ABSTRACT

Title of Document:	ROLE OF NF-κB/REL PROTEINS IN MEDIATING INNATE IMMUNE RESPONSES IN *DROSOPHILA MELANOGASTER*
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In order to survive, the fruit fly *Drosophila melanogaster* needs to have a robust immune system to protect itself from numerous pathogenic microorganisms that are ubiquitous in its natural habitat. In two related projects, we studied the role of Rel transcription factors in mediating these immune responses. There are three Rel transcription factors, closely related to mammalian NF-κB: Dorsal, Dif, and Relish. They play an important role in mounting aspects of this response, including the inducible expression of antimicrobial peptides. To study the roles of these transcription factors *in vivo*, we used microarrays to determine the effect of null mutations in individual transcription factors on larval immune gene expression. Of the 188 genes that were significantly up-regulated in wildtype larvae upon bacterial challenge, overlapping but distinct groups of genes were affected in the Rel mutants. We also ectopically expressed Dorsal or Dif and used cDNA microarrays to

determine the genes that were up-regulated in the presence of these transcription factors. Combining this data, we also identified novel genes that may be specific targets of Dif. In a related project, we observed that injection of a fungal secondary metabolite, Destruxin A reduced expression of various antimicrobial peptide genes. This reduction appeared to be mediated through suppression of the IMD pathway, through Relish. Destruxins are a class of cyclic depsipeptides produced by various fungi including the entomopathogen *Metarhizium anisopliae*. Though a direct relationship has been established between Destruxin production and fungal virulence, their *in vivo* mode of action during pathogenesis remained largely uncharacterized. To explore these effects, we looked at changes in gene expression following injection of Destruxin A into *Drosophila*. Microarray results revealed reduced expression of various AMPs that play a major role in the fly's humoral immune response. Flies co-injected with Destruxin A and the Gram-negative bacteria *E.coli*, showed increased mortality and an accompanying increase in bacterial titers. This mortality was rescued through ectopic activation of IMD pathway components upstream of Relish that are responsible for AMP induction. Together, these results suggest a novel role for Destruxin A in specific suppression of the humoral immune response in insects.

ROLE OF NF-κB/REL PROTEINS IN MEDIATING INNATE IMMUNE
RESPONSES IN *DROSOPHILA MELANOGASTER*

By

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Dedication

To my Parents

Debajyoti and Dipti Rekha

and my wife Anyesha

For love, patience and support.

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List of Abbreviations

AMP: Antimicrobial Peptide

ChIP: Chromatin Immunoprecipitation

dHPLC: Denaturing High Performance Liquid Chromatography

DmIKK: Drosophila melanogaster I-kappa kinase

Dscam: Down Syndrome Cell Adhesion Molecule

GNBP: Gram negative binding proteins

GO: Gene Ontology

HS: Heat shock

IRAK: IL-1 Receptor Associated Kinase

I-κB: Inhibitor of kappa-B

JAK: Janus Kinase

LPS: Lipopolysaccharides

MAST: Motif Alignment and Search Tool

NF-κB: Nuclear Factor Kappa B

PAMP: Pathogen Associated Molecular Patterns

PGRP: Peptidoglycan Recognition Receptor

PPO: Prophenoloxidase

PRR: Pattern Recognition Receptors

Q-PCR: Quantitative PCR

RNAi: RNA interference

ROS: Reactive Oxygen Species

STAT: Signal Transducers and Activators of Transcription

SELEX: Systematic evolution of ligands by exponential enrichment

TEP: Thiol Ester Protein

TIR: Toll-IL-1 Receptor

TLR: Toll-like Receptors

TNF-α: Tumor Necrosis Factor alpha

Chapter 1: Lessons from the fly: An overview of *Drosophila* immunity

Abstract

Drosophila have a variety of innate immune strategies for defending itself from infection, including humoral and cell mediated responses to invading microorganisms. At the front lines of these responses, are a diverse group of pattern recognition receptors that recognize pathogen associated molecular patterns. These patterns include bacterial lipopolysaccharides, peptidoglycans, and fungal β-1,3 glucans. Some of the receptors catalytically modify the pathogenic determinant, but all are responsible for directly facilitating a signaling event that results in an immune response. Toll and IMD are two major signaling pathways that are activated during humoral responses, and they in turn regulate the activation of Drosophila NF-κB proteins Dorsal, Dif, and Relish. In this chapter, we review what is known about innate immunity in Drosophila, and how those lessons may be applied towards a broader understanding of immunity.

1.1 Drosophila as a model for studying immunity

The fruit fly, *Drosophila melanogaster*, can sustain themselves on a wide variety of nutrients. They can live off slime from the drainage system and fermenting food discarded in a garbage can. They particularly like decaying fruit. The Drosophila life cycle involves laying eggs in the fruit, from which larvae emerge about a day later. These larvae continuously feed over the next week before they start forming pupae, readying themselves for metamorphosis into adult flies. As a result of their versatility and survival skills, fruit flies are found outdoors almost all over the world. At the same time, their life-style exposes them to a variety of microorganisms some of which are insect pathogens.

To survive, flies therefore need a robust immune system that can protect them from the variety of threats that they might face in nature. This system needs to efficiently recognize invading pathogens and mount an effective response that subdues the infection. Components of such a system, broadly termed innate immunity, have been identified in Drosophila with several key features conserved between the various metazoans, ranging from mammals to plants (Hoffmann, 2003; Hoffmann and Reichhart, 2002). Prior to the discovery of the role of these innate immune pathways however, classical ideas in immunology focused on the vertebrate adaptive system. In vertebrates, lymphocytes recognize pathogens based on the expression of highly variable immunoglobulin molecules that are generated through genetic recombination. This system has a measure of immunological "memory" and it learns from previously encountered infectious agents (Ahmed and Gray, 1996). This is mediated by keeping memory lymphocytes in

circulation that have encountered a non-self antigen associated with infection. By contrast, the innate immune system relies on an ancestral, heritable memory that is used to code pattern recognition receptors (PRRs) that recognize conserved constituents of microorganisms. Bacterial lipopolysaccharides (LPS), CpG DNA, and flagellin, fungal β-1,3 glucans, and viral double-stranded RNA are among the pathogen associated components directly recognized by various innate immune receptors (Barton and Medzhitov, 2002; Brown and Gordon, 2005). Upon recognition of Pathogen Associated Molecular Patterns (PAMPs), the PRRs initiate an immune response conferring protection to the host. In mammals, these receptors are responsible for initiating signaling cascades that lead to the production of immune effectors such as antimicrobial peptides and cytokines, and trigger the activation of phagocytosis and proteolytic cascades (Medzhitov and Janeway, 2000a). In Drosophila, conserved pathways are involved in inducing a class of cationic peptides that have antimicrobial activity. They are also involved in activating various cellular defense responses such as the initiation of melanization reactions that are toxic to microbes and activation of hemocytes that can phagocytose foreign cells.

Drosophila has been a particularly attractive model system to study innate immunity because it presents researchers with a simpler immune system than mammals, to study conserved signaling pathways. Flies are a well established genetic system and have a short generation time of 14 days. This enables the use of forward genetic screens to identify mutants necessary for the immune response. The Drosophila genome has been sequenced, which also expedites identification of genes and comparative genomics

analyses (Adams et al., 2000). Finally, it is relatively easy to incorporate and regulate the expression of transgenes in flies, providing valuable opportunities for characterizing the role of genes *in vivo* (Brand et al., 1994; Rubin and Spradling, 1982). Thus, the ability to combine genetic and molecular approaches has made fruit flies a powerful system to study innate immunity.

This chapter will summarize the current state of knowledge on Drosophila immunity. The first section will describe the basic strategies used by Drosophila to protect themselves from invading microorganisms. In the next section, we will examine the receptors involved in recognition of pathogenic determinants and initiating the signaling pathways. Then, we will describe in detail the signal transduction pathways responsible for mediating the strategies and compare them with conserved signaling pathways in other organisms. Finally, we will examine some of the pathogens that are known to infect flies and stimulate immune responses, with special emphasis on entomopathogenic fungi. This class of fungi is relevant to the work described in this thesis, and as a result will help put the work in context.

1.2 The Immune Response in the Fly

To protect them from infection, Drosophila employ a variety of immune strategies which can broadly be subdivided into two major categories: passive barriers and active responses. The passive barriers to infection include physical barriers such as the Drosophila exoskeleton, peritrophic membrane, and tracheal lining. The vast majority of microbes in the environment are stopped at this stage, and relatively few bacteria, viruses, or fungi are capable of breaching this barrier (either through ingestion or through penetration through the cuticle) to enter the body. In addition, passive chemical microenvironments such as lysozymes in the midgut, low pH in the digestive tract, and production of antimicrobial peptides (AMPs) in barrier epithelia help neutralize pathogens that enter these areas and discourage their entry into the hemocoel (Basset et al., 2000; Kocks et al., 2005; Tzou et al., 2000).

Considerable work however has focused on the active responses mounted by the Drosophila innate immune system. These responses to infection can be divided into a combination of three main strategies:

1.2.1   **Proteolytic cascades and melanization.** When the fly cuticle is damaged, a set of proteolytic reactions involving its blood proteins cause a rapid clotting and deposition of melanin around the site of the breach in order to heal the wound. This involves a cascade of events marked by the proteolysis of Prophenoloxidase (PPO) and generates reactive oxygen species (ROS) (Soderhall and Cerenius, 1998). Both ROS and melanin are toxic to invading pathogens (Braun et al., 1998; Rizki and Rizki, 1984). Curiously, flies with mutations in the

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PPO cascade alone, are not more susceptible to bacterial infection, which suggests that other cell mediated and humoral responses may be playing a more crucial role to *Drosophila* immunity *in vivo* (Leclerc et al., 2005). However, the bacteria used in this study are not known to cause melanization to begin with, casting doubts about melanizations role in clearing the bacteria.

1.2.2   **Phagocytosis and cellular responses.** *Drosophila* have three major kinds of blood cells, as characterized by morphology and function. Nearly 90% of all blood cells are plasmatocytes, which demonstrate a macrophage-like behavior by phagocytosing invading bacteria (Holz et al., 2003; Lanot et al., 2001; Rizki and Rizki, 1984). Lamellocytes work together to encapsulate larger invaders that cannot be phagocytosed, e.g. the eggs of endoparasitoid wasps. Finally, crystal cells provide enzymes required for melanization reactions. Embryos and larvae produce crystal cells until the pupal stage, after which no evidence of hematopoiesis has been found (Rizki and Rizki, 1984). Adult flies have a fixed population of sessile blood cells localized mainly on the anterior portion of the dorsal side of their abdomens. These hemocytes are capable of phagocytosing bacterial invaders to protect the host (Elrod-Erickson et al., 2000; Lanot et al., 2001).

1.2.3   **Antimicrobial peptides and humoral response.** There are seven known classes of inducible antimicrobial peptides (AMPs) in *Drosophila*: Attacin, Cecropin, Defensin, Diptericin, Drosocin, Drosomycin, Metchnikowin (Bulet et al., 1999; Meister et al., 2000). Several of the peptides work by disrupting bacterial membranes (Moore et al., 1996). They are primarily produced

by the fat body, the flies' functional analog of the mammalian liver, within hours of an immune challenge. The AMPs are then secreted into the hemocoel where they block the proliferation of microorganisms (Bulet et al., 1999). Drosomycin for example, demonstrates antifungal activity and is preferentially induced upon fungal infection (Lemaitre et al., 1997). Similarly, Defensin and Metchnikowin have activity against Gram-positive bacteria and Attacin, Cecropin, Diptericin, and Drosocin are effective against Gram-negative bacterial challenge (Bulet et al., 1999; Meister et al., 2000). The transcription of these AMPs is regulated by two major signal transduction pathways: Toll and IMD (Figure 1-1) (De Gregorio et al., 2002). Both these pathways have elements conserved with mammalian pathways regulating NF-κB, a transcription factor important for mediating numerous immune responses (Khush et al., 2001). The Toll pathway is central to Drosophila immune responses. It is involved in the induction of AMPs through the regulation of Dif, an NF-κB-like transcription factor. *Toll* mutant flies are particularly susceptible to fungi and Gram-positive bacteria, and fail to induce Drosomycin (Gobert et al., 2003; Lemaitre et al., 1996). The IMD pathway on the other hand broadly detects Gram-negative bacterial determinants through its receptor PGRP-LC (Choe et al., 2005; Choe et al., 2002; Werner et al., 2003). Mutants in the IMD pathway fail to activate Relish, another NF-κB-like protein, that is responsible for the transcription of Diptericin (Hedengren et al., 1999). The selective activation of the Toll or IMD pathways appears to confer some specificity of response against Gram-positive bacteria and fungi, or Gram-negative bacteria respectively. However, some lines of evidence suggest that IMD

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is also important for resistance to Gram-positive *Micrococcus luteus* infection, (Hedengren-Olcott et al., 2004; Leulier et al., 2000) while Toll is also important for responses to Gram-negative *E. coli* and *Pseudomonas aeroginosa* and the virus, Drosophila X virus (De Gregorio et al., 2002; Lau et al., 2003; Zambon et al., 2005). Thus there may be some functional overlap between the roles of these two pathways in regulating responses to different microbes.

Before any of these immune signaling pathways can be activated, an infection first needs to be identified. Upon recognition, one or more of these pathways to combat the infection can be activated. Pattern Recognition Receptors (PRRs) are involved in recognizing pathogens, and are thus at the front lines of immune defenses.

1.3 The Pattern Recognition Receptors

1.3.1 Toll Receptors.

Drosophila Toll was initially identified as one of 12 maternal effect genes that function in a pathway required for dorsal-ventral axis formation in fly embryos (Anderson and Nusslein-Volhard, 1984; Belvin and Anderson, 1996). Interestingly, Drosophila Toll and the mammalian IL-1 receptor share a conserved intracellular domain named the Toll-IL-1-Receptor (TIR) domain, and regulate orthologous signaling pathways (O'Neill and Greene, 1998).

Eleven Toll-Like-Receptors (TLRs) have been identified in mammals, and they are specialized for detection of different PAMPs, often with the help of other proteins (Barton and Medzhitov, 2002). TLR4 for example is involved in the direct detection of bacterial Lipopolysaccharides (LPS) in a complex with helper proteins CD14 and MD2 (Akashi et al., 2000). TLR2 recognizes a broad array of ligands including bacterial lipoproteins, peptidoglycan, and yeast zymosan. TLR2 achieves this range of ligand specificities, by forming heterodimers with TLR1 or TLR6. The TLR 1/2 heterodimer for example, binds triacylated lipopeptides, whereas the TLR 2/6 combination is specific for diacylated lipopeptides (Ozinsky et al., 2000). Other TLRs appear to act alone as homodimers. For example, TLR5 recognizes flagellin, the protein that makes up bacterial flagella (Smith et al., 2003). TLR9 recognizes unmethylated CpG DNA characteristic of bacterial genomes, and TLR3 binds to double-stranded RNA (Alexopoulou et al., 2001; Hemmi et al., 2000). Upon detection of their PAMPs, different TLRs activate combinations of downstream components, leading to potentially complex signaling outcomes. In mammalian dendritic cells for example, the TLRs 5, 7, and 8 signal through

MyD88, an adaptor protein traditionally activated by mammalian Toll, to produce a pro-inflammatory reaction (Barton and Medzhitov, 2003). TLR3 on the other hand signals through MyD88 and an additional adaptor protein complex TICAM/TRIF to activate an antiviral response (Muzio et al., 2000). Since TLR3 detects double stranded RNA of potentially viral origin, this may represent an appropriate immune response for the host.

In contrast to mammalian TLRs, Drosophila Toll appears not to be directly involved in pathogen recognition as a PRR. It does however, play a central role in mediating responses to multiple types of infections. Fungal, Gram-positive bacterial, and viral responses require the Toll pathway (De Gregorio et al., 2002; Lemaitre et al., 1996; Tauszig-Delamasure et al., 2002; Zambon et al., 2005). In the case of fungi and Gram positive bacteria, upstream PRRs recognize PAMPs and trigger a proteolytic cascade that activates Toll and results in AMP production (Filipe et al., 2005; Gobert et al., 2003; Michel et al., 2001; Pelte et al., 2006). Eight other Toll receptors have been identified in *Drosophila,* but they appear to have significant functional differences with mammalian TLRs. Foremost, Drosophila Tolls have not been shown to detect PAMPs so far. Instead some of them such as Toll-2 (also known as 18-wheeler) and Toll-8 have developmental and neural functions respectively (Eldon et al., 1994; Seppo et al., 2003). Most Tolls do not appear to be upregulated upon infection, and none of them have been identified through screens for mutants with immunodeficiency (De Gregorio et al., 2001; Irving et al., 2001). The possible exception 18-wheeler, is expressed in larval fat body upon infection but its role in immunity is not clear (Ligoxygakis et al., 2002a). The induction of several AMPs were affected in *18-wheeler* mutants, including a 95% reduction in Attacin and 65% reduction in Cecropin expression, and these flies were susceptible to *E.*

coli (Williams et al., 1997). However, these mutants appear to have defects in fat body development which may also cause the aberrant AMP production (Eldon et al., 1994; Ligoxygakis et al., 2002a). Constitutive expression of another fly Toll receptor, Toll-9 has been shown to induce Drosomycin expression in Drosophila S2 cell lines. It uses components of the traditional Toll pathway like the adaptor protein MyD88, to mediate this induction (Bettencourt et al., 2004b; Ooi et al., 2002). Thus, analogous to some mammalian TLRs, Toll-9 may also be using shared signaling components to affect AMP expression *in vivo*. The isolation and characterization of mutations affecting the other fly Toll receptors will likely shed more light on the role and regulation of this receptor family.

1.3.2 Peptidoglycan Recognition Proteins (PGRPs)

Peptidoglycans are polymers of N-acetylglucosamine and N-acetylmuramic acid that are present in bacterial cell walls. In Gram-positive bacteria a Lys-type stem peptide is involved in crosslinking these monomers, and the resulting peptidoglycan constitutes nearly half of the exposed bacterial cell wall mass. Gram-negative bacteria, have a diaminopimelic acid (DAP) stem peptide connecting the monomers, and their peptidoglycan is present in a relatively thin layer underneath an LPS outer membrane (Doyle and Dziarski, 2001; Schleifer and Kandler, 1972). Peptidoglycan Recognition Proteins (PGRPs) are a class of PRRs that recognize this conserved determinant of all bacteria. These receptors were first characterized in moths *Bombyx mori* and *Trichoplusia ni* (Kang et al., 1998; Liu et al., 2000). Thirteen PGRP genes have since been identified in *Drosophila*, 7 in *Anopheles* mosquitoes, and 4 in mammals (Christophides et al., 2002; Dziarski, 2004; Liu et al., 2001). However, alternative splicing may generate a larger

functional repertoire. In *Drosophila* alone, the 13 PGRP genes transcribe at least 17 proteins (Werner et al., 2000).

Some mammalian PGRPs have bacteriostatic functions. PGRP-S is involved in the intracellular killing of bacteria in mouse polymorphonuclear leukocytes, though the mechanism by which they do this is not clear (Dziarski, 2003). A bovine PGRP-S orthologue, also known as oligosaccharide binding protein (OBP) has shown activity against various types of pathogens including Gram-negative, Gram-positive bacteria and certain fungi (Tydell et al., 2002). Thus some PGRPs have evolved to recognize a very wide range of ligands, some of which are not even peptidoglycans. Drosophila PGRPs have not however been demonstrated to have intrinsic antimicrobial properties, and so far have only been shown to activate immune signaling pathways.

Drosophila have 7 short PGRPs, (SA, SB1, SB2, SC1a, SC1b, SC2, and SD) that lack a transmembrane domain, and are predicted to be secreted (Figure 1-2) (Werner et al., 2000). They also have 6 long PGRPs, (LA, LB, LC, LD, LE, and LF) that are predicted to be membrane bound (Werner et al., 2000). Drosophila PGRPs can also be classified according to function, as catalytic or non-catalytic. All PGRPs share homology with N-acetylmuramoyl-L-alanine amidases, which cleave peptidoglycan at the lactylamide bond between the glycan backbone and the stem peptides (Kang et al., 1998). Some PGRPs such as PGRP-SC1a and SC1b, retain this function and have been demonstrated to cleave peptidoglycan *in vitro* (Mellroth et al., 2003). However, other receptors such as PGRP-LC, LE, SA, and SD are non-catalytic due to the lack of a critical cysteine in the conserved catalytic domain (Mellroth et al., 2003).

Both catalytic and non-catalytic PGRPs have been shown to play a crucial role in Drosophila immune recognition. Non-catalytic PGRP-LC (also known as ird7 or totem) responds primarily to DAP-type peptidoglycan found in Gram-negative bacteria, and activates the IMD pathway (Choe et al., 2002; Gottar et al., 2002; Kaneko et al., 2004; Lim et al., 2006; Ramet et al., 2002b). PGRP-LC has also been shown to activate phagocytosis because RNA inhibition of the gene in Drosophila S2 cells causes a reduction in the phagocytosis of Gram-negative *E. coli*, but not Gram-positive *S. aureus* (Ramet et al., 2002b). PGRP-LE also appears to detect Gram-negative bacteria, and its overexpression in larvae activates the IMD pathway as well as the PPO cascade (Takehana et al., 2002). PGRP-LC, PGRP-LE (loss of function) double mutants show a more dramatic susceptibility to Gram-negative bacterial infection than either mutation alone. Recent evidence suggests that the PGRP-LE protein can act as an intracellular receptor for monomeric peptidoglycan, while also strengthening PGRL-LC's peptidoglycan recognition at the cell surface (Kaneko et al., 2006). Thus, the two PGRPs may be acting together for peptidoglycan recognition (Kaneko et al., 2006; Takehana et al., 2004). The study of loss-of-function mutants in PGRP-SA (also known as Semmelweis) reveal its role in activating the Toll pathway in response to Gram-positive bacterial, but not fungal, challenge (Michel et al., 2001). *PGRP-SD* mutant flies are also susceptible to Gram-positive bacterial challenge and PGRP-SA and PGRP-SD appear to act together to recognize Lys-type peptidoglycan and activate the Toll pathway (Bischoff et al., 2004; Michel et al., 2001). PGRPs therefore mediate specificity of immune response to Gram-positive or Gram-negative bacteria, by recognizing the bacterial

peptidoglycan and differentially activating the Toll or IMD pathway respectively (Lemaitre, 2004; Leulier et al., 2003).

Catalytic PGRPs also play a crucial role in immune recognition, and appear to chemically modify peptidoglycans. *In vitro*, PGRP-SC1b cleaves staphylococcal peptidoglycan and the resulting products exhibit a reduced ability to activate AMP genes in Drosophila blood cell lines (Mellroth et al., 2003). This suggested the catalytic PGRPs may be acting as scavengers to limit an inflammatory response to free peptidoglycan (Mellroth et al., 2003). Another recently identified receptor, PGRP-LB is induced by the IMD pathway, and has amidase activity that is involved with degradation of gram-negative peptidoglycan. It also appears to down-regulate IMD pathway responses, producing a potential negative feedback loop that may be instrumental in stabilizing its levels (Zaidman-Remy et al., 2006). RNA inhibition of *PGRP-SC* 1 and 2 appears in fact to cause a higher activation of the IMD pathway, suggesting a potentially suppressive role for these proteins (Bischoff et al., 2006). Recent work with PGRP-SC1a however suggests the catalytic processing may be required for initiating both cellular and humoral responses *in vivo* (Garver et al., 2006). *PGRP-SC1a* mutants are unable to activate the Toll pathway or phagocytose *S. aureus*, suggesting it may have a role in mediating both these responses. A targeted mutation in the PGRP-SC1a catalytic domain rendering it able to bind peptidoglycan but not cleave it, affects phagocytosis but not Toll signaling *in vivo* (Garver et al., 2006). This suggests that peptidoglycan cleavage products may be important for other receptors to activate phagocytosis, in a potentially multi-step recognition process (Filipe et al., 2005; Kaneko et al., 2004).

1.3.3 Gram Negative Binding Proteins (GNBPs)

GNBPs are small 50 kDa proteins containing a C-terminal β-glucanase-like domain (Werner et al., 2000). They often share structural similarity to PGRPs. They were initially isolated from immune challenged silkworm *Bombyx mori*, as binding to Gram-negative bacterial surface, but not significantly to Gram-positive bacteria (Lee et al., 1996). Three GNBP family members have been identified in *Drosophila*, but no corresponding homologs exist in mammals (Medzhitov and Janeway, 2000b). They have high affinity for bacterial LPS and fungal β-1,3 glucans *in vitro*. While silkworm GNBPs has glucanase activity, the critical amino acids required for activity are not conserved in fly GNBPs. In contrast to expectation, mutations in GNBP1 (also known as Osiris), the best studied of these receptors in *Drosophila*, indicated that it is involved in Gram-positive bacterial recognition (Gobert et al., 2003; Pili-Floury et al., 2004). PGRP-SA and GNBP1 act in a complex together to activate the Toll pathway (Ferrandon et al., 2004). While the mechanism of activation is not fully understood, the hemocoel of wild type, but not GNBP1 mutant flies, can hydrolyze Gram-positive peptidoglycan (Filipe et al., 2005). GNBP1 facilitates the cleavage of peptidoglycans *in vivo*, to possibly generate products that are recognized by PGRP-SA. A multistep process that recognizes and processes peptidoglycan may thus be responsible for Toll pathway activation (Filipe et al., 2005; Gobert et al., 2003).

1.3.4 Thiol ester proteins (TEPs)

In vertebrates, the complement system is an important part of innate immune responses. The C3 protein of this system binds to the pathogen surface via a thioester bond, and initiates a cascade of events leading to phagocytosis or lysis of the invader (Volanakis,

1990). Related to mammalian C3, a class of α-macroglobulins has been identified in invertebrates such as horse-shoe crabs (Armstrong and Quigley, 1987). These proteins act as protease inhibitors, in response to proteases secreted from tissues damaged from infection. Because of their potential role in the immune response, there was a search for similar C3-α-macroglobulin like molecules in *Drosophila*.

Four proteins of this family have been identified in *Drosophila*. They contain highly conserved thioester motifs, and have been named Thioester containing proteins (Tep 1-4) (Lagueux et al., 2000). They contain a signal peptide suggesting that they are secreted. Tep2 has 5 splicing isoforms, while the others have single splicing forms, and all of them have a basal level of expression throughout *Drosophila* development (Lagueux et al., 2000). The Tep1, Tep2, and Tep4 genes are upregulated in the fat body during immune challenge by bacteria. The JAK/STAT pathway, important for hemocyte development and immune regulation, has been implicated in this induction; gain-of-function JAK causes induction of Tep factors (Lagueux et al., 2000). Gain-of-function *Toll* mutants also cause induction of Tep genes, suggesting that Toll may also play a role in their regulation (Lagueux et al., 2000).

In a recent screen in Drosophila S2 cell lines using RNAi libraries for phagocytosis defects, the inhibition of a number of genes were identified. Among them, TepII inhibition affected phagocytosis of the Gram[-] *Escherchia coli* (but not the fungi *Candida albicans* or the Gram[+] *Staphylococcus aureus*) while TepIII affected phagocytosis of *S. aureus* (but not the other two microorganisms) (Stroschein-Stevenson et al., 2006). No mutations in Teps have been identified in *Drosophila*, and as a result their role *in vivo* is

not yet clear. However, in the mosquito *Anopheles gambiae* RNA inhibition has been used to reduce Tep gene expression and examine their immune function. Tep1 appears to be important for recognition of *E. coli* and *S. aureus*. Tep receptors have been shown to bind to both kinds of bacteria *in vitro* and *in vivo*, with functional thioester bonds and promote phagocytosis (Levashina et al., 2001). Interestingly, Tep1 also binds to the rodent malaria parasite *Plasmodium berghei*, and affects the vectorial capacity of mosquito (Blandin et al., 2004). Teps thus provide the first evidence of complement-like activity in insects (Levashina et al., 2001).

1.3.5 Scavenger Receptors

Scavenger receptors are a class of pattern recognition receptors with broad specificity. Mammalian scavenger receptors are macrophage cell surface molecules associated with the endocytic uptake of lipoproteins (Pearson, 1996). Some of them are membrane-bound, while others are secreted (Pearson, 1996).

In *Drosophila*, four Class C scavenger receptors have been identified. Of these, DScr-C1 is expressed on embryonic hemocytes and is important for phagocytosis (Pearson et al., 1995) of both Gram-positive and Gram-negative bacteria, but not yeast (Ramet et al., 2001). The gene loci encoding these genes have been found to be highly polymorphic between *Drosophila* species. This polymorphism may be the result of fly populations subjected to different selection pressures from exposure to different pathogens (Schlenke and Begun, 2005).

The *Drosophila* scavenger receptor Croquemort is required for phagocytosing dying cells in the *Drosophila* embryo (Franc et al., 1999). Its mammalian homologue, CD36 has

been implicated in recognizing and internalizing primarily Gram-positive bacteria and signaling through Toll-like receptors 2 and 6. Mice with a critical mutation in the signaling domain, fail to clear *S. aureus* efficiently (Stuart et al., 2005). *In vitro*, *Drosophila* Croquemort appears to be similarly involved in the recognition and phagocytosis of *S. aureus* (Stuart et al., 2005). However, *in vivo* data so far is restricted to its role as an apoptotic marker during the phagocytosis of dying cells. Determination of whether Croquemort also facilitates phagocytosis of pathogens and the activation of the Toll pathway analogous to CD36 in mammals remains to be established. Peste, another CD36-like protein, appears to be responsible for the phagocytosis of mycobacteria and listeria, but not other bacteria like *E. coli* or *S. aureus.* Peste has been identified through a genome-wide RNA interference screen in a Drosophila cell line using *Mycobacterium fortuitum* (Philips et al., 2005). The mechanism by which it mediates this distinction as well as its role *in vivo*, however remain to be characterized.

Another protein with an interesting role is a novel *Drosophila* receptor named Eater. The extracellular domain of Eater contains Epidermal Growth Factor-like repeats. These domains are similar to scavenger receptors P120 and CED-1/SREC, in flesh flies and humans respectively (Hori et al., 2000; Kocks et al., 2005). Like scavenger receptors, Eater seems capable of binding multiple ligands including lipids and carbohydrates. Cell line evidence indicates that RNAi silencing of this receptor causes roughly 50% reduction in binding and internalization of *E. coli* and *S. aureus* (Kocks et al., 2005). *In vivo*, Eater is only expressed in hemocytes and its deficiency does not appear to affect Toll or IMD pathway signaling. However, phagocytosis of *S. aureus*, and to a lesser extent *E. coli*, is impaired in Eater deficient flies. This phenotype can be reversed by the expression of an

Eater transgene, suggesting that it is specifically responsible for facilitating phagocytosis of bacteria (Kocks et al., 2005). Eater-deficient flies are also more susceptible to Gram-negative *Serratia marcesens* infection (a natural pathogen of *Drosophila*) presumably due to their impaired ability to phagocytose.

1.3.6 Down Syndrome Cell Adhesion Molecule (Dscam)

The *Drosophila* homolog of the human Down Syndrome Cell Adhesion molecule has been recently identified as playing a role in immunity, and it presents the intriguing possibility for recognizing a variety of pathogens. Dscam can potentially generate a staggering 18,000 variations of its extracellular domain through alternative splicing, and these isoforms are temporally and spatially regulated (Schmucker et al., 2000; Watson et al., 2005). Dscam and its human homolog were originally studied for their role in axon guidance in humans and embryonic central nervous system in flies. In *Drosophila* it interacts with the adaptor Dreadlocks (Dock) and serine/threonine kinase signaling protein Pak (Ang et al., 2003). Dscam is important for the pathfinding of the Bolwig nerve, and embryonic brain development. Alternative splicing leads to tremendous diversity of this protein which helps in neuronal connectivity (Schmucker et al., 2000).

More recently, it was found that Dscam isoforms are produced in *Drosophila* fat body, hemocytes, and secreted into hemolymph serum. Certain splicing forms were found to bind preferentially to *E. coli*. Further, RNAi knockdown of specific isoforms of Dscam receptors in cell lines, reduced the ability of these cells to phagocytose *E. coli* by 30% (Watson et al., 2005). This raises the possibility that different splice-forms of Dscam might act like mammalian immunoglobulins, by detecting specific pathogenic epitopes

and marking them for phagocytosis. However, it is not yet clear whether Dscam receptors bind pathogens *in vivo*. Furthermore, no evidence to date has shown the clonal expansion of selected hemocytes in Drosophila in response to any pathogen. Thus, the mechanism of how an adaptive process involving Dscam diversity might work in flies remains an open question.

1.3.7 Other potential Pattern Recognition Receptors

Drosophila express classes of proteins which are predicted to be PRRs based on similarities with such proteins in other organisms. One such class is the hemomucins, some members which are secreted while others are transmembrane glycoproteins. In mammals, they are known to play a role in the immune response, by enabling leukocyte attachment during inflammation. A *Drosophila* hemomucin has been isolated using affinity purification techniques, and two splicing isoforms have been identified (Theopold et al., 1996). But a specific role in pattern recognition has not been demonstrated yet. Another class of proteins, galectins have also been identified as potential PRRs (Fujita et al., 1998). In mammals, various classes of lectins work together to mark a non-self carbohydrate for attack by complement or humoral immune responses (Vasta et al., 1999). Similar genes in the flesh fly *Sarcophaga peregrine* are induced in larvae upon injury (Fujita et al., 1998). In Drosophila one galectin has been identified, and it is predicted to bind to galactose of presumably non-self origin (Pace and Baum, 2004; Pace et al., 2002) but relatively little is known about its mode of action. We can look forward to better characterization of these, and other potential PRRs in the future.

Much of the molecular mechanisms by which PRRs detect PAMPs and mediate immune responses remain to be clarified. Structural analysis of more PRRs and the characterization of the mechanisms by which they detect and signal will likely yield greater insight into this mechanism (Kim et al., 2003; Reiser et al., 2004; Swaminathan et al., 2006). Relatively new techniques such as RNA interference screens and proteomic analyses have helped identify new components important for pathogen detection and immune signaling (Foley and O'Farrell, 2004; Philips et al., 2005; Ramet et al., 2002b). These along with traditional approaches like forward genetic screens may help identify novel genes, which can lead to a better understanding of the role of these proteins *in vivo*. This understanding should also shed light on complex signaling events that appear to tailor specific immune responses to different classes of pathogens. The insect immune response has historically impressed us with startling insights into its elegance and effectiveness, and this trend can be predicted to continue in *Drosophila*, its best established model system.

1.4 Signaling Pathways in Drosophila Immunity

Innate immune signaling pathways are activated by detection of pathogenic determinants by Pattern Recognition Receptors (PRRs), and lead to the activation of specific sets of transcription factors that are responsible for the induction of genes important for the response. The main signaling pathways involved with innate immunity in Drosophila are:

1.4.1 The Toll pathway: This pathway is named after its receptor Toll, which was identified in a genetic screen for developmental defects (Nusslein-Volhard and Wieschaus, 1980). Toll, which means "cool" in German, was apparently the exclamation uttered by its discoverers upon seeing the startling defect in dorso-ventral polarity resulting from a null mutation in this receptor. In embryos, cleavage of pro-spätzle in a gradient dependent manner along the ventral side of the embryo results in the formation of spätzle, a ligand that binds and activates the Toll receptor (Morisato and Anderson, 1994; Schneider et al., 1994; Weber et al., 2003). Toll dimerizes as a result, and recruits a complex consisting of adaptor proteins dMyD88, kinase Pelle, and the adaptor protein Tube. This complex results in the phosphorylation of Pelle and initiates a series of events that results in the phosphorylation of Cactus, a homologue of the mammalian IκB (Belvin et al., 1995; Bergmann et al., 1996). The identity of the kinase responsible for phosphorylating Cactus has eluded researchers. Cactus sequesters the NF-κB protein Dorsal in the cytosol, and phosphorylation marks Cactus for ubiquitination and degradation by the proteosome (Belvin and Anderson, 1996). This releases the NF-κB transcription factors to translocate to the nucleus and activate transcription of a variety of genes including those involved in dorso-ventral patterning during development.

Mammalian NF-κBs were discovered as a family of nuclear factors that interacted with various κB enhancer sequences found near genes coding for immunoglobulins (Sen and Baltimore, 1986). Similar motifs were identified in genes regulated by the Toll pathway in Drosophila (Uvell and Engstrom, 2003). Homology between Toll and IL-1 signaling were also noted. Further experiments revealed that the Toll pathway played a crucial role in defending flies from fungal infections (Lemaitre et al., 1996). Subsequently, other components of the Toll pathway have been identified, helping to elucidate its role during an immune response in greater detail. During the immune response, fungal components are recognized by pattern recognition receptors that activate serine proteases such as Persephone, and the inhibitor Necrotic (Ligoxygakis et al., 2002b; Michel et al., 2001; Pelte et al., 2006). This proteolytic cascade leads to the cleavage of spätzle, and Toll activation. Gram-positive bacterial determinants have been found to activate the Toll pathway through a peptidoglycan recognition protein (PGRP-SA) known as Semmelweis.

The Drosophila Toll pathway had been fairly well characterized by the time its role in the immune response was discovered, and this expedited the identification of homologous mammalian Toll like receptors (TLRs) (Rock et al., 1998). These TLRs were implicated in various aspects of immune recognition (Aderem and Ulevitch, 2000; Barton and Medzhitov, 2002). The intracellular domain of the IL-1 receptor was also found to be similar to a domain in the drosophila Toll receptor, and aptly named the Toll-IL-1 Receptor (TIR) domain (Xu et al., 2000). The pathways involved in signaling were also found to be surprisingly conserved (Belvin and Anderson, 1996). Adaptor proteins MyD88 and TRAF had been identified in mammals before they were identified in Drosophila. Upon comparison, Mammalian MyD88, IL-1 Receptor associated kinase

(IRAK), and TRAF were found to be similar to Drosophila dMyD88, Pelle, and dTRAF respectively. The mechanism by which mammalian I-κB is degraded in a signal dependent manner to free active NF-κB was first identified in mammals and later found to be conserved with Drosophila Cactus/Rel regulation (Nicolas et al., 1998). The Drosophila and mammalian Toll/IL-1 signaling pathways therefore together served to help expedite the elucidation of each other, and reveal a critical pathway for innate immune signaling that has been broadly conserved among metazoans.

1.4.2 The IMD pathway

The IMD pathway regulates the NF-κB protein Relish, and resembles the mammalian pathway responsible for tumor necrosis factor-α (TNF-α) signaling (Barton and Medzhitov, 2003; Hultmark, 2003). It is activated primarily by Gram-negative bacteria, through the peptidoglycan recognition protein PGRP-LC receptor. PGRP-LE also acts as an intracellular receptor responsible for recognizing bacterial peptidoglycan, to enhance PGRP-LC's ability to activate the IMD pathway (Kaneko et al., 2006). This recruits imd, a death domain containing protein with a TNF-α receptor interacting protein (RIP) domain. This sets into motion the two signaling cascades: the activation of DREDD, a caspase-8 homolog in Drosophila, and the phosphorylation of Relish through the activation of I-κB kinase, DmIKK. DmIKK is composed of three major subunits, a structural DmIKKγ component (also known as Kenny), and catalytic component DmIKKβ (ird5). The beta subunit and gamma structural protein are required for phosphorylation of Relish (Silverman et al., 2000; Wu et al., 2001). Other factors such as the adaptor dFADD and the kinase dTAK are involved in the signaling pathways

responsible for DmIKK activation. DREDD is believed to cleave phosphorylated Relish (Stoven et al., 2003). Relish is a compound protein consisting of an NF-κB-like Rel DNA-binding domain and an inhibitory I-κB domain. Cleavage results in the release of the Rel domain that can translocate to the nucleus and initiate gene transcription. The IMD pathway and Relish play a critical role in humoral immunity, by inducing a large number of AMP genes including Diptericin, Cecropin, and Drosocin (Hedengren et al., 1999).

