2010-03-29

A View of the IMD Pathway from the RHIM

Kamna Aggarwal
University of Massachusetts Medical School

A VIEW OF THE IMD PATHWAY FROM THE RHIM

A Dissertation Presented

By

Kamna Aggarwal

Submitted to the Faculty of the University of Massachusetts Medical School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MARCH 29, 2010

INTERDISCIPLINARY GRADUATE PROGRAM

A Dissertation Presented
By
Kamna Aggarwal

Approved as to style and content by:

Neal Silverman, Ph.D., Thesis Advisor

Michael Brodsky, Ph.D., Member of Committee

Katherine Fitzgerald, Ph.D., Member of Committee

Scot Wolfe, M.D., Member of Committee

François Leulier, Ph.D., Member of Committee

Michelle Kelliher, Ph.D., Chair of Committee

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program
March 29, 2010

Acknowledgments

There are many people I would like to thank for making my graduate school journey so memorable. Foremost I would like to thank my advisor Dr. Neal Silverman who is not only a gifted scientist, but also a great mentor and teacher. I have learnt from him that there is no substitute for a well planned and structured approach for testing a hypothesis. He also taught me to think critically and to plan every experiment and to think about all the controls as every control is important for proper analysis of the data. I am particularly grateful to him for being extremely patient with me and allowing me the freedom to work independently. I can say with confidence that Neal is one of the best mentors I will have ever worked with.

It was an honor to have Drs, Michelle Kelliher, Katherine Fitzgerald, Michael Brodsky and Scot Wolfe on my thesis committee. Their comments and suggestions were highly valuable and I thank them for their time. I am also grateful to Dr. François Leulier for taking the time to evaluate my dissertation.

I was incredibly lucky in training in an environment so well stocked in scientists with diverse interest and expertise in their field. I would especially like to thanks the past and present members of the Silverman. In particular I would like to thank Takashi Kaneko, Charles Sweet, Nicholas Paquette and Deniz Hasdemir for teaching me various techniques in the lab and also for providing a great working environment.

My graduate school journey would have been tough without the love and good will of the following people. So a big thank you to all my friends and family members for being a part of my life and in particular, to listening to my woes (Kayla Morlock, Florentina Rus and my loving sister Aparna Aggarwal) and for providing me with a home away from home (Sujan Rafi-ul Kabir, Hema Bashyam, the Jain family and the WCBA family).

Although I have had a great time at UMass one of the most difficult thing about this journey has been to be away from my family. They are my pillars of strength and responsible for all the success that has come my way. I am indebted to them for supporting me in all my endeavors and having faith in times when even I lacked it. I am also thankful to my parent in laws for loving me like a daughter and letting me be a part of their family.

Last but definitely not the least I would like thanks my husband Tathagat Dutta Ray. I lack the words to express my love for him. Without him this journey would have been impossible. I thank him for giving me endless encouragement, help and advice and for always being there besides me. I am very lucky to share my life with him.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF PUBLICATIONS

Aggrawal K, Silverman N. RYBP: a new regulator of the IMD pathway. In preparation.

Ganesan S, Aggarwal K, Paquette N and Silverman N. NF-κB/Rel proteins and the humoral immune responses of *Drosophila melanogaster.* Curr Topics in Microbiol and Immunol. (In press)

Silverman N, Paquette N, Aggarwal K. Specificity and signaling in the *Drosophila* immune response. ISJ 6: 163-174, 2009.

Paquette N, Broemer M, Aggarwal K, Husson M, Ertürk-Hasdemir D, Reichhart J-M, Meier P, Silverman N. Immune-induced IMD Cleavage, DIAP2 Association, and K63-Ubiquitination Drives Innate Immune Signaling in *Drosophila*. Mol Cell. 2010 Jan 29;37(2):172-82.

Aggarwal K, Rus F, Vriesema-Magnuson C, Ertürk-Hasdemir D, Paquette N, Silverman N. Rudra interrupts receptor signaling complexes to negatively regulate the IMD pathway. Plos Pathogen 2008 Aug 8, 4(8): e1000120.

Aggarwal K, Silverman N. Positive and negative regulation of the Drosophila immune response. BMB Rep. 2008 Apr 30;41(4):267-77.

Aggrawal K, Silverman N. Peptidoglycan recognition in Drosophila. Biochem. Soc Trans. 2007 Dec;35(Pt 6):1496-500.

Ertürk-Hasdemir D, Paquette N, Aggarwal K, and Neal Silverman. Bug versus Bug: Humoral Immune Responses in *Drosophila melanogaster*. Book chapter in *Innate Immunity in Plants, Animals, and Man*: 2007.

Kaneko T, Yano T, Aggarwal K, Lim JH, Ueda K, Oshima Y, Peach C, Ertürk-Hasdemir D, Goldman WE, Oh BH, Kurata S, Silverman N. PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. Nat Immunol. 2006 Jul;7(7): 715-23.

Lim JH, Kim MS, Kim HE, Yano T, Oshima Y, Aggarwal K, Goldman WE, Silverman N, Kurata S, Oh BH. Structural basis for preferential recognition of diaminopimelic acid-type peptidoglycan by a subset of peptidoglycan recognition proteins. J Biol Chem. 2006 Mar 24;281(12):8286-95.

ABSTRACT

Innate immunity is the first line of defense against invading pathogens. It functions to eliminate pathogens and also to control infections. The innate immune response is also important for the development of pathogen-specific adaptive immune responses. As a result, the study of innate immune signaling pathways is crucial for understanding the interactions between host and pathogen. Unlike mammals, insects lack a classical adaptive immune response and rely mostly on innate immune responses.

Innate immune mechanisms have been widely studied in the fruit fly, *Drosophila melanogaster*. The genetic and molecular tools available in the *Drosophila* system make it an excellent model system for studying immunity. Furthermore, the innate immune signaling pathways used by *Drosophila* show strong homology to those of vertebrates making them ideal for studying these pathways. *Drosophila* immunity relies on cellular and humoral innate immune responses to fight pathogens. The hallmark of the *Drosophila* humoral immune response is the rapid induction of antimicrobial peptide genes in the fat body. The production of these antimicrobial peptides is regulated by two immune signaling pathways-Toll and Immune Deficency (IMD) pathways.

The Toll pathway responds to many Gram-positive bacterial and fungal infections , while the IMD pathway is potently activated by DAP-type peptidoglycan (PGN) from Gram-negative bacteria and certain Gram-positive bacteria. Two receptors, PGRP-LC and PGRP-LE, are able to recognize DAP-

type PGN at the cell surface or in the cytosol, respectively, and trigger the IMD pathway. Upon binding DAP-type PGN, both PGRP-LC and PGRP-LE dimerize/ multimerize and signal to the downstream components of IMD pathway. It is unclear how the receptor activates its downstream components.

My work has focused on understanding the molecular events that take place at the receptors following there activation. In these studies I have identified a common motif in the N-terminal domains of both the receptors, known as the RHIM-like domain. The RHIM-like domain is critical for signaling by either receptor, but the mechanism(s) involved remain unclear. IMD, a downstream component of the pathway, associates with both PGRP-LC and -LE but the interaction of PGRP-LC with IMD is not mediated through its RHIM-like domain. Also, mutations affecting the PGRP-LC RHIM-like motif are defective in all known downstream signaling events. However, the RHIM-like mutant receptors are capable of serving as a platform for the assembly of all known components of a receptor proximal signaling complex. These results suggest that another, unidentified component of the IMD signaling pathway may function to mediate interaction with the RHIM-like motif.

I performed a yeast two-hybrid screen to identify proteins that might interact with the receptor PGRP-LC through its RHIM- like domain. With this approach, two new components of the IMD pathway were identified. The first component I characterized is called Rudra and it is a critical feedback inhibitor of peptidoglycan receptor signaling. The other factor is known as RYBP, it includes

a highly conserved ubiquitin binding motif (NZF), and RNAi studies suggest it is a critical component of the IMD pathway. The identification and characterization of these two new components of the IMD pathway has provided a new insight into the molecular events that take place proximal to the receptor.

PREFACE TO CHAPTER I

Portions of this chapter have been published separately in:

1. Deniz Ertürk–Hasdemir, Nicholas Paquette, **Kamna Aggarwal**, and Neal Silverman. *Bug versus Bug: Humoral Immune Responses in Drosophila melanogaster*. Chapter in Innate Immunity in Plants, Animals, and Man, Nucleic Acids and Molecular Biology, volume 21, Edited by Holger Heine

Kamna Aggarwal, Deniz Erturk Hasdemir, Nicholas Paquette and Neal Silverman all contributed to the manuscript equally.

2. **Kamna Aggarwal** and Neal Silverman. Positive and negative regulation of the Drosophila immune response. BMB Rep. 2008 Apr 30;41(4):267–77.

INTRODUCTION

Insects rely primarily on innate immune responses to fight pathogens, and have developed multiple mechanisms to recognize and respond to infection. Even without an adaptive immune response, insects respond effectively to a wide range of pathogens. The insect and mammalian innate immune responses exhibit a great deal of evolutionary conservation. One of the best examples of this conservation was provided by the discovery of the Toll pathway as a key component of the immune system in the fruit fly *Drosophila melanogaster*, and the subsequent identification of the mammalian Toll–like Receptors (TLRs). In addition, both the insect and mammalian immune response relies on the evolutionarily conserved NF–κB signaling cascades for the control of immune system induced gene expression. *Drosophila* employ several distinct effector mechanisms for immune protection including clotting, melanization, encapsulation, cell–based phagocytosis and the inducible production of a battery of antimicrobial peptides (AMPs). This AMP response is critical for protection against many microbial pathogens. Two signaling pathways regulate the production of these AMPs in *Drosophila* – the IMD and Toll pathways.

Drosophila is a favorite model system for studying the immune response. Although *Drosophila* are evolutionarily separated from vertebrate organisms, many major pathways, including those involved in immunity, DNA damage repair, and neurodegeneration, are reasonably well conserved. Further, while vertebrate systems have multiple redundant proteins which can make analysis difficult, *Drosophila* often do not employ the same level of redundancy, thus making the analysis easier. A variety of genetic, genomic, and molecular tools are also available for studying *Drosophila.* Well established tools such as balancers and P–elements allow for easy genome wide

screening. A number of well established *Drosophila* cell culture lines are also available. Finally, *Drosophila* have a short generation time and are both cheaper and easier to maintain than most mammals. Overall *Drosophila* provide versatility and control that are ideal for scientific research.

Overview of the *Drosophila* Immune Response

The immune response against microbes in *Drosophila* is a multi–layered system. Structural barriers such as a chitin–based exoskeleton and the endothelia form the first layer of defense. If a pathogen breaches these barriers, several immune effector mechanism are activated, including cellular responses (i.e. phagocytosis, encapsulation, melanization) and humoral responses (i.e. AMPs). This AMP response is critical for protection against many microbial pathogens. AMPs are found both locally, at the site of infection, and systemically in the insect sera, or hemolymph. The local response induces AMP gene expression in epithelial tissues, like the trachea and the gut while the systemic humoral response induces AMP production in the fat body. Other tissues, including the malphigian tubules and circulating blood cells, known as hemocytes also contribute to AMP production during the humoral response (Ferrandon et al., 1998; Liehl et al., 2006; Tzou et al., 2000).

The *Drosophila* humoral immune system responds to microbial challenge by triggering the expression of anti-microbial peptide genes through NF-κB signaling pathways. In fact, NF-κB/Rel proteins control the transcription of almost one half (162/400) of the immune responsive genes, including the collection of cationic antimicrobial peptides (AMPs) (Irving et al., 2001; De Gregorio et al., 2001; De Gregorio et al., 2002). Genes from many different functional classes are up-regulated in

response to immune challenge. These categories included actin-associated, calcium binding, cell adhesion, chaperones, heat-shock proteins, enzyme inhibitors, growth factors, carrier proteins, motor proteins, nucleic acid-binding factors, structural proteins (cytoskeleton, cuticle, and muscle), transcription factors, and others. As a result of infection-induced NF-κB activation, AMPs that are undetectable in the hemolymph (blood) of unchallenged flies are rapidly elevated to concentrations up to 100μM (Hoffmann and Reichhart, 2002). As best we know, the regulation of AMP production occurs at the level of gene expression. AMP genes are direct targets of NF-κB activation and their transcription is induced to very high levels rapidly after infection. Although the bulk of AMP production occurs in the insect fat body (similar to the mammalian liver), AMP genes are also expressed in circulating phagocytic hemocytes and local epithelial tissues, particularly, the gut and the trachea (Ferrandon et al., 1998; Liehl et al., 2006; Tzou et al., 2000). Two NF-κB signaling pathways control AMP gene expression– the Toll and the IMD pathways. These pathways are activated by microbial cell walls and/or other virulence determinants by circulating, cell surface, and/or cytosolic receptors. Each pathway responds to distinct microbial components and induces the expression of somewhat overlapping subsets of AMP and other immune responsive genes. For example, the anti fungals *Drosomycin* and *Metchnikowin* are strongly induced by the systemic Toll pathway, while the IMD pathway induces antibacterial peptides such as *Diptericin (Lemaitre et al., 1997)*(Tzou et al., 2002). On the other hand, some AMP genes such as *Cecropin* and *Attacin* are co-operatively regulated by both the pathways (Manfruelli et al., 1999). Details on these regulatory events are discussed in more detail below.

The main focus of this thesis is to understand how the receptor signals to its downstream components. The introduction to this work is designed to provide an overview of each component of the two different immune signaling pathways and how they are activated and regulated, in insects. Also included are some aspects of how these signaling pathways function in mammals.

Microbial Recognition–the Peptidoglycan Recognition Proteins

Recognition of pathogens is the first step in a cascade of events that leads to either the Toll or IMD–driven immune responses. Microbial products, often cell wall components, are detected by recognition receptors, which in turn stimulate signaling pathways that culminate in the induction of AMP gene expression. Peptidoglycan recognition proteins (PGRPs), are the key receptors that recognize peptidoglycan (PGN) and trigger the Toll and IMD pathways.

PGRP was first identified as a PGN binding factor from the hemolymph of the silkworm *Bombyx mori*, and was shown to be involved in activating the melanization cascade *in vitro* (Yoshida et al., 1996). PGRPs were subsequently cloned from both *Bombyx mori* and the moth *Trichoplusia ni (Kang et al., 1998; Ochiai and Ashida, 1999)*.

Drosophila encodes 13 PGRP genes that are spliced into at least 17 PGRP proteins (Werner et al., 2000). All PGRP proteins contain a domain homologous to bacteriophage type–2 amidases, enzymes that cleave the lactyl group in acetylmuramic acid and L–alanine in the stem–peptide of PGN (Mellroth et al., 2003) (**Figure 1.1a**). Six of the *Drosophila* PGRPs (PGRP–SB1,–SB2,–SC1a/b,–SC2,–LB) are either known or predicted to be type 2 amidases that are involved in degrading PGN and dampening immune activation. The other seven lack type 2 amidase activity but function through

binding PGN. In particular, 4 PGRPs (PGRP–SA,–SD,–LC, and–LE) function as receptors in the IMD or Toll pathways, as detailed below (Mellroth et al., 2003; Bischoff et al., 2004)(Zaidman-Remy et al., 2006). PGRP–LF seems to function as a decoy receptor, binding PGN but not activating immune signaling (Persson et al., 2007; Maillet et al., 2008), while the functions of PGRP–LA and–LD remain elusive (Royet and Dziarski, 2007).

The *Drosophila* PGRPs can also be classified as either short PGRP proteins (seven different genes encoding seven proteins) or long PGRP proteins, with extended N–termini (ten genes encoding thirteen proteins; (Werner et al., 2000)). Most short PGRP proteins have a signal sequence, lack a transmembrane domain, and are likely to be secreted (SA, SB1, SB2, SC1a, SC1b, SC2, SD). Some long PGRP proteins have a single–pass transmembrane domain and are likely transmembrane proteins (LAa, LAb, LCa, LCx, LCy, LD, LF). However, some long PGRP proteins lack both a signal peptide and a transmembrane domain (LAc, LB, LE), and are likely intracellular proteins, or they could be secreted by a non–canonical mechanism (Takehana et al., 2002).

Mammals encode four PGRPs, termed PGRP–S, PGRP–L, PGRP–Iα, PGRP–Iβ (also referred to as PGLYRP–1, PGLYRP–2, PGLYRP–3, PGLYRP–4). Of these, only PGRP–L has amidase activity (Gelius et al., 2003; Kim et al., 2003). Mammalian PGRPs are expressed in a variety of tissues including bone marrow (PGRP–S), skin and intestinal tract (PGRP–Iα, PGRP–Iβ) and liver (PGRP–L; (Kang et al., 1998; Lo et al., 2003; Lu et al., 2006; Mathur et al., 2004)). Unlike the insect PGRPs, the non–catalytic mammalian PGRPs are bactericidal (Cho et al., 2005; Dziarski et al., 2003;

Gelius et al., 2003; Liu et al., 2000; Lu et al., 2006; Tydell et al., 2002; Wang et al., 2003). Mice deficient for PGRP–S display increased susceptibility to intraperitoneal infections with non–pathogenic Gram–positive bacteria (Dziarski et al., 2003). PGRP–S is present in neutrophil granules and is involved in the intracellular killing of bacteria. It is also found associated with DNA nets released by activated neutrophils, where it is implicated in direct bacterial killing, acting synergistically with lysozyme (Cho et al., 2005; Liu et al., 2000). PGRP–Iα and–Iβ are secreted as di–sulfide hetero–and homodimers that are bactericidal against both pathogenic and non–pathogenic Gram–positive bacteria but are only bacteriostatic against other normal–flora bacteria. They are also bacteriostatic against some Gram–negative bacteria (Lu et al., 2006). The bactericidal and bacteriostatic mechanisms of the mammalian PGRPs are not yet clearly defined. PGRP–L is produced by the liver and secreted into the bloodstream (Zhang et al., 2005). Recent work by Dizarski and colleagues demonstrates that PGRP-L also functions to modulate the immune response by co-operating with other innate immune pathogen recognition receptors. In contrast to previous speculations, Saha et al., demonstrate that PGRP–L is pro inflammatory and along with Nod2 and TLR4 is required for the development of peptidoglycan induced local inflammation and arthritis (Saha et al., 2009)(Fritz et al., 2007; Uehara et al., 2006).

Peptidoglycan

Drosophila recognizes bacteria by detecting specific forms of bacterial PGN via the PGRP receptors. PGN is a polymeric glycopeptide that forms the cell wall of most bacteria. PGN contains long glycan chains usually composed of alternating residues of N–acetylglucosamine and N–acetylmuramic acid (MurNAc), with short stem–peptides of

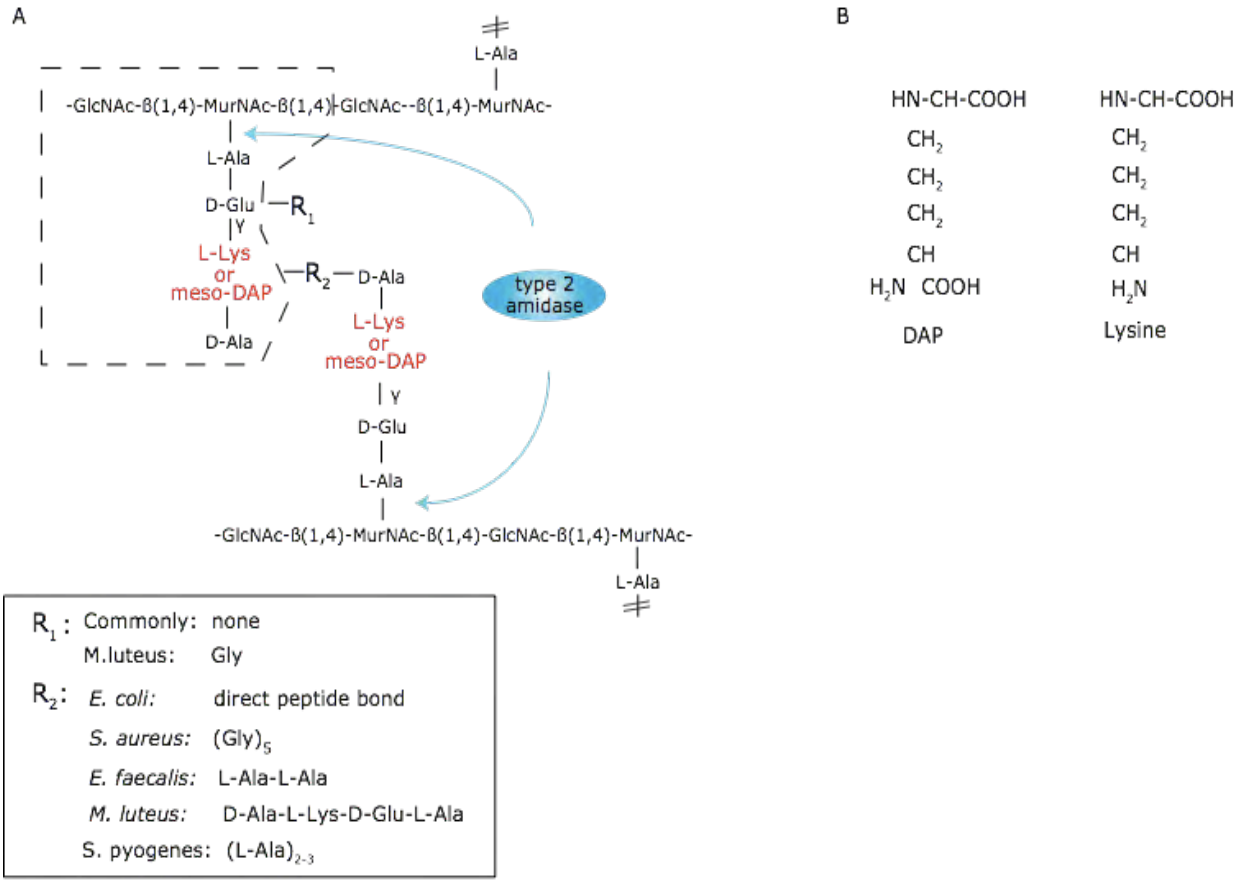


Figure 1.1: Peptidoglycan structure.
(a) As shown, peptidoglycan has a common core structure with a great deal of inherent variation. Most notably, the constituent of the third position of the stem–peptide can vary, and are most commonly L–lysine or meso–DAP. In addition, the amount and exact chemical nature of the crosslinking bridges can vary, with some examples noted in the box below. TCT is a monomeric unit of the DAP–type peptidoglycan chain, and is indicated in the dashed box.
(b) Structures of lysine and DAP.

alternating L–and D–amino acids attached to the lactyl group of MurNAc. These stem–peptides are often cross–linked to each other to stiffen the cell wall; the precise nature of these cross–linking structures is highly variable. The stem–peptides also display a great deal of variation in their amino acid constituents. The carbohydrate backbone is more constant but also can be modified by various chemical substitutions, such as acetylation (Schleifer and Kandler, 1972; Mengin-Lecreulx and Lemaitre, 2005). PGN from Gram–negative bacteria and certain Gram–positive bacteria (e.g. *Bacillus* spp, *L. monoctyogenes*) contain *meso*–diaminopimelic acid (DAP) at the third position of the stem–peptide chain, while other Gram–positive PGN contain lysine at this position **(Figure 1.1)**. Also, the structure and degree of cross–bridging peptides is highly variable. The difference in the amino acid at position 3 in the stem peptide along with the amount and type of cross–linking accounts for much of the variability in the structure of PGN produced by different bacteria (Schleifer and Kandler, 1972). Another major difference between Gram–positive and Gram–negative bacteria is the localization of the PGN in the cell wall. Gram–negative bacteria include a thin layer of PGN, which is concealed in the periplasmic space between the inner and outer membranes. In contrast, Gram–positive bacteria contain a thick, multilayered PGN cell wall at their surface.

The Toll pathway is activated by lysine–type PGN, while the IMD pathway is activate by DAP–type PGN (Kaneko et al., 2004; Leulier et al., 2003). Also, the IMD pathway is activated by both polymeric DAP–type PGN and a monomeric fragment of DAP–type PGN, known as tracheal cytotoxin [TCT; (Kaneko et al., 2004; Stenbak et al., 2004)]. TCT is a disaccharide tetra–peptide fragment of DAP–type PGN with a 1,6

anhydro–arranged muramic acid that is released in large quantities by some Gram–negative bacteria, like *Bordetella pertussis*, *Neisseria gonorrhoeae*, and *Vibrio fischeri* [(Cookson et al., 1989a; Goldman et al., 1982; Melly et al., 1984; Rosenthal, 1979); **Figure 1.1**]. TCT is linked to the cytopathology caused by *Bordetella* and *Neisseria* infection, and it is implicated in the developmental tissue degeneration caused by successful symbiosis of the squid *Euprymna scolopes* with *V. fischeri* (Koropatnick et al., 2004).

