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Transcriptional Regulation of the Drosophila Peptidoglycan Sensor PGRP-LC by the Steroid Hormone Ecdysone: A Masters Thesis

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TRANSCRIPTIONAL REGULATION OF THE *DROSOPHILA* PEPTIDOGLYCAN SENSOR PGRP-LC BY THE STEROID HORMONE ECDYSONE

A Masters Thesis Presented

By

MEI TONG

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Abstract

Drosophila is host to the steroid hormone ecdysone, which regulates development and immune functions using a common group of transcription factors. Developmentally-induced ecdysone pulses activate the expression of the EcR, BR-C, HR46, Eip74EF, Eip75B, Eip78C, and Eip93F, which assume control of hundreds of other genes involved in the transition from larva to pupa stage. Many of the transcription factors are related to mammalian nuclear hormone receptors by homology. In addition to these transcription factors, the ecdysone-regulated GATA factors SRP and PNR are required for the proper expression of the peptidoglycan sensor PGRP-LC, which belongs to a conserved class of proteins in innate immunity. Although the transcriptional network has been elucidated in development, it is unclear why ecdysone control of *PGRP-LC* gene activity involves these nine transcription factors and how ecdysone is regulated in the context of an infection *in vivo*.

An ecdysone-activated enhancer was located upstream of the *PGRP-LC* locus using a reporter plasmid. Female flies that lacked the enhancer had reduced *PGRP-LC* expression, but survived infection. Male flies did not experience these changes. Therefore, *PGRP-LC* enhancer appears to be a female-specific cis-regulatory element. The lack of survival phenotype could be caused by using an improper injection site. Bioinformatics software was used to identify putative individual and overlapping binding sites for some transcription

factors. Site-directed mutations of the motifs reduced *PGRP-LC* promoter activity without abolishing the signal. These results suggest that the transcription factors assemble at multiple locations on the *PGRP-LC* enhancer and form strong protein-protein bonds. Septic injury led to elevated ecdysone in whole flies, which could be a neuroendocrine response to stress similar to the mammalian system. Steroid hormone regulation of immune receptors is a common theme in humans and flies, and these results could advance our understanding of the transcriptional regulation of related genes and gender differences observed in innate immune responses at the transcriptional level. ■

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List of Abbreviations & Nomenclature

AEL	After Egg Laying
AF-1, AF-2	Transactivation Function
AMP	Antimicrobial Peptide
bHLH-PAS	Basic Helix-loop-helix-per-arnt-sim
BR-C	Broad-complex
BTB/POZ	BR-C, ttk and bab / Pox virus and Zinc finger
CBP	CREB-binding Protein
CRE/Enhancer	*PGRP-LC* cis-regulatory element in the *PGRP-LA 3' UTR*
DSCP	*Drosophila* Synthetic Core Promoter
Ecdysone/20E	20-Hydroxyecdysone
EcR	Ecdysone Receptor
Eip	Ecdysone-induced Protein
ETS	E-twenty-six
EST	Expressed Sequence Tag
Fbp1	Fat Body Protein 1
FTZF1	Fushi Tarazu Factor 1
FXR, LXR	Farnesoid X Receptor, Liver X Receptor
HR46	Hormone Receptor-like in 46
IMD	Immune Deficiency
IL-1R	Interleukin-1 Receptor
JH	Juvenile Hormone III
l(2)mbn	Lethal(2) Malignant Blood Neoplasm

LRH-1, SF-1	Liver Receptor Homolog 1, Steroidogenic Factor 1
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NHR	Nuclear Hormone Receptor
NOD2	Nucleotide-binding Oligomerization Domain-containing Protein 2
PAMP	Pathogen-Associated Molecular Pattern
PGRP/PGLYRP	Peptidoglycan Recognition Protein
PNR	Pannier
PTTH	Prothoracicotropic Hormone
REV-ERBA	Reverse ErbA
RHIM	RIP Homotypic Interaction Motif
RORB	RAR-Related Orphan Receptor Beta
RXR	Retinoid X Receptor
S2* cells	Schneider 2* cells
SRP	Serpent
SMRTER	SMRT-related Ecdysone Receptor-interacting Factor
TIR	Toll-IL-1R
TCT	Tracheal Cytotoxin
TNFR	Tumor Necrosis Factor Receptor
TLR	Toll-like Receptor
USP	Ultraspiracle

Preface

This thesis is the original work of the author Mei Tong with exceptions. Figure 1.1A was created by former graduate student Li Chen. Figure 1.1B, 1.1C, 1.1D, 1.1E, and 1.1F are the published work of former postdoctoral researcher Florentina Rus, who also generated the unpublished data from Figure 3.3A. The published figures are from Rus et al. (2013). EMBO J. *32*, 1626–1638 of which Mei Tong is co-author. ■

CHAPTER I: Introduction

1.1 Literature Review

Hormones regulate the physiology and behavior of all multicellular organisms, from humans to flies and plants. In humans, these are molecules released by endocrine glands in order to transmit messages created from endogenous and environmental signals. The hormones travel using the blood vessel network and can reach distantly located tissues or organs. The output of these hormone signals allow an individual to cope with stressful events or undergo developmental changes. Other vital functions include regulating sleep, appetite, homeostasis, metabolism, and the immune response.

Most hormones in humans are composed of peptides or derived from cholesterol (steroid hormones). (A handful of hormones are amino acid or fatty acid derivatives.) In order for peptide hormones (*e.g.*, insulin and growth hormone) to transmit a signal across the plasma membrane, they bind to cell surface receptors, which activates a cascade of biochemical reactions inside the cell. Most circulating steroid hormones are associated with plasma proteins, which aid their transport in a water-soluble medium, but limit their access to target cells until they are released in the extracellular fluid. The small and lipophilic nature of steroid hormones such as glucocorticoids and estrogens allow them to passively diffuse across the plasma membrane and bind to free nuclear hormone receptors (NHRs) located inside the cell. These receptors directly bind to DNA to control genomic responses.

Activated peptide hormone receptors employ enzymes and second messengers in the cellular response. Enzymes can process multiple substrates and elicit parallel signaling cascades. This effectively amplifies a signal originating from an individual receptor. Non-genomic responses such as the activation of Protein kinase A and the opening of intracellular calcium channels occurs rapidly (**Norman et al., 2004**). Hormone receptors located at the plasma membrane can also control transcription and translation by regulating transcription factor or ribosome activity (**Magnuson et al., 2012**). In contrast, NHRs possess both DNA-binding and ligand-binding domains and are functional transcription factors. Although canonical steroid hormone pathways have a direct mode of action, there is growing evidence that steroid hormones can activate rapid non-genomic responses through novel membrane-bound receptors or NHRs tethered to the membrane (**Norman et al., 2004**).

Some unoccupied NHRs remain in the inactive state in the cytoplasm (*e.g.*, glucocorticoid receptor) and associated with chaperone proteins. When the receptor encounters their cognate ligand, they disassociate from the chaperone to expose their nuclear localization signal and are escorted to the nucleus. There are other NHRs that can be found resting in the nucleus (*e.g.*, retinoid X receptor), bound to chromatin and interacting with repressor proteins (**Glass and Ogawa, 2005**). The activation of these receptors is followed by an exchange between repressor and activator proteins. In addition, some NHRs form

homodimers or heterodimers upon their activation, which occurs through the ligand-binding or DNA-binding domains (**Aranda and Pascual, 2001**).

The cellular mechanism of peptide hormones was solved in part by the advent of second messengers and secondary signaling molecules that function at the plasma membrane, which helped propel the discovery of peptide hormone receptors (**Tata, 2005**). The development of gene cloning and sequencing technologies provided fundamental tools for identifying and characterizing hormone receptors (**Tata, 2005**). The first NHR, estrogen receptor, was identified in rat uterine homogenate using an estradiol radioisotope, which formed a complex with the receptor in the nuclear fraction in a sucrose gradient (**Toft and Gorski, 1966**). Prior to this work, the molecular function of steroid hormones eluded early endocrinologists until studies of hormone action on insect developmental was brought to the forefront.

The fruit fly *Drosophila melanogaster* have three major stages of development—embryo, larva, and pupa—and a generation time of 9 days at 25ºC. The larval stage is a 4 day period of rapid growth fueled by copious feeding on decomposed plant material. However, larva possess a protective outer cuticle that must be shed periodically for larvae to continue growing and reach adulthood. This process is driven by pulses of the steroid hormone ecdysone (20-hydroxyecdysone), a major molting hormone in arthropods. *Drosophila* also experiences late larval and prepupal ecdysone pulses, which trigger a series of puffs—transcriptionally active genes—in the polytene chromosomes of the

salivary glands in a precise sequence. These giant chromosomes are composed of thousands of chromatin strands created by endoreplication and the puffs encode for material that makes the larva-pupa phase transition possible.

Chromosome puffs formed in the salivary glands during the late larval phase could be induced *ex vivo* by purified ecdysone extracts (**Ashburner, 1990**). The puffs observed *ex vivo* were coordinated in their response, appearing in a specific order and time. A small number of puffs became active within minutes of their exposure to ecdysone, followed by a larger set of puffs that manifested hours later; these were termed early and late puffs or genes (**Ashburner, 1990**). Importantly, the early puffs could be activated in the presence of protein synthesis inhibitors, but the late puffs were dependent on protein synthesis (**Ashburner, 1990**). A model developed to describe these observations proposed that ecdysone binds to an intracellular receptor that directly interact with DNA to control transcription of the early puffs (**Ashburner, 1990**). Moreover, the formation of late puffs would depend on the protein products encoded by the early puffs. These results provided direct evidence that steroid hormones could function at the gene transcription level.

The ecdysone receptor (EcR) was cloned and characterized (**Koelle et al., 1991; Yao et al., 1993**) and is among 18 *Drosophila* NHRs identified to date. Ecdysone influences the gene activity of its own receptor and half of the NHRs, including those encoded in the early puffs located at loci 75B (*Eip75B*), 78C (*Eip78C*), and 46F (*Hr46*) (**King-Jones and Thummel, 2005**). (*Hr46* puff

appears after the early puffs because it also depends on early gene products and is technically an early-late puff.) These are zinc-finger transcription factors with a ligand-binding pocket, but most NHRs are “orphans” and do not have a defined ligand. The exceptions are the EcR and Eip75B, which binds heme and responds to nitric oxide and carbon monoxide (**Reinking et al., 2005**). NHR cross-regulation presumes that a preexisting low level of one NHR in the presence of its cognate ligand can activate the expression of another NHR. This phenomenon has been detected in the tissues of other animals (**Tata, 2002**) and could be an effective way to activate many genes necessary for a specific biological outcome.

Receptors for glucocorticoid and estrogen were among the first mammalian NHRs cloned and they belong to a superfamily of 48 genes encoding NHRs. Common features found in invertebrate and vertebrate NHRs include the isoform-specific transcription activation domain (AF-1), DNA-binding domain, ligand-binding domain, and a second transcription activation domain (AF-2). In addition to their transactivation activity, AF-1 and AF-2 associate with co-activators (**Aranda and Pascual, 2001**). Although not all mammalian NHRs are represented in *Drosophila*, every *Drosophila* NHR has one or more mammalian orthologue. For example, the EcR is related to FXR (farnesoid X receptor) and LXR (liver X receptor), but there is not a genetic equivalent to the glucocorticoid receptor in *Drosophila* (**King-Jones and Thummel, 2005**). The conservation of these genes makes it possible to use invertebrate models to study the biological and molecular function of their mammalian counterparts.

Many developing and adult tissues express the EcR, and immunostained tissues from larva, prepupa, and adult flies show the EcR is mostly nuclear (**Koelle et al., 1991; Talbot et al., 1993; Schwedes et al., 2011**). The pairing of EcR with USP (ultraspiracle)—a NHR related to RXR (retinoid X receptor)—forms a dimer capable of interacting with EcR response elements and this interaction becomes more stable in the presence of ecdysone (**Yao et al., 1993**). During the resting state, the unoccupied EcR-USP complex binds to repressor protein SMRTER (SMRT-related ecdysone receptor-interacting factor), which becomes displaced when the EcR bonds with a compatible ligand (**Tsai et al., 1999**). The ecdysone pathway is involved in numerous cellular and developmental activities including systemic remodeling of tissues during metamorphosis; larval organs are degraded (**Yin and Thummel, 2004**) and adult precursor tissues undergo proliferation and differentiation. These tissue-specific responses are partly driven by differentially expressed EcR protein isoforms (**Talbot et al., 1993**).

Ecdysone is the only physiologically active steroid hormone in *Drosophila* (**King-Jones and Thummel, 2005**). It is synthesized from cholesterol derived from plant sterols acquired from a diet of decayed organic matter such as fruit. Therefore, unlike in humans, steroid hormones cannot be generated *de novo* in flies. Ecdysone biosynthesis happens in the larval-specific prothoracic gland, which is located adjacent to the brain. The neuropeptide PTTH (prothoracicotropic hormone) stimulates ecdysone secretion into the hemolymph

—insect blood in an open circulatory system—where it may encounter target tissues such as the salivary glands. Shortly before the adult emerges from the pupal case (eclosion), the prothoracic gland becomes fully degraded (**Dai and Gilbert, 1991**).

It is known that the ecdysone pathway has a role in adult functions such as the immune response and reproduction (**Meister and Richards, 1996; Flatt et al., 2008; Rus et al., 2013**), but details of ecdysone production are unclear. Ecdysone was detected in male and female hemolymph (**Handler, 1982**), ovaries (**Bownes et al., 1984**), and nutritional deprivation can enhance ecdysone production in female reproductive organs (**Terashima, 2005**). However, expression of ecdysone metabolic enzymes occurs in adult peripheral tissues such as the malpighian tubules (functionally similar to mammalian kidneys) and fat body (the insect liver) (**Petryk et al., 2003**), which suggest that adult flies have multiple tissues that produce ecdysone.

Ecdysone coordinates with juvenile hormone III (JH), another lipid hormone (sesquiterpene), to regulate developmental outcomes. JH levels are generally high during the initial larval phase, becoming low or absent in late larvae and pupae (**Dubrovsky, 2005**). According to studies in the moth *Manduca*, the magnitude of the ecdysone and JH response determines the timing of the larva-pupa transition (**Riddiford et al. 2003**). Ecdysone-triggered pupation occurs at the appropriate time because JH limits ecdysone activity in larvae and

prevents premature pupation and precocious metamorphosis (**Riddiford et al. 2003**).

JH is generated by a conserved metabolic pathway (mevalonate pathway) and produced by the corpus allatum. The corpus allatum and prothoracic gland are part of the same organ complex that is collectively known as the ring gland. Unlike the prothoracic gland, the corpus allatum does not undergo degradation during metamorphosis (**Dai and Gilbert, 1991**). JH has functions in adult flies and can inhibit ecdysone-mediated activation of the humoral response (**Flatt et al., 2008**). There have been many efforts to identify the JH receptor and it was discovered that Met and Gce, paralogous bHLH-PAS (basic helix-loop-helix-per-arnt-sim) transcription factors, assumed redundant roles as JH receptors (**Abdou et al., 2011; Jindra et al., 2015**). (bHLH-PAS transcription factors are not related to NHRs.) The hormone-binding domain is probably served by the PAS domain and it was demonstrated that a high concentration of endogenous or exogenous JH in fat body cells promotes nuclear import of Met (**Charles et al., 2011; He et al., 2014**).

Thus far, the role of steroid hormones in *Drosophila* development have been discussed in some detail and their role in adult immunity was briefly touched upon. Development and immunity are actually coupled systems in the developing fly, and the ecdysone pathway was adapted for simultaneous gene activation in the two systems. The first evidence of this dual role appeared in transgenic animals with the *β-galactosidase* gene reporter under the control of

the antimicrobial peptide (AMP) *Diptericin* promoter. The animals were infected at different developmental stages, but reporter activity only began to show 96 hours after the embryos were laid (AEL) (**Reichhart et al., 1992; Meister and Richards, 1996**); this corresponded with the timeframe in which many ecdysone-induced early genes are expressed in late larva (**Reichhart et al., 1992; Andres et al., 1993**). The reporter was active in the fat body, a major source of endogenous AMPs. Initially (96 h AEL), the reporter pattern was mosaic, but 24 h later all the fat body cells were stained (**Reichhart et al., 1992**). However, exposing infected larvae to exogenous ecdysone significantly increased the ratio of animals with fully stained fat bodies to mosaic fat bodies at 96 h AEL (**Meister and Richards, 1996**). Therefore, ecdysone actively enhanced *AMP* expression in the fat body of infected larva and could be a critical factor for combating infection.

Drosophila relies on early forms of chemical and cellular defenses to survive systemic infections including AMP secretion and phagocytosis (**Defaye et al., 2009**). These are rapid non-adaptive responses that are activated by common molecular constituents in bacteria and fungi such as peptidoglycan and β-glucans. AMPs are small peptides (<10 kDa) produced by the Toll and IMD (immune deficiency) pathways, which uses PGRP (peptidoglycan recognition protein) receptors to sense gram-positive or gram-negative peptidoglycan. There are 20 genes encoding AMPs with a broad range of targets (**Lemaitre and Hoffman, 2007**). For example, Diptericin is effective against gram-negative

bacteria (**Wicker et al., 1990**), Defensin is active against gram-positive bacteria (**Cociancich et al., 1993**), and Drosomycin is an effective anti-fungal (**Fehlbaum et al., 1994**). The mode of action for most *Drosophila* AMPs has yet to be elucidated, but models developed from studies of human AMPs suggest they may disrupt membrane integrity or interfere with intracellular processes (**Cociancich et al., 1993; Brogden, 2005**).

Human innate immunity also depends on AMPs for host defense, which has grown into library of 103 peptides and 14 proteins including lysozyme and members of cathelicidins, defensins, and histatins (**Wang et al., 2014**). These AMPs display a mixed spectrum of activities against bacteria, fungi, viruses, and parasites. They are secreted constitutively or induced under inflammatory conditions from exocrine glands, epithelial tissue, and immune cells (**Wang et al., 2014**). The regulation of human AMPs are not fully understood, but some AMPs are under the direct control of vitamin D_3, and NF-κB via NOD2 and TLR signals (**Wang et al., 2004; Liu et al., 2006; Wang et al., 2010**). In *Drosophila*, the fat body is a major source of AMPs present in the hemolymph. Blood cells (hemocytes) and the local epithelia such as the gut and trachea also produce AMPs. Septic injury with any microorganism generates a battery of AMPs, but gram-negative bacteria selectively trigger a robust and sustained *Diptericin* expression, which reaches an optimal peak 6 h after infection (**Lemaitre et al., 1997**). The DAP-type peptidoglycan present in all gram-negative bacteria and certain gram-positive bacteria is recognized by the PGRP-LC and PGRP-LE

receptors of the IMD pathway, and *AMP* gene activation is carried out by the NF-κB-related transcription factor Relish (PGRP-LE not shown) (**Figure 1.1A**).

Drosophila hemocytes can secrete AMPs and participate in other facets of immune defense including phagocytosing microorganisms by plasmatocytes. Larvae have special defenses against larger parasitic objects (*e.g.*, wasp eggs), which become encapsulate by lamellocytes and melanin produced by crystal cells. Open wounds in the cuticle barrier are immediately sealed with clotting fibers and melanin deposited by the hemocytes. Depending on the stage of development, 95-100% of circulating hemocytes are professional phagocytes (**Lanot et al., 2001**). Hematopoiesis only occurs twice and the cells are derived from the embryonic head mesoderm or larval lymph gland. As a result, all adult hemocytes have embryonic or larval origins. Multiple hemocyte cell lines have been established including l(2)mbn, a mixed population of tumorous blood cells derived from the larval stage. It was determined that 24 h ecdysone pretreatment of these cells was necessary to achieve robust *Diptericin* expression in response to an infectious agent (**Dimarcq et al., 1997**). These results have been replicated in S2* cells, a phagocytic cell line derived from late embryos (**Rus et al., 2013**).