1.4.3 Mammalian NF-κB Transcription Factors

The Toll and IMD pathways together regulate the three Drosophila NF-κB proteins: Dorsal, Dif, and Relish. On the other hand, mammals express five NF-κB proteins: RelA, RelB, c-Rel, p50, and p52, which are regulated by orthologous signaling pathways (Baeuerle and Henkel, 1994). Among these, NF-κB usually refers to the p50-p65 heterodimer; it is the most abundant, and best studied. Because of significant conservation between mammalian and Drosophila NF-κB proteins, the fruit fly has been an attractive model to study the role of these transcription factors in a simpler system. Many different agents can induce NF-κB activity in mammalian cells: pro-inflammatory cytokines like tumor necrosis factor and interleukin-1, bacterial LPS, dsRNA, some viruses, and ionizing radiation (Baeuerle and Henkel, 1994; Karin, 1999a; May and Ghosh, 1998) (Table 1-2). Toll-like Receptors (TLRs) specifically recognize some of these agents. For example, TLR-4 seems to physically interact with bacterial LPS (Poltorak et al., 2000), while ds-RNA stimulates TLR3 (Alexopoulou et al., 2001), and CpG DNA activates TLR9 (Kim et al., 2000). Other important receptors include the

interleukin receptors, mannose-binding receptors, and scavenger receptors. The signals mediated through these multiple receptors, apart from producing their own independent effects, also converge to induce NF-κB activity, through the activation of the I-κB Kinase (IKK) complex (Ghosh and Karin, 2002). Activated IKK phosphorylates two conserved serines in the N-terminal regulatory domain of IκB (Karin, 1999b).

Six vertebrate IκBs have been identified: IκBα, IκBβ, p105, p100, IκBε, and Bcl-3 (Baeuerle and Baltimore, 1996). They are characterized by multiple (6-8) ankyrin repeats that are required for their interaction with the Rel Homology domains in NF-κB. IκBα and IκBβ also have a C-terminal PEST sequence that causes any unbound protein to get degraded (Karin, 1999b). Phosphorylated IκB undergoes ubiquitination-mediated proteosomal degradation (Spencer et al., 1999). The NF-κB released goes on to transcribe a variety of genes ranging from cytokines, chemokines, and adhesion molecules, to regulators of apoptosis and cell proliferation (Table 1-2) (Ghosh and Karin, 2002). IκBα-deficient mice display skin defects, increased number of granulocytes, and die within 10 days of birth (Klement et al., 1996). There is increased NF-κB activity in some cells (such as hematopoietic cells) but not in others, suggesting that other IκBs may be compensating for this loss. Bcl-3 knockout mice show altered lymph node architecture and p100 mice show severe gastric hyperplasia (Baeuerle and Baltimore, 1996).

Mammalian NF-κBs are responsible inducing a variety of genes of significant biomedical interest, including inflammatory cytokines, chemokines, interferons, MHC proteins, growth factors, cell adhesion molecules, and viral genes. Their misregulation has been

linked to a number of diseases ranging from Alzheimers (Tanaka et al., 2002) and asthma (Xiong et al., 2002) to cancer (Rayet and Gelinas, 1999). NF-κB proteins can also be co-opted by pathogens for their own ends, for example HIV uses NF-κB to induce expression of its viral genes (Arenzana-Seisdedos et al., 1993; Nabel and Baltimore, 1987). A greater understanding of the mechanisms by which NF-κB proteins regulate these processes can potentially lead to novel approaches to therapies for these challenging public health issues of our time.

1.4.4 JAK-STAT Pathway

Janus Kinase (JAK), and Signal Transducers and Activators of Transcription (STAT) act together to induce genes in the fat body and hemocytes in response to infection. According to their canonical pathway, the binding of ligands to extracellular receptors causes dimerization and autophosphorylation of JAK proteins to create active SH2 docking sites for STAT proteins. The STAT proteins are normally present in the cytoplasm as inactive monomers, and activation by JAK causes them to dimerize, translocate to the nucleus, and activate transcription of a variety of genes (Vinkemeier, 2004). In mammals, JAK-STAT misexpression has been linked to a variety of malignancies (Calo et al., 2003). Drosophila have a simpler JAK-STAT pathway with just one JAK and one STAT isoform required for multiple biological processes. Recent work including evidence from an RNAi screen on insect cell lines suggest conservation with mammals on critical aspects by JAK-STAT regulation including cellular proliferation, immune response, and stem cell maintenance (Bach et al., 2003; Mukherjee et al., 2005). On the whole however, compared to the Toll and IMD pathways, immune

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responses mediated through the JAK-STAT pathway appear to be relatively late and short-lived (Boutros et al., 2002).

A variety of stimuli are involved in activating the JAK-STAT pathway. In hemocytes for example, the cytokine-like *upd3* is responsible for activating *domeless* receptor. This leads to the activation of the JAK-STAT pathway (Agaisse and Perrimon, 2004). However, cytokines involved in activating mammalian JAK-STAT pathways do not appear to have clear homologues in *Drosophila*. BRWD3, a Drosophila homolog of the human BRWD3 is a large WD40 protein. This was also identified as capable of stimulating JAK-STAT signaling (Muller et al., 2005). This is particularly interesting because a defect in the human homolog BRWD3 has been identified in a large proportion of individuals suffering from B-cell chronic leukocytic leukemia (B-CLL). This suggests that aberrant JAK-STAT signaling may be a potential reason for the pathology.

In mammals, mutations in JAK-STAT pathway leads to defects in B-cell and T-cell proliferation and results in severe immunodeficiency such as SCID (severe combined immunodeficiency)(Schindler, 2002). Various mammalian cytokines are known to activate this signaling pathway, and conversely compounds known as Suppressors of Cytokine Signaling (SOCS) are known to inhibit JAK-STAT activation (O'Shea et al., 2002). JAKs are known to bind to certain cell surface cytokine receptors, where their dimerization leads to STAT phosphorylation and activation. Among the identified targets induced by this pathway in Drosophila immunity, are the stress-induced gene *totA*, and the thiolester containing protein TEP-1 which is believed to play a role in opsonizing pathogens (Agaisse and Perrimon, 2004; Agaisse et al., 2003; Boutros et al., 2002;

Lagueux et al., 2000). The pathway has also been implicated in mediating Drosophila antiviral responses (Dostert et al., 2005). JAK-STAT is also likely to regulate hematopoiesis during development. Activation of the pathway leads to hemocyte overproliferation and melanotic tumors (Luo et al., 1997).

1.4.5 JNK Pathway

The c-Jun N-Terminal Kinase pathway (JNK) pathway is a highly conserved signaling pathway that controls cytoskeletal remodeling and transcriptional regulation in response to a wide variety of signals. They belong to a broader class of Mitogen Activated Protein (MAP) Kinases, that are activated in mammals in response to a variety of stimuli including cytokines, UV radiation, heat shock, and mitogens (Pearson et al., 2001). Functional characterization of Drosophila JNK mutants has been relatively hard *in vivo* because disruption of this signaling pathway results in embryonic lethality (Noselli and Agnes, 1999). Bacterial infection has been observed to activate JNK pathways, through dTAK1 that is also involved in IMD pathway activation (Delaney et al., 2006; Silverman et al., 2003). In S2 cells the JNK pathway has been found to regulate AMP expression (Kallio et al., 2005). dTAK1 mutants however continue to demonstrate Relish activation, the transcription factor activated by the IMD pathway (Delaney et al.,2006). This suggests that other possibly redundant mechanisms may be acting in parallel to enable Relish mediated AMP expression. dTAK1 however has been shown to act upstream of Relish, while Relish has been shown to induce processes that inhibit dTAK1, suggesting a possible feedback loop important for Drosophila immune regulation (Park et al., 2004). This mode of regulation appears to be conserved between flies and mammals, and may be

a process by which inflammation-induced apoptosis is regulated in other organisms. On the other hand, dTAK1 mutants do fail to activate JNK pathway components, suggesting that JNK/NF-κB pathways may be working together to activate AMPs during the Drosophila immune response (Delaney et al., 2006). The JNK pathway is also required for the expression of other immune induced genes such as Punch, malvolio, and sulfated which may play a role in the cellular immune response (Delaney et al., 2006; Silverman et al., 2003). The JNK pathway is also involved in efficient wound healing (Ramet et al., 2002a). Thus, the JNK pathway plays a significant role in mediating distinct aspects of the Drosophila immune response.

Like mammals, Drosophila expresses three classes of MAP kinases: JNK has already been discussed, Extracellular Regulated Kinases (ERKs), and p38 isoforms. D-MKK4, D-MKK3, D-MKK7, and D-p38 were identified based on homology to ERK type and p38 type mammalian MAP kinases respectively. D-MKK4 has been implicated in the activation of Drosophila JNK pathways, while D-p38 kinases were efficiently activated by D-MKK3 (Han et al., 1998). D-MKK3 and D-p38 therefore appear to belong to a distinct Drosophila p38 pathway, and further experiments revealed that they may be involved in attenuating AMP expression after exposure to LPS (Han et al., 1998). Apart from this role in immunity, Drosophila MAP kinases have been observed to have distinct roles during Drosophila embryogenesis and wing development. By contrast, mammalian MAP kinases demonstrate considerable redundancy. Upon activation, multiple mammalian JNKs are involved with expression of a variety of genes involved in cell differentiation, apoptosis, and immunity including RANTES, IL-8, and GM-CSF. Other mammalian MAP kinases such as those belonging to the ERK or p-38 classes work

similarly, to mediate varied gene expression in response to mitogens. They have therefore been extensively studied as an important set of signal transduction pathways for activating immune responses.

1.5 Fungal infection in Drosophila

Recognition of pathogens, signaling, and the orchestration of means to neutralize them are part of the host organisms arsenal for protecting itself. The evolutionary war between host and pathogen is however, played out in cycles: surviving pathogens possess the ability to colonize or infect a host, while the surviving host has favorable mutations that help it overcome the infectious load. In one experiment, above a hundred different wildtype *Drosophila melanogaster* strains isolated from different populations were in-bred to produce genetic homogeneity. A distinct correlation was observed between the presence of polymorphisms in certain pattern recognition and signal transduction factors and their efficiency in overcoming infection from the gram-negative bacteria, *Serratia marcesens* (Lazzaro et al., 2004; Schlenke and Begun, 2003). These studies provide a genetic basis for understanding natural selection of Drosophila in response to microbial threats. Just as selection pressure has enriched resistance to bacteria in some fly populations, various pathogens are also likely to specialize in infecting flies. Few studies of innate immunity in Drosophila however, have focused on the use of natural pathogens that infect them in nature, or used natural means of infection. This is mostly because of a broader failure to identify pathogens that can produce robust and reproducible host immune responses. In addition, most bacterial or viral pathogens would likely be ingested orally in nature, and this method has traditionally produced a low rate of infection in the lab. Efforts have therefore focused on injecting these pathogens, which result in measurable and reproducible perturbations to the immune response. Entomopathogenic fungi on the other hand are likely to penetrate the insect cuticle, and shaking Drosophila on spores of fungi has been an attractive natural infection method. These methods have

been especially useful in identifying flies lacking anti-fungal responses (Lemaitre et al., 1996).

Metarhizium anisopliae is an entomopathogenic fungus that can infect a relatively broad range of insect hosts. The study of this fungus has gained increasing prominence due to its popularity as a biological pest control agent, to reduce the population of insect pests, particularly grasshoppers, termites and thrips (Ansari et al., 2004; Lord, 2005; Wang and St Leger, 2005; Wekesa et al., 2005). Recently, this fungus has been shown to reduce populations of the malaria causing *Anopheles* mosquito (Riba et al., 1986; Scholte et al., 2006). Different *Metarhizium* isolates can be very diverse, having different host specificities and virulence characteristics. To date none are known to specialize in infecting Drosophila, but evidence from our lab (Appendix D) and other groups suggest that some *Metarhizium* isolates can be lethal to fruit flies as well. *Metarhizium* causes "green muscardine", a disease characterized by the covering of the insects' body with green fungal conidia. These asexual spores can come into contact with other insects and attach to their cuticle. There, they germinate hyphae that penetrate the cuticle. Several toxins secreted during this penetration are known to aid in infection, and cause insect mortality while the fungus proliferates using the insect as a source of nutrition. Some of these toxins are discussed below.

1.5.1 Destruxins

Destruxins (Drx) are a class of cyclic depsipeptides that have been isolated from several *Metarhizium* strains. Depsipeptides are a class of substances that are usually cyclic containing ester bonds and peptides, which are made naturally by some bacteria and

fungi. They are a class of secondary metabolites, produced through a non-ribosomal biosynthetic process comprised of multiple enzymes (Jegorov et al., 1993). Analogues have been isolated from other entomopathogenic fungi including *Aschersonia*, *Beauveria*, and *Nigrosabulum* (Pedras et al., 2002). They are composed of an alpha-hydroxy acid and five amino acid residues in cyclic structure and are broadly classified into five chemical groups, labeled A through E. Drxs A, B, and E have been well characterized as virulence factors, and their secretion is believed to be an important cause of insect mortality during infection. Destruxin producing fungi have been observed to cause insect mortality faster than similar fungal strains that produce less of this toxin. Destruxins have strong Ca^{2+} chelation properties, and this is believed to cause muscular depolarization in insects by opening Ca^{2+} channels in membranes (Dumas et al., 1996; Samuels et al., 1988). The muscles lose control as a result, and the affected insect undergoes paralysis or tetanus of voluntary muscles (Samuels et al., 1988). In Drosophila, this can often be seen in *Metarhizium* infected animals as an unnatural stiffening of the wing (unpublished observations). Apart from this toxic effect, Destruxins have also been reported to affect host cellular responses. Destruxin-treated immunocompetent insect cells appeared to be deficient in encapsulation and phagocytosis at non-lethal doses of around 0.8 microgram/gram of body weight (Vey et al., 1985; Vilcinskas et al., 1997a). Destruxins are also particularly toxic to mammalian leukemia cells and spleen lymphocytes and have demonstrated anti-proliferative activity on mouse neoplasms in vitro (Pedras et al., 2002). Destruxins A, B, and E have also been shown to have antiviral properties in insect and human cell lines (Huxham et al., 1989; Quiot et al., 1980; Vey et al., 1985). Apart from

their ability to open calcium channels, the mechanisms by which Destruxins achieve these varied biological activities remain to be studied *in vivo*.

1.5.2 Other secondary metabolites

Swainsonines are indolizidine alkaloid molecules with a fused piperidine and pyrrolidine ring system, which is produced by a variety of fungi including the *M. anisopliae* strain F-3622 (Hino et al., 1985). They have inhibitory activity on lysosomal α-mannosidase and on the Golgi complex α-mannosidase II, involved in the cellular degradation of polysaccharides. The inhibition leads to the formation of autophagic vacuoles in various tissues, particularly the liver, and decreases the capacity of endosome-lysosome fusion (Tulsiani and Touster, 1992). This inhibitory activity by itself can be toxic to a host, causing a lysosomal storage disease known as locoism, as well as teratogenicity and birth defects in mammals. Interestingly, Swainsonine has been documented to prevent tumor proliferation and metastasis in mammals, and has been clinically tested as an anticancer drug with considerable promise (Nemr, 2000). It is also known to stimulate the immune system, but little is know about the mechanism by which it does this.

Apart from these toxins, *Metarhizium* and other fungi produce a vast array of secondary metabolites which remain to be characterized. A group of compounds known as viridoxins for example have been found to be toxic to insects, but their mechanism of action is not understood (Gupta et al., 1993). Like Destruxin and Swainsonine, a number of these toxins have documented effects on host immune responses. An isolate of Metarhizium produced a novel compound which resembles a known immunosuppressive called Ovalicin. This compound, named Mer-f3 was found to have a similar activity *in*

vitro, but was found to be less toxic than ovalicin on mammalian cells (Zimmermann and Hartmann, 1981). This pattern of immunosuppressive activity present in numerous fungal metabolites leads to a broader evolutionary question: could the fungi be producing these compounds to suppress host immune responses in order to improve their chances of infecting the insect?

1.6 Scope of research: Innate immune responses in Drosophila

Insect pathogens have been under-exploited as a resource of medically active compounds, but this is changing with the realization that they are exceptionally rich sources of novel biologically active substances (Isaka et al., 2005). In a preliminary screen (Appendix D), we observed that Destruxin producing fungi appeared to be more virulent to Drosophila, in agreement with published reports in other insects. To explore the role of Destruxin further, we used cDNA microarrays to explore the effects of non-lethal doses of the toxin on Drosophila gene expression. This effect had never been studied before on a genomic level. With the advent of relatively new technologies such as cDNA microarrays, new approaches of studying the processes by which such compounds may facilitate fungal pathogenesis are now within reach. Following these approaches to their logical conclusions, we first identified the genes that were affected in Destruxin-injected flies. A number of genes involved in the Drosophila humoral immune response, particularly the AMPs, were down-regulated. This Destruxin induced immunesuppression coincided with greater bacterial proliferation and higher insect mortality in infected insects, when compared to control flies that had not been injected with Destruxin. Two known pathways are involved in regulating the AMPs—the Toll and IMD signaling pathways. Using ectopic activation of components in this pathway to rescue immune suppression, we inferred that the IMD pathway was being suppressed in Destruxin injected flies.

The use of microarrays also enabled us to investigate other aspects of the Drosophila immune response. NF-κB transcription factors are at the heart of both Drosophila humoral responses and mammalian innate immunity. Drosophila expresses three NF-κB proteins: Dorsal, Dif, and Relish. However, despite the extensive study, little is known

about the broader genomic roles of these proteins. Thus, one of the objectives of my research was to use microarrays to look at changes in gene expression, either when these three factors are absent, or when they are ectopically expressed. Together these experiments reveal the distinct but overlapping functions *in vivo*, of these homologous proteins. The data was also used to identify Dif specific target genes that can serve as a useful marker for assaying Toll pathway activation.

Perhaps the uniting theme of this dissertation then, is the use of Drosophila genomics tools to study the role of Rel/NF-κB proteins in mediating innate immune responses. Injection with a fungal effector, a mutation in a Rel protein, or ectopic expression of a Rel, affected gene Drosophila gene expression. A study of this data helped characterize the role of the Rel/NF-κB proteins in different aspects of the immune response. The goal of the current thesis was to examine gene expression from microarrays, to try and make sense of them, generate hypotheses, and further investigate the validity of those hypotheses with experiments. Through these experiments, we hoped to distill some of the findings into useful results that broaden our knowledge of innate immunity.

Pathogens and their determinants

Toll Pathway   IMD Pathway   JAK/STAT Pathway

Gram+ Bacteria   Fungi   Gram– bacteria   Injury
Lys-PGN   β1,3 Glucans   DAP-PGN

GNBP-1   PGRP-SA
         PGRP-SD

Spätzle

Toll   PGRP-LC
              PGRP-LE   upd3

imd

Cytoplasm   MyD88   TAK   dome
                                              Hop(dJAK)
Tube Pelle   IKK
                         dredd

Cactus Dorsal   Cactus Dif   Relish   dSTAT

Nucleus   Dif   Relish   dSTAT

Drosomycin, AMPs   Diptericin, AMPs   TEP1
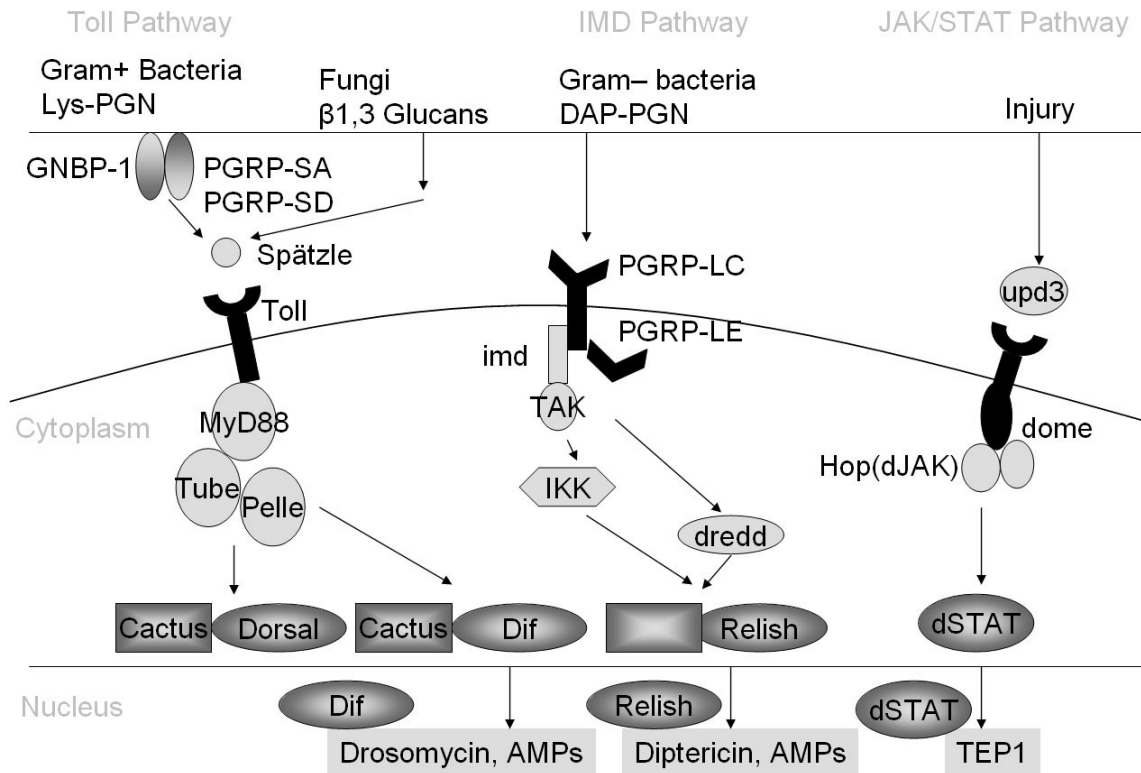
Figure 1-1 : Immune pathways in Drosophila. Gram-positive bacteria are detected by PGRP-SA and –SD. This results in the cleavage of Spätzle, and the activation of the Toll pathway. Gram-negative bacteria activate the IMD pathway through the receptor PGRP-LC and PGRP-LE. Septic injury can lead to the activation of the JAK-STAT pathway. There may be considerable cross talk between all these signal transduction pathways.
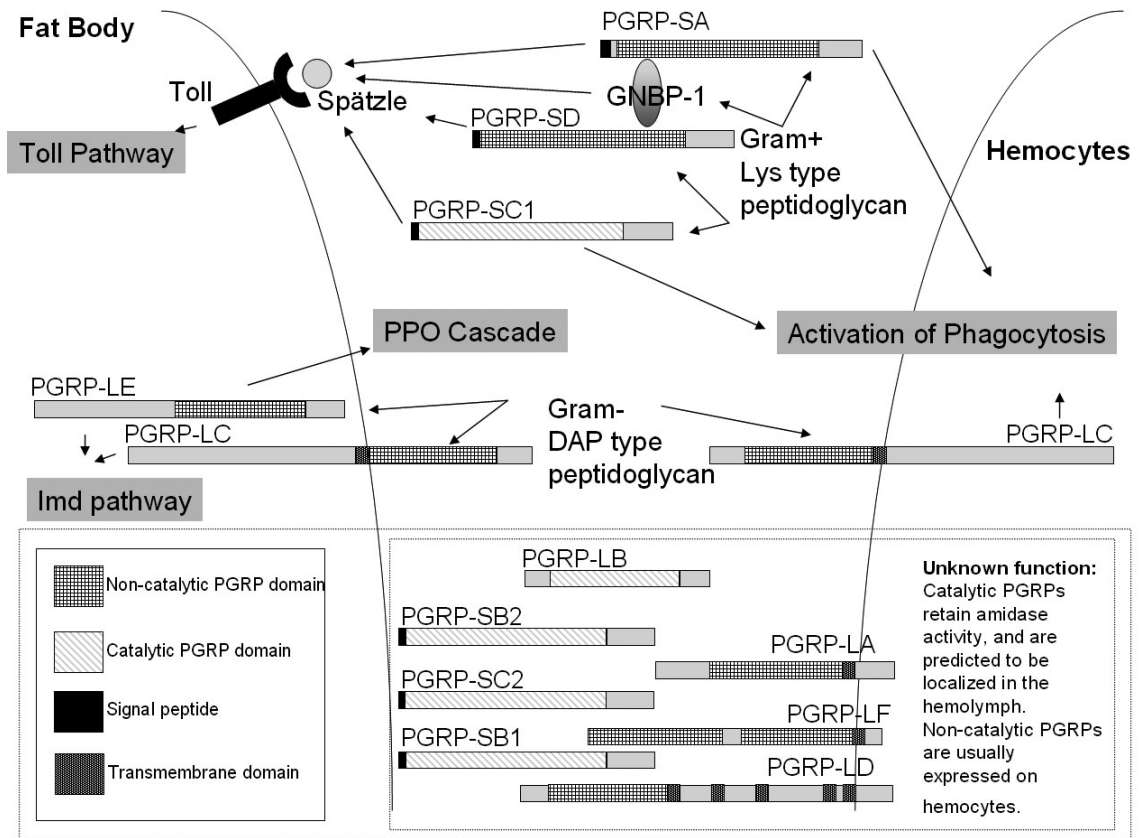
Figure 1-2: The Peptidoglycan Recognition Receptors. The receptors are arranged based on domains and probable function. Known signaling pathways are shown using arrows.

Gene	Type of protein	Putative Ligand	Evidence
18 wheeler, Toll-2	Toll-like Receptor	Unknown	Expression of Attacin affected in mutant flies.
Toll-9	Toll-like Receptor	Unknown	Protein activates Drosomycin in S2 cells through MyD88.
Ird7, totem, PGRP-LC	Peptidoglycan Recognition Protein	DAP-type peptidoglycans, G- bacteria	Activate IMD pathway *in vivo*. Phagocytosis of *E. coli* in S2 cell lines, affected upon RNAi.
PGRP-LE	Peptidoglycan Recognition Protein	DAP-type peptidoglycans, G- bacteria	Overexpression activates PPO cascade in cell lines. Help PGRP-LC recognize peptidoglycans.
semmelweis, PGRP-SA	Peptidoglycan Recognition Protein	Lys-type peptidoglycans, G+ bacteria	Activation of Toll and phagocytosis of *S. aureus* pathway affected in mutants.
PGRP-SD	Peptidoglycan Recognition Protein	Lys-type peptidoglycans G+ bacteria	Activation of Toll pathway affected in double mutants with PGRP-SA mutants.
picky, PGRP-SC1a	Peptidoglycan Recognition Protein	G+ bacterial peptidoglycans	Activation of Toll pathway and *S. aureus* phagocytosis affected in mutants.
PGRP-SC1b	Peptidoglycan Recognition Protein	G+ bacterial peptidoglycans	Cleaves *S. aureus* peptidoglycans.
osiris, GNBP1	Gram Negative Binding Protein	Potentially G+ bacterial determinants.	Hydrolyzes G+ peptidoglycan. Acts in complex with PGRP-SA to activate Toll pathway.
TEPs	Thiolester containing proteins	Possibly binding to Gram-negative bacterial surface.	RNAi of homologous mosquito gene reduces phagocytosis of Gram-negative bacteria. Plasmodia population larger in *Tep1* mutant mosquito, causing higher vectorial capacity.
dSR-C1	Scavenger Receptor	Possibly both G+ and G- bacteria.	RNAi of S2 cells reduces phagocytosis of bacteria.
Crq	Scavenger Receptor, CD36 like.	Apoptotic cells and possibly G+ bacteria.	Phagocytosis of *S. aureus* impaired in cell lines.
Peste	Scavenger Receptor, CD36 like	Mycobacteria	RNAi of S2 cells reduces phagocytosis of Mycobacteria.
Eater	Scavenger Receptor, Epidermal Growth Factor like.	Possibly G+ and G- bacteria.	Reduction in phagocytosis of *S. aureus* and *E. coli in vitro* in cell-line mutants and *in vivo* in deficiency. Increased susceptibility to natural infection *in vivo*.
DScam	Immunoglobulin superfamily	Diverse potential ligands.	RNAi of some Dscam isoforms reduces ability to phagocytose bacteria.

Table 1-1: Pattern recognition receptors in Drosophila. The gene, type of protein, putative ligand, and evidence suggesting its role in pattern recognition are provided.

Stimuli that can induce NF-κB in mammals.	Phorbol esters, proinflammatory cytokines like tumor necrosis factor (TNF) and interleukin 1 (IL1), bacterial LPS, ds RNA, some viruses, Tax protein of HTLV-1, ionizing radiation, CpG DNA
Some of the Genes Induced by NF-κB in mammals.	**Cytokines**: IL-1, IL-2, IL-6, IL-12, TNF-α, LT-α, LT-β, and GM-CSF. **Chemokines**: IL-8, MIP-1α, MCP1, Rantes, eotaxin. **Adhesion molecules**: ICAM, VCAM, E-Selectin. **Acute phase proteins**: SAA **Inducible effector enzymes**: iNOS, CoX-2. **Antimicrobials**: β-defensin **Adaptive immune response important genes**: MHC proteins, IL-2, IL-12, IFN-β. Some cytokines stimulate migration and maturation of lymphocytes. **Apoptosis/Cell proliferation**: c-IAP-1, c-IAP-2, A1 (Bfl1), Bcl-X, Fas ligand, c-myc, cyclin D1.

Table 1-2: Table showing list of stimuli that can induce NF-κB in mammals and the genes induced as a result.

Chapter 2: Microarray analyses reveal distinct roles for Rel proteins in the *Drosophila* immune response

Abstract

The fruit fly *Drosophila melanogaster* express three closely related NF-κB-like transcription factors: Dorsal, Dif, and Relish. They share significant sequence identity in their DNA binding Rel homology domain and bind similar DNA sequence motifs. These factors play an important role in the transcription of Drosophila humoral immune genes such as antimicrobial peptide genes during infection. To study their roles *in vivo*, we used microarrays to determine the effect of null mutations in individual Rel transcription factors on larval immune gene expression. Of the 188 genes that were significantly up-regulated in wildtype larvae upon bacterial challenge, overlapping but distinct groups of genes were affected in the Rel mutants. A mutation in *Relish* affected expression of the largest percentage of genes involved in Humoral and Defense responses, compared to *Dorsal* and *Dif*. We also ectopically expressed Dorsal or Dif and used cDNA microarrays to determine the genes that were up-regulated in the presence of these transcription factors. This expression was sufficient to drive expression of some immune genes, suggesting redundancy in the regulation of these genes. Combining this data, we also identified novel genes that may be specific targets of Dif.

2.1 Introduction

NF-κB transcription factors play a central role in both mammalian and insect innate immune responses. Because of significant conservation in the signaling pathways responsible for NF-κB activation, the fruit fly *Drosophila melanogaster* has been an attractive model to study these transcription factors. The fruit fly expresses three Rel/NF-κB proteins: Dorsal, Dif, and Relish. These proteins share a Rel homology domain that dimerizes to bind DNA and initiate transcription. Two signal transduction pathways, Toll and imd, are involved in activating these three Rel proteins (Hoffmann, 2003; Khush et al., 2001). Recognition of pathogenic determinants by upstream pattern recognition receptors causes the activation of these pathways in immune tissues of Drosophila. Toll activation causes phosphorylation and degradation of an inhibitor of κB (IκB), Cactus. Cactus sequesters Dorsal and Dif in the cytosol, and its degradation releases them to translocate to the nucleus and initiate transcription of genes (Belvin et al., 1995; Govind, 1999). The imd pathway regulates Relish activation in a similar way: phosphorylation and cleavage of the inhibitory ankyrin repeat domain of Relish leads to release of the Rel domain responsible for transcription (Hedengren et al., 1999; Stoven et al., 2000; Stoven et al., 2003). During an immune response, differential Toll and imd pathway activation of the three NF-κB proteins is believed to cause different transcriptional outcomes. *In vitro* evidence demonstrates heterodimerization and homodimerization of the three Rel proteins can create transcription factors with different target specificities (Han and Ip, 1999). SELEX assays have identified that Dorsal and Dif/Relish have greater affinity for different 9-12 base pair sequences (Senger et al., 2004). Due to the presence of cis-acting

transcription factor binding sites with affinity for different dimer combinations, it is predicted that different sets of genes may thus be controlled by the three Rel proteins.

The antimicrobial peptides (AMPs) in the fat body are an important class of proteins differentially regulated in this way (Lemaitre et al., 1997). The humoral immune response is characterized by the induction of these AMPs in the fat body of the fly in response to infection. These peptides have antimicrobial activity and help subdue the infection. The fruit fly also mounts cellular responses, such as phagocytosis or encapsulation of microbes by circulating hemocytes, and proteolytic cascades that result in melanization toxic to infectious agents at wound sites (Lanot et al., 2001; Nappi et al., 2005; Tang et al., 2006). Rel proteins have been well characterized for their central role in mediating the humoral response, but they may also induce genes involved with these other aspects of the Drosophila immune response. However, studies of Rel transcription factors have usually focused on their role in regulating expression of the antimicrobial peptide (AMP) genes.

Flies lacking Dorsal do not appear to be compromised in their ability to induce any of the known AMPs (Lemaitre et al., 1995b). Whereas *Dif* mutant flies have greatly reduced abilities to induce *Drosomycin* and *Defensin* (Meng et al., 1999; Rutschmann et al., 2000) and *relish* mutant flies are incapable of inducing *Diptericin* and *Cecropin* during infection (Hedengren et al., 1999). On the other hand, expression of Rel proteins in tissue culture cells reveals that Dif and Relish together form the most potent activator of *Drosomycin*, *Defensin*, and *Attacin* (Han and Ip, 1999). Expression of Dif alone can

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induce *Cecropin* and *Diptericin* expression, while expression of Dorsal or Relish alone, does not result in induction of any of these AMPs. While these studies hint at considerable regulatory complexity resulting from the heterodimerization of Rel proteins, they have focused solely on the induction of AMP genes. The broader effects of Rel mutations in regulating global gene expression during an immune response are not known, so we sought to explore this question *in vivo*.

Using a microarray approach, we examine the effect on immune gene expression when the Rel proteins are absent due to mutation or when they are ectopically expressed. Using Affymetrix Drosophila GeneChips, we identified 188 genes that were induced upon Gram$^-$ bacterial infection in wildtype OregonR (OR) larvae. Among these genes, overlapping but different subsets of these genes failed to induce to wildtype levels in *dorsal*, *Dif*, and *relish* mutant larvae. A substantial percentage of these affected genes were involved with mediating the flies' immune responses, with most of the known AMPs affected by *Relish* or *Dif* mutations. Redundancy between Rel proteins may account for failure to see the effects of single mutants, so we also looked at global gene expression resulting from ectopic expression of Dorsal or Dif. A number of genes important for immunity were induced in both cases, including some that were not identified from the loss-of-function experiments. A comparison of these datasets enabled a global characterization of the role of the Rel proteins in mediating gene expression in an immune response. It also enabled the identification of putative target genes. To date, understanding the Toll pathway has been limited because the only known target gene has

been *Drosomycin*. Here we report the identification of several novel Toll target genes that are induced more rapidly, and are more specific indicators of Toll pathway activation.

2.2 Experimental Procedure

Larval staging and infection

Larvae were accurately staged to roughly 80 hours, as described (Andres and Thummel, 1994). The adult flies used were more than 5 days old. For infection, 1 ml of an overnight culture of *E. coli* DH5α or *M. luteus* was spun down and resuspended in 1 ml of PBS. Approximately 0.5 μl of this suspension was injected into flies using a pico-pump. For each sample, following injection with bacteria or PBS, 20 larvae or adults were homogenized and their RNA extracted using STAT-60 following the manufacturer's (Isotex diagnostics) protocol.

Microarray experiments

Affymetrix microarray experiments were conducted using commercially available Drosophila GeneChips (Affymetrix, California). RNA was extracted from 50 larvae for each experimental replicate, repeated in triplicate for each genotype. Calculations were performed according to laboratory methods from the Affymetrix GeneChip manual. Genes which were induced greater than 2-fold with a p-value < 0.01 in at least 2 out of 3 replicates in wildtype *E. coli* injected larvae, were selected as induced during the immune response (GEO Acc. No. GSE5489). The average expression of all three data points is used as fold-change for the gene (Table 2-1).

The cDNA microarray comprised of 464 genes, selected based on previous results with Affymetrix chips, combined with genes selected from other published microarray studies as being induced during Drosophila immune responses (Boutros et al., 2002; De Gregorio et al., 2001; Irving et al., 2001). We used Primer3 (http://www-

genome.wi.mit.edu/genome_software/other/primer3html) to design primers to amplify unique 200-600 bp regions of the selected genes (primer sequences available at GEO Acc. No. GPL4064). Fragments were amplified from whole genomic DNA of wildtype larvae in a 96 well format. Printing, hybridization and scanning of slides were performed with an Affymetrix 417 Arrayer and 418 Scanner (see http://www.umbi.umd.edu/~cab/macore/macorestart.htm for detailed protocols).

For the cDNA microarray experiments, RNA was extracted from a pooled sample of 20 larvae with STAT-60 buffer, according to the manufacturer's protocols (Isotex Diagnostics). The RNA was further purified using the Qiagen RNAeasy purification kit, and directly labeled using Amersham Biosciences Cyscribe First-Strand Labeling Kit, according to manufacturer's protocols. The raw scanned image files were analyzed using Spotfinder (TIGR), and data normalization, quality assurance and control, filtering, and clustering was performed using MIDAS (TIGR) and MS-Excel (Saeed et al., 2003). Standard Deviation normalization and Lowess transformation was performed on the data using MIDAS (http://www.tm4.org/midas.html). The affected genes were then classified according to Gene Ontology, and the major groups are presented in Figure 2-2 (GEO Acc. No. GSE5469).

Quantitative PCR

The RNA was subjected to reverse transcription using Superscript II (Invitrogen) and the resulting cDNA was quantified by real-time PCR using LUX probes (Invitrogen) or SYBR Green (Applied Biosystems) on an ABI 5700 and 7300. Gene expression was

normalized using RP49 as an endogenous control. The data presented in this paper has been further normalized to set uninjected or heat shock induced wildtype levels as the calibrator. For our work, a $p<0.05$ is deemed to be a significant difference in gene expression using an unpaired homoscedastic student's T-test.

2.3 Results and Discussion

2.3.1 Rel proteins have overlapping but distinct functions

For an unbiased genomic level perspective on the roles that Dorsal, Dif, and Relish play during an immune response, we used Affymetrix oligonucleotide microarrays to study gene expression in flies' mutant for these transcription factors. These Affymetrix experiments were performed by Junlin Wu in our lab. Third instar larvae were accurately staged and injected with *E. coli*. Two hours later, RNA was extracted, labeled, and hybridized to the microarrays. Along with the wildtype larvae, we injected and studied gene expression in Rel mutant larvae *dorsal[1]*, *Dif[1]*, and *relish[E20]*. Of the 13,500 transcripts represented on the microarray, 188 genes were significantly induced (greater than 2-fold in at least 2 out of 3 replicates with a p-value<<0.01) by infection in wildtype flies. For these genes, the effect of a given Rel mutation was calculated as an expression ratio, comparing expression in the Rel mutant to wildtype levels (Table 2-3). A selection of genes induced or repressed in the Rel mutants is presented (Table 2-1). Other groups have performed similar microarray experiments to study gene expression during infection in adults and *Drosophila* S2 cells (Boutros et al., 2002; De Gregorio et al., 2002; Irving et al., 2001) but this is the first time a comparison of the effect of specific Rel mutations on gene expression has been examined.

All studies to date have explored the role of Rel proteins on the induction of AMP genes. We found a number of AMP genes that are poorly expressed in *relish* mutants including *Diptericin*, *Cecropin*, *Defensin*, *Attacin*, and *Drosomycin*, confirming the key role played by Relish in the humoral immune response. Other transcripts affected in Relish mutants

that are categorized as being important for the defense response by Gene Ontology (GO:0009607) include: the peptidoglycan recognition receptor (PGRP) SA involved in recognition of Gram+ bacterial peptidoglycan; CG9733, a protein with serine protease activity that is not known to play a role in Toll signaling or melanization (Kambris et al., 2002); CG13422 a protein with predicted glucosidase activity believed to play a role against Gram- bacteria; and the heat shock protein Hsp70 that is likely to be part of the stress response to pathogenic challenge (Ashburner and Bonner, 1979; De Gregorio et al., 2001; De Gregorio et al., 2002; Irving et al., 2001; McGraw et al., 2004; Ross et al., 2003). More than half of all genes failing to induce in Relish mutants, belong to GO categories for humoral immune response (GO:0006959) and defense response (GO:0009607) (Figure 2-1). Relish therefore appears to play a fairly specific role in regulating genes in this functional category. By contrast, less than a quarter of genes affected in *Dif* and *Dorsal* mutants fall in those two GO categories, and therefore these factors appear to play a relatively less specific role in mounting a humoral immune response (Table 2-1).

The AMPs *Defensin* and *Cecropin* as well as immune induced molecules IM1 and IM23 whose function in the immune response is not known, express at lower levels in response to a mutation in the *Dif* gene (Boutanaev et al., 2002; Uttenweiler-Joseph et al., 1998). CG13422 and CG9733 induced during an immune response, appear to require both Dif and Relish for their expression, along with 8 other genes (Table 2-1). Heterodimerization between the Dif and Relish may be required for optimal expression of these genes in response to bacterial infection. We find other immune-induced genes that also require

more than one Rel for expression. This suggests that other possible dimer combinations between Dorsal, Dif, and Relish may occur to produce functional transcription factors. Dif mutants also show a lowered expression of several genes that are not known to be involved in a defense response: CG3523, predicted to be involved in fatty acid biosynthesis; Cytochrome P450 involved in steroid metabolism; and RSG7, important for regulating G-protein mediated signaling. Dif therefore maybe mediating more physiological changes associated with the immune response.

As previously reported, mutation of *dorsal* does not affect expression of known AMP genes. Dorsal was therefore believed not to play a role in the immune response. However, in a broader genomic context the *dorsal* mutation does affect expression of a number of genes such as IM1, IM23, and CG6429 predicted to play a role in the defense response (De Gregorio et al., 2001; De Gregorio et al., 2002). Like *Dif* however, *dorsal* also affects a number of proteins not directly associated with immunity: CG14762, predicted to be involved in cell adhesion, and Acp1, a structural constituent of the adult cuticle expression. *dorsal* mutants have decreased phenoloxidase activity and exhibit less melanization (Bettencourt et al., 2004a). But our results with *dorsal* do not indicate significant changes in expression of genes associated with melanization, suggesting that these processes may not be primarily regulated at the transcriptional level.

While *dorsal* and *Dif* loss-of-function mutations affect the induction of fewer immunity genes compared to *relish*, their absence results in the up-regulation of a relatively larger percentage of immunity genes by comparison. The Toll pathway regulates both Dorsal

and Dif, (Lemaitre et al., 1995b) and loss of expression of either factor leads to the induction of some genes important for the Defense response including: Glutathione S-transferase D2; CG5550, which contains fibrinogen domains that may interact with extracellular matrix or receptors involved with the immune response; Peptidoglycan Recognition Receptor protein PGRP-SC2 that plays a role in the recognition of bacterial peptidoglycan and may suppress signaling through the imd pathway (Bischoff et al., 2006); and Heat shock proteins HSP23 and HSP70 which are possibly induced as a response to stress during infection (De Gregorio et al., 2002; Irving et al., 2001; Singh et al., 2001). It is known that optimal expression of the Toll target gene, *Drosomycin* occurs 24 hrs post-infection, indicating that Toll pathway activation may affect an immune response days after infection. This is consistent with the observation that *dorsal* and *Dif* mutants show higher expression of some immune response genes --suggesting that the Toll pathway may indeed be important for fine tuning later aspects of the immune response. On the other hand, in *relish* mutants, relatively few defense response genes are up-regulated, and instead we see no specific pattern by GO category. In sum, mutations in the three Rel proteins affect expression of distinct but overlapping groups of genes (Figure 2-1). This suggests that the use of multiple Rel proteins with distinct but overlapping functions may contribute to the complexity and regulation of distinct aspects of the immune response.

2.3.2 Ectopic Dorsal and Dif regulate different genes

Having examined loss of function mutations, we decided to ectopically express individual Rel proteins, to study their individual effect on immune gene expression in the absence of

infection. We used a heat shock (HS) driven promoter system to induce expression of transgenic Rel proteins in the absence of infection. For HS-dorsal and HS-Dif, exposure to heat shock resulted in a strong induction of the transgenic protein (Figure 2-2). For Relish, a heat shock driven Gal4 transcription factor was used to bind an upstream activation sequence (UAS) and drive Relish expression. This HS-Gal4;UAS-Relish system however did not produce reproducibly high levels of induction that would enable the study of Relish effects independent of Dif and Dorsal (Figure 2-2). Unlike HS-Dl and HS-DIF, the Relish transcript expression is driven using a UAS-Gal4 system, and this indirect mode of activation may account for its lower expression. Further, larvae that did express Relish transcript upon heat shock did not show a corresponding induction of the established Relish target gene *Diptericin* (data not shown). This suggests that other signaling events like the cleavage of Relish, or presence of cofactors, might be necessary for optimal transcription (Dushay et al., 1996; Stoven et al., 2000). By contrast, the effect of Dorsal and Dif expression proved to be easier to study, because of robust transgenic expression and their simpler mechanism of regulation. These transcription factors are also interesting, because both of them are regulated by the same pathway and bound by the same IκB, Cactus. Yet there is clear evidence that they may be regulated differentially (Bettencourt et al., 2004a; Wu and Anderson, 1998), and that once released they mediate transcription of different subsets of genes, as supported by our microarray studies. The distinct roles of Dif and Dorsal in the immune response were less apparent than that of Relish. Thus, the ectopic expression of these factors is likely to provide more insight into the possibility of redundancy between them, and help better elucidate their respective roles in an immune response.

To examine the classes of genes activated by Dorsal and Dif *in vivo*, RNA was extracted from heat shock induced wildtype, HS-dorsal and HS-Dif larvae, labeled and hybridized onto cDNA microarrays constructed in our lab. These customized microarrays enable the study of 464 Drosophila genes selected based on previous work (Boutros et al., 2002; De Gregorio et al., 2001; Irving et al., 2001) and from our own microarray experiments, as being induced during the immune response. Genes that were significantly affected in HS-dorsal and HS-Dif larvae in comparison to wildtype were further classified based on available Gene Ontology information (Figure 2-3). Both Dorsal and Dif are capable of inducing a large number of genes involved in the humoral and defense responses. Over 25% of the genes induced when either Dorsal or Dif is expressed, belong to this category. Interestingly, ectopic Dorsal causes the induction of the AMP genes *Defensin*, *Diptericin*, *Attacin*, and *Metchnikowin*. *Dorsal* mutant larvae do not fail to induce any of these AMPs, suggesting that Dorsal may be acting redundantly with other Rel factors. Dorsal expression also induces components of the Toll pathway such as Dif, Cactus, and Pelle, suggesting a possible explanation for the induction of these components during infection (Table 2-2). Ectopic Dif on the other hand notably induces Gram⁻ binding proteins (GNBP) 2 and GNBP3 which are involved with recognition of bacteria and fungi, and the AMPs *Attacin* and *Drosomycin* that have activity against these pathogens. Our results indicate that both Dorsal and Dif are regulated by the Toll pathway, and despite that play distinct roles as evidenced by their ability to upregulate distinct groups of immune genes.

2.3.3 Identifying Rel-specific target genes

In the past, target genes have been essential for identifying components of signaling pathways. Most components of the imd pathway for example, have been identified using genetic screens for mutations that failed to express its target gene *Diptericin* (Vidal et al., 2001; Wu et al., 2001). Similarly, the use of *Drosomycin* led to the seminal discovery of the importance of the Toll pathway in Drosophila immune responses (Lemaitre et al., 1996). *Drosomycin* expression has been the sole target gene used to assay the activation of the Toll pathway. However, *Drosomycin* is not an ideal target gene. Other groups have reported that the imd pathway influences *Drosomycin* expression, and our data supports this finding. *relish* mutant larvae showed a significant lowering of *Drosomycin* expression compared to *dorsal* or *Dif* mutants alone. *In vitro* overexpression experiments have suggested that a Relish-Dif heterodimer is most effective at inducing *Drosomycin*, and this may explain these observations (Han and Ip, 1999). Further, *Drosomycin* is expressed at a high basal level and typically induces only up to two to six fold, often with peak induction at 24 hours after infection. With this long time frame, it is likely that pathways other than Toll may be activated and contribute to *Drosomycin* expression. Ideally, a Toll target gene would be highly inducible at an earlier time for a more clear and specific readout of Toll pathway activation.

From our microarray data we looked for genes which might be regulated specifically by a particular Rel protein. A Dorsal target gene would, for example, not be expressed in *dorsal* mutant larvae, and induced when Dorsal is expressed ectopically. This regulation should also ideally be specific, such that a mutation in other Rel proteins should not affect its expression. From the microarray results we identified CG7214 as a putative

Dorsal target gene, and CG15065 and CG13422 as putative Dif targets (Table 2-1). We used Q-PCR to confirm the expression of these genes in HS-dorsal and HS-Dif larvae (Figure 2-4). CG7214 failed to induce during ectopic Dorsal expression, while the putative Dif target genes CG15065 and CG13422 did induce to significant levels. Using Q-PCR we assayed the expression of these putative target genes in *Dif* and *dorsal* mutant flies (Figure 2-5). Flies with mutations in both the imd and Toll pathways (*imd;spätzle)*, was used as a negative control while wildtype was used as a positive control. CG7214 was induced upon *M. luteus* infection, a known Gram$^+$ bacterial activator of the Toll pathway. However, both *Dif* and *dorsal* mutant flies failed to express CG7214 suggesting that both proteins may be required for its induction, and that CG7214 is a non-specific target gene for Dorsal. On the other hand, both potential Dif targets were induced in wildtype *E. coli* and *M. luteus* infected flies within 2 hours but failed to be induced in *Dif* mutants. CG13422 is particularly attractive as a potential target, because it can be detected easily, inducing up to 20-fold within 2 hours after infection.

Following that, we analyzed up to 2kb upstream of all genes whose induction was affected in Rel mutants for putative NF-κB sites using Target Explorer (Sosinsky et al., 2003). The presence of these sites suggests a possibility of direct binding and cis-activation of the gene by Dif, and we found sites matching predicted κB motifs upstream of both CG15065 and CG13422 (Figure 2-6). These sites are also broadly conserved between related Drosophila species, suggesting that they may have been selected for through evolution. In combination with data from Figure 2-4 and 2-5, this suggests that CG15065 and CG13422 may be direct targets of Dif. We speculate that unlike other

genes regulated by the Rel proteins, these target genes are directly bound by Dif, and specifically induced when Dif is activated in the nucleus. The use of these genes may therefore be used to assess Dif activation, and might help identify novel components of the Toll pathway in the future.

2.4 Conclusion

Here, we have presented the effect of Rel proteins on Drosophila immune gene expression *in vivo*, either when they are absent or when they are ectopically expressed. In this context, Relish plays a fairly focused role in mediating humoral and defense responses, while Dorsal and Dif are involved with inducing genes with a variety of functions. Some genes may be induced redundantly by the different Rels, and our ectopic expression experiments helped to identify genes that could be induced by Dorsal or Dif overexpression. Our data gives insight into possible heterodimer combinations that may be responsible for inducing different subgroups of genes. This data may help elucidate the distinct transcriptional roles of Rel family members. Finally, the identification and characterization of new target genes should facilitate the identification of novel components of the Toll pathway.
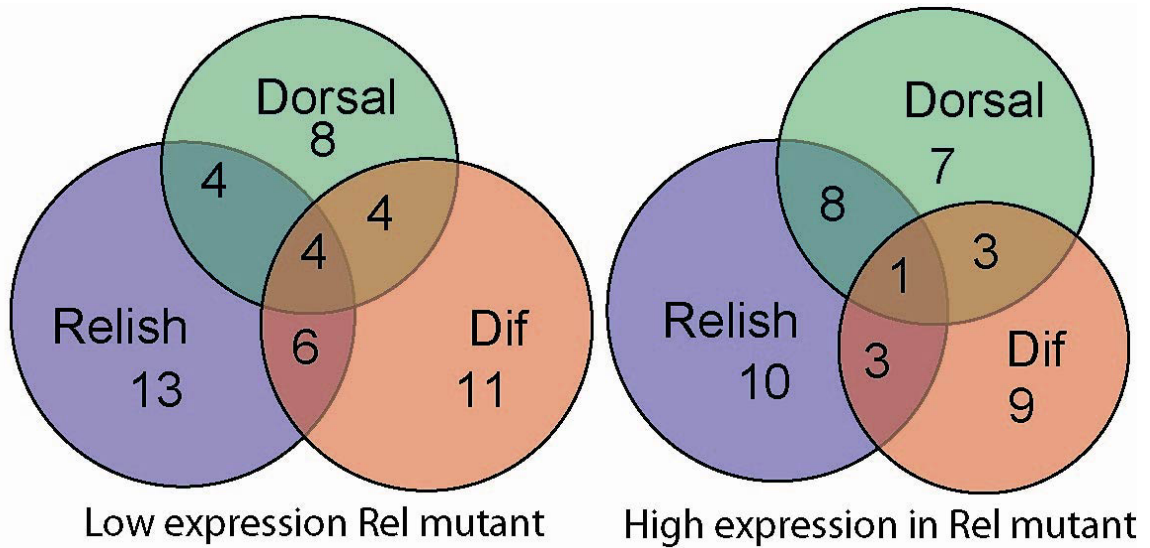
Figure 2-1: Venn diagrams of the numbers of genes significantly down-regulated (left) and up-regulated (right) in Rel mutant larvae. Among the 188 genes induced in wildtype, the number of genes that had significantly lower induction (left) in *dorsal[1]* (Green), *Dif[1]* (Orange), and *relish[E20]* (Blue) larvae, or numbers of genes that were significantly up-regulated in these mutants (right) are shown. These Affymetrix chip experiments were performed by Junlin Wu.
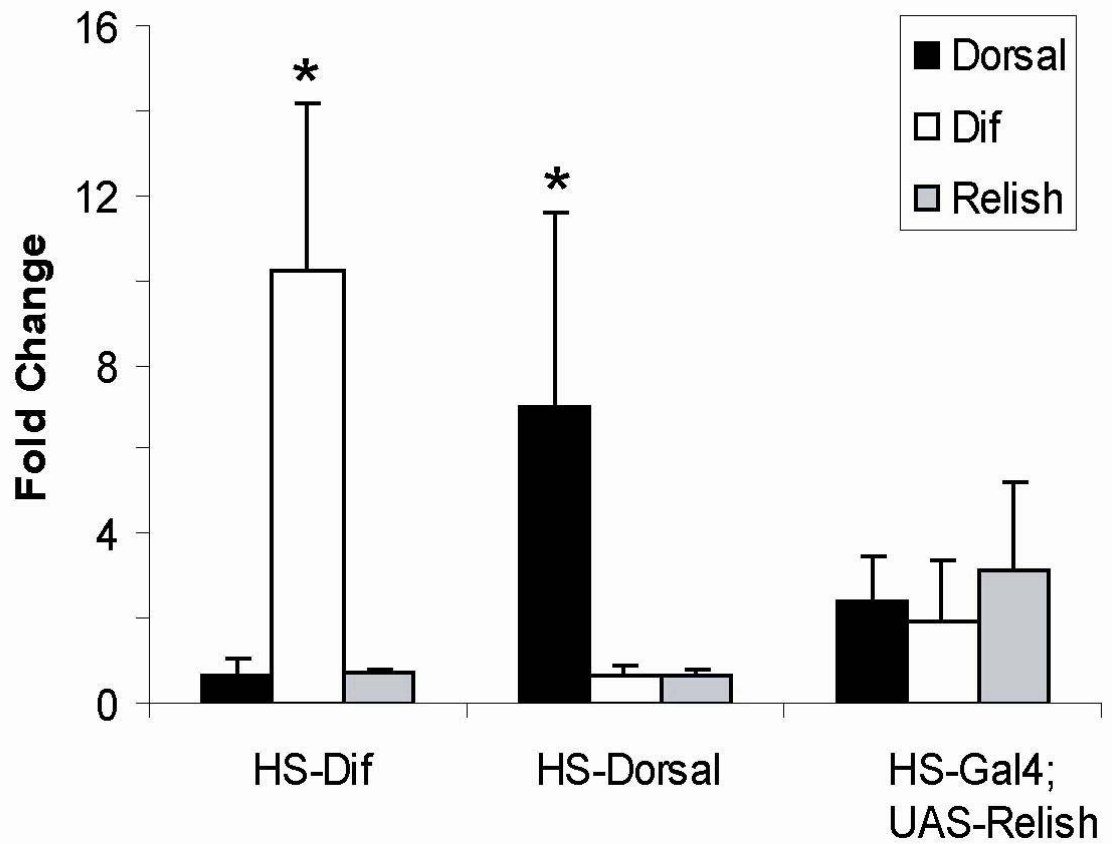
Figure 2-2: Heat shock induces specific ectopic expression of Dorsal and Dif in HS-dorsal and HS-Dif larvae. Quantitative PCR measuring *dorsal*, *Dif*, and *relish* transcript levels in HS-dorsal, HS-Dif, and HS-Gal4;UAS-Relish larvae after exposure to 37°C for 1 hour relative to heat shock treated wildtype larvae. Error bars represent SD of at least three biological replicates, and (*) denotes statistically significant induction with p-value<0.05 for two-tailed T-test.
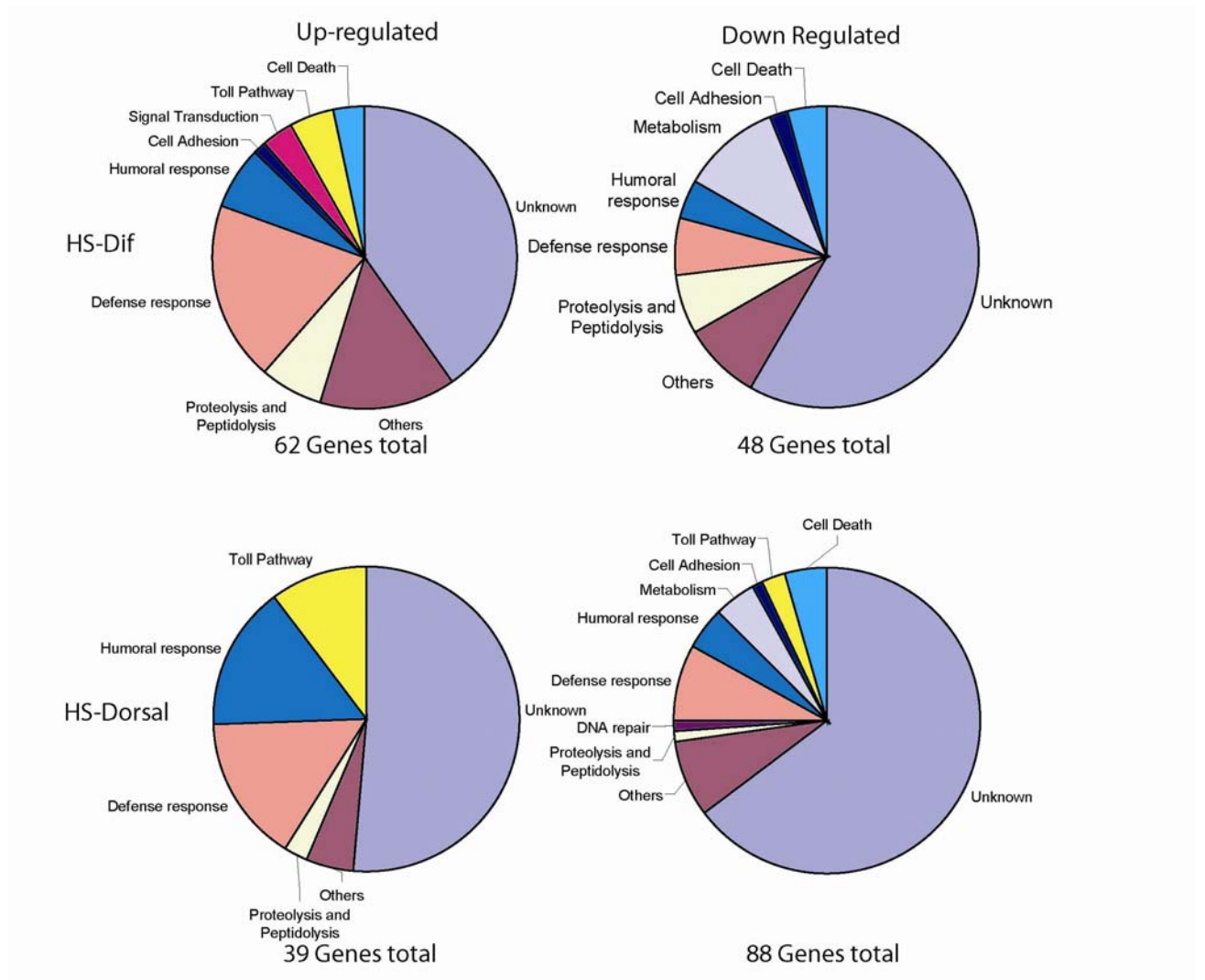
Figure 2-3: Functional composition of genes significantly affected upon ectopic expression of Dorsal and Dif based on Gene Ontology. Genes significantly up-regulated (left), and down-regulated (right), in HS-Dif (top) and HS-dorsal (bottom) larvae after exposure to heat shock.
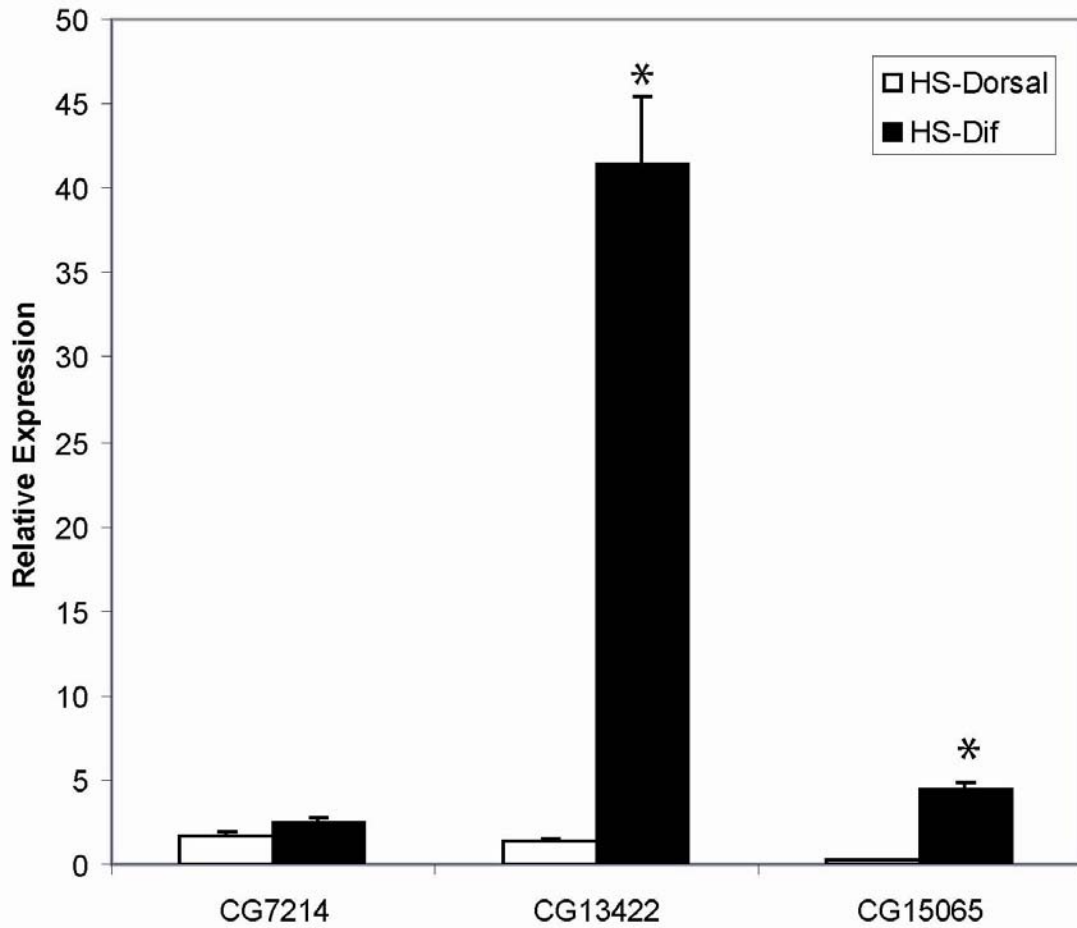
Figure 2-4: Q-PCR verification of expression of predicted target genes. Quantitative PCR showing CG7214, CG13422, and CG15065 transcript levels in HS-dorsal and HS-Dif larvae after exposure to heat shock. Error bars represent SD of at least three replicates and (*) denotes statistically significant induction with p-value<0.05 for two-tailed T-test.
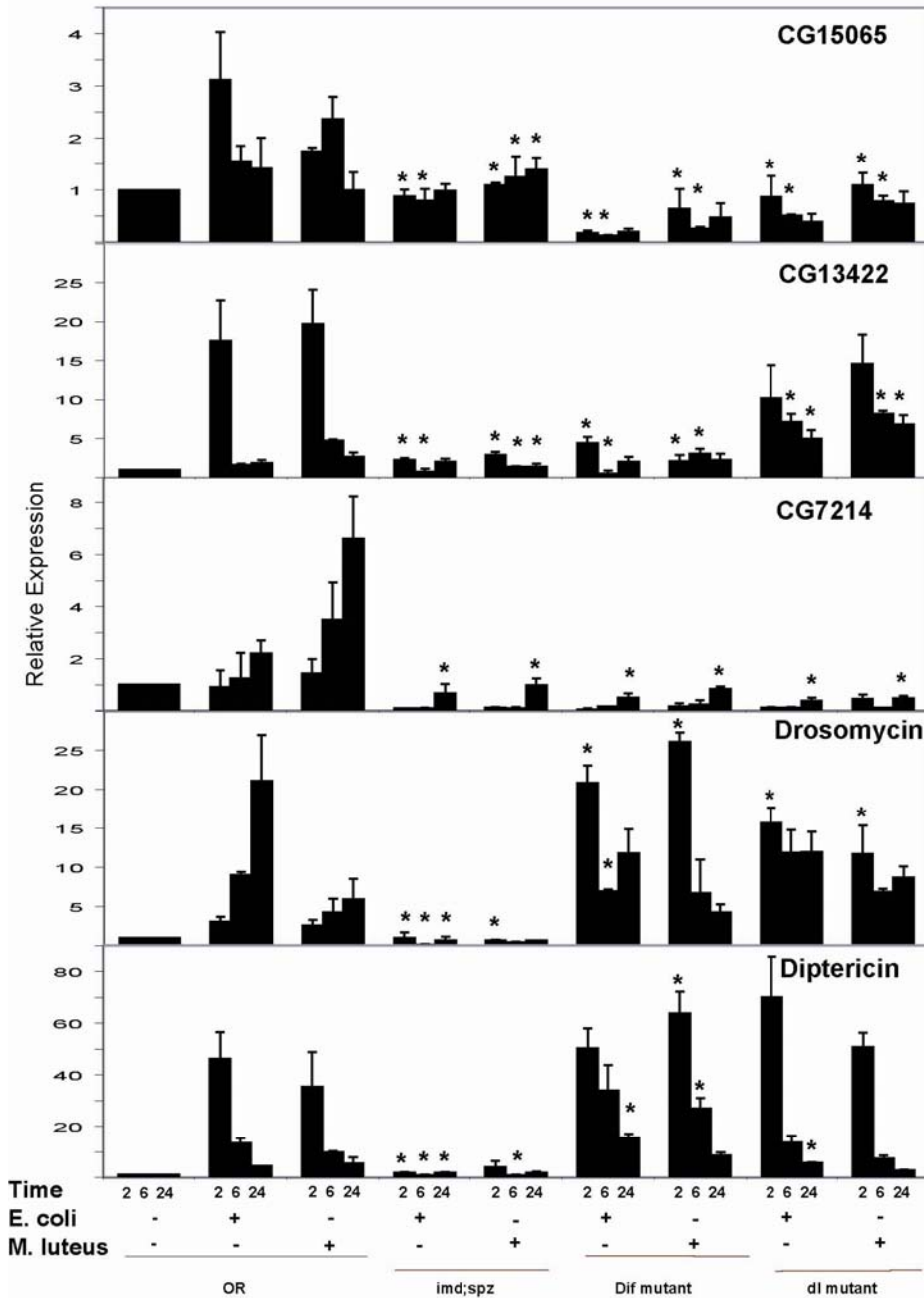
Figure 2-5: Expression of predicted target genes upon bacterial infection. Quantitative PCR measuring CG15065, CG13422, CG7214, Drosomycin, and Diptericin transcript levels in wildtype, *imd;spz*, *Dif[1]*, and *dorsal[1]* / Deficiency J4. Flies are injected with *E. coli* or *M. luteus*, and harvested at 2, 6, or 24 hours after injection to examine gene expression. (*) denotes significant difference in expression from OR flies with same infection and time point with a p-value<0.05 for two-tailed unpaired T-test.
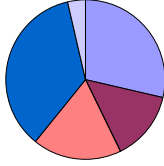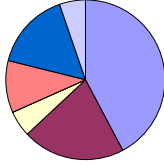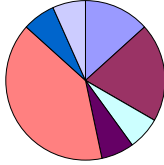
Figure 2-6: Conservation of putative NF-κB binding site upstream of CG15065 and CG13422 between related Drosophila species. The sites were identified using Target Explorer, 198 bp (CG15065) and 119 bp (CG13422) upstream of their respective start sites. Clustal alignments of available sequences from related species show conservation of the binding site.

	Gene	GO Category	Wt	Wl	Dl1	Dif1	RelE 20	HS-Dl	HS-Dif
Genes requiring Dorsal expression									
21 Genes Total	CG18239	Unknown: Inparanoid orthology to Dictyostelium discoideum Cyp-12Dl	2.04	11145	0.04	0.09	0.03	1.08	1.22
	IM1	Defense response	3.23	6993	0.07	0.04	0.31	0.89	1.02
	CG14419	Unknown: no matches	2.12	4529	0.13	0.29	0.22	1.04	0.63
	CG14481	Unknown: no matches	2.43	2901	0.13	0.62	0.43	0.40	0.29
	CR14499	Unknown: pseudogene no matches	1.74	908	0.15	0.45	0.15	0.92	0.98
	Acp1	Others: Adult cuticle protein	1.58	15977	0.17	0.44	0.42	0.83	0.92
	CG17104	Unknown: Tribolium castaneum similar to CG5343-PA, e=2.1	1.77	793	0.18	0.64	0.77	1.11	1.23
	IM23	Defense response	3.64	942	0.19	0.15	0.56	1.15	1.13
	Ccp84Ab	Others: larval cuticle	1.72	1184	0.20	1.35	0.72	1.02	1.02
	CG6429	Defense response	4.38	1779	0.21	0.77	0.37	0.74	1.42
	Faa	Metabolism: amino acid catabolism	1.57	1413	0.22	1.08	0.62	0.80	1.16
	CG7214	Unknown	1.45	9524	0.24	0.64	0.59	1.39	0.74
	CG14762	Others: Nerve impulse transmission	1.75	1105	0.26	0.43	0.12	1.02	1.00
	CG14850	Unknown: Drosophila melanogaster Adult cuticle protein 1 CG7216-RA (Acp1), e=4e-11	1.98	27734	0.32	0.12	0.76	1.09	1.16
Genes requiring Dif Expression									
26 Genes Total	CG17105	Unknown: no matches	1.44	11542	2.51	0.02	2.81	1.10	1.00
	CG13135	Unknown: no matches	2.99	8609	1.47	0.02	0.34	1.03	1.05
	IM1	Defense response	3.23	6993	0.07	0.04	0.31	0.89	1.02
	CG18067	Unknown: Rhizobium meliloti partial galactoglucan operon, strain EFB1, e=0.33	3.45	8295	1.09	0.08	0.94	0.72	1.25
	CG18239	Unknown: Inparanoid orthology to Dictyostelium discoideum Cyp-12Dl	2.04	11145	0.04	0.09	0.03	1.08	1.22
	CG14850	Unknown: Aspergillus nidulans FGSC A4 hypothetical protein, e=2.9	1.98	27734	0.32	0.12	0.76	1.09	1.16
	BcDNA:GH07626	Metabolism: fatty acid biosynthesis	1.75	3850	0.73	0.12	1.45	0.85	0.93
	CG13461	Unknown: no matches	1.64	3817	2.10	0.13	0.59	1.36	1.03
	Def	Humoral response	7.07	1644	1.02	0.14	0.04	1.08	0.95
	CG13422	Defense response	4.26	3048	2.20	0.14	0.23	1.03	1.43
	CG15065	Unknown: Drosophila melanogaster Immune induced molecule 1 CG18108-RA (IM1), e=2e-05	3.12	14514	1.74	0.14	0.64	0.94	1.35
	IM23	Defense response	3.64	942	0.19	0.15	0.56	1.15	1.13
	CG15067	Unknown: no matches	2.23	1420	1.20	0.16	0.20	0.51	0.98
	Cyp4p1	Others: Cytochrome electron transport	1.93	1026	1.60	0.17	1.10	0.96	0.78
	CG9733	Unknown: Manduca sexta mRNA for prophenoloxidase activating protease I ,e=1e-63	17.81	971	0.97	0.18	0.19	1.11	1.18

Legend (pie charts):
- Unknown
- Others
- Proteolysis and Peptidolysis
- Cell Proliferation
- DNA repair
- Defense response
- Humoral response
- Metabolism

Gene	Description							
CG6906	Others	1.77	3046	0.83	0.21	0.62	1.31	0.88
CG8087	Unknown: Burkholderia xenovorans LB400 chromosome 1, e=0.95	1.38	9746	1.25	0.24	0.40	0.88	0.88
CG14419	Unknown: no matches	2.12	4529	0.13	0.29	0.22	1.04	0.63
CecB	Humoral response	31.91	4981	1.33	0.29	0.08	0.70	0.81

Genes requiring Relish Expression

28 Genes Total

Gene	Description							
Diptericin	Humoral response	33.85	12251	1.70	1.39	0.01	1.00	0.91
CecA2	Humoral Response	55.02	4284	2.37	0.61	0.02	1.11	1.16
CG18239	Unknown: Inparanoid orthology to Dictyostelium discoideum Cyp-12Dl	2.04	11145	0.04	0.09	0.03	1.08	1.22
Def	Humoral response	7.07	1644	1.02	0.14	0.04	1.08	0.95
CecC	Humoral response	11.62	3822	1.55	0.60	0.04	0.90	0.69
DptB	Humoral response	7.07	12703	1.90	1.58	0.06	1.44	0.98
Rel	Defense response	5.78	4654	1.15	1.02	0.06	1.09	0.96
AttD	Humoral response	2.62	2517	1.11	0.99	0.07	0.61	0.40
CecB	Humoral response	31.91	4981	1.33	0.29	0.08	0.70	0.81
CecA1	Humoral response	15.93	9810	2.80	0.61	0.08	1.02	0.99
CG14762	Others: Cell adhesion	1.75	1105	0.26	0.43	0.12	1.02	1.00
CR14499	Unknown: pseudogene no matches	1.74	908	0.15	0.45	0.15	0.92	0.98
Drosomycin	Humoral response	8.97	8581	2.06	0.49	0.15	1.11	1.73
Hsp70 BC	Defense response	5.92	854	1.19	7.90	0.18	1.41	1.04
CG9733	Defense response	17.81	971	0.97	0.18	0.19	1.11	1.18
CG15067	Unknown: no matches	2.23	1420	1.20	0.16	0.20	0.51	0.98
CG9080	Unknown: no matches	3.05	11125	3.36	2.03	0.22	1.86	1.21
CG14419	Unknown: no matches	2.12	4529	0.13	0.29	0.22	1.04	0.63
CG13422	Defense response	4.26	3048	2.20	0.14	0.23	1.03	1.43
PGRP-SA	Defense response	2.69	9727	0.63	0.67	0.27	3.25	1.00

Genes upregulated in Dorsal mutants

19 Genes Total

Gene	Description							
Cyp4e3	Others: Electron transport	1.49	1855	10.14	0.62	1.81	1.09	0.82
PGRP-SC2	Defense response	1.37	6088	4.31	3.01	5.21	0.99	1.08
Hsp23	Defense Response	1.94	2226	3.77	6.00	1.12	1.03	1.43
CG9080	Unknown: no matches	3.05	11125	3.36	2.03	0.22	1.86	1.21
Attacin-C	Humoral response	18.72	7473	3.32	2.10	0.35	1.87	1.56
Mtk	Humoral response	7.99	8593	3.07	1.50	0.41	1.63	0.89
CecA1	Humoral response	15.93	9810	2.80	0.61	0.08	1.02	0.99

Genes upregulated in Dif mutants

16 Genes Total

Gene	Description							
Hsp70 BC	Defense response	5.92	854	1.19	7.90	0.18	1.41	1.04
Hsp23	Defense response	1.94	2226	3.77	6.00	1.12	1.03	1.43
CG12505	Unknown: no matches	1.63	8898	1.24	3.36	0.44	0.90	1.13
GstD2	Defense response	1.78	9626	0.59	3.31	1.64	1.81	1.16
CG5550	Defense response	2.96	3754	1.92	3.05	0.85	1.00	0.00
PGRP-SC2	Defense response	1.37	6088	4.31	3.01	5.21	0.99	1.08

Genes upregulated in Relish mutants
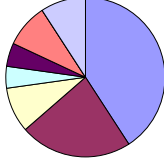
	Gene	GO Category	Wt	WI	Dorsal	Dif	Relish		
	PGRP-SC2	Defense response	1.37	6088	4.31	3.01	5.21	0.99	1.08
	CG17105	Unknown: no matches	1.44	11542	2.51	0.02	2.81	1.10	1.00
	InR	Others: Insulin Receptor	3.16	310	1.87	2.29	2.58	1.02	0.90
	CG13686	Others: Spermatogenesis	2.28	59	2.29	1.47	2.30	1.15	1.40
22 Genes total	CG13905	Unknown: no matches	3.10	3017	2.12	1.91	2.28	1.20	1.08

Table 2-1: Selected list of genes regulated by Dorsal, Dif, and Relish. 188 Genes were significantly induced in wildtype larvae upon *E. coli* infection. Among them, genes which which were significantly affected in specific Rel mutants are listed. Genes affected by a specific Rel mutation are grouped together, and pie charts (Column 1) represent the relative functional compositions of these genes based on available Gene Ontology information. (Columns 2-5) Gene, wildtype induction (Wt), and wildtype average intensity (WI) are provided. GO Categories best fitting the gene is provided, as categorized in column 1. Thus if a gene is classified as "Defense Response" in the pie charts of column 1, it is described in the same way in column 2. "Others" represents genes for which GO information is available but they represent a too small a percentage of genes to be given a separate classification in the charts in Column 1. Their GO function is listed where available. If the gene has not been classified based on GO categories yet, its best match obtained through protein blast searches of all available genomes is given along with the expectation (e-value) of the match. (Columns 6-8) Ratios of mutant final expression intensity divided by wildtype intensity are presented as ratios for Dorsal, Dif, and Relish mutant lines. If the ratio is more than one standard deviation below the mean ratio for all genes in the category, it is colored Green to indicate significant reduction. Red denotes significant expression, with ratios over 1 SD above the wildtype. (Columns 8-9) The fold change of genes when Dorsal or Dif are ectopically expressed. The values are colored Green if they represent a significant reduction and Red if there is significant induction.