Recent data show that PGRPs employ several strategies to recognize and discriminate between different types of PGN. One strategy discriminates between DAP versus lysine in the third position of the stem–peptide, while another detects the presence or absence of a cross–bridging peptide. The C–terminal PGRP domain of PGRP–Iα (PGRP–IαC) binds lysine–type PGN, with or without a penta–glycine cross–bridge [as seen in *S. aureus*; (Schleifer and Kandler, 1972)]. PGRP–S and PGRP–LCx preferentially recognize uncross–linked DAP–type PGN (Swaminathan et al., 2006; Takehana et al., 2002). PGRP–IαC forms several van der Waals contacts with the lysine through two amino acids, Asn236 and Phe237. By comparison, the corresponding residues that recognize DAP–type PGN are Gly–Thr (in PGRP–LCx,–LE and human PGRP–S). Swap experiments demonstrated that these positions are involved in determining lysine versus DAP–type binding properties. In addition, the structures of PGRP–LC or PGRP–LE bound to TCT indicate that another important residue making specific contact with the DAP residue is a highly conserved arginine found at the bottom of the PGN docking groove. The side–chain carboxylate of DAP forms a bidendate salt bridge with the guanidium group of this Arg (residue 254 in

PGRP–LE, 413 in –LC). All PGRPs that have the conserved Gly–Thr also have this Arg residue. Thus, these residues likely function together to stabilize the interaction with DAP–type PGN (Chang et al., 2006; Guan and Mariuzza, 2007; Lim et al., 2006).

The Toll Pathway

The Toll pathway responds to Gram–positive bacterial and fungal infections (Lemaitre et al., 1996). Unlike human TLRs, *Drosophila* Toll does not directly bind pathogens or microbe–derived compounds. Instead, Toll functions more like a cytokine receptor, binding a processed form of the cytokine Spätzle, a member of the cysteine knot family of growth factors and cytokines (Weber et al., 2003; Hu et al., 2004; Hoffmann et al., 2008). Spätzle is made as a pro–protein that circulates in the hemolymph. Upon immune activation (or the perception of developmental cues), serine protease cascades are triggered that culminate in the cleavage of Spätzle. Once processed, mature Spätzle binds to and dimerizes the transmembrane receptor Toll, initiating the intracellular signaling pathway. Four different serine protease cascades appear to converge on the cleavage of Spätzle. In early development, the protease Easter is responsible for cleaving Spätzle. During the immune response, bacterial PGN, fungal β–glucans, and microbial proteases are sensed by three distinct mechanisms, but converge upon activation of one serine protease, known as the Spätzle processing enzyme (SPE), which in turn cleaves and activates Spätzle (**Figure 1.2**).

The serine protease Persephone appears to function as a sensor for proteases secreted by both fungal and bacterial pathogens (Gottar et al., 2006; El Chamy et al., 2008). Persephone is likely activated by cleavage after a histidine residue, unlike the

other proteases involved in the Toll signaling pathways, and maybe a good target for subtilisin–like proteases produced by microbial pathogens (El Chamy et al., 2008). The activation of the Persephone–Toll pathway by microbial proteases occurs independently of recognition of microbial cell wall material, which can also stimulate the Toll pathway through more classical receptor–mediated recognition.

For example, β–glucans from the cell wall of yeast are recognized by Gram–negative binding protein 3 (GNBP3) (Gottar et al., 2006), while two secreted PGRP receptors and GNBP1 are involved together in PGN recognition (Gobert et al., 2003; Bischoff et al., 2004; Wang et al., 2006; Wang et al., 2008). Despite their name, none of the GNBPs are known to recognize Gram–negative bacteria, but GNBP1 and GNPB3 are involved in the recognition of fungal or Gram–positive bacterial cell walls. The N–terminus of GNBP3 binds to long β–1,3 glucans, common to the cell walls of many types of fungi, especially yeast (Mishima et al., 2009). GNBP1, on the other hand, is involved in PGN recognition, although its role is controversial. Lysine–type PGNs, common to many Gram–positive bacteria, are potent agonist of the Toll pathway. As in the IMD pathway, PGRP receptors are critical for the recognition of lysine–type PGN. In particular, two secreted PGRPs, PGRP–SA and–SD, are involved in the Toll pathway. Genetic studies have shown that some Gram–positive bacteria and the PGN from these same species, such as *M. luteus,* are sensed through PGRP–SA (Michel et al., 2001). In fact, the structure of PGRP–SA bound to a lysine–containing muropeptide has been solved. PGRP–SA can also bind DAP–type PGN, albeit to a lesser degree. However, PGRP–SA appears to be able to specifically cleave DAP–type muropeptides, removing the final amino acid in the stem–peptide. It has been postulated that this

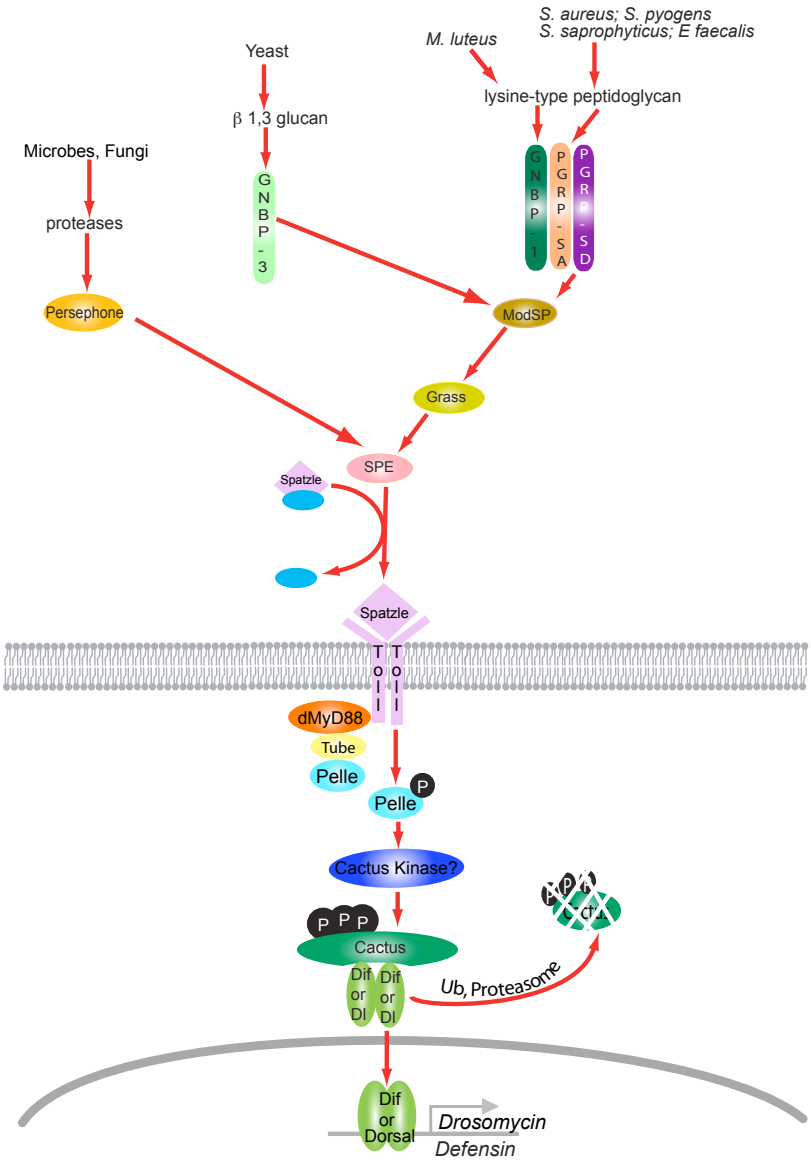


Figure 1.2: Toll signaling pathway.
The Toll signaling pathway and its multiple modes of activation in the *Drosophila* immune response. Three distinct mechanisms of microbial recognition, leading to the cleavage of Spätzle and activation of Toll, are illustrated. The mechanisms include peptidoglycan detection, by PGRP–SA, PGRP–SD and GNBP1, β–glucan detection through GNBP3, and protease activity sensing via the serine protease Persephone. All these detection modalities lead to the activation of the Spätzle processing enzyme (SPE) which converts this cytokine into its active form, for binding and activating Toll. The intracellular signal transduction downstream of Toll is very similar to the MyD88–dependent pathway, which functions downstream of most mammalian TLRs. The key features of this pathway include a trimeric receptor associated complex, containing MyD88, Tube and Pelle, which ultimately lead to the phosphorylation and degradation of the IκB homolog Cactus and the nuclear translocation of NF–κB homologs DIF and Dorsal. In this schematic representation of the Toll pathway. Red arrows indicate the Toll signaling cascade, including the three microbial recognition systems and the intracellular signaling cascade, while the black arrows highlight the negative regulators and their likely targets. See text for more details.

carboxypeptidase activity prevents DAP–type PGN from stimulating the Toll pathway via PGRP–SA (Chang et al., 2004).

Interestingly, not all lysine–type PGN require PGRP–SA to trigger the Toll pathway. In particular, *S. aureus, E. faecalis, S. pyogenes,* and *S. saprophyticus* infections still produce strong AMP gene responses in *PGRP–SA* mutant (*seml)* flies. Response to these bacteria or their PGNs instead requires either PGRP–SA or PGRP–SD (Bischoff et al., 2004). The mechanism of PGRP–SD–mediated recognition of some, but not all, lysine–type PGN producing bacteria remains unclear. One possibility is a structural difference common to those PGNs sensed by PGRP–SD, which prevents detection by PGRP–SA, or vice versa. However, biochemical studies of PGRP–SD do not support the notion that it is involved in recognizing lysine–type PGN. Crystallographic studies show that PGRP–SD has a deep PGN binding cleft, typical of all PGRPs, and this binding cleft includes an arginine (Arg90) in the key position typical of DAP–PGN specific recognition. In fact, binding studies confirm that PGRP–SD binds DAP–type PGN, from *B. subtilis*, but not lysine–type from *S. aureus* (Leone et al., 2008). In addition, the moderate induction of *Drosomycin* observed following either *B. subtilis* or *E. coli* infection, which is Toll dependent (Leulier et al., 2003), required both PGRP–SA and PGRP–SD. So, somehow PGRP–SA and PGRP–SD function together in the recognition of DAP–type PGN, for moderate Toll induction, but function in a more redundant manner for the recognition of certain lysine–type PGN, for robust Toll induction. However, the recognition of *M. luteus* PGN appears to be more simple and requires only PGRP–SA. The molecular mechanisms involved in the recognition of various PGNs by PGRP–SA and/or PGRP–SD remain to be determined. As mentioned

above, GNBP1 also functions in PGN recognition and Toll signaling. In fact, PGRP–SA,–SD, and GNBP1 form a trimeric complex together in the presence of PGN fragments (Wang et al., 2008). Some groups have reported that GNBP1 provides a critical PGN processing activity to this complex, required to generate small PGN fragments which are bound by PGRP–SA and/or PGRP–SD for Toll activation (Filipe et al., 2005; Wang et al., 2006). However, another group has reported that they do not observe a similar PGN digesting activity associated with GNBP1, in *Drosophila* or *Tenebrio molitor* (Buchon et al., 2009). Instead, this group proposes that GNBP1 serves to link the PGRPs with the downstream serine protease cascade, described below. Thus, it appears that GNBP1 functions in a complex with the PGN sensing receptors PGRP–SA and PGRP–SD, but the biochemical mechanism by which it contributes to immune recognition or Toll signaling are not yet clear.

Both the GNBP3–mediated recognition of β–glucans and the PGRP–SA/SD/GNBP1–mediated recognition of bacterial PGNs trigger Toll signaling through the same serine protease cascade. This cascade involves the modular serine protease (ModSP), which is probably directly activated by these microbial sensing receptor complexes, and at least two downstream CLIP–domain serine proteases–Grass and SPE. As mentioned above, SPE cleaves and thereby activates Spätzle, the ligand for Toll. Another protease, known as Spirit may function between Grass and SPE, and other non–catalytic serine–protease homologs, Sphinx 1/2 and Spheroide, are also implicated in this pathway by RNAi based studies (Kambris et al., 2006). However, the assignment of these factors to this pathway requires further genetic and biochemical characterization. The pathways presented in **Figure 1.2** suggests a protease cascade

that is consistent with the genetic analysis of mutants in *Drosophila* and the biochemical analysis of the cascade from the hemolymph of *Tenebrio.* However, biochemistry of the *Drosophila* serine protease cascade still requires further study, as several issues remain unresolved, including the role of Spirit. In addition, the predicted specificity of the *Drosophila* ModSP does not match the predicted cleavage site of the downstream serine protease Grass, and ModSP does not cleave Grass *in vitro (Buchon et al., 2009)*. Thus, it remains possible that other factors may be involved. Currently, it is not clear how (or even if) PGN binding to PGRP–SA/SD/GNBP1 leads to the activation of ModSP, and, as mentioned above the exact biochemical role of GNBP1 remains controversial. The protease cascade culminates in SPE directly cleaving pro–Spätzle, releasing the active C106 fragment (Jang et al., 2006; Kambris et al., 2006).

Spätzle binding induces dimerization of the Toll receptor. Although the ligand is a symmetric dimer, biophysical studies indicate that the Spätzle–induced Toll dimer is asymmetrical (Weber et al., 2003). It is not yet clear whether the asymmetric aspect of the ligand–induced Toll dimer is critical for the activation of intracellular signaling. Dimerization of the Toll receptor is believed to recruit a pre–existing Myeloid differentiation primary response gene 88 (Myd88)/Tube complex that ultimately recruits the kinase Pelle, which is homologous to the mammalian IRAK family of kinases. The assembly of the resulting receptor complex occurs via two distinct functional domains. While the interaction between Toll and Myd88 occurs via their Toll/IL–1R (TIR) domains, Myd88, Tube and Pelle interact in a trimeric complex via death domains (DD) found in each protein (Sun et al., 2002b; Sun et al., 2002a; Tauszig-Delamasure et al., 2002; Towb et al., 1998). Although the DDs of these proteins are necessary for their

interactions, Myd88 and Pelle do not interact directly; Tube acts as the core of the trimeric complex (Sun et al., 2002a). Thus the activated Toll receptor interacts directly with Myd88, which interacts with Tube, which ultimately recruits the kinase Pelle. Similar IRAK–kinase recruitment via an adapter complex is seen in mammalian Myd88–dependent TLR signaling.

Drosophila TNF–receptor–associated factor 2 (dTRAF2), the homolog of mammalian TRAF6, may also play a role in Toll signaling; however its role is unclear. In transiently transfected *Drosophila* cells, Pelle interacts with dTRAF2 and co–expression of Pelle and dTRAF2 synergistically activates the Toll pathway target gene *Drosomycin* (Shen et al., 2001). However RNAi to *dTraf2* shows no suppression of AMP gene expression after stimulation of the Toll or IMD pathways (Sun et al., 2002a; Zhou et al., 2005). In adult flies, over–expression of dTRAF2 is able to induce AMP gene expression and nuclear translocation of DIF as well as Relish. Interestingly, *dTraf2* null larvae exhibited reduced, but not abolished, levels of AMP gene expression following *E. coli* infection (Cha et al., 2003). These data suggest that dTRAF2 may function in both the IMD and Toll pathways, but bypass mechanisms may be present which circumvent dTRAF2 in both cases.

Infections by Gram–positive bacteria and fungi culminate in the nuclear translocation of NF–κB proteins DIF and/or Dorsal. DIF is the main regulator of Toll signaling in both adults and larvae, whereas Dorsal is specifically required for the immune response in larvae. Dorsal was first identified for its role in dorso–ventral patterning in the developing embryo (Santamaria and Nusslein-Volhard, 1983). The intracellular signaling components that lead to activation of Dorsal are the same in both

early embryo and in the immune response (Drier and Steward, 1997). DIF/Dorsal is sequestered in the cytoplasm by its interaction with the IκB protein Cactus. In the embryo, Cactus and Dorsal are found in a complex in which one homodimer of Dorsal interacts with one molecule of Cactus (Isoda and Nusslein-Volhard, 1994). Upon signaling, Cactus is degraded and DIF or Dorsal translocate to the nucleus (Belvin et al., 1995; Bergmann et al., 1996; Gillespie and Wasserman, 1994; Reach et al., 1996; Wu and Anderson, 1998). Cactus degradation, like IκB degradation, is controlled by phosphorylation and ubiquitin/proteasome–mediated degradation (Bergmann et al., 1996; Reach et al., 1996; Fernandez et al., 2001; Liu et al., 1997). However, neither of the two *Drosophila* IKK–related kinases (IKKε, IKKβ) are required for Toll–mediated Cactus phosphorylation and degradation. Although *Drosophila* IKKβ can phosphorylate Cactus *in vitro* (Kim et al., 2000), it is not required for *Drosomycin* expression in cells or in flies (Silverman et al., 2000; Lu et al., 2001; Rutschmann et al., 2000). Although the sequence motifs that are phosphorylated are very similar to those critical for IκBα phosphorylation in human cells, the kinase that phosphorylates Cactus is yet to be identified. Once phosphorylated, Cactus is likely ubiquitinated via the Slimb–SCF E3–ligase complex. *Drosophila* embryos mutant for *slimb*, the βTrCP homolog, are unable to activate the Dorsal target genes *twist* and *snail* (Spencer et al., 1999). Interestingly, Cactus degradation is required but not sufficient for efficient nuclear translocation of Dorsal during development (Bergmann et al., 1996).

Degradation of Cactus and nuclear translocation of DIF (and Dorsal) leads directly to the transcriptional induction of many immune responsive genes (De Gregorio et al., 2001; De Gregorio et al., 2002; Irving et al., 2001). For example, the well

characterized AMP genes *Defensin*, *Drosomycin*, *Cecropin* and *Metchnikowin* are activated by Toll signaling. The promoter/enhancer regions of all these AMP genes include κB–sites where DIF or Dorsal bind (Senger et al., 2004). In addition, Toll signaling leads to the activation of other less well characterized genes, some of which may be AMPs while others may control different facets of the immune response. For example, Toll signaling is linked to the activation of the cellular immune response and the proliferation of hemocytes (Zettervall et al., 2004; Qiu et al., 1998). Also, many components of the Toll pathway are regulated by Toll signaling itself (De Gregorio et al., 2002; Lemaitre et al., 1996). Most notably, Cactus is up–regulated in response to immune challenge via the Toll pathway. This generates a negative feedback loop to down–modulate the cascade (Nicolas et al., 1998).

The Toll and IMD pathways are thought to be activated independently and initiate specific responses to different microorganisms. However, some AMPs are activated by both the Toll and IMD pathways. Tanji *et al*. (2007) have demonstrated that some AMP genes have distinct κB elements in their enhancer region (*e.g. Drosomycin*). These elements respond to either Relish or DIF, with optimal gene induction occurring only when both the Toll (DIF) and IMD (Relish) pathways are activated, suggesting synergistic regulation of AMPs by two pathways (Tanji et al., 2007)**.**

The IMD Pathway

The IMD pathway is potently activated by DAP–type PGN derived from Gram–negative bacteria and certain Gram–positive bacteria, such as *Bacillus* spp. Initial studies suggest that the IMD pathway is activated preferentially by Gram–negative bacteria, which lead many to assume that LPS, the most potent activator of the

mammalian innate immune response, would be the main agonist of this pathway (Werner et al., 2003; Samakovlis et al., 1992). However, a careful analysis of published results suggested otherwise. In addition to Gram–negatives, certain Gram–positive bacteria, e.g., *Bacillus spp*, are also IMD pathway activators (Lemaitre et al., 1997). Subsequently, Lemaitre's group showed that DAP–type PGN, from *E. coli* or *B. thurengensis*, activate the IMD pathway, while our group demonstrated that purified LPS samples are unable to trigger the IMD pathway, and IMD agonistic activity could be traced to DAP–type PGN (Leulier et al., 2003; Kaneko et al., 2004). Lemaitre's group also show that the Toll pathway is activated by PGN, but in this case lysine–type PGN from Gram–positives like *M. luteus* and *E. fecalis* was more potent (Leulier et al., 2003).

Recognition of DAP–type PGN involves the receptors PGRP–LC and PGRP–LE (Takehana et al., 2002; Leulier et al., 2003; Ramet et al., 2002; Gottar et al., 2002; Choe et al., 2002). *PGRP–LC* encodes three alternatively spliced transcripts *PGRP–LCa*,*–LCx*,*–LCy*. All three isoforms encode single–pass transmembrane cell surface receptors. They each have distinct extracellular domains, which include a PGRP motif, anchored to the identical transmembrane and cytoplasmic domains (Werner et al., 2000). *PGRP–LE* encodes only one protein, which lacks both a signal sequence and a transmembrane domain. Although *PGRP–LC* null flies, which lack all three isoforms, produce dramatically reduced levels of AMPs following infection with Gram–negative bacteria such as *E. coli* and *A. tumefaciens*, they are not particularly susceptible to infection by all Gram–negative bacteria. For example, *PGRP–LC* mutants are sensitive to *A. tumefaciens*, *E. carotovora carotovora*, and *E. cloacae*, but not *E. coli* and *B. megaterium*. (Gottar et al., 2002; Choe et al., 2002; Takehana et al., 2002). In contrast,

mutants that abolish signaling through the IMD pathway, such as null alleles in IKK genes, are highly susceptible to all Gram–negative bacteria. Therefore, it was hypothesized that another receptor must also recognize and respond to Gram–negative bacteria. Moreover, it was suggested that relatively low levels of AMP gene induction, as observed in *PGRP–LC* mutants, are sufficient to protect against infection with many Gram–negative bacteria. Genetic experiments suggested that PGRP–LE is the alternate receptor for the IMD pathway. Double *PGRP–LC*, *PGRP–LE* mutants are hypersusceptible to most Gram–negative bacteria, similar to other null mutants in the IMD pathway, and these double mutants do not induce detectable level of AMP genes following infection. Over–expression of either PGRP–LC or PGRP–LE, in flies or in cell culture, is sufficient to drive AMP expression through the IMD pathway. PGRP–LE over–expression also activates the phenoloxidase cascade (Park et al., 2007; Takehana et al., 2002). While both PGRP-LC and PGRP-LE are potent activators of the IMD pathway, PGRP-LE can also trigger an autophagic response through a Relish (NF-κB)-independent pathway that is critical to protect the animal against intracellular pathogens like *Listeria* (Yano et al., 2008).

TCT binds PGRP–LCx directly, and then this ligand/receptor complex interacts with PGRP–LCa (Chang et al., 2005; Mellroth et al., 2003). The crystal structure of TCT bound to the ectodomains of PGRP–LCx and –LCa has been solved. TCT binds in the deep PGN binding cleft of PGRP–LCx, typical of PGRP–muropeptide interactions. The disaccharide unit of TCT makes important contributions to the interactions between PGRP–LCx (bound to TCT) and PGRP–LCa (Chang et al., 2006). The interactions responsible for TCT–induced PGRP–LE multimerization are very similar in molecular

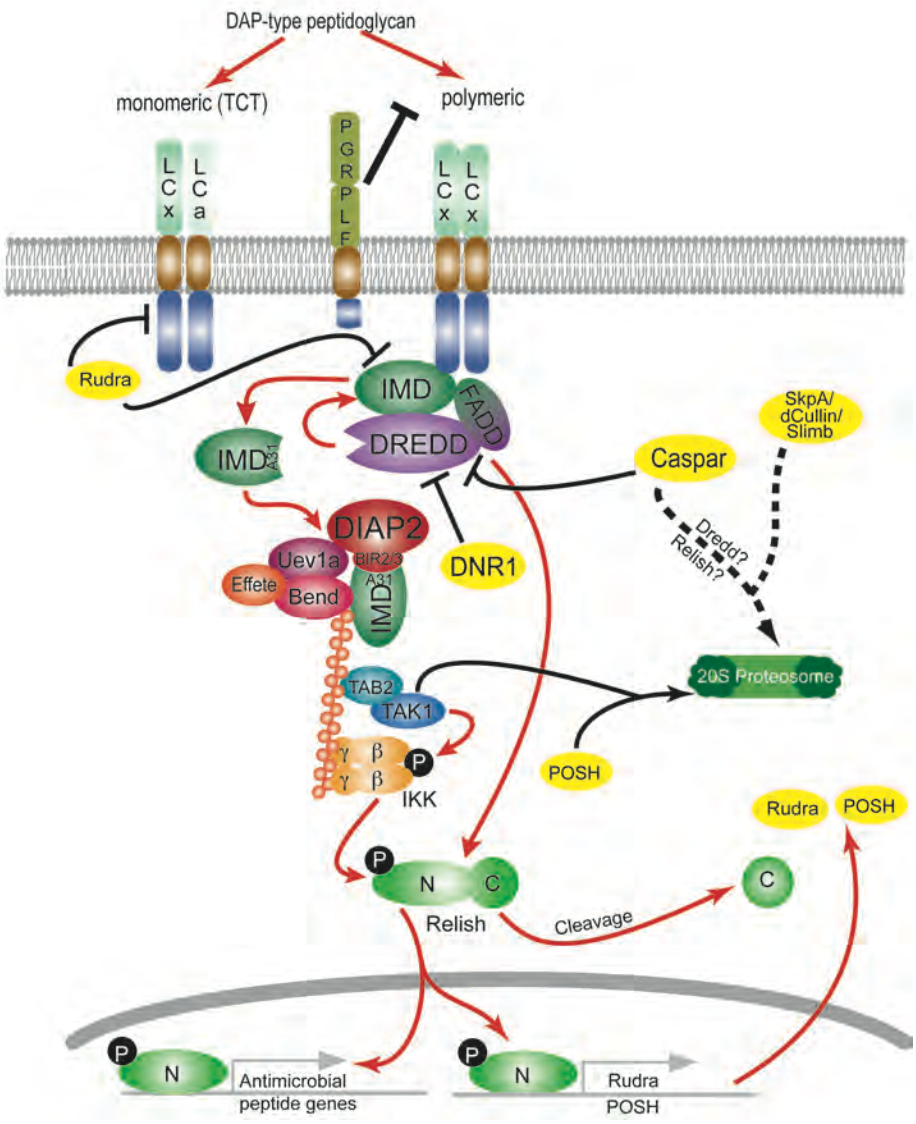


Figure 1.3: IMD signaling pathway.
This pathway is preferentially triggered by DAP–type peptidoglycan, common to Gram–negative bacteria and certain Gram–positives, especially *Bacillus spp.* DAP–type peptidoglycan can be recognized by different receptors, depending on its location and size. In addition, DAP–type peptidoglycan that reaches the cytosol can trigger another receptor, PGRP–LE. Both PGRP–LC and PGRP–LE trigger a similar intracellular signal transduction pathway, as outlined here, that culminates in the activation of the NF–κB precursor Relish. In addition, recognition of intracellular DAP–type peptidoglycan by PGRP–LE also triggers an autophagic response, which is important in the protection against intracellular bacteria. After activation of the receptor, IMD is cleaved by the caspase DREDD. Cleavage of IMD leads to its interaction with the ubiquitin E3 ligase DIAP2 and in concert with the E2 conjugating enzymes IMD is then K63–polyubiquitinated. K63 poly–ubiquitnated IMD acts as a scaffold to recruit TAK1 and IKK. TAK1/TAB2 are responsible for the activation of the NF–κB/Relish branch of the IMD pathway which is critical for the induction of AMP gene expression. In this schematic representation of the IMD pathway. Red arrows indicate the IMD signaling cascade while the black arrows highlight the negative regulators and their likely targets. The dashed black line suggests a hypothetical interaction between Caspar and the SCF complex, as discussed in the text.

detail to those responsible for the TCT–mediated PGRP–LCx/LCa dimer. Because PGRP–LCa cannot bind TCT in a typical PGN binding cleft [the LCa cleft is occluded; (Chang et al., 2006; Chang et al., 2005)], the LC complex is limited to a dimeric form, while PGRP–LE forms a head–to–tail multimer, with each subunit binding to TCT and interacting with another subunit (Lim et al., 2006). The molecular mechanism by which PGN binding to either PGRP–LC or PGRP–LE leads to activation of the IMD pathway is the subject of my thesis work.