The transcriptional profile of ecdysone-treated S2* cells was analyzed on a DNA microarray and among the key components of the IMD pathway, the *PGRP-LC* was upregulated by ecdysone (**Figure 1.1B**). In the developing fly, the ecdysone pulses also correspond with spontaneous *PGRP-LC* expression (**Figure 1.1C**). Nine transcription factors that were upregulated by ecdysone in

the S2* cells (*EcR* is not shown) (**Figure 1.1D**) were selected for further analysis based on previous studies that implicated them in the ecdysone pathway in development or the immune response. EcR, Eip75B, Eip78C, and HR46 are NHRs encoded by the early puffs or early-late puffs. The EcR is related to FXR and LXR, which bind to sterols and bile acids to regulate cholesterol and fat metabolism in the liver (**Kalaany and Mangelsdorf, 2006**). Eip75B and Eip78C are duplicated genes that share homology with REV-ERBA, while HR46 is homologous to RORB. Both REV-ERBA and RORB are orphan NHRs and are involved in circadian rhythm among other functions. For example, oscillating cytokine expression to endotoxin becomes impaired in *Rev-erbα* mutant mice (**Gibbs et al., 2012**).

BR-C (broad-complex), Eip74EF, and Eip93F are also encoded by early genes. BR-C is a zinc-finger transcription factor that possess a conserved BTB/POZ protein-protein binding domain. Eip74EF is related to the ETS family of protooncogenes in mammals by the ETS helix-turn-helix DNA-binding domain. Eip93F contains a helix-turn-helix DNA-binding domain. Finally, SRP (serpent) and PNR (pannier) belong to a family of zinc-finger transcription factors that interact with a GATA consensus sequence. A previous report showed that SRP was directly involved in *AMP* expression the the larval fat body (**Petersen et al., 1999**).

The selected transcription factors were required for ecdysone-mediated *PGRP-LC* expression in S2* cells (**Figure 1.1E**). The exception was *Eip75B*

knockdown samples that displayed enhanced *PGRP-LC* levels, suggesting it holds an inhibitory role in *PGRP-LC* gene activity. It is not clear how these transcription factors would interact to regulate *PGRP-LC*. There are few examples that show cooperation between these transcription factors to regulate gene transcription. For example, binding sites for the EcR and SRP were located on a 70 bp enhancer for *Fbp1*, which encodes for a protein transporter in the fat body. (**Brodu et al., 1999**); EcR interacts with HR46, which represses EcR transactivation activity (**White et al., 1997**); and Eip75B interacts with HR46 to inhibit HR46 activation of the mid-prepupal gene *FTZF1* (*LRH-1/SF-1*) (**White et al., 1997**). In addition to forming a multi-protein activator complex, the transcription factors could also be involved in a hierarchical transcriptional network similar to the early and late gene model.

Alternative splicing of the *PGRP-LC* locus give rise to multiple transcripts that encode for three major isoforms that share a common intracellular domain that contains a RHIM (RIP homotypic interaction motif)-like motif (**Kaneko et al., 2006**) and transmembrane domain, but possess variable extracellular PGRP domains. The PGRP domain determine the capacity of each isoform to recognize polymeric or monomeric (tracheal cytotoxin, TCT) peptidoglycan fragments. While *PGRP-LCx* is required for the *AMP* response to polymeric peptidoglycan or TCT, *PGRP-LCa* is only necessary for TCT recognition (**Kaneko et al., 2004**). However, PGRP-LCx possess a peptidoglycan docking groove that is missing from PGRP-LCa, which itself cannot bind to peptidoglycan (**Chang et al., 2005;**

Mellroth et al., 2005). Instead, PGRP-LCx-PGRP-LCa heterodimer formation only occurs in the presence of TCT (**Chang et al., 2005; Mellroth et al., 2005**). PGRP-LCx also forms homodimers that recognize polymeric peptidoglycan structures, and this interaction may occur through the RHIM-like motif (**Kaneko et al., 2004; Choe et al., 2005; Lim et al., 2006**). Although PGRP-LCy can interact with PGRP-LCx and PGRP-LCa, its role in the IMD pathway is still under investigation (**Kaneko et al., 2004**).

The *PGRP-LC* locus is situated in-between *PGRP-LA* and *PGRP-LF*. PGRP-LA and PGRP-LF isoforms are transmembrane proteins that lack residues responsible for peptidoglycan binding in PGRP-LCx and have opposing functions in the IMD pathway (**Chang et al., 2006; Gendrin et al., 2013; Basbous et al., 2011**). Although the systemic *AMP* response does not require *PGRP-LA, PGRP-LA* deficient animals have an impaired *AMP* response in the larval respiratory tract and adult intestinal tract (**Gendrin et al., 2013**). Ubiquitous overexpression of a PGRP-LA isoform that lacks the PGRP domain can drive *Diptericin* expression *in vivo* (**Gendrin et al., 2013**). This particular isoform possess the RHIM-like motif that could support intracellular PGRP-LC signaling complexes. In contrast, PGRP-LF binds to the PGRP-LC-TCT complex and antagonizes the IMD pathway in the adult fat body and hemocyte cell culture (**Persson et al., 2007; Maillet et al., 2008; Basbous et al., 2011**). Independent studies that analyzed PGRP-LF binding to peptidoglycan reported different results. The binding was not detected using the hold-up assay (**Basbous et al., 2011**), but the

presence of the V5-His tag increased the affinity of the PGRP domain to peptidoglycan in pull-down assays (**Persson et al., 2007; Basbous et al., 2011**). Ecdysone-treated S2* cells upregulated *PGRP-LA*, but *PGRP-LF* was not regulated by ecdysone (data not shown).

There are a total of 13 PGRP genes in *Drosophila* that encode 19 proteins that have enzymatic activity and/or function as peptidoglycan receptors (**Royet and Dziarski, 2007**). PGRP-LB, -SB1, -SB2, -SC1, and -SC2 have a conserved amidase active site that has been proven or is predicted to cleave the peptidoglycan to inactivate it. They could be secreted in the hemolymph or gut lumen to limit Toll and IMD signaling and maintain intestinal microbiota homeostasis. PGRP-SA and PGRP-SD are secreted sensors of the Toll pathway that can recognize (Lys-type) peptidoglycan in gram-positive bacteria and activate a serine protease cascade that catalyses pro-spätzle proteolysis (**Michel et al., 2001; Bischoff et al., 2004**). Spätzle binding to the Toll receptor activates the NF-κB-related transcription factors Dorsal and Dif, which activates *AMP* gene expression.

The PGRP receptors of the Toll pathway function independently, synergistically, or in a redundant manner in response to different bacteria. For example, PGRP-SA is responsible for the recognition of *M. luteus* (**Michel et al., 2001; Bischoff et al., 2004**). *S. pyogenes*-mediated *AMP* response in wildtype flies is comparable to *PGRP-SA* or *PGRP-SD* single mutants, but became downregulated in double mutants, which suggested redundant roles for these

receptors in *S. pyogenes* recognition (**Bischoff et al., 2004**). Although PGRP-SA and PGRP-SD can bind peptidoglycan derived from *E. coli* and other gram-negative bacteria (**Mellroth et al., 2005; Basbous et al., 2011**), these mutant flies were not susceptible to *E. coli* infection (**Bischoff et al., 2004**).

PGRP-LE is an intracellular receptor in the IMD pathway that responds to TCT (**Kaneko et al., 2006)**. However, PGRP-LE can recognize DAP-type peptidoglycan (**Takehana et al., 2002**) and recognition of the intracellular pathogen *L. monocytogenes* by PGRP-LE is crucial for the autophagy-mediated defense mechanism in hemocytes (**Yano et al., 2008**). In addition, PGRP-LE is involved in melanin production in larvae, which serves to heal wounds or encapsulate bacteria (**Takehana et al., 2002**). Besides *PGRP-LC* and *PGRP-LA*, *Toll* is the only other aforementioned immune receptor that is upregulated by ecdysone in S2* cells (**Dimarcq et al., 1997**).

Similar to the origins of ecdysone, initial Toll studies began in the field of development, but focused on its role in establishing dorsal-ventral polarity in embryos (**Lemaitre, 2004**). It was later discovered that the Toll pathway resembles signaling pathways initiated by the mammalian IL-1R and the TLR (Toll-like receptor) family of pathogen recognition receptors (**Lemaitre, 2004**). These membrane-associated receptors share a conserved cytoplasmic TIR (Toll-IL-1R) domain that binds to homologous adapter proteins (MyD88~dMyD88) and activates related kinases (IRAK~Tube/Pelle) and transcription factors (NF-κB~Dorsal/Dif) (**Dunne and O'Neill, 2003**). However, sequence deviation in the

ectodomains reflect the different types of ligands recognized by these receptors. While Toll and IL-1R binds to cytokines (*i.e.*, Spätzle, IL-1), TLRs senses PAMPs (pathogen-associated molecular patterns) such as lipoproteins (TLR1, TLR2, TLR6) and lipopolysaccharide (TLR4) (**Kawai and Akira, 2007**). Foreign nucleic acids activates receptors confined to the endosomal compartments (TLR3, TLR7, TLR8, TLR9) and even structural proteins such as flagellin have dedicated receptors (TLR5). The IL-1R and TLR signaling cascades promote production of cytokines and chemokines that control cellular and antibody responses specific to the invading microbe.

The *Drosophila* IMD pathway is commonly compared to the TNFR (tumor necrosis factor receptor) pathway in humans due to the presence of conserved players, which also appear in the TLR pathways. However, PGRP-LC and the cytokine receptor TNFR does not share sequence homology. The human genome contains 4 “PGLYRP” genes that encode for proteins that possess peptidoglycan amidase activity or are directly involved in bacteria killing (**Royet and Dziarski, 2007**). PGLYRPs function in the form of disulphide-linked dimers and bind gram-negative or gram-positive peptidoglycan. PGLYRP-1 is expressed in neutrophils and has bacteriolytic or bacteriostatic activities against gram-positive bacteria and *L. monocytogenes* (**Liu et al., 2000; Osanai et al., 2011**); PGLYRP-1 mutant mice are susceptible to gram-positive bacteria infections and their neutrophils become deficient to kill intracellular bacteria (**Dziarski et al., 2003**). PGLYRP-2 is constitutively expressed in the liver and secreted in the

bloodstream, where may hydrolyze Lys-type peptidoglycan (**Wang et al., 2003; Zhang et al., 2005**). PGLYRP-2 is also upregulated by peptidoglycan *in vivo* and has pro-inflammatory role in peptidoglycan-induced arthritis in mice (**Saha et al., 2009**) Recombinant PGLYRP-3 and PGLYRP-4 are secreted in the supernatant and the formation of heterodimers are favorable (**Lu et al., 2006**). They are bacteriostatic and bacteriolytic against both gram-postive and gram-negative bacteria, and are expressed in many tissues including the skin, eyes, and intestinal tract, and could be induced with bacteria in keratinocyte cell culture (**Lu et al., 2006**).

In *Drosophila*, *PGRP-LC* expression becomes enhanced upon infection and the mechanism by which this occurs remains undetermined (**Figure 1.1F**). The ecdysone-regulated transcription factors were generally required for the basal and induced *PGRP-LC* level with the exception of *Eip75B* knockdown flies, which experienced elevated *PGRP-LC*. *AMPs* such as *Diptericin* were sensitive to the changes in the corresponding *PGRP-LC* level. Consequently, the *PGRP-LC* deficient flies became immunodeficient, while *Eip75B* knockdown flies had better survival to infection than wildtype flies (**Rus et al., 2013**). These results demonstrate that the IMD pathway is regulated by canonical and novel ecdysone-regulated transcription factors in the *Drosophila* fat body and *PGRP-LC* is subject to positive and negative regulation. However, the molecular mechanism of hormone-mediated regulation of key components of the IMD pathway including *PGRP-LC* continues to be under investigation. *Drosophila* is

an established model in innate immunity research. In addition to their high fecundity and quick generation time, there are economical benefits of working with insects. The conservation of these genes combined with a genetically tractable system makes *Drosophila* a valuable model to study the steroid hormone regulation of the innate immune response *in vivo*. ■

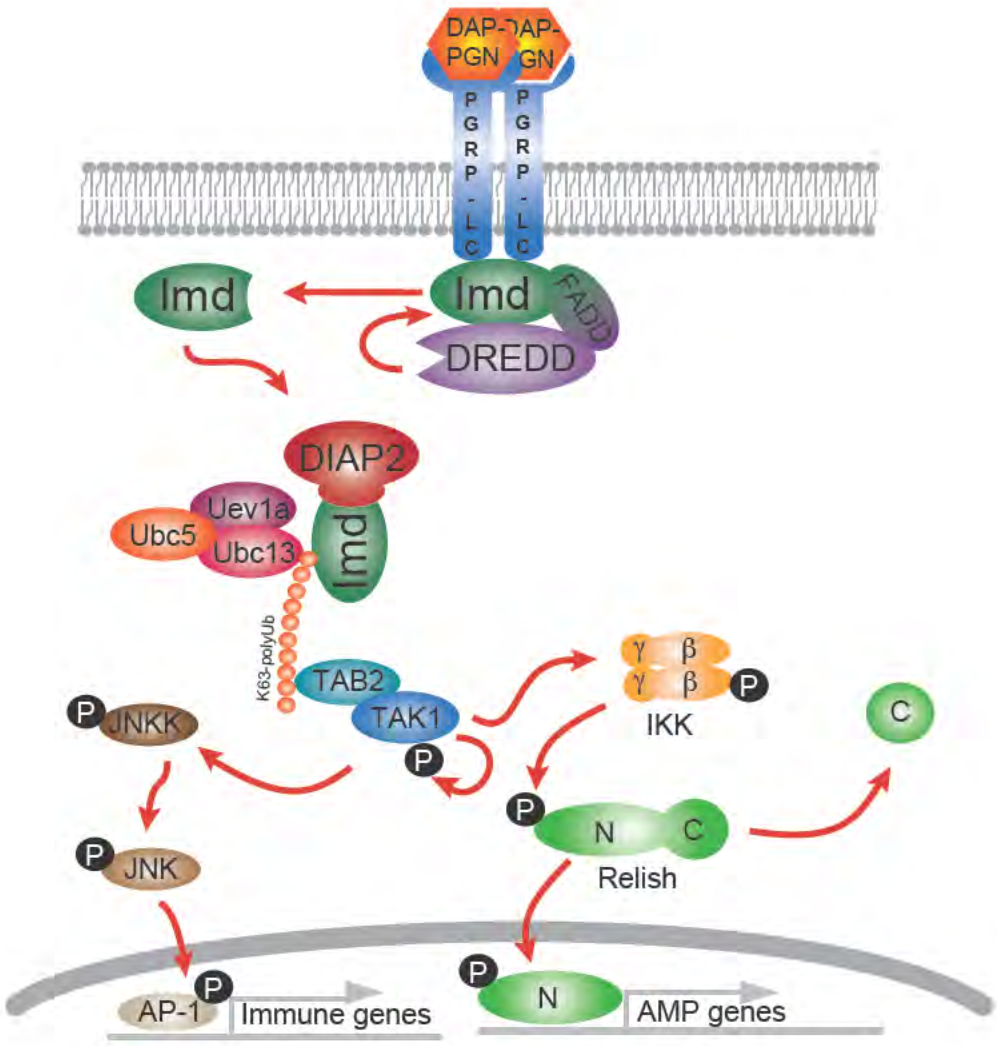


Figure 1.1A: In the IMD pathway, *AMP* gene expression is activated by DAP-type peptidoglycan binding to the PGRP-LC receptor. IMD is cleaved by the caspase DREDD, exposing a binding site for the ubiquitin ligase Diap2. A trio of ubiquitin-conjugating enzymes promote IMD polyubiquitination, which activates downstream kinases that support cleavage and nuclear transport of the NF-kB homologue Relish. This figure was provided by Li Chen. ■

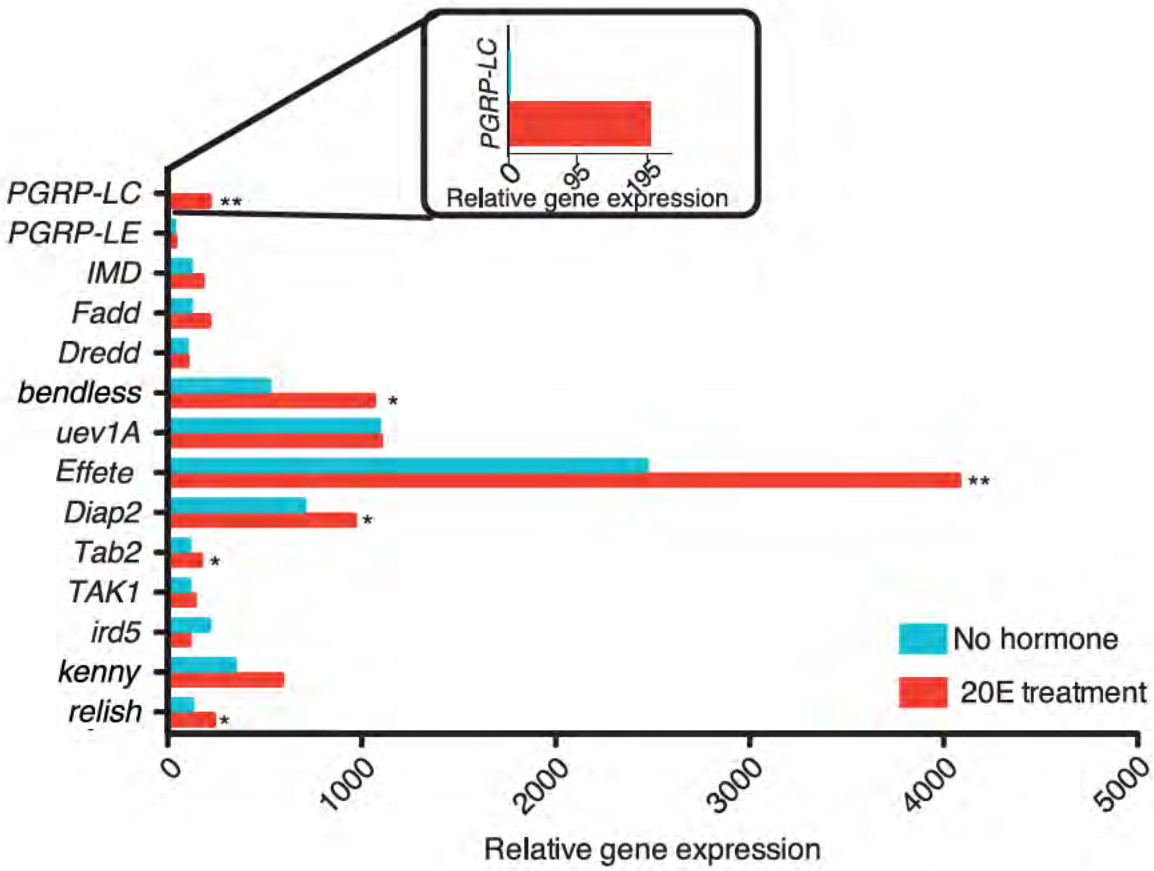


Figure 1.1B: *PGRP-LC* expression is significantly induced by ecdysone (20E) in hemocyte cell culture. Other genes in the IMD pathway are shown. Experiments were performed in S2* cells untreated or treated with 1 uM ecdysone for 24 h. RNA harvested from these samples were applied to Affymetrix *Drosophila* 2.0 Chips in triplicate. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. This figure was provided by Florentina Rus (Rus et al., 2013) and modified to improve clarity. ■

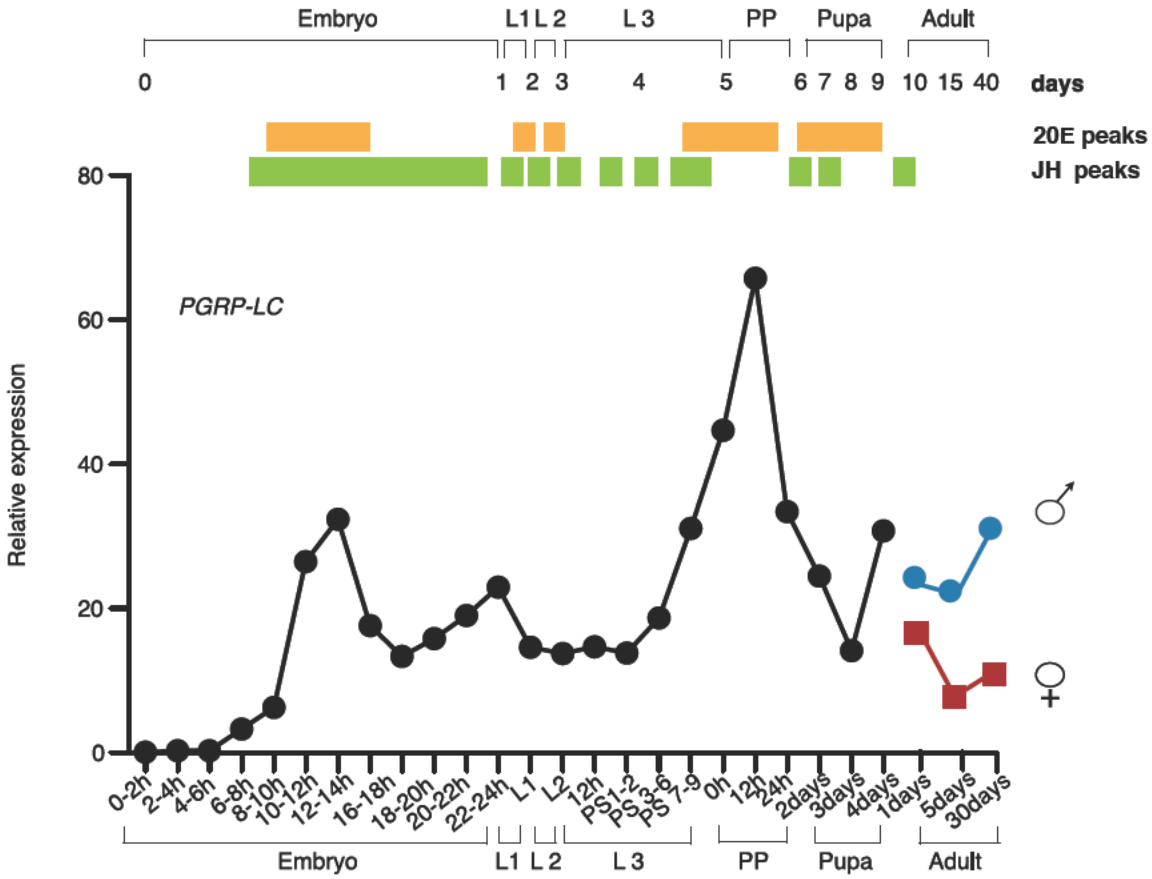
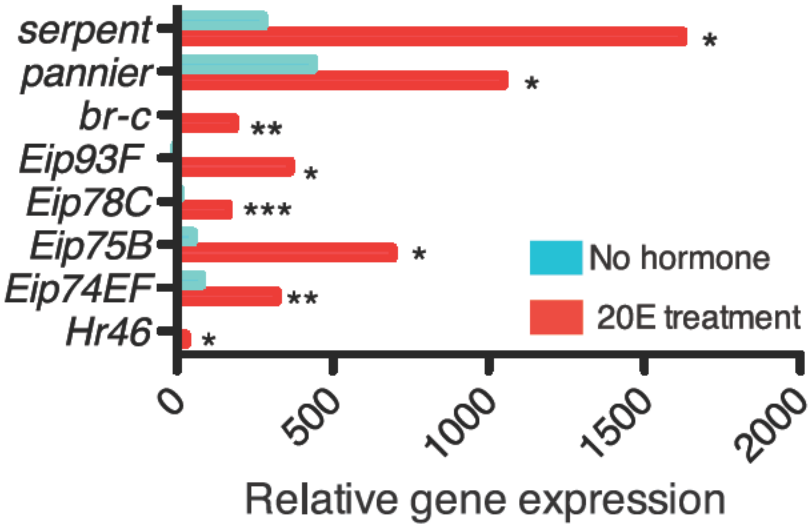


Figure 1.1C: Ecdysone pulses correspond with spontaneous *PGRP-LC* expression in the developing fly. Ecdysone (20E) and juvenile hormone (JH) peaks are shown in orange and green (Dubrovsky, 2005). The timespan of each developmental stage appears at the top as days after egg laying and at the bottom as separate units for each discrete stage. The *PGRP-LC* profile is based on modENCODE RNA-Seq temporal expression data (Graveley et al., 2011). This figure was provided by Florentina Rus (Rus et al., 2013). ■


serpent
pannier
br-c
Eip93F
Eip78C
Eip75B
Eip74EF
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No hormone
20E treatment
0
500
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2000
Relative gene expression

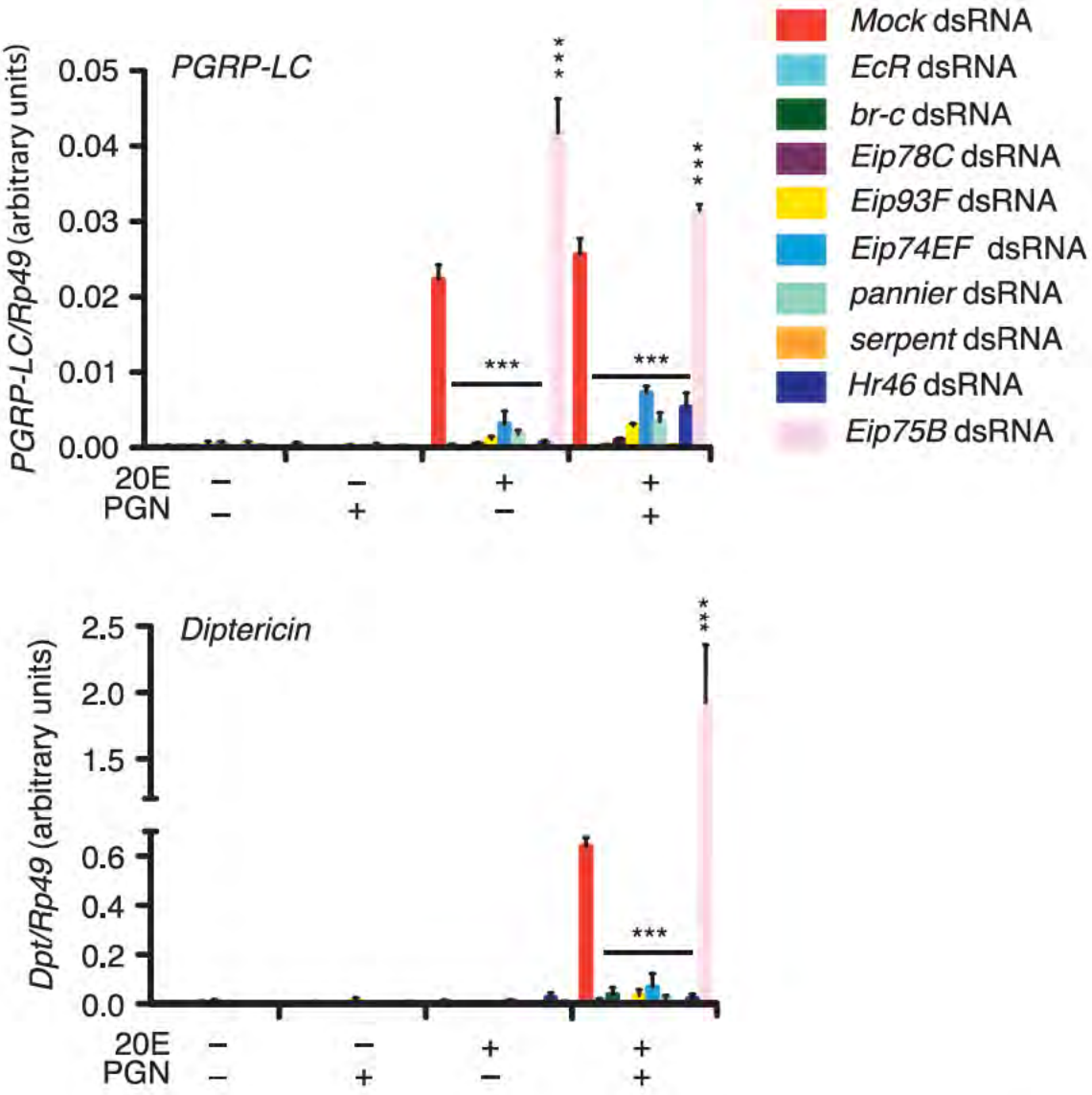


Figure 1.1E: Ecdysone-regulated transcription factors are required for *PGRP-LC* expression (top) and ecdysone (20E) treatment is prerequisite for peptidoglycan-mediated *AMP* expression (bottom). The enhanced phenotype displayed by *Eip75B* knockdown samples suggest it holds an inhibitory role in *PGRP-LC* gene activity. S2* cells transfected with dsRNA for 24 h were untreated or treated with 1 uM ecdysone for 24 h and/or stimulated with 2 mg/ml peptidoglycan for 6 h. ***$P<0.001$. This figure was provided by Florentina Rus (Rus et al., 2013). ■

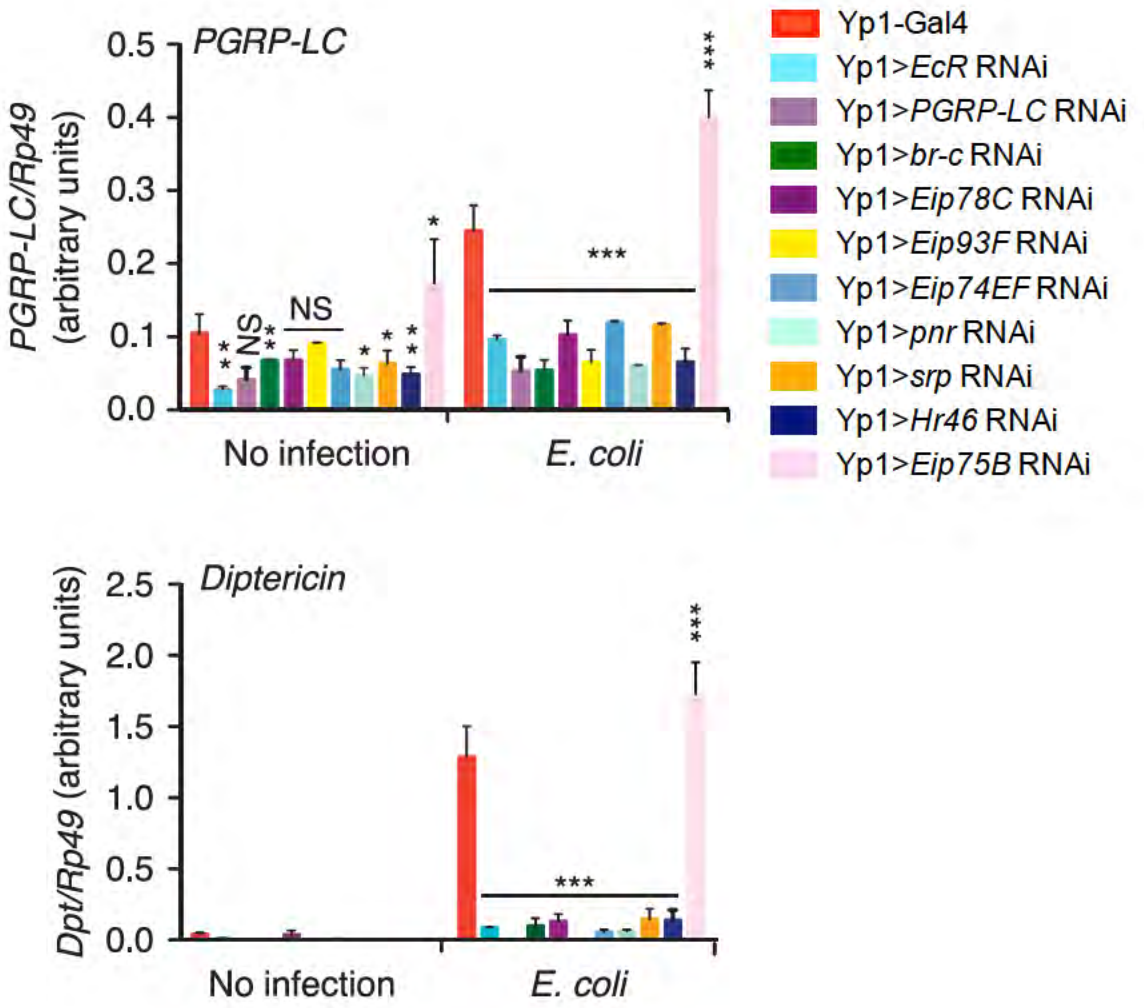


Figure 1.1F: Infected flies have upregulated *PGRP-LC* expression, which becomes impaired by the knockdown of ecdysone-regulated transcription factors (top). *AMP* expression is sensitive to *PGRP-LC* transcriptional level (bottom). The enhanced phenotype displayed by *Eip75B* knockdown samples suggest it holds an inhibitory role in *PGRP-LC* gene activity *in vivo*. *Yp1 (yolk protein 1)-GAL4* is a female-specific fat body driver. Flies were uninfected or pricked in the abdomen with a concentrated *E. coli 1106* pellet and infected for 24 h. **P*<0.05, ***P*<0.01, ****P*<0.001. This figure was provided by Florentina Rus (Rus et al., 2013). ■

1.2 Thesis Rationale

The major *Drosophila* immune receptors PGRP-LC and Toll are transcriptionally regulated by ecdysone, but the mechanism of this control remains enigmatic (**Figure 1.1B; Dimarcq et al., 1997**). Proper expression of *PGRP-LC* by ecdysone requires at least nine transcription factors including EcR, BR-C, HR46, Eip74EF, Eip75B, Eip78C, Eip93F, SRP, and PNR (**Figure 1.1E**), which are related to mammalian counterparts by orthology or protein domain. However, the role of these nine transcription factors in *PGRP-LC* gene activity is undetermined. There are a few examples of a parallel phenomenon in mammals. Glucocorticoids activate *TLR2* in lung epithelial cells (**Hermoso et al. 2004, Homma et al., 2004**) and *NLRP3* in human macrophages (**Busillo et al. 2011**). In addition, *NOD2* is activated by vitamin D_3 in primary human keratinocytes (**Wang et al., 2010**). Thus, steroid hormone regulation of immune receptors is a common theme in both humans and flies. Elucidating the transcriptional network of the ecdysone pathway on *PGRP-LC* could uncover novel regulatory pathways of related genes in other organisms.

The neuroendocrine response to stress have immunomodulating functions in vertebrates. Stressors stimulate the hypothalamic–pituitary–adrenal axis and the adrenal gland releases glucocorticoids into the blood to systemically control the immune system (**Sternberg, 2006**). Although glucocorticoids are pharmacological inhibitors of cytokine production in humans (**Ashwell et al. 2002**), there is evidence that acute stress enhances cell-mediated immunity

(**Dhabhar and Mcewen, 1997**). In the *Drosophila* system, several studies have demonstrated that certain kinds of stress such nutritional or sleep deprivation elevates ecdysone (**Terashima, 2005; Ishimoto and Kitmoto, 2010**), but it is unknown whether flies undergoing these kinds of stresses have enhanced immunity. Flies infected with bacteria experience an increase in *PGRP-LC* (**Figure 1.1F**) and it is not clear whether infection engages the neuroendocrine axis to upregulate ecdysone production or if the immune response has a role in this function. Similarly, *PGRP* is also upregulated in larvae from a moth species challenged with gram-negative bacteria (**Kang et al., 1998**). Exploring ecdysone regulation using an *in vivo* model in the context of infection would help elucidate a complex, but conserved interplay between the neuroendocrine and immune systems. ■

1.3 Thesis Objectives

In order to identify ecdysone-activated *PGRP-LC* cis-regulatory elements, I used a reporter assay to measure promoter activity driven by inserts cloned from the *PGRP-LC* locus. These experiments were carried out in *Drosophila* S2* cells treated with ecdysone. ChIP-seq data was mined from modENCODE to look for enrichment of activator proteins and ecdysone-regulated transcription factors. To validate the role of the upstream *PGRP-LC* enhancer *in vivo*, I generated mutant flies using the CRISPR-Cas9 method and measured *PGRP-LC* expression and their survival to bacteria infection.

To characterize the *PGRP-LC* enhancer element, I used bioinformatics software to search for conserved regions along a *Drosophila* genome alignment and putative binding sites for nine transcription factors regulated by ecdysone. These motifs were mutated in the reporter plasmids and I looked for changes in the reporter activity. To help determine the transcription factors that act on the *PGRP-LC* promoter insert, the nine transcription factors were knocked down in S2* cells and the reporter activity was compared to endogenous *PGRP-LC* gene activity. Finally, enzyme immunoassay was used to quantify ecdysone in infected flies and assess the effect of infection on hormone production. ■

CHAPTER II: Materials & Methods

Drosophila Cell Culture Maintenance

S2* cells were maintained on Schneider's *Drosophila* Medium (Thermo Fisher) at 27ºC. Additives to medium include 10% Fetal Bovine Serum (Valley Biomedical or ATLANTA biological), 1% GlutaMAX (Gibco), and 0.2% Penicillin-Streptomycin (Gibco).

Construction of Reporter Plasmids

The control plasmid pGL3-Basic (pGL3-Luciferase) was a gift from the Kate Fitzgerald lab (Umass Medical School). Luciferase reporters driven by inserts derived from the *PGRP-LC* promoter, except F19b, were created by digesting pGL3-Diptericin promoter-Luciferase (**Tauszig et al., 2000**) with NheI and NcoI and exchanging the *Diptericin* promoter insert. In order to make F19b, F3 was digested with BglII, the ends blunted with T4 DNA Polymerase (NEB), and self-ligated. pGL3-Hsp70-Luciferase was created by digesting pGL3-Per-E-box-Hsp70-Luciferase with KpnI and XhoI to remove Per-E-box insert, blunting ends with T4 DNA Polymerase (NEB), and self-ligating ends. Hsp70-Luciferase reporters driven by upstream inserts derived from the *PGRP-LC* cis-regulatory element was created by exchanging the Per-E-box insert. Hsp70-luciferase reporter with the downstream insert was cloned with SalI and BamHI restriction sites. The pGL3-Per-E-box-Hsp70-Luciferase plasmid was a gift from the Patrick Emery lab (Umass Medical School). The Inserts were cloned from genomic DNA

extracted from *D. melanogaster* Oregon R strain or *D. simulans*, *D. yakuba*, *D. pseudoobscura*, or *D. virilis* genomic DNA ordered from the UCSD *Drosophila* Species Stock Center (https://stockcenter.ucsd.edu/index.php?table=GenomicDNA). The copia-Renilla luciferase plasmid was given to the Silverman lab from the Michael Rosbash lab (Brandeis University).

RNAi

Liner DNA templates were generated by PCR using *D. melanogaster* Oregon R genomic DNA with Phusion High-Fidelity DNA Polymerase (NEB). RNAi (dsRNA) for *GFP*, *EcR*, *br-c*, *srp*, *pnr*, *Hr46*, *Eip74EF*, *Eip75B*, *Eip78C*, and *Eip93F* were synthesized using the T7 RiboMAX Express Large Scale RNA Production System (Promega). The dsRNA were purified with the RNeasy Mini Kit (Qiagen).

Luciferase Assay

S2* cells were plated in 6-well plates (1x10^6 cells/mL with 3 mL per well) in the afternoon and incubated at 27ºC overnight. The next morning, 1.5 ug firefly luciferase plasmid, 1.5 ug *Renilla* luciferase plasmid, and/or 1.5 ug dsRNA were delivered to the cells by calcium phosphate transfection, and the plates were incubated at 27ºC for 24 h. The cells were split in 96-well plates (1x10^5 cells/mL with 100 uL per well) into 6 or 12 wells per sample, and incubated at 27ºC for 24 h. Half of the replicates were treated with 1 uM ecdysone (Sigma) diluted with Schneider's *Drosophila* Medium; an equal volume of Schneider's *Drosophila* Medium was added to the other half. These plates were incubated at 27ºC for 24 h. To process cells for reading, plates were spun down in a 4ºC centrifuge at

1000 RPM for 5 min, and the supernatant was aspirated. Cells were lysed with 60 uL/well Passive Buffer (Promega) diluted with sterile water on a shaker on slow for 15 min. 20 uL lysate was added to duplicate white reading plates with 20 uL luciferin (Biosynth, prepared in-house at 150 ug/mL) or 20 uL coelenterazine (Biotium); coelenterazine was 1 mg/mL dissolved in 100% ethanol and then 5 ug/mL diluted with sterile Dulbecco's Phosphate-Buffered Saline (Corning cellgro). The plates were immediately scanned uncovered in the 2102 EnVision Multilabel Reader (PerkinElmer) for 0.1 s. Each firefly luciferase reading was normalized to the corresponding *Renilla* luciferase reading; the mean and standard deviation of the replicate values were used for analysis.

RNA extraction and qRT-PCR

To prepare samples for dual assays in Figure 3.2D, transfection of S2* cells was carried out as previously described and incubated at 27ºC for 24 h. The cells were split in 96-well plates for luciferase assay (protocol described above and and continued from there) and 6-well plates (1x10^5 cells/mL with 3 mL per well) into 2 wells per sample for qRT-PCR, and incubated at 27ºC for 24 h. Half of the replicates were treated with 1 uM ecdysone and these plates were incubated at 27ºC for 24 h. To process samples for RNA extraction, the cells were transferred to conical tubes and spun down in a 4ºC centrifuge at 1000 RPM for 1 min, and the supernatant was aspirated. The cells were lysed with 500 uL TRIzol Reagent (Ambion), transferred to microcentrifuge tubes, and incubated at room temperature for at least 5 min. Lysates were mixed with 100 uL chloroform

(Fisher Scientific), incubated at room temperature for 2 min, and the tubes were spun down in a 4°C centrifuge at 12000*g* for 15 min. 150 uL of the aqueous layer was transferred to a clean microcentrifuge tube, mixed with 150 uL isopropanol (Sigma), and incubated at room temperature for 10 min before tubes were spun down in a 4°C centrifuge at 12000*g* for 10 min and the supernatant was aspirated. The pellet was washed with 500 mL 75% ethanol, the tubes were spun down in a 4°C centrifuge at 12000*g* and for 5 min, and the supernatant was aspirated. The pellet was briefly dried at room temperature and resuspended in 30 uL nuclease-free water. 1 ug RNA was treated with DNase I, Amplification-Grade (NEB) at 25ºC for 30 min and deactivated at 65ºC for 10 min with 2.5 mM EDTA. Half of RNA sample was converted to cDNA using iScript cDNA synthesis kit (BioRad), and then quantified with the C1000 Thermal Cycler (BioRad) with SYBR Green (BioRad) using the default 2-step melting curve program. qRT-PCR primers were specific to *PGRP-LCx*, *Diptericin,* or *Rp49*. Cycle number from qRT-PCR reading was converted to copy number according to the standard curve trendline and normalized to Rp49 values. Efficiency of DNase I treatment was quantified by measuring Rp49 expression in the other half of RNA sample diluted in water up to the same volume as the SYBR Green reaction. Primer amplification specificity was determined by analyzing the melting curve.

CRISPR Flies (Figure 3.1G) and Infection

[G0: generating sgRNA germline, balancing sgRNA] 205 *v*– embryos with the nanos promoter driving integrase expression in the primordial germ cells from the

X-chromosome and possessing the attP40 insertion site on the 2nd were injected with a plasmid containing the *v*+ gene and attB-U6-sgRNA (left)-U6-sgRNA (right). There were 160 surviving larvae, but only about 88 adults eclosed and were mated with *v*– flies with CyO/Sco (2nd). Many crosses were discarded due to mite infestation, leaving about 12 crosses remaining. [G1: recovering sgRNA transformants, introducing Cas9] 3 CyO or Sco *v*+ male or female flies were collected. These flies were mated with *w*– flies with vasa promoter driving Cas9 (X) in the primordial germ cells. Extra sgRNA transformant lines were mated with CyO/Sco (2nd) flies for backup. [G2: generating mutant germline, balancing mutation, removing Cas9] 3 *w*– non-CyO or non-Sco male flies were recovered and mated with *w*– flies with If/CyO (2nd), TM6Tb/TM3Sb (3rd). [G3: recovering mutant fly, removing sgRNA] 15 *w*– CyO (2nd), TM3Sb (3rd) male flies were collected from each cross and mated with the same female flies in G2. The males were collected for PCR screening with primers overlapping the deleted mutant region. Control DNA was extracted from yw, Cas9 male flies. [G4: propagating mutant chromosome] If/CyO (2nd), TM3Sb (3rd), non-TM6Tb flies from confirmed mutant vial was intercrossed. Abbreviations: G = generation, sgRNA = synthetic guide RNA, *v* = *vermillion*, *y* = *yellow*, *w* = *white*, – = mutant, + = wildtype. X, 2nd, or 3rd in parenthesis represent chromosome number. sgRNA target sites were generated and analyzed using online tools (http://www.flyrnai.org/evaluateCrispr/) and CRISPRseek (**Zhu et al., 2014**). Cloning the sgRNAs into the pCFD4 plasmid was carried out by Mike Brodsky (UMass Medical School). Rainbow

Transgenic Flies (http://www.rainbowgene.com/) were commissioned for their injection services. yv; CyO/Sco and yw, vas-Cas9 stocks were provided from Mike Brodsky. Infected flies were pricked in left or right prothorax with microsurgery needle dipped in a concentrated pellet of *E. coli 1106* or *Ecc15* and maintained on low yeast food at 25ºC. The bacteria was grown in LB Broth (Fisher Scientific) with (*Ecc15*) or without (*E. coli 1106*) 100 ug/mL ampicillin overnight in a shaker. Flies harvested after 6 h (3 groups of 5 flies per genotype) were processed for RNA extraction and qRT-PCR, which were carried out as described above. For survival experiments, 45 uninfected or infected flies per genotype were distributed across 3 vials to avoid overcrowding and tracked daily for total death count for 1 week.

Motif Prediction Software

Drosophila genome alignment in MAF format was downloaded from the UCSC Table Browser (http://genome.ucsc.edu) with the following settings: clade: Insect, genome: D. melanogaster, assembly: April 2006 (BDGP R5/dm3), group: Comparative Genomics, track: Conservation, table: multiz15way, and region: chr3L:9331030-9331450. Conversion from MAF format to FASTA file, with one sequence per species output, was performed with Galaxy (https://usegalaxy.org/). To improve alignment precision, unrelated species were deleted (*e.g. A. gambiae*, *A. mellifera*, *T. castaneum*) and gaps were removed (using any word processor “Find and Replace”) to make unaligned sequences. Multiple alignment was performed with MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/)

with Pearson/FASTA output format. The newly aligned file was uploaded to Twine (version 1.0, August 2, 2013) (http://labs.bio.unc.edu/crews/twine/Twine_main.html) for analysis. The DNA binding specificity data was provided by the FlyFactorSurvey database (release March 2013) (http://mccb.umassmed.edu/ffs/).

***Drosophila* Sequence Alignment**

The same steps were taken as described above to download the *Drosophila* genome alignment of the region chr3L:9331030-9331511, convert file formats, and remove extraneous species and gaps. Multiple alignment was performed with MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/) with ClustalW output format.

Enzyme Immunoassay

Infected flies were pricked in the abdomen with microsurgery needle dipped in a concentrated pellet of *E. coli 1106*. Total body ecdysteroids were extracted from 25 adult female or male flies using 250µL 100% methanol. Ecdysone levels were determined by competitive enzyme immunoassay (ACE Enzyme Immunoassay; Cayman Chemical) using 20-hydroxyecdysone EIA antiserum (Cayman Chemical). Calibration curves were generated using 20-hydroxyecdysone (Sigma). This assay was performed by Florentina Rus (UMass Medical School).

Statistical analysis

All data is presented as the mean of biologically independent samples, unless stated otherwise, and error bars represent standard deviation. To calculate statistical significance, unpaired *t*-test was performed. ■

Table 1: Cloning primers used to make inserts in Figure 3.1A. Primers are preceded by a 4 bp extension (CATC) and NheI site (forward primer) or NcoI site (reverse primer).

	Forward Primer (5'-3')	Reverse Primer (5'-3')
F3	CATC-GCTAGC-TATGCTGGCTTCGAAACCAA	CATC-CCATGG-TTTAAATTGCCGACGAAAAC
F14	CATC-GCTAGC-AATAATGATGTTTTATTTTT	CATC-CCATGG-TTTAAATTGCCGACGAAAAC
F19b	Not Applicable	Not Applicable
F4	CATC-GCTAGC-TATGCTGGCTTCGAAACCAA	CATC-CCATGG-GCGATCAAATCGCAGCGGCC
F22	CATC-GCTAGC-AGATCTTTTGAGAAATCACT	CATC-CCATGG-TTTAAATTGCCGACGAAAAC

Table 2: Cloning primers used to make inserts in Figure 3.1B, Figure 3.1D, and Figure 3.2A. Primers are preceded by a 4 bp extension (CATC) and KpnI site (forward primer) or XhoI site (reverse primer). ^F57 primers include BamHI site (forward primer) or SalI site (reverse primer).

	Forward Primer (5'-3')	Reverse Primer (5'-3')
F38 (*D. mel.*)	CATC-GGTACC-AATGGAGCTTTCTTTTAATC	CATC-CTCGAG-ATCATTGTACATATATTCAC
F39	CATC-GGTACC-AATGGAGCTTTCTTTTAATC	CATC-CTCGAG-GATCACAACATCGTCTATAT
F40	CATC-GGTACC-AATGGAGCTTTCTTTTAATC	CATC-CTCGAG-AGACACACACAAGATCGATG
F41	CATC-GGTACC-AATGGAGCTTTCTTTTAATC	CATC-CTCGAG-AAGGGGTACTGGTATTGGCA
F42	CATC-GGTACC-AATGGAGCTTTCTTTTAATC	CATC-CTCGAG-CTGCGGCTTGGGAATTTCCA
F44	CATC-GGTACC-AATGGAGCTTTCTTTTAATC	CATC-CTCGAG-GGTTTCGAAGCCAGCATAAC
F45	CATC-GGTACC-AAAGTAAACCGGTTGGGAGC	CATC-CTCGAG-ATCATTGTACATATATTCAC

F46	CATC-GGTACC-TTTATTTATAGTCTCTCTTC	CATC-CTCGAG-ATCATTGTACATATATTCAC
F47	CATC-GGTACC-TCAACCTCTATCAGTGCCAA	CATC-CTCGAG-ATCATTGTACATATATTCAC
F48	CATC-GGTACC-TTATTCCCTCATAGACTTTA	CATC-CTCGAG-ATCATTGTACATATATTCAC
F49	CATC-GGTACC-GTGTGTGTGTGTAAATATAT	CATC-CTCGAG-ATCATTGTACATATATTCAC
F50	CATC-GGTACC-GCTGTGGCTGCCTTTGTCAT	CATC-CTCGAG-ATCATTGTACATATATTCAC
F57^	CATC-GGATCC-AATGGAGCTTTCTTTTAATC	CATC-GTCGAC-ATCATTGTACATATATTCAC
F58 (*D. sim.*)	CATC-GGTACC-ATTTGAGCTTTCTTTTAATC	CATC-CTCGAG-TATTATCACTGTATATGCAC
F59 (*D. yak.*)	CATC-GGTACC-AGTCGAGCTTTCTTTTAATC	CATC-CTCGAG-ATATTGCATGTTATATGCAC
F60 (*D. pse.*)	CATC-GGTACC-ACACGAGCTTTCTTTTTTCT	CATC-CTCGAG-TTGTTGTACAGTATTTTAAT
F61 (*D. vir.*)	CATC-GGTACC-ACGTAAGTTTTCGCTTCTTG	CATC-CTCGAG-CTTTTAATTAACTCACAAAC

Table 3: sgRNA target sites in Figure 3.1F were cloned into the pCFD4-U61-U63 plasmid according to the CRISPR fly design protocol (http://www.crisprflydesign.org/).

	Left Target Site	Right Target Site
sgRNA	GTAAACCGGTTGGGAGCAAG	AATCCTATTTACAGGAAGAC

Table 4: qRT-PCR primers used in Figure 3.1H and Figure 3.2D.

	Forward Primer (5’-3’)	Reverse Primer (5’-3’)
PGRP-LCx	CGGGGAGGAGCTCTACAAGA	CTTATTAGATTTCGTGTGACCAGTGC
Diptericin	CATTGCCGTCGCCTTACTT	TAGGTGCTTCCCACTTTCCA

Rp49	GCACTCTCTGTTGTCGATACC CTTG	AGCGCACCAAGCACTTCATC

Table 5: Site-directed mutagenesis PCR primers used to make constructs in Figure 3.2C. Nucleotides in bold differs from the wildtype sequence.

	Forward Primer (5’-3’)	Reverse Primer (5’-3’)
F67 (F3 br-Z3 mut.)	GTTTTACTGTCGCTCAA AA**CT**CTGGAAATTCCCA AGCCG	CGGCTTGGGAATTTCCA G**AG**TTTTGAGCGACAGT AAAAC
F65 (F3 Hr46 mut. #1)	CAAGCCGCAGTCAACC **GA**TATCAGTGCCAATAC	GTATTGGCACTGATA**TC** GGTTGACTGCGGCTTG
F66 (F3 Hr46 mut. #2)	CGCAGTCAACCTCT**CG**C AGTGCCAATACC	GGTATTGGCACTG**CG**AG AGGTTGACTGCG
F63 (F3 srp mut. #1)	GCCAGTACAACATCA**GG** ATCGGTAGCTTCAC	GTGAAGCTACCGAT**CC**T GATGTTGTACTGGC
F64 (F3 srp mut. #2)	GCCAGTACAACATCATTA **GA**GGTAGCTTCACTTCA CTTC	GAAGTGAAGTGAAGCTA CC**TC**TAATGATGTTGTAC TGGC
F61 (F3 pnr mut. #1)	GCTGCCTTTGTCAT**GG**A TCTGCCTTTCAACG	CGTTGAAAGGCAGAT**CC** ATGACAAAGGCAGC
F62 (F3 pnr mut. #2)	GCTGCCTTTGTCATTTA **GA**TGCCTTTCAACGTGA ATA	TATTCACGTTGAAAGGC A**TC**TAAATGACAAAGGC AGC
F72 (F47 Hr46 mut. #1)	CGATAGGTACCTCAACC **GA**TATCAGTGCCAATAC C	GGTATTGGCACTGATA**T** **C**GGTTGAGGTACCTATC G
F70 (F47 srp mut. #1)	GCCAGTACAACATCA**GG** ATCGGTAGCTTCAC	GTGAAGCTACCGAT**CC**T GATGTTGTACTGGC
F71 (F47 srp mut. #2)	GCCAGTACAACATCATTA **GA**GGTAGCTTCACTTCA CTTC	GAAGTGAAGTGAAGCTA CC**TC**TAATGATGTTGTAC TGGC
F68 (F47 pnr mut. #1)	GCTGCCTTTGTCAT**GG**A TCTGCCTTTCAACG	CGTTGAAAGGCAGAT**CC** ATGACAAAGGCAGC

F69 (F47 pnr mut. #2)	GCTGCCTTTGTCATTTA **GA**TGCCTTTCAACGTGA ATA	TATTCACGTTGAAAGGC A**TC**TAAATGACAAAGGC AGC
F73 (F50 pnr mut. #1)	GCTGCCTTTGTCAT**GG**A TCTGCCTTTCAACG	CGTTGAAAGGCAGAT**CC** ATGACAAAGGCAGC
F74 (F50 pnr mut. #2)	GCTGCCTTTGTCATTTA **GA**TGCCTTTCAACGTGA ATA	TATTCACGTTGAAAGGC A**TC**TAAATGACAAAGGC AGC

Table 6: RNAi primers used to make dsRNA in Figure 3.2D. Primers are proceeded by a 6 bp extension (GGATCC) and the minimal T7 RNA polymerase promoter (TAATACGACTCACTATAGG).

	Forward Primer (5'-3')	Reverse Primer (5'-3')
GFP	GGATCC-TAATACGACTCACTATAGG-AGCCGCTACCCCGACCACAT	GGATCC-TAATACGACTCACTATAGG-TTGCTCAGGGCGGACTGGGT
EcR	GGATCC-TAATACGACTCACTATAGG-TTCTCCTCCTGGGTAATCTG	GGATCC-TAATACGACTCACTATAGG-TGCTCGTCGGAGGTGA
br-c	GGATCC-TAATACGACTCACTATAGG-GCCCTGGTGGAGTTCATCTA	GGATCC-TAATACGACTCACTATAGG-AGCAGCTGGTTGTTGATGTG
srp	GGATCC-TAATACGACTCACTATAGG-TCTTGGGTCAACATGAGCAG	GGATCC-TAATACGACTCACTATAGG-TCGATTTTATGCTGTTGGCA
pnr	GGATCC-TAATACGACTCACTATAGG-GCCGTCAAGATGTACCACAG	GGATCC-TAATACGACTCACTATAGG-GTCCATAGCGCTCTCGTAGG
Hr46	GGATCC-TAATACGACTCACTATAGG-GAAGACGGGCTCCTTTGA	GGATCC-TAATACGACTCACTATAGG-CAGCACTAAGCTCTGATACA
Eip74EF	GGATCC-TAATACGACTCACTATAGG-CCCAGAGTGTTATCCAACCG	GGATCC-TAATACGACTCACTATAGG-GTATGCCGCGCTGGTAGTAG

Eip75B	GGATCC-TAATACGACTCACTATAGG-CAATCACAATCAGGTGGTGC	GGATCC-TAATACGACTCACTATAGG-AATATCGCTGCGCTTCATCT
Eip78C	GGATCC-TAATACGACTCACTATAGG-GCTTCTTCGAGGTCTGGTTG	GGATCC-TAATACGACTCACTATAGG-CCAGTTCATCCGTAGCCAGT
Eip93F	GGATCC-TAATACGACTCACTATAGG-AGAACGCGTTGCTGAAGAAT	GGATCC-TAATACGACTCACTATAGG-CGGTGTTGGTGTACGTGATG

CHAPTER III: Results

3.1 Ecdysone activated enhancer controls the *PGRP-LC* promoter

Identifying enhancers for specific genes can be a challenging task since the location and spatial arrangement of enhancers relative to genes they regulate is variable. We took an unbiased approach to search for enhancer elements upstream of the *PGRP-LC* coding sequence and cloned about 2.6 kb of the *PGRP-LC* promoter region from a *D. melanogaster* strain and inserted it upstream of the luciferase reporter (**Figure 3.1A**). The 2.6 kb promoter fragment includes part of the *PGRP-LA 3' UTR* (357 bp), an intergenic region that separates the *PGRP-LA* and *PGRP-LC* loci (473 bp), the first putative exon of one *PGRP-LCx* transcript renamed "*exon 1.1*" (57 bp), an intron (1571 bp), and the first exon of multiple transcripts, including *PGRP-LCx* and *PGRP-LCa*, called "*exon 1.2*" (152 bp). The evidence for the transcription start site in *exon 1.1* is limited to EST (expressed sequence tag) sequencing, while the *exon 1.2* transcription start site is supported by gene annotation based on mRNA and cDNA evidence.