Genes with lower expression in HS-Dif

Name	HS-Dif	HS-Dorsal
CG14481	0.286059	0.402039
CG7629	0.397915	0.607683
CG9928	0.434766	0.95731
(JAK)HOP	0.437707	0.905984
Mdr49	0.460794	0.606285
CG10872	0.527395	0.705642
CG13095	0.572535	1.080685
CG11459	0.59486	0.590664
CG6675	0.600114	0.660407
CG15404	0.604703	0.578759
CG9334	0.611252	0.982368
CG7294	0.61138	0.717341
CG7722	0.616609	1.063651
CG6468	0.626712	0.727038
CG14419	0.634058	1.040995
Lectin-galC1	0.637823	1.089405
Ice	0.638166	0.483647
SRP	0.641986	0.781718
CG6277	0.651589	0.586988
Jon25Biii	0.670554	1.318459
CecC	0.691755	0.899893
CG1241	0.695409	0.629609
CG15493	0.695754	1.036747
CG13324	0.710512	0.63989
CG13218	0.715583	0.968552
ninA	0.717585	1.170885
plx	0.729191	1.078995
CG16756	0.734394	0.750848
CG13323	0.73469	0.727035
BSK	0.735457	0.882481
GNBP1	0.736255	1.014735
CG7422	0.737571	1.179135
CG7214	0.738001	1.394506
LAR	0.739731	1.087688
CG4428	0.756158	0.700501
CG16794	0.76614	0.814348
Lip1	0.77491	1.054933
twi	0.775216	0.918839
CG13805	0.775511	0.624995
Uro	0.776039	0.963502
CG10337	0.780433	1.039203
PMKK4	0.781839	0.634444
Cyp4p1	0.783702	0.962406
CG5429	0.785465	0.878527
CG6289--GH21475	0.786336	0.876944

Genes with higher expression in HS-Dif

Name	HS-Dif	HS-Dorsal
CYNS	1.27046	1.396898
CG13422	1.27254	0.836798
GNBP3	1.273326	0.892613
lectin-28C	1.275161	0.952517
CG16713	1.277481	1.151837
CG12965	1.280764	1.03453
crq	1.282127	1.238859
CG3212	1.28538	0.62
CG6194	1.28607	0.910396
CG16832	1.290911	0.684378
CG7695	1.291336	0.906561
CG7219	1.29162	1.107995
chn	1.294407	0.766271
CG6639	1.2947	0.863098
aay	1.298154	0.986588
GNBP2	1.303212	0.981341
CG12821	1.315124	0.998285
CG6361	1.327116	0.840081
CG4823	1.327798	0.747894
CG11100	1.330227	0.887766
gcm2	1.341502	0.731
CG8492	1.344502	1.031992
CG15065	1.348298	0.943599
BCDNA	1.351541	0.977088
CG5514	1.35233	1.178114
GRIM	1.354482	1.019885
PGRP-LE	1.354704	1.09489
Dif	1.355604	1.388225
CG1865	1.366485	0.955415
CG11842	1.371405	0.862376
CG6687	1.371647	0.84021
CG4757	1.379807	0.692276
lectin-21Cb	1.397192	1.148401
CG2217	1.403112	0.805409
CG2056	1.405747	1.019117
CG15068	1.407527	0.736358
CG6524	1.409895	1.134934
Thor	1.410986	1.239137
CG6429	1.421062	0.739947
Hsp23	1.425436	1.026728
CG13422	1.427161	1.033454
rhea	1.434684	0.800928
ATTA	1.438773	1.458796
CG9631	1.446057	1.066207
CG7219	1.448544	0.648131
IM3	1.486066	0.754915
CG15306	1.520883	0.869433
Hsp68	1.527147	0.796453

Name	HS-Dif	HS-Dorsal
Zip3	1.545325	1.056589
AttC	1.56219	1.874335
CG1021	1.566074	0.982577
star1	1.591747	0.939132
Drs	1.611614	1.065313
CG2839	1.612283	1.07934
CG8137	1.674576	0.78915
PGRP-SD	1.693568	0.958849
CG8678	1.717122	1.386712
Drs	1.725372	1.107277
nec	2.055722	1.004523
CG9877	2.203088	3.178848

Genes with lower expression in HS-Dorsal

Name	HS-Dif	HS-Dorsal
CG14481	0.286059	0.402039
TepI	0.94383	0.473189
CG12334	0.841468	0.475395
Ice	0.638166	0.483647
CG15067	0.984503	0.505878
SCA	0.863379	0.540373
CG14852	0.802617	0.55898
CG6680	1.016704	0.57103
CG15404	0.604703	0.578759
dream	1.031186	0.578957
CG6277	0.651589	0.586988
CG11459	0.59486	0.590664
CG4115	0.890443	0.602831
Mdr49	0.460794	0.606285
CG7629	0.397915	0.607683
CG3212	1.28538	0.62
CG13805	0.775511	0.624995
CG1241	0.695409	0.629609
PMKK4	0.781839	0.634444
CG13324	0.710512	0.63989
CG6271	0.912741	0.642271
CG7219	1.448544	0.648131
alpha actinin	1.173221	0.651246
CG8866	1.006474	0.652695
CG6675	0.600114	0.660407
SR-C1	1.065039	0.665095
CG15046	1.064429	0.671121
PUCKERED	1.047413	0.671141
CG13245	1.122173	0.671751
CG3348	0.965489	0.675953
Ca1.1	0.993903	0.682023
CG12780	1.047858	0.684177
CG16832	1.290911	0.684378
lectin-46Ca	1.12003	0.685455
ndl	1.138867	0.689091
CG4757	1.379807	0.692276
CG4428	0.756158	0.700501
CecB	0.810259	0.702424
CG10872	0.527395	0.705642
CG11819	1.137447	0.708067
Toll-9	1.163456	0.715
DIM2A	1.048244	0.71734
CG7294	0.61138	0.717341
CG1342	0.789141	0.721891
CG18067	1.25105	0.723247
CG13323	0.73469	0.727035
CG6468	0.626712	0.727038
gcm2	1.341502	0.731
Rep4	0.907661	0.732954
CG14567	0.901388	0.733388
CG15098	0.880895	0.733916
CG15068	1.407527	0.736358
GNBP3	1.032586	0.736714
CG12789	1.152691	0.737936
CG10035	1.244915	0.738455
TEPII	1.149833	0.738696
yellow-f	0.842223	0.739125
CG6429	1.421062	0.739947
RHEA	0.925332	0.744817
CG4823	1.327798	0.747894
Toll-6	0.882194	0.750459
CG16756	0.734394	0.750848
CG15293	0.978405	0.751216
CG16844	1.486066	0.754915
GM04155	1.053699	0.759048
CG11798	1.294407	0.766271
CG14230	0.920229	0.770319

Genes with higher expression in HS-Dorsal

Name	HS-Dif	HS-Dorsal
CG6906	0.879874	1.314529
Jon25Biii	0.670554	1.318459
TEPI	1.048554	1.323929
PGRP-LC	1.019833	1.331207
CG2444	1.203586	1.339855
Def	0.979814	1.351746
CG13461	1.027122	1.356343
WNT8C	1.173612	1.370727
dl	1.016603	1.374146
CG8678	1.717122	1.386712
DIF	1.355604	1.388225
CG7214	0.738001	1.394506
PGRP-SB1	1.178597	1.396581
PGRP-SB1	1.21186	1.396761
CYNS	1.27046	1.396898

CG6489	1.036286	1.406213	PGRP-SC1b	1.115278	1.53241
CG9568	1.02911	1.425891	IM10	1.22334	1.569477
DIPTERICIN	0.975042	1.436655	GATAe	1.181696	1.579864
CG15126	1.030181	1.43698	dl	0.90312	1.59105
CACTUS AK	1.158576	1.454381	Mtk	0.885591	1.625062
AttB	1.180562	1.45459	lectin-29Ca	1.119684	1.77547
AttA	1.438773	1.458796	GstD2	1.161475	1.814964
CG15282	0.840936	1.469411	CG9080	1.21223	1.857088
CG17738	0.871685	1.479298	AttC	1.56219	1.874335
PGRP-LB	1.264347	1.485897	CG9877	2.203088	3.178848
PELLE	0.894506	1.52868	PGRP-SA	0.99736	3.250137

Table 2-2: Supplementary data of gene expression in HS-Dorsal and HS-Dif larvae
Significant gene expression changes occurring in HS-Dorsal and HS-Dif lines obtained using cDNA microarrays. Genes are grouped based on those expressing significant higher or lower upon ectopic induction of Dorsal and Dif.

Gene Name	CG Number	Wildtype Intensity	Wildtype Induction	Ratio induced in Dorsal mutant	Ratio induced in Dif Mutant	Ratio induced in Relish mutant
CecA2	CG1367	4284.2	55.0	2.4	0.6	0.0
Dpt	CG12763	12251.3	33.8	1.7	1.4	0.0
CecB	CG1878	4981.1	31.9	1.3	0.3	0.1
CG4740	CG4740	7473.4	18.7	3.3	2.1	0.3
CG9733	CG9733	971.3	17.8	1.0	0.2	0.2
CecA1	CG1365	9810.4	15.9	2.8	0.6	0.1
CecC	CG1373	3822.0	11.6	1.5	0.6	0.0
CG14567	CG14567	9224.5	10.7	0.7	1.1	0.5
CG14934	CG14934	184.3	10.4	0.4	0.3	3.0
Dro	CG10816	8581.9	9.0	2.1	0.5	0.2
CG8348	CG8348	220.8	8.8	2.4	1.9	2.0
AttA	CG10146	20830.0	8.8	2.1	2.1	0.7
RSG7	CG9108	198.6	8.3	0.7	0.3	0.3
Mtk	CG8175	8593.6	8.0	3.1	1.5	0.4
DptB	CG10794	12703.2	7.1	1.9	1.6	0.1
Def	CG1385	1644.9	7.1	1.0	0.1	0.0
b	CG7811	461.9	6.9	2.1	1.6	0.3
RpS3	CG6779	30460.5	6.2	2.0	2.7	2.3
alpha-Est9	CG1128	606.3	6.1	1.2	1.0	0.3
CG6489	CG6489	854.2	5.9	1.2	7.9	0.2
Rel	CG11992	4654.1	5.8	1.1	1.0	0.1
CG18672	CG18672	198.1	5.5	0.9	1.8	1.0
CG11945	CG11945	154.8	5.5	1.5	1.4	0.9
Syx13	CG11278	751.6	5.3	1.4	2.0	1.1
CG7502	CG7502	575.5	5.1	1.1	1.3	0.9
betaTub60D	CG3401	238.5	4.8	2.5	1.2	0.7
CG15248	CG15248	466.1	4.8	1.9	0.9	1.3
CG6390	CG6390	596.3	4.7	1.7	1.3	0.6
CG6429	CG6429	1779.2	4.4	0.2	0.8	0.4
CG12054	CG12054	271.7	4.3	1.7	1.2	2.2
CG13422	CG13422	3048.9	4.3	2.2	0.1	0.2
yellow-c	CG4182	3430.2	3.9	0.9	1.0	0.7
dm	CG10798	205.5	3.9	2.5	1.6	1.6
CG11765	CG11765	10885.9	3.7	1.9	1.5	1.2
CG14895	CG14895	412.9	3.7	2.7	3.6	1.8
CG15066	CG15066	942.1	3.6	0.2	0.1	0.6
CG7219	CG7219	3450.7	3.6	1.4	0.3	0.7
CG2121	CG2121	1097.1	3.5	5.1	3.1	1.3
CG18067	CG18067	8295.5	3.4	1.1	0.1	0.9

g	CG13868	8385.9	3.4	1.4	0.7	1.8
Hmgcr	CG10367	330.8	3.3	1.9	0.9	1.2
IM1	CG18108	6993.0	3.2	0.1	0.0	0.3
CG5834	CG5834	581.6	3.2	1.4	2.4	0.3
InR	CG18402	310.2	3.2	1.9	2.3	2.6
CG15065	CG15065	14514.7	3.1	1.7	0.1	0.6
CG11806	CG11806	197.7	3.1	1.6	1.9	1.8
CG13905	CG13905	3017.2	3.1	2.1	1.9	2.3
CG9080	CG9080	11125.2	3.1	3.4	2.0	0.2
IM2	CG18106	1963.3	3.0	2.0	0.4	0.4
CG13135	CG13135	8609.4	3.0	1.5	0.0	0.3
Oli	CG5545	173.8	3.0	1.5	0.2	0.9
CG5550	CG5550	3754.1	3.0	1.9	3.1	0.8
CG18342	CG18342	4485.6	2.8	2.0	1.5	1.0
CG11709	CG11709	9727.2	2.7	0.6	0.7	0.3
sop	CG5920	29817.3	2.7	1.6	2.3	1.7
yellow-b	CG17914	2016.7	2.6	1.9	0.7	1.3
tamo	CG4057	403.8	2.6	1.4	1.6	1.3
AttD	CG7629	2517.4	2.6	1.1	1.0	0.1
TepII	CG7052	1402.8	2.6	1.2	1.1	0.6
aay	CG3705	3847.2	2.5	2.1	0.5	1.0
CG16844	CG16844	26823.1	2.5	1.5	0.6	0.7
CG14481	CG14481	2901.7	2.4	0.1	0.6	0.4
PGRP-LF	CG4437	438.6	2.4	1.7	1.6	0.9
CG13680	CG13680	215.8	2.4	1.1	1.5	1.3
CG13686	CG13686	59.1	2.3	2.3	1.5	2.3
CG18279	CG18279	7837.0	2.3	1.7	0.6	0.4
CG15067	CG15067	1420.7	2.2	1.2	0.2	0.2
ird7,PGRP-LC	CG4432	278.9	2.2	1.9	0.6	0.3
enb	CG15112	233.6	2.2	1.1	2.2	2.0
CG2444	CG2444	7889.2	2.2	1.3	0.3	0.4
CG5246	CG5246	1880.4	2.2	2.3	0.8	1.1
TepII	CG18589	5174.5	2.2			
CG4096	CG4096	431.6	2.1	1.3	1.6	1.5
CG14419	CG14419	4529.7	2.1	0.1	0.3	0.2
CG18239	CG18239	11145.4	2.0	0.0	0.1	0.0
CG4618	CG4618	1475.5	2.0	1.2	1.3	0.9
Galpha73B	CG12232	461.9	2.0	1.9	0.7	1.2
Sur-8	CG5407	367.4	2.0	2.1	2.4	1.4
Beach1	CG9011	242.3	2.0	1.6	2.3	1.6
CG6142	CG6142	1656.9	2.0	0.9	0.7	0.1
serpin-27A	CG11331	1530.2	2.0	1.0	1.2	1.0
CG14439	CG14439	3456.2	2.0	1.1	1.1	0.7

CG14850	CG14850	27734.7	2.0	0.3	0.1	0.8
CG2471	CG2471	3381.5	2.0	2.9	0.8	1.3
CG15745	CG15745	320.0	1.9	2.9	1.7	3.4
Hsp23	CG4463	2226.0	1.9	3.8	6.0	1.1
Cyp4p1	CG10842	1026.4	1.9	1.6	0.2	1.1
CG17738	CG17738	36052.3	1.9	1.2	1.4	0.8
CG5322	CG5322	242.5	1.9	2.1	2.1	1.4
CG9584	CG9584	495.3	1.8	1.3	1.8	1.2
CG11819	CG11819	1141.9	1.8	1.2	0.9	1.0
Gld	CG1152	1473.5	1.8	1.6	2.3	0.9
CG14205	CG14205	1142.7	1.8	3.4	2.1	2.8
CG13324	CG13324	12506.1	1.8	0.9	1.0	0.9
syx1A	CG5448	942.8	1.8	1.4	1.9	2.3
CG9759	CG9759	840.2	1.8	0.8	1.3	0.9
CG4181	CG4181	9626.4	1.8	0.6	3.3	1.6
CG6906	CG6906	3046.5	1.8	0.8	0.2	0.6
CG11073	CG11073	2337.9	1.8	1.2	1.3	0.8
CG17104	CG17104	793.2	1.8	0.2	0.6	0.8
CG13321	CG13321	10612.9	1.8	0.9	2.4	1.4
CG4427	CG4427	945.6	1.8	1.3	0.9	1.5
CG14762	CG14762	1105.1	1.8	0.3	0.4	0.1
CG8547	CG8547	2065.7	1.8	1.3	1.8	0.9
BcDNA:GH07626	CG3523	3850.2	1.7	0.7	0.1	1.4
CG14499	CG14499	908.2	1.7	0.1	0.5	0.2
CG5810	CG5810	312.2	1.7	2.1	3.3	2.4
Ccp84Ab	CG1252	1184.8	1.7	0.2	1.4	0.7
kel	CG7210	2427.9	1.7	1.2	1.0	0.9
sls	CG18242	1360.9	1.7	0.9	1.2	0.8
CG9877	CG9877	46007.9	1.7	1.5	2.0	1.0
CG18107	CG18107	177.8	1.7	3.0	1.1	2.0
CG18153	CG18153	471.4	1.7	1.0	0.9	0.3
CG15678	CG15678	463.8	1.7	2.2	1.7	0.7
CG5391	CG5391	3243.5	1.7	1.8	0.3	0.9
CG17107	CG17107	41266.1	1.7	1.4	1.9	1.6
CG9568	CG9568	20507.4	1.7	2.0	1.7	1.4
Drs	CG10810	16618.4	1.7	1.1	1.3	0.8
Ag5r	CG9538	7782.7	1.7	2.7	0.5	1.1
Or92a	CG17916	586.7	1.7	0.5	0.6	0.0
Picot	CG8098	3626.4	1.7	2.6	1.2	1.3
CG4392	CG4392	2763.7	1.6	1.0	0.9	1.5
CG8629	CG8629	38806.0	1.6	0.5	0.5	0.9
CG6687	CG6687	3780.0	1.6	1.1	0.9	1.2

CG8129	CG8129	2272.7	1.6	1.0	1.1	1.1
CG13461	CG13461	3817.3	1.6	2.1	0.1	0.6
CG12505	CG12505	8898.3	1.6	1.2	3.4	0.4
CG6263	CG6263	1022.3	1.6	1.4	1.0	1.6
CG16750	CG16750	1379.4	1.6	1.7	1.7	1.5
IM4	CG15231	11800.3	1.6	1.9	0.9	1.2
CG13323	CG13323	25721.3	1.6	1.3	1.1	1.3
CG2226	CG2226	417.2	1.6	1.0	2.0	1.1
Talin	CG6831	1119.7	1.6	1.3	1.0	1.1
CG3884	CG3884	2072.6	1.6	1.1	2.2	1.4
Acp1	CG7216	15977.2	1.6	0.2	0.4	0.4
sym=CG17090	CG17090	1084.1	1.6	1.5	1.3	2.3
Faa	CG14993	1413.1	1.6	0.2	1.1	0.6
CG17839	CG17839	1133.1	1.6	2.1	1.5	0.9
CG10924	CG10924	1756.3	1.6	3.9	2.8	2.5
CG9336	CG9336	8882.9	1.6	1.3	0.9	0.8
MESR3	CG15162	657.8	1.5	2.1	1.9	1.8
CG6183	CG6183	3657.0	1.5	2.9	2.0	3.1
CG17524	CG17524	19110.2	1.5	1.3	1.9	1.0
CG15784	CG15784	17072.1	1.5	1.2	1.6	1.5
CG14852	CG14852	12567.1	1.5	2.2	0.7	1.1
BG:DS01759.2	CG15293	6221.7	1.5	0.6	0.4	0.5
CG13482	CG13482	16388.1	1.5	2.3	1.6	1.0
CG3541	CG3541	4450.6	1.5	1.1	0.9	0.8
Tequila	CG18403	2450.3	1.5	0.6	0.9	0.5
CG5530	CG5530	389.8	1.5	1.1	1.4	1.1
CG13846	CG13846	386.1	1.5	1.5	1.5	0.9
CG11796	CG11796	3468.8	1.5	0.6	2.2	0.5
Cyp4e3	CG4105	1855.5	1.5	10.1	0.6	1.8
rho-4	CG1697	580.0	1.5	1.7	1.9	1.7
CG14963	CG14963	1833.3	1.4	3.6	0.8	1.4
CG7214	CG7214	9524.8	1.4	0.2	0.6	0.6
CG17105	CG17105	11542.1	1.4	2.5	0.0	2.8
CG14608	CG14608	740.7	1.4	1.7	1.8	1.0
bun	CG5461	1182.0	1.4	1.5	1.4	1.7
CG13335	CG13335	1152.4	1.4	2.0	2.1	1.0
Men	CG10120	3827.8	1.4	1.4	1.1	1.8
Nrg	CG1634	1993.0	1.4	1.0	1.4	1.5
CG18649	CG18649	3111.9	1.4	1.3	0.5	1.0
CG8502	CG8502	13333.1	1.4	2.3	1.1	0.9
Reg-5	CG2928	4937.1	1.4	1.0	0.6	0.8
BG:DS07486.3	CG4793	407.9	1.4	2.2	0.9	1.0
CG12074	CG12074	4096.2	1.4	1.2	1.9	0.9

CG8087	CG8087	9746.4	1.4	1.2	0.2	0.4
Paip2	CG12358	4941.1	1.4	0.5	1.1	0.3
CG17362	CG17362	14798.8	1.4	1.3	0.2	0.8
PGRP-SC2	CG14745	6088.4	1.4	4.3	3.0	5.2
sog	CG9224	279.6	1.4	1.5	1.8	1.5
CG13805	CG13805	11957.1	1.4	1.6	2.0	1.9
CG9090	CG9090	12226.6	1.4	0.6	0.9	0.8
Amy-d	CG17876	14899.3	1.3	2.3	2.6	1.8
CG7017	CG7017	13464.5	1.3	1.0	0.7	0.8
CG15309	CG15309	1251.1	1.3	1.8	1.0	1.8
bent	CG10285	762.6	1.3	0.6	1.4	1.7
CG10332	CG10332	458.5	1.3	1.0	1.9	1.0
CG1534	CG1534	15440.5	1.3	1.4	1.3	1.2
shot	CG18076	1593.1	1.3	1.3	0.7	1.1
CG3672	CG3672	5263.6	1.3	3.4	1.9	2.4
CG12969	CG12969	3788.6	1.3			
CG10725	CG10725	15989.7	1.3	0.8	0.6	1.4
CG15762	CG15762	221.9	1.3	1.6	1.0	0.9
BG:DS00941.14	CG7953	25496.3	1.3	1.7	0.6	1.4
CG18282	CG18282	18970.7	1.3	1.9	2.2	2.1
CG11912	CG11912	7730.0	-1.9	3.2	2.2	2.2
CG5656	CG5656	1035.7	-2.3	2.8	0.9	1.1
CG4471	CG4471	899.3	-4.0	4.0	1.6	2.0

Table 2-3 Supplementary Table showing affect of Rel mutant on gene expression as a ratio of wildtype expression. The gene name, CG Number, Wildtype final intensity, wildtype induction fold change is given. A ratio comparing the final wildtype induction level to that observed in Dorsal, Dif, and Relish mutants is also shown. A green color is used to denote a significant lowering of expression compared to wildtype induction, while red is used to denote a higher expression. These Affymetrix chip experiments were performed by Junlin Wu and the initial statistical analysis was done by Jun Li.

Chapter 3: Fungal peptide Destruxin A plays a specific role in suppressing the innate immune response in Drosophila melanogaster

Abstract

Destruxins are a class of cyclic depsipeptides produced by various fungi including the entomopathogens *Metarhizium anisopliae* and *Aschersonia alyrodis*. These peptides are produced during infection of an insect host and cause muscular paralysis and death. Though a direct relationship has been established between Destruxin production and fungal virulence, their *in vivo* mode of action during pathogenesis remains largely uncharacterized. To explore these effects, we looked at changes in gene expression following injection of Destruxin A into the fruit fly *Drosophila melanogaster*. Microarray results revealed reduced expression of various antimicrobial peptides (AMPs) that play a major role in the fly's humoral immune response. Flies co-injected with Destruxin A and the Gram-negative bacteria *E.coli*, showed increased mortality and an accompanying increase in bacterial titers. This mortality was rescued through ectopic activation of components in the fly's signal transduction pathways that are responsible for AMP induction. Together, these results suggest a novel role for Destruxin A in specific suppression of the humoral immune response in insects.

3.1 Introduction

Insects are the most diverse and prolific land animals and a variety of pathogens have specialized to infect them. Unlike bacteria or viruses that usually need to be ingested, certain fungal species can directly breach the insect cuticle to cause disease. Fungi are the most commonly observed insect pathogens in nature, causing the largest percentage of deaths due to infection. As a result, methods of controlling insect populations using live fungal insecticides have attracted medical and agronomical interest (Blanford et al., 2005; Wright et al., 2005). The ascomycete *Metarhizium anisopliae* is already in commercial use to control termites, grasshoppers, and thrips (Hunter et al., 2001; Maniania et al., 2002; Wright et al., 2005). In some fungi, success in infecting a wide variety of insects can at least in part be attributed to secretion of virulence factors during pathogenesis. Destruxins were initially identified as toxic compounds secreted by *Metarhizium* and were later characterized as important virulence factors accelerating the deaths of infected insects (Dumas et al., 1994; Fargues et al., 1985; Kershaw et al., 1999; Kodaira, 1961).

Chemically, Destruxins are cyclic hexadepsipeptides composed of an alpha-hydroxy acid and five amino acid residues. Five natural analogues (labeled A-E) have been isolated (Kodaira, 1961; Païs et al., 1981; Suzuki et al., 1970). These forms differ in the R-group of the hydroxyl acid residue and appear to have overlapping but different biological effects. Primarily however, injection, ingestion, or topical application of a Destruxin on insects causes tetanic paralysis (Samuels et al., 1988). Destruxin-induced membrane depolarization due to the opening of Ca^{2+} channels has been

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implicated as a cause of paralysis and death (Samuels et al., 1988). Destruxin also causes signaling changes, through the phosphorylative activation of certain proteins in lepidopteran and human cell lines. In addition, Destruxins cause morphological and cytoskeletal changes in insect plasmatocytes *in vitro*, and this adversely affects insect cellular immune responses such as encapsulation and phagocytosis (Vey et al., 2002; Vilcinskas et al., 1997a; Vilcinskas et al., 1997b). These could be indirect results of a calcium influx (Dumas et al., 1996). Destruxins also show biological activities against non-insects. They are particularly toxic to mammalian leukemia cells and spleen lymphocytes, and have demonstrated anti-proliferative activity on mouse neoplasms *in vitro* (Pedras et al., 2002). Destruxins A, B, and E have also been shown to have antiviral properties in insect and human cell lines (Huxham et al., 1989; Quiot et al., 1980; Vey et al., 1985). For example, Destruxin B has demonstrated a suppressive effect on Hepatitis B surface antigen expression (Chen et al., 1997; Yeh et al., 1996).

Aside from their ability to open calcium channels, the mechanisms by which Destruxins achieve their varied biological activities have not been studied *in vivo*. Therefore, we used *Drosophila melanogaster* as an insect model to characterize the range of functions affected by Destruxins. Among insects *Drosophila* has the best characterized immune response and because of similarities in key signaling pathways has been an invaluable model for understanding innate immunity in humans. Flies have three active innate immune mechanisms for dealing with an invading microorganism. Proteolytic cascades triggered by microbial determinants lead to the formation of melanotic clots at the site of infection (Soderhall and Cerenius, 1998).

The toxic melanin along with encapsulating lamellocytes that circulate in the *Drosophila* hemolymph, can neutralize many foreign microorganisms (Braun et al., 1998; Rizki and Rizki, 1984). Other hemocytes actively phagocytose invading pathogens (Elrod-Erickson et al., 2000; Lanot et al., 2001). In addition, pathogenic determinants activate Drosophila Pattern Recognition Receptors (PRRs) which initiate signal transduction cascades that trigger a humoral immune response. This response is marked by the production of antimicrobial peptides (AMPs) that have activity against the invading pathogen. Two key pathways, named Toll and IMD, have been identified that mediate AMP expression (De Gregorio et al., 2002; Hoffmann, 2003; Lemaitre et al., 1996). The former involves the activation of the Toll receptor due to detection of primarily fungal or Gram-positive bacterial determinants by upstream PRRs (Gobert et al., 2003; Levashina et al., 1999; Ligoxygakis et al., 2002b). The activation of the Toll pathway leads to the phosphorylation of several adaptor proteins that culminates in the phosphorylation of Cactus, an IκB homologue in Drosophila (Belvin et al., 1995; Bergmann et al., 1996; Reach et al., 1996). This leads to degradation of Cactus, freeing the NF-κB proteins Dorsal and Dif to translocate to the nucleus. There they activate transcription of a variety of genes important for the immune response, including the antimicrobial peptide Drosomycin (Lemaitre et al., 1995a; Meng et al., 1999). In the IMD pathway, activation of the receptor Peptidoglycan Recognition Protein LC (PGRP-LC) by Gram-negative bacterial peptidoglycan (Choe et al., 2002; Kaneko et al., 2004), leads to the phosphorylation of the adaptor IMD (Georgel et al., 2001) and subunits of Drosophila IKK (DmIKKβ and Kenny/IKKγ) (Rutschmann et al., 2000; Silverman et

al., 2000), and finally the cleavage of the NF-κB like protein Relish. Relish is responsible for the transcription of many proteins important for the immune response, including the antimicrobial peptide Diptericin (Lemaitre et al., 1995a; Meister et al., 1997). Drosomycin and Diptericin are often used as target genes to assay for the activation of the Toll and IMD pathways respectively. Flies mutant in both the IMD and Toll pathways are unable to produce any of the characterized AMPs and are highly susceptible to infection from normally innocuous bacteria or fungi (De Gregorio et al., 2002; Lemaitre et al., 1996).

Here we present evidence that Destruxin A suppresses the Drosophila humoral immune response. We used cDNA microarrays and quantitative PCR to examine the effect of Destruxin A on adult Drosophila gene expression. The data revealed a significant proportion of AMP genes were down-regulated suggesting that Destruxin may be suppressing components of the Drosophila immune system. The data further showed Destruxin had the ability to lower the expression of AMPs even when an immune response had been activated by Gram-negative bacterial infection. Destruxin also increased susceptibility of the fly to bacterial infection. The susceptibility could be rescued by ectopic expression of components of the IMD pathway. This result suggests that Destruxin mediates the specific down-regulation of AMPs through targeting a Drosophila innate immune signaling pathway, and is the first evidence of such a phenomenon *in vivo*. In the evolutionary arms race between insect and fungus, Destruxins may thus be playing a novel role in facilitating fungal survival through specific suppression of host immune response components.

3.2 Experimental Procedure

Spotted Microarray Construction and Analysis

From previous Affymetrix chip based microarray experiments and a survey of the literature, we selected 464 genes important for Drosophila immune responses. We used Primer3 (http://www-genome.wi.mit.edu/genome_software/other/primer3html) to design primers to amplify unique regions of the selected genes, generating fragments between 200-600 bp in length (specific primer sequences can be obtained upon request). Fragments were amplified from whole genomic DNA of wild-type, Oregon R strains of *Drosophila melanogaster* in a 96-well format. The reaction mixture to produce each amplicon contained 50 ng Drosophila genomic DNA, 1 μM forward primer, 1μM reverse primer, 1xTitanium Taq (Invitrogen), and 0.5 mM dNTP. The following PCR protocol was used: an initial 95ºC denaturation step for 5 minutes, followed by 20 cycles of 30 seconds of 95ºC denaturation, 30 seconds of 60ºC annealing, and 45 seconds of 75ºC extension. PCR products were run on agarose gels to confirm amplification success and specificity.

Printing, hybridization and scanning of slides were performed with an Affymetrix 417 Arrayer and 418 Scanner at the University of Maryland Biotechnology Institute's Microarray Core facility located at the Center for Biosystems Research. PCR products were spotted in triplicate on poly-lysine coated glass slides, with a mean spot diameter of 100 μm and a spot spacing of 375 μm. Following printing and cross-linking, slides were washed with 1% SDS to remove background, treated with blocking solution (0.2 M succinic anhydride, 0.05 M sodium borate, prepared in 1-

methyl 2-pyrrolidinone) and washed with 95ºC water and 95% ethanol. After drying, slides were kept in the dark at room temperature. See http://www.umbi.umd.edu/~cab/macore/macorestart.htm for detailed protocols.

For microarray experiments, RNA was extracted from a pooled sample of 20 flies with STAT-60 buffer, according to the manufacturers protocols (Isotex Diagnostics). They were further purified using the Qiagen RNAeasy purification kit, and directly labeled using Amersham Biosciences Cyscribe First-Strand Labeling Kit, according to manufacturer's protocols. The raw scanned image files were analyzed using Spotfinder (TIGR), and data normalization, quality assurance and control, filtering, and clustering was performed using MIDAS (TIGR) and MS-Excel (Saeed et al., 2003). Standard Deviation normalization and Lowess transformation was performed on the data using MIDAS (http://www.tm4.org/midas.html). Experiments were done in triplicate, and genes that had at least two readable spots were selected. A criteria of one standard deviation above or below the mean induction of all genes, was used to select for up or down-regulated genes. On normalized data, this represents the top (and bottom) 16% of all genes on the array. The genes were then classified according to available Gene Ontology classifications, and the major groups are presented in Table 3-1.

Fly Stocks

OregonR flies were used as wild-type. For ectopic expression of Toll and IMD pathway components, we used the transgenic c564-Gal4 line of flies that express

Gal4 in various tissues throughout the fly, particularly in the lymph gland, fat body, salivary glands, imaginal discs, gut, and brain (Harrison et al., 1995). The c564-Gal4 flies were crossed into transgenic flies expressing UAS-DmIKKβ (provided by K. V. Anderson); UAS-PGRP-SA (provided by J. Royet); UAS-IMD, UAS-Diptericin, and UAS-Drosomycin (provided by B. Lemaitre) to ectopically express these components in the fly.

Bacterial infection, survival and proliferation assays- *E.coli* DH5α strains were grown up in LB media overnight, resuspended in an equal volume of filter-sterilized Phosphate Buffered Saline (PBS). About 0.5μL of the bacteria was then injected into the abdomens of female adult flies using a pneumatic picopump PV820 (World Precision Instruments) apparatus. A solution of 86 μM of Destruxin A (Sigma-Aldrich) in 1xPBS was used for Destruxin injection. For gene expression studies, RNA was extracted 4 hours after injection. The experiment was repeated a minimum of three times.

Each survival experiment was performed with at least 20 flies and repeated 3 times. The total number of flies in each treatment was assessed for survival periodically over a 5 day period. The Kaplan-Meier statistical model was used to compare fly survival. This is a widely used statistical model to analyze survival data for actuarial data for vertebrate studies, because it can take into account test subjects leaving the experiment due to factors other than the ones being studied in the experiment. For example in human studies, statistically significant results can be derived from lower

numbers of subjects even when some patients leave the experiment if they choose to do so. At each time point, the probability for an individual to survive is calculated and graphed in a step function. The probability of survival on a given day for example, is calculated by dividing the number of test subjects who have survived beyond the day by the number of subjects that were alive at the beginning of the day. This method automatically takes into account any subjects who were removed from the experiment for unrelated reasons. A Mantel-Cox Log-Rank statistic is used to calculate the expectation that a given control and experimental population have the same survival characteristics. In this method, differences between observed and expected values of for the control and experimental population are added for all time points. A chi-square distribution is used to determine the probability of this calculated total difference occurring purely by chance, using a null hypothesis that there is no survivability difference between control and experimental treatments. A low expectation denoted by a low P-value therefore signifies a significant difference in survival between the populations that are being compared. For our studies, p-values<0.05 were deemed significant.

For the bacterial proliferation assay, Ampicillin-resistant *E.coli* were used. Twenty-four hours post-injection, the flies were anesthetized, surface sterilized by dipping in 95% ethanol, and homogenized in 1 ml of LB media containing 1% Triton-X100 and 100μg/ml Ampicillin. The homogenized media was incubated at 37ºC for 1 hour and 50μL was plated on LB-Amp plates representing colony forming units in 1/20th of a fly. Colonies were counted following an overnight incubation of the LB-Amp plates

at 37°C. The experiment was independently repeated 10 times for each treatment, and the error bars show standard error of the mean.

Phagocytosis assay

The assay was performed as described (Elrod-Erickson et al., 2000). Adult flies are injected in the abdomen with fluorescein-labeled *E.coli* particles (Molecular Probes). After 30 minutes, trypan blue is injected to quench extracellular fluorescence. If the hemocytes are able to take up the fluorescent particles, the fluorescence can be visualized through the cuticle on the dorsal side of the abdomen. In cells deficient for phagocytosis, the fluorescence of the particles remains outside the phagocyte and is quenched by the trypan dye. This results in reduced visualization of particles inside flies deficient in phagocytosis.

Quantitative PCR

RNA was isolated using STAT-60 buffer according to manufacturer's protocol (Isotex Diagnostics). The RNA was digested with RNAse-free DNAse, and subjected to reverse transcription using Superscript II (Invitrogen). The resulting cDNA was quantified using real-time PCR using LUX probes (Invitrogen) on an ABI 5700 following manufacturer's protocols. Gene expression was normalized using RP49 as an endogenous control. The data presented in this paper has been further normalized to set uninjected wild-type levels as the calibrator. The specific primers used can be obtained upon request. The experiments were repeated a minimum of three times and in some cases over 5 times.