Upon binding PGN, the PGRP–LC receptor leads to the activation of downstream signaling events in which the adaptor protein IMD is cleaved by the caspase Death related ced–3/Nedd2–like protein (DREDD). Cleavage of IMD exposes an Inhibitor of apoptosis (IAP) binding motif allowing IMD to interact with the Baculovirus IAP Repeat (BIR) 2 & 3 domains of the ubiquitin E3 ligase *Drosophila* Inhibitor of apoptosis 2 (DIAP2). In concert with the E2 conjugating enzymes, Uev1a, Bendless and Effete, the E3 ligase DIAP2 leads to K63 polyubiquitination of IMD. In a manner similar to mammalian NF–κB signaling, it is then proposed that this K63–polyubiquitin chain acts as a scaffold to recruit downstream kinase TGFβ activating kinase 1 (TAK1 (Paquette et al., 2010)). TAK1 binding protein (TAB2) contains a highly conserved K63–polyubiquitin binding domain and is required for the activation of the downstream Janus kinase (JNK) and Relish/NF–κB arms of the IMD pathway. TAB2 is spliced into two different splice forms, while both arms can utilize the long (full length) TAB2 isoform, the short isoform is only able to promote activation of the Relish/NF–κB pathway (Alain C unpublished). In addition, auto–phosphorylation of S176, within the activation loop of TAK1, is also required for activation of the kinase (Paquette N unpublished). In concert with TAB2,

activated TAK1 can then initiate signaling to one or both downstream arms of the IMD pathway.

The JNK arm of the IMD pathway is activated by the TAK1–mediated signaling to Hemipterous, the *Drosophila* MKK7/JNKK homolog (Sluss et al., 1996; Holland et al., 1997; Chen et al., 2002). Hemipterous then goes on to phosphorylate the *basket* protein (JNK), which activates *Drosophila* Activator protein 1 (AP–1). Signaling through the IMD/JNK pathway has been linked to the up–regulation of wound repair and stress response genes (Boutros et al., 2002; Silverman et al., 2003). Yet, the precise role that JNK signaling plays in the IMD pathway is controversial. Several reports have concluded that JNK signaling is not involved in AMP gene induction. Instead, AMP gene expression relies entirely on the NF–κB/Relish branch of the IMD pathway (Boutros et al., 2002; Silverman et al., 2003). In fact, it is proposed that an unidentified product of the Relish branch of the IMD pathway inhibits JNK signaling (Park et al., 2004), while the JNK pathway is proposed to directly inhibit AMP gene expression by recruiting histone deacetylases (Kim et al., 2005). However, Delaney and colleagues (2006) have demonstrated that the TAK1/JNK branch of the IMD pathway is critical for AMP gene induction, at least in clones of JNK–deficient cells within the larval fat body (Delaney and Mlodzik, 2006). The role of the JNK pathway in antimicrobial gene expression remains controversial and further work will be necessary to clarify whether JNK has a positive and/or, negative role in the process.

In parallel to JNK activation TAK1 is also required for induction of the NF–κB/ Relish branch of the IMD pathway, through activation of the *Drosophila* IKK complex (Vidal et al., 2001; Silverman et al., 2003). The *Drosophila* IκB kinase (IKK) complex

contains two subunits: a catalytic kinase subunit encoded by *ird5* (IKKβ) and a regulatory subunit encoded by *kenny* (IKKγ; (Silverman et al., 2000; Rutschmann et al., 2000)). In S2 cells, it was clearly demonstrated that the IKK complex is activated rapidly following immune stimulation and this activation requires TAK1 (Silverman et al., 2003; Silverman et al., 2000). Activated IKK complex can directly phosphorylate Relish. Relish is a bipartite protein similar to mammalian NF–κB precursors p100 and p105. It contains an N–terminal Rel homology domain (RHD) and an inhibitory IκB domain with six ankyrin repeats that retain the protein in the cytoplasm. Upon infection with Gram–negative bacteria, *Relish* expression is strongly induced in adult flies (Dushay et al., 1996). *Relish* mutant flies show extreme sensitivity to infections and fail to induce antimicrobial genes after bacterial infection (Hedengren et al., 1999). Although the *Relish* locus encodes an embryo specific isoform, *Relish* does not appear to have a role in development as homozygous Relish mutants continue to be viable and fertile.

In mammals, the NF–κB precursors p100 and p105 are processed by the proteasome and their C–terminal region is degraded to produce p50 and p52, respectively. This processing is regulated by phosphorylation of C–terminal serine residues, which leads to ubiquitination and partial proteasome degradation of the C–terminus (Perkins et al., 1997). In contrast, Relish processing does not depend on proteasomal degradation. Relish is instead endo–proteolytically cleaved by a caspase, producing a N–terminal RHD transcription factor module that translocates to the nucleus to activate immune genes, while the stable C–terminal domain remains in the cytoplasm (Stöven et al., 2000). Relish cleavage occurs after residue D545, within a typical caspase target motif, $_{542}$LQHD$_{545}$. DREDD, in addition to its previously mentioned role

upstream in the pathway, also appears to function downstream and is a likely candidate for the caspase responsible for cleavage of Relish. DREDD and Relish physically interact under cell culture conditions and *Dredd* RNAi prevents AMP gene expression induced by an activated allele of TAK1 (Zhou et al., 2005). *Dredd* mutants also fail to cleave Relish, are unable to induce AMP gene expression and are highly sensitive to Gram–negative bacterial infections (Stöven et al., 2003; Leulier et al., 2000). Furthermore, over expression of DREDD is sufficient to cause Relish cleavage and purified DREDD cleaves Relish *in vitro* (Erturk-Hasdemir et al., 2009).

Phosphorylation of Relish occurs in a signal dependent manner by the *Drosophila* IKK complex and the C–terminus of Relish is required for both its phosphorylation and cleavage (Stöven et al., 2003). 2 serine residues, (528 and 529), in the N–terminal of Relish are phosphorylated by *Drosophila* IKKβ. However, these residues are not required for Relish cleavage. Instead, phosphorylation of these residues appears critical for the proper transcriptional activation of Relish targets via efficient recruitment of RNA polymerase II to the promoters of AMP genes. Apart from its role in phosphorylation, the IKK complex also functions non–catalytically in the cleavage of Relish (Erturk-Hasdemir et al., 2009). In total, Relish activity is coordinately regulated by 2 distinct mechanisms; cleavage by DREDD and phosphorylation by the IKK complex (**Figure 1.3**).

Many questions remain unanswered about molecular mechanisms required for IMD signaling. It is clear that DAP–type peptidoglycan is the major activator of this pathway, through PGRP–LC and PGRP–LE. It is also well established that many of the components involved are homologous to factors involved in mammalian NF–κB

pathways. In particular the involvement of a RIP1–like molecule, IMD, highlights similarity to the TNFR and TRIF–dependent TLR pathways. However, more study is needed to elucidate the molecular events proximal to the receptor as well as mechanisms required for TAK1 activation, Relish cleavage, and the transcriptional activation of AMP genes, all of which occurs very rapidly after PGN recognition.

Recent work has shown that the intensity and duration of the *Drosophila* immune response is tightly regulated. As in mammals, hyper–activated immune responses are detrimental, and the proper down–modulation of immunity is critical for protective immunity and health. In order to keep the immune response properly modulated, the Toll and IMD pathways are controlled at multiple levels by a series of negative regulators. In the next few pages, we focus on recent studies identifying and characterizing the negative regulators of these pathways.

Down–regulation of Immune Signaling Pathways by PGN Digesting Enzymes

The PGRP family of proteins are similar in structure to N–acetylmuramoyl–L–alanine amidases (NAMLAA), enzymes that degrade PGN by removing the stem–peptide from the glycan backbone of PGN (Mellroth et al., 2003). The PGRP proteins that function as receptors in the IMD and Toll pathways lack a critical cysteine residue that is required for catalysis and thus function only as PGN binding receptors. On the other hand, PGRP–LB,–SB1, and–SC1 encode active amidases, and PGRP–SB2 and–SC2 are predicted amidases (Royet and Dziarski, 2007; Mellroth et al., 2003; Werner et al., 2000). Digestion of PGN with type 2 amidases, like these PGRPs, significantly reduces its immunostimulatory activity (Werner et al., 2003; Kaneko et al., 2004). PGRP–LB digests only DAP–type PGN, whereas PGRP–SC digests both DAP–type

and lysine–type PGN (Zaidman-Remy et al., 2006)(Mellroth et al., 2003). Depletion of *PGRP–SC1/2* or *PGRP–LB* by RNAi *in vivo* leads to markedly higher levels of *Diptericin* expression following infection. PGRP–LB and PGRP–SC are expressed in the gut epithelial and PGRP–LB regulates the immune reactivity of the gut to ingested bacteria. In the gut, the IMD pathway is activated only when the PGN–degrading activity of PGRP–LB is saturated or inactivated. Also in infected larvae, RNAi knockdown of *PGRP–SC1/2* leads to increased developmental defects and lethality (Bischoff et al., 2006). This further supports the idea that PGN–degrading PGRPs prevent potentially pathological consequences to host tissues because of prolonged immune activity.

PGRP–LB and PGRP–SC are both targets of IMD signaling (like the AMP genes) and thus form a critical link in a negative feedback loop whereby activation of IMD signaling, by PGN, leads to the production of enzymes which digest this stimulating microbial compound. Additionally, another report shows that *PGRP–SC1* mutants affect Toll signaling and may also play a role in phagocytosis of *S. aureus* (Garver et al., 2006).

Although PGRP–LF is a non–catalytic PGRP, it also seems to play an inhibitory role in immunity. PGRP–LF is a transmembrane receptor but it contains only a 23 amino acid intracellular tail. PGRP–LF encodes two PGRP domains in its extracellular portion, which show different affinity for DAP–type PGN and low affinity towards lysine–type PGN. Depletion of PGRP–LF leads to infection independent activation of the IMD pathway in cells and flies. It also leads to the activation of the JNK pathway and hence developmental deffects (Persson et al., 2007; Maillet et al., 2008).

Negative Regulation in the Toll Pathway

In addition to the control of the upstream serine proteases by the serpin necrotic, the Toll pathway is repressed by an intracellular negative feed–back loop controlled by wnt inhibitor of Dorsal (WntD), a member of the wnt family of ligands. Activation of the Toll pathway leads to the transcription of *wntD* (Gordon et al., 2005; Ganguly et al., 2005). The earliest role of WntD in the embryo is to restrict the field of Dorsal activation by inhibiting its translocation into the nucleus. This ensures the establishment of a proper boundary between the developing ventral and terminal domains. WntD is able to block the translocation of Dorsal in *cactus* mutants. Therefore, WntD blocks nuclear translocation of Dorsal downstream of, or in parallel to Cactus. In addition to its role in embryonic patterning, WntD also regulates the Toll pathway in the context of immunity. For example, wntD mutants induce higher levels of some AMP genes. *wntD* mutants are also more sensitive to infection with *L. monocytogens*. It is hypothesized, that the *wntD* mutants have a higher mortality following infection due to the hyper–activation of Dorsal target genes (Gordon et al., 2005).

Negative Regulation in the IMD Pathway

Recent work has demonstrated that IMD is cleaved in a signal dependent manner and then K63 polyubiquitnated. K63 polyubiquitnation of IMD is required for the activation of downstream signaling (Paquette et al., 2010). Thevenon *et al* (2009) have characterised a deubiquitnating enzyme, *Drosophila* Ubiquitin specific protease 36 (dUSP36), that negatively regulates the IMD pathway. *dUsp36* mutant flies show constitutive activation of the IMD pathway and are more sensitive to infection. dUSP36 is required for immune tolerance in that it prevents the activation of IMD pathway in

response to environmental bacteria. Further, they show that dUSP36 blocks signaling at the level of IMD. dUSP36 interacts with the N terminus of IMD removing K63 polyubiquitin chains from IMD and promoting K48 polyubiquitnation and subsequent degredation (Thevenon et al., 2009).

TAK1 plays a critical role in the IMD pathway, serving as the branch point for JNK and NF–κB/Relish activation. In turn, these two modules of the IMD pathway negatively regulate each other. IMD regulates the JNK branch by inducing certain genes, via Relish, that lead to the degradation of dTAK1 (Park et al., 2004). JNK activation is prolonged when the Relish branch of the IMD pathway is inhibited. Relatedly, Tsuda et al. (2006) showed that Plenty of SH3 (POSH) regulates the termination of IMD–>JNK signaling. POSH mutant flies also exhibit increased mortality following *E.coli* infection, possibly because of a hyperactive immune responses (Tsuda et al., 2006). POSH encodes a Really interesting new gene (RING) finger, a signature ubiquitin E3–ligase motif, and is auto–ubiquitnated. Also POSH immunoprecipitates with TAK1 and over–expression of POSH reduces the stability of TAK1. Thus, it is hypothesized that POSH negatively regulates the IMD–>JNK pathway by regulating the stability of TAK1 via the ubiquitin/proteosome degredation pathway. On the other hand, JNK signaling also inhibits Relish–mediated transcriptional activation, via the recruitment of a 'repressosome' to AMP genes, as discussed in more detail below (Kim et al., 2007; Kim et al., 2005).

The IMD pathway may also be inhibited by another E3 protein, know as Defense repressor 1 (Dnr1), a conserved protein with an N–terminal ezrin/radixin/moesin domain and a C terminal RING finger. *Dnr1* RNAi stimulated a *diptericin–lacZ* reporter, even in

the absence of immune stimulation, in *Drosophila* cells. Dnr1 appears to have a complex relationship to the caspase DREDD. On one hand, Dnr1 is believed to function as an inhibitor of DREDD. On the other hand, immune stimulation stabilizes Dnr1, in a DREDD–dependent manner. Therefore, Dnr1 may provide another feedback loop where immune stimulation, via DREDD, promotes accumulation of a DREDD inhibitor (Foley and O'Farrell, 2004)(Guntermann et al., 2009).

Another negative regulator of IMD signaling is Caspar. Interestingly, Caspar is homologous to human Fas associated factor 1 (hFAF1), which associates with various components of the TNF/NF–κB pathway like FAS, Fas–Associated protein with Death Domain (FADD), caspase–8 and NF–κB (Park et al., 2004; Chu et al., 1995; Ryu et al., 2003). *caspar* mutant flies show constitutive expression of *Diptericin*, even in the absence of infection. And, mutation of *caspar* actually protects flies against mildly pathogenic bacteria (Kim et al., 2006), unlike several other mutants where hyper–activation of IMD signaling causes hyper–susceptibility to infection (Kim et al., 2007; Thevenon et al., 2009; Tsuda et al., 2006)(Zaidman-Remy et al., 2006). Over–expression of Caspar inhibits AMP gene induction and causes decreased viability after infection with these same mildly pathogenic bacteria. It is hypothesized that Caspar blocks Relish cleavage by interfering with DREDD. Caspar contains two ubiquitin related domains: a so–called UAS (or UBA) domain and a ubiquitin–like domain (UBx), as well as FAS–and DED–interaction domains, all of which are found in hFAF1. These later two domains may mediate interaction between Caspar and either DREDD or dFADD, but this has not yet been demonstrated. The ubiquitin related domains suggest that Caspar may regulate protein degradation of IMD pathway components, but this also

awaits experimental data.

The possible connection between Dnr1 or Caspar and the Ub/proteasome mediated degradation of IMD components is reminiscent of an earlier publication. Khush *et al*., (2002) found that mutations in *skpA* constitutively induce IMD signaling, but not the Toll pathway (Khush et al., 2002b). SkpA is the homolog of the human Skp1 protein, a subunit of SCF–E3 ubiquitin ligase which targets substrates for K48–polyubiquitination and degradation by the 26S proteasome. Other mutants that also effect the *Drosophila* SCF component, i.e. slimb and dCullin1, also induce *Diptericin* expression in the absence of infection. In cells, RNAi targeting *skpA* or *slimb* leads to the accumulation of both the full length and cleaved forms of Relish. Therefore, it was hypothesized that the SkpA, Slimb and dCullin regulate the IMD pathway by controlling Relish stability. Potentially these SCF–dependent effects could be mediated through Caspar (see dashed line in **Figure 1.3**).

Transcriptional regulation of the *Drosophila* immune response

Proper regulation of NF–κB transcription factors is critical for health. The lack of NF–κB signaling or inappropriate activation of NF–κB can lead to serious conditions such as cancer, (auto) inflammatory diseases or developmental defects. Consequently, animals have evolved complex mechanism to keep NF–κB, per se, in–check once it is activated. For example, the JNK branch of the IMD pathways appears to induce the formation of a repressing complex, the ‘repressosome,’ that inhibits AMP genes.

As mentioned above the JNK and Relish branches of the IMD pathway are thought to mutually inhibit each other (Park and Gambhir, 2005). The down–regulation of the IMD pathway by the JNK pathway involves the transcription factor dAP–1, a well

established target of JNK signaling in flies and mammals (Kim et al., 2005; Davis, 1999). AP–1 functions together with the *Drosophila* Signal transducers and activator of transcription (STAT) protein, Stat92E, to negatively regulate Relish and AMP gene expression. Stat92E is a transcription factor that is activated by the JAK/STAT pathway. Bacterial infections are known to induce this transcription factor via the production of Unpaired–3 (UPD3), a ligand for the JAK/STAT pathway receptor Domeless. UPD3 production following infection requires IMD–>Relish signaling in hemocytes (Agaisse et al., 2003). Several Relish–dependent AMP genes, especially *AttacinA*, have AP–1 and Stat92E binding sites, in addition to κB sites, in their promoter/enhancer regions. In some cases the Stat92E site overlaps the Relish binding site. Mutation of the AP1 or Stat92E binding site leads to 3–fold higher levels of an *AttacinA* reporter (Kim et al., 2007). AP1 and Stat92E bind the *AttacinA* promoter cooperatively, with the aid of the High mobility group (HMG) protein Dispersed 1 (Dsp1), and then recruit the histone deacetylase dHDAC1 to form a repressosome complex. Flies with reduced levels of Stat92E, Jun (known as Jun related antigen, Jra, in *Drosophila*) or Dsp1 induce higher levels of *AttacinA* transcript following bacterial infection. Also, these mutants display reduced survival following infection with mildly pathogenic bacteria like *E.coli*. Heterozygosity for a Relish null allele suppresses these phenotypes, consistent with the idea that the respressosome competes with Relish. For example, Relish is recruited quickly to the *AttacinA* promoter, within 15 minutes of PGN stimulation. But, after longer periods of stimulation, loss of Relish binding is observed while the repressosome shows sustained binding. Repressosome recruitment to the *AttacinA* promoter also leads to its hypoacetylation. The repressosome–mediated down regulation of Relish–mediated

transcription of *AttacinA* is not unique; the AMP genes *AttacinB*, *Cecropin A1*, *Drosocin* and *Metchnikowin* show a pattern similar to *AttacinA.* Thus, the inhibitory affect of the repressosome complex on NF–κB is important for maintaining a balanced immune response (Kim et al., 2005; Kim et al., 2007).

Tissue specific immune regulation

In most of the work discussed above, the systemic *Drosophila* immune response was studied. This response relies primarily on the inducible expression of AMP genes in the fat body, the insect liver. In addition, many studies have also been performed in immune responsive *Drosophila* cell lines, which are hemocyte–derived. However, IMD and Toll signaling also occurs in many other tissues. For example, the Toll pathway plays critical roles in hematopoeitic development and function. The transcription factors DIF and Dorsal are required for hemocyte proliferation and to prevent apoptosis. DIF and Dorsal also contribute to the control of systemic infections by regulating phagocytosis (Matova and Anderson, 2006). The IMD pathway is present and functional in almost all epithelial cells. However, the responses in these tissues are not identical to that observed in the fat body (or in cell culture). In particular, the outputs of IMD signaling are significantly modified in the gut.

Although *Drosophila* harbor a substantial number of resident bacteria in their alimentary tract (Ryu et al., 2008; Ren et al., 2007; Cox and Gilmore, 2007), AMP genes are not expressed in the gut epithelia of healthy animals. Recent studies have shown that these resident microbes still induce IMD signaling in the gut–as assayed by Relish translocation and expression of the PGN–digesting PGRP–LB and–SC. However, other Relish target genes, especially the AMP genes, are mostly silent. Ryu et al. (2008)

further showed that the gut specific inhibition of AMP gene expression is mediated by the homeobox transcription factor Caudal (Cad). Caudal is best known for the critical role it plays in development of the mesoderm and gut (Lengyel and Iwaki, 2002). A Caudal–response element, essential for the suppression of *CecropinA* expression in the gut, was characterized in the promoter/enhancer region of this AMP gene. Moreover, knockdown of *caudal* in the gut, by tissue–specific RNAi, caused constitutive expression of *Cecropin* in this tissue. AMP gene expression required IMD signaling components, like TAK1 or DREDD, as well as resident gut bacteria. Germ–free animals showed little or no AMP expression, even in the absence of Caudal. Expression of AMPs in the gut, with *caudal* RNAi, caused a shift in the bacterial populations resident in the gut. In particular, one *Acetobacteraceae* species was greatly reduced while a *Gluconobacter* species flourished. The *Gluconobacter sp*. was pathogenic, causing significant gut epithelial apoptosis and reduced survival (**Figure 1.4**). These findings not only characterized a novel means of regulating immune signaling in a tissue specific manner, but also highlight the critical importance that proper immune regulation plays in health and disease, even in the fruit fly (Ryu et al., 2008).

Several studies using a gut infection model system have shown that the redox system plays an essential role in host survival by generating microbicidal effectors such as reactive oxygen species (ROS) (Ha et al., 2005a; Ha et al., 2005b). In this redox system, dual oxidase (DUOX), a member of the nicotinamide adenine dinucleotide phosphate (NADP)H oxidase family, is responsible for the production of ROS in response to gut infection (Ha et al., 2005a). Following microbe–induced ROS generation, ROS is eliminated by immune–regulated catalase (IRC), thereby protecting

the host from excessive oxidative stress (Ha et al., 2005b). The DUOX–dependent ROS serves on the front line of microbicidal function and the IMD pathway functions as a second line of defense in the gut. DUOX expression and activity are regulated in response to the microbial fluctuation in the gut.

DUOX activity is regulated by the Gαq–PLCβ–Ca2+ signaling pathway. Basal Gαq–PLCβ–DUOX activity is sufficient for host survival. Under conventional conditions, PLCβ is constitutively active at a low level and this helps maintain a low DUOX activity. This DOUX activity is essential for normal host survival. However, in the case of high bacterial burden the DOUX–ROS system is strongly activated for full microbicidal activity. Flies that have an impaired Gαq–PLCβ–Ca2+–DUOX have a shorter life span under natural rearing conditions and also under gut infection studies. However, flies with impaired Gαq–PLCβ–$Ca2^{+}$–DUOX pathway survive normally following septic injury indicating that the systemic immune response is distinct from the mucosal immune pathway. It is not clear how Gαq and PLCβ induced $Ca2^{+}$ modulates DUOX enzyme activity (Ha et al., 2009a). In addition to regulation of its enzymatic activity, DUOX expression is also regulated.

DUOX expression pathway is activated when the host encounters bacterial invasion in the gut epithelia. p38 pathway is mostly responsible for the activation of DUOX expression through activation of the transcription factor ATF2. p38 mediated activation of DUOX is critical, because loss of p38 pathway components leads to host death following oral infection. Unlike the DOUX activity pathway, the DOUX expression is activated only in response to infection. Another important difference between the two pathways is that the DOUX activity pathway functions in an IMD independent way.

However, the DOUX expression pathway functions in both IMD dependent and independent ways. PLCβ mediates the PGN independent pathway and PGRP–LC and IMD but not Relish are required for the PGN mediated activation of the p38 pathway. Thus, the PGN independent PLCβ and PGN dependent IMD pathway merge at MEKK1 (MAPKK) to create a new p38–dependent DUOX expression pathway. Like the DOUX activation pathway the DOUX expression pathway is not required to fight systemic infections. However, MEKK1 and p38 are indispensable for host resistance to gut infection (**Figure 1.4**).

ROS is a highly toxic and diffusible molecule and thus ROS generation is tightly regulated. Cross–talk between the DUOX expression and activity pathways help fine tune ROS production depending on whether the gut encounters infectious or commensal microbes. In the presence of a normal bacterial burden, PLCβ is activated at low levels by the basal concentration of non–PGN ligands and it subsequently down–regulates PGN–dependent activation of p38 through the sequential induction of calcineurin B (CanB) and MAP kinase phosphatase–3 (MKP3) (Ha et al., 2009b). Maintenance of DUOX expression at the basal level is important to reduce the oxidative damage that occurs during the commensal–gut interaction. And under, high microbial burdens, DUOX–activity and DUOX–expression pathways co–operate for full activation of DUOX which leads to high levels of ROS production.

Recent studies have revealed striking similarities and dissimilarities in the signaling pathways used by humans and flies to activate their innate immune responses. In both cases, infection leads to the activation of TLRs, which in turn initiate intracellular signaling cascades that culminate in the activation of NF–κB/Rel family of

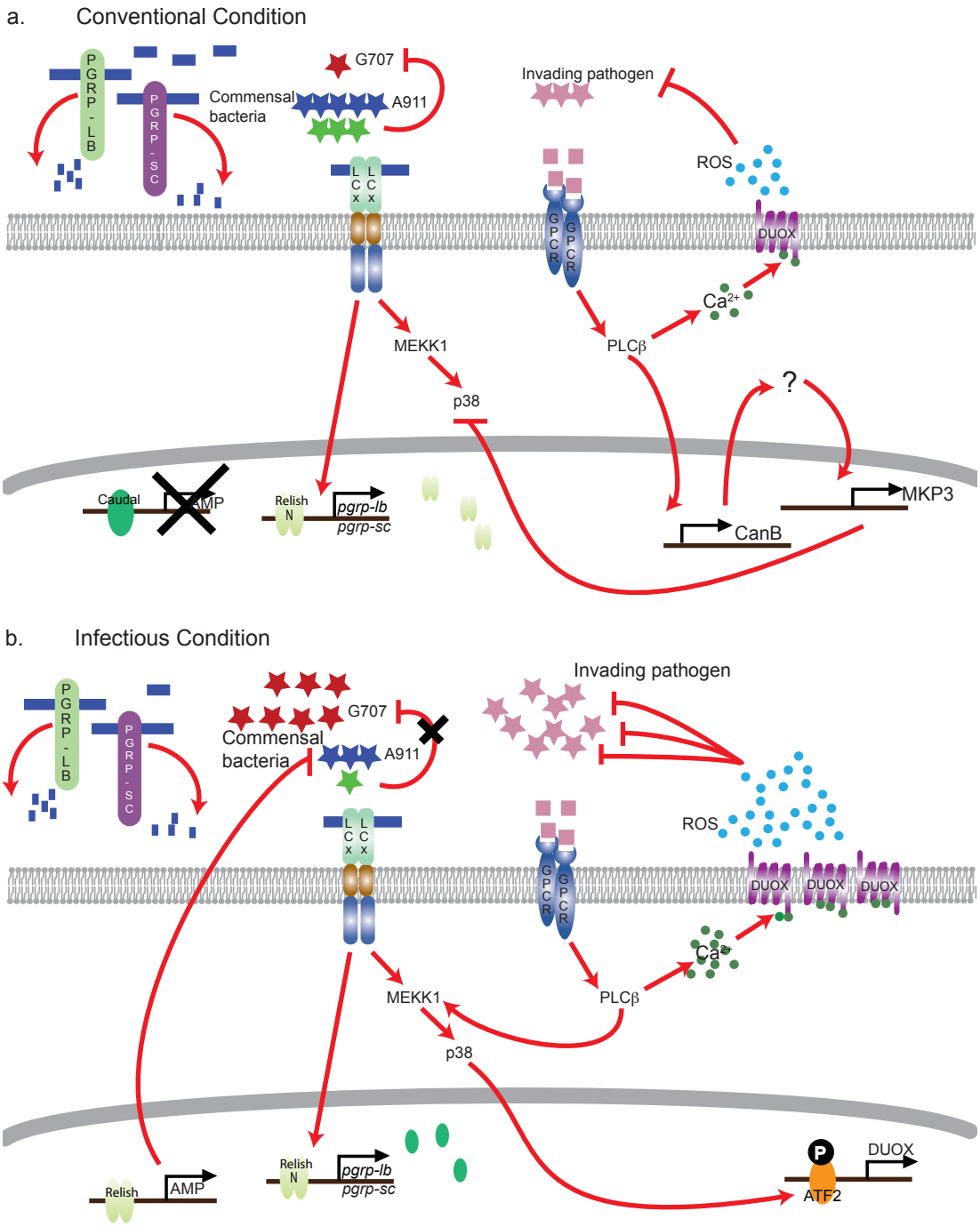


Figure 1.4: Gut immunity.
(a) Schematic representation of the signaling cascade activated in the gut under normal and infectious conditions. Under conventional conditions IMD and MEKK1–p38 pathway is activated by commensal bacteria and PLCβ is also activated at the basal level by non PGN ligand. In this condition PGRP amidases, Caudal and PLCβ–CanB–MKP3 helps down regulate AMP and DUOX expression. **(b)** However, under infectious conditions high concentration of PGN overcomes the negative regulation and leads to AMP production by IMD pathway and also DUOX expression. In addition non PGN ligands increase ROS production by activating both DUOX activity and induction pathway. See text for more detail.

transcription factors. However, the *Drosophila* Toll functions as a cytokine receptor while the mammalian TLRs are directly involved in microbial recognition. To understand these similarities and dissimilarities better the next few pages, highlight the innate immune signaling pathways present in mammals.

Mammalian NF–κB signaling

Mammals utilize an arsenal of defense mechanisms to combat infection. The innate immune system is the first line of defense. Innate immunity functions to eliminate pathogens and also to control infections. The innate immune response is also important for the development of pathogen specific adaptive immune response which is mediated by the B and T cells (Pasare and Medzhitov, 2004).

The immune cells express different Pathogen Recognition Receptors (PRRs) like Toll–like receptors (TLRs), Retinoic acid–inducible gene (RIG)–I–like receptors (RLRs) and Nucleotide oligomerization domain (NOD)–like receptors (NLRs) in various compartments and trigger the release of inflammatory cytokines and type I interferons in response to an infection (Beutler, 2009; Medzhitov, 2007; Janeway and Medzhitov, 2002; Akira et al., 2006).

In mammals, 12 different members of the TLR family have been identified. TLRs are type I integral member glycoproteins. The extracellular N–terminal domain of TLRs contain Leucine Rich Repeats (LRR) which recognize and bind different Pathogen associated molecular patterns (PAMPs) and activate downstream signaling which culminates in the translocation of NF–κB into the nucleus. Two other signaling pathways the tumor necrosis factor receptors (TNFR) and the interleukin–1 receptor (IL–1R) also signal through the canonical NF–κB pathway. Unlike the TLRs, TNFR and

IL–1R do not bind PAMPs but instead bind the cytokines TNF and IL–1, respectively, which are produced during an infection. Although the extracellular domains of TLRs and IL–1R show striking differences, there intracellular domain show striking homology and is called the Toll/IL–1 receptor (TIR) domain (O'Neill, 2003; Dunne and O'Neill, 2003). And even though these receptors bind different ligands they are able to activate similar downstream signaling pathways.

TLRs family members can be categorized into two sub–populations depending on their cellular localization. TLR–1,–2,–4,–5,–6 and–11 are expressed exclusively on the cell surface and recognize microbial components like lipids, lipoproteins and proteins. On the other hand TLR–3,–7,–8 and –9 are localized in intracellular vesicles such as endosomes and the endoplasmic reticulum and mostly recognize microbial nucleic acids (Nishiya et al., 2005; Latz et al., 2004).

Upon ligand binding TLRs oligomerize causing a conformational change which results in the recruitment of the downstream adaptor proteins such as Myeloid differentiation primary response gene 88 (MyD88), toll–interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP also called Mal), TIR–domain–containing adapter–inducing interferon–beta (TRIF), and TRIF–related adaptor molecule (TRAM) (Horng and Medzhitov, 2001; Fitzgerald et al., 2001; Yamamoto et al., 2003a; Yamamoto et al., 2002; Wesche et al., 1997; Muzio et al., 1997). Binding of TLR–1,–2,–4,–5,–6,–7,–8,–9 and–11 to their respective ligands recruits MyD88 (Wesche et al., 1997; Burns et al., 1998). In addition to MyD88, TLR1,–2,–4 and–6 recruit TIRAP which serves as a linker protein between the TIR domain of TLRs and MyD88 (Fitzgerald et al., 2001; Schilling et al., 2002; Yamamoto et al., 2002). Ligand binding by TLR–3 and–

4 leads to the recruitment of TRIF (Yamamoto et al., 2002; Yamamoto et al., 2003b). TLR–4 recruits TRIF through TRAM which again acts as a linker protein to bring together the TIR domains of TLR and TRIF (Fitzgerald et al., 2003; Yamamoto et al., 2003a). The TLR signaling pathways are categorized as MyD88 dependent and independent pathways.

The MyD88 dependent pathway is utilized by all TLR's except TLR–3. Ligand binding to the receptor leads to activation of IKK and NF–κB via tumor necrosis factor receptor associated factor 6 (TRAF6), which is recruited to the receptor in a MyD88 and IL–1 receptor–associated kinase (IRAK) dependent manner .(Cao et al., 1996a; Cao et al., 1996b). IRAK4 is initially activated by phosphorylation followed by the sequential activation of IRAK1 and IRAK2 (Suzuki et al., 2002; Qin et al., 2004). The activation of IRAKs results in TRAF6 activation which once activated interacts with the ubiquitin E2 complex of Uev1A and Ubc13. Using its own RING finger to act as an E3 ligase, TRAF6 promotes its own K63–polyubiquitination (Deng et al., 2000; Lamothe et al., 2007; Wang et al., 2001; Wooff et al., 2004).

Polyubiquitinated TRAF6 acts as a scaffold to recruit downstream kinases. The MAP3 kinase TGFβactivated kinase1 (TAK1) is recruited by ubiquitin–binding motifs in its binding partners TAK1–binding protein 2 (TAB2) and TAB3 (Kanayama et al., 2004). TAB2 and TAB3 serve as adaptors between TRAF6 and TAK1. The TAB2 and TAB3 proteins contain two ubiquitin–binding motifs, an N–terminal CUE domain and a C–terminal nuclear protein localization four zinc finger (NZF) domain (Ishitani et al., 2003; Cheung et al., 2004; Kanayama et al., 2004; Takaesu et al., 2000). Although the NZF appears to play a significant role in binding TAB2/3 to ubiquitinated TRAF6, the CUE

domain appears to be dispensable for NF–κB signaling (Kanayama et al., 2004; Kishida et al., 2005).

Binding of the TAB2/3–TAK1 complex to ubiquitinated TRAF6 leads to activation of TAK1, and initiation of downstream signaling pathways (Takaesu et al., 2001; Jiang et al., 2002; Wang et al., 2001; Sakurai et al., 2000; Kishimoto et al., 2000; Xia et al., 2009). TAK1 phosphorylates the multi–protein IKK complex composed of three proteins; two catalytic subunits, IKKα and IKKβ, and a scaffold protein IKKγ (NEMO) (Hacker and Karin, 2006; Mercurio et al., 1997; Rothwarf et al., 1998; Zandi et al., 1997). IKKγ is necessary for activation of the classical NF–κB pathway. Of the two catalytic subunits IKKβ is the major kinase. Once activated by phosphorylation and perhaps ubiquitination, the IKK complex phosphorylates the NF–κB inhibitory protein IκB leading to its K48–polyubiquitination and degradation by the proteosome (Chen, 2005; Hacker and Karin, 2006). NF–κB can then freely translocate into the nucleus where it binds any number of κB sites activating various inflammatory cytokines and cell survival genes (Hoffmann and Baltimore, 2006).

Activated TAK1 not only activates NF–κB signaling but also phosphorylates various MAP2 kinase proteins, such as MKK3,–4,–6, and–7 (Chang and Karin, 2001). Phosphorylation of these proteins leads to the activation of both p38 and c–Jun N–terminal kinase (JNK). This results in the activation and nuclear translocation of activator protein 1 (AP1), which promotes the induction of various pro–inflammatory cytokines (Chang and Karin, 2001; Shim et al., 2005).

MyD88–independent signaling pathways are also present downstream of TLR–4 (and TLR–3). The TLR–4 pathway can lead to the activation of NF–κB or the activation

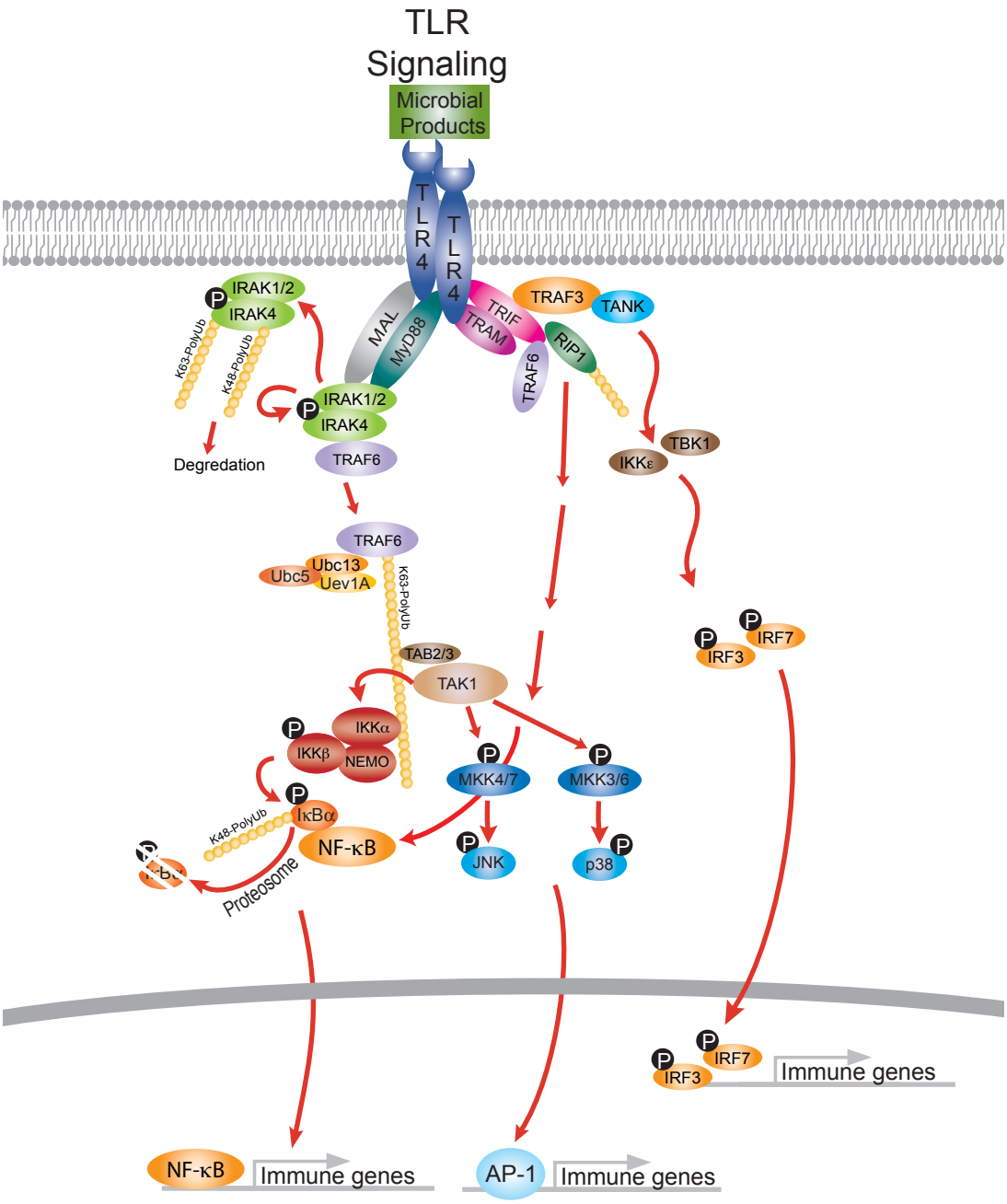


Figure 1.5 Mammalian NF–κB signaling
Upon recognition of microbial products TLR4 dimerizes and leads to the recruitment of the adaptor proteins MyD88 and Mal. MyD88 interacts with IRAK via homeotypic death domain interactions and binds TRAF6. Activated IRAK auto–phosphorylates and TRAF6 is then released into the cytoplasm. In the cytoplasm, TRAF6 interacts with the E2 enzymes Uev1a, Ubc13, and Ubc5 promoting its own K63–polyubiquitination. K63–polyubiquitination of upstream pathway members leads to the recruitment of TAK1 via ubiquitin binding domains found in TAB2/3. Activated TAK1 then phosphorylates and activates the IKK complex resulting in the phosphorylation and K48–polyubiquitin mediated degradation of IκBα, releasing NF–κB into the nucleus. TAK1 also phosphorylates MKK3,–4,–6 and–7 activating JNK/p38 mediated AP1 signaling. In a second MyD88 independent pathway TLR4 activation leads activation of NF–κB or the activation of the transcription factors IRF3 and IRF7. It is unclear how TRAF6 or RIP1 activate NF–κB.

of the transcription factors interferon regulatory factor 3 and 7 (IRF3 and IRF7). In case of IRF3 and IRF7 activation, the adaptors TRIF and TRAM associate with the proteins TRAF3 and TANK to induce signaling to the IKK–like proteins TBK1 and IKKε (Yamamoto et al., 2003b; Hacker and Karin, 2006; Oganesyan et al., 2006; Sato et al., 2003). However, recent work by Kawagoe et al shows that TANK is not required for the type I interferon pathway. Analysis of *Tank*$^{-/-}$ mice shows TANK is instead an essential negative regulator of the canonical NF–kB signaling pathway (Kawagoe et al., 2009). Once activated IKK and TBK1 phosphorylate the transcription factors IRF3 and IRF7, causing them to dimerize and translocate into the nucleus where they activate the production of type I interferons (Sharma et al., 2003; Fitzgerald et al., 2003).

The MyD88–independent signaling pathway activates NF–κB signaling through TRAF6 or RIP1. The N–terminal of TRIF contains 3 putative motifs that bind TRAF6 and activates NF–κB signaling (Sato et al., 2003). An alternative pathway leading to NF–κB activation involves the C–terminal of TRIF, which contains the RIP–homotypic interaction motif (RHIM). TRIF interacts with RIP1 through its RHIM domain and recruits it to the receptor and activates NF–κB signaling. RHIM mutant of TRIF is unable to activate NF–κB and IFN–β signaling. RIP3 negatively regulates this signaling pathway by inhibiting the interaction of RIP1 and TRIF. RIP1 gets poly ubiquitnated when it is recruited to the receptor (Cusson-Hermance et al., 2005; Meylan et al., 2004). However, it is unclear how TRAF6 or RIP1 activate NF–κB.

In addition to TLRs that detect PAMPs either on the cell surface or in the lumen of intracellular vesicles there are other PRRs present that detect intracellular PAMPs. NLRs and RLRs are two such family of proteins that recognize intracellular danger

signals (Dinarello, 2002; Dinarello, 2005). NLRs are a family of receptors that are characterized by the presence of NOD domains. NLRs are tripartite proteins that have a C terminal leucine rich repeat domain, a central NOD domain and a variable N terminal domain that can be either a caspase activation and recruitment domain (CARD), a Pyrin domain (PYD) or a baculovirus inhibitor of apoptosis repeat domain (BIR) (Martinon and Tschopp, 2005; Franchi et al., 2009). The leucine rich repeats are implicated in recognizing the ligands. The NOD domains are similar to NB–ARC motif of APAF1. APAF1 is a protein involved in apoptosis and the NB–ARC motif mediates ATP dependent oligomerization of APAF1 upon binding cytochrome c and activates the apoptosis cascade. The CARD, PYD and BIR domains mediate protein–protein interaction and facilitate downstream signaling. Most NLRs are involved in activating the inflammasome but some NLRs like NOD1 and NOD2 also activate NF–κB signaling pathway. NOD1 binds γ–D–glutamyl–diaminopimelic acid (iE–DAP) and NOD2 binds muramyl dipeptide (MDP) and activate downstream signaling by recruiting RIP2 via the CARD domain. RIP2 is polyubiquitinated by cIAP1 or cIAP2. This modification of RIP2 allows TAK1 binding which leads to IKK activation and subsequent activation and translocation of NF–κB (Inohara et al., 2003; Inohara et al., 1999; Ogura et al., 2001; Viala et al., 2004; Hasegawa et al., 2008)(Bertrand et al., 2009).

Recent work has shown that DNA–dependent activator of IRFs (DAI) functions as a cytosolic receptor for DNA. However, DAI is not the only cytosolic receptor that recognizes DNA since DAI knockout mice are not essential for either innate or adaptive immune responses to B-DNA or DNA vaccination. DAI binds to transfected DNA and activates IFN and NF-κB signaling. DAI interacts with TBK1 to activate IRF3 signaling.

DAI contains two RIP homotypic interaction motifs (RHIM) and these are important for recruitment of RIP1 and RIP3 and activation of NF-κB signaling. DAI and RIP1/3 interact with each other through the RHIM domain and both the RHIM domains of DAI are important for the activation of NF-κB signaling (Rebsamen et al., 2009; Ishii et al., 2008).

RLRs recognize intracellular RNA patterns produced by viruses. The RLR family has 3 members retinoic acid inducible gene I (RIG–I), melanoma differentiation–associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP–2) (Meylan et al., 2006). All the three proteins have a DExD/H box RNA helicase domain. In addition, RIG–I and MDA5 have two N terminal caspase activation and recruitment domains (CARD). CARDs mediate the interaction of activated RIG–I and MDA5 with the adaptor mitochondrial antiviral signaling (MAVS, also known as IPS–1, VISA, and Cardif), which localizes to the outer mitochondrial membrane. MAVS in turn mediates downstream signaling by activating the kinases TANK binding kinase 1 (TBK1) and IKKε which in turn activate the transcription factors IRF3, IRF7 and NF–κB (Meylan et al., 2005)

Negative Regulation in the TLR Pathway

TLR signaling pathway is activated in response to various pathogens and stress proteins, hence if left unchecked TLR signaling pathways lead to an over–activated immune response. Therefore, it is necessary to keep the TLR pathways under tight regulation. To this effect TLR signaling pathways employ many different negative regulators. In the following section I will summarize some of the important negative

regulators of TLR signaling based on there location and function (Lang and Mansell, 2007; Wang et al., 2009; Liew et al., 2005).

Extracellular regulation

The first line of regulation is provided by the soluble decoy receptors. Soluble TLRs act as decoy receptors by competing with TLR agonists. sTLR–2 is a naturally occurring soluble TLR2 that is constitutively released by normal monocytes and is present in the human plasma and breast milk. sTLR–2 not only functions as a decoy receptor but also inhibits TLR signaling by binding CD14 the co–receptor for TLR2 signaling (LeBouder et al., 2003; Iwaki et al., 2002). Even though there is a single copy of the TLR–4 gene, multiple mRNA products have been detected in mouse and human. This includes an mRNA encoding a soluble TLR–4 (Qureshi et al., 1999). *In vitro* studies using recombinant sTLR–4 have shown that it can block LPS induced NF–kB activation and TNF production. The mechanism by which sTLR–4 inhibits signaling is unclear, however, it is hypothesized that like sTLR–2 it acts as a decoy receptor and also blocks the interaction between TLR–4 and its co–receptors CD14 and MD2 (Iwami et al., 2000).

Transmembrane Protein Regulators

Another strategy involving negative regulators is transmembrane proteins that inhibit signaling by either sequestering the adaptor proteins or by interfering with binding of the ligand to the specific TLR. Several transmembrane proteins like Suppressor of tumorigenicity 2 (ST2), Single immunoglobulin IL–1 related protein (SIGIRR), Radioprotective 105 (RP105) and TNF–related apoptosis–inducing ligand receptor (TRAILR) belong to this family (Brint et al., 2004; Schmitz et al., 2005; Divanovic et al.,

2005a; Divanovic et al., 2005b; Diehl et al., 2004). One of the best characterized member of this family is SIGIRR.

SIGIRR is also a member of the TIR superfamily like ST2. SIGIRR is expressed by epithelial cells and immature dendritic cells (Thomassen et al., 1999; Wald et al., 2003; Garlanda et al., 2004). Over–expression of SIGIRR by dendritic cells leads to inhibition of NF–kB activation by IL–1 and IL–8, and SIGIRR knockout mice have increased cytokine production in response to TLR–4 and TLR–9 ligands but not TLR–3 (Garlanda et al., 2004). Data suggests that SIGIRR interferes with the receptor proximal complex by interacting with TRAF6 and IRAK1 also, SIGIRR has been shown to interact with IL–IR and TLR–4 (Wald et al., 2003). However, the exact mechanism is unclear and more work is needed to elucidate how SIGIRR functions as a negative regulator.

Intracellular Protein Regulators

The intracellular regulators down modulate TLR signaling using various different mechanisms. For example IRAKM, MyD88s,Toll interacting protein (TOLLIP), and Sterile–alpha and armadillo motif protein (SARM) negatively regulate TLR signaling by interacting with various components of the TLR pathway and inhibiting there function (Janssens et al., 2002; Burns et al., 2003; Carty et al., 2006; Burns et al., 2000).

In particular, IRAKM is a member of the IRAK family of kinases that is composed of four members. In contrast to IRAKM, the other three family members IRAK1, IRAK2 and IRAK4 are activators of TLR signaling. IRAKM shares about 30–40% homology with the other IRAK members and unlike the other IRAKs it has restrictive expression (Wesche et al., 1999). IRAKM is expressed predominantly in the monocytes/

macrophages and weakly in other tissues and IRAKM expression is upregulated in response to LPS. *In vitro*, macrophages from IRAKM deficient mice produce higher levels of cytokines in response to TLR–4 and TLR–9 ligands. Additionally IRAKM deficient mice have an impaired LPS tolerance against endotoxin shock when compared to wild–type mice. Over–expression studies suggest that IRAKM downregulates TLR signaling by inhibiting the dissociation of IRAK1 and IRAK4 from the TLR complex by either stabilizing the TLR–MyD88–IRAK4 complex or by inhibiting the phosphorylation of IRAK1 and IRAK4 (Kobayashi et al., 2002). In addition to IRAKM two of the four splice variants of IRAK2 (IRAK2c and IRAK2d) also down modulate TLR signaling when over–expressed in 3T3 fibroblasts. IRAK2c and IRAK2d lack a death domain and IRAK2c is upregulated when RAW264.7 cells are stimulated with LPS for 1 and 3 hours indicating a negative feedback mechanism (Hardy and O'Neill, 2004).

Another strategy utilized by intracellular proteins is to function as a transcriptonal repressor. Activating transcription factor 3 (ATF3) is a member of the CREB family of basic leucine zipper transcription factors (Mayr et al., 2001). ATF3 has been demonstrated to be both an activator or repressor of transcription depending on the cell type and stimulus (Kawauchi et al., 2002). In the context of TLR signaling Gilchrist *et al* took a system biology's approach and showed that ATF3 represses the transcription of *Il6* and *Il12b.* IL6 and IL12b among 30 other genes were identified to have a putative ATF3 site within 100 base pairs of a NF–κB site and 500 base pairs of a transcriptional start site. They further demonstrated that ATF3 deficient mice have atleast 10 times higher levels of circulating IL6 and IL12 in response to LPS when compared to wild type mice. Also ATF3 deficient mice succumb to endotoxic shock with 24 hours of

intrperitoneal LPS administration. ATF3 exerts transcriptional repression by chromatin remodeling. ATF3 in conjunction with HDAC deacetylates histones and thus alters chromatin structure to limit transcription factors like NF–κB from DNA (Gilchrist et al., 2006).

Intracellular Proteins that negatively regulate TLR signaling by degradation

One of the most commonly utilized mechanisms for regulating TLR signaling is the degradation or destabilization of components of the TLR signaling pathway. Several negative regulators of TLR signaling function as ubiquitin modifying enzymes. For example, Suppressor of cytokine signaling–1 (SOCS–1), Pin1 and Triad3A negatively regulate TLR signaling pathways by targeting various components of the TLR pathway for polyubiquitnation followed by proteosomal degradation. SOCS–1 is one of the best characterized negative regulator in this category and is described in detail below.

SOCS–1 is one of the 8 members of the SOCS family. SOCS–1 was first identified as an intracellular protein that negatively regulates cytokine production by the JAK/STAT pathway (Alexander, 2002). SOCS–1 deficient mice die within 3 weeks of birth due to multi– organ inflammation and this phenotype can be rescued by crossing to IFNγ deficient mice (Starr et al., 1998; Naka et al., 1998). However, these double deficient mice are still hypersensitive to LPS. Also, SOCS–1 deficient mice are highly susceptible to sepsis and SOCS–1 deficient macrophages produce elevated levels of cytokines such as IL–6 and TNF in response to LPS. All this data together indicates that SOCS–1 negatively regulates TLR signaling pathway (Nakagawa et al., 2002; Kinjyo et al., 2002). Work by Mansell et al shows that SOCS–1 negatively regulates TLR–2 and TLR–4 pathway by targeting the adaptor protein Mal for polyubiquitnation

followed by proteosomal degradation. SOCS–1 inhibits Mal mediated phosphorylation of the p65 subunit of NF–κB and hence its transactivation (Mansell et al., 2006).

Intracellular Proteins that negatively regulate TLR signaling by deubiquitnation

Protein ubiquitination not only leads to the degradation of proteins but it also plays a key role in regulation of TLR signaling. Like phosphorylation, protein ubiquitination is also reversible and involves deubiqitinases, specialized proteases that act on ubiquitin. Several deubiquitinases like A20, deubiquitnating enzyme A (DUBA) and cylindromatosis protein (CYLD) also function in the TLR pathways. A20 was the first deubiquitinase to be discovered in the TLR pathway (Kayagaki et al., 2007; Regamey et al., 2003; Trompouki et al., 2003; Yoshida et al., 2005).

A20 is a negative regulator that down modulates both TNFR1 and TLR mediated NF–κB pathways. A20 is expressed in many cell types and its expression is rapidly upregulated by LPS and TNF (Krikos et al., 1992; Opipari et al., 1990). Work by Ma and colleagues shows that TNFR1 and A20 doubly deficient mice develop spontaneous inflammation like the A20 deficient mice indicating that A20 regulates TNF independent pro–inflammatory signals. Also, mice reconstituted with hematopoietic stem cells from A20 deficient mice rapidly die following LPS stimulation. This data shows that A20 is important for protecting the host from endotoxic shock. However, A20 does not play an important role in LPS tolerance. A20 is a cysteine protease deubiquitinating enzyme and it removes K63–linked ubiquitin molecules from TRAF6 and hence down modulates TLR signaling. TRAF6 is a component used by all TLR signaling pathways hence A20 regulates both TLR–dependent and independent pathways (Boone et al., 2004).

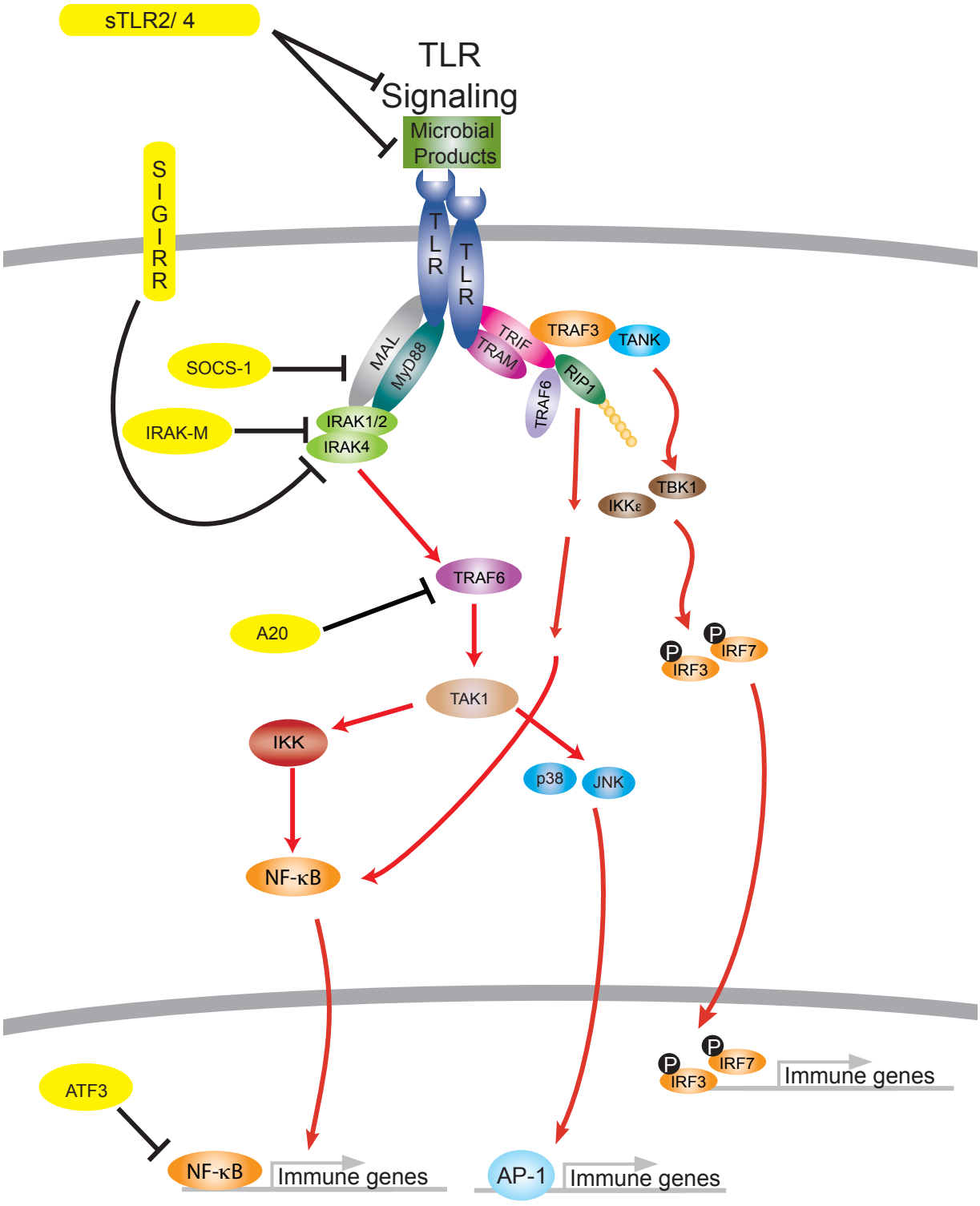


Figure 1.6: Negative Regulators of Toll–like receptor. Schematic representation of the TLR pathway showing only the basic outline. The black arrows highlight the negative regulators that are discussed in the text and their likely targets.

Thesis objective

Currently there are a lot of open questions about what regions of the innate immune receptors are important for signaling and how the signal is transmitted to it down stream components. In the work presented here I use *Drosophila* as a model system to try and answer some of these questions. In particular, this thesis elucidates some receptor proximal events that regulate IMD signaling both positively and negatively.

In these studies I have identified a common motif in the N-terminal domains of both the receptors, known as the RHIM-like domain. The RHIM-like domain is critical for signaling by either receptor, but the mechanism(s) involved remain unclear. IMD, a downstream component of the pathway, associates with both PGRP-LC and -LE but the interaction of PGRP-LC with IMD is not mediated through its RHIM-like domain. Also, mutations affecting the PGRP-LC RHIM-like motif are defective in all known downstream signaling events. However, the RHIM-like mutant receptors are capable of serving as a platform for the assembly of all known components of a receptor proximal signaling complex. These results suggest that another, unidentified component of the IMD signaling pathway may function to mediate interaction with the RHIM-like motif.

I performed a yeast two-hybrid screen to identify proteins that might interact with the receptor PGRP-LC through its RHIM- like domain. With this approach, two new components of the IMD pathway were identified. The first component I characterized is called Rudra and it is a critical feedback inhibitor of peptidoglycan receptor signaling. The other factor is known as RYBP, it includes a highly conserved ubiquitin binding motif (NZF), and RNAi studies suggest it is a critical component of the IMD pathway. The

identification and characterization of these two new components of the IMD pathway has provided a new insight into the molecular events that take place proximal to the receptor. By studying *Drosophila* innate immune signaling I hope to contribute to the ever growing body of knowledge regarding innate immunity.

PREFACE TO CHAPTER II

This chapter was previously published in Nature Immunology (2006):

Takashi Kaneko, Tamaki Yano, **Kamna Aggarwal**, Jae-Hong Lim, Kazunori Ueda, Yoshiteru Oshima, Camilla Peach, Deniz Erturk-Hasdemir, William E Goldman, Byung-Ha Oh, Shoichiro Kurata & Neal Silverman. PGRP-LC and PGRP-LE play essential yet distinct roles in the *Drosophila* immune response to monomeric DAP-type peptidoglycan. Nat Immunol. 2006 Jul;7(7):715-23.

Kamna Aggarwal performed all PGRP-LE RHIM-like domain related cell culture experiments (Figure 2.7d, Figure 2.8b) and characterized the PGRP-LCx transgenic flies (Figure 2.9c). Also, I wrote the manuscript with Takashi Kaneko and Neal Silverman.

CHAPTER II

PGRP-LC and PGRP-LE play essential yet distinct roles in the *Drosophila* immune response to monomeric DAP-type peptidoglycan

Abstract

Studies of *Drosophila* immunity in tissue culture suggest that recognition of polymeric peptidoglycan (PGN) requires only peptidoglycan recognition protein-LCx (PGRP-LCx), while recognition of monomeric PGN requires a heterodimeric complex of PGRP-LCa and PGRP–LCx. Here we present *in vivo* studies indicating that either *PGRP-LC* or *PGRP-LE* is sufficient to mediate responses to monomeric PGN, while recognition of polymeric PGN depends solely on PGRP-LC. In addition, we demonstrate that full-length PGRP-LE acts as an intracellular receptor for monomeric PGN. However, a version of PGRP-LE containing only the PGRP domain functioned extracellularly, as a CD14-like accessory factor capable of enhancing PGRP-LC-mediated PGN recognition. Interaction with IMD was not required for PGRP-LC signaling. Instead, PGRP-LC and PGRP-LE signal through a RHIM-like motif.

Introduction

Infection with gram-negative bacteria results in activation of the *Drosophila* IMD signaling pathway, which, via activation of the NF-κB homolog Relish, drives expression of genes encoding antimicrobial peptides(Kaneko and Silverman, 2005). Bacterial peptidoglycan (PGN) are responsible for the activation of the IMD pathway (Leulier et al., 2003; Kaneko et al., 2005; Kaneko et al., 2004). Specifically, diaminopimelic acid-containing PGN (DAP-type) potently activates the IMD pathway while lysine-containing PGN (Lys-type) activates the Toll innate immune signaling pathway. Host cell recognition of PGN requires peptidoglycan recognition proteins (PGRPs). PGRP-SA and PGRP-SD recognize Lys-type PGN, which is produced by many Gram-positive bacteria, and PGRP-LC and PGRP-LE recognize DAP-type PGN, which is produced by Gram-negative bacteria (Kaneko and Silverman, 2005).

The *Drosophila* genome encodes 17 PGRP gene products which are often characterized as either short or long form PGRPs. Short form PGRPs consist solely of a PGRP domain, and usually contain a signal sequence, which results in production of secreted proteins such as PGRP-SA and PGRP-SD. Long form PGRPs contain an extended N-terminal region, which often includes a transmembrane domain, in addition to their C-terminal PGRP domain. For example, PGRP-LC encodes three distinct proteins, generated via alternative splicing, each of which contain identical N-terminal cytoplasmic signaling and transmembrane domains but unique C-terminal extracellular PGRP domains (Werner et al., 2003).

In contrast, the long form PGRP-LE contains no predicted transmembrane domain or signal sequence, and is thus predicted to be an intracellular PGRP. With the

exception of their PGRP domains, PGRP-LC and PGRP-LE share little apparent homology (Werner et al., 2003) . PGRP-LE binds specifically to DAP-type but not to Lys-type PGN (Takehana et al., 2002). Surprisingly, the PGRP-domain of endogenous PGRP-LE (PGRP-LE^{pg}) can be found outside the cell, in the hemolymph. Consistent with this extracellular localization of PGRP-LE^{pg}, IMD signaling induced by PGRP-LE over-expression occurs in a non-cell autonomous manner and depends in large part on PGRP-LC (Takehana et al., 2004). PGRP-LE also exhibits PGRP-LC-independent activity, but it is not clear if this occurs within the cell or via an alternate cell surface receptor.

PGRP-LC mutant flies exhibit greatly reduced antimicrobial peptide gene expression following infection with *E. coli* (Gottar et al., 2002; Choe et al., 2002; Ramet et al., 2002), and are hyper-susceptible to infection with some gram-negative bacteria, such as *Erwinia caratova*. However, *PGRP-LC* mutant flies exhibit normal resistance to infection with *E. coli* (Gottar et al., 2002). *PGRP-LE* mutant flies display normal antimicrobial peptide gene expression after, and are resistant to, *E. coli* infection. However, flies expressing mutant forms of both *PGRP-LC* and *PGRP-LE* are highly susceptible to *E. coli* and other DAP-type PGN-containing bacteria (Takehana et al., 2004). These results suggests that PGRP-LC and PGRP-LE have some redundant functions and are individually capable of preventing *E. coli*-induced lethality, although why *PGRP-LC* mutants withstand *E. coli* infection, even when their antimicrobial peptide gene expression is severely reduced, is not clear.

In addition, the mechanisms by which PGRP-LE- or PGRP-LC-induced signals are transmitted through the cell interior are not completely understood. A recent report

suggested that the first 263 amino acids of the cytoplasmic domain of PGRP-LC are required for signaling, and that this cytoplasmic domain of PGRP-LC interacts with IMD (Choe et al., 2005). However, the inactive mutant analyzed in this report lacks almost the entire PGRP-LC cytoplasmic domain. Therefore, specific motif(s) involved in PGRP-LC signaling were not defined. Moreover, interaction between IMD and PGRP-LC was shown only when these proteins were over-expressed, and this interaction was not functionally linked to PGRP-LC signal transduction.

Here, we demonstrate that recognition of the monomeric DAP-type PGN tracheal cytotoxin (TCT) requires either PGRP-LC or PGRP-LE. PGRP-LE-mediated recognition of TCT can occur intracellularly, and extracellular PGRP-LE^{pg} may stimulate IMD signaling by facilitating the interaction of PGN with PGRP-LC, in a CD14-like manner. In addition, we define a previously-undetected RHIM-like motif in the cytoplasmic domains of PGRP-LC and PGRP-LE that is essential for signaling, but dispensable for interaction with IMD, suggesting that interaction with IMD is not critical for PGRP-LC and PGRP-LE signaling.

Results

PGRP-LE and PGRP-LC recognize TCT

TCT is a dissacharide-tetrapeptide fragment of PGN that contains a 1,6-anhydro arranged muramic acid and a DAP residue at the third position of the stem-peptide (Cookson et al., 1989b), and activates the IMD pathway in *Drosophila* and in a *Drosophila* cell line (S2*) (Kaneko et al., 2004). RNA interference studies demonstrated that TCT-mediated IMD activation in S2* cells requires two splice-isoforms of PGRP-LC,

PGRP-LCa and PGRP–LCx. To examine the role of various PGRP-LC isoforms in TCT recognition *in vivo*, two *PGRP-LC* mutant strains were injected with TCT. *PGRP-LC*^{1} contains an 858 base pair insertion in the exon encoding the common N-terminal domain of PGRP-LC, and is therefore thought to be a null allele. *PGRP-LC*^{2} contains a point mutation specifically affecting the PGRP-LCx isoform (Choe et al., 2002). Both mutants responded to TCT challenge with induction of the antimicrobial peptide genes *diptericin* (**Figure 2.1a**), *cecropin* and *attacin* (data not shown). In contrast, both PGRP-LC mutants failed to upregulate antimicrobial gene expression after infection with *E. coli* or injection of polymeric *E. coli* PGN. It remained possible that neither mutation generated a complete null PGRP-LC allele. Therefore, we assessed flies carrying the *PGRP-LC*$^{\Delta E}$ allele, which lacks the entire PGRP-LC locus, and noted similar responsiveness to monomeric TCT (**Figure 2.1b**). Dose response analysis demonstrated that the *PGRP-LC* mutants were approximately 50% less responsive to TCT, compared to wild-type (WT) flies (**Figure 2.2**). However, TCT responsiveness was completely dependent on the IMD pathway. Neither *kenny*^{1} nor *imd*^{Shadok} mutant flies both null alleles of essential components in the IMD pathway) responded to TCT challenge (**Figure 2.1c**). These results suggest the existence of another receptor capable of recognizing TCT and activating IMD signaling. PGRP-LE is known to bind DAP-type PGN and also protect flies from gram-negative infection, thus it is a candidate for this alternate TCT receptor.

To test the possibility that PGRP-LE is also capable of recognizing TCT and activating IMD signaling, *PGRP-LE* mutant and *PGRP-LE, PGRP-LC* double mutant flies were injected with TCT. While *PGRP-LE* mutants exhibited normal responses, the

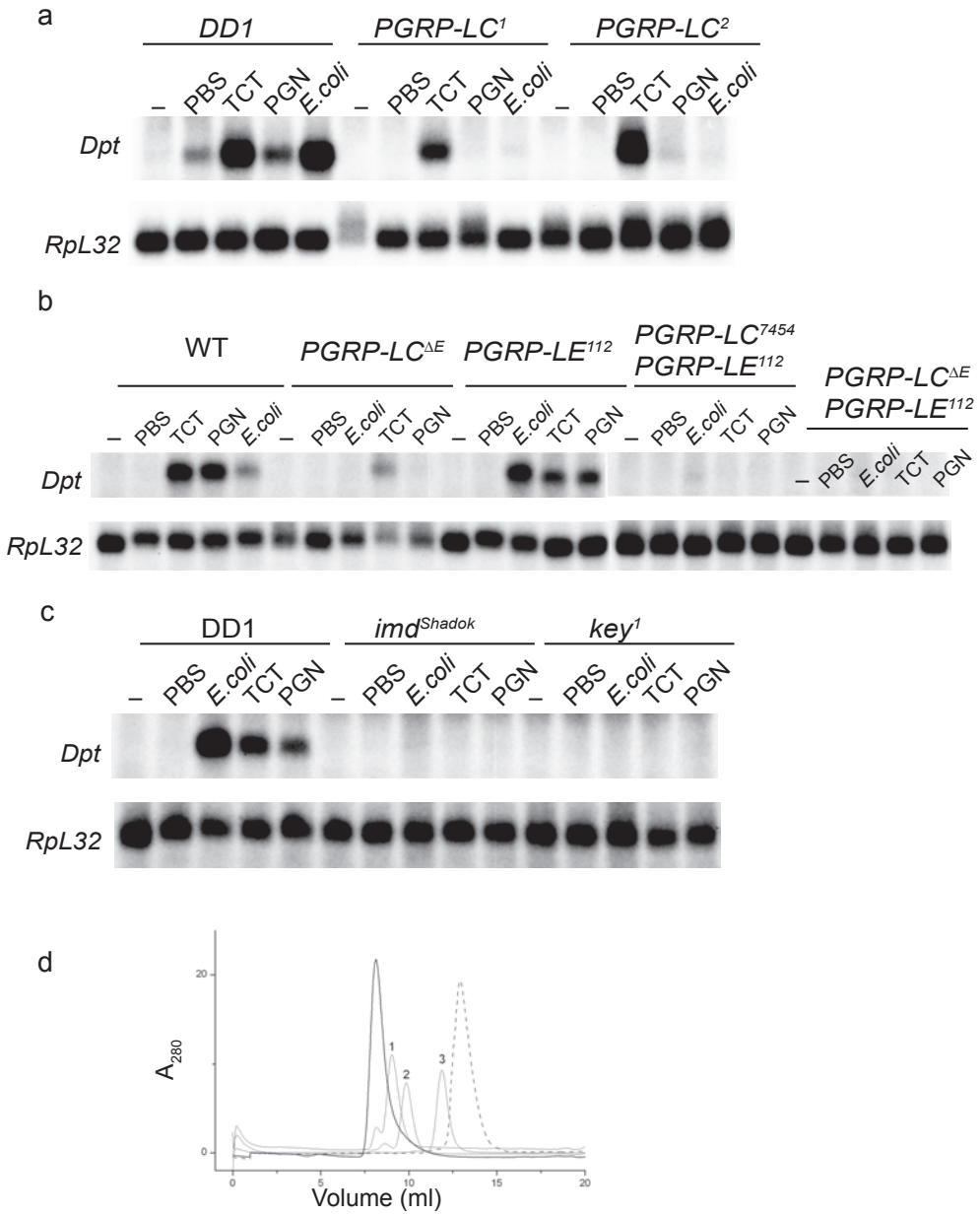


Figure 2.1: PGRP-LE is a second receptor for monomeric DAP-type peptidoglycan.

(a-c) Indicated adult flies (2-5 days old) were injected with monomeric DAP-type peptidoglycan (TCT), polymeric DAP-type peptidoglycan (PGN) or live *E. coli*. RNA was extracted 24 h after injection, and expression of genes encoding the antimicrobial peptide gene *diptericin* and the ribosomal protein *RpL32* (loading control) was assessed by Northern blot. *PGRP-LC*^{1} and *PGRP-LC*$^{\Delta E}$*, PGRP-LC* null alleles; *PGRP-LC*^{2}*, PGRP-LCx*-specific allele; *PGRP-LE*^{112}, *PGRP-LE* null allele; *imd*^{shadok}*, imd* null allele; *key*^{1}*, kenny* null allele. WT strain in panel b is an isogenic *white* strain. (d) Oligomerization of recombinant purified PGRP-LE^{pg} was assessed in the presence (solid line) and absence (dashed line) of TCT by size exclusion chromatography (Superdex 75). Lighter solid lines indicate elution of (1) BSA (67kDa), (2) ovalbumin (43kDa) and (3) chymotripsinogen A (25kDa) controls. For all panels, results typical from at least two independent experiments.

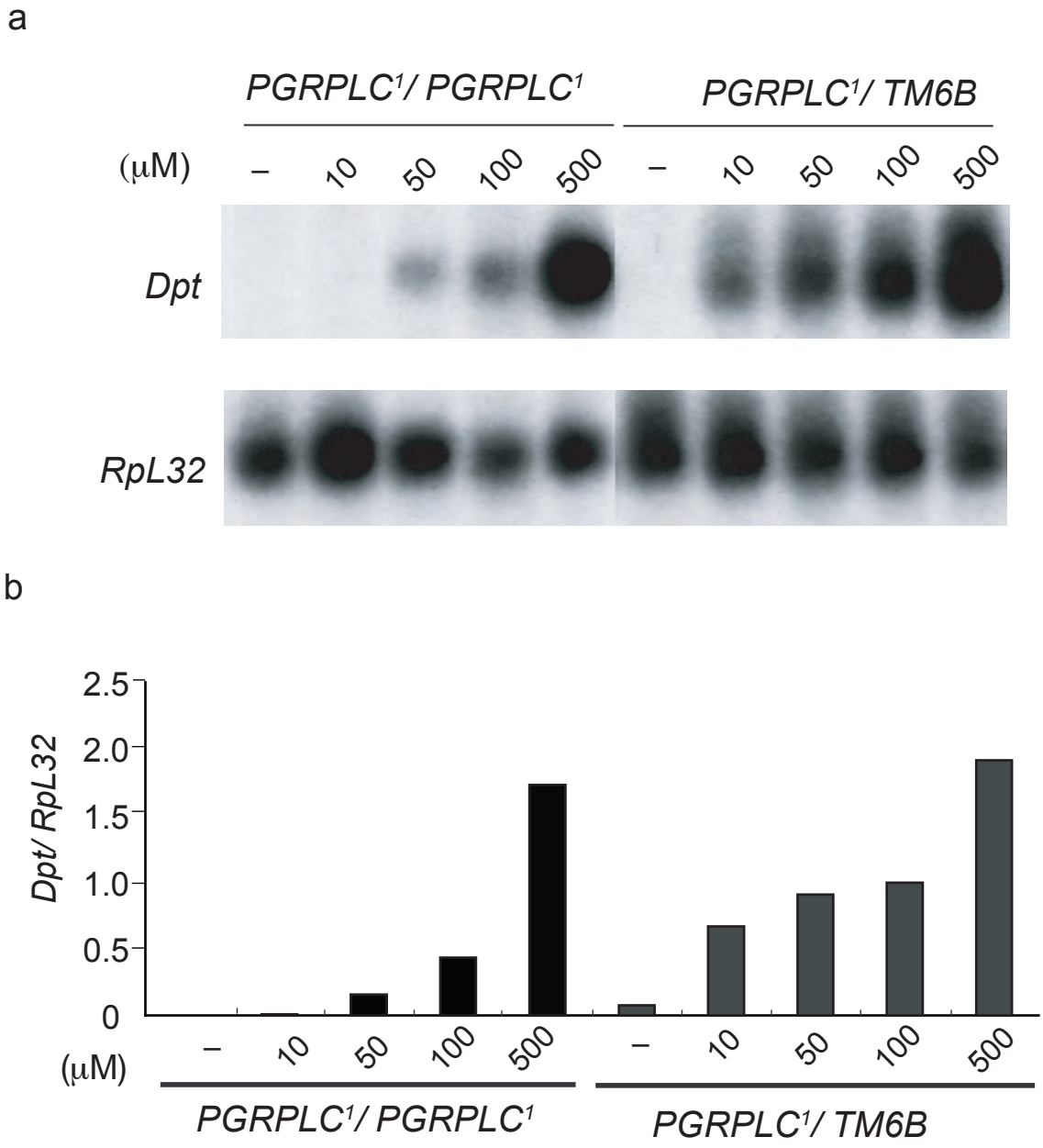


Figure 2.2 TCT-induced immune activation is reduced but not eliminated in *PGRP-LC* mutant flies.
(**a**) Northern blots depict *diptericin* induction following injection of increasing doses of TCT into *PGRP-LC*^{1} homozygous mutants or their heterozygous (*PGRP-LC*^{1}*/TM6B*) siblings. (**b**) Phosphoimager quantification of the data presented in (**a**). Representative of two independent experiments.

double *PGRP-LC, PGRP-LE* mutants failed to respond to TCT, *E. coli* PGN or live *E. coli* (**Figure 2.1b**). These results suggest that PGRP-LE is a second receptor for TCT. S2* cells do not express PGRP-LE. Therefore, in S2* cells, the response to TCT is strictly dependent on PGRP-LC (**Figure 2.3a**).

Consistent with the notion that PGRP-LE is a second TCT receptor, recent studies demonstrated that PGRP-LE^{pg} binds TCT, with an apparent K_d of ~27 nM. In addition, the recently-solved structure of a co-crystal of PGRP-LE^{pg} bound to TCT suggested that TCT binding results in PGRP-LE^{pg} multimerization (Lim et al., 2006). To determine whether TCT binding induced PGRP-LE^{pg} multimerization, PGRP-LE^{pg}, with or without TCT, was fractionated by gel filtration chromatography. In the presence of TCT, PGRP-LE^{pg} eluted near the void volume with the molecular weight marker thyroglobulin on a Superdex 75 column, suggesting a molecular weight of at least 669 kDa (the molecular weight of thyroglobulin), while in the absence of TCT, PGRP-LE^{pg} eluted at ~25 kDa, near the predicted monomeric size of 20 kDa. Together, the results presented here and in our previous report clearly show that TCT-binding induces PGRP-LE^{pg} oligomerization, and suggest that ligand-induced oligomerization may play a role in PGRP-LE signaling (Lim et al., 2006).

PGRP-LE, an intracellular TCT receptor

PGRP-LE functions independently of PGRP-LC in responding to TCT (**Figure 2.1b**), and *PGRP-LE* encodes neither a signal peptide nor a transmembrane domain. Therefore, PGRP-LE might function as an intracellular receptor for TCT. However, a previous report clearly demonstrated that PGRP-LE can function in a cell non-autonomous manner when over-expressed in the fat body, and PGRP-LE^{pg} can be

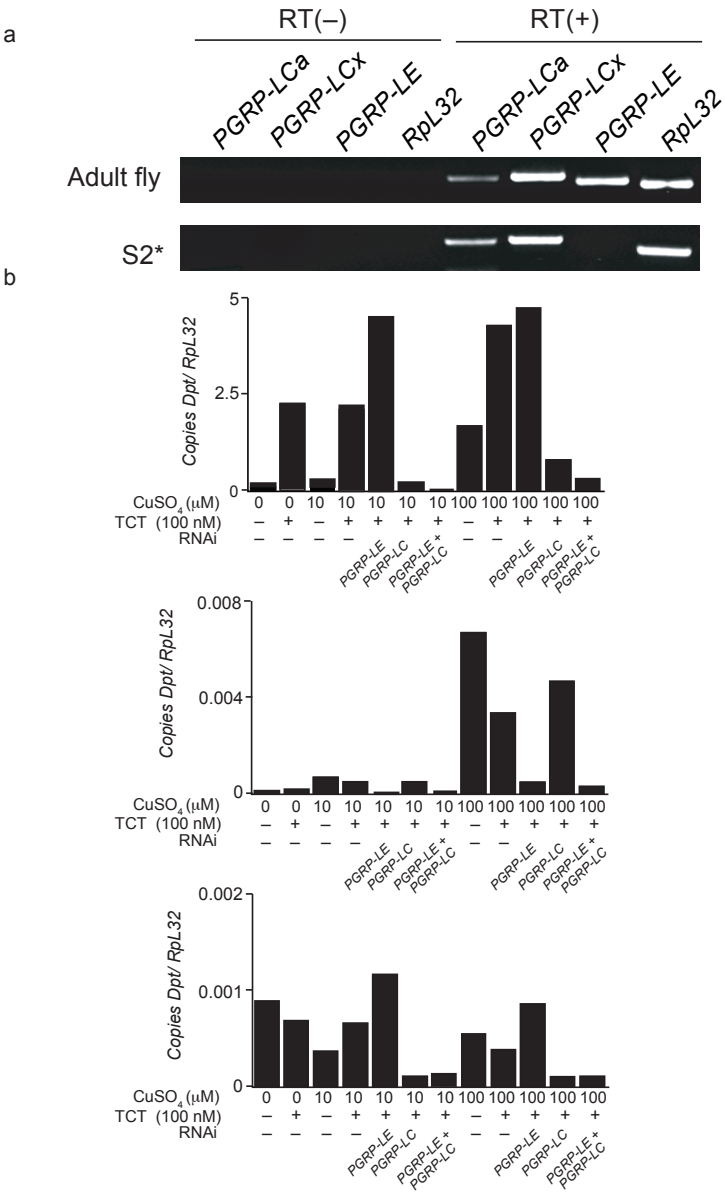


Figure 2.3: PGRP-LE is not expressed in cultured S2* cells.

(**a**) Total RNA was extracted from adult flies or S2* cells, and PGRP-LE transcripts were detected by RT-PCR. *RpL32*, *PGRP-LCx* and *PGRP–LCa* are presented as controls. No reverse transcriptase (RT(–)) control is indicated. (**b**) *Diptericin* (top), *PGRP-LE* (middle) and *PGRP-LC* (bottom) transcripts were detected by real-time RT-PCR (normalized to *RpL32* transcripts) in S2 cells stably transfected with a metallothionein promoter driven PGRP-LE expression construct. PGRP-LE was expressed in two quantities, by addition of either 10 or 100 μM copper sulfate for 40 hours, then cells were stimulated (or left unstimulated) with 100 nM TCT for 12 hours. RNAi to PGRP-LC and/or PGRP-LE was used to determine the relative contribution of these two receptors to TCT-mediated *diptericin* induction, as indicated. In these conditions, the response to TCT is almost entirely dependent on PGRP-LC, even when PGRP-LE is expressed in either low or high amounts. Representative of least three independent assays.

found in the hemolymph despite lacking a predicted signal sequence (Takehana et al., 2004). These data do not preclude the possibility that PGRP-LE also functions, in other situations, intracellularly in a cell-autonomous manner. To examine this possibility, PGRP-LE was expressed within clones of cells in the malphigian tubules (the insect kidney) using a combination of the flp-FRT and GAL4-UAS systems (Ito et al., 1997). Malphigian tubules are an immune responsive organ with autonomous IMD signaling activity (McGettigan et al., 2005). In this system, an actin promoter-driven GAL4 transgene is nonfunctional due to the insertion of transcriptional termination signals flanked by two FRT sequences in the initial state (Ay-GAL4). Expression of the flp recombinase induces recombination between these the two FRT sequences and removes the terminator, generating a functional actin-GAL4 that drives the expression of PGRP-LE and GFP transgenes, which are controlled by a UAS_{Gal} promoter. Expression of flp recombinase is controlled by the hsp70 promoter and is induced clonally by a brief heat shock. *Diptericin* expression was monitored with a *dipt-LacZ* reporter transgene, while PGRP-LE expression was reported by a UAS-GFP transgene. In malphigian tubules, expression of PGRP-LE was sufficient to induce *diptericin* expression, which occurred exclusively in a cell-autonomous manner (**Figure 2.4a**). The *dipt-LacZ* reporter was only expressed in cells expressing PGRP-LE, suggesting that PGRP-LE may function as an intracellular TCT receptor in this tissue.

To directly test this possibility, whole organs were collected from *dipt-LacZ* larvae, washed extensively to remove all hemolymph, and stimulated with TCT. IMD activation was assayed by staining for β-galactosidase activity (Yajima et al., 2003). TCT induced *dipt-LacZ* reporter expression in 96% of malphigian tubules from WT flies (**Figure 2.4b**).

This response was completely dependent on the addition of TCT (**Figure 2.4c**). Moreover, this response was strongly dependent on PGRP-LE and only mildly involved PGRP-LC. 27% of *PGRP-LE*, and 79% of *PGRP-LC,* mutant malphigian tubules exhibited *dipt-LacZ* expression (**Figure 2.4d**). Expression of the *diptericin* reporter was detected in only 6% of malphigian tubules from double *PGRP-LC, PGRP-LE* mutants. The data in **Figure 2.4d** represent the percentage of tubules that contained one or more cells expressing the dipt-*lacZ* reporter. Consistent with these results, anti-PGRP-LE antisera detected full length (45 kDa) PGRP-LE in immunoblots from lysates from WT, but not *PGRP-LE*^{112}, malphigian tubules (**Figure 2.4e**).

S2 cells do not express PGRP-LE (**Figure 2.3a**). In most cases, expression of PGRP-LE was not sufficient to allow them to respond to TCT, independently of PGRP-LC, when TCT was added directly to the culture media (**Figure 2.6a** and **Figure 2.3b**). We speculated that this poor response was due to a failure of S2 cells to import TCT into the cytoplasm, where it can be recognized by intracellular PGRP-LE. To test this hypothesis, TCT was delivered directly into the cytoplasm of S2 cells by transfection with calcium phosphate. TCT induced *diptericin* and *drosomycin* transcription only in S2 cells expressing PGRP-LE (**Figure 2.4f**). In the copper treated cells, the response to TCT was largely independent of PGRP-LC, consistent with intracellular recognition (data not shown). Note that the relatively unresponsive S2 cells, not the highly responsive S2* sub-line, were used for these experiments (Samakovlis et al., 1990).

Next, we directly demonstrated that PGRP-LE is expressed within cells of the malphigian tubules and in the cytoplasm of S2 cells. PGRP-LE was detected intracellularly in malphigian tubules by confocal immunofluorescence with anti-PGRP-

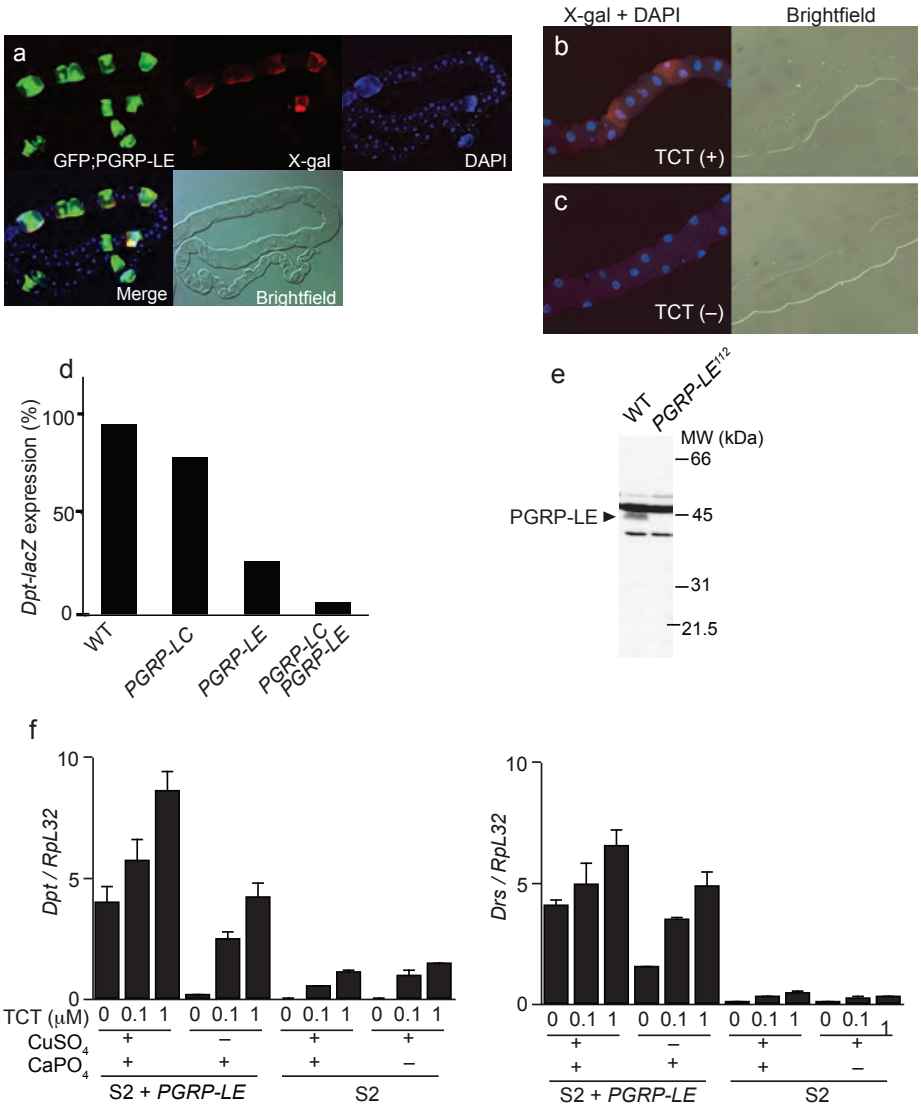


Figure 2.4: PGRP-LE is an intracellular receptor for TCT.

(**a**) Immunofluorescence analysis of *diptericin* expression (via *diptericin-LacZ* reporter transgene) following clonal expression of PGRP-LE (monitored via UAS-GFP reporter transgene) in malphigian tubules. Representative of ten mosaic tubules. **(b-d**) After extensive washing to remove hemolymph, malphigian tubules from *diptericin-LacZ* larvae were challenged in culture with 10 μM TCT. The immune response was monitored by staining with X-gal and DAPI. Malphigian tubules from WT or mutant were compared, and the percentage of tubules with one or more *LacZ* expressing cells is indicated in **(d).** 48 tubules were examined for each genotype. **(e**) PGRP-LE expression in WT and *PGRP-LE*^{112} malphigian tubules was monitored by immunoblotting with anti-PGRP-LE. Representative of at least two independent immunoblots. (**f**) Parental (PGRP-LE-deficient) S2 cells and S2 cell lines stably transfected with a metallothionein-PGRP-LE construct were treated with 100 μM copper sulfate for one hour (or left untreated), after which they were transfected with the indicated amounts of TCT. Twelve hours later RNA was extracted and expression of *diptericin*, *drosomycin* and *RpL32* (normalization control) was assessed by real-time RT-PCR. Note, the sub-line of S2 cells used in this experiment responded poorly to TCT, compared to the S2* cells used elsewhere in this study and previously[2]. Error bars indicate s.d. Representative of two independent experiments, each in duplicate. **(a-c)** , magnification 200x.

LE (**Figure 2.5a**). When expressed in S2 cells, V5-tagged PGRP-LE was observed in the cytoplasm, as detected by whole cell immunofluorescent microscopy (**Figure 2.5b**). When expressed in S2* cells, YFP-tagged PGRP-LE was found to be cytoplasmic by confocal fluorescent microscopy (**Figure 2.5c**). These localization and functional data clearly illustrate that full-length PGRP-LE can function as an intracellular receptor for TCT.

CD14-like activity of PGRP-LE^{pg}

Unlike full-length intracellular PGRP-LE, a truncated form of PGRP-LE, which consists of the PGRP-domain (PGRP-LE^{pg}), is easily detected extracellularly in hemolymph (Takehana et al., 2004). To compare the activity of full-length PGRP-LE and PGRP-LE^{pg}, both forms were stably expressed from a copper-inducible promoter in S2 cells (**Figure 2.6a**). Full-length PGRP-LE potently induced *diptericin* expression, in a manner independent of PGRP-LC but dependent on IMD (**Figure 2.6**, left). In contrast, PGRP-LE^{pg} did not induce *diptericin* expression, but instead robustly enhanced TCT-mediated *diptericin* induction in a PGRP-LC-dependent manner (**Figure 2.6a**, right). Full-length PGRP-LE did not enhance TCT-mediated *diptericin* induction (**Figure 2.6a**, left).

The requirement for PGRP-LC in the PGRP-LE^{pg}-enhanced response to TCT suggests that PGRP-LE^{pg} functions outside the cell. Consistent with this possibility, PGRP-LE^{pg} was detected in significant quantities in the conditioned media of PGRP-LE^{pg}-expressing S2 cells (**Figure 2.6b**). Moreover, after addition of TCT, soluble PGRP-LE^{pg} associated with S2 cells (**Figure 2.6c**), and this cell association required PGRP-LC (data not shown). Serine 232 in PGRP-LE and serine 391 in PGRP-LCx are critical for

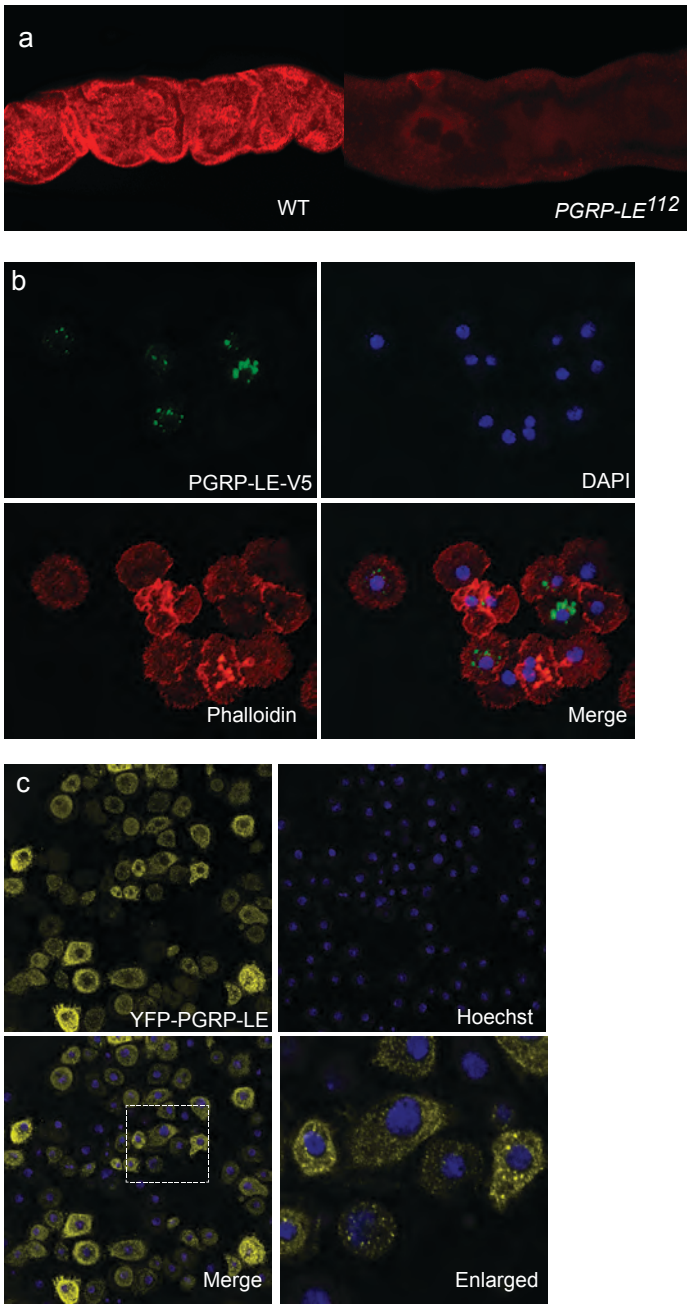


Figure 2.5: Intracellular localization of PGRP-LE.

(**a**) Endogenous PGRP-LE in WT or *PGRP-LE*^{112} malphigian tubules was detected by staining with anti-PGRP-LE (which was pre-absorbed with *PGRP-LE*^{112} tissues). Staining was visualized by confocal microscopy. Magnification 200x. Representative of two independent experiments (**b**) S2 cells engineered to express high amounts of V5-tagged PGRP-LE were stained with anti-V5 (to detect PGRP-LE), DAPI, and phalloidin (to detect the actin network). Staining was visualized by whole cell microscopy. Magnifcation 1200x. Representative of six independent experiments (**c**) S2* cells engineered to express YGP-tagged PGRP-LE were stained with Hoechst. Staining and YFP expression was visualized by fluorescent confocal microscopy. Magnification 63x and then enlarged another 3.44x. Representative of three independent experiments.

TCT-induced dimerization of two PGRP-domains (Lim et al., 2006) (Chang et al., 2006). The PGRP-LE^{pgS232E} mutant failed to enhance TCT-induced *diptericin* expression (**Figure 2.6d**) and failed to associate with S2 cells after addition of TCT (data not shown). However, PGRP-LE^{pgS232E} was detected in the cell culture media in amounts similar to WT PGRP-LE^{pg} (**Figure 2.6b**). These results suggest that PGRP-LE^{pg} functions in a CD14-like manner by binding TCT in solution and carrying it to the cell surface, where it interacts with PGRP-LC to induce signal transduction.

PGRP signaling through a RHIM-like motif

The molecular mechanisms by which PGRP-LC and PGRP-LE activate intracellular signaling are unclear. Overexpression of either *PGRP-LC* or *PGRP-LE* in S2* cells or *in vivo* is sufficient to activate IMD signaling and drive antimicrobial peptide expression in the absence of microbial challenge. Using *PGRP-LC* overexpression-induced *diptercin* expression as an assay, we identified regions within the intracellular domain of PGRP-LCx that are required for signaling. A series of seven internal deletions, each removing approximately 50 amino acids, and in total spanning the entire intracellular domain, were expressed using a copper-inducible promoter in stable S2* cell transfectants. Two adjacent deletions, Δ5 and Δ6, failed to induce diptericin, while the other deletions behaved similarly to WT PGRP-LCx (**Figure 2.7a**). These results are supported by and expand upon the recent findings of Choe *et al*, who assayed the effects of large N-terminal PGRP-LC deletions (Choe et al., 2005). All deletion mutants presented here were expressed on the cell surface, as analyzed by live cell flow cytometry (data not shown). The region defined by the two signal deficient deletions (residues 175-243) was further dissected with five additional ~20 amino acid deletions

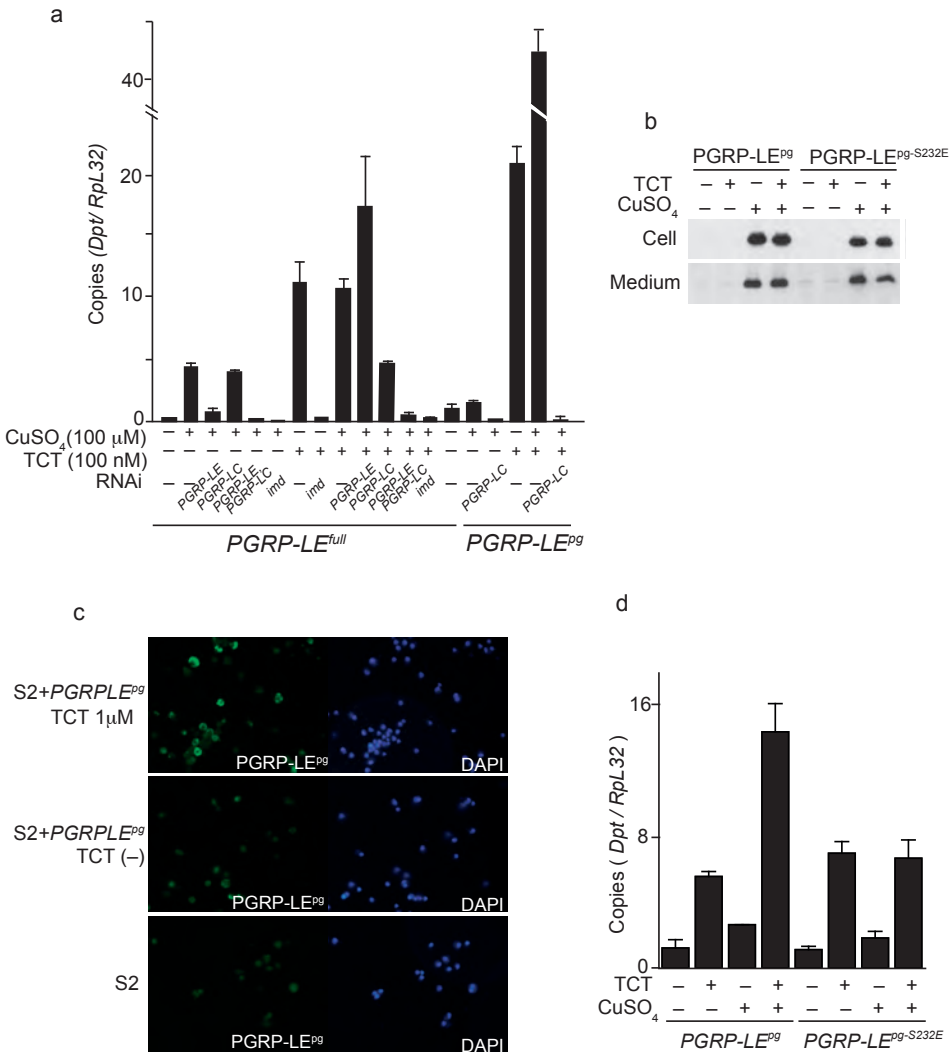


Figure 2.6: CD14-like activity of the PGRP domain of PGRP-LE.

(**a**) S2 cells stably transfected with metallothionein promoter-driven transgenes encoding either full length PGRP-LE (*PGRP-LE^{full}*) or the PGRP domain of PGRP-LE (*PGRP-LE^{pg}*), were transfected with the indicated dsRNAs and treated with copper for 40 h, followed by TCT stimulation for an additional 12 h, as indicated. RNA was extracted and *diptericin* and *RpL32* were quantified by real-time RT-PCR. *PGRP-LE^{full}* induced *diptericin* expression and this activity did not require *PGRP-LC*. Expression of *PGRP-LE^{pg}* did not activate *diptericin* expression but robustly enhanced the PGRP-LC-dependent response to TCT. Representative two independent experiments, both performed in duplicate. (**b**) Extracellular PGRP-LE^{pg} was detected by anti-V5 immunoblot of lysates and culture medium from S2 cells stably expressing V5-tagged PGRP-LE^{pg} or PGRP-LE^{pgS232E}. Representative of two independent assays. (**c**) TCT-induced cell surface association PGRP-LE^{pg} was detected by staining S2 cells stably expressing V5-tagged PGRP-LE^{pg}, in the presence or absence of 1μM TCT, with anti-V5 and DAPI. Representative of five experiments. (**d**) Requirement for interaction between TCT-bound PGRP domains was detected treating S2 cells stably expressing copper-responsive transgenes encoding PGRP-LE^{pg} and PGRP-LE^{pgS232E} as in (**a**). Error bars indicate s.d. Representative of three independent experiments, each performed in duplicate.

(Δ11-Δ15). Deletions 13, 14 and 15 failed to induce diptericin when overexpressed (**Figure 2.7b**). These results clearly define a region, within residues 205-242, as critical for IMD pathway activation. Interestingly, this region contains a stretch of amino acids bearing similarity to other PGRP proteins, including the *Anopheles* mosquito PGRP-LC homolog and *Drosophila* PGRP-LAa and PGRP–LE (**Figure 2.7c**). The homology between these proteins, outside of their C-terminal PGRP domains is limited to just this short stretch. A PGRP-LE deletion mutant removing 15 residues spanning this conserved motif (Δ98-113), failed to induce *diptericin* expression when overexpressed in S2* cells (**Figure 2.7d**). WT and mutant versions of PGRP-LE were expressed in similar amounts in these stable transfectants. These results define a conserved motif found in PGRP-LC and PGRP-LE which is required for signal transduction.

To define more precisely which residues within this PGRP signaling motif are critical for signaling, a series of alanine substitution mutants in PGRP-LC and PGRP–LE were similarly assayed. Using stable transfectants and a copper inducible promoter, thirteen PGRP-LC substitution mutants, focusing on the conserved residues indicated in **Figure 2.7c**, were overexpressed in S2* cells. Five residues (L210, T217, F218, G219 and G226) were found to be absolutely essential for IMD signaling. In addition, three other residues (V216, D220, and V228) appeared to contribute to IMD signaling, as S2* cells expressing these mutants produced significantly fewer diptericin transcripts when compared to S2* cells expressing WT PGRP-LCx (**Figure 2.8a**). Note, V228A was variable, ranging from reduced to abolished diptericin induction. These results clearly define a core motif of four amino acids ($_{216}$VTFG$_{219}$) as well as some outlying residues (L210, D220, G226, and V228) that are critical for PGRP-LC-induced IMD activation.

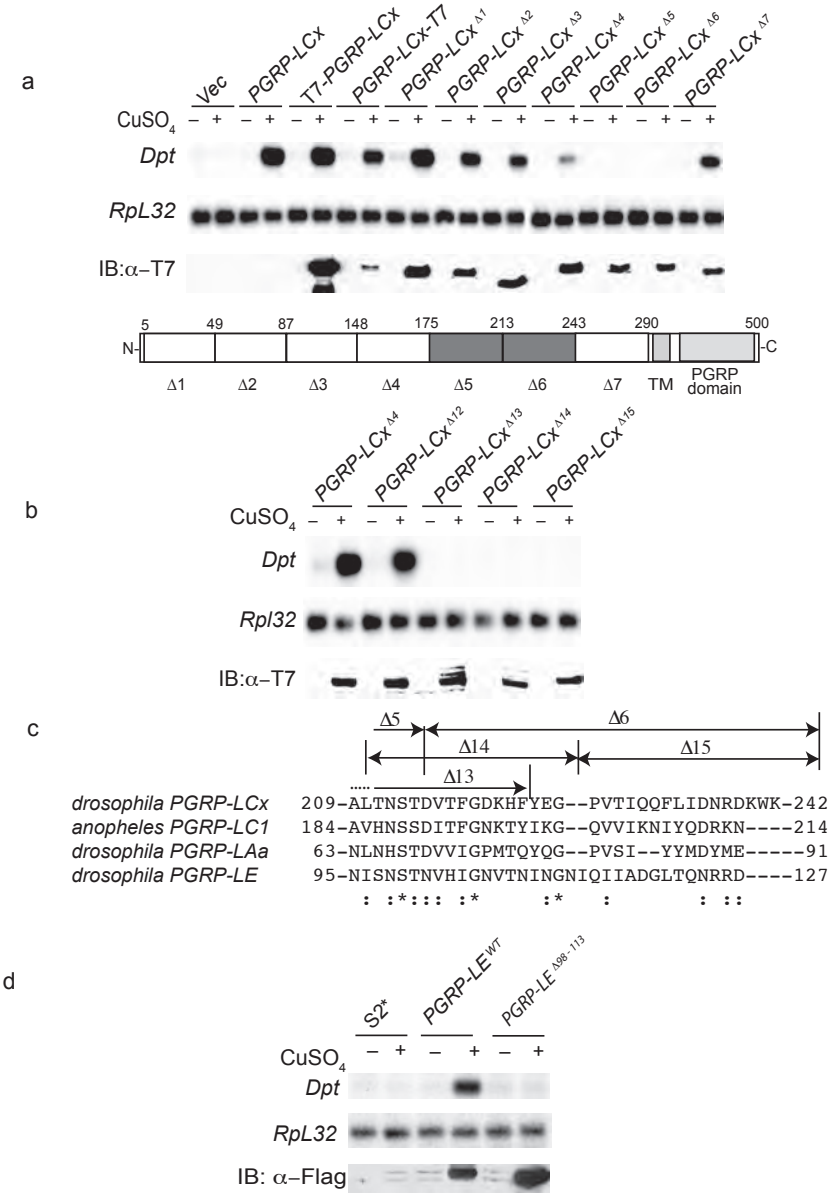


Figure 2.7: PGRP-LC and PGRP-LE signal via a conserved motif.

(**a**) S2* cells stably expressing metallothionein promoter-driven transgenes encoding T7-tagged full length and deleted versions (diagram, bottom) of PGRP-LCx were treated with copper, and *diptericin* expression was assayed by Northern blot. Expression of each transgene-encoded construct was detected by anti-T7 immumoblot. All deletion mutants carried a C-terminal T7 tag, and WT PGRP-LCx was tested without a tag as well as with either N-or C-terminal T7 tags, as indicated. (**b**) S2* cells stably expressing metallothionein promoter-driven transgenes encoding a PGRP-LC genes containing smaller deletions spanning the region deleted in Δ5 and Δ6 constructs were analyzed as in (**a**). (**c**) Conservation of this signaling motif in mosquito PGRP-LC1 and in *Drosophila* PGRP-LE and PGRP-LAa. Clustal X alignments are shown and the regions of the deletions used in (**b**) are indicated. (**d**) S2* cells stably transfected with metallothionein promoter driven transgenes encoding Flag-tagged WT PGRP-LE or PGRP-LE lacking the conserved signaling motif were analyzed as in (**a**). **(a)** and **(b-c)** representative of at least three independent assays.

Alanine substitutions of PGRP-LE revealed similar requirements ($_{102}$VHIG$_{105}$ in PGRP-LE), further highlighting the importance of this conserved core motif (**Figure 2.8b**).

This core motif displays sequence similarity with the recently-defined RIP homotypic interaction (RHIM) motif (**Figure 2.8c**). This domain was identified in TRIF and RIP1 and is required for the interaction between these two proteins and for TRIF and TLR3-induced NF-κB activation (Meylan et al., 2004; Sun et al., 2002b). The RHIM core amino acid motif (VQIG) resembles the motif in the center of the signaling region of PGRP-LC (VTFG). Additional similarities between the RHIM domain and the PGRP-LC signaling domain are found outside this core motif, including identity at residues L210, N212, S213, and T214. Likewise, the region of PGRP-LE that is homologous to PGRP-LC and is required for LE-mediated IMD activation contains similar homologies to the RHIM motif, including a VHIG motif, with homology extending beyond this core motif. Although the homologies between *Drosophila* PGRPs and the mammalian RHIM domain proteins are weak, the fact that they coincide with residues required for signaling is highly suggestive of a conserved structural element involved in signal transduction in mammalian TLR3 (MyD88-independent) and *Drosophila* IMD signaling pathways.

IMD–PGRP-LC interaction is dispensable for signaling

Among the known intracellular components of the IMD pathway, the IMD protein is thought to function most proximal to the receptor(s). To examine the potential interaction between IMD and PGRP-LC more precisely, the collection of in-frame PGRP-LC deletion mutants (**Figure 2.7a**) was co-expressed with IMD in S2* cells by transient transfection, and their interaction was examined by co-immunoprecipitation. In

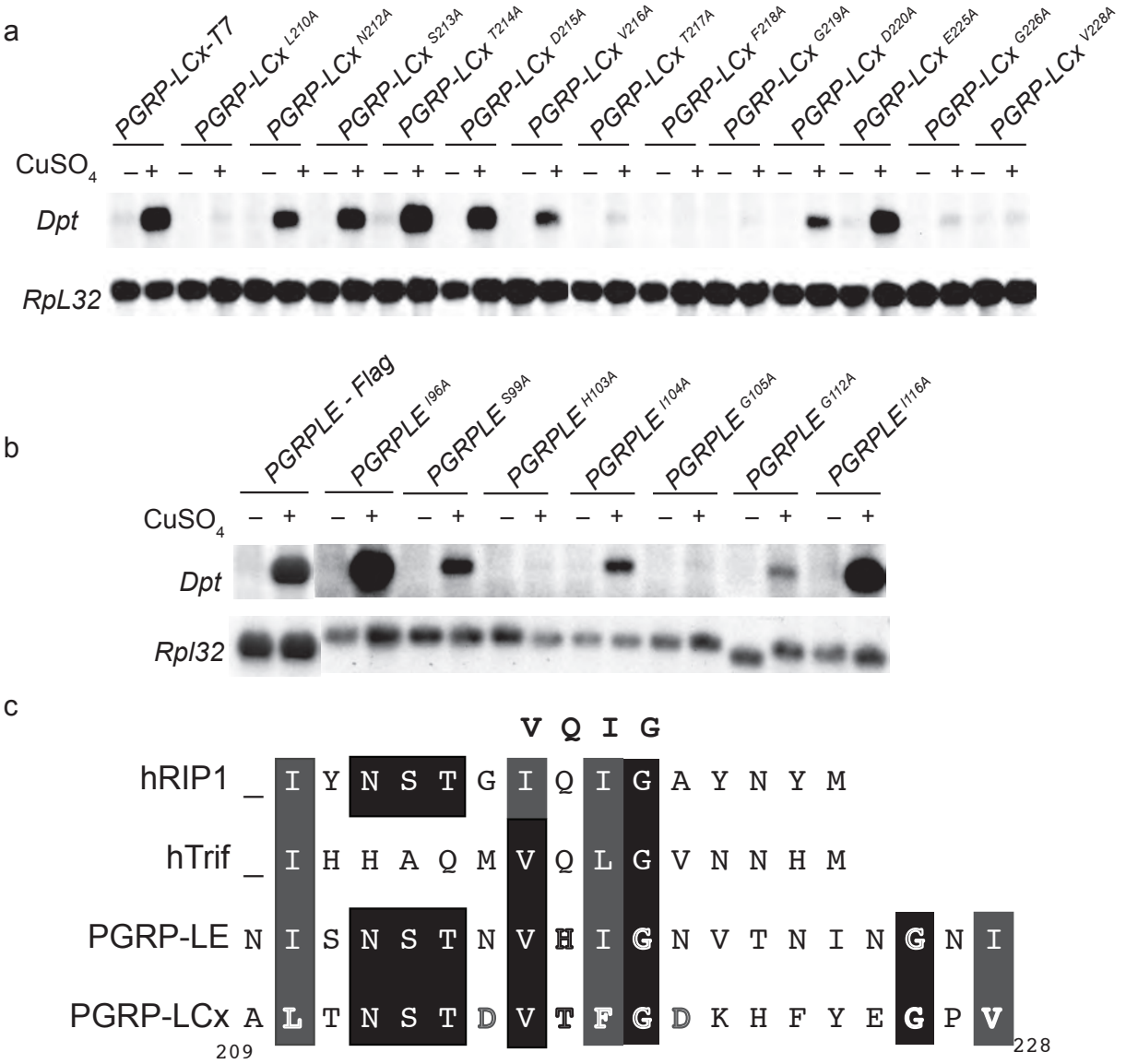


Figure 2.8: RHIM-like features of the conserved PGRP signaling motif.

(**a**,**b**) S2* cells stably transfected with metallothionein promoter-driven transgenes expressing PGRP-LC (**a**) or PGRP-LE (**b**) genes containing the indicated alanine substitutions were analyzed as in **Figure 2.7a**. (**c**) Clustal alignment of the signaling motif conserved in PRGP-LC and PGRP–LE with the RHIM motifs in human RIP1 and TRIF. Box shading depicts degree of conservation (gray, similar amino acid; black, identical amino acid). Amino acids critical for signaling are highlighted in bold, as determined in (**a**) and (**b**). **(a)** and **(b)** representative of at least three independent assays.

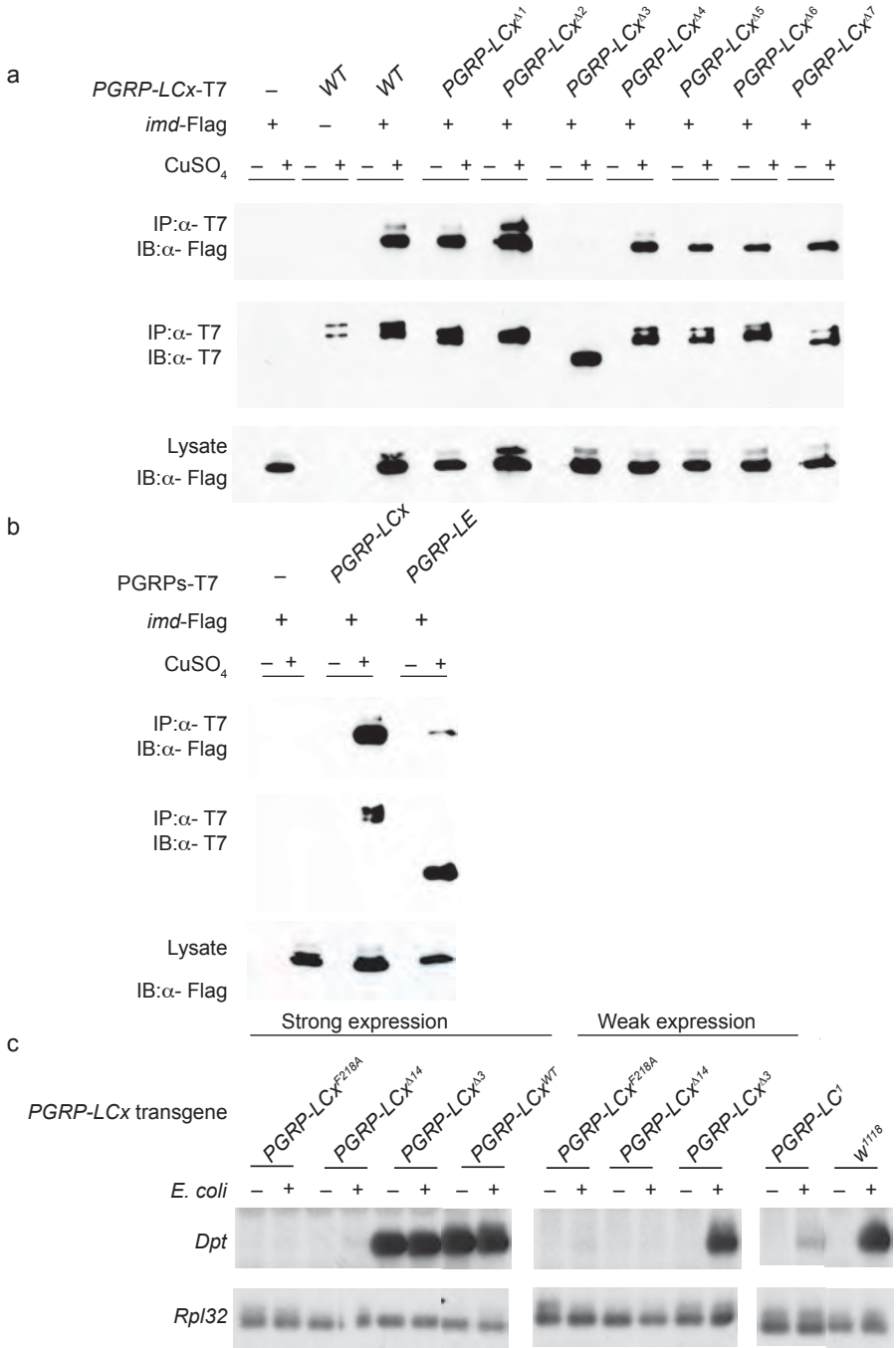


Figure 2.9: The interaction between PGRP-LC and IMD is not essential for signal transduction.

(**a**,**b**) S2* cells were transiently transfected with metallothionein promoter expression plasmids for T7-tagged PGRP-LCx (**a**) or T7-tagged PGRP-LE (**b**) and Flag-tagged IMD, and treated with copper, where noted. Cell lysates were subjected to indicated immunoprecipitations and immunoblotting. (**c**) *E. coli* was injected, where noted, into *PGRP-LC*^{1} adult flies carrying UAS promoter-driven transgenes encoding indicated *PGRP-LC* mutants. Strong expression was achieved with the C564 Gal4 driver, and low expression without a Gal4 driver. Eight hours after infection, RNA was isolated, and *diptericin* and *RpL32* expression was assessed by Northern blot. All panels representative of at least three independent assays.

agreement with previous reports (Choe et al., 2005), we detected a clear interaction between IMD and WT PGRP-LC (**Figure 2.9a**). IMD also interacted with PGRP-LE, although not as robustly as with PGRP-LC (**Figure 2.9b**). However, PGPR-LC deletions that interrupt the RHIM-like domain (Δ5 and Δ6) interacted with IMD, while a deletion removing amino acids 87-148 (Δ3), which retained the ability to induce *diptericin* expression (**Figure 2.7a**), failed to interact with IMD (**Figure 2.9a**). Similarly, strong expression of the Δ3 PGRP-LC deletion, but not the RHIM-like mutants Δ14 and F218A, in adult flies drives robust *diptericin* expression (**Figure 2.9c**). Moreover, when expressed in lower amounts, the Δ3 PGRP-LC deletion, but not the Δ14 or F218A mutants, supported *E.coli* infection-induced *diptericin* expression. These results demonstrate that IMD–PGRP-LC interaction is not essential for activation of IMD signaling, while the RHIM-like motif is critical *in vivo*.

Discussion

In our model (**Figure 2.10**), intracellular PGRP-LE oligomerizes following TCT binding and is sufficient to mediate TCT-induced IMD activation, while extracellular PGRP-LE^{pg} serves to enhance PGPR-LC-mediated TCT-induced IMD activation. The RHIM-like domains of PGPR-LC and PGRP-LE play an important role in activation of the IMD signaling pathway.

In cultured *Drosophila* cells, TCT is recognized by a heterodimeric PGRP-LCa–PGRP-LCx receptor complex (Kaneko et al., 2004). TCT binds to the PGRP domain of PGRP-LCx and recruits the PGRP-LCa PGRP domain (Chang et al., 2006; Chang et al., 2005; Mellroth et al., 2005). In addition to inducing this dimerization of PGRP-LCa and PGRP-LCx, TCT induced oligomerization of PGRP-LE^{pg}. These TCT-induced

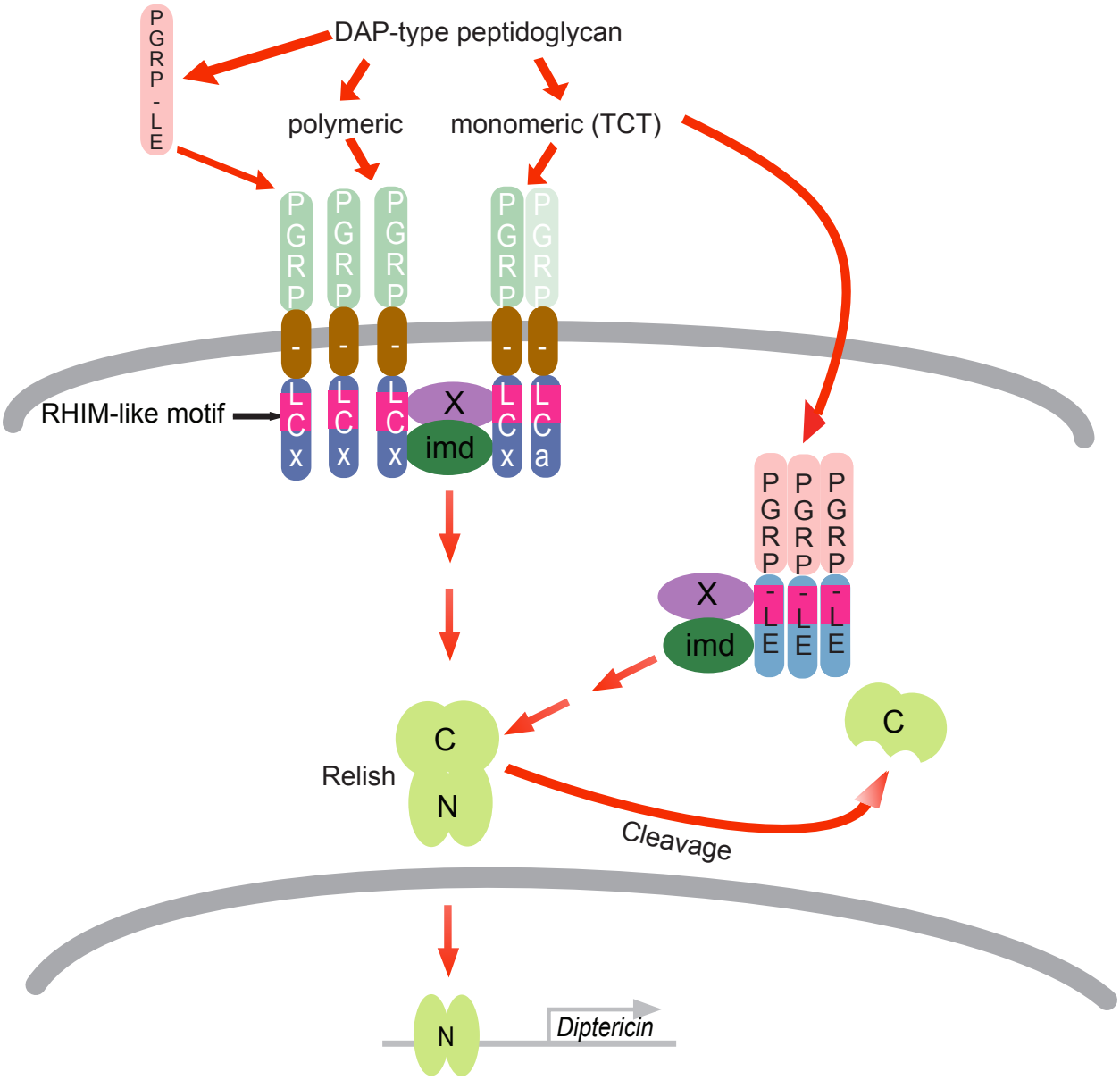


Figure 2.10: Model of PGRP-LC and PGRP-LE-mediated recognition of monomeric and polymeric DAP-type peptidoglycan.

Monomeric DAP-type PGN (TCT) is recognized by a heterodimer of PGRP-LCa and PGRP–LCx on the cell surface, or by PGRP-LE within the cell. TCT binding to PGRP-LCx causes its dimerization with PGRP-LCa, while TCT binding to PGRP-LE causes its oligomerization. Subsequent intracellular signaling is transduced through the RHIM-like motif found in all PGRP-LC isoforms and in PGRP-LE, although the function and/or binding partner (factor 'X' in diagram) of this motif is not yet identified. PGRP-LC and PGRP-LE also interact with IMD, although this interaction does not appear to be essential for signal transduction. Polymeric PGN requires only PGRP-LCx for signaling, and the polymeric repetitive nature of this ligand may be sufficient to cluster this receptor and activate signaling. The PGRP-domain of PGRP-LE can also function outside of the cell in a CD14-like manner, by presenting PGN to cell surface PGRP-LC receptors.

oligomers are very large, fractionating in (or near) the void volume during gel filtration, and, like the PGRP-LC dimers, they may play an important role in activating IMD signal transduction.

The data presented here clearly demonstrate that PGRP-LE functions by two distinct mechanisms. Unlike PGRP-LC and the mammalian Toll-like receptors, PGRP-LE is not a transmembrane protein. PGRP-LE was predicted to reside in the cytoplasm (Werner et al., 2000). Here, we demonstrated that PGRP-LE is found in the cytoplasm and can function as an intracellular TCT receptor. In addition, we showed that a fragment of PGRP-LE, corresponding to the PGRP domain alone, was found and functioned extracellularly to enhance PGRP-LC dependent recognition of TCT. This is consistent with the non-autonomous manner in which PGRP-LE acted when overexpressed in the fat body. In fact, the predominant form of PGRP-LE found in the cell-free fraction of hemolymph *in vivo* was a fragment consisting of the PGRP domain. Full-length PGRP-LE is found in the malphigian tubules as well as in hemocytes. We propose that the extracellular activity of PGRP-LE is transduced to the cytoplasm by PGRP-LC. As an extracellular PGN receptor, PGRP-LE^{pg} appears to function analogously to CD14, in that it can bind PGN in the circulation (or culture media) and carry it to the appropriate cells (fat body and hemocytes), where the PGN–PGRP-LE complex then binds the cell surface receptor PGRP-LC and activates IMD signaling (Gioannini et al., 2004; Visintin et al., 2003).

As an intracellular receptor, PGRP-LE functions independently of PGRP-LC. It is not clear how TCT gains access to intracellular PGRP-LE. Perhaps certain cell types (e.g. malphigian tubules) express transporters capable of importing small fragments of

PGN across the plasma membrane. As intracellular receptor, PGRP-LE may have evolved to detect intracellular bacteria, bacteria that pump PGN into the cytoplasm via specialized secretions systems, and/or PGN actively imported into certain cell types, in a manner similar to that proposed for mammalian NOD proteins (Strober et al., 2006). PGRP-LE must have its own intrinsic ability to interact with and activate Imd pathway components. This notion is supported by the essential role of the conserved RHIM-like motif of PGRP-LE.

Surprisingly, the interaction between IMD and PGRP-LC, which is detected in transfection experiments, is not physiologically relevant or is superfluous for PGRP-LC-mediated antimicrobial peptide gene induction. Interaction with IMD requires a region (residues 87-148) within the cytoplasmic domain of PGRP-LC that is not required for PGRP-LC-mediated IMD pathway activation. The RHIM-like domain of PGRP-LCx is also not required for PGRP-LCx homodimerization (data not shown). Instead, we predict that in order to transduce a signal, the RHIM-like motifs of PGRP-LC and PGRP-LE interact with an unidentified component of the IMD pathway, whose characterization will be the focus of future work.

Methods

Reagents

Insoluble peptidoglycan from *E.coli* was purchased from Invivogen. Isolation of TCT from *Bordetella pertusis* was described previously reported (Cookson et al., 1989a).

Fly stocks and microinjections

All fly strains used were previously published: *PGRP-LC*^{1} and *PGRP-LC*$^{2\ 9}$, *PGRP-LC*$^{\Delta E}$, *imd*^{shadok}, *key*^{1}, and DD1 strains (Rutschmann et al., 2000; Gottar et al., 2002), and the *PGRP-LE*^{112} and double *PGRP-LC* and *PGRP-LE* mutant flies (*PGRP-LC*$^{\Delta E,}$*PGRP-LE*^{112}, *PGRP-LC*^{7454},*PGRP-LE*^{112}) (Takehana et al., 2004). Microinjections into 2- to 5-day-old adults were performed as described previously (Kaneko et al., 2004; Wu et al., 2001). Twenty-four hours after infection, flies were harvested and stored at -80°C.

RNA analysis

Total RNA from flies or cultured cells was isolated using the TRIzol reagent (Invitrogen), as described previously, and expression of the antimicrobial peptide gene *diptericin* and control *RpL32* genes was analyzed by Northern blot, as previously described (Kaneko et al., 2004; Silverman et al., 2000). Quantitative analysis of *diptericin* expression was analyzed by phosphoimager (Fuji) normalizing to the level of *RpL32* expression. In all S2* based experiments, cells were pretreated with 1 μM 20-hydroxyecdysone for 24-40 h prior to a 7-8 h treatment with 500 μM copper sulfate, to induce expression of PGRPs and/or IMD. Real-time RT-PCR using Light Cycler (Roche Diagnostics) was performed as described previously (Choe et al., 2002).

DNA cloning

A DNA fragment encoding the PGRP domain of PGRP-LE (residues 173-339) was amplified from a Berkeley Drosophila Genome Project cDNA EST clone and subcloned into a bacterial expression vector for recombinant protein production. DNA

fragments encoding C-terminal V5-tagged WT PGRP-LE and PGRP-LE^{PG} (residues 172-345) were subcloned into pMT-V5-His vector (Invitrogen). These vectors were cotransfected with pCoHygro (Invitrogen), and stably transfected cells were selected with Hygromycin B (Invitrogen) according to the manufacturer's protocol.

C- or N-terminal T7-tagged WT *PGRP-LC*, N-terminal Flag-tagged *imd,* or C-terminal T7- or Flag-tagged *PGRP-LE* were PCR amplified from cDNA clones and subcloned into pRmHa3 (Bunch et al., 1988), for copper inducible expression, by standard techniques. These constructs were transfected into the *Drosophila* S2* cells and stably transfected cells were selected with G418 (Invitrogen) or Hygromycin (Calbiochem) as described previously (Wu et al., 2001).

Mutagenesis of the PGRP-LC and PGRP-LE RHIM-like domains was performed using PCR-based QuikChange® II XL Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. Mutagenesis of PGRP-LE^{S232E} was performed with PCR-based LA PCR *in vitro* Mutagenesis kit for pBluescript II (TAKARA BIO Inc.), and subcloned into pMT-V5-His vector. All constructs were verified by sequencing.

The YFP-PGRP-LE fusion was constructed by standard protocols, by cloning the PGRP-LE coding region into a modified version of pPAC-PL that contains mYFP at the N-terminus, which allowed expression of the YFP-PGRP-LE fusion from the actin promoter. The modified version pPAC-PL containing mYFP was constructed by standard protocols subcloning mYFP (gift of E. Latz) into pPAC-PL.

To create PGRP-LE^{S232E} site-directed mutagenesis was performed with LA PCR *in vitro* Mutagenesis kit for pBluescript II (TAKARA BIO Inc.). The mutagenized fragment was subcloned into pMT-V5-His vector, and stably transfected in S2 cells.

Protein production and purification

The detailed procedure for the production of PGRP-LE^{pg} was recently reported (Lim et al., 2006).

Gel filtration

Samples were injected into a Superdex 75 10/300 GL column (Amersham Phamacia) pre-equilibrated with buffer A. The protein content was monitored by absorbance at 280 nm.

Clonal analyses and immunohistochemistry

Clonal analysis of PGRP-LE over-expression and immunohistochemistry of malpighan tubules were performed as previously described (Takehana et al., 2004).

Whole organ culture

Whole organ culture of *Diptericin-LacZ*-carrying third instar larvae were performed as previously described[16]. TCT, to a final concentration of 10 μM, was added directly to the culture. *LacZ* staining was performed as previously described (Takehana et al., 2004).

Localization studies

A cell line stably expressing YFP-PGRP-LE was generated, via G418 selection. For confocal microscopy, stable cells were treated for 24 h with 20-hydroxyecdysone, then plated on Concanavalin A-treated 35 mm glass-bottom culture dishes, and

observed by fluorescent microscopy under the 63X objective of a Leica SP2 AOBS laser scanning microscope. Nuclei were stained with Hoechst 34580 (Invitrogen), and images were produced by sequential scanning with a 514 nm laser excitation and a 522-599 nm emission window for YFP and 405 nm laser excitation and a 523-600 nm emission window for Hoechst 34580.

For immunohistochemistry of V5-tagged PGRP-LE, the protein was induced by 24 h treatment with 100 μM $CuSO_4$ in stably transfected S2 cells. The cells were cultured for 1.5h on Concanavalin A-treated glass slides, fixed with 2% PFA, treated with PBS containing 1% BSA and 0.1% Triton X-100 for 1h, and stained with mouse monoclonal anti-V5 (Invitrogen), FITC-labeled anti-mouse IgG (Jackson Immunologicals), DAPI (Sigma), and rhodamine-labeled phalloidine (Molecular Probes).

V5-tagged PGRP-LE^{pg} was induced in stably transfected S2 cells with 24 h treatment with 100 μM $CuSO_4$, after which cells were subjected to treatment of 1μM TCT for 4 h. Cells were then fixed with 2% PFA, incubated with PBS containing 1% BSA for 1h, and stained with anti-V5 (Invitrogen), FITC-labeled anti-mouse IgG, and DAPI.

RNAi, TCT transfection and real-time RT-PCR

dsRNAs used in RNAi experiments were synthesized and purified as described previously (Silverman et al., 2000). Templates for dsRNA for PGRP-LE were amplified using primers 5'-TAATACGACTCACTCACTATAGGGAGACCACAAAGTTGAGCCAGG-3' and 5'-TAATACGACTCACTCACTATAGGGAGACCACGA-3'. dsRNAs were transfected into

S2* cells cultured in Schneider's Drosophila medium containing 10% fetal bovine serum, as previously described (Silverman et al., 2000). After 42 h, cultures were diluted to 1×10^6 cells/ml and treated with 1 μM 20-hydroxyecdysone and 100 μM $CuSO_4$ for 40 h prior to treatment with 100 nM TCT. Twelve hours later, total RNA was isolated, as described above, and expression of antibacterial peptides was quantified by RT-PCR using a Light Cycler (Roche Diagnostics) as described previously(Takehana et al., 2002).

For TCT transfections, pMT-V5-PGRP-LE stable S2 cells were first stimulated with copper sulfate (100 μM) for 1h or left untreated. TCT was prepared by dilution into BBS pH 6.94, followed by the addition of $CaCl_2$, and thoroughly vortexed, per standard transfection protocols. After 15 min incubation at room temperature the suspension was added dropwise to the copper-treated or untreated S2-PGRP-LE cells. Total RNA was extracted from the cells after 12 h incubation at 25°C and assayed by real-time RT-PCR.

Immunoblot and coimmunoprecipitation

Cell lysates from S2* cells stably transfected with epitope-tagged PGRPs were prepared and protein expression was monitored by immunoblot using monoclonal anti-Flag (M2 or M5, Sigma) or anti-T7 (Novagen) and anti-IgG-HRP secondary antibody (Amersham). Images were visualized with West Pico SuperSignal (Pierce).

For the analysis of the interaction between PGRPs and IMD, S2* cells were transiently co-transfected with both pRmHa3-PGRPs-T7 and pRmHa3-Flag-IMD for 3d and stimulated with 500 μM of copper for 5-7h. After preparing cell lysates in a non-ionic

detergent lysis buffer, coimmunoprecipitations were performed as described previously with Flag-M2-agarose (Sigma) or anti-T7 agarose (Novagen) (Silverman et al., 2000).

Transformation and analysis of UAS-PGRP-LC mutants

Mutated *PGRP-LC* genes were amplified by PCR and subcloned into the *Eco*RI and *Bgl*II sites of pUAST (Brand and Perrimon, 1993). After sequence verification, P-element mediated transformation was carried by standard techniques, at the Model Systems Genomic Center (Duke University). For strong expression in the fat body, the C564 Gal4 driver was used (Harrison et al., 1995). For low expression no Gal4 driver was used. Flies were infected, by pricking with an *E. coli* laden needle, RNA was extracted 8 h later and assayed by Northern blot for *diptericin* and *RpL32* expression (Choe et al., 2002).

CHAPTER III

Searching for new interactors of the RHIM-like motif through a yeast two-hybrid screen

Abstract

Two receptors, PGRP-LC and PGRP-LE both activate the IMD signaling pathway in response to DAP-type PGN. These two receptors utilize a common motif in their N-terminal domains, known as the RHIM-like domain, to trigger signal transduction. Genetic studies indicate that the IMD protein functions immediately downstream of the PGRP-LC receptor, yet the RHIM-like motif is not essential for IMD association with PGRP-LC. Thus, the mechanism by which the RHIM-like domain functions in signaling is unclear, and I hypothesize that the RHIM-like domain interacts with an unidentified protein to mediate downstream signaling. To test our hypothesis, I performed a yeast two-hybrid screen with the N-terminal, cytosolic domain of PGRP-LC as bait. 24 strong positive interactors were then counter screened with baits lacking a functional RHIM-motif, thereby identifying 5 potential RHIM-interactors for further study.

Introduction

Insects rely primarily on innate immune responses to fight pathogens and one of the key features of this response is the production of antimicrobial peptides (AMP) following an infection. In *Drosophila,* two signaling pathways regulate the production of these antimicrobial peptides - the IMD and Toll pathways (Aggrawal and Silverman, 2007). Two receptors, PGRP-LC and PGRP-LE, are able to recognize DAP-type PGN at the cell surface or in the cytosol, respectively, and trigger the IMD pathway (Kaneko et al., 2006; Werner et al., 2000; Gottar et al., 2002; Ramet et al., 2001; Choe et al., 2002; Takehana et al., 2002).

Upon binding DAP-type PGN, both PGRP-LC and PGRP-LE get activated and signal via a common motif in their N-terminal domains, known as the RHIM-like domain (Kaneko et al., 2006; Choe et al., 2005; Lim et al., 2006). The RHIM-like domain is critical for signaling by either receptor, but the mechanism(s) involved remain unclear (Kaneko et al., 2006). Genetic experiments suggest that the *imd* protein functions immediately downstream of PGRP-LC and upstream of all other known components of the pathway (Georgel et al., 2001). IMD associates with both PGRP-LC and -LE but the interaction of PGRP-LC with IMD is not mediated through its RHIM-like domain (Kaneko et al., 2006). Therefore I hypothesize that an unidentified protein interacts with the RHIM- like domain of PGRP-LC and is important for signaling.

In this study I performed a yeast two-hybrid screen to identify proteins that might interact PGRP-LC through its RHIM- like domain. With this approach, I have identified five new proteins as potential RHIM-dependent PGRP-LC interactors.

Results and Discussion

In order to find potential partners and regulators of the IMD pathway receptors, a yeast two-hybrid screen was performed with the cytoplasmic domain of PGRP-LC as bait and a *Drosophila* third instar cDNA library as prey (James et al., 1996; Silverman et al., 2000). The *Drosophila* genome has approximately 15000 genes . The cDNA library is composed of approximately 1 x 10^7 cDNA clones and we screened about 3x10^7 clones. From this, 242 PGRP-LC interacting clones were identified with a Ade2 reporter gene selection scheme.

A second round of assays was performed on all 242 interacting clones, taking advantage of a *LacZ* reporter gene to provide a more quantitative metric of interaction. In this case, β-galactosidase activity (produced from *LacZ*) is proportional to the strength of the protein-protein interaction monitored in the two-hybrid assay. Colorimetric X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) substrate was used to monitor β-galactosidase activity, and the degree of blue X-gal staining was recorded, as indicated in the chart in **Figure 3.1b**. The 24 strongly interacting clones were analyzed further.

A third round of screening was performed, on these 24 strongest hits to confirm the interaction was dependent on the PGRP-LC bait and to determine if the interaction involved the RHIM Type to enter text-like domain. For this, the clones were selected on FOA plates. The presence of URA3 containin Type to enter text g plasmids allows for negative selection. U*RA3* encodes orotidine-5'phosphate decarboxylase, an enzyme which is required for the biosynthesis of uracil. *Ura3*$^-$ (or *ura5*$^-$) cells can be selected on media containing FOA. The *URA3*$^+$ cells are killed because FOA is converted to the

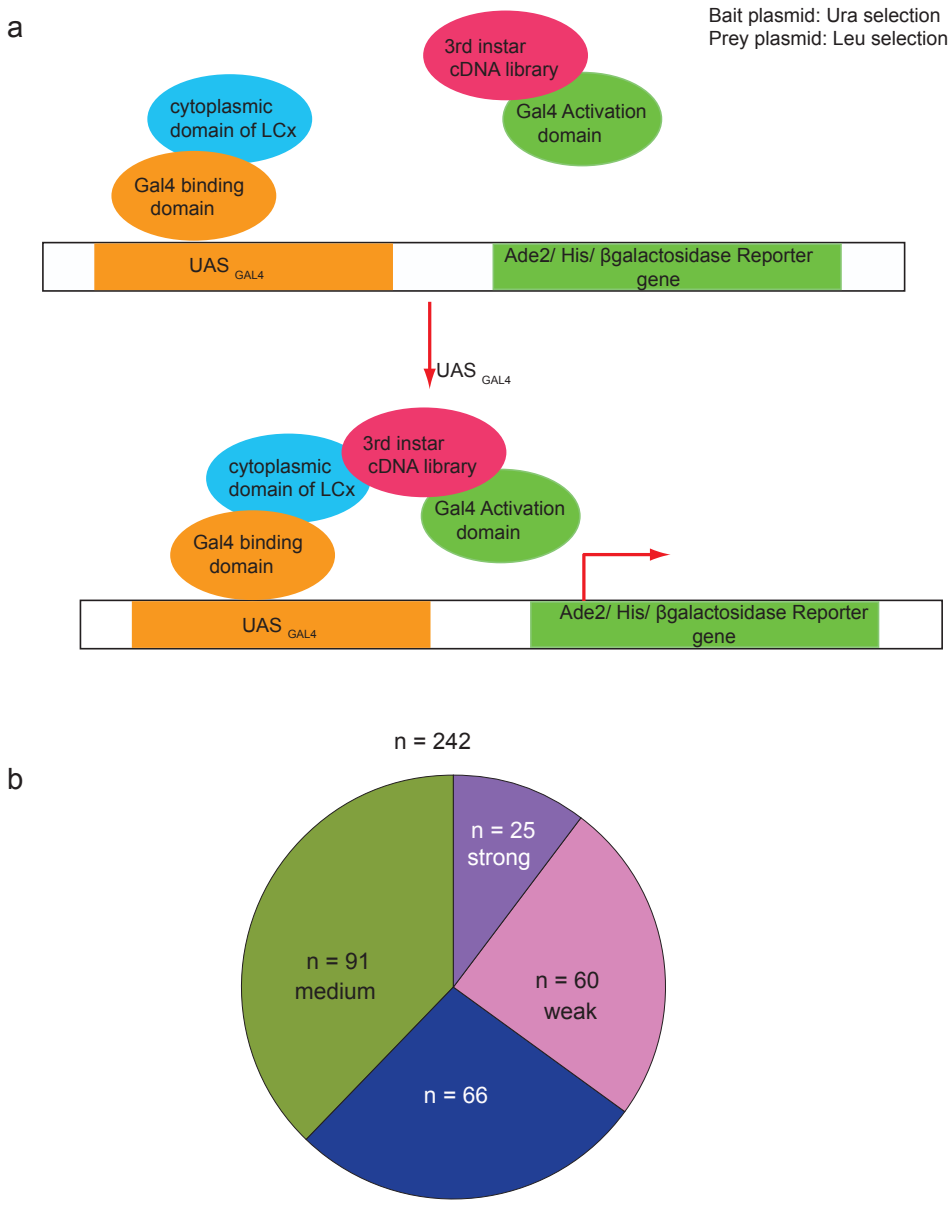


Figure 3.1: **X gal screening**
(a) Schematic representation of the yeast two hybrid screen and also the markers and reporters that are utilized in the screen.
(b) Quantitative analysis of X-gal screening. Clones that interacted with cytoplