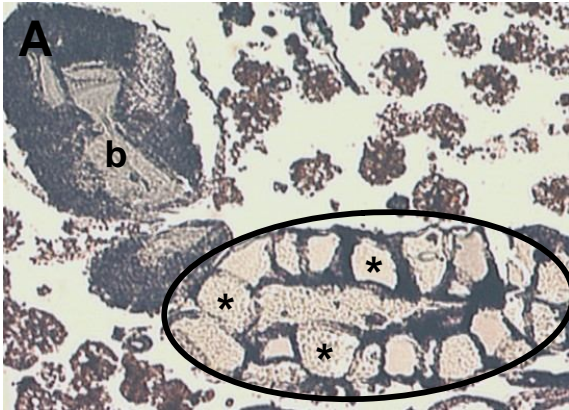
3.4 Autophagic Vacuoles Persist Throughout the Degradation of Larval Salivary Glands in *Relish* Mutants

In order to determine at what point salivary gland destruction is disrupted in *Relish* mutants, pupae from this line as well as wildtype pupae were examined at 12, 14, and 16 hours apf. Histological examination of dying salivary glands throughout this time period made it possible to determine approximately when the morphology of the mutant glands deviates from the normal progression of destruction *via* autophagy.

Beginning at 12 hours apf, dying glands display three distinct phases (Fig. 8). At the onset of cell death, glands are extremely large, occupying a large portion of the thorax. The cytoplasm of individual cells is dominated by extremely large autophagic vacuoles (Fig. 8A). By 14 hours apf, glands appear to condense. The overall size of the gland is considerably reduced, and the autophagic vacuoles have begun to shrink and disappear (Fig. 8B). By 16 hours apf, all that remains of the larval salivary glands are small, scattered fragments. Autophagic vacuoles are no longer present, and individual cells are detached from one another and appear to be dispersing throughout the thorax and abdomen (Fig. 8C).

The early stages of salivary gland destruction appear to progress normally in *Relish* mutants. Glands contain autophagic vacuoles at 12 hours apf. By 14 hours, however, the *Relish* mutant phenotype has begun to appear in a small portion of pupae (26.7%, n=15). The majority of *Relish* mutant pupae (68.4%, n=19) display persistent salivary gland remnants bearing large autophagic vacuoles by 16 hours apf (Fig. 8D). Based on these results, it seems that dying *Relish* mutant salivary glands

fail to lose their autophagic vacuoles. Glands do not properly condense, and when they finally break apart, the fragments are abnormally large and persist well beyond the point at which they are absent in wildtype pupae.



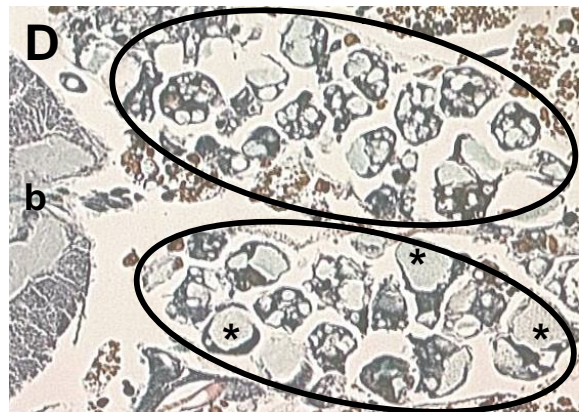
Wildtype 12 hrs apf



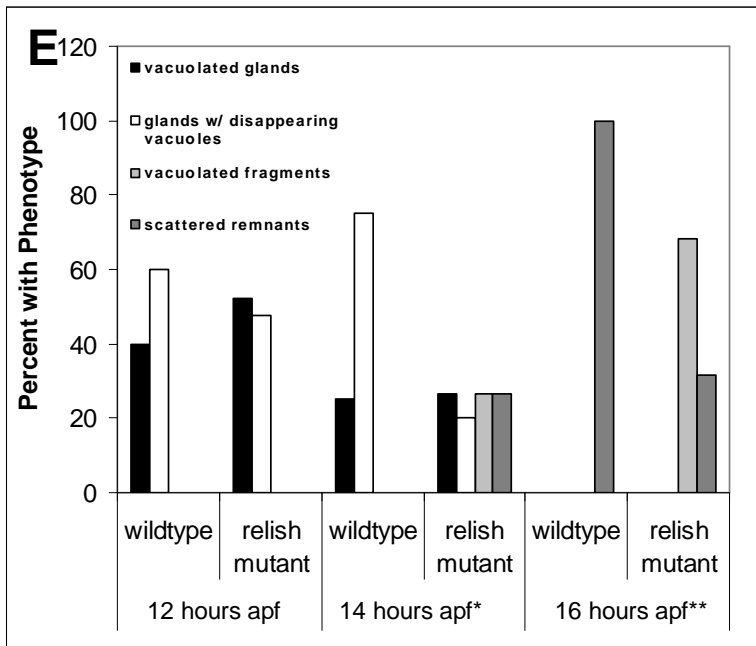
Wildtype 14 hrs apf



Wildtype 16 hrs apf



Relish mutant 16 hrs



*p<0.05

**p<0.0005

Fig. 8: Salivary Gland Destruction Does Not Proceed Normally in *Relish* Mutants. (A-D) Wildtype and *Relish* mutant pupae were examined at time points throughout salivary gland destruction. (A) Wildtype glands first appeared vacuolated at 12 hours apf. (B) By 14 hours apf, they became condensed and vacuoles began to disappear. (C) By 16 hours apf, glands began to break apart and disperse throughout the thorax. (D) In *Relish* mutants, autophagic vacuoles persisted even when glands began to break apart. (E) Percentages of flies with each phenotype are shown throughout the time frame in which wildtype glands are destroyed. b= brain; g= gut; asterisk= autophagic vacuole

3.5 *Relish* Mutants Have Reduced Levels of AMP Transcripts

The fact that *Relish* is required for salivary gland destruction does not necessarily mean that AMPs are also required for this process. It is possible that the *Relish* transcription factor has other targets in addition to the AMP genes, and that these other targeted genes are somehow involved in autophagic cell death. In order to better understand the relationship between AMP expression and the *Relish* mutant phenotype, qRT-PCR was used to compare the transcription levels of AMPs before and during salivary gland cell death in wildtype and *Relish* mutant pupae.

mRNA was extracted from wildtype and *Relish* mutant salivary glands at 6 and 13.5 hours apf. cDNA was generated using RT-PCR, and qRT-PCR was used to measure the relative expression of each antimicrobial peptide. Each reaction was performed in triplicate, and the experiments were performed three times. Rp49 was used as the control.

The dramatic induction of AMP gene transcription during salivary gland destruction in wildtype flies (Fig. 6) is severely impaired in *Relish* mutants (Fig. 9). Nearly all AMP transcripts showed a reduced level of induction in *Relish* mutants when compared to the induction seen in wildtype salivary glands. The induction of *Attacin*, *Cecropin*, *Diptericin*, and *Drosocin* transcription in *Relish* mutant salivary glands was only 37.5% ($p \leq 0.05$), 12.1% ($p \leq 0.10$), 15.2% ($p \leq 0.10$) and 12.7% ($p \leq 0.05$) that of wildtype glands, respectively. *Drosomycin* was the only AMP whose induction was not reduced in *Relish* mutants. This is not, however, entirely

unexpected, as *Drosomycin* transcription is entirely independent of the Imd pathway in salivary glands (Tzou et al., 2000).