3.3 Results

3.3.1 Destruxin injection causes a reduction in expression of some antimicrobial peptide genes.

To test the effect of non-lethal doses of Destruxin on Drosophila gene expression, we used cDNA microarrays to compare wild-type flies injected with 86 μM Destruxin A to flies injected with PBS. The dose was determined experimentally. Different 10x dilutions of Destruxin A were injected in flies. 0.5 μL of 86 μM could be injected into flies without causing significant difference in mortality compared to PBS-injected flies within 5 days, while the next higher concentration (860 μM) caused significant mortality (data not shown). The custom-made microarrays enable the study of 464 Drosophila genes selected from an extensive literature survey of data collected by other groups through microarray experiments on genes predicted to be important for the immune response (De Gregorio et al., 2002; Irving et al., 2001). Genes that were significantly up or down-regulated upon Destruxin injection were classified based on known gene ontology (GO) information (Table 3-1). MIAME compliant raw data can be accessed from NCBI GEO website (Acc. no. GSE5767). Categories of genes for general metabolism were not affected by Destruxin. By contrast, 52% of all Antibacterial Humoral Response genes (GO: 0006961) on the array were down-regulated in Destruxin-injected flies (Table 3-1). This represents nearly 23% of all down-regulated genes on the microarray (Figure 3-1B), and represents the largest category in the chart. None of the genes in this category were found to be induced upon Destruxin injection (Figure 3-1A and Table 3-1). Similarly, 34% of genes having Peptidoglycan Receptor Activity (GO:0004867) were down-regulated

representing 15% of all down-regulated genes (Figure 3-1B). Only 5% of genes in this category were induced (Table 3-1). Proteolysis and Peptidolysis genes (GO: 0006508) represented the largest percentage of all up-regulated genes (Figure 3-1A), but only 10% of all genes in this category were up-regulated (Table 3-1). Since 11% of these genes were down-regulated, there was no significant shift in either direction for this category (Table 3-1). Of note, most genes (nearly 85% of the genes on the array) were not affected by Destruxin injection, suggesting that the down-regulated antibacterial humoral genes represent a specific phenomenon, and are not the result of general ill-health brought about by Destruxin injection. Thus compared to other categories, we observed the most significant and specific down-regulation of the antibacterial peptide response.

To confirm that Destruxin caused down-regulation of antimicrobial peptide genes, Quantitative RT-PCR was used to examine Diptericin, Cecropin, Attacin and Metchnikowin expression (Figure 3-2). In all cases the injected flies had a significantly lower AMP production than PBS-injected flies within 4 hours of injection (using a one-tailed T-test cut-off of $p<0.05$), as predicted by the microarrays. Quantitative RT-PCR confirmed Destruxin's suppressive effects on these AMPs in the absence of infection. We used the Gram-negative bacteria *E.coli* to determine whether Destruxins can also reduce AMP expression when the immune response has been activated. Co-injection of Destruxin A with the bacteria significantly reduced expression of Diptericin, Attacin, and Drosomycin in these immune-stimulated flies at 4 hours as compared to injection with *E.coli* (Figure 3-2).

To address the question of whether Destruxin is inhibiting or merely delaying the expression of these AMPs, we also examined their expression at 8 and 24 hrs. For Drosomycin, Destruxin appears to suppress expression at the earlier time point, but not at the later timepoints. Drosomycin expression typically peaks at 24 hours after infection, and it is possible that the injected Destruxin is no longer effective at this later time point. Inhibition of Diptericin and Attacin expression by Destruxin was easier to interpret because of the relative transience in their expression. Inhibition by Destruxin also appeared to be specific to these three AMPs, as levels of some others such as Cecropin, Drosocin, and Metchnikowin induced by bacterial injection were not significantly affected by Destruxin.

3.3.2 Destruxin injected flies are more susceptible to bacterial infection.

We performed survival assays to determine whether the decrease in antimicrobial peptide expression produced by Destruxins leaves flies more vulnerable to microbial infection. Wild-type flies injected with *E.coli*, Destruxin A, or PBS alone show very low mortality rates. However, co-injection of Destruxin A with *E.coli* causes a significant decrease in survival compared to control flies, with over 70% dead within 5 days (Figure 3-3A). *E.coli* is not naturally lethal to the fly, and flies injected with the bacteria alone do not demonstrate significant mortality. To test whether the lower expression of AMP genes also resulted in greater bacterial proliferation within the fly, we performed bacterial survival counts from infected flies. Drosophila were injected with Ampicillin-resistant *E.coli* with or without Destruxin, and incubated at 29°C for

24 hours. They were then homogenized in LB media and spread on LB-Ampicillin plates. A count of the resulting colonies revealed a higher titer of live colonies inside the Destruxin and *E.coli*-injected flies compared to flies injected with *E.coli* alone (Figure 3-3B). This is consistent with the hypothesis that a lowering in AMP production brought about by Destruxin allows *E.coli* to proliferate and colonize the fly to accelerate its demise.

Interestingly, Destruxin does not appear to affect Drosophila cellular immune responses *in vivo*. Destruxin-injected flies continue to demonstrate melanization spots at the site of injection, suggesting that components of the prophenoloxidase cascade remain unaffected (data not shown). While Destruxins have been shown to affect hemocytes ability to phagocytose in cell culture (Vilcinskas et al., 1997a; Vilcinskas et al., 1997b), we saw no such effect *in vivo* in Drosophila. Phagocytosis of *E.coli* bacterial particles in Destruxin-injected flies appeared to be the same as that seen in PBS-injected flies (Figure 3-4). Thus, proliferation of bacteria in the Destruxin injected flies appears to be a consequence of the lower expression of antimicrobial peptide genes.

3.3.3 Ectopic expression of components of the IMD pathway rescues bacterial susceptibility in Destruxin-injected *Drosophila*.

Antimicrobial peptides are induced through the activation of the Toll and IMD signaling pathways. Thus we hypothesized that Destruxin might be affecting these signaling pathways to mediate a specific down-regulation of antimicrobial peptide

genes. To test this hypothesis, we ectopically expressed various components of the Toll or IMD pathway using a UAS-Gal4 promoter system (Brand et al., 1994). In this system, we used the Drosophila c564 line to drive expression of a yeast transcription factor Gal4, in various Drosophila tissues including those that normally express AMP genes, the fat body and the lymph glands (Harrison et al., 1995). Gal4 protein binds Upstream Activating Sequence (UAS), to transcribe genes that have been placed downstream of the UAS regulatory element. In this experiment, components of the Toll and IMD pathway are expressed through this system independent of their activation by infection. We tested whether the system was working, by looking at the expression levels of the target genes Diptericin and Drosomycin (Figure 3-5). DmIKKβ and Imd expression led to significant levels of Diptericin expression in the absence of infection, and these expression levels continued to remain higher than wild-type even upon Destruxin injection. This confirms the activation of the IMD pathway through independent expression of these components (Figure 3-5). In some cases however, this approach was not as straightforward. Imd expression for example, also managed to induce relatively high levels of Drosomycin, a target of the Toll pathway. Similarly, ectopic expression of PGRP-SA which is known to be upstream of Toll (Michel et al., 2001) was better at inducing Diptericin than Drosomycin. Thus, there may be cross-talk between the Toll and IMD pathways.

When flies ectopically expressing components of the IMD pathway were co-injected with *E.coli* and Destruxin, they survived significantly better than wild-type flies (Figure 3-6). Imd and DmIKKβ expression significantly rescued mortality caused by

Destruxin co-injected with bacteria. Ectopic expression of Toll pathway components appeared to have the same effect. PGRP-SA-expressing flies co-injected with Destruxin and *E.coli* survived significantly better than wild-type flies given the same treatment (Figure 3-6). However, because PGRP-SA weakly activated targets of the IMD pathway (Figure 3-5), it was unclear which pathway was responsible for mediating the observed rescue.

To resolve this issue, we ectopically expressed the target antimicrobial peptides Drosomycin and Diptericin. Ectopic Drosomycin expression alone failed to rescue the survival of *E.coli* and Destruxin co-injected flies (Figure 3-6). Drosomycin, an important target of the Toll pathway, has lower expression in Destruxin injected flies (Figure 3-2). However, in the absence of rescue, it remains unclear whether this lowering is mediated through the Toll pathway. On the other hand, the expression of the IMD pathway effector Diptericin was sufficient to significantly rescue survival of flies, albeit to a lower level compared to DmIKKβ. These results further validate our model that Destruxin-induced Drosophila mortality is due to bacterial proliferation resulting from immune suppression, because the expression of an antibacterial peptide makes them significantly less vulnerable to infection. This also indicates that Destruxin acts upstream of the IMD pathway to mediate a lowering of Diptericin expression.

3.4 Discussion

Destruxins are secreted by a variety of fungi and are best known for their insecticidal and phytotoxic activity during the establishment of fungal infection (Kershaw et al., 1999; Kodaira, 1961). They are also gaining utility as lead compounds for the generation of anti-viral and anti-cancer agents for human therapies (Chen et al., 1997; Pedras et al., 2002). There is evidence suggesting that their biological role may involve manipulating host signaling (Dumas et al., 1996). We used cDNA microarrays to test the effect of injecting Destruxin A into adult *D. melanogaster*. Injection of Destruxin may be representative of the physiological scenario, as fungal hyphae secrete it after penetrating the cuticle. Injection also allowed us to isolate Destruxin's effect on Drosophila gene expression and survival in a controlled way, independent of other components of a natural fungal infection. Microarray results showed the Antibacterial Humoral Response genes are disproportionately down-regulated compared to other categories of genes, and this was the most significant difference in gene expression patterns. Though the array is enriched for immunity genes, this finding still represents a specific shift in expression of genes compared to PBS-injected controls.

We did not observe any failure in the phagocytosis or melanization machinery *in vivo*. Thus, Destruxin appears to be specifically affecting components of the humoral immune response. Furthermore, this effect is sufficient to cause greater susceptibility to bacterial infections. This mortality is accompanied by a greater proliferation of *E.coli* in Destruxin-injected flies, suggesting that the suppression by Destruxin of the

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immune response genes makes the fly unable to clear a bacterial infection. To determine the mechanism by which Destruxin mediates suppression of AMPs, we ectopically expressed components of the Toll and IMD pathways. Imd, DmIKKβ, and Diptericin producing flies were significantly less susceptible to mortality from coinjection of *E.coli* and Destruxin. Since expressing components of the IMD pathway alone facilitates fly survival, Destruxin may suppress the IMD pathway upstream of the components we have tested. That this suppression is specific to the IMD pathway is suggested by the failure of Destruxins to inhibit metabolic housekeeping genes and other aspects of the immune response.

Suppression of a host immune response would have obvious benefits for a pathogenic fungus such as *Metarhizium anisopliae*. Flies lacking the ability to produce AMPs due to mutations in both the Toll and IMD pathways are extremely vulnerable to fungal challenge (Lemaitre et al., 1996; Tzou et al., 2002), and these innate immune pathways are conserved between insects. Thus, the ability to reduce AMP production is likely to aid fungal survival in a variety of insect hosts.

It is noteworthy that Drosomycin, the only AMP with known inhibitory activity to *M. anisopliae* was one of the AMPs suppressed by Destruxin. Insects also seem to have pathways responsible for Destruxin biotransformation (Roberts, 1966). Studies with Destruxin E in locusts suggests the existence of pathways that metabolize the toxin and produce a population of secondary compounds (Cherton et al., 1991; Lange et al., 1992). This may be indicative of a co-evolutionary history between fungi producing

these toxins and insects evolving biochemical means to detoxify them. Recently, Varroa mites were shown to suppress humoral AMP expression in honey bees (Gregory et al., 2005). Similar suppression by fungi expressing Destruxins adds a new dimension to the dynamics of host-pathogen interactions.

Insect pathogenic fungi have been under-exploited as a resource of medically active compounds but this is changing with the realization that they are exceptionally rich sources of novel biologically active substances (Isaka et al., 2005). To date, the only commercialized example is Cyclosporine, a undecapeptide from the mosquito pathogenic fungi *Tolypocladium inflatum* that prevents IL-2 expression in mammalian cells by inhibiting calcineurin (Motta et al., 1991). Cyclosporin A is therefore used extensively in current human therapies such as organ transplantation which require suppression of the host immune response (Herndon, 2000). Destruxins are also candidates for medical use, particularly as therapeutic agents for viral diseases and cancer. But as this study shows, Destruxins have complex effects on cells and more research is required to predict and improve cellular responses to them.

ACKNOWLEDGEMENTS

Microarray facility and Alvaro Godinez for help with microarray printing, hybridization, and data acquisition.

BIOLOGICAL PROCESSES		
CG Number	Name	Fold Change
Antibacterial Humoral Response	**GO:0006961**	**17 Total**
Down Regulated		*52%*
CG1373	Cecropin C	0.5756
CG1878	Cecropin B	0.5926
CG1367	Cecropin A2	0.5961
CG4740	Attacin	0.6056
CG1365	Cecropin A1	0.6453
CG8175	Metchnikowin	0.6705
CG12763	Diptericin	0.699
CG8846	Thor	0.78
CG10363	TEP IV	0.8131
Proteolysis and Peptidolysis	**GO:0006508**	**42 total**
Down Regulated		*11%*
CG4096		0.699477
CG9733		0.790125
CG9455		0.793
CG6361		0.8045
CG16705		0.8065
Up Regulated		*10%*
CG5909		1.284
CG1857	necrotic	1.444
CG4804		1.45
CG7788	ice	2.34
Heatshock	**GO:0009408**	**5 total**
Down Regulated		*20%*
CG4466	Hsp27	0.798
Up Regulated		*40%*
CG6489	Hsp70-BC	1.29
CG4463	Hsp-23	1.64
Toll Pathway	**GO:0008063**	**17 Total**
Down Regulated		*5%*
CG5974	Pelle	0.79
Up Regulated		*17%*
CG1857	Nec	1.44
CG16844	IM3	1.46
CG5490	Toll	1.51
Jak Stat Signaling	**GO:0007259**	**3 Total**
Down Regulated		*33%*
CG15154	Socs36E	0.758526
Apoptosis	**GO:006915**	**25 total**
Down Regulated		*4%*
CG4319		0.78
Up Regulated		*12%*
CG4280	Crq	1.24
CG4345	Grim	1.26
CG7788	Ice	2.34
Polysaccharide Metabolism	**GO:0005976**	**11 Total**
Down Regulated		*18%*
CG13422		0.69
CG4144	GNBP2	0.756
Up Regulated		*18%*
CG5008	GNBP3	1.25
CG12780		1.4370
Lipid Metabolism	**GO:0006629**	**7 Total**
Down Regulated		*28%*
CG6675		0.7045
CG6271		0.76
Spermatogenesis	**GO:0007283**	**12 Total**

Down Regulated		*8%*
CG15378	Lectin 22C	0.806
MOLECULAR FUNCTION		
Peptidoglycan Receptor Activity	**GO:0016019**	**18 Total**
Down Regulated		*34%*
CG9681		0.62
CG14745		0.64
CG4437	PGRP-LF	0.66
CG9681	PGRP-SB1	0.74
CG7496	PGRP-SD	0.75
CG14704	PGRP-LB	0.79
Up Regulated		*5%*
CG4432	PGRP-LC	1.40
Serine Protease Inhibitors	**GO:0004867**	**25 Total**
Down Regulated		*12%*
CG3604		0.75
CG9455		0.79
CG5794	Pelle	0.79
Up Regulated		*20%*
CG16713		1.32
CG12172	Spn43Ac	1.36
CG1859		1.37
CG1857	Nec	1.44
CG4804		1.45
Serine type endopeptidase activity	**GO:0004252**	**20 Total**
Down Regulated		*10%*
CG9733		0.79
CG6361		0.804
Up Regulated		*5%*
CG5909		1.28
Scavenger Receptor	**GO:0005044**	**11 Total**
Up Regulated		*18%*
CG4280	Crq	1.24
CG7244		1.43
Others	**Not available**	**191**
Down Regulated		*3%*
CG13323		0.6627
CG13324		0.694
CG9616		0.725
CG12821		0.74
CG17738		0.749
CG7267		0.788
CG15126		0.799
Up Regulated		*6%*
CG15068		1.23
CG14073		1.24
CG3838		1.318
CG1600		1.32
CG16794		1.394
CG14419		1.465
CG13905		1.48
CG16836		1.49
CG9568		1.62
CG14481		1.667
CG7629		2.409

Table 3-1: Genes affected by Destruxin A, grouped according to available Gene Ontology information.
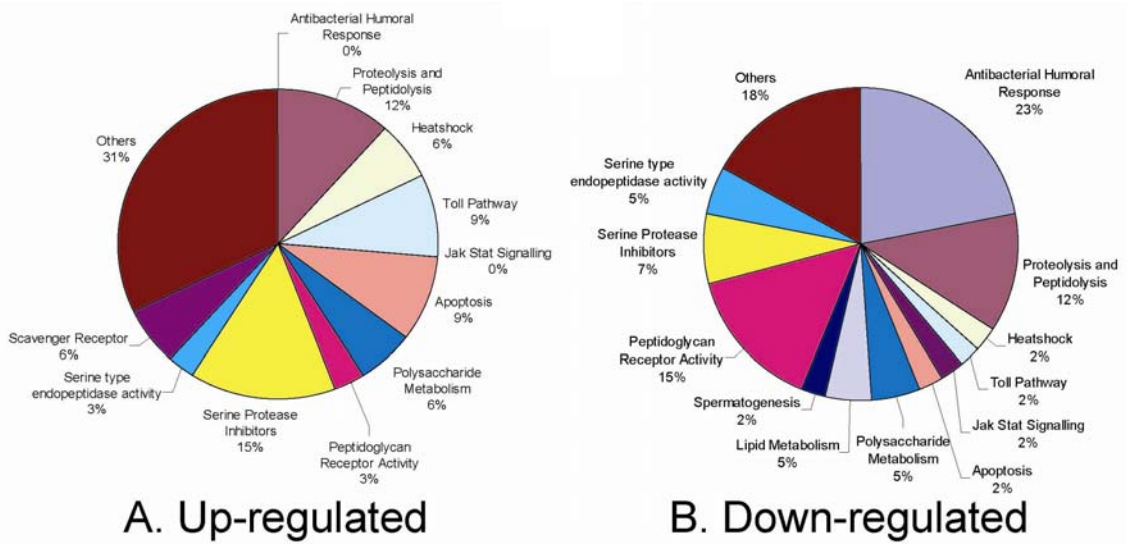
Figure 3-1. Pie Charts showing Gene Ontology Composition of Genes Affected by Destruxin. Genes that were A. Up-regulated, and B. Down-regulated to a significant degree upon Destruxin injection. The categories were selected based on available Gene Ontology information. The percentage of genes in each class among the total number of affected genes is also provided.
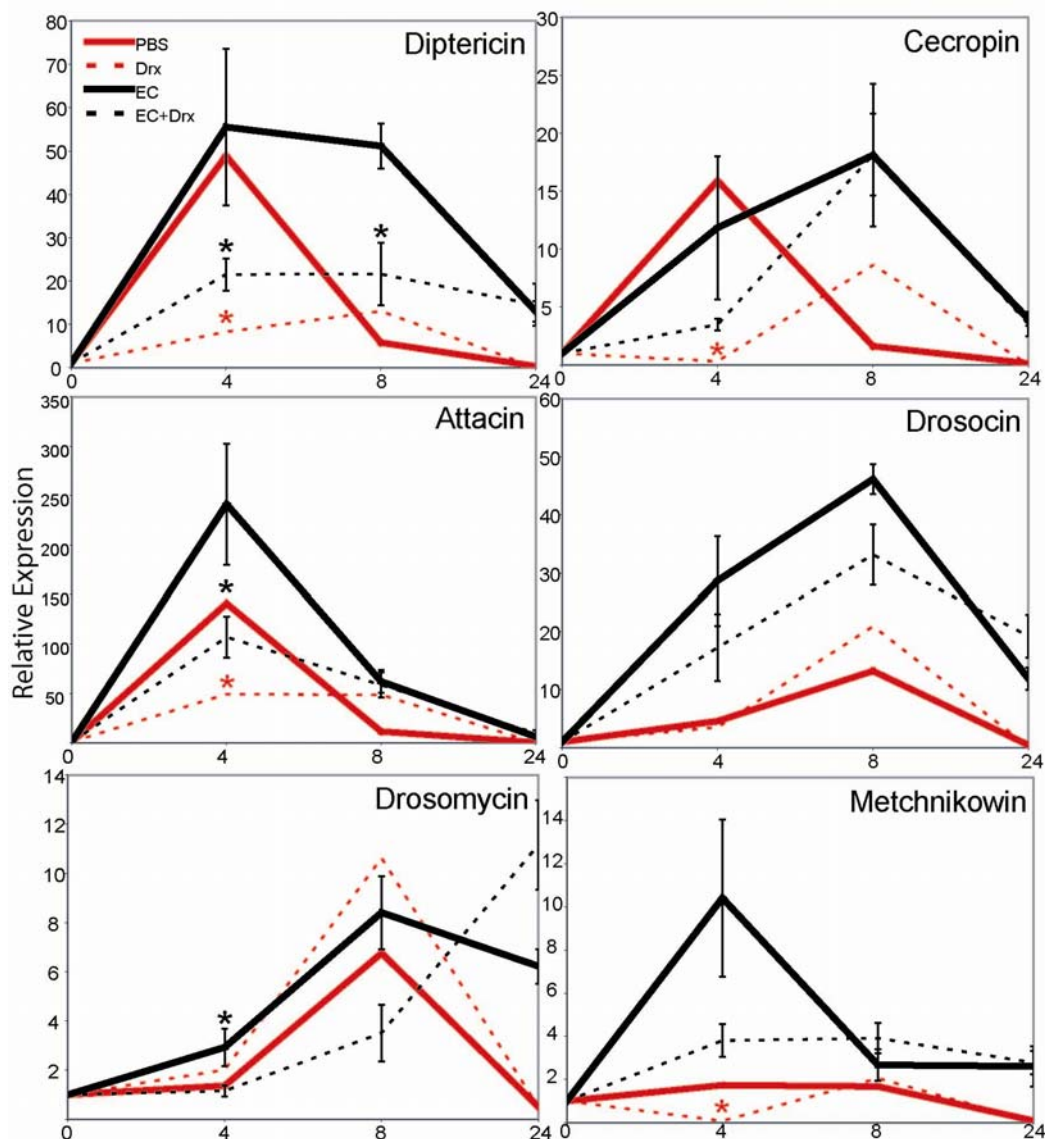
Figure 3-2. Quantitative RT-PCR looking at the effect of Destruxin on specific Antimicrobial peptides. Co-injecting Destruxin with *E.coli* causes a lowering of Drosomycin, Diptericin, and Attacin gene expression compared to plain *E.coli* injected flies (P<0.05 using a one-tailed T-test). Adult flies more than 5 days old were injected with PBS, *E.coli*, 86 μM Destruxin, or *E.coli* + Destruxin. RNA was isolated from pooled samples of 20 flies four hours after injection, and Reverse Transcription and Quantitative RT-PCR were done to examine gene expression. The data was normalized using Drosophila RP49 as an endogenous control, and the y-axis represents relative expression compared to uninjected transcript levels, set as 1. The experiment was repeated at least three times and in some cases over 5 times. The error bars represent standard error of the mean. Statistically significant differences in the antimicrobial peptide expression (Diptericin, Attacin, Drosomycin) are marked with asterisk marks (*) having a one-tailed T-test p-value<0.05.
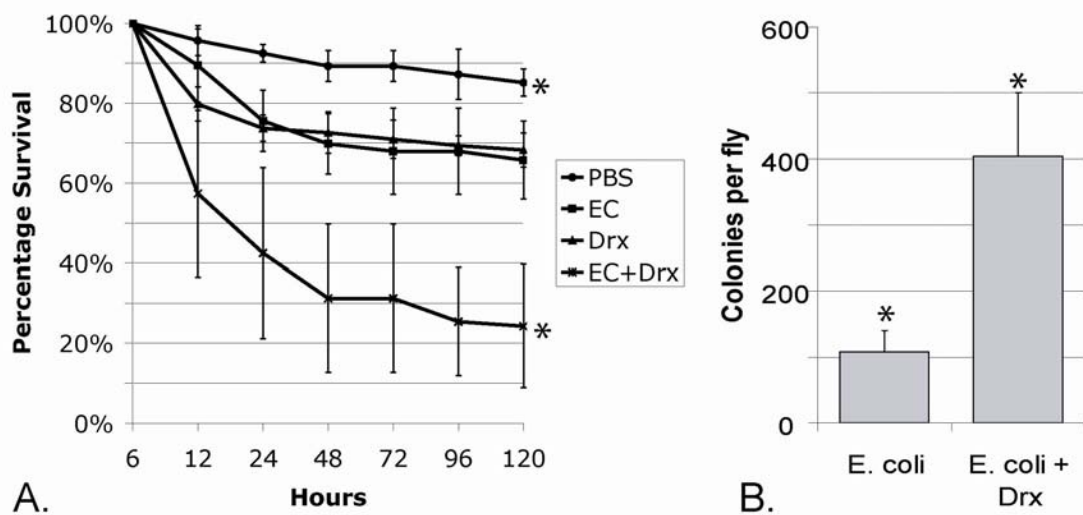
Figure 3-3. Coinjection of Destruxin with *E.coli* causes Drosophila mortality. A. Thirty flies or more were injected with PBS, *E.coli*, Destruxin, or *E.coli* and Destruxin and the number of flies alive in the vial was counted over the course of the next five days. The survival curve shows significant fly mortality in *E.coli*+Destruxin injected flies, but not control animals (* indicates that these curves show significant differences, represents $p \ll 0.05$ using Mantel-Cox Log-Rank statistics). The experiment was repeated at least three times and error bars represent standard error of the mean. B. Destruxin injected and control flies were assayed for proliferation of live bacteria. Bacterial colony counts shows lower bacterial titers upon *E.coli* injection, compared to Destruxin+*E.coli* injected flies (* represents $p < 0.05$ using a two tailed T-test). Error bars represent standard error of mean of more than 10 independent experiments.
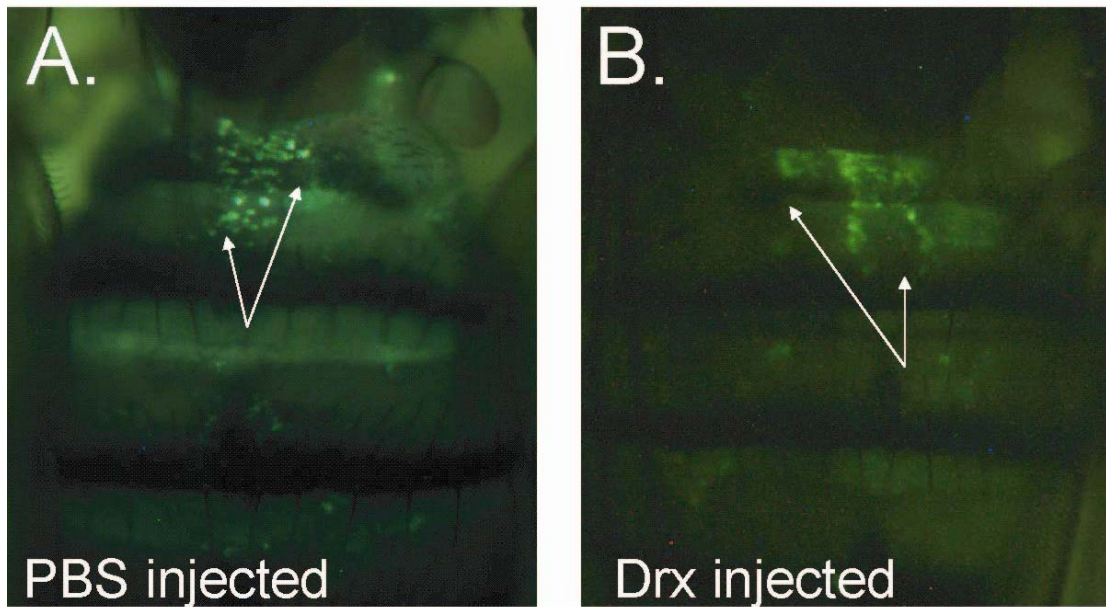
Figure 3-4. Phagocytosis assay. Flies were injected with fluorescein *E.coli* particles and the signal was quenched 30 minutes later with Trypan blue dye. Any visible fluorescence is a result of phagocytosed bacterial particles. A. Flies that were injected with control PBS and B. Flies that were injected with Destruxin A, show no observable difference in degree of phagocytosis. This suggests that Destruxin injection does not cause a defect in Drosophila phagocytosis responses. This experiment was done by Melek Erdinc.
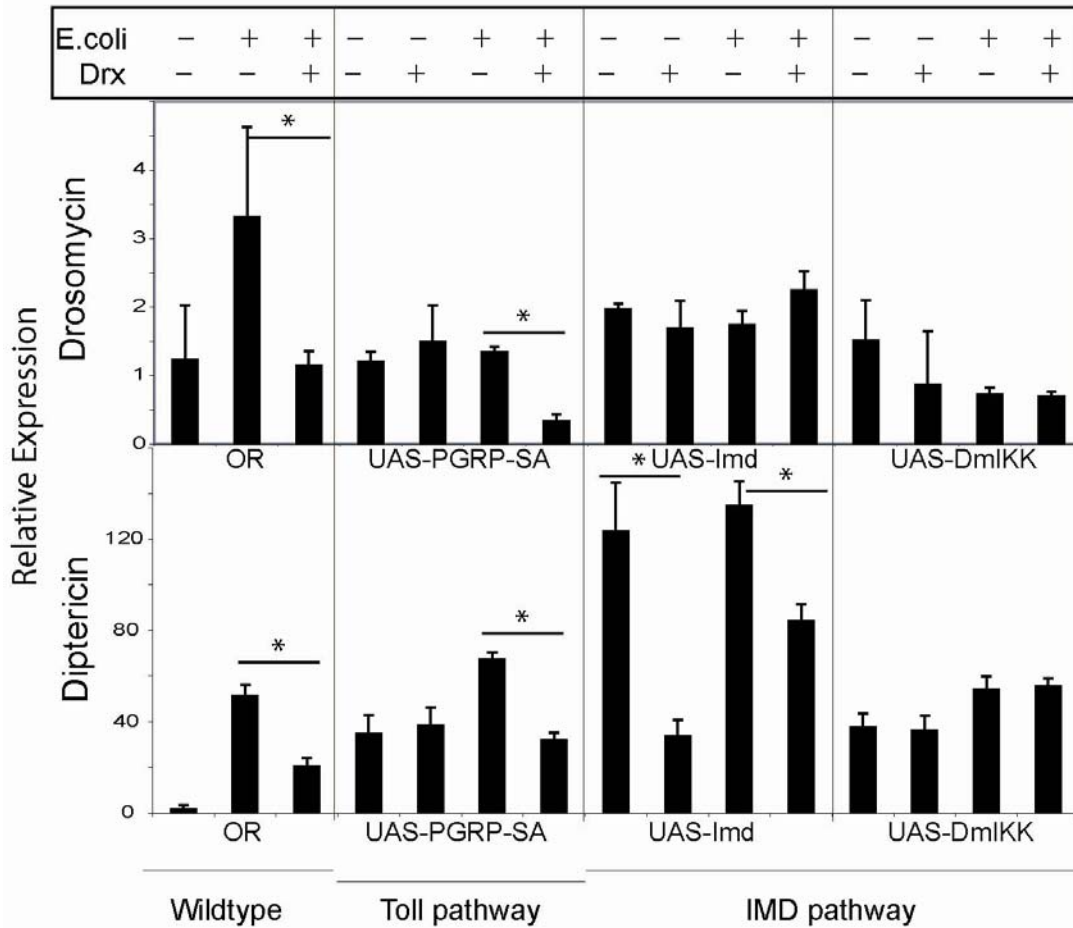
Figure 3-5. Quantitative PCR showing expression of Drosomycin (Top) and Diptericin (Bottom). Relative expression of target genes Drosomycin and Diptericin are given, using RP49 as an endogenous control and using uninjected wildtype transcript levels as a calibrator, set to 1. Wildtype uninjected, *E.coli* injected, and *E.coli* + Destruxin injected transcript levels are provided as reference. Transgenic Drosophila ectopically expressing components of the Toll pathway (PGRP-SA) or IMD pathway (IMD and DmIKKβ) are given. Ectopic activation of various IMD pathway components activates the IMD target Diptericin compared to OR uninjected controls in a statistically significant manner (p<<0.05 for Diptericin expression for UAS-PGRP-SA, UAS-IMD, and UAS-DmIKK). Drosomycin expression is not significantly induced upon ectopic expression of Toll or IMD pathway components. A T-test comparison between samples representing a statistically significant changes are shown using a bar (* represents p<0.05 with a two tailed T-test). Experiments were repeated at least three times.
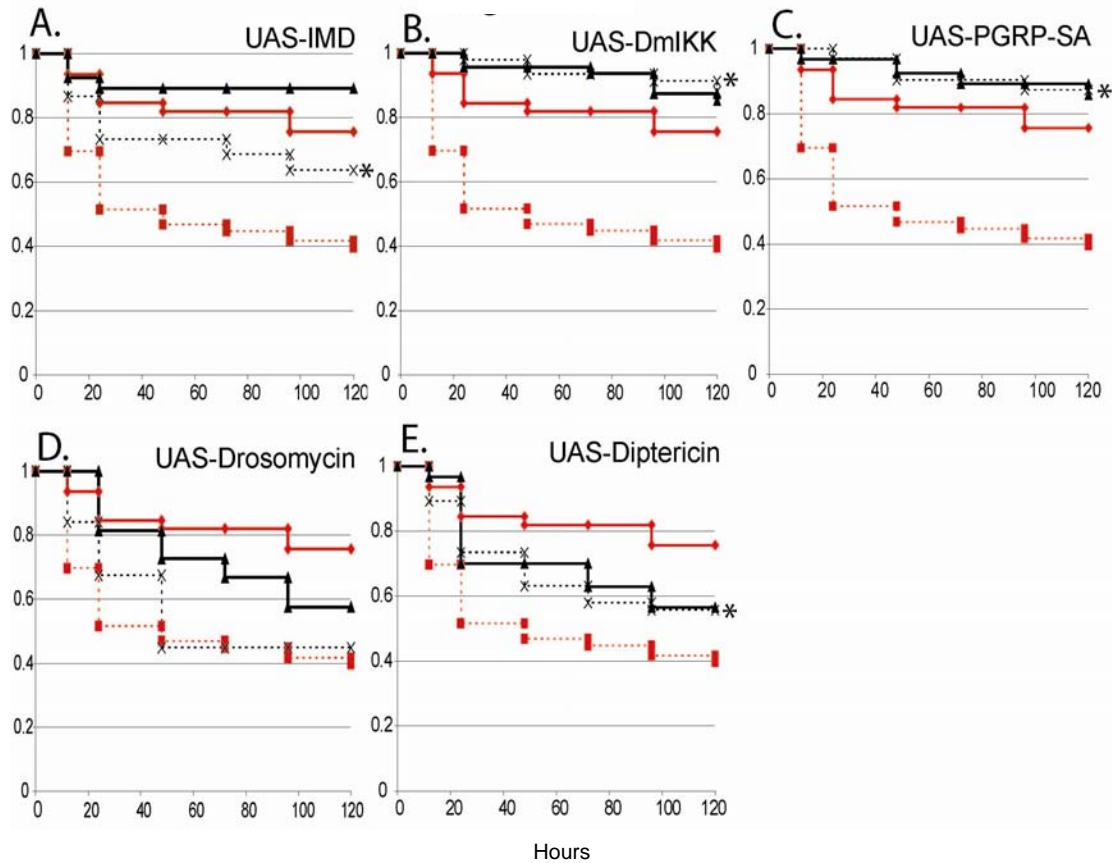
Figure 3-6. Graphical analysis of survival assay using ectopic expression of Toll and IMD components. UAS-Gal4 system is used to drive the expression of components of the Toll and IMD pathway. The Drosophila c564 line is used to drive expression of IMD pathway genes A. UAS-Imd, B. UAS-DmIKKβ, Toll pathway genes C. UAS-PGRP-SA, and antimicrobial effectors D. UAS-Drosomycin, and E. UAS-Diptericin. Survival of IMD, DmIKKβ, PGRP-SA, and Diptericin expressing lines produced a statistically significant rescue compared to wildtype *E.coli*+Destruxin coinjected flies (* represents p<<0.05 using a Mantel-Cox log rank test) when comparing the dotted black with the dotted red line (*E.coli*+Destruxin injected flies in wildtype and UAS-Toll/IMD pathway).The survival rates of wildtype flies injected with *E.coli* (solid red line with ♦),or with *E.coli* and Destruxin (dotted red line with ■), transgenic Gal4-UAS flies expressing components of the Toll or IMD pathway injected with *E.coli* (solid black line with ▲), or with *E.coli* + Destruxin (dotted black line with ✗).

Gene	Gene Name	Fold Change
CG10332		0.484
CG2956	Twist	0.507
CG12763	Diptericin	0.542
CG5974	Pelle	0.553
CG1373	Cecropin C	0.576
CG1878	Cecropin B	0.593
CG1367	Cecropin A2	0.596
CG4740	Attacin-C	0.606
CG9681		0.625
CG1365	Cecropin A1	0.645
CG14745		0.650
CG14745		0.662
CG13323		0.663
CG4437	PGRP- LF	0.667
CG9681	PGRP-SB1	0.670
CG8175	Metchnikowin	0.671
CG13324		0.694
CG4096		0.699
CG13422		0.700
CG12763	Diptericin	0.700
CG6675		0.705
CG3666	Transferrin 3	0.712
CG9616		0.725
CG6898	Zip-3	0.733
CG9681	PGRP-SB1	0.748
CG3604		0.749
CG12821		0.749
CG7496		0.755
CG4144	GNBP-2	0.756
CG15154	Socs36E	0.759
CG6271		0.764
CG17738		0.773
CG1365		0.782
CG4319	Reaper	0.784
CG11374		0.785
CG8846	Thor	0.788
CG7267		0.789
CG14704	PGRP-LB	0.790
CG9733		0.790
CG5974	Pelle	0.790
CG9455		0.793
CG4466	Heat shock protein 27	0.798
CG15126		0.800
CG11299		0.800
CG11459		0.804
CG6361		0.805
CG16705		0.807
CG15378	Lectin-22c	0.807
CG17814	Peritrophin-15a	0.810
CG10363	Thiolester containing protein IV	0.813
CG15358		0.815
CG10956		0.818
CG3858		0.819
CG1021		0.819
CG7219		0.826
CG10278		0.826
CG1652		0.829
CG1093	Pollux	0.830
CG18316		0.830
CG10383		0.831
CG3410		0.831
CG13095		0.832
CG18067		0.834
CG4267		0.834
CG7722		0.834
CG14230		0.836
CG15046		0.837
CG8091	Nedd2-like caspase	0.843
CG12826		0.845
CG8215		0.845
CG18372	Attacin-B	0.846
CG4269		0.847
CG1342		0.848
CG9414		0.850
CG14302		0.854
CG7279	Lipase 1	0.855
CG8492		0.855
CG10198	Nup98	0.856
CG14499		0.858
CG15306		0.859
CG10872		0.859
CG15066	Immune induced molecule 23	0.860
CG14027	Turandot M	0.861
CG6277		0.869
CG11825		0.870
CG6449	Ninjurin A	0.871
CG5791		0.874
CG12052	Longitudinals lacking	0.874
CG13805		0.876
CG6524	Chorion protein 19	0.877
CG1385	Defensin	0.878
CG15422		0.879
CG11709	PGRP-SA	0.881
CG14438		0.883
CG1093	Pollux	0.883
CG1887		0.886
CG13422		0.891
CG7052	Thiolester containing protein II	0.892
CG5370	Death caspase-1	0.894
CG4427		0.900
CG17278		0.900
CG11529		0.902
CG15010	Archipelago	0.903
CG3365	Drongo	0.905
CG13135		0.905
CG7763		0.906
CG13461		0.908
CG8896	18W	0.908
CG18402	Insulin-like receptor	0.908
CG6829	Apaf-1-related-killer	0.909
CG17105		0.911
CG1102		0.911
CG3132		0.911
CG2958		0.913
CG9441	Punch	0.913
CG6266		0.916
CG9434	Frost	0.916
CG5550		0.916
CG10146	Attacin-A	0.917
CG8577		0.920
CG7171	Urate oxidase	0.921
CG18030	Jonah 99Fi	0.922
CG7219		0.923
CG7710		0.923
CG3212		0.923
CG10697	Dopa decarboxylase	0.925
CG7863		0.926
CG11841		0.928
CG5493		0.928
CG7052	TEPII	0.928
CG6483	Jonah 65Aiii	0.929
CG8137		0.929

Gene	Name	Value		Gene	Name	Value
CG5778		0.930		CG11992	Relish	0.991
CG8995	PGRP-LE	0.930		CG11331	Serpin-27A	0.991
CG13249		0.931		CG8871	Jonah 25Biii	0.993
CG8825	Glaikit	0.933		CG7738		0.993
CG6289		0.934		CG11765	Peroxiredoxin 2540	0.995
CG18108	Immune induced molecule 1	0.934		CG2839		0.995
				CG9144		0.995
CG6528		0.934		CG5848	Cactus	0.996
CG17061	Methuselah-like 10	0.937		CG5514		0.996
CG5523		0.937		CG9095		0.998
CG10039		0.938		CG18096	Thiolester containing protein I	0.998
CG7068	TEPIII	0.941				
CG12505		0.941		CG6134	Spatzle	1.000
CG7503	Connectin	0.942		CG18525	Serine protease inhibitor 5	1.000
CG14746		0.944		CG15065		1.001
CG9080		0.945		CG15043		1.001
CG3978	Pannier-SD02611	0.946		CG4183	Heat shock protein 26	1.002
CG14852		0.947		CG3359	Midline fasciclin	1.003
CG7227		0.947		CG2045		1.003
CG10363	Thiolester containing protein IV	0.947		CG8913		1.010
CG11709	PGRP- SA	0.947		CG6680		1.010
CG14567		0.949		CG15067		1.010
CG4199		0.950		CG18550	Yellow-f	1.011
CG13845		0.950		CG14957		1.011
CG5528		0.951		CG10810	Drosomycin	1.013
CG5429		0.952		CG5489		1.013
CG8678		0.952		CG6457	Yippee interacting protein 7	1.013
CG17107		0.954		CG8157		1.013
CG9972		0.955		CG2444		1.015
CG6687		0.956		CG11159		1.016
CG7695		0.957		CG7986		1.016
CG5413		0.957		CG9336		1.016
CG18563		0.959		CG15423		1.017
CG1576		0.959		CG2275	Jun-related antigen	1.019
CG6127	Serrate	0.960		CG14762		1.021
CG14132		0.961		CG1385	Defensin	1.021
CG6531	Wengen	0.962		CG4757		1.023
CG5140		0.962		CG5915	Rab-protein 7	1.024
CG10035		0.963		CG1241		1.026
CG11501		0.965		CG7294		1.026
CG17797	Acp 29AB	0.965		CG6468		1.027
CG7635		0.967		CG5819		1.028
CG3505		0.970		CG6906		1.029
CG7876		0.971		CG5123	Wrinkled	1.031
CG12318		0.972		CG7996	Snake	1.034
CG11331	Serpin-27A	0.972		CG10842	Cytochrome P450	1.034
CG3801	Acp76A	0.972		CG6687		1.034
CG7393		0.973		CG2081		1.035
CG30080		0.975		CG8595	Toll-7	1.035
CG3066		0.975		CG8856	Src-II	1.036
CG4115		0.975		CG7798		1.037
CG7850	Puckered	0.976		CG16712		1.040
CG16832		0.977		CG6667	Dorsal	1.041
CG4144	GNBP-2	0.977		CG9453		1.044
CG4823		0.980		CG18337		1.044
CG11819		0.980		CG3829		1.045
CG16926		0.981		CG12334		1.047
CG10126		0.984		CG10363	TEP4	1.047
CG1857	Necrotic	0.984		CG6794	Dorsal-related immunity factor	1.048
CG6717		0.985		CG3705	Astray	1.048
CG13837		0.985		CG5848	Cactus	1.049
CG2056		0.986		CG10910		1.051
CG10812	Drosomycin-5	0.986		CG17011		1.052
CG4428		0.987		CG16978		1.052
CG6877		0.987		CG4105	Cytochrome P450-4e3	1.053
CG13218		0.987		CG4432	PGRP-LC	1.054
CG10337		0.989		CG6822	Rhea	1.055
CG32672		0.990		CG3615		1.055
CG17104		0.990		CG14850		1.057

Gene	Name	Value		Gene	Name	Value
CG9598		1.059		CG6194		1.147
CG5730	Annexin IX	1.059		CG7106		1.159
CG7000		1.060		CG5436	Heat shock protein 68	1.159
CG11211		1.062		CG7250	Toll-6	1.163
CG18241	Toll-4	1.062		CG18096	Thiolester containing protein I	1.163
CG9697		1.062		CG12965		1.166
CG5750	immune deficiency	1.063		CG9976	Galactose-specific C-type lectin	1.166
CG12789		1.063		CG15098		1.169
CG4144	GNBP-2	1.065		CG7214		1.169
CG1643		1.066		CG18318		1.171
CG5399		1.068		CG15231	Immune induced molecule 4	1.172
CG9645		1.070		CG4181	Glutathione S transferase D2	1.172
CG11798	Charlatan	1.072		CG5576	Immune deficiency imd	1.175
CG4998		1.073		CG5848	Cactus	1.179
CG8087		1.073		CG14866		1.180
CG5909		1.073		CG3523		1.181
CG10641		1.074		CG9877		1.183
CG18106	Immune induced molecule 2	1.076		CG1252		1.186
CG4559	Imaginal disc growth factor 3	1.077		CG4716		1.190
CG16756		1.077		CG1865		1.196
CG13686		1.079		CG9454		1.201
CG14704	PGRP- LB	1.082		CG5246		1.205
CG10861		1.083		CG6667	Dorsal	1.205
CG4859	Matrix metalloproteinase 1	1.084		CG1594	Hopscotch	1.205
CG6895	GNBP-1	1.085		CG1656		1.210
CG18589		1.085		CG15493		1.211
CG5773		1.086		CG17799		1.216
CG9631		1.086		CG2217		1.216
CG9584		1.086		CG7445	Fightin	1.216
CG2826		1.091		CG9978		1.217
CG12111		1.091		CG15068		1.236
CG13429		1.092		CG4280	Croquemort	1.241
CG7228		1.093		CG14073		1.242
CG9334		1.093		CG6117	cAMP-dependent protein kinase	1.245
CG9649		1.093		CG5008	GNBP-3	1.251
CG5335		1.095		CG10967	Atg-1	1.255
CG9134		1.095		CG18239	Cyp12d1-d	1.265
CG6639		1.096		CG4345	Grim	1.268
CG11100		1.099		CG5909		1.285
CG10367	HMG Coenzyme A reductase	1.100		CG6489	Heat-shock-protein-70Bc	1.290
CG4472	Imaginal disc growth factor 1	1.101		CG15293		1.311
CG3348		1.101		CG3838		1.318
CG4257	D-STAT	1.102		CG1600		1.321
CG9453	Serine protease inhibitor 4	1.105		CG16713		1.322
CG5123	HID	1.107		CG5178	Actin 88F	1.346
CG4319	Reaper	1.110		CG12172	Serine protease inhibitor 43Aa	1.363
CG4437	PGRP-LF	1.114		CG1859		1.379
CG14993	Fumarylacetoacetase	1.116		CG16794		1.394
CG11992	Relish	1.117		CG11172		1.403
CG12780		1.118		CG4432	PGRP-LC	1.406
CG4384		1.123		CG7496		1.434
CG9456		1.126		CG12780		1.437
CG4376	Alpha actinin	1.128		CG7422		1.438
CG3962		1.128		CG1857	Necrotic	1.444
CG15404		1.129		CG4804		1.452
CG8369		1.130		CG14419		1.466
CG4665	Dihydropteridine reductase	1.130		CG16844	Immune induced molecule 3	1.469
CG3615		1.131		CG3201	Myosin light chain 2	1.485
CG18279	Immune induced molecule 10	1.131		CG13905		1.490
CG8343		1.132		CG16836		1.492
CG10118	Pale	1.137		CG17916	Odorant receptor 92a	1.510
CG6794	Dorsal-related immunity factor	1.138		CG5490	Toll	1.516
CG5008	GNBP-3	1.142		CG7285	Allatostatin C receptor 1	1.570
CG11842		1.143				

CG9568		1.621	CG7788	Ice	2.340
CG4463	Heat shock protein 23	1.650	CG7216	Adult cuticle protein 1	2.376
CG14481		1.668	CG7629		2.409
CG3879	Multi drug resistance 49	2.145			

Table 3-2: Supplementary figure showing gene expression during Destruxin injection: Microarray gene expression upon Destruxin injection. To construct the cDNA microarray, we selected 464 genes important for the Drosophila immune response based on a literature survey. CG Number, and Gene name (when available) are provided for spots on the cDNA microarray as well as normalized fold change resulting from Destruxin injection, compared to PBS-injected controls. The microarray experiments were done in triplicate and genes that had at least two readable spots were selected for analysis. Criteria of one standard deviation above or below the mean induction level of all genes on the array has been used to select for up or down-regulated genes respectively. The genes are presented in ascending order of destruxin-induced fold changes and are colored green if there is a statistically significant up-regulation, black if there is no significant effect and red if there is a significant down-regulation.

Chapter 4: General Discussion

Abstract:

Drosophila immune responses, particularly the humoral responses mediated through the Toll and IMD pathways, have been studied extensively. This thesis has made two contributions to the understanding of these immune responses. First, I have studied the transcription factors regulated by the Toll and IMD pathways: Dorsal, Dif, and Relish. Their absence or ectopic expression causes gene expression changes that have been studied using a microarray approach. These results give us important insights into the roles of this important class of transcription factors during an immune response. This also enabled us to identify target genes for assaying Toll pathway activation. Second, I have taken an entomopathogenic fungal toxin that is expressed during pathogenesis and characterized its ability to suppress these humoral immune response pathways in Drosophila. This toxin, a secondary metabolite called Destruxin, appears to be acting through the IMD pathway though by specifically mediating immunosuppression remains to be characterized. While these results together advance our understanding of Drosophila immunity, various open questions remain. This chapter will address how our results affect our broader understanding of immunity, some of the questions they raise, and future experiments which can be done to address these questions.

4.1 Discussion on Rel transcription factors

While many groups have extensively studied Rel proteins in both Drosophila and mammalian systems, this is the first time their individual effects on gene expression have been examined *in vivo*. The Rel proteins are so named because they share a Rel homology domain that is involved in DNA binding. Because of this conservation, the three Drosophila Rel proteins are known to bind very similar motifs. Considerable work has gone into determining how this group of closely related transcription factors mediates diverse transcriptional outcomes. *In vitro* evidence suggests that the Rel proteins dimerize, and different dimers have affinities for different DNA binding sites. These lines of evidence hint at a process, whereby genes are regulated based on the nature of Rel DNA-binding motif present in their regulatory regions. The identification of other conserved motifs such as R1 and GATA binding sites upstream of some of the AMPs (Uvell and Engstrom, 2003), and their requirement in wildtype induction of these peptides, suggests that the Rel proteins may need to interact with other partners to recruit the transcription complex. In this context, we decided to identify the genes that each Rel transcription factor was responsible for regulating. Such a study could provide insight into the distinct roles of Rel proteins in the innate immune response, thereby helping to answer how diverse transcriptional outcomes are mediated.

The initial use of the Affymetrix Drosophila GeneChip meant that the study was as comprehensive as possible. Over 13,500 genes are represented in the Affymetrix Drosophila GeneChips and covered almost all known genes at the time. During

infection of wildtype larvae with *E. coli*, 188 of these genes were significantly up-regulated. The effect of Rel mutations on these genes was examined. Some genes failed to induce to wildtype levels in the Rel mutant. We attributed this difference in expression to the main variable in the experiment-- the presence of the Rel mutation, and inferred that the Rel transcription factor was involved in the genes' wildtype induction. Genes were grouped based on the Rel mutation that affected it.

We were interested in studying the DNA binding motifs which may cause different genes to be regulated by different Rels. We examined a part of their regulatory regions, a 2 kb stretch immediately upstream of their transcription start site, using the Motif Alignment and Search Tool (MAST) algorithm (Appendix B). This approach however failed to identify unique differences in the Rel binding site that might cause one group of genes to be regulated differently from another. In fact, the highest scoring recurring motifs identified in these regulatory regions were not NF-κB or other known regulatory motifs. This may be because the sequences used are likely to consist of a mixture of some direct target genes of Rel and some indirect targets. This would introduce a measure of noise into the system, making it harder for MAST to identify repetitive NF-κB sequences as statistically significant. It is clear that their presence and repetition represent too much of a coincidence to appear purely by chance, but their function *in vivo* remains to be characterized. Other approaches involved a more direct search for NF-κB binding sites. Using motifs identified through *in vitro* SELEX studies and other data (Senger et al., 2004), these direct search techniques attempted at differentiating between a Dif/Relish, Relish, and

Dorsal binding sites. While these attempts were useful in the broad identification of NF-κB binding motifs, no observable correlation was identified between specific dimer motifs defined *in vitro* and groups of similarly regulated genes from our microarray data (data not shown). Ideally, knowledge of these motifs should be adequate to identify a Dif/Relish, Relish, or Dorsal site by scanning the Drosophila genome, but this seems beyond capabilities of present bioinformatic approaches. Further, the cellular transcriptional regulation is very likely to be a more complex phenomenon, involving multiple transcription factors and co-factors. Thus, small differences in one motif may not be sufficient to account for much of the regulatory diversity demonstrated by genes regulated by Rel proteins *in vivo*.

Our methods were however fairly successful at identifying broad NF-κB binding motifs. We went on to test these motifs for conservation between closely related Drosophila species. As new sequence data becomes public, it provides the ability to compare genomes for these conserved sites. Conservation suggests that some evolutionary mechanism discourages random mutations at those sites. In the case of putative transcription factor binding sites, this is further circumstantial evidence that the sites identified are indeed important for mediating normal gene expression. Future work however does need to be done to establish that these sites are biologically active NF-κB binding sites *in vivo*. To test this, initial Electrophoretic Mobility Shift Assays can be performed with the sequences and purified Rel proteins. Transgenic Drosophila lines can be constructed containing reporter genes fused to these putative NF-κB promoter elements. An assay of the reporter in specific tissues during NF-κB

activation can reveal its biological relevance *in vivo*. By looking at activation of these transgenes in Rel mutants, one can also dissect the role of individual Rel proteins. These experiments would therefore test the predictions generated by computational searches of NF-κB site, but broader questions remain regarding the biological role of Rel proteins.

Despite the need for more experiments, this thesis makes a significant contribution to the understanding of the role of Rel factors. In combination with data obtained from ectopic expression of Dorsal and Dif, it identifies genes that are affected when the Rels are absent or present ectopically. Dorsal and Dif upregulate distinct groups of immunity genes, though they are regulated by the same Toll pathway. Dorsal has a well characterized role during development. Some evidence suggests that Dif can act redundantly with Dorsal in larvae. But Dif mutant flies have no developmental defects suggesting that *Dif-* flies do not play a critical role in this process. On the other hand, *Dif* mutants fail to induce a variety of immune genes including *Drosomycin*, an important AMP in the humoral response. *dorsal* mutants do not appear compromised in their ability to induce any of the AMPs, and were therefore believed to not play a significant role during the immune response. Our work demonstrates that *dorsal* mutants do affect other genes believed to be involved in Drosophila immune responses, and that ectopic expression of Dorsal is sufficient to induce a number of immunity genes including many AMPs. Interestingly, some AMPs express to a higher than wildtype level in *dorsal* mutant larvae. This hints at a far broader role for Dorsal in immunity than previously believed.

In addition to the generation of these descriptive data sets, we also identified target genes that appear to be regulated specifically by Dif. We show that they induce faster and appear to be regulated more specifically than *Drosomycin*, making them a more favorable target gene. Much of what we know about the Toll pathways role during Drosophila immune response has been obtained through the study of a single target gene, *Drosomycin*. This target gene may not be particularly specific to the Toll pathway, and has been shown to be affected by the IMD pathway. Further, Drosomycin expression peaks up to 24 hours after infection, suggesting other regulatory processes may be taking place within that time window. The availability of target genes without some of these drawbacks, may therefore aid in the identification of novel components in the Toll pathway. For example, they could be used in genetic screens for defects in Toll activation. Certain components of the Toll pathway have not been identified yet, most notably the kinase responsible for phosphorylating Cactus. If multiple kinases act redundantly in a pathway, that usually hampers ones ability to identify it from a straight forward screen. However, if the Cactus kinase does not act in this way, and its identification so far has been impeded because of relatively non-specific induction of target genes such as Drosomycin, then these new targets may prove to be a big step towards the elucidation of this important signaling pathway. Specific targets can also help determine how recognition of different pathogenic determinants propagates through the signaling pathways into the activation of transcription factors.

An experimental approach to identifying target genes of Rel proteins involves Chromatin immunoprecipitation (ChIP). This experiment may be performed, to obtain a clearer understanding of the mechanisms involved by which Rel proteins regulate gene expression. Nobody has to date successfully published a CHIP assay *in vivo* using Drosophila, so protocols will need to be adapted from plant or tissue culture experiments. The quality of antibodies currently available for Dif and Relish also poses a challenge, and these issues will need to be addressed before this experiment can be performed succesfully. In the experiment, Drosophila would be subjected to infection, their proteins would be crosslinked to bind DNA and lysed. The DNA would be sheared using sonication, and antibodies against Dorsal, Dif, or Relish would be used separately to precipitate fragments of DNA bound by the three proteins. On the one hand, this may enable the identification of DNA binding motifs specific to the three transcription factors based on an analysis of the sequences precipitated. On the other, this method may provide another means of identifying genes regulated by specific Rel proteins. Usually, the amount of precipitate is not adequate for sequencing or cloning, and PCR amplification must instead be used to assay for the presence of a specific gene in the precipitate. Therefore data obtained from our studies using microarrays may be used as a starting point for selecting candidate genes to amplify and test in this manner. Some recent approaches have involved ChIP followed by amplification of the precipitate using random primers, and hybridization onto custom-made genomic microarrays. These elegant approaches may also provide important insights into Rel protein targets *in vivo*.

The understanding gained from this study of Rel proteins may also translate to applications in human health. Mammalian IL-1 and TLR signaling proceeds through signaling components that are remarkably conserved with flies and a study of Drosophila Rel proteins may be helpful to mammalian researchers studying the role of NF-κB. Mammalian NF-κB proteins are responsible for inducing a variety of genes of significant biomedical interest, including inflammatory cytokines, chemokines, interferons, MHC proteins, growth factors, cell adhesion molecules, and viral genes. Their misregulation has been linked to a number of diseases ranging including Alzheimers (Tanaka et al., 2002), asthma (Xiong et al., 2002), cancer (Rayet and Gelinas, 1999) and AIDS (Nabel and Baltimore, 1987). A greater understanding of the mechanisms by which NF-κB regulate different classes of genes, may help in the development of therapeutic strategies. Drugs are unlikely to be designed to directly target the NF-κB because they regulate too many critical biological processes. Downstream processes however, that are regulated by these factors and mediate some of these pathologies, could make effective targets. Thus the understanding of NF-κB/Rel factors has various potential applications for human health.

4.2 Discussion on Destruxin as an immunosuppressive

Studying a biological process can sometimes result in unforeseen biotechnological applications. Curing a disease, making a pest resistant crop, or engineering a pathogen that is more harmful to its intended victim are all examples of such biotechnology applications. In the case of Rel transcription, one may want to enhance or suppress the activity of the signaling pathways leading to their activation. Though it was not the original intended aim of the study, characterization of a fungal secondary metabolite called Destruxin A revealed that it could suppress IMD signaling and expression of various AMPs. Destruxins are increasingly gaining utility as lead compounds for the generation of antiviral and anti-cancer agents for human therapies (Chen et al., 1997; Pedras et al., 2002). Other groups have reported that their biological role may involve the manipulation of host signaling (Dumas et al., 1996).

Using cDNA microarrays, we tested the effect of non-lethal Destruxin A doses on Drosophila gene expression. Our results indicated that compared to other genes, antimicrobial humoral response genes were the largest group to be down-regulated. That this suppression is specific is suggested by Destruxins failure to inhibit metabolic and housekeeping genes. This was accompanied by an increased susceptibility of the Destruxin-injected flies to bacterial infection. There was no observed failure in phagocytosis or melanization reactions, leading us to believe that the susceptibility was caused as a result of a reduction in AMP expression. Further, *E. coli* and Destruxin co-injected flies had a greater proliferation of bacteria than flies

injected with *E. coli* alone. This observation hinted at the model wherein a reduction of AMPs caused by Destruxin allows greater *E. coli* proliferation, and results in Drosophila mortality. To test this model, we ectopically expressed components of the Toll or IMD pathways to see whether we this could rescue the susceptibility phenotype. Expression of various IMD pathway components including IMD and DmIKKβ rescued the mortality of *E. coli* and Destruxin coinjected flies observed in wildtype. In fact, the expression of the AMP Diptericin alone was sufficient for rescue. This confirmed our hypothesis that Destruxin's suppression of expression of some AMPs is mediated through the IMD pathway and that the loss of AMP expression was the most likely cause of increased mortality.

The mechanism by which Destruxin mediates this suppression remains to be characterized. Future experiments will need to address this mechanism. Destruxin's role in binding Calcium has been well studied in other insect systems, and it is believed to disturb cellular membrane potential at doses approximating physiological levels during fungal infection. Studying the role of intracellular calcium in mediating Drosophila immune signaling may well be the one most promising lead at this point, though no evidence of this exists so far. Use of a Calcium channel blocking drug could be used to determine whether this activity is enough to mimic Destruxins immunosuppressive effect. Suppression of host immune responses would have obvious benefits for an entomopathogen like *Metarhizium anisopliae*. Flies lacking the ability to produce AMPs due to to mutations in both the Toll and IMD pathways are extremely vulnerable to fungal challenge (Lemaitre et al., 1996; Tzou et al.,

2002), and these innate immune pathways are conserved between insects. Thus, the ability to reduce AMP production is likely to aid fungal survival in a variety of insect hosts. One idea for biological pest control could involve engineering fungi that overexpress Destruxin, though whether this might affect fungal fitness remains to be determined. The toxic and immunosuppressive effects of Destruxin might then work together to help eliminate the targeted insect species. Before one can implement this idea, further experiments need to be done on Destruxins toxicity and environmental impact. On the whole, fungal secondary metabolites remain an underexploited resource for interesting compounds with potentially useful activities.

In the past, it was a fungal metabolite, the antibiotic Penicillin that revolutionized the field of medicine. More recently Cyclosporin, another fungal compound, is used in human therapies as an immunosuppressive for its inhibitory activity on NFAT signaling. It is currently used during organ transplants to prevent tissue rejection (Herndon, 2000). Destruxins may therefore be serving a similar role with its ability to inhibit NF-κB type pathways. In future, I expect more experiments on compounds secreted by fungi will reveal fascinating chemical and biomedical properties.

4.3 Conclusion

Over the past 100 years, biology has made amazing progress using a reductionist approach. We began understanding complicated biological processes as interactions between various chemicals. We explored and characterized the smallest units of inheritance and now have a far better idea of how it happens. As technology improved, it gave us the ability to look at multiple proteins or multiple gene transcripts simultaneously resulting in mega-experiments that generate a lot of data. To make sense of all this data, an integrative systems biology approach is increasingly gaining center stage. Part of this ability rests on more recent advances in information technology. It is an attempt to understand and integrate vast amounts of genomic, proteomic, and metabolomic data and get working models that facilitate understanding of biological complexity and provide us with predictive power. The experiments described in this thesis fit into this larger narrative by giving us a genomic overview of the role of Rel/NF-κB proteins during Drosophila innate immune responses. In exploring gene expression in wild type flies, Rel mutants, flies ectopically expressing Rel proteins, or the effects of suppressing Rel activation through Destruxin injection, this thesis contributes towards a broader understanding of how these transcription factors and signaling pathways mediate innate immune reactions.

Appendix A: Construction of cDNA microarray

A.1 Introduction

Microarray technology enables researchers to look at the relative levels of large numbers of transcripts in one experiment. There are two main variants of this technology:

- **cDNA microarrays**: cDNAs of about 250-5000 bp in length are spotted in microscopic quantities using a robotic arm onto a solid surface, such as a poly-lysine coated glass slide (Ekins and Chu, 1999). Two RNA samples of interest (one experimental, and the other a control reference) are labeled using separate dyes (e.g. Cy3 and Cy5). When these labeled samples are hybridized on the microarray, the relative difference in the amount of the two dyes is used to detect the relative fold change in the transcript level.

- **Oligonucleotide microarray**: Oligonucleotides about 20-80 bp in length are synthesized on a chip using a photolithographic technique. Labeled sample DNA is hybridized on these microarrays to determine relative transcript abundance. This method is used by Affymetrix Inc., to create their GeneChip line of products.

In order to explore the effects of null mutations in Rel proteins on Drosophila gene expression during infection, we used the Affymetrix Drosophila GeneChip version 1.0 (Chapter 2). These commercially available arrays have several advantages: they represent over 14,100 transcripts covering almost all known Drosophila genes and they come with statistical tools and internal controls to facilitate robust statistical

analysis of the gene expression data. They are however very expensive. Each GeneChip can cost up to $300, making the cost of one experiment done in triplicate (necessary for statistical confidence) including labor and reagents easily exceed $2,000. DNA microarrays by contrast cost about $25 to print and hybridize (not including the labor and reagents required to make cDNA libraries), and approximately $185 per slide to hybridize (source: UMBI Microarray core facility). Thus, when it comes to array construction and hybridization, DNA microarrays are almost a third of the price of commercially available Affymetrix Drosophila GeneChip microarrays today.

There can be however, some potential disadvantages to manufacturing ones own cDNA microarrays. Among them are the various sources of systemic variation inherent in this technology that can contribute to noise when measuring gene expression. On the surface, the dye intensities should be directly proportional to the quantity of labeled transcript bound by it. Complications arise however, because the two fluorescent dyes Cy3 and Cy5 are known to label with differing efficiencies. Direct labeling chemistries (e.g. Amersham Biosciences Cyscribe First Strand Labelling Kit) are known to reduce some of this dye incorporation bias, when compared to the older indirect labeling method (e.g. Ambion's amino allyl cDNA labeling kit). At other times, using a flip-dye approach, where the control and experimental samples are labeled with the other dye can help identify and resolve this bias. Sometimes noise arises that can be correlated with spot-diameter variations or location on the microarray slide. To account for all these sources of variation from the "true" signal, considerable data normalization is often required. Normalization refers

to a mathematical process of removing these sources of noise, based on assumptions of what the data's distribution should look like. If the system is noisy, numerous replicates can aid in gaining greater confidence about the reproducibility of the results. Carefully designed in-built controls comprised of genes whose regulation has been well studied can also help greatly during data processing. The field of microarray data analysis is an evolving one and there is no standardized method for processing this kind of data. The responsibility of designing a microarray and a pipeline of statistical processes that provides meaningful gene expression data lies with the experimentalist. In other words, commercial microarrays come with guarantees and quality assurances (backed by a large corporation) and substantial research, while home-grown cDNA microarrays do not.

Despite their challenges, cDNA microarrays represented an attractive approach for us. Therefore, in order to pursue our research interest and examine the regulation of Drosophila immunity genes in greater detail, we decided to construct a DNA microarray. This would have the added advantage, of enabling us to do more experiments using a variety of conditions, with a focus on genes of our interest. From previous Affymetrix chip based microarray experiments and a survey of the literature, we selected 464 genes important for Drosophila immune responses. Most of these selected genes have been induced in response to infectious challenge, either in our own Affymetrix microarray experiments, or reported by other groups performing similar experiments in adult flies or S2 cell lines (Boutros et al., 2002; De Gregorio et al., 2002; Irving et al., 2001). Here we report the procedures used to amplify and

purify the unique DNA segments associated with these selected genes, and the construction of our cDNA microarrays.

A.2 Methods: Amplifying unique regions of selected genes

We used Primer3 (http://www-genome.wi.mit.edu/genome_software/other/primer3html) to design primers to amplify unique regions of the selected genes, generating fragments between 200-600 bp in length (Table A-1). Fragments were amplified from whole genomic DNA of wild-type, Oregon R strains of *Drosophila melanogaster* in a 96-well format.

The reaction mixture to produce each amplicon contained:

50 ng Drosophila genomic DNA

1 μM forward primer

1μM reverse primer

1xTitanium Taq (Invitrogen)

0.5 mM dNTP

The following PCR protocol was used: an initial 95ºC denaturation step for 5 minutes, followed by 20 cycles of 30 seconds of 95ºC denaturation, 30 seconds of 60ºC annealing, and 45 seconds of 75ºC extension. PCR products were run on agarose gels to confirm amplification success and specificity (Figure A-3). The products were subsequently purified using a QIAGEN 96-well DNA purification kit.

Five 96-well plates were used to handle all the samples, including some that were designed and amplified by Junlin Wu in our lab.

Printing, hybridization and scanning of slides were performed with an Affymetrix 417 Arrayer and 418 Scanner at the University of Maryland Biotechnology Institute's Microarray Core facility located at the Center for Biosystems Research. PCR products were spotted in triplicate on poly-lysine coated glass slides, with a mean spot diameter of 100 μm and a spot spacing of 375 μm. Following printing and cross-linking, slides were washed with 1% SDS to remove background, treated with blocking solution (0.2 M succinic anhydride, 0.05 M sodium borate, prepared in 1-methyl 2-pyrrolidinone) and washed with 95ºC water and 95% ethanol. After drying, slides were kept in the dark at room temperature. See http://www.umbi.umd.edu/~cab/macore/macorestart.htm for detailed protocols.

A.3 Data Normalization:

After hybridization of labeled samples onto the microarray, a scan for intensities in the Cy3 and Cy5 channels generates two image files. These image files were read using Spotfinder (TIGR), and the intensities of the spots were converted into numerical values that could be processed further. This raw data is likely to suffer from certain sources of noise (Figure A-1). A series of data normalizations therefore need to be performed to eliminate some of these biases. These statistical operations were performed using MIcroarray Data Analysis System (MIDAS) from The Institute

of Genomics Research (TIGR), and the following operations were used to normalize all cDNA microarray data described in this thesis.

First we performed a total intensity normalization to ensure that the total signal derived from both channels (Cy3 and Cy5) is equal. The assumption made here is that the total signal should be equal if equal quantities of labeled RNA were used, or if labeling efficiencies were optimal (Quackenbush, 2002).

For our microarray data, we used a Standard Deviation Normalization method to remove differences in variance between different block locations of the array. The assumption made here is that the variance within locations is supposed to approximate one another (Quackenbush, 2002; Yang et al., 2002). This normalization method is therefore meant to reduce position dependent noise (Figure A-2).

We also used a Lowess Normalization method to remove any differences in the spread of intensities between the Cy3 and the Cy5 channel. The assumption made here is that, on average, as intensities of Cy3 and Cy5 increase, the number of Red and Green spots should be roughly equal. Higher intensities should not correlate with upregulation (or down-regulation) of genes; in fact a higher intensity spot should be as likely to be an induction as a repression (Quackenbush, 2002; Yang, 2002). This normalization method is therefore meant to reduce intensity dependent noise (Figure A-2).

Raw data	Intensity plot	RI plot
	ChannelB Intensity vs. ChannelA Intensity	Log Intensity-Ratio vs. Log Intensity-Product
	- Total data point number: 1440	
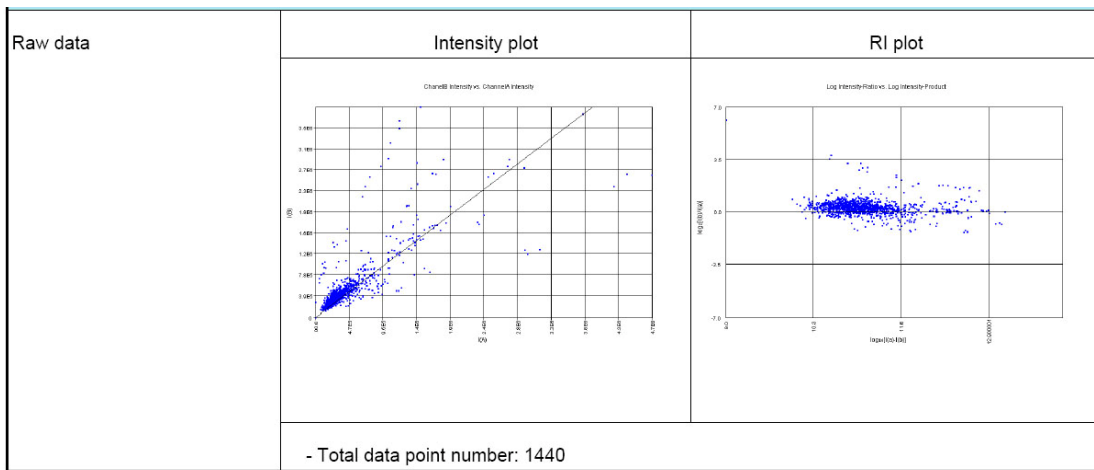
Figure A-1: Raw data derived from a cDNA microarray. The intensity plot demonstrates the intensities for each plot on the Cy3 and Cy5 labels, in the X and Y axis respectively. The Ratio Intensity (RI) plot shows the same data with the log of the difference between Cy3 and Cy5 (ratio) intensities on the Y-axis and intensity on the X-axis. This particular sample shows one microarray slide comparing a Destruxin injected sample to wildtype PBS injected controls.

Standard deviation regularization (Block mode)	pre-normalization Box plot	post-normalization Box plot

- Averaged block log-ratio SD: 0.41377607
- Pre-normalization slide log-ratio SD: 0.49835333
- Post-normalization slide log-ratio SD: 0.43469676

	pre-normalization RI plot	post-normalization RI plot
Lowess normalization	pre-normalization RI plot	post-normalization RI plot

- Processed data point number: 1198

Figure A-2: Standard deviation and Lowess Normalization. The standard deviation values of all the blocks in different locations of the cDNA microarray before and after normalization are shown at the Top. This process reduces variations in overall standard deviation between blocks. A Ratio-Intensity plot (before and after) the Standard Deviation Normalization and Lowess Normalization are shown at the bottom. The Lowess Normalization process removes intensity dependent biases in data, causing a more uniform spread.

Gene Name	Left Primer	Right Primer	Expected Size (in bp)
CG10812	TGCAGATCAAGTTCCTGTACCT	CTCGCACCAGCACTTCAGA	200
CG10810	AGTACTTGTTCGCCCTCTTCG	TTAGCATCCTTCGCACCAG	200
CG18106	TGTGGTAATCAACGGGGACT	CATTTTATTTGTAATTGGCGATG	200
CG10126	GCCGCAGAGTCGTTTGAATA	CATCCAGGTTTCCTCGACCT	200
CG14481	TGATCTCGACGGTTCAACG	GTGGCAACTGTTGAATCTGGT	200
CG9441	GAAGTGCACGTTCCACCAC	TCGAGACTCTGGTCGTAGCC	201
CG15493	TGTCAATGGCCATAAGGTTG	TCGTCGAACTCCTCGAACTT	201
CG9568	TCAACGAAACGAATGTCTGC	TGTTTAGCTCCGTCGTCAGAT	201
CG9616	CACCTTGTTTGCGTTATTGG	CGCTTTCCTTGCATTTCG	201
CG14302	TGCAGTCTTCAACCTGCTGT	GGGATAGATGGTCGACGTTT	202
CG16712	ACTGCCCGCTGGTATTGAT	TGAATATTTTGTAGAGCATAGAGGTCA	202
CG8825, CG8826 Glaikit	TCTATCCCAGCTACGGCAAC	CACCGACTGATCCTCCAGAT	203
CG3666	CGTCTCGTCACTGGACTCAA	CGTGGTGCAGAATACAATGG	204
CG6429	TTTTTCGTACTCGGGCTCAC	GTCGCCAGGTATGGTCAATC	204
CG3727	CTGGTACAAAGCTCGCAACA	GCCGGCTTATTACCCTCATT	204
CG14027 TotM	TGCTGGGAAAGGTAAATGCT	AACGCCTCAAGAGATCATGG	204
CG9928	TATAAACCTCGGTGGCGAAG	TTTGAGTCTCCTGCGGATCT	204
CG15066	GTAGTCGGTGGCCAATCCTA	CCACGCAATCCTTATTGTGA	205
CG6426	ATTGCGTGAACGATCCCTAC	GTCGATCTGCTGGAACGAAT	206
CG3879	CAACGTGGTCGAGGAGGTAT	GCGTCACACCATACCAGATG	206
CG14704	GCCCTACGTCATCATCCATC	TTGTACTTGGGTGCATGAGC	207
CG5974 pelle	ACTACCGCAGTCCCAACATC	GTGGGTTTTGTGCCTTATGC	207
CG14567	CATTGCCATGACTTTGTTGG	GGCTCCAAACGGTAGATTGA	208
CG7052	CTGGAGATCGGCTACCAGAG	CGCCACTCTCCTTCTGTTTC	208
CG12763	ACATGACCATGAAGCCCACT	TTTCCAGCTCGGTTCTGAGT	209
CG9681	TCCATCATTCGGACAATCCT	GCCAATGCTCTTGCGATTAT	209
CG18402	GGCCACCATCCTAACGACTA	TGTCCTCGTCGGAGCTACTT	209
PGRP-SB1	TCCATCATTCGGACAATCCT	GCCAATGCTCTTGCGATTAT	209
CG18030	ACCTGGTCAACAAGGTGGAG	CGTTGGTGTTGATGCAAATC	210
CG10842	GGATCGATACCGGGAGAACT	AGCGGTGCTTGAACTTTTGT	211
CG17738	GTCGTCGGTGGAAAAGGATA	CCTCCGTAGAAGGGTCTTCC	213
CG7267	CTCAACTCGCTGGTGGAGA	ATCTTTACGTCCGGCAGATG	214
CG13249	GATGGATCCGTTCAATCCAC	CGTCGTAGCGACATCTCTTG	215
CG12821	TTGTTCTTCCAGGCACACAG	CATGGATGCAATGCCATAAA	215
CG10861	TGGTTCCAAAAGTGAGAATCAA	AATTTGCTCGCTGGCATC	215
CG5778	AGTCTACACAGGTGGCAACG	TAAGACCGCCATCATCGTAG	215
CG16704	AATCTGTGGCGAGGAGTTTG	GGATAACAAAACCGAACTTCCA	216

CG12826	GTTAACGGACAACCGCCTTA	AAAGAAGTTGGGCACGAAGA	217
CG10039	TCGTCGTGGCCTTCTCTACT	ATCGTACTTCGGTGGCAGAG	217
CG7171	CTATTTGCTGGCGAAAAAGC	TGTCAATGGAGCTGAAGTCG	218
CG3365	AGCAGCACCAGCAGAATCTC	ATTGTTGGCCATCACAGGAG	218
CG6483	GAGGAACGACATTTCCCTGA	TGTTCTGGCATTGAGAGACG	218
CG15043	CCAGACAGCCAACACTTACG	GCCTGGTATGAGAAGTTCTTGG	219
CG12052	GGTCAATATCTCGCAGGATCA	ACATCGCCAGACGTATCCAT	219
CG18337	CCAAGCACAACCGAAGAAAT	GTCCGTAGTTGCCATGACCT	219
Mlc2	CCGATCAACTTCACCCAGTT	TCTTGTCGTCGATCACCATC	220
CG15282	TCGTTTTTGTGGCCCTTATC	AAGAAGCCTCCTTGTTGCTG	220
CG11501	GGAGCTGGTGGAATTCAAGA	CGTACTGCTGGTTCCTCCTC	221
CG18372	TACTCCCACATCAACGGACA	TAACCGAAGTGGTGGGAAAG	221
CG10641	GAGGTGGACGAGGATAACGA	CGAATCTCGTCGTGGAACTT	221
CG9434	AGTGGAATCCAAATGGCAAC	ATCCTCGGTGGTCAACTCAG	221
CG15098	CCGGCGAACTGAAGTAAAAT	AGAATCTTCCGATCCCTTGG	221
CG32672	TGAGAATGTTTACGGCATGG	GCCCTGCGTATCAGATCAAT	221
CG17916	TGGGATCGGAAATCTTTGAG	CTTCGTAGGCCTCCAAATCA	222
CG14993	TATGTTCCGTGGACCAGACA	CCAGCTGATTTCCCTTACCA	224
fln	AAGATCCATGGGGTTTCGAC	CACCGGTTTCCTGTAACCAT	224
CG4199	GTCGTGAAAACGTCCCACTT	AAATTGGGTGTTGAGCTTGG	224
CG8913	ATCTTCATGCGCAATCACAA	ACCGCAAACTCATTGGAGAC	224
CG5493	CTATCAGCCGCTGGAGAAAC	AACTTTCCATTGCCAGCATC	225
CG3348	GCTGCCAGGGTCTAGATGAG	TTGTGGACACCGATTGACTG	225
CG4144	CCCGGCTCTAATGGACTACA	CACTCGCCCTCTCCTGTAAG	225
CG10337	GTAGCGTTCTGAGCGGTCTT	CTCCGGAGTTCGTAATTTGC	226
CG3066	ATACCAGCAAGGACGTGGAC	AGAGCTCCCCGGTATTGATT	228
CG2081	GGAACTCTGGAGCAGACCAA	TTCCGCAATAAACTCCATTTG	228
CG5915	GCCCAACTCGTTCAAGAATC	ACTTGAAACGCCATCTCCAC	228
CG8238	CGGGTGGTGAGTTCCATACT	TCTCGTTAATCCACGGAACC	228
upheld (troponin T)	CCAGAGTTCATCAAGCGTCA	GCTTCTTCTCCTCAGCCTCA	229
CG7710	ACATCTATGCCGAGCCCAAT	AGAGTGCGAAAGTTGGCTGT	229
CG14499	TGGGCGGAAGTGAAGATTAC	TGCGTCTTGAAGATTTGCAG	229
CG17814	TTGATCTGCCTTGCCTTCTT	CCATGAAACGCACTCCTTCT	231
CG4269	CGTACTCAGTTCGGATGTCG	CCAGTCCTTGATGCCATCTT	232
CG16705	CTGGGGACTTACCGAGAACA	GCCGGCAATGTAGAAGACAT	232
CG7986	ACTGCTGGCGAGGTACAGAT	TAAACGACAGCGAAACGATG	232
CG5576 imd	GAACCTGTTGCCCACAATCT	AATGCACCACATCCATTGAA	233
CG14132	CAAGTGCTTCGACAAGCTGA	TAAAGTCCTTGCCGGTCTTG	233
CG16978	TTACTGACCGACACGATCCA	TCATGAACTCTTCGGCACTG	235
CG18279	AAATTTAGGGCCCAATGGAG	CCACATGGAACTGTGGACTG	236
CG4665	CGATCTCATGTGGAAGCAGA	ACGGGAAGTATGGACACAGC	237

CG5773	GATGGTTTCCAGAGCCAGAC	GCACCTGGTTGTTCCTCATT	238
CG4472	TACTGGGCTTGCTCAGTGTG	GGCGGTGATGTCCTTGTAGT	239
CG17105	GGTATACACGGTGGCTTTGG	TAAGCACCCGGACTGTAACC	239
CG15404	ATGGTTGCCAAGATTCTGCT	ATTTCTCTTGTCGCGCACTT	240
CG11841	GTGTGGATGCGAATGATGAG	CCCTTGATCCAGTTGAGGAA	241
CG6361	GCCGTATCAAAGATCCTGCT	CGAGGAGATTACGCCTACGA	243
CG16926	CGACAAGCTGATGAACTCCA	TTACCAAAGTGTTATGTGCGTTT	243
Actin 88F	TGATGCGGGTGCATTAGTTA	CTTCTCCATGTCGTCCCAGT	244
CG8215	AATGCCTGGATGGAAGACAG	CTCCTCGCAATCCATATCGT	246
CG4466	GACTGGGTCGTCGTCGTTAT	AAGTGACGCTGGATCATTCC	247
CG4437	TTACCCGACCCTATTGGTTG	ACAGCTGTGATCCCAACCTC	248
CG7788	TCAACCATGAGCACTTCGAG	CTGTGTGTCCTTGGCGTAGA	248
CG8091	ATGGTGGGGATAGTGCCATA	TCTGCACATACGACGAGGAG	249
CG7629	AGCCAATCTCTTCCAGAGCA	AGGTGATGATTGGCACTTCC	249
CG15422	TCCATGTGGCTATCCTCCTC	TGATACTCGTACGCCTGCTG	249
CG5413	GACCCCTCTTCCGGTTACTC	GTCCAAATCGGTTAGCAGGA	249
CG8157	TCAAGGAGGATTTGGTGGTC	CTCCATGGTGTCTGTGGTGT	251
CG11992	GCCATCCAGACGATACAGGT	ACGGTTGGCACTCCATTAAG	251
CG15423	GGTGGAAGGCCAATTGTAGA	TGCTGAACGATGATGACCTC	254
CG18589	AAAGGGTCATGTGGAGTTCG	TTCGCAGAGTTTGTGACTGG	255
CG4096	AGGATGTGGATCAGGAGTCG	CTGGGTATCGTGTTCCGAGT	255
CG5550	AAGAAGAGCGGTGTCCAGAA	CAGCAAAGTCCACCAACTCA	256
CG4740	CAACGGTGTTCACAATCTGG	AGGGTCCACTTGTCCACTTG	257
CG1021	ATTTCTTGGGTCCACAGCAG	GATGGACTCCTTGGTGCATT	257
CG1643	TTTTCGATTCCAGCAAATCC	CGCAACTAAAGTCGGCAGAT	258
CG9733	GGATACCGGCTATGTGAGGA	CTGGCCATCGTAAATCCTGT	259
CG7863	ACCACTACGACGACCACCTC	TTGAGCTGTTTGCAGTGGAC	259
CG7635	CGCCATCTTCATATGCTTCA	ACCTGGATGACCGCATAAAG	261
CG12780	TCCGGTTATCAAGTCCCACT	GGCCATTATACCGCACTGTT	262
CG18525	TGCGCAGTGCCTATATTCTG	GGAGTGATTTCATCGGCACT	262
CG1576	GCCTTCAGAAGCTGGACATC	CTCCTGCATCTTACGCATCA	263
CG7695	CTCATCTGTTTGGGATGCAA	CGCACAGACTGGGTCATAGA	264
CG7738	TACGCCTTGGATGATTCTGA	CTATCGGCCATACCAACCAC	264
CG17107	TTCTAATCTTCGCCCTGGTG	TATCCATGTCTGCCATGTCC	265
CG11709	GTTCGATTCGGATCTCCTTG	TGATGATACGCCTGCATGTT	266
CG13422	TCAACTCACCGAAGGGATTC	CTGCCCAACCACATACCTCT	266
CG6667	GATCTGAATTCGGTGCGATT	AAAGGCCTCCCAAACACTCT	267
CG7216	GTCGCCGTTATCTTCACCTT	GACGACTGAGGGAGCATGAG	267
CG3505	TAGCCATCGAGGAGTTGCTT	GCTGGCGTACTTCCTTTGAC	269
CG6134 spz	AAGTATCGGCCACCACAATC	TTTTGGGTACACCAGCTTCC	270
CG4105	GGCTGGGTCATGGTCTTCTA	ATGGAGGAGTCACGCTGTTC	271
CG14762	TTGTGATGGACCAGGTGCT	CTGTTGTGGATGGTCAGGTG	274

CG4319	CGGGAGTCACAGTGGAGATT	TGGCTCTGTGTCCTTGACTG	274
CG9336	CGGGGACATCAACAATATCC	AACCATTCGTCGTGCATTTA	275
CG4757 GH05741	CTGGGAGATTTGCGATTTGT	ATACCAACACGCAATCACGA	277
CG13135	CATCGATTGCACAGATACCG	TTGGCTCCTCCTATTGTTCC	277
CG6898	TTCTCATGTGCTGTGGCTTC	TTGTTGCTGTGGCTTGGTAG	278
CG2217	TCAAGGAGGCCATTATCGAC	GGCCTGAATGATTTCCAAGA	278
CG6449	CCGATGATGATGACAACGAC	AAGTGCCAGGTCCATCATTC	278
CG11529	CTCGGACTCGATGCAGTACA	GGCTACCAATCTTGCTCTCG	278
CG4183	TACGAGCTTGGACTGGGATT	ACCAAGATGGAGTCGTCCAC	281
CG3615	TCGTCTGGCTACTTGCCTTT	CCAGATCCGAATCCTCGATA	282
CG3615	TCGTCTGGCTACTTGCCTTT	CCAGATCCGAATCCTCGATA	282
CG7496	AACGGAGCCATAGACAGCAT	CCCGTTCTTCGAAGTTACCA	283
CG9080	AGATCACGCTGGAAGATGCT	TTTACGTCCCCAACTGGAAC	284
CG11825	ATTCGAAACCGACGAAGATG	ATTCGTCTGGCCTGGTATTG	284
CG6906	AATGACGAACAACGGTCACA	GAAAGAAGAAGGCCATCACG	284
CG9877	TCGTTGTGATTTTCGCACTC	TTGAATTGAACAGGCTTAGGG	286
CG6639	AAACCGAACTTGTGGTCAGG	AGGACGGTGGAGTATCGTTG	286
CG7850 puc	GGAACGGGGTAAATCCAAGT	AGCAGATTTGGCTTGCTGAT	288
CG13245	TCCCACATCCGTTTCCTAAC	TAAGACCGCATTTCCTCCTG	288
CG13845	TCCCACATCCGTTTCCTAAC	TAAGACCGCATTTCCTCCTG	288
CG8087	CATTGACTGCACCACCAATC	CACGTTGACACGTCTGGAGT	289
CG2444	AGTGTGTGCTGGACACGAAC	TGCCCATTGCCTCCTAGTTA	289
CG17061	GCGGCAGGTCTTTAACTCTG	TATGTCGGAACCATGCAGAA	291
CG15306	AGCCTACCAGCTGAGGTCAA	CTCGTCCTGGTTAGCTTTGC	294
CG7279	ATCACCTGCCACCTCGTTAC	ATGAATTTTCCCCGGATTTC	295
CG4432	GAATCGTCGCAGAAGTGTGA	CCGCCTTAAAATCGGTTACA	295
CG5399	ACGTGGCTTTCTGGAACAAC	ACGCCTTCCGGAGTATAGGT	295
CG18563	AAGTGAGCCCCAAAACAATG	CGGCCCTAACCACAATTCTA	296
CG3132	GCTCTACGAGACCTGGTTGC	GTGGGAATTGGGTCATGTTC	296
CG6489	ACAAGGGTCAGATCCACGAC	TAGTCTGCTTGCACGGAATG	297
CG14850	GTCGCTTGCCTATTGAGGAG	TTGTTTGAAGTGGGTGTGGA	299
CG1252	ACAACAAGGGACAGGTGGAG	CAGTCTTGACCACAGCAGGA	300
CG13461	CCAGCTGAGGATGAGGAGTC	AATCTGGTTGTTGGGTGGAG	300
CG4267	GGAGCAAGACTTCCACGAAC	TGGTTTGAATGGACTCCACA	303
CG4716	CCAGCTGCGGTAGAAGAAAC	GCTGGTGTTGTCCTTGTCCT	303
CG5370	GGAACGATATGCAAGCGAAT	CGTATCCTTGGCGTACAGGT	304
CG15067	GCTCCCATGATCAAACGACT	GGAATCGATGGTGGTTATGC	305
CG5848	CGACGAAGAAGAGGATCAGG	TCCCAGGCGTTCATGATATT	306
CG5123	TCGCCAAGCGTTTTAGTTCT	ATCCCCTTGCTCACTTGATG	307
CG3604	TAGTATTCGCGGCTGTTCTG	GGAAACCGCAGACTTTACCA	308
CG5514	GAGCGAAAGAACTCGGTGTC	CGGTGGCTCAATTGTATGTG	309

CG15046	TACTTCCACCACAACCACCA	AGATTGAGCGGACAGCAGAT	312
CG13218	TGAGGTGCGATCTTCAAGTG	AGCTTTCCTTGTAGCGTCCA	312
CG6457	GATCCAGACTTCGTCCGTGT	ATGAGGACACCATCGAGAGC	312
CG18550	GATTTGAGATTCCGCAGAGC	CTGGCCATAGGATGGAAGAA	313
CG7294	GATTCGGACGACCAGGATT	GTAGAAACCACCACCGCTTG	317
CG1241	GCTGTGGTGGAGGACATTTT	AGGGCGTTATTGTGGTTCAG	317
CG10035	TCCTGGTGCCCACTAATACC	CACTCGTTCCGGAGTCATCT	320
CG9453	CTCAGAGCGTGGACTTTTCC	GTCCTTGTAGGGCAGTTCCA	320
CG9584	GACTCGGAGGCACTTACTCG	TAGGCGATCCGTAGAGTGCT	321
CG18067	GGTCACGGTGGGAGCTATAA	CGTCCACCTATCTTGGCAAT	323
CG11459	GGGAAAGGTTGCATTCAAGA	AGGGAACACAGTCCACCAAG	326
CG11100	CTTTAACGAGTCCGCCTCAG	GGCATCCAACTCCTCCACTA	328
CG6829	GGAGACCGTGCTAGAGATGC	CTTAACCTCATCCGGCGTAA	328
Hsp23	AAAGGATGGCTTCCAGGTCT	CCTCCTTGGGATTCTCCTTC	329
CG18316	CCGCGTAAAATAAAGGGTGA	TTGGGCACTTTTGAAGAACC	331
CG13324	ATGCGTCTTCTGCTCATCCT	TAACGACCCCAGATCTCGAC	338
CG5730	TCTGCACGCTGTCCAACTAC	CTTGATGGCCTTCTCGATGT	338
CG13323	ATGCGTCTTCTGCTCATCCT	TAACGACCCCAGATCTCGAC	338
CG3705	ATGGCCATAATCTGCTGACC	CTCCTGGATGAAATCCCTCA	341
CG6531	TGGCCACCACTTAAACACAA	ATGGTGTCCGTGTGTAGCTG	345
CG4427	GAAATAGCGGTTCCCAACAA	TGGTGTCTCGGGTGTTTGTA	348
CG2056	CTAAAGGTGCCGCTGAAAAG	GATCGAATTCGGGACTGAAA	349
CG6794	AGCCAAGTGCAAGAAGTGGT	AGCTTCTTGGCGCACTGTAT	352
CG6877	GCACACTCCAGATAGCGACA	CTGCGAGACATCCTCGTACA	352
CG11765	CTGGGTGGTGCTCTTCTCTC	GGACATGGGGTAGAACATGG	358
CG4428	GGCTATGTGGACGATGAGGT	AAAGAGTCCGAATCCGAGGT	362
CG14438	GAGCAAGACTTCCCAACAGC	ACGTCATTGTGTCGTCCAAA	363
CG8871	CAAGCATTCCAACGCTGATA	TTGGAGGAGACGAAGTTGCT	363
CG12505	AGGAGTTCATCGGCAACATC	GATGTGCTTGCGGTACTTGA	365
CG11299	CTCACCAAGGGGAAGAACAG	TATCCGTTAAGGGTCGTTGC	365
CG11798	GTGCACATCCAGAAGCGTTA	ATACTGCTCGAGGTGGGATG	365
CG14419	AAAGCCCAAGTCAGAGTCCA	CAGTTCCGATAGTGCCGATT	370
CG30080	CGTTCAACATTAGGGGCTGT	TGATTGCCAGTGAGAGTTCG	370
CG7219	TTAAGGCATTCTGGGAAACG	CATTCGCTGCATGACTGTCT	371
CG6194	TCATGAACGCCTTCAGTCAG	AGGGCTGGATACAAGGAGGT	376
CG6524	ACAGCGATCTTACGGTCAGG	TGGTTCTCTGGGACGAAAAG	378
CG1857	GGACCCAGAACAATCAGGAA	GCTCGCGGTTGTAGTAGACC	378
CG7214	GCCGTAGTCATGTTCGTGTG	GGAGGTGGCAGAGTTGATGT	381
CG14745	ACCACACCGCTGGAAACTAC	CGGATCTCGTTCCAGATGTT	382
CG6117	GTCGGCGTACAAAATTCCAT	CTCCTCGTCATCGGTTTCAT	394
CG4998	CGGGATAAGCTGTGTGGATT	GGTGGCTTCGTGTAGGTGAT	401
CG6528	TCATCTGTGTGCCCGTTAAA	GTAGGCCACGATTAGGGTCA	404

CG5909	CGAGGAGTACGGCATAGAGC	CTGGACCACTCGGTAGGTGT	406
CG14852	ATGGAAGCACAACGCCTACT	CTGTTATTGCGACGGTTCCT	406
CG18239	ATATCGACGCCCACCTACAG	CTCATGGTTCCAAAGCCATT	416
CG1102	GGCGGATCTCTAATCAACCA	TCCAACTCAGCCTTCAGCTT	419
CG9414	AATGGTCCACTTCCTTGTGC	GGACTTCCATCAGCTCTTGC	423
CG9645	TGGACGCCCTCCTACATATC	CGCTGAAGACTTTTCCCTTG	431
CG5490	CTAACCATCCAGCCCAAAGA	GCCTCAGCTTATTGCTACCG	433
CG1093	GCTGACCGAAAGAGACGAAC	GCATGGTGCTGTGGTAATTG	439
CG4181	CAATCCACAGCACACCATTC	TCTCATCCCATCCTGGAGTC	449
CG15154	CAGCAGTTCCGTGAGCAATA	GTCCATCTTGCCCCAGTAGA	463
CG11842	ATTTAGCTACCCGGCGATTT	GCTGCTGCTTAATCCAGTCC	470
CG12334	TTCGAACCGTATTCCAGTCC	TTGAGCGAGTAGTGCCAATG	471
CG10910	TTCAGCTCAGTGCGTCCTTA	ATGGGCCTTGAGTAGTGGTG	472
CG9649	GAGGAGCCAGTTCAAAGACG	AGTGTAGCTCCCGACGAAAA	473
CG4559	GGCAGTTGGACAAAATCGTT	GGAAATCGTCTTGGGTCAGA	476
CG11819	CCTTGAGTGCGGAGAAGAAC	CTACACTCTCCGGCACTTCC	480
CG5489	CATTCCGCTATAGGCACCAT	AGGCAGCATAGTTTCGCATT	481
CG3962	CCAACTTCCTCAAGGAGCAG	CGTGTTGTGGTACTCCATGC	482
CG5246	AATCATCATTTGGGCCATGT	GAGACTGGCAGTTGTCGTGA	496
CG7503	CCTTTCGCTCCTGCTAATTG	TCACAATGCTGCTCAGGTTC	503
CG4859	CTTCTATCGCGGCTTTGAAC	AACTTGCTGCCCTTGAAGAA	519
CG3359	CTGGACCTCACCTTCTGCTC	GACGCACACTAGATCCAGCA	520
CG2275	ACTCACCGGATCTGTCATCC	CTAGCCAGGTCGACGTTCTC	526
CG13905	AACTTTGCCTTGGAGCTGAA	GGATAAGGGCGTCTACACCA	529
CG7876	TGGTCTGGTCTTCAGCTCCT	GTGTTGGGCTCATCGGTACT	531
CG10118	TCTCACCGAGGACGAGATTT	CCAGGTCTTGACCTCCACAT	532
CG10967	GCAGCCAATTAGCGTAAAGC	TCAGCTTGGACAATGTCTCG	536
CG6675	GATATTTTGGCAAGCCTGGA	CTGAGCTCCCAAGGAGTGAC	537
alpha actinin	GATTGTGAAGCAGCACCAGA	TGTCGAAGTGGTTGAAGCTG	548
CG5429	GGCACTGGTTTTATGCTGGT	CATGCCAGATGTGAAAGGTG	550
CG13805	AGAGGTCCCTTCAGCTGTCA	AGGATCTCAAAGTCCGAGCA	551
CG11331	GAGAACCAGCTCCACGAGAC	CAGCGTCACCTTCACTTTCA	552
CG17104	GTGCAACAGCTTGAGATGGA	ACGATAGGAGCTGCATTGCT	554
CG15293 BG:DS01759.2	TTTCCGAGATTTCCGAATTG	TGAGATCCTTCGTTGGTTCC	560
CG2045	ACAATGGGCAGGAATCTCAC	CCAGTCCATGTAGCTGCTGA	621
CG5436	GCAAGCAGTCCAAGACCTTC	ACCCTTATAACCGCCAGCTT	626
CG6277	AAGTCCATTGATGCCTCCAG	TCCTCGTAGTCTCCGCACTT	632
CG10872	GGATCGCCCTTTCAGATACA	TATAGTCGGACGGGTTGAGG	634
CG8678	GGAGATATTCGCCAAGGACA	CTTTCAGCTCAACCGTCTCC	659
CG9631	CCACCTCAACGCCAATTAGT	CCGCTTCATCTCCTTGAGAC	665
CG3838	TTTGGAGGGGAACAACAGTC	GACGTTGCTGTGGAGTGAGA	680

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CG10383	CACTGCTCGACAGGATTTCA	TGCTAAATCTGAGGGCGACT	681
CG10198 Nup98	CACCAATGTTTTTGGCAGTG	GGTGTGGCACCAAAAAGAGT	702
CG1600	ATCCCGATCACCATAATCCA	CTCCAGTTGTGGCAAAGTGA	702
CG5819	CTCAAGCATTGGTGCAAAGA	CTTTAGGCGCTTCAACTTGG	706
CG6271	CGCCACTACCAAGTCCATTT	ACGGGCACATAGAAGTCACC	738
CG3523	TCGATTTGAGCAACAACAGC	GCAGGAAACACTGGAGAAGC	758
CG17278	GCATCCATCCATCCCAATAC	TCAGAATTCCGATTCCGTTC	773
CG4823	GGAGCCTTTAAGTGCGAGTG	AACAGGCTCCACATCCATTC	804
CG10363	TTGGACACTCGCATCAAGAG	CTTGTTGGAAACGGTCACCT	822
CG10697	AGTGAAGCCTGGCTACCTGA	CGAGCAGTCAAAGTTCACCA	838
CG13095	CAGCTGTCCAACTCGATGAA	AAGTCGGTGCCCATGTACTC	854
CG6687	GCCCTCCTTCCCGTAGTAAC	TAACGGAATCGGCATTTAGC	868
CG10367	CCCTGCTGTTAGACGGAGAG	ACGATCTGTGCCAGCTTCTT	895
CG14073	CTGCCAAACAGTCAGCGATA	GTGGAACCGGTTGGCTACTA	925
CG9598	GGTCACCACCGAGTTGAGTT	GTGGTGTCAACGATGTCCTG	953
CG4280	CCTATGAAAGCTGGCTGGAG	GCTTCATTCCACTGACAGCA	963
CG15126	CACCACCACACGGAGGAC	ATGAAATTGTGCAGCAAAGG	162
CG1365, Cecropin A1	CTCTCATTCTGGCCATCACC	CTTGTTGAGCGATTCCCAGT	192
CG15068	CTTTTGGCTTTGGCTAGTGg	GCCGCGTATATTGCAAACTT	163
CG1367, Cecropin A2	TCTTCGTTTTCGTCGCTCTC	GTGTGCTGACCAACACGTTC	162
CG16713	TCTGTGGTCTACCCCATTCC	TCTTCACAGATTTCCCTCGAA	163
CG16836	CGGAACTTTGATGCTGCTTT	TCCAAAGAAGATGGCTCCAG	253
CG1878, Cecropin B	TCGTCTTTGTGGCACTCATC	TCCCTGGTATGCTGACCAAT	164
CG8175, Metchnikowin	CCACCGAGCTAAGATGCAA	AATAAATTGGACCCGGTCTTG	170
CG8369	TGAAGTCTACGAGCCCGTTT	CTCGAATGctgcaaaaggat	178
CG16844	GCTAATACGCGGGAGTCAAG	GGAGTGGctgtgttggaaat	179
CG10332	GGTCGGCATTCAGATTCACT	TGGAGACCTGAACGGATTTC	181
CG1373	CATCAGTCGCTCAGTTTCCA	GCCAAGTTTCTTCAGCCAAC	183
CG18108	TGTGGCCAATGgtgagtaaa	TTTTTCGAATCCTTGGGTTG	187
CG15065	TTCAACCGTTGCCAACAATA	TACAATGACGGCAATCTCCA	191
CG1385, Cecropin C	GGATGCAACCATTCAAGGAC	AAGCTAGTTTATTTCTATGGCTGGT	193
CG14957	CTGCGCTAAAGGATCTGGAC	CGGTCTTGCAGCTTTTGATT	226
CG15231	CAGACCGACAACACCCAGTA	CGAAATTTGCACTGACTTTGA	237
CG12965	CGTTCAACATTAGGGGCTGT	TGATTGCCAGTGAGAGTTCG	370
CG8846, Thor	TTATCTACGAGCGGGCTTTC	CGGTTTTAGTGGGTGCATCT	437
CG5791	CGTGAGAGTCGATACGGTCA	CAGTGCTGACATCTGCCATT	443
CG16756	tcgcgttcgcttgattcta	caaaccttcgcacttggca	300
CG11159	agcatggccagtcacttg	tgaaacagaccgtagctga	300
CG9656	tgtgctgatgatagctggga	cagagggatacagaaccgt	310
LYSA	gctttcatcgttctggttg	atcagtttagcgatggacg	320

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CG11211	cttgaagctaaccatgtatgca	tatactctcttgtccagcc	400
CG12780	gttatcaagtcccactagc	tttgcaagccgcttagttta	400
CG12111	cacttaatgtaggtgaacttg	gcattccatcggagattgata	400
CG6266	tcctggcgaattggtggta	aggtaaggaggagatgatg	418
CG13422	ttggagccgttgacatcga	tcagttctcggcaaatcca	450
CG11709	acatcaacatcatgcagcc	ccacaaagttgccgataaag	450
CG9681	tcagctctatccgccaatg	atctcgttgtacagagcatc	450
CG13429	ctaacatgcgtcctcttgga	atgtggattgtcgtaactgg	458
CG7798	agttaccattagaagcctgc	ttaccgatgctcaacccga	480
CG16832	gattttgacattgccgcac	ctctcaataacaaataccga	480
CG14745	actgcatttccttagtgctc	acaatcgatccgcaaccatg	480
CG7496	cgaaaccttatgcaatgcac	ccagtcagttgacagacac	500
CG9978	ggcggttatacttgttacattc	cttaaacaagctactcgcg	550
CG17011	actgctcggcttgacatatg	cttctcgtgatgttctcg	550
CG17797--ACP29AB	AAAcactcatcataggacatc	acctcttatacctgttggca	554
CG9697	ccatctttcatcaacactcg	tacagttggctctcgttcta	600
CG8856	aataactggctcgcagggaa	Cctgta atgtgctggctac	600
CG1887	agtacgtttcgtgcttctc	ggtaacggctgctcaattca	600
CG12318	gattcaactagttccgtctga	gcatgatcttaatgggagcta	600
CG6127--SER	atggacttgcagttgatcga	gtgtactttcagtcgtccag	600
CG10363	actcccaccgtgactacaa	gacgacattgaactcgcca	600
CG17799	tcgatattcaggcccattg	cctcgaaatgtacaagtacg	620
CG13686	cttgtacctaatcgtcctg	ctacacagcttggcaaatga	650
SR-C1	ctgtagtctttcggtactg	gagcacacgatcagcattga	650
CG15378	atgctaaaatccgcaagtgc	agtggcatgggtaatcgta	650
CG9455	atccgtccaaagtgcattga	tacctgcttgaggtccac	660
CG5750	caaatgcggaatagcaacg	tctggctctatgtacaaggta	660
CG6717	cttgtgtaagcggtcaacga	gcagaatgatgcgcataga	660
CG2839	aactcggtggtcacattga	tgttcgaatgcgcgaccta	700
CG2826	gctgcactttccatccatca	tagcactgaatgttccagca	700
CG18318	atagtcgtcggttcggatgca	ccattgctgtggaagttgag	700
CG3858	gtatgttttgtgctctttggc	gacttcttggcgactgatc	700
CG7422	atctctgagaatgatcctgg	cccatatagttgtgaatgaaac	700
CG7000	agagatcgctggagaatgc	cgaagatcccgaagatctg	700
CG7219	tatgagtccgtagaagagc	ctgcagattcaggatttcga	720
CG9454	ccatttcagctaaccagca	agtgctggcctaaacatca	740
CG3410	gaacttactccttgttagcca	aactggctagatgccatac	750
CG13837	catcgatgaagtcagtctaca	cgctaggtgtacatgtttagc	750
CG18096--TEPI	tgaagtctcagtcagcctga	gacagcacaagacgttgaag	750
CG3212	gcattgcctattgcgatgg	aggtacgacagtcacattac	780
CG10956	atccagcatgctgttcatcta	tcctgaaactcaaaggtgac	780

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CG3801--ACP76A	ctgataagggaaatcgataga	atggttgactcggaagttg	800
OS	gagaacagagactatgtgc	accgcaacatgcaaacttac	800
CG7285	gtcagtaccagtcgagtga	ttgaactggacagcgctta	800
CG18241	cttctacgcttcagtcatca	tcgaaacaacaagagcacc	800
CG15358	acggaatatgcagaagctca	gtatttagcggccactctta	800
TEPII4	gctgtctcgctttgtgtga	agccgtaatctgggcatatg	800
CG14866	atggaccgctcagctatca	gatcttcttgatcttgttgtcc	800
CG11374	gtagtgttgccttggattgc	agcaagtgtatgagcgtgta	800
CG5528	gttcggcagcagttcattga	atggcatcgctaacactctg	820
CG18096	ctgacagcacaagacgttga	ctacggtctaacagcgctta	840

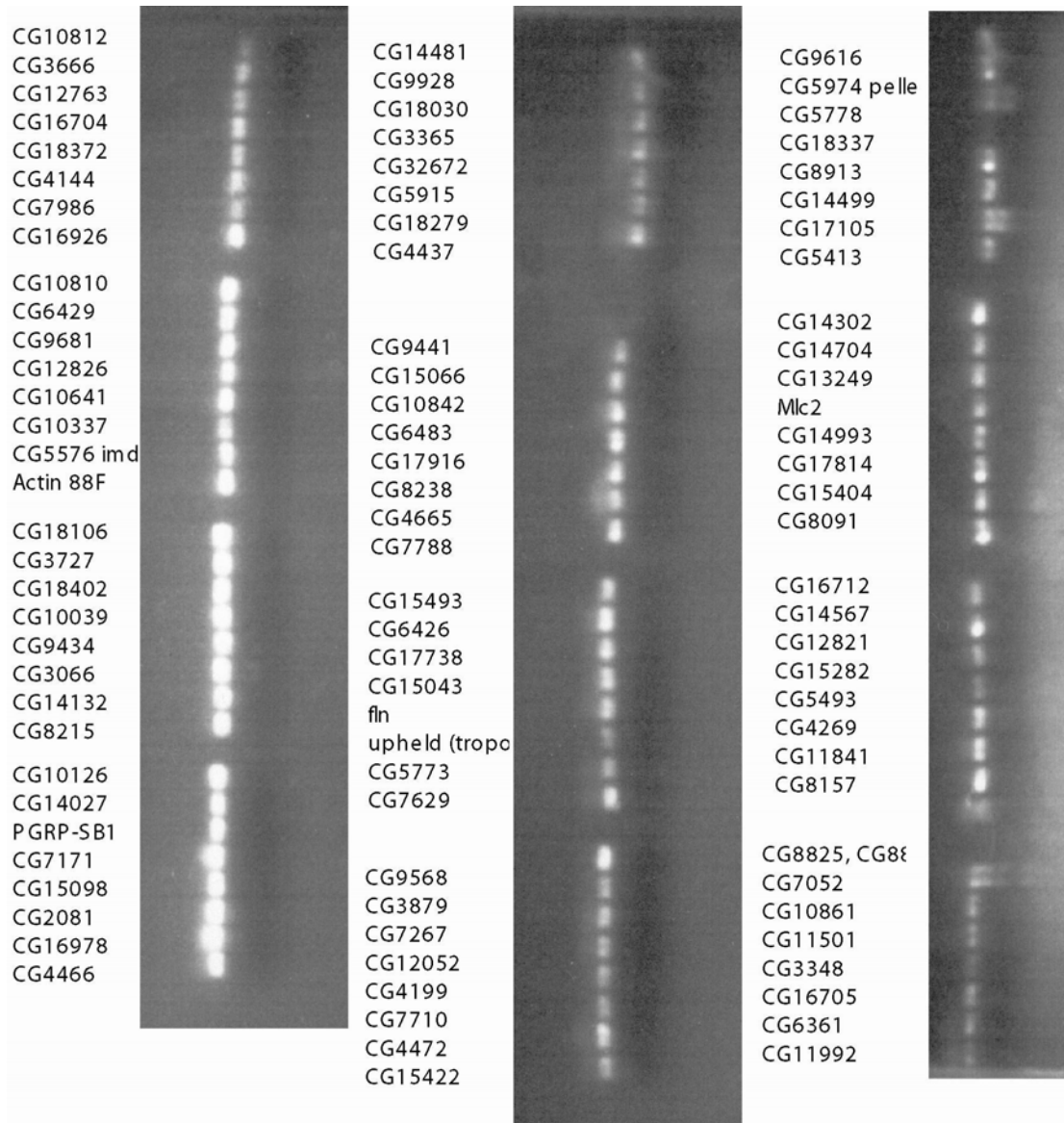Table A-1: Primers designed using Primer3 to amplify unique regions of selected genes

Figure A-3A: Plate 1 Showing PCR amplification of 96 genes CG10812-CG11992. Approximately 4 μl of PCR product was run on the gel. The remaining product was subsequently purified using a resin-based PCR purification kit before spotting on the glass slide.

Figure A-3B: Plate 2 Showing PCR amplification of genes CG15423-CG11798.
Approximately 4 μl of PCR product was run on the gel. The remaining product was subsequently purified using a resin-based PCR purification kit before spotting on the glass slide.

Figure A-3C: Plate 3 Showing PCR amplification of genes CG14419-CG14957. Approximately 4 μl of PCR product was run on the gel. The remaining product was subsequently purified using a resin-based PCR purification kit before spotting on the glass slide.
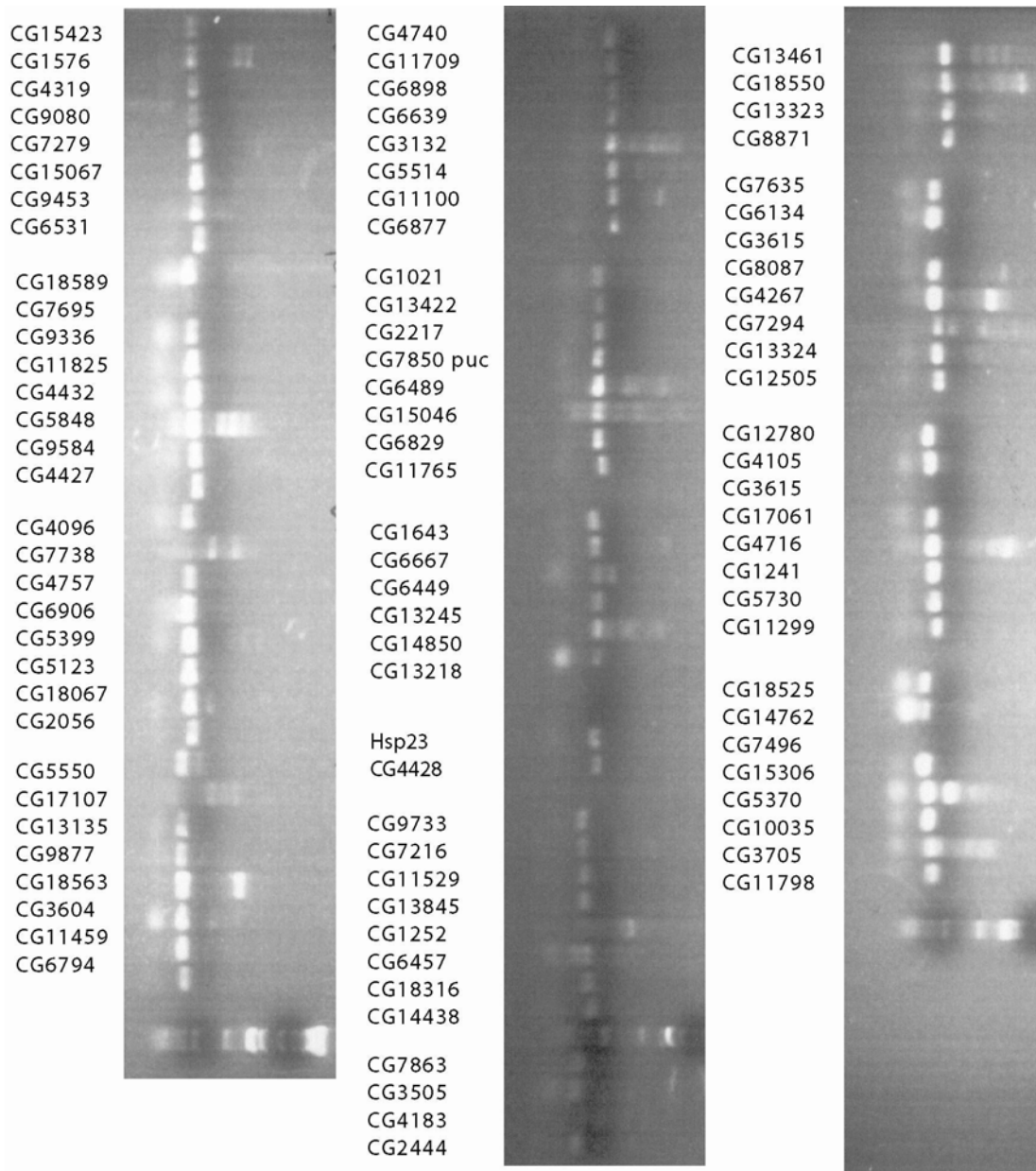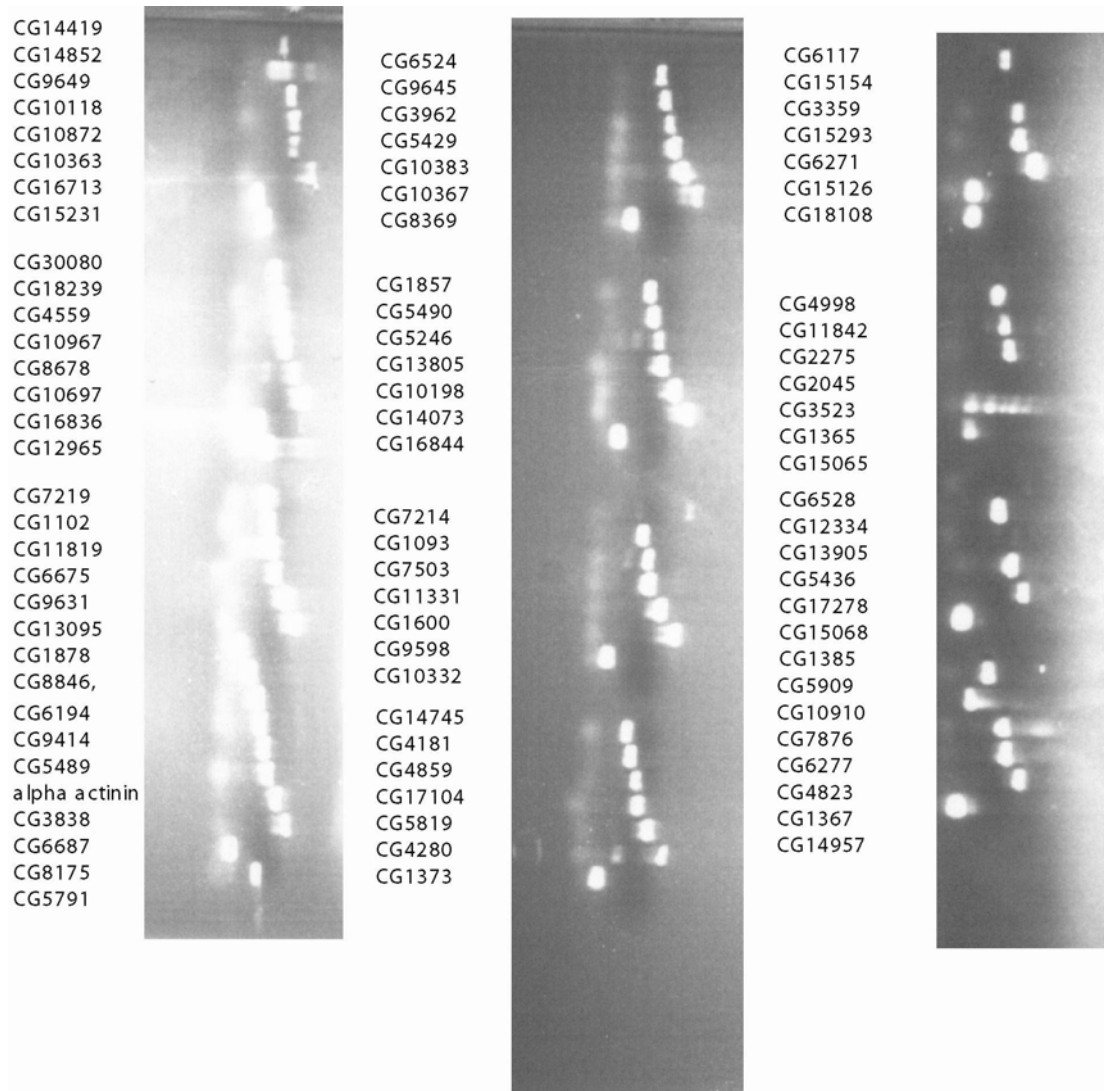
Appendix B: Identification of putative NF-κB binding sites

B.1 Introduction

The NF-κB/Rel transcription factors in Drosophila share significant structural similarity, particularly in the conserved Rel homology domain, used to bind DNA. Drosophila express three Rel proteins: Dorsal, Dif, and Relish, and they are believed to dimerize to initiate transcription of a wide variety of genes. These factors appear to be regulated by just two signal transduction pathways: Toll and imd. Antimicrobial peptides are an important class of proteins induced by these transcription factors, and these proteins fail to express in a fly mutant for both Toll and IMD pathway components. Further, there appears to be differential induction of AMPs depending on the pathogen responsible for infection, and the resulting pathways that have been activated (Lemaitre et al., 1997). This suggests that differential activation of the two pathways, and the three Rel proteins, can lead to diverse outcomes in gene expression. *In vitro* studies have shown that Dif and Relish together form the most potent heterodimer, capable of inducing Drosomycin, Defensin, and Attacin (Han and Ip, 1999). Expression of Dif alone induces Cecropin and Diptericin, while Dorsal or Relish did not have a remarkable effect on any of the AMPs by themselves.

The mechanism, by which this diversity of responses is mediated, might be due to different affinities for cis-acting NF-κB binding sites by different dimer combinations of Rel proteins. Using a SELEX based approach, unique 9-12 bp sequences preferentially bound by Dorsal or Dif/Relish, have been identified (Senger et al., 2004). These motifs have been identified upstream of many genes believed to be

regulated by Rel proteins, including AMP genes, which is further evidence for this direct regulation. Electrophoretic mobility shift assays represent some of the best evidence of direct DNA binding by Rel proteins, and the sequences that they have an affinity for *in vivo*. They have been used to demonstrate direct binding between certain Rel protein and these upstream regions, in the case of AMPs Diptericin and Cecropin (Petersen et al., 1995; Reichhart et al., 1992; Samakovlis et al., 1992). A variety of computational tools have been developed to help identify potential transcription factor binding sites within DNA sequence data. These algorithms tend to be good at finding statistically significant matches between the query consensus sequence of the transcription factor, and the genomic DNA search space. Finding TF-binding sites exclusively *in silico* however remains a challenging problem. Today, the understanding of NF-κB factors or TF-binding search algorithms is adequate to make good predictions as to the most likely binding sites, which need to be verified at the bench.

We identified genes that induced in wildtype flies during infection, but failed to be induced in the various Rel mutants (Chapter 2). The absence of a Rel causes a significant reduction in transcript levels of these affected genes post-infection, suggesting that the transcription factor may be responsible for induction of the gene during a normal wildtype response. We therefore decided to analyze the upstream binding sequences of all genes whose expression was affected in Rel mutants, in hopes of identifying the binding sites. In conjunction with the gene regulation data provided by the microarray experiments, presence of these sites would lead to a

hypothesis of direct interaction between transcription factor and DNA. Analysis of these sites could further help identify sequence difference between genes regulated by different Rel proteins, e.g. Dif vs. Dorsal. A characterization of these differences could also provide insight into the mechanism by which Relish, Dif, and Dorsal differentially induce different genes based on different upstream binding affinities.

B.2 Motif Alignment and Search Tool

In order to identify unique motifs present upstream of genes apparently regulated by individual Rel proteins, we used the Motif Alignment and Search Tool (MAST) (Bailey and Gribskov, 1998). The algorithm can be used at http://meme.sdsc.edu/meme/mast-intro.html. We let the algorithm search the input sequences for recurring motifs—sequences that appear repetitively. For example, we ran up to 2kb of upstream sequence from all genes that failed to induce significantly in the *dorsal* mutant (and that were induced in wildtype flies) during infection. This resulted in the algorithm identifying two unique motifs that appeared repetitively within this set of input sequences:

1. TTCCGCCCCCCAAGCCGCCCGCCGCCCCCACACCCA

2. GGTCCGGGGCGTGGGCCTCTGCTGACCGG

Figure B-1 shows a graphical depiction of the locations of these motifs in the upstream sequences for *dorsal*, *dif*, and *relish*. These motifs represent the best matches, and the likelihood of them matching by chance is extremely small (p<<0.01).

The NF-κB site is fairly well characterized and broadly has a generic GGGGATCCCC motif. Based on EMSA assays, the NF-κB element binds to GCAAAATCCCC upstream of Cecropin, and GGGAAATTCT upstream of Diptericin. Interestingly, none of the motifs identified by the MAST algorithm are similar to the known motifs such as Rel, GATA or R1 that are known to regulate target genes like Cecropin (Uvell and Engstrom, 2003). This may be because the sequences we used consist of a mix, with some genes directly bound by the Rel proteins, while others are not. This would introduce noise into the system, making it trickier for the algorithm to identify repetitive NF-κB sequences as statistically significant. This "black box" approach however, does allow the unbiased identification of any highly represented motif, without prejudging what a certain binding site should look like.

B.3 Target Explorer

For a more directed search, we used Target Explorer, (http://trantor.bioc.columbia.edu/Target_Explorer/) a web-based system that can perform a variety of tasks, including the creation of customized library of binding sites for known transcription factors through the prediction and annotation of putative target genes that are potentially regulated by these factors (Sosinsky et al., 2003). Using a population of putative NF-κB sites selected from upstream sequences from genes selected from our microarray data, we constructed the position weight matrices.

A matrix for example was generated from the following sequences found upstream of

genes that failed to induce in Relish mutant larvae:

```
Training set:
>a                          CAGAATTTAGTTTCCCA           GACGGGAATTTTTTGCTG
CTCTGCCAAACGGGAGCA          >l                          >3
CGTCCGTTGAGGCTCA            TGGGGATGGCGTAAT             CGGGACAAAAGTTCACCA
>b                          >m                          G
AAAATTGGGAGCATATGC          CGGGGAACTTTTCGCAGC          >4
CAGTGGTTC                   >n                          AAGACAAGCCTCCCA
>c                          AGTGGATCCCCTTC              >5
ACGATCCCGTGG                >k                          CAGACAAAATCCCAT
>d                          GGGGGATGGCCCAGG             >6
TAGGGATAAGCCCAGGC           >o                          AGAAATCCCC
>e                          GAAGATGGGCGCCCTG            >7
CAGATGAGATTCCCC             >p                          CGGGAAATAACGC
>f                          AGGGAGCCATTCGGC             >8
TTGGGAATCCCTGTCAA           >q                          GGCGATTCCCA
>g                          CAGGCATCCCC                 >9
AGTGGATCCCCACAT             >r                          TGGGATTGCGATG
>h                          GGAATCTAAATTCCCAT           >10
AAGTGAATCCCATC              >1                          TGGCGTTCCCG
>i                          GAAGACCATCAGCCCACC
TTGGGATCCAT                 T
>j                          >2
```

This led to the generation of the following matrix:

```
Matrix name: Relishtraining
Score matrix:
A  -2.30  -2.30 -2.30 -0.27 -2.30   0.10 -2.30  -2.30  -2.30  -2.30
C  -2.30  -2.30  1.41  0.10 -2.30  -2.30 -2.30   0.10   1.28   1.28
G   1.53   1.53 -0.51  0.47  1.53  -2.30 -2.30   1.28   0.10   0.10
T  -2.30  -2.30 -2.30 -0.27 -2.30   0.74  1.13  -2.30  -2.30  -2.30
```

Using this matrix, we could scan through the Drosophila Genome and come across

genes that appeared to have an NF-κB motif present upstream of its start site (Complete

list given in Table B-1). It is not surprising that some of the highest scores were received by

genes used to train the matrix in the first place, given below:

```
Alignment matrix:
AttacinDfly3R_13451005D.melanogasterChromosome3R : GGCGGTTGCC score=12.18
CecropinA1D.melanogasterChromosome3R : GGCTGTTCGC score=9.08
CecropinA2fly3R_26026126D.melanogasterChromosome3R : GGCAGTTGCG
score=10.26
CecropinBfly3R_26027365D.melanogasterChromosome3R : GGCGGTTCCG score=9.82
CecropinCfly3R_26030372D.melanogasterChromosome3R : GGGGGATGGC score=8.44
Defensinfly2R_5113723D.melanogasterChromosome2R : GGCCGTTGCC score=11.81
Diptericingi|24655337|ref|NM_079063.2|DrosophilamelanogasterCG10794-
```

```
PA(DptB) : GGCTGTTGCC score=11.44
DiptericinBfly2R_13929910D.melanogasterChromosome2R : GGCAGATGCC
score=10.80
Drosomycingi|414661|emb|X75595.1|DMMRCRPD.melanogaster(OregonR)mRNAforcys
teine-richpeptide : GGCCGATGCC score=11.17
Max score: 12.18
(sum of maximal scores at each position)
Min score: -20.97
(sum of minimal scores at each position)
Min score for the training set of sequences: 8.44
```

It was however, a proof of principle that the matrix could select the same genes out from the Drosophila genome. This method was refined to identify putative Rel binding sites, both in the Drosophila genome, and upstream of genes identified from our microarray results. A complete list of genes with putative Rel binding sites is given in Table B-1.

B.4 Manual Search

We also manually searched through upstream regions of our selected genes, for NF-κB binding sites. This was done through looking for specific motifs for Dorsal, Relish, and Dif/Relish as defined by SELEX experiments (Senger et al., 2004). Some of the queries used in Microsoft word to find the different sequences are given below. Using these queries and wildcards through the "Find" function of MS-word, and using other objective criteria was used to identify putative binding sites:

```
GGGA??[C-T]C[C-T] Relish Forward

[A-G]G[A-G]??TCCC Relish Reversed

GGGAAA*TTT*C[C-T]? Dorsal Forward only if * is made of 3-5 A's, and
T* is 3-5 bases in length.

?[G-A]G*AAA*TTTCCC Dorsal Reversed only if * is made of 3-5 A's, and
T* is 3-5 bases in length.

G?GA?TCCC Dif/Rel Reversed

GGGA?TC?C  Dif/Rel Forward
```

```
GGGA*T*C[C-T] Dorsal Forward. [A-G]GA*T*CCC Dorsal reverse.
We picked sequences that are less than 15 bp and/or look like NF-κB
binding sites empirically, that have been picked out by this query.
```

B.5 Conclusions

Finally, we put together our various results from the different search techniques in a graph. Each time a gene an NF-κB binding site was identified upstream of a gene, it was given a point regardless of which method was used to identify the site. This was done without judgement as to the best approach for finding these sites; each hit was given an equal weight. When the scores are added up, the highest scoring genes can be predicted to be most likely to have NF-κB sites. These predicted sites will need to be validated in future for actual binding to Rel proteins, by Electrophoretic Mobility Shift Assays, or mutation analysis.

Dorsal Motifs
1   36   TTCCGCCCCCCAAGCCGCCCGCCGCCCCCACACCC
2   29   GGTCCGGGGCGTGGGCCTCTGCTGACCGG

Dif Motifs
1   29   GTGGGCGGTGTGCCGGCTGCGGCCGCTGG
2   50   TGTCTCCGGGTTTGGAGACTGAGTCCACTGGCCAATCCATGGGATGGAAC
3   21   TTTTTTTTTGATTTCTATTTT

Relish Motifs
1   29   TTTTCTAAAAATAAATACAATATATAAAG
2   11   CCACCGCCGCC
3   48   TGGTCCTGCTGTACGGCCACGTGCAGAGGAGTCGGACCGGCCGCGTTC

Figure B-1: Locations of motifs identified upstream of genes believed to be predicted by specific Rel proteins. The motifs identified for each Rel protein is given. The numbered boxes represent the location of the motif. Plus and minus is used to denote the orientation of the motif in the sequence. The first 700 base pairs upstream of each gene are shown. The identified motifs thus appear repetitively with a p<<0.01.

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Table B-1: List of all genes in the Drosophila genome with putative NF-κB binding sites. Position weight matrices trained using putative NF-κB sites identified from cDNA microarray data, were used to scan the Drosophila genome for other targets with similar motifs. Many of these genes are also represented on the cDNA microarray and upregulated by infection.

Acp36DE	CG12911	CG15380	CG2841
Apc	CG12912	CG15382	CG2887
AttA	CG12975	CG15429	CG2970
BG:DS02740.2	CG1299	CG15480	CG3029
BG:DS04095.3	CG13101	CG15897	CG3116
BG:DS07473.1	CG1314	CG15909	CG3124
BcDNA:GH02288	CG13184	CG15924	CG3140
BcDNA:GH07485	CG13189	CG1672	CG3229
BcDNA:LD21720	CG13282	CG16783	CG3280
Bub3	CG13311	CG16805	CG3358
CG10014	CG13318	CG16836	CG3387
CG10078	CG13344	CG16844	CG3448
CG10091	CG13345	CG16867	CG3483
CG10123	CG13362	CG1698	CG3494
CG10165	CG1339	CG17116	CG3549
CG10284	CG13482	CG17177	CG3566
CG10406	CG13509	CG17179	CG3591
CG10473	CG13541	CG17188	CG3700
CG10474	CG13565	CG17189	CG3701
CG10489	CG13609	CG17210	CG3792
CG10541	CG13667	CG17269	CG3878
CG10630	CG13685	CG17390	CG3907
CG1079	CG13782	CG17487	CG4095
CG10794	CG13806	CG1749	CG4154
CG10847	CG13918	CG17530	CG4226
CG10877	CG13928	CG17533	CG4286
CG10879	CG13948	CG17534	CG4291
CG10912	CG13966	CG17593	CG4335
CG10955	CG14111	CG17637	CG4384
CG11127	CG14112	CG17738	CG4423
CG11305	CG14129	CG17754	CG4465
CG11335	CG14165	CG17796	CG4523
CG11406	CG14343	CG17826	CG4530
CG1147	CG14374	CG17977	CG4548
CG11490	CG14413	CG18089	CG4558
CG11603	CG14487	CG18304	CG4665
CG11617	CG14506	CG18432	CG4674
CG12009	CG14525	CG18485	CG4679
CG12099	CG14529	CG18538	CG4751
CG12118	CG14730	CG18539	CG4752
CG12170	CG14754	CG18558	CG4792
CG1218	CG14758	CG1857	CG4814
CG12208	CG14778	CG18606	CG4848
CG12370	CG14834	CG18608	CG4896
CG12496	CG14873	CG1883	CG5001
CG12539	CG14945	CG1911	CG5116
CG12706	CG14969	CG2070	CG5124
CG1273	CG14972	CG2104	CG5164
CG12809	CG14980	CG2158	CG5171
CG12852	CG15094	CG2257	CG5207
CG12856	CG15104	CG2269	CG5302
CG12858	CG15344	CG2650	CG5443

|			
CG5792	CG7699	CG9649	ImpL3
CG5824	CG7702	CG9681	Mst35Bb
CG6052	CG8010	CG9725	Nle
CG6124	CG8044	CG9780	Pox-m
CG6290	CG8192	CG9796	Reg-2
CG6306	CG8386	CG9826	RnrS
CG6365	CG8398	CG9958	Su(fu)
CG6426	CG8458	CG9960	Thiolase
CG6429	CG8462	CLIP-190	Tpi
CG6435	CG8613	CecA1	Xpac
CG6554	CG8632	CecA2	amon
CG6631	CG8773	Chi	ast
CG6693	CG8784	CstF-64	bt
CG6792	CG9001	CycJ	esc
CG6803	CG9007	Cyp313a2	fl(2)d
CG6911	CG9021	Cyp6d5	gol
CG6985	CG9034	Cyp6g2	ken
CG7006	CG9139	Cyp6t3	m1
CG7045	CG9146	Dro	msl-2
CG7163	CG9149	EG:22E5.10	prod
CG7222	CG9300	EG:80H7.2	qm
CG7240	CG9331	EG:EG0007.11	salr
CG7298	CG9346	Eip78C	sna
CG7443	CG9351	Ercc1	spn-E
CG7629	CG9525	Glu-RI	sut4
CG7637	CG9646	HLHm7	vg
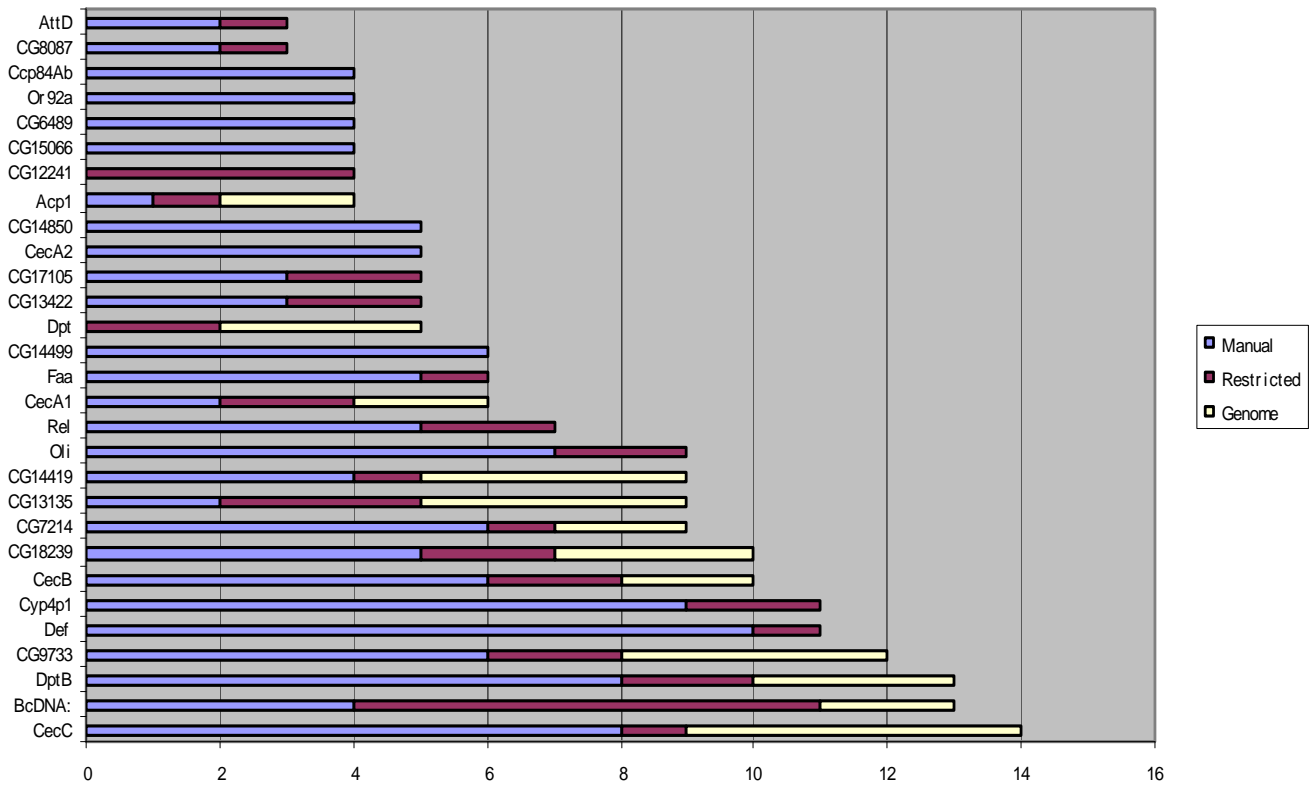
Figure B-2: Graph showing results from search for putative NF-κB binding sites. Sites identified through manual search (blue), use of position weight matrices on genes selected from microarray results (red), and use of position weight matrices on the whole Drosophila genome (yellow) are graphed, and arranged in increasing order.

Appendix C: Screening for Mutations in Cactus using Denaturing HPLC

Components of the Toll pathway are required for proper immunity of Drosophila against fungal and gram-positive bacterial pathogens. Activation of this pathway leads to the signal-induced degradation of Cactus. Cactus sequesters Rel transcription factors, Dif and Dorsal in the cytosol. Cactus degradation releases the transcription factors, which then go on to induce genes important for the immune response. Dif and Dorsal are regulated differently however, raising the interesting possibility that Cactus degradation may be signaled through multiple pathways. While the effect of Toll signaling on Cactus has been extensively studied in the context of embryonic development, this effect during immune challenge is not as well understood. The signal dependent release and activation of the Dorsal morphogen on the ventral side of developing embryo causes proper dorso-ventral patterning. Dif on the other hand does is not known to play a role in the embryo and transcribes genes important for the immune response in larvae and adults. To date, all mutations in Cactus have been isolated from genetic screens affecting dorso-ventral patterning, which inevitably favors finding mutations affecting interactions between Cactus and Dorsal. Here we report an unbiased approach to finding Cactus alleles that may regulate Dif in the immune response, through the use of denaturing HPLC.

The DHPLC system is an ion-pair reversed-phase HPLC system, that separates DNA based on size and the formation of heteroduplexes (Bentley et al., 2000). A WAVE DNA-fragment analysis system by Transgenomics is used, with a polystyrene-

divinylbenzene polymer DNAsep column. The DNA is bound to the column using an ion pairing agent (TEAA) and subsequently eluted using an organic solvent (Acetonitrile). The system is sensitive enough to detect mutations in 1 out of 3 fly lines, and this allows us to pool 3 lines for faster screening.

We looked at the first and sixth exon in the Cactus gene for this screen (Figure C-1). The first exon of the Cactus gene codes for this N-terminal regulatory region, which may be responsible for the differential regulation of Cactus bound to different Rel proteins (Fernandez et al., 2001; Nicolas et al., 1998). The Cactus protein also has two splicing isoforms: maternal 71 KDa and a maternal/zygotic 69KDa. The maternal form includes the 6th Exon of the Cactus gene. Analysis of the Cact^{Su} mutation, that produces a transcript very similar to the maternal form, suggests that the maternal form has a weaker affinity for Dorsal. We therefore screened for mutations in these two exons (1 and 6) because they seemed most likely to affect Cactus interaction with a specific Rel protein. Upon identification of new mutants, the alleles will need to be characterized. Defects in Cactus-Dl interactions should be detectable from defects in Drosophila development. Defects in Dif regulation will need to be assayed by measuring the induction of antimicrobial peptides specifically induced by Dif. Q-PCR using Dif specific target genes, such as those identified in Chapter 2 could be used in assaying this induction.

We used EMS mutagenized lines with second chromosome mutations also known as the "Zuker" lines, along with along with a number of known Cactus alleles from Dr.

Nusslein-Volhard (Koundakjian et al., 2004; Nusslein-Volhard and Wieschaus, 1980; Tsunoda et al., 1997). Over 2000 fly lines were screened using using the dHPLC approach. This project was done in collaboration with Dr. Charles Dearolf: we amplified PCR fragments of the regions and sent it to his lab, where the dHPLC assay was performed. The screen identified 4 mutations in Exon 1 and none in Exon 6 (Figure C-2). The locations of the mutations are shown in Figure C-2. A total of 6000 lines were originally planned on being screened, covering all the Zuker lines with second chromosome mutations. However, covering a third of the total screen yielded only one mutation, and the screen was therefore discontinued. Other groups are currently pursuing a TILLING project with the Zuker lines to identify novel alleles using a similar reverse genetic approach (Henikoff et al., 2004; Till et al., 2003). From our data, it is unclear how successful this approach will be for identifying novel mutations.
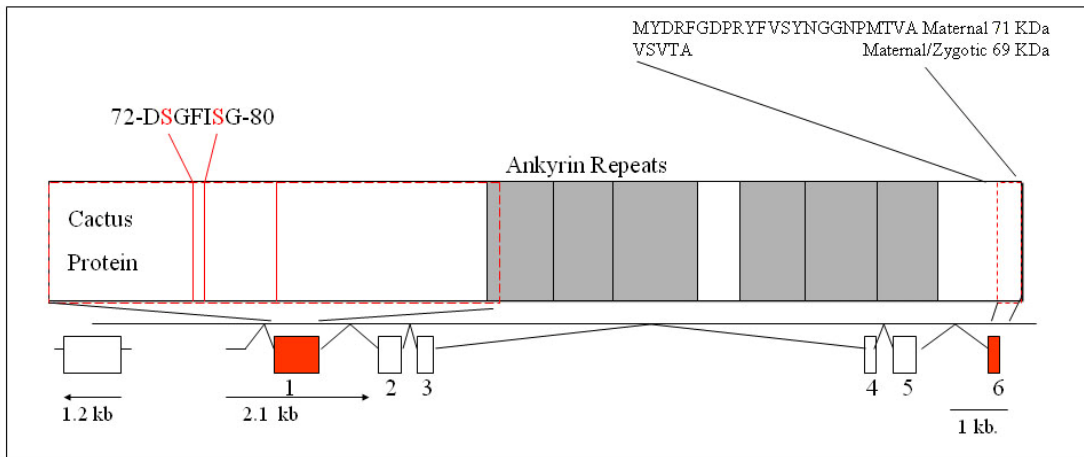
Figure C-1: Cactus sequesters Rel proteins in the cytosol with its ankyrin repeat domains. Phosphorylation at key serines in its N-terminal regulatory region exposes Cactus to enzymes that ubiquitinate it at specific Lys-sites in the same region. Proteosomal degradation of cactus causes it to release the Rel proteins.

Drosophila Cactus	MPSPTKAAEA ATKATATSDC SCSAASVEQR APSNAANPSS SLATSGKIGG	2596 K to K
Mouse IkBe	....	
Human IkBa	....	4300 S to S
Consensus		
Drosophila Cactus	KTQDQTAAIN KQKEFAVPNE TSDSGFISGP QSSQIFSEEI VPDSEEQDKD	
Mouse IkBe	....MS	
Human IkBa	....	
Consensus		
Drosophila Cactus	QQESAPQKEQ PVVLDSGIID EEEDQEEQEK EEEHQDTTTA TADSMRLKHS	
Mouse IkBe	DARKGPDEAD DSQCDSGIES LRSLRSLPEP TAAPGSGSSQ SGCPQPWRHA	
Human IkBa	....MFQA	
Consensus	.....p.... ....dsgi.. ....e. ....ha	
Drosophila Cactus	ADTGIPQWTV ESHLVSRGEQ LNNLGQSSST QITGRSKVQS STASTGNANP	879 G to A
Mouse IkBe	PET....... ..HKEPEKED ADGERADSTY ASSSLTESFP LLERPEAKDP	
Human IkBa	AERPQEWAME GPRDGLKKER LLDDRHDSGL DSMKDEEYEQ MVKELQEIRL	
Consensus	aet....... ..h....ke. l...r.ds.. .s....e... ....p	
Drosophila Cactus	SGSGATSSAP PSSINIMNAW ERFYQQNDDG DT	M716 S to S
Mouse IkBe	SPPAPGSPLP PAGVLSPQQL EALTYISEDG DT	
Human IkBa	EPQEVPRGSE PWK....... ...QQLTEDG DS	
Consensus	sp....s..p p......... e...q...edg dt	

Figure C-2: The mutations identified using Denaturing HPLC. The Zuker collection of Fly lines with EMS mutations in the second chromosome were used. The genomic DNA was extracted and the exons of interest were PCR amplified.

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Appendix D: Screen for flies susceptible to fungal infection

Fungi represent one of the most potent threats to insect populations in nature. In order to identify potentially novel Drosophila immune pathways that may be involved in mediating an anti-fungal defense, we screened selected P-lines that had been reported to be deficient in defensin production. Toll mutant flies were used as a negative control, because of their known susceptibility to even innocuous fungi. We set out to identify fungi that could be used for the screen. A majority of strains were not naturally pathogenic to Drosophila. *Metarhizium anisopliae* strain 2575 was selected over three other Metarhizium strains for infecting Drosophila within 5 days (Figure D-1). The infection method was standardized before the screen and involved:

1. Anesthetize more than 30 flies using CO_2.

2. Drop them into a sabouraud-agar plate inoculated with *Metarhizium*, between 21 -30 days old.

3. Shake flies for one minute until visibly covered with spores.

4. Drop flies back into fly food vial.

Over a period of 5 days, immunodeficient flies such as those deficient in the Toll pathway (*Toll*) or in both Toll and IMD pathways (*imd-spz*) showed significant mortality compared to wildtype flies. We could therefore use this method to screen other flies, and look for other mutants which succumbed easily to fungal infection.

Table D-1 shows a list of flies infected, and the percentage survival at each day after infection. Flies with less than 60% survival after five days are colored Red, and lines

that met this criterion were chosen as being potentially immunodeficient. Greater numbers of these flies will need to be screened to establish in a statistically rigorous manner that these flies have a reproducible phenotype consistent with this criteria. The Kaplan-Meier log-rank statistic can be used to determine significance for mortality rates. Subsequently, crossing these flies to deficiencies could serve as a method to map the location of these mutations. Future work would then focus on characterizing the mutation, through identification of the genes affected. The momentum gained in other areas of research however, prompted me to discontinue this project and pursue other promising projects.

Day 2   Day 6   Day 10

imd-
spz
flies

Figure D-1: Me-1 (Metarhizium anisopliae ARSEF 2575) kills flies within five days. Fungal hyphae germinate on the fly cuticle, and within a week have started to grow over the insect.

Fly stock	Nearby Gene	Day 2	Day 3	Day 4	Day 5
IMD-Spz		100%	100%	0%	0%
14816	CG5522	72.82%	38.83%	32.04%	32.04%
Toll-		92.00%	68.00%	64.00%	36.00%
12670	CG3960	100.00%	96.67%	81.21%	39.88%
13973	CG1600	67.39%	47.83%	44.57%	44.57%
13765	CT30997	80.46%	64.37%	44.83%	44.83%
12598	CG8772	69.77%	48.84%	46.51%	46.51%
Toll-		100.00%	79.97%	55.03%	46.91%
12712	CG4532	68.13%	63.74%	50.55%	47.25%
12632	CG9990	85.87%	71.74%	63.04%	51.09%
12804	CG8524	60.67%	60.67%	52.81%	51.69%
12670	CG3960	92.98%	75.44%	59.65%	52.63%
14816	CG5522	100.00%	72.57%	58.52%	54.22%
13973	CG1600	100.00%	80.24%	57.75%	54.24%
12917	CG6145	100.00%	81.70%	71.54%	62.25%
13765	CT30997	100.00%	89.33%	74.41%	62.42%
12917	CG6145	80.60%	73.13%	64.18%	64.18%
12539	CG3365	93.44%	68.85%	67.21%	67.21%
12712	CG4532	100.00%	84.42%	77.93%	67.68%
12676	CG1534	82.28%	79.75%	70.89%	69.62%
12767	CG7701	80.00%	80.00%	80.00%	70.00%
12785	CG7590	85.48%	82.26%	74.19%	70.97%
12807	CG13650	97.10%	84.06%	75.36%	71.01%
12943		100.00%	88.90%	75.69%	72.27%
Wildtype Infected		100.00%	86.00%	86.00%	73.00%
12598	CG8772	100.00%	85.58%	75.44%	73.42%
12750	CG9584, CG9585	100.00%	93.76%	93.76%	74.97%
13604	CG14745	100.00%	93.06%	81.39%	75.83%
12676	CG1534	100.00%	91.11%	91.11%	78.19%
Toll- Uninfected		100.00%	100.00%	100.00%	80.00%
12459	CG5683	96.81%	86.17%	86.17%	80.85%
12566	CG2471	97.47%	91.14%	81.01%	81.01%
12830	CG4181	100.00%	98.48%	95.45%	81.84%
12459	CG5683	100.00%	95.89%	87.42%	82.38%
13223	CG5953	100.00%	98.29%	93.25%	83.11%
12562	CG2221	100.00%	90.20%	86.68%	83.17%
Toll- Uninfected		100.00%	100.00%	100.00%	83.33%
12566	CG2471	100.00%	97.27%	93.62%	83.64%
12866	CG4427	100.00%	97.16%	92.81%	83.73%
12572	CG7876	100.00%	90.89%	85.75%	84.31%
12631	CG10716	100.00%	90.63%	89.06%	84.38%
12804	CG8524	100.00%	98.48%	98.48%	84.50%
12643	CG18240	100.00%	97.62%	95.24%	84.52%
12637		100.00%	85.71%	84.56%	84.56%
12694		100.00%	94.82%	89.16%	84.82%
12756	CG13245	100.00%	96.25%	93.78%	85.00%
12730	CG5966	93.75%	91.67%	91.67%	87.50%
12903	CG5317	100.00%	96.67%	87.50%	87.50%
12632	CG9990	100.00%	96.30%	96.30%	87.78%
12441	CG15096	100.00%	99.17%	89.17%	88.33%
12767	CG7701	100.00%	96.67%	92.50%	89.17%

12974		100.00%	98.33%	98.33%	89.74%
12669	CG13512	100.00%	98.11%	91.14%	91.14%
12409	CG3171	100.00%	96.38%	91.58%	91.58%
12457	CG4212	100.00%	91.67%	91.67%	91.67%
12516	CG3168	100.00%	98.33%	95.00%	91.67%
12669	CG13512	100.00%	97.37%	97.37%	92.11%
12482	CG4070	100.00%	95.75%	92.83%	92.93%
12795		100.00%	97.65%	94.12%	92.94%
12785	CG7590	100.00%	99.17%	94.17%	93.33%
12562	CG2221	96.97%	95.45%	93.94%	93.94%
12730	CG5966	100.00%	97.52%	94.65%	94.65%
12542	CG4463	100.00%	94.97%	94.97%	94.97%
13120	CG3845	100.00%	99.17%	97.50%	95.83%
12631	CG10716	100.00%	100.00%	96.36%	95.89%
12807	CG13650	100.00%	99.17%	98.33%	96.67%
12542	CG4463	100.00%	100.00%	100.00%	100.00%
12647	CG6528	100.00%	100.00%	100.00%	100.00%
Toll10b		100.00%	100.00%	100.00%	100.00%
Wild-type Uninfected		100.00%	100.00%	100.00%	100.00%
Wild-type Uninfected		100.00%	100.00%	100.00%	100.00%

Table D-1: Mortality of flies infected with Metarhizium anisopliae strains over a five day period. Survival below 60% is marked in Red. More than 30 flies were used in each sample, and mortality is given as a percentage of surviving flies with the number of flies surviving after infection on day 1 set at 100%.
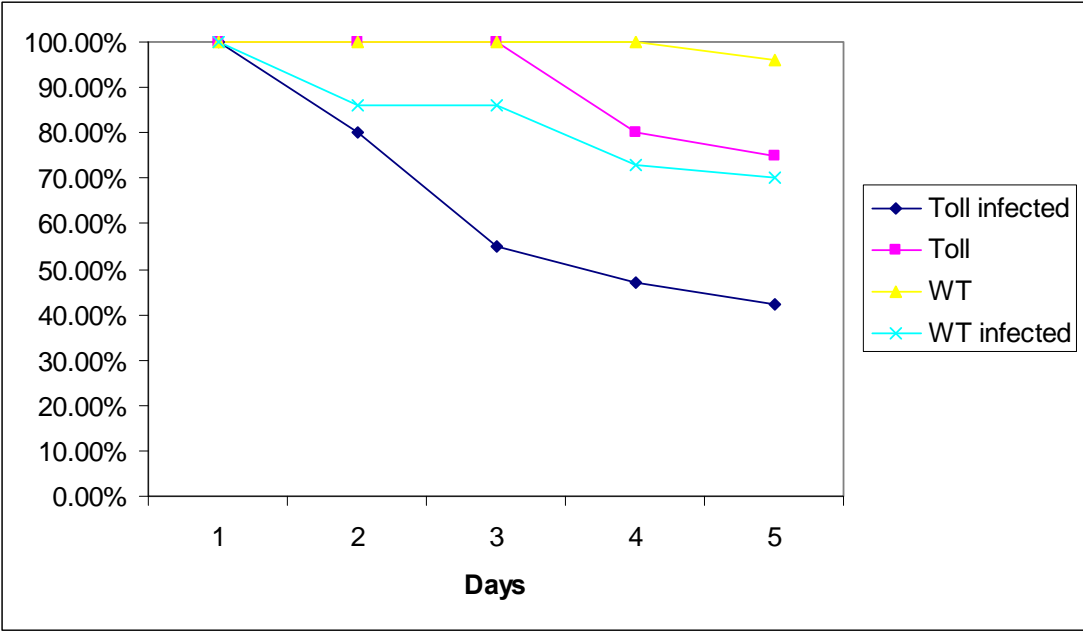
Figure D-2: Survival of wildtype and Toll mutant flies upon natural infection with
Metarhizium anisopliae ARSEF 2575

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