The *PGRP-LC* promoter reporter was transfected into S2* cells and the addition of ecdysone triggers a significant increase in reporter activity from the 2.6 kb fragment, F3 (**Figure 3.1A**). However, when the *PGRP-LA 3' UTR* was deleted from the insert in F14, the activity was reduced by two-thirds and the remnant activity was narrowed down to the *exon 1.2/intron* in F22. Joining the

PGRP-LA 3' UTR to *exon 1.2/intron* (F19b) could partially restored the full activity observed for F3. Furthermore, the *PGRP-LA 3' UTR* was not sufficient to drive the *exon 1.1* promoter if the *exon 1.2* promoter is disrupted (F4). Although unifying the region that is partially required for reporter activity (*PGRP-LA 3' UTR*) and the smallest region that could respond to ecdysone (*exon 1.2/intron*) in F19b did not fully account for all the activity observed in F3, this data suggest that the *PGRP-LA 3' UTR* could function as an ecdysone-activated enhancer for the *exon 1.2 PGRP-LC* promoter, which may contain additional ecdysone-responsive elements. Statistically significant RNA polymerase II ChIP-seq peaks in the *exon 1.2* region supports the existence of a promoter located immediately upstream of the transcription start site (**Nègre et al., 2011**).

To further investigate the enhancer potential of the *PGRP-LA 3' UTR* (conveniently renamed the *PGRP-LC* CRE (cis-regulatory element) or enhancer), this fragment was cloned directly upstream (F38) or downstream (F57) of the minimal promoter *Hsp70* and luciferase (**Figure 3.1B**). The size of the CRE tested here contains an additional 23 bp on the left side compared to Figure 3.1A, but this did not significantly impact the reporter activity (data not shown). The *PGRP-LC* CRE responded to ecdysone treatment when placed upstream, but not downstream of the reporter gene. It is unclear why this is the case, but it is possible that some enhancers function strictly upstream of promoters. Whether or not *PGRP-LC* CRE in the inverted position could still promote gene transcription has not been tested.

CBP (CREB-binding protein)-P300 is a pair of related coactivators that possess intrinsic acetyltransferase activity and participate in transcriptional activation or repression, including nuclear hormone receptor transactivation. (**Chakravarti et al., 1996; Vo and Goodman, 2001**). Multiple protein-binding domains allow CBP-P300 to cooperate with numerous transcription activators and members of the general transcription machinery. Active cis-regulatory elements are generally enriched with CBP-P300, a finding that was validated in *Drosophila* and its CBP-P300 homologue, Nejire (**Nègre et al., 2011**). Nejire was associated with the *PGRP-LC* enhancer in both the *PGRP-LA 3' UTR* and *exon 1.2* with significant peaks in adult flies and the pupae (**Figure 3.1C**). In the latter stage, ecdysone pulses reaches a peak and this corresponds with robust *PGRP-LC* expression in the same stage (**Figure 1.1C**). It is also striking that the antibody against the broad-complex core associates with the *PGRP-LC* CRE and *exon 1.2* in 16-24 h embryos (**Figure 3.1C**). However, the EcR did not bind in this region in a significant manner.

PGRP-LA and *PGRP-LC* and their relative locations are conserved within the *Drosophila* genus. To demonstrate conservation of ecdysone action on the *D. melanogaster PGRP-LC* enhancer among other species, this region from an aligned sequence of four other *Drosophila* species was cloned into the Hsp70-Luciferase reporter (**Figure 3.1D, Figure 3.1E**). DNA was sampled from a genetically diverse group of flies including *D. simulans* and *D. yakuba*, which both belong to the same group as *D. melanogaster*, and the more evolutionary

distant *D. pseudoobscura* and *D. virilis*. All of the *PGRP-LC* CRE reporters were responsive to ecdysone treatment and luciferase activities were significantly upregulated (**Figure 3.1D**). Thus, the ecdysone activation of the *PGRP-LC* CRE is a conserved pathway and could have biologically relevant functions in the *Drosophila* immune system.

In order to test the biological function of the *PGRP-LC* CRE *in vivo*, the *PGRP-LC* CRE was targeted for deletion using the genome editing tool CRISPR-Cas9 to make precise DNA breaks (**Figure 3.1F**) (**Bassett et al., 2013**). The deletion is based on the location of putative binding sites that was identified using the motif prediction software Twine (**Figure 3.2B**). Two suitable sgRNAs were generated and analyzed using online tools and CRISPRseek (**Zhu et al., 2014**). The mating scheme to generate mutant flies is described in Figure 3.1G and the deletion was validated by PCR and sequencing (**Figure 3.1G**).

Female and male control and mutant flies (ΔCRE) were infected with live *E. coli* and harvested 6 h later for RNA extraction. *PGRP-LCx* and *Diptericin* expression level was measured using qRT-PCR. Bacterial infection upregulated *PGRP-LCx* in female mutant flies, but it was less than infected control flies (**Figure 3.1H**). The decrease in *PGRP-LCx* expression was only half of control, but this was sufficient to achieve a lethal phenotype to infection in a previous report (**Rus et al., 2013**). The male mutant flies did not experience significant changes in either *PGRP-LCx* or *Diptericin* expression. The average female *Diptericin* level did show a reduction in the absence of the *PGRP-LC* CRE, but it

was not significant because the data distribution range was wide across all genotypes. However, the pattern of expression appears to be heading toward the same direction as *PGRP-LCx* and only one set of experiments was carried out so these data has yet to be validated.

To test whether the mutant flies would survive systemic infection with pathogenic bacteria, female and male control and mutant flies were infected with the gram-negative bacteria *Ecc15* (**Figure 3.1I**). The total number deaths were counted each day and after a week it became apparent that both female and male mutant flies continued to be as active as control flies. Under the hands of another member of the lab, the control flies experience nearly a 50% drop in survival in a week after *Ecc15* infection (**Rus et al., 2013**). However, this did not occur in this case and any deaths that did take place were mostly due to flies getting stuck in condensation. Therefore, the results were inconclusive since it appears the bacteria that was used for infection that day was not pathogenic or technical differences in which the experiments were carried out limited the pathogenic capacity of *Ecc15*. ■

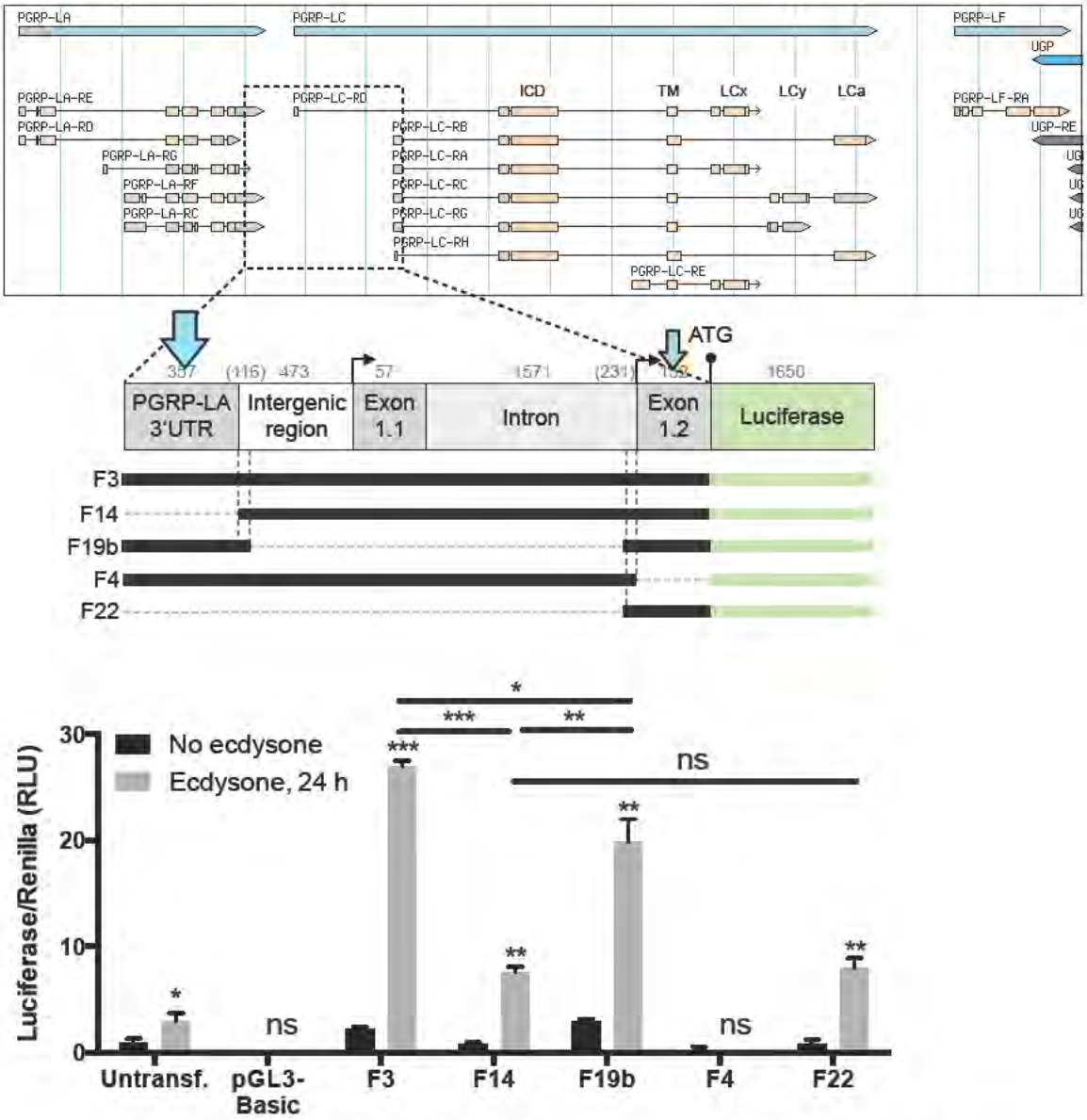


Figure 3.1A: Ecdysone-controlled elements in the *PGRP-LA 3' UTR* and *exon 1.2* contribute to the activation of the *PGRP-LC* promoter. There was a reduction in ecdysone-mediated reporter activity from F3 to F14, and residual activity was narrowed down to *exon 1.2* (F22). The blue arrows show the locations of the putative *PGRP-LC* cis-regulatory elements. S2* cells cotransfected with the firefly and *Renilla* luciferase reporters were treated with 1 uM ecdysone or an equal volume of media for 24 h. Data is presented as the mean of triplicate samples and error bars represent standard deviation. Regional sizes above the reporter diagram are in basepair. *P<0.05, **P<0.01, ***P<0.001, ns = not significant. Abbreviations: RLU = relative light units, untransf. = untransfected, ICD = intracellular domain, TM = transmembrane, LCx = *PGRP-LCx*, LCy = *PGRP-LCy*, LCa = *PGRP-LCa*, F = insert fragment. Figure uses a modified screenshot from the *D. melanogaster* Genome Browser (http://flybase.org/). ■

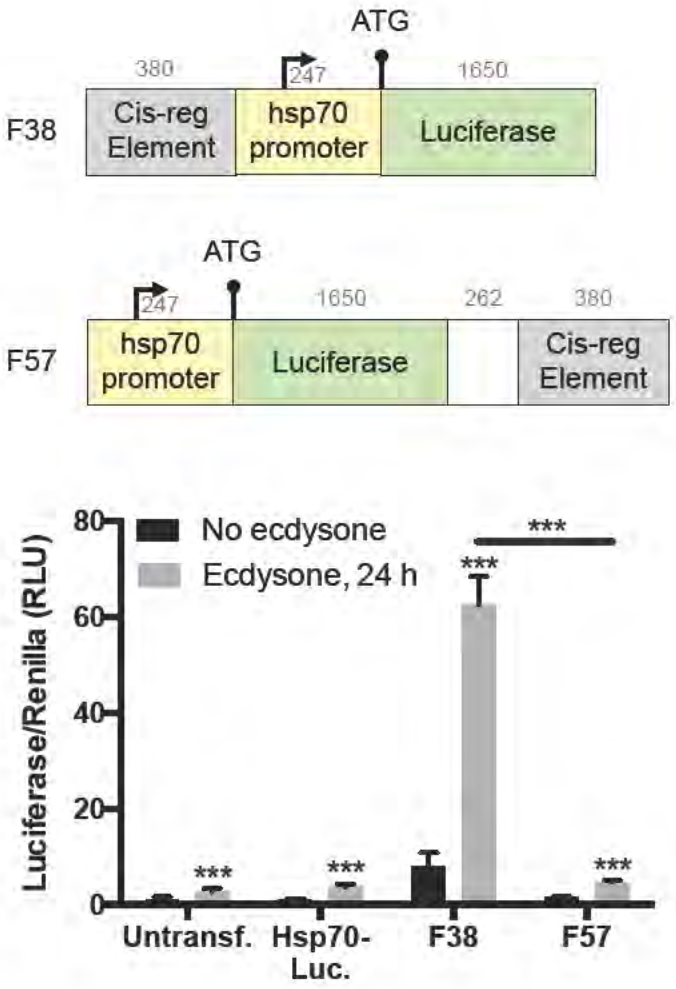


Figure 3.1B: The *PGRP-LC* cis-regulatory element display enhancer potential when placed upstream (F38), but not downstream (F57) of the *Hsp70* minimal promoter. The cis-regulatory element comes from the *PGRP-LA 3' UTR* in Figure 3.1A, but is extended 23 bp on the left side. S2* cells cotransfected with the firefly and *Renilla* luciferase reporters were treated with 1 uM ecdysone or an equal volume of media for 24 h. Data is presented as the mean of 6 samples and error bars represent standard deviation. Regional sizes above the reporter diagram are in basepair. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Abbreviations: cis-reg element = cis-regulatory element, RLU = relative light units, untransf. = untransfected, F = insert fragment. ■

Figure 3.1C: Enhancer-associated coactivators p300-CBP (Neijire) bind to the *PGRP-LC* cis-regulatory elements located in the *PGRP-LA 3' UTR* and *exon 1.2* in 16-20 h embryos, pupae, and adult flies (top). (The developmental stages are out of order.) EcR and br-c core ChIP-seq are displayed below. (Latter samples were derived from 16-24 h embryos.) Highlighted yellow boxes represent statistically significant peaks. The blue arrows show the locations of the putative *PGRP-LC* cis-regulatory elements as in Figure 3.1A. Figure uses modified screenshots from the modENCODE *D. melanogaster* Genome Browser (http://gbrowse.modencode.org/fgb2/gbrowse/fly/). ■

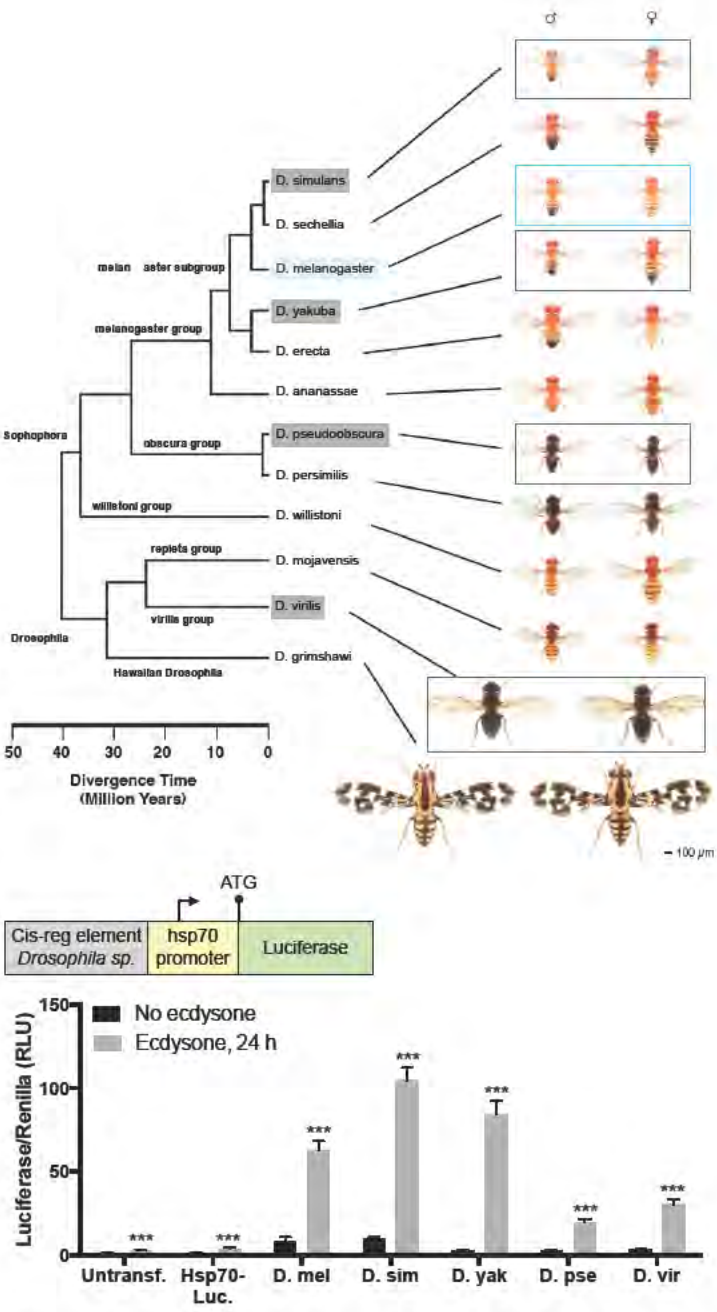


Figure 3.1D: The ecdysone-induced *PGRP-LC* enhancer activity is conserved among multiple *Drosophila* species. "D. mel" corresponds with the F38 reporter (Figure 3.1B). Inserts were produced by PCR with *Drosophila* genome DNA templates (Figure 3.1E). S2* cells cotransfected with the firefly and *Renilla* luciferase reporters were treated with 1 uM ecdysone or an equal volume of media for 24 h. Data is presented as the mean of 6 samples and error bars represent standard deviation. *P<0.05, **P<0.01, ***P<0.001. Abbreviations: cis-reg element = cis-regulatory element, RLU = relative light units, untransf. = untransfected, F = insert fragment. Figure uses a modified screenshot from FlyBase (http://flybase.org/). ■