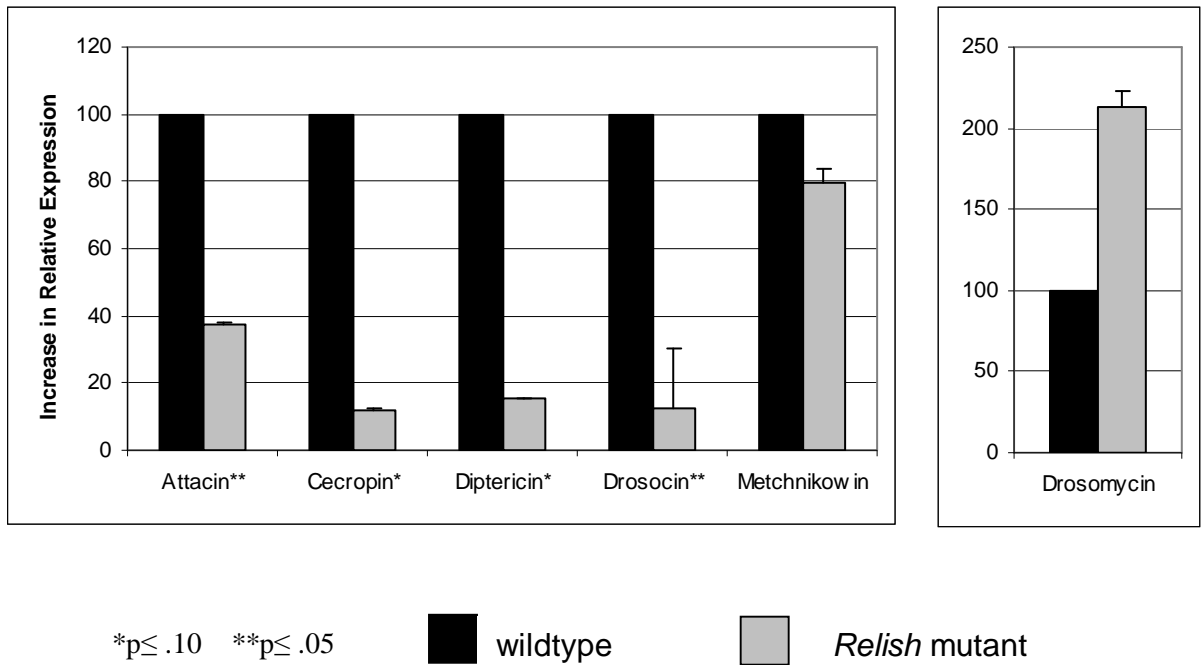


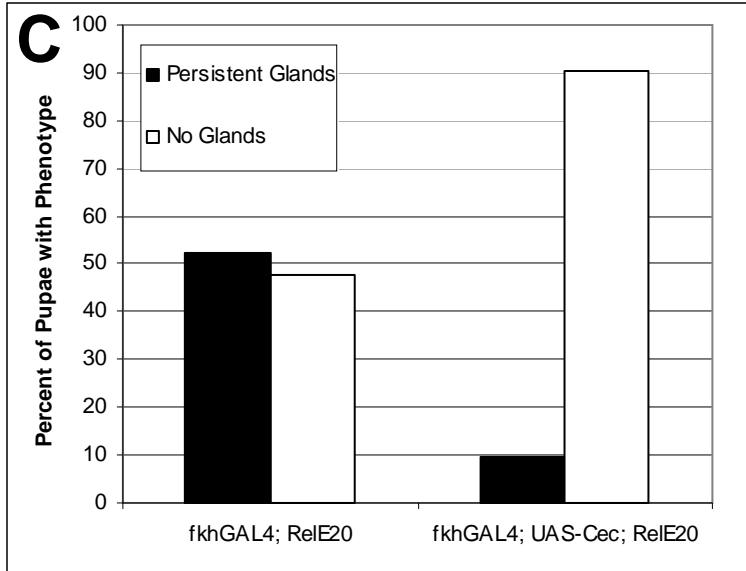
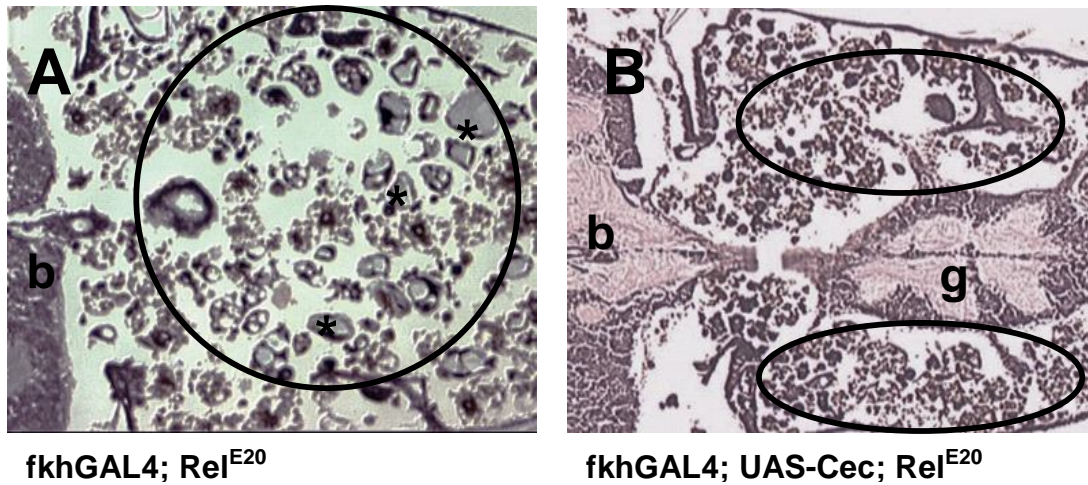
Fig. 9: Relish is Required for the Increase in Antimicrobial Peptide Transcription During Salivary Gland Destruction. Quantitative Real-Time PCR was used to measure mRNA levels for each AMP gene in salivary glands collected from both wildtype (Oregon-R) and *Relish* mutant pupae before and during salivary gland destruction (i.e., at 6 and 13.5 hours apf). The increase in transcription levels of each AMP gene during salivary gland cell death was compared in wildtype and *Relish* mutant glands. Increases in transcription levels in *Relish* mutant glands are expressed as percentages of the increases in wildtype glands. Results depicted are means calculated from three independent experiments. Each reaction was performed in triplicate, and Rp49 was used as a control.

3.6 Tissue-Specific Expression of the AMP Cecropin Rescues the *Relish* Mutant Phenotype

It has been previously demonstrated that driving the expression of a single AMP is enough to restore the wildtype immune response to certain bacteria in immune-deficient flies, as indicated by a normal survival rate upon infection (Tzou et al., 2002). This was accomplished using the GAL4/UAS system of targeted gene expression (Brand & Perrimon, 1993). Under this system, the yeast GAL4 transcriptional activator is placed under the control of a tissue-specific fly cis-regulatory element, and is then crossed to another transgenic line containing the GAL4 target sequence (UAS) as the cis-regulatory element of the gene of interest. Progeny containing both transgenes exhibit directed expression of the targeted gene in the desired tissue.

It is possible to assess the relative importance of individual AMPs in salivary gland cell destruction by using the GAL4-UAS system to determine if driving expression of one AMP in the salivary glands is sufficient to rescue the *Relish* mutant phenotype. Standard crosses were used to generate fly lines containing both the salivary gland-specific GAL4 construct, *forkhead (fkh) GAL4* (Henderson & Andrew, 2000), and the UAS-*Cecropin* target sequence construct in a *Relish* mutant background. Offspring of these crosses should constitutively express Cecropin in salivary gland cells despite the absence of a functional Relish transcription factor. Histological analysis of *fkhGAL4; UAS-Cecropin; Rel^{E20}* pupae at 24 hours apf showed that driven expression of the *Cecropin* gene in salivary gland cells is

sufficient to rescue the *Relish* mutant phenotype (Fig. 10). Only a small portion (9.7%, n=31) of pupae expressing Cecropin in a *Relish* mutant background contained vacuolated salivary gland fragments at 24 hours apf. In contrast, the majority (52.2%, n=23) of control *fkhGAL4; Rel^{E20}* pupae still contained persistent vacuolated gland fragments at this same time point. The frequency of persistent vacuolated fragments detected in *fkhGAL4; Rel^{E20}* control pupae was nearly identical to that of *Relish* null mutants (55%, n=20, Fig. 7).



$p < 0.001$

Fig. 10: Constitutive Expression of Cecropin in Salivary Glands Rescues the *Relish* Mutant Phenotype. (A-C) Expression of the AMP Cecropin was driven using the salivary-gland specific enhancer, *forkhead(fkh)-GAL4* in order to determine whether expression of a single AMP was sufficient to restore salivary gland destruction in *Relish* mutants. (A) Control pupae contained persistent vacuolated salivary gland fragments at 24 hours apf, a time at which glands are completely destroyed in wildtype pupae. (B) Pupae in which Cecropin expression was driven in a *Relish* mutant background exhibited no sign of salivary gland fragments at 24 hours apf. (E) Percentages of pupae with and without persistent gland fragments are shown at 24 hours apf (n>20). b= brain; g= gut; asterisk= autophagic vacuole

Chapter 4: Discussion & Conclusions

4.1 Antimicrobial Peptides are Required for Autophagic Cell Death in Larval Salivary Glands

The results of this research project have provided strong evidence that AMPs are involved in the destruction of salivary glands *via* autophagic cell death during metamorphosis. It has been shown that there is a strong induction in transcription of each of the AMPs upon the onset of salivary gland death (Figs. 5 & 6), and that this induction is controlled by the NF κ B transcription factor, Relish (Fig. 9). Disruption of this induction as the result of a mutation abolishing Relish function leads to a phenotype in which pupae fail to properly and completely degrade larval salivary glands. *Relish* mutants display persistent fragments of larval glands long past the point at which these structures are absent in wildtype pupae (Fig. 7). The persistent fragments contain large autophagic vacuoles, and it appears that this is due to a failure to collapse the vacuoles at the time when dying glands typically begin to condense before finally breaking apart (Fig. 8). This process is clearly dependent upon the presence of AMPs, as experiments in which a single AMP was ectopically expressed in salivary glands in the presence of a Relish mutant background restored successful salivary gland destruction to near wildtype levels (Fig. 10). The AMP transcription required for salivary gland destruction is independent of the Toll pathway, as only pupae with mutations in *Relish*, the Imd pathway transcription

factor, exhibit the persistent salivary gland phenotype. This is not surprising, as it has been previously established that AMP transcription is under the exclusive control of the Imd pathway in epithelia (Tzou et al., 2000).

4.2 A Possible Role for Antimicrobial Peptides in Autophagic Cell Death

It is not presently known how AMPs function in salivary gland destruction. Although their role in the immune response is still not fully understood, it is believed that (in most cases) their cationic nature enables them to permeabilize the membranes of invading pathogens, which eventually leads to lysis of the targeted microbe (Bulet et al., 1999). An intriguing possibility is that this same mechanism is used to eliminate the membranes of autophagic vacuoles during salivary gland cell death. This would explain the persistence of vacuoles in salivary glands from pupae in which AMP transcription has been disrupted.

In mammalian macrophages, it has been established that antimicrobial peptides can be stored in active or inactive states within cytosolic granules. Upon the activation of an immune response, the granules can fuse with phagosome containing pathogens which are then degraded by the active peptides. It is possible that a similar mechanism of delivery exists for autophagosomes. In such a case, AMPs would enter the autophagic vacuoles of dying salivary gland cells and degrade the vacuoles from the inside out.

4.3 Future Research into the Role of Antimicrobial Peptides in Autophagy

Further study is necessary to elucidate how AMPs effect salivary gland destruction. Characterization of the contents of autophagic vacuoles could determine whether AMPs are present within these structures. This could be accomplished by the use of immunocytochemistry. If AMPs do in fact localize to autophagic vacuoles in wildtype salivary glands, this would provide strong evidence that the peptides serve to degrade the membranes of these compartments, thereby allowing cell death to progress normally. Antibodies to the *Drosophila* AMPs are not currently commercially available, but for the purposes of this study, experimental antibodies to Drosocin and Cecropin were obtained from the laboratory of David Schneider (Stanford University). Unfortunately, despite repeated attempts, it was not possible to successfully visualize AMPs in salivary glands using these particular antibodies.

An alternative approach to demonstrate whether AMPs degrade autophagic vacuoles would be to inject AMPs into *Relish* mutant flies. In this way, it may be possible to promote the elimination of persistent vacuoles. Further genetic experiments will also help to understand the relationship between NF κ B/AMPs and autophagy. For example, driving expression of *Relish* (and therefore the AMPs) in salivary glands may be sufficient to restore normal salivary gland cell death in *atg* mutants, which have a phenotype similar to that of *Relish* mutants (Berry & Baehrecke, 2007).

If AMPs do not appear to degrade autophagic vacuoles, they may function in a manner that is also utilized in apoptotic cell death. It would be interesting to profile

AMP expression levels in apoptotic cells, and to look for defects in the progression of apoptosis in AMP mutant cells.

Another avenue of research that should be explored is whether AMPs are utilized in autophagy as part of the starvation response. It seems likely that, given the fact that autophagy can function in both cell death and survival, such a dual role can exist for molecules involved in this process, such as the AMPs. This seems particularly plausible since a microarray analysis of starved and fed larvae showed that nutrient deprivation leads to expression of AMPs (Zinke et al., 2002). Furthermore, a later study confirmed this trend through the use of qRT-PCR, and demonstrated that the Imd pathway is involved in this response (Wu et al, 2007).

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