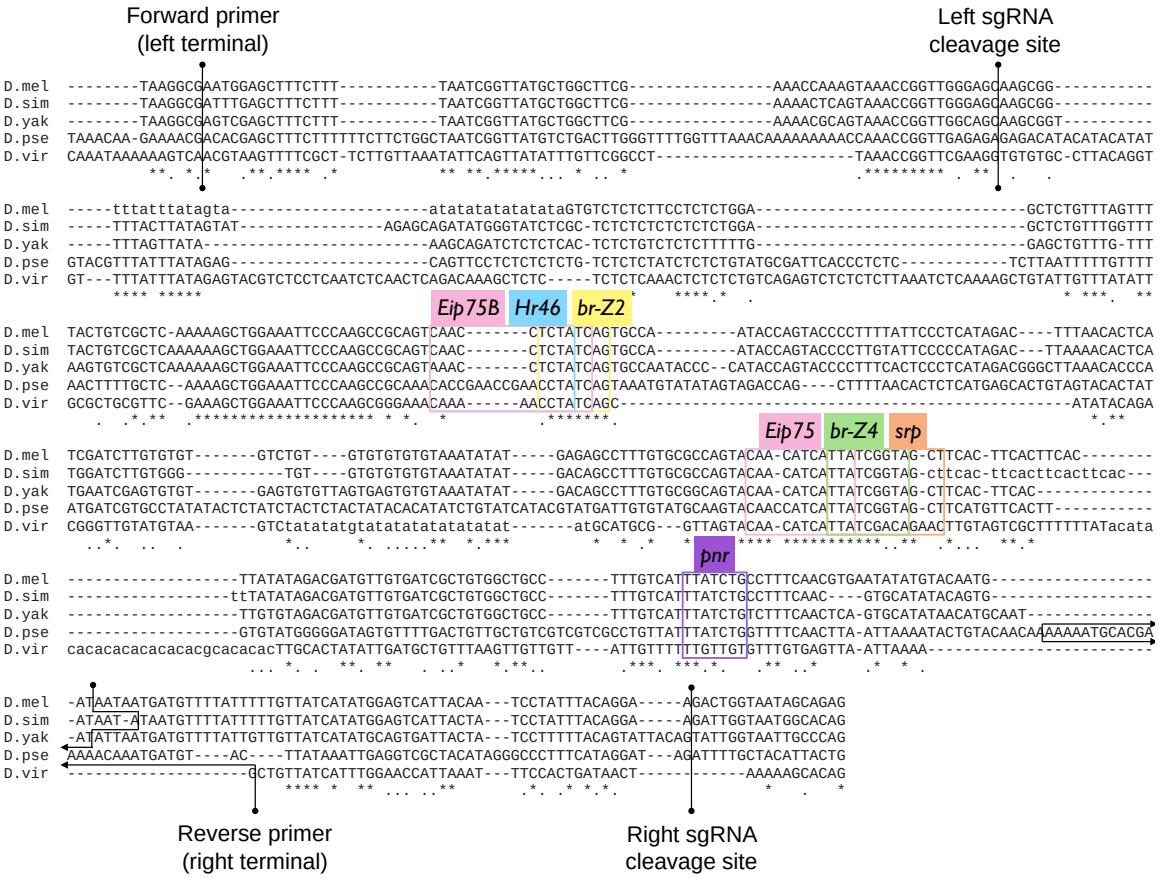


Figure 3.1E: *Drosophila* genome alignment of the *PGRP-LC* cis-regulatory element in the *PGRP-LA 3' UTR*. The extremes of the left and right cloning primers are labeled accordingly. Location of the left and right sgRNA cleavage sites are shown. The putative binding sites for ecdysone-regulated transcription factors is well conserved among divergent *Drosophila* species. *Drosophila* genome alignment was downloaded from the UCSC Table Browser (http://genome.ucsc.edu) and multiple alignment was performed with MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/). ■

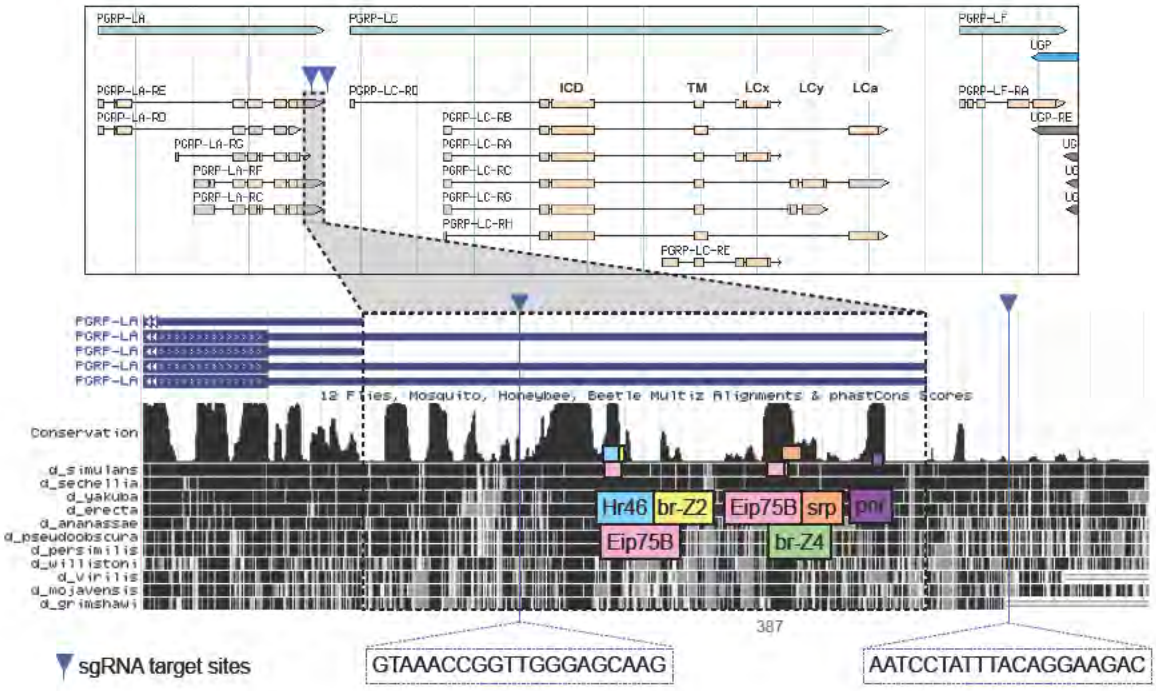
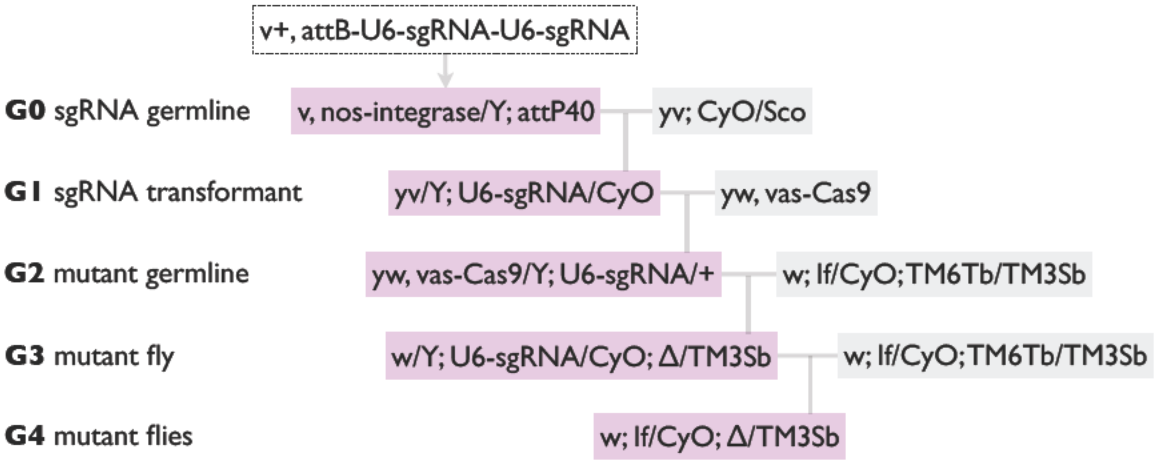


Figure 3.1F: Targeting a cluster of putative binding sites on the *PGRP-LC* enhancer for deletion on the 3rd chromosome. The deletion was carried out using the CRISPR-Cas9 method and sgRNA target sites are represented by inverted blue triangles. Two sgRNAs were generated and analyzed using online tools and CRISPRseek (Zhu et al., 2014). The deleted region is 387 bp and contains a cluster of putative motifs for ecdysone-regulated transcription factors (colored blocks). Black peaks are locations of conserved regions in a *Drosophila* genome alignment compared to *D. melanogaster*. Twine was used to identify the motifs and the process of selecting the motifs is described in Figure 3.2B. The mating schematic is described in Figure 3.1G. Abbreviations: sgRNA = synthetic guide RNA. Figure uses a modified screenshot from the *D. melanogaster* Genome Browser (http://flybase.org/) and UCSC Genome Browser (*http://genome.ucsc.edu*). ■




```
PGRP-LC cis-regulatory element   : [1 : 380]
Intergenic Region                : [381 : 853]
Hr46                             : [150 : 159]
E75                              : [150 : 160]
br-Z2                            : [155 : 162]
E75                              : [269 : 279]
srp                              : [277 : 288]
br-Z1                            : [278 : 285]
pnr                              : [345 : 351]
Left sgRNA target site           : [45 : 64]
Right sgRNA target site          : [422 : 441]

Wildtype 1     AATGGAGCTTTCTTTTAATCGGTTATGCTGGCTTCGAAACCAAAGTAAACCGGTTGGGAG  60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CRISPR   1     AATGGAGCTTTCTTTTAATCGGTTATGCTGGCTTCGAAACCAAAGTAAACCGGTTGG---  57

Wildtype 61    CAAGCGGTTTATTTATAGTCTCTCTTCCTCTCTGGAGCTCCGTTTAGTTTTACTGTCGCT  120
CRISPR   58    ------------------------------------------------------------  57

Wildtype 121   CAAAAAGCTGGAAATTCCCAAGCCGCAGTCAACCTCTATCAGTGCCAATACCAGTACCCC  180
CRISPR   58    ------------------------------------------------------------  57

Wildtype 181   TTTTATTCCCTCATAGACTTTAACACTCATCGATCTTGTGTGTGTCTGTGTGTGTGTGTA  240
CRISPR   58    ------------------------------------------------------------  57

Wildtype 241   AATATATGAGAGCCTTTGTGCGCCAGTACAACATCATTATCGGTAGCTTCACTTCACTTC  300
CRISPR   58    ------------------------------------------------------------  57

Wildtype 301   ACTTATATAGACGATGTTGTGATCGCTGTGGCTGCCTTTGTCATTTATCTGCCTTTCAAC  360
CRISPR   58    ------------------------------------------------------------  57

Wildtype 361   GTGAATATATGTACAATGATAATAATGATGTTTTATTTTTGTTATCATATGGAGTCATTA  420
CRISPR   58    ------------------------------------------------------------  57

Wildtype 421   CAATCCTATTTACAGGAAGACTGGTAATAGCAGAGAAATATATTAAAAATGAAATGGATA  480
                                     ||||||||||||||||||||||||||||||||||||
CRISPR   58    ----------------------TAATAGCAGAGAAATATATTAAAAATGAAATGGATA  93
```

Figure 3.1G: *Drosophila* mating schematic to generate CRISPR-mediated deletion of the *PGRP-LC* cis-regulatory element in the *PGRP-LA 3' UTR* (top). Flies with transgenic sgRNA germ line was created by injecting integrase expressing attP40 embryos with the attB-U6-sgRNA-U6-sgRNA plasmid (G0). Transgenic offspring of G0 flies was mated with Cas9 flies (G1) to generate the deletion (Δ) of the *PGRP-LC* cis-regulatory element in the primordial germ cells (G2). The offspring of G2 was mated with flies with 3rd chromosome balancers, where the *PGRP-LC* locus is located (G3). The male parent of G3 was screened for the deletion by PCR and sequencing (bottom panel). The deletion was 387 bp; although it was accurate, it was imprecise. The offspring of G3 from the confirmed mutant vial is intercrossed to propagate the mutant chromosome (G4). Abbreviations: G = generation, sgRNA = synthetic guide RNA, *v* = *vermillion*, *y* = *yellow*, *w* = *white*, Y = male sex chromosome. ■

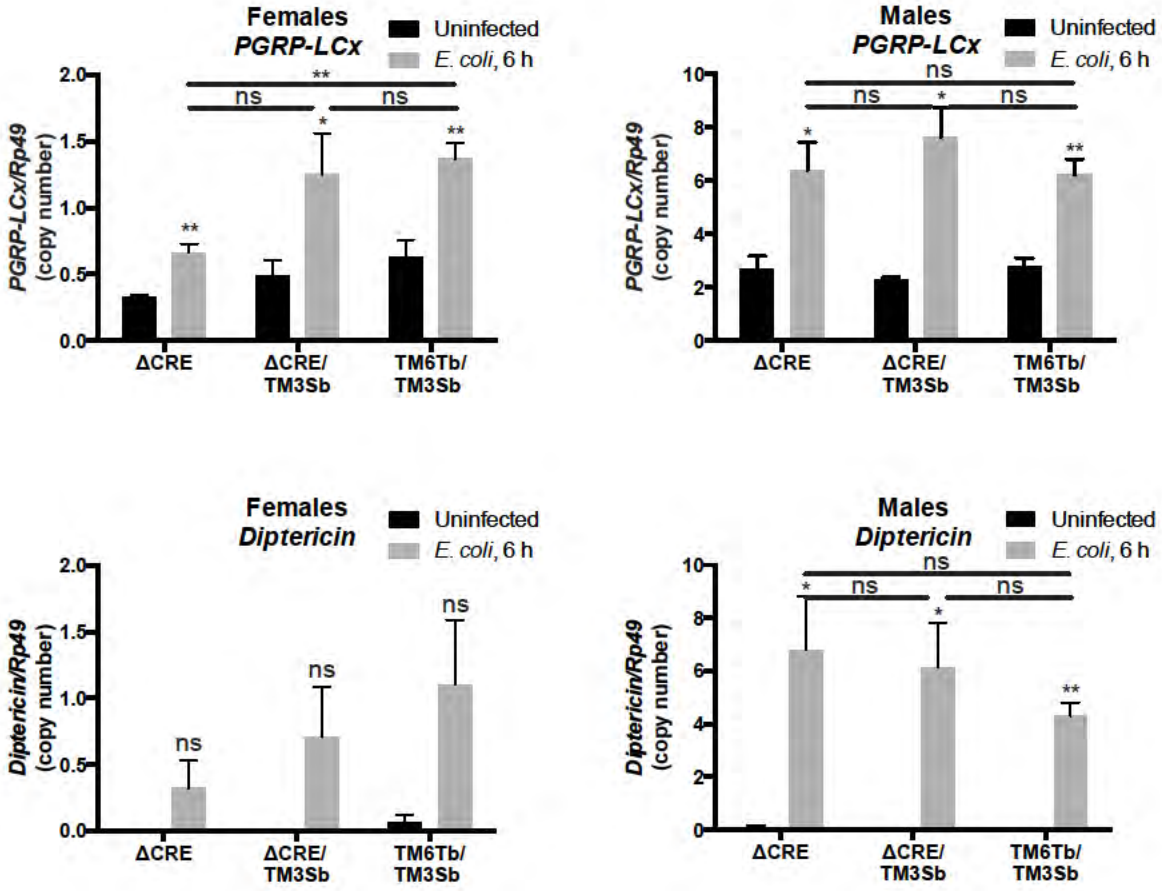


Figure 3.1H: Infection-induced *PGRP-LC* expression is significantly reduced in female but not male ΔCRE flies (top). The corresponding *AMP* expression was not significant, but is trending toward the same outcome (bottom). Flies were uninfected or pricked with a concentrated *E. coli 1106* pellet and infected for 6 h. RNA was harvested from whole flies and the same samples were used for *PGRP-LC*, *Diptericin*, and *Rp49* qRT-PCR reactions. Data is presented as the mean of 3 groups of 5 flies and error bars represent standard deviation. Cycle number from qRT-PCR data was converted to copy number according to the standard curve trendline for *PGRP-LCx*: $y = -2.9917x + 38.983$, $R^2 = 0.99356$; *Diptericin*: $y = -3.6761x + 33.724$, $R^2 = 0.98461$; and *Rp49*: $y = -3.4583x + 32.394$, $R^2 = 0.99872$. **P*<0.05, ***P*<0.01, ****P*<0.001, ns = not significant. Abbreviations: ΔCRE = deleted cis-regulatory element in the *PGRP-LA 3' UTR*. ■

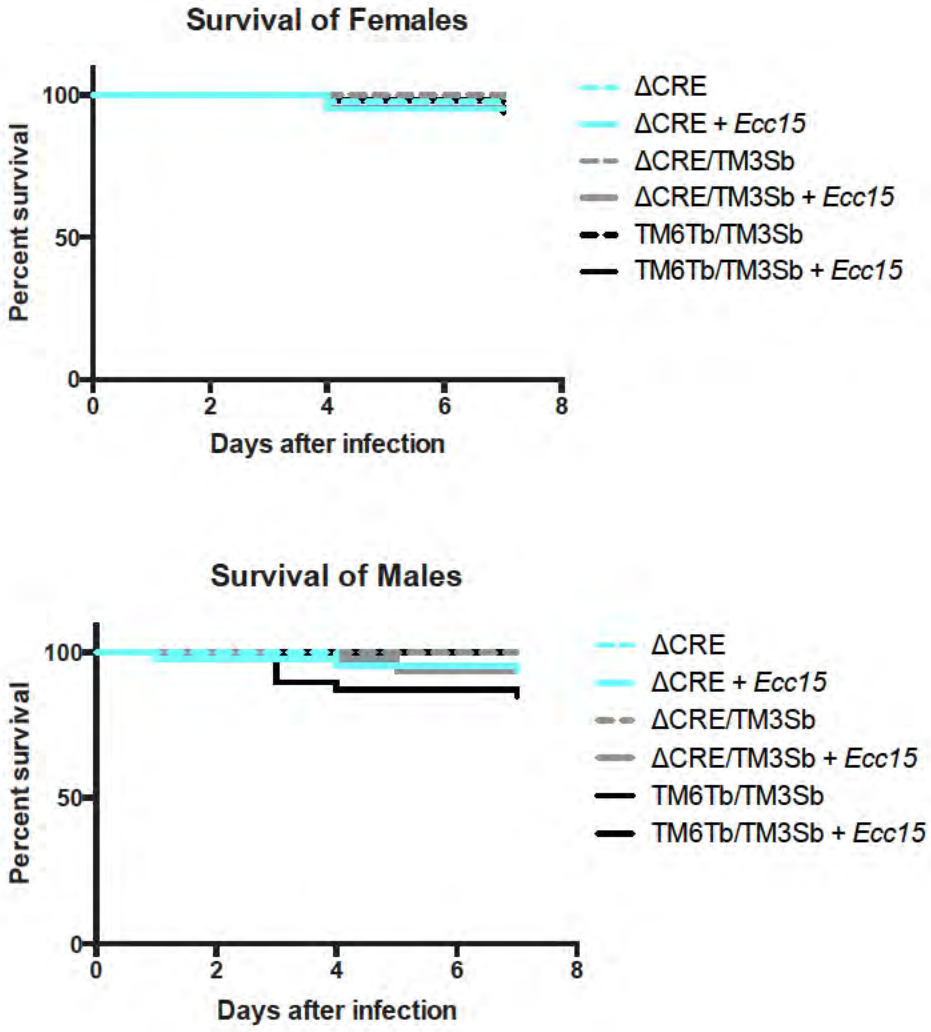


Figure 3.1I: The ΔCRE genotype did not impair female or male survival to *Ecc15* infection. Flies were uninfected or pricked with a concentrated *Ecc15* pellet and the number of deaths were tallied each day for a week. Each line represent data from 45 flies. Abbreviations: ΔCRE = deleted cis-regulatory element in the *PGRP-LA 3' UTR*, *Ecc15* = *Erwinia carotovora carotovora 15*. ■

3.2 Characterizing the *PGRP-LC* enhancer

The ecdysone pathway has been studied extensively in the developing fly, but details of its role in regulating the IMD pathway is limited. It was demonstrated that nine transcription factors were required for *PGRP-LC* expression in ecdysone-treated S2* cells (**Figure 1.1E**) and in infected flies (**Figure 1.1F**). There was one exception: the knockdown of the nuclear hormone receptor Eip75B enhanced *PGRP-LC*. In order to help piece together the ecdysone signaling network in controlling *PGRP-LC* gene activity, bioinformatic software was used to identify putative motifs within the 380 bp *PGRP-LC* CRE and it was expected that mutating these binding sites would affect the enhancer activity on the promoter.

Initially, elements in the *PGRP-LC* CRE were analyzed using the UCSC Genome Browser (http://genome.ucsc.edu/), which provided the *Drosophila* genome alignment and conservation data from 11 *Drosophila* species compared to *D. melanogaster* (**Figure 3.2A**). Since critical motifs that serve a protective role in nature would most likely be located within the most conserved regions, truncations made from the left and right of the *PGRP-LC* CRE were made based on these regions represented by the black peaks. The truncated inserts were cloned in the Hsp70-Luciferase reporter.

Samples transfected with reporters that had deletions that beginning on the right side of the insert (F39-44) showed an immediate reduction in activity that remained consistently low compared to the full-length fragment, F38 (**Figure**

3.2A). On the other hand, deletions beginning from the left side did not affect the reporter activity until F48, and the reduction occurred gradually. F38 and F47 displayed similar activity level, and deletion of regions possessed by the F47 insert diminish the ecdysone-mediated *PGRP-LC CRE* activity. This data suggest that there are multiple elements that are located on the right side of the *PGRP-LC* CRE, which is contained in the F47 insert. The firefly luciferase data were normalized to *Renilla* luciferase readings, but the average of ecdysone-treated samples were not normalized to the corresponding untreated samples. Interpretation of the data changes when analyzed in terms of fold-change due to the variable background that appears in untreated cells (**Figure 3.2A**), but the original analysis is valid.

The software Twine allows in-depth analysis of candidate motifs in the *PGRP-LC* CRE in a systematic and rigorous manner; it works in conjunction with multiple alignment data. *Drosophila* genome alignment was downloaded from the UCSC Genome Browser and after removing unrelated species (*e.g. A. gambiae*) and gaps to improve alignment precision, multiple alignment was performed with online tools. This newly aligned file was uploaded to Twine, which was preloaded with libraries of binding specificity data in the form of position frequency matrices. Data for the nine ecdysone-regulated transcription factors were analyzed on the *PGRP-LC* CRE multiple alignment. The number of candidate motifs were narrowed down using the motif cutoff, which is based on the log-odds 0 equivalent and is unique for each binding specificity data (**Figure 3.2B**). Motifs

that were selected for experimental testing were chosen based on relative conservation among *Drosophila* species (not shown).

Four target sites composed of individual or a cluster of three overlapping binding sites were selected to be mutated and their relative positions in the *D. melanogaster PGRP-LC* CRE are shown (**Figure 3.2B**). These included binding sites for several BR-C isoforms, HR46, Eip75B, SRP, and PNR. (Binding sites for the EcR, Eip74EF, Eip78C, and Eip93F were not found in the alignment.) These target sites were individually mutated in three reporters: F3, which possess the endogenous *PGRP-LC* promoter, and F47 and F50, in which the luciferase gene is under the control of the *Hsp70* promoter. Each independent mutant reporter is composed of two nucleotide substitutions and for some target sites, two mutant reporters were made (**Figure 3.2B**).

All of the target sites exists in the F3 reporter, while F47 lacks the *br-Z3* binding site and F50 possess only one candidate motif for PNR (**Figure 3.2C**). Mutation of the F3 *br-Z3* and *Hr46* (mut. #1) motifs did not change the ecdysone-mediated reporter activity observed in wildtype F3. This was true in the F47 reporter that also had *Hr46* mut. #1. The F3 *Hr46* (mut. #2) motif includes a highly conserved nucleotide and mutating this motif significant reduced the activity, although the signal was not abolished. Mutations in the serpent and pannier binding sites downregulated the activity of all reporters tested, but these reporters were still activated by ecdysone. These results could suggest that both

protein-DNA interactions and protein-protein contacts between adjacent transcription factors help maintain a protein complex on the *PGRP-LC* CRE.

It is unclear whether the F3 reporter activity is equivalent to the endogenous *PGRP-LC* activity induced by ecdysone. To help determine whether ecdysone action on the endogenous *PGRP-LC* locus accurately reflects the ecdysone activity on the *PGRP-LC* promoter reporter, we wondered whether knocking down the nine transcription factors would also adversely affect the reporter activity. S2* cells, cotransfected with the F3 reporter and dsRNA, were divided into separate plates. The plates were independently treated with ecdysone and the cells were process for luciferase reading or qRT-PCR. *PGRP-LCx* expression was measured to indirectly quantify dsRNA knockdown efficiency, but only the *EcR*, *br-c*, and *srp* dsRNA effectively silenced *PGRP-LCx* gene activity, while *Eip93F* dsRNA partially reduced it (**Figure 3.2D**). The reporter activity of corresponding samples treated with *EcR*, *br-c*, and *srp* dsRNA were downregulated. However, the *Eip93F* dsRNA did not prevent reporter activation by ecdysone. These results suggest that ecdysone activation of the *PGRP-LC* promoter reporter, F3, involves the EcR, BR-C, and SRP, but could be missing binding sites for Eip93F. ■

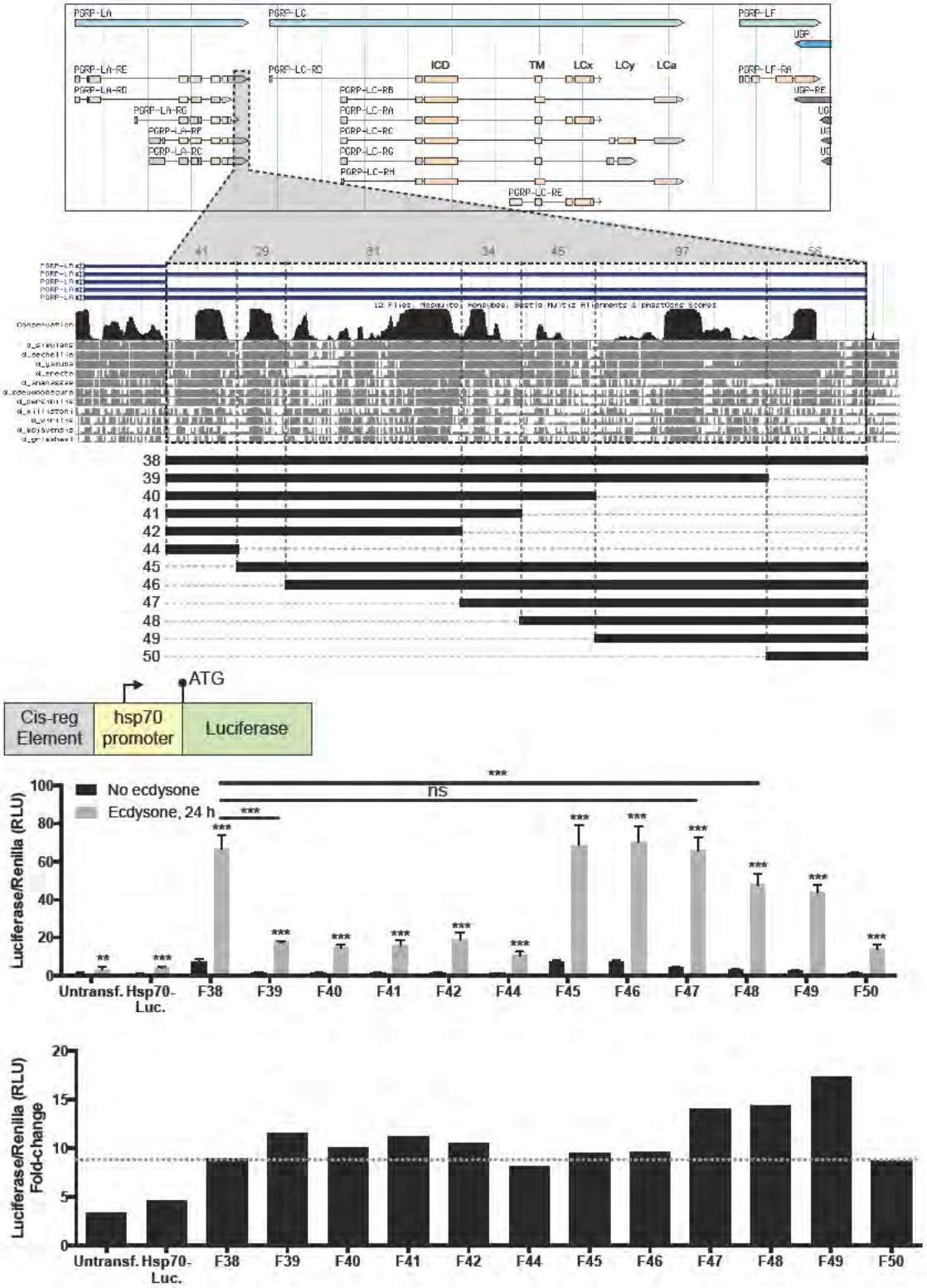

PGRP-LA
PGRP-LC
PGRP-LF
UGP
PGRP-LA-RE
PGRP-LA-RD
PGRP-LA-RG
PGRP-LA-RF
PGRP-LA-RC
PGRP-LC-RD
ICD
TM
LCx
LCy
LCa
PGRP-LC-RB
PGRP-LC-RA
PGRP-LC-RC
PGRP-LC-RG
PGRP-LC-RH
PGRP-LC-RE
PGRP-LF-RA
UGP-RE
41
29
81
34
45
97
38
39
40
41
42
44
45
46
47
48
49
50
ATG
Cis-reg Element
hsp70 promoter
Luciferase
No ecdysone
Ecdysone, 24 h
ns
Luciferase/Renilla (RLU)
Untransf.
Hsp70-Luc.
F38
F39
F40
F41
F42
F44
F45
F46
F47
F48
F49
F50
Luciferase/Renilla (RLU) Fold-change

Figure 3.2A: Multiple elements throughout the enhancer are important for robust *PGRP-LC* promoter activity as induced by ecdysone (top). Compared to F38, there is significantly less activity in reporters from F39-44 and F48-50. The same graph presented in terms of fold-change (below). The reporter activity in the smallest fragments, F44 and F50, were comparable to the full fragment in F38. Truncations are based on location of highly conserved regions in a *Drosophila* genome alignment compared to *D. melanogaster*. S2* cells cotransfected with the firefly and *Renilla* luciferase reporters were treated with 1 uM ecdysone or an equal volume of media for 24 h. Data is presented as the mean of 6 samples and error bars represent standard deviation. Regional sizes above the alignment are in basepair. *P<0.05, **P<0.01, ***P<0.001, ns = not significant. Abbreviations: RLU = relative light units, untransf. = untransfected, F = insert fragment. Figure uses a modified screenshot from the *D. melanogaster* Genome Browser (http://flybase.org/) and UCSC Genome Browser (*http://genome.ucsc.edu*). ■

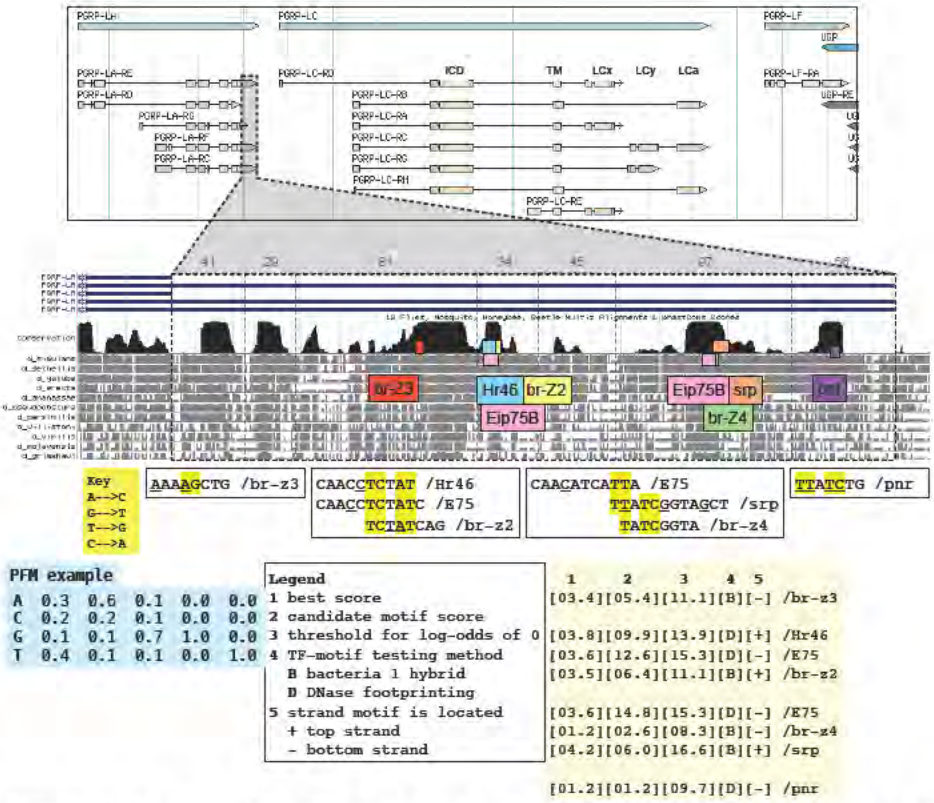


Figure 3.2B: Location and sequence of putative binding sites for ecdysone-regulated transcription factors in the *PGRP-LC* enhancer. Colored blocks represent the motifs and their general position in *D. melanogaster PGRP-LC* CRE. Above, conservation in a *Drosophila* genome alignment compared to *D. melanogaster* (black peaks). Motifs were selected based on frequency of appearance in the multiple alignment. Below, motif sequence; nucleotides highlighted in yellow were mutated; there are two nucleotide substitutions in each independent mutant. (The key for substitutions is on the left.) Underlined nucleotides have 100% frequency among experimental binding sites tested. Binding specificity data for the nine transcription factors were analyzed using the software Twine. The number of candidate motifs were narrowed down using the motif cutoff, which is based on the log-odds 0 equivalent and is unique for each binding specificity data (column 3); compared to the motifs score (column 2). (Twine takes the negative natural logarithm of the values so they appear inverted.) The score is the product of the base frequency at each position in the PFM. The log-odds of 0 means the probability of the motif appearing in real binding site is equal to the probability of the motif appearing in a sequence with equiprobable nucleotides; candidate motifs scores that fell below the log-odds of 0 are more likely to represent a functional motif than a random sequence. Column 1 shows the best score from each binding specificity data. Columns 4 and 5 show the method used to generate the PFM and the strand the motif is located. Regional sizes above the alignment are in basepair. Abbreviations: PFM = position frequency matrix. Figure uses a modified screenshot from the *D. melanogaster* Genome Browser and (http://flybase.org/) UCSC Genome Browser (*http://genome.ucsc.edu*). ■

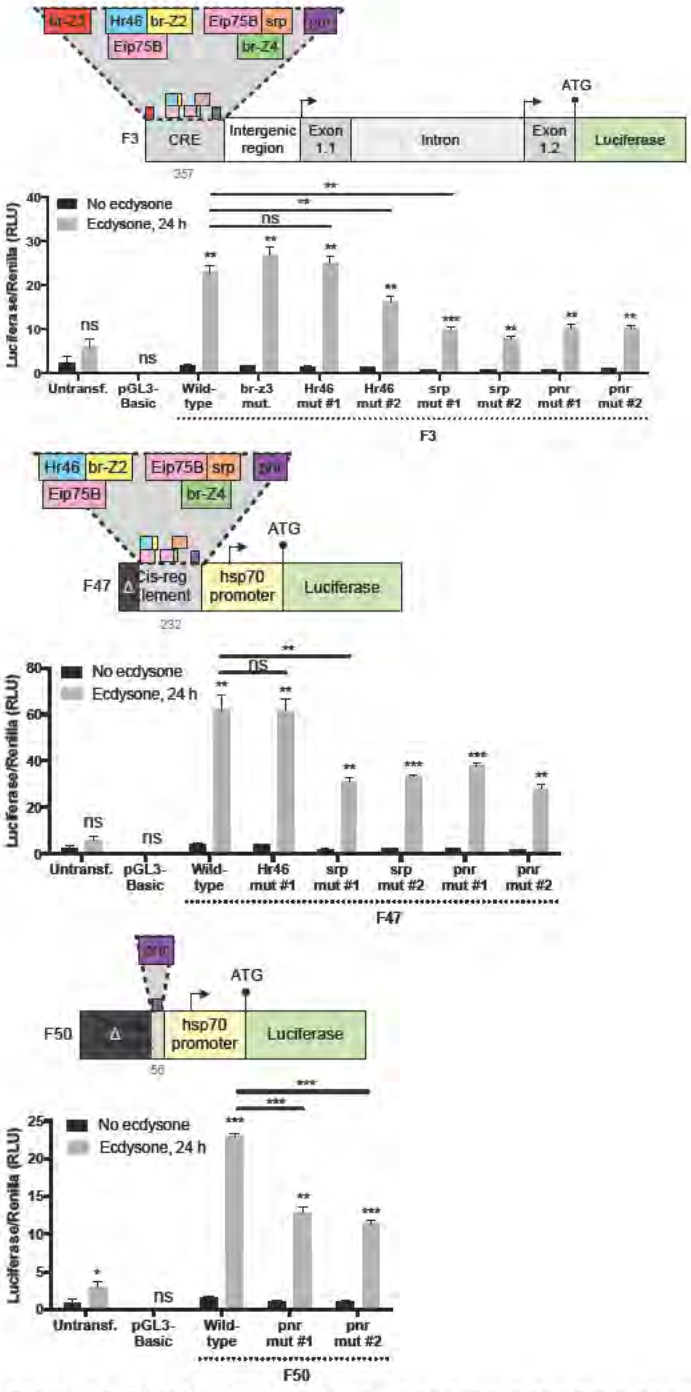


Figure 3.2C: Mutations in the candidate binding sites reduce, but doesn't eliminate the reporter activity. S2* cells cotransfected with the firefly and *Renilla* luciferase reporters were treated with 1 uM ecdysone or an equal volume of media for 24 h. Data is presented as the mean of triplicate samples and error bars represent standard deviation. Delta (Δ) represent deleted regions in F38. Regional sizes above the reporter diagram are in basepair. **P*<0.05, ***P*<0.01, ****P*<0.001, ns = not significant. Abbreviations: RLU = relative light units, F = insert fragment. ■

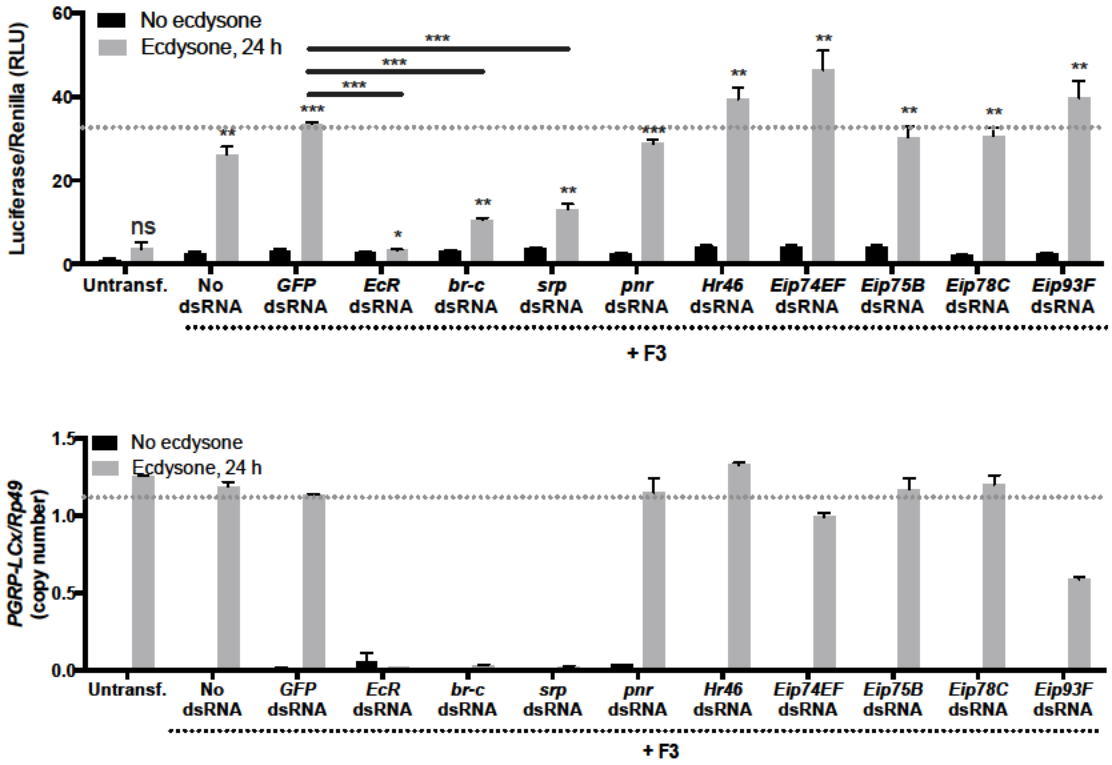


Figure 3.2D: Ecdysone-activated *PGRP-LC* promoter activity depends on ecdysone-regulated transcription factors (top). *RNAi* (dsRNA) knockdown efficiency was indirectly confirmed by measuring *PGRP-LCx* expression in the same cells (bottom). Experiments performed in S2* cells, cotransfected with F3 and *Renilla* luciferase reporters and dsRNA, were split into plates for luciferase reading or qRT-PCR. The samples were treated with 1 uM ecdysone for 24 h. Data is presented as the mean of triplicate samples and error bars represent standard deviation. Cycle number from qRT-PCR data was converted to copy number according to the standard curve trendline for *PGRP-LCx*: y = -3.1307x + 38.25, R^2 = 0.97782 and *Rp49*: y = -3.3909x + 31.315, R^2 = 0.99302. **P*<0.05, ***P*<0.01, ****P*<0.001, ns = not significant. Abbreviations: RLU = relative light units, F = insert fragment. ■

3.3 Proposing model for ecdysone regulation of *PGRP-LC* in infected flies

Most of the work completed *in vivo* has focused on the ecdysone pathway and its role in regulating the immune response. There is limited information about the upstream signals and pathways that regulate hormone production in flies that experience a systemic infection following cellular injury. In the adult fly, *PGRP-LC* exists at a basal level, which doubles in expression after 6 or 24 h of infection with gram-negative bacteria (**Figure 1.1F; Figure 3.1H**). To investigate ecdysone production in these flies, ecdysone was measured at regular time intervals over 24 h in female or male flies infected with *E. coli*. The ecdysone level in female flies doubles after 3 h of infection and males experience a slight increase after 6 h (**Figure 3.3A**). It is unclear whether cellular injury and/or dissemination of bacteria into the hemolymph contributes to ecdysone biosynthesis.

In the proposed model, there are at least two scenarios by which ecdysone production could occur in the infected adult fly (**Figure 3.3B**). Stressful conditions such as cell injury may engage the nervous system to communicate with an unidentified endocrine synthesizing tissue to initiate ecdysone production (IMD-independent route). Subsequently, ecdysone activates the ecdysone pathway in the fat body, which induces a transcription factor complex (*e.g.*, BR-C, SRP, *etc.*) that interacts with an upstream enhancer to control *PGRP-LC* transcription in female flies. If bacteria enters through the lesion, the binding of peptidoglycan to PGRP-LC would activate expression of AMPs that target the invading bacteria. It is also possible that the activated IMD pathway could

promote ecdysone biosynthesis in the fat body (IMD-dependent route) with or without the stress-induced ecdysone production. ■

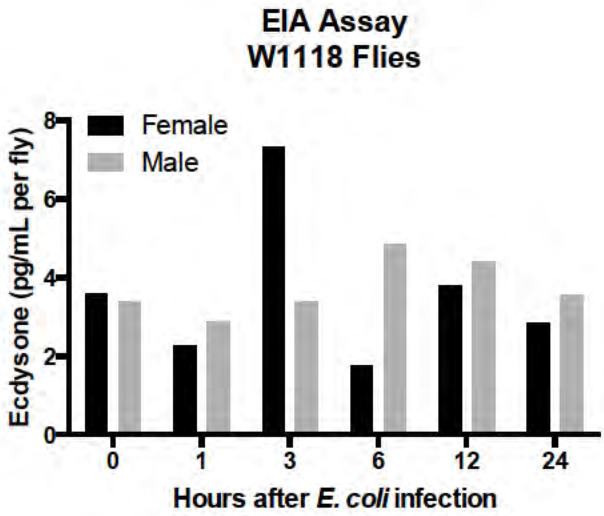


Figure 3.3A: Ecdysone production in wildtype female and male flies are influenced by gram-negative bacteria infection after 3 h and 6 h, respectively. Flies were pricked with a concentrated *E. coli 1106* pellet and infected for the displayed amount of time. The ecdysone concentration was calculated according to the standard curve trendline equation $y = -0.779\ln(x) + 4.6782$, $R^2 = 0.97466$. Each bar represent 25 flies in one sample. Data was generated by Florentina Rus (unpublished). ■

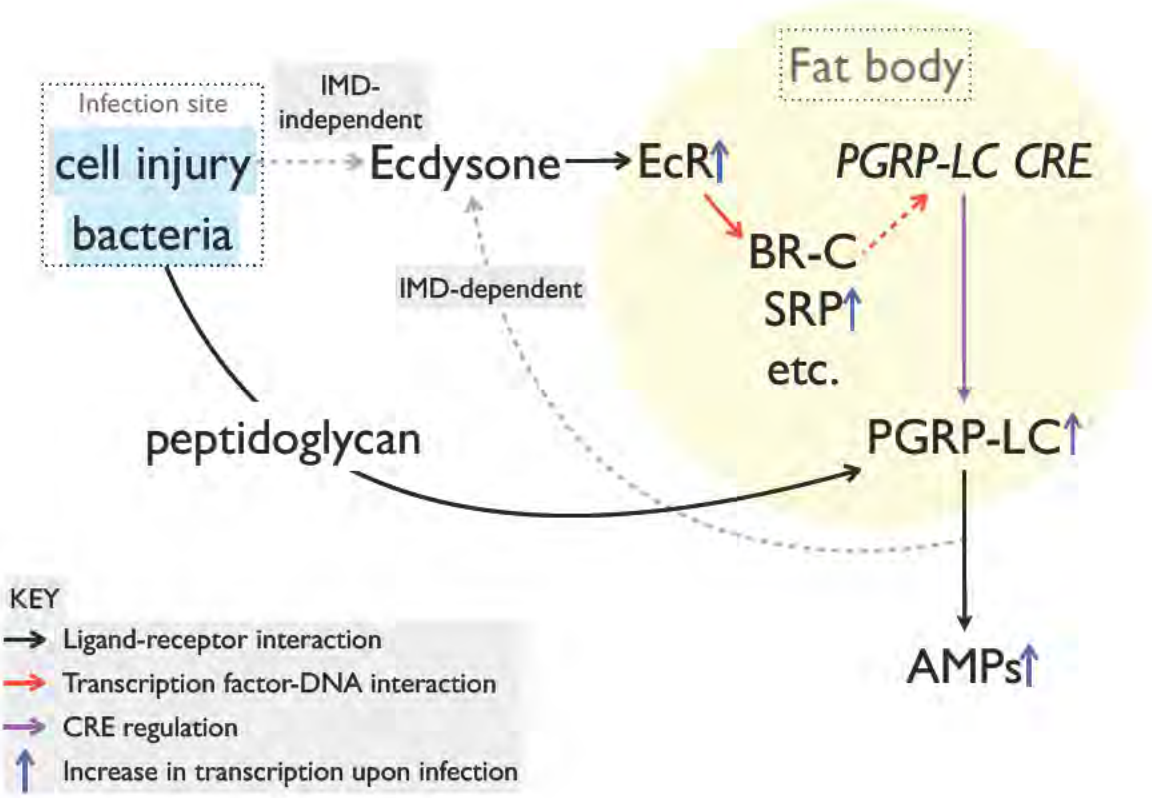


Figure 3.3B: In the proposing model, infection-induced *PGRP-LC* expression in flies depends on ecdysone production by an unidentified tissue. The activated ecdysone pathway and transcription factors interact with a female-specific cis-regulatory element (CRE) in the *PGRP-LA 3' UTR* to regulate *PGRP-LC* expression. Ecdysone production could be activated by the neuroendocrine response to cell injury (IMD-independent route). In the presence of bacteria, the IMD pathway becomes activated and could also promote ecdysone biosynthesis to reinforce PGRP-LC production (IMD-dependent route). Dotted lines represent pathway interactions under investigation. ■

CHAPTER IV: Conclusion

The *Drosophila* system host the steroid hormone ecdysone, which regulates development and immune functions using a common group of transcription factors. The larva-pupae transition and metamorphosis are initiated by ecdysone, which activates a small group of genes that encode for transcription factors including EcR, BR-C, HR46, Eip74EF, Eip75B, Eip78C, and Eip93F. Subsequently, these transcription factors regulate hundreds of genes for a specific developmental outcome. In addition to these transcription factors, the ecdysone-regulated GATA factors SRP and PNR are required for the expression of the immune receptor PGRP-LC. While the transcriptional network has been elucidated in development, it is unclear why these transcription factors are involved in the activation of *PGRP-LC* and how the ecdysone pathway is regulated in the context of an immune response *in vivo*.

Flies maintain a basal level of *PGRP-LC* expression, which increases by two-fold when infected with the gram-negative bacteria *E. coli* (**Figure 3.1H**). In female flies, this induction is partly mediated by an ecdysone-responsive element located about 2 kb upstream from the *exon 1.2* transcription start site (**Figure 3.1H**). Male flies that lacked the *PGRP-LC* CRE did not experience these changes (**Figure 3.1H**). It is unclear why gender-specific phenotypes exists in the immune system, but differences in the immune response have been observed in the human population. For example, females generate more pro-inflammatory

cytokines in response to endotoxin (**van Eijk et al., 2007**) and certain autoimmune diseases are more prevalent in one gender across the world (**Ngo et al., 2014**). In flies, topical application of a pathogenic species of fungus led to lower survival rates to infection in female flies compared to males (**Taylor and Kimbrell, 2007**). Furthermore, the number of basal and induced *PGRP-LCx* transcripts (unnormalized) are remarkably higher in control male flies compared to female flies (data not shown). Thus, it is possible that the *PGRP-LC* regulation differs between male and female flies with the *PGRP-LC* CRE representing one variation. On the other hand, the *PGRP-LC* CRE reporter plasmids were activated by ecdysone in S2* cells, which were derived from male animals. There could be endogenous controls in flies that may not exist in a *in vitro* system. Additional work is necessary to resolve these discrepancies, including comparing *PGRP-LC* expression in male S2* cells and female Kc cells that lack the *PGRP-LC* CRE.

A second element located in *PGRP-LC exon 1.2* or part of the preceding intron was partially activated by ecdysone in S2* cells (**Figure 3.1A**). It is not clear why multiple distinct elements exist in the *PGRP-LC* locus, but they could be involved in different roles in transcriptional control or necessary to maintain *PGRP-LC* expression under different conditions. For example, *exon 1.2* could be involved in the initiation of transcription or basal expression, while the *PGRP-LC* CRE would be necessary to induce *PGRP-LC* in response to infection. Future studies of *exon 1.2* will include identifying binding sites for ecdysone-regulated

transcription factors, gel-shift assays, and precise nucleotide mutations in the F3 reporter plasmid and *in vivo* since large deletions could disrupt the core promoter sequence.

The *PGRP-LC* promoter is defined by RNA polymerase II *in vivo* ChIP-seq data, which binds to *exon 1.2* (**Nègre et al., 2011**). Other *in vivo* ChIP-seq data shows the activator CBP-P300 homologue Nejire and BR-C are bound to *PGRP-LC* CRE and *exon 1.2* in a discrete and significant manner *in vivo* (**Figure 3.1C**). These results support the presence of cis-regulatory elements in *PGRP-LC* CRE and *exon 1.2*. Furthermore, the *PGRP-LC* CRE displayed enhancer potential when it was joined to a minimal promoter, although it failed to activate the promoter in the downstream position. Equivalent regions of the *PGRP-LC* CRE in other *Drosophila* species, as defined by genome alignment, were also activated by ecdysone (**Figure 3.1D**). Collectively, these results suggest that the ecdysone pathway interacts with a conserved enhancer element upstream of *PGRP-LC* to control *PGRP-LC* gene activity.

Although female ΔCRE flies experienced a reduction in bacteria-mediated *PGRP-LC* induction, their survival to the pathogenic bacteria *Ecc15* was not significantly impaired (**Figure 3.1I**) and any deaths that occurred were mostly due to flies getting stuck in condensation. However, the results were not conclusive since it appears the the pathogenicity of *Ecc15* was limited the day the flies were infected. In a previously publish report, control flies infected with the same bacteria were susceptible to infection (**Rus et al., 2013**), which did not

occur in this study. Flies in the previous study were infected in the abdomen, while flies in this study were infected in the prothorax. The abdomen cuticle can be difficult to penetrate so the shoulder was chosen as the site of infection for this study. The site of infection can result in different mortality rates (**Chambers et al., 2014**) and it is possible that flies infected in the shoulder could be more resistant to *Ecc15*-mediated lethality compared to those infected in the abdomen for reasons unknown. In future studies, the flies will be infected in the abdomen and other pathogenic bacteria will be tested such as *P. aeruginosa*.

Binding sites for nine ecdysone-regulated transcription factors in the *PGRP-LC* CRE were analyzed using bioinformatic software and four target sites composed of individual or a cluster of binding sites were identified (**Figure 3.2B**). These included binding sites for two BR-C protein isoforms, HR46, Eip75B, SRP, and PNR. In the two clusters, individual motifs have overlapping sequences, which could result in competitive binding between transcription factors (*e.g.*, Eip75B prevents HR46 binding in the second target site). The development of cooperative binding is also possible such that the binding one transcription factor induces changes in the DNA conformation, which creates an optimal binding position for the next transcription factor (*e.g.*, IFN-β enhanceosome) (**Panne, 2008**). If the transcription factors bind to the opposite faces of the DNA helix, it is possible to accommodate multiple transcription factors at the same motif.

Generally, mutations in the last three target sites led to a reduction in reporter activity, but the signal was not completely abolished (**Figure 3.2C**).

These results could suggest that both protein-DNA interactions and protein-protein contacts between adjacent transcription factors help stabilize a multi-protein complex on the *PGRP-LC* CRE. In this scenario, mutations that disrupt some DNA contacts destabilize the formation of an activator complex and weakens *PGRP-LC* CRE activity, but other DNA contacts and protein-protein bonds prevent the complex from disassembling. The motifs that displayed partial response were located within the F47 insert, which contains important elements necessary to respond to ecdysone (**Figure 3.2A**). The luciferase data were normalized to *Renilla* luciferase reading, but treated samples were not normalized to the corresponding untreated samples. When the ecdysone-treated samples were normalized to the untreated samples, any downregulation that occurred were not as striking (**Figure 3.2A**). This would suggest that the smallest fragments tested, F44 and F50, both contain the necessary ecdysone-regulated elements sufficient for reporter activity. Although interpretation of the data changes when analyzing the samples in terms of fold-change, the comparisons between treated samples are still meaningful since the treated samples is significantly upregulated compared to untreated samples.

The dichotomy in the luciferase assay analysis occurred sometimes when the average of the ecdysone-treated samples were normalized to untreated samples. The background activity in untreated samples was detected in cells transfected with constructs that possess either the *PGRP-LC* or *Hsp70* promoter. For reasons unknown, there was even ecdysone-induced activity in

untransfected cells, which became exacerbated when the cells were not properly maintained at a density below 10x10^6 cells/mL (data not shown). Therefore, it was critical that the cells were split frequently during maintenance and a few times before using when the cell population exceeded the recommended maximum density. This helped reduce the background activity, but did not necessarily eliminate it. Adjusting the ratio of firefly to *Renilla* luciferase plasmid such that only minimal amounts of each are used could help diminish the activity in untreated samples (**Schagat et al., 2007**). In addition, a different minimal promoter, such as the *Drosophila* synthetic core promoter (DSCP), can be used in place of the *Hsp70* promoter (**Pfeiffer et al., 2008**). The size of the insert fragment did not correlate with the magnitude of the background level (**Figure 3.2A**).

Putative binding sites for EcR, Eip74EF, Eip78C, and Eip93F were not found in the *PGRP-LC* CRE. These motifs could be located in regions that were not analyzed such as *exon 1.2* or outside of the *PGRP-LC* CRE. For example, *Eip93F* knockdown did not downregulate the F3 reporter activity, suggesting the *Eip93F* binding site could be located outside of the extended *PGRP-LC* promoter region (**Figure 3.2D**). (Incidentally, the dsRNA designed in this study are different from Figure 1.1E, and was added at concentration of 0.5 ug/mL compared to 2 mg/mL in the previous study. A combination of these factors could cause variations in *PGRP-LC* expression.) If the transcription factors form a higher-order protein complex then their function at the *PGRP-LC* CRE may not require

contact with DNA. Alternatively, some transcription factors may regulate *PGRP-LC* expression indirectly by activating other genes required for *PGRP-LC* expression since it takes 18 h of ecdysone treatment in S2* cells to generate robust *PGRP-LC* levels (**Rus et al., 2013**). Performing co-immunoprecipitation, gel-shift assay, and measuring transcription factor expression over time-course treatment with ecdysone in S2* cells will help elucidate the transcription factor network.

Developmentally-induced ecdysone pulses control immune gene activity as they drive developmental functions. Neuropeptide signals activate ecdysone synthesis and secretion from the prothoracic gland, which corresponds with spontaneous *PGRP-LC* induction in the developing fly (**Figure 1.1C**). In contrast, it is not understood how the ecdysone biosynthesis pathway is regulated in the context of an infection *in vivo*. When adult flies are infected with gram-negative bacteria, the preliminary results show ecdysone level increasing in female and male flies (**Figure 3.3A**). Several studies have demonstrated that certain kinds of stress such nutritional or sleep deprivation increases ecdysone levels in flies (**Terashima, 2005; Ishimoto and Kitmoto, 2010**). Therefore, elevated ecdysone in infected flies could be an neuroendocrine response to stress. Further work is necessary to validate these experiments, and distinguish between stressed-induced ecdysone production by cell injury and a potential positive feed-back loop from the IMD pathway following its activation by bacteria. ■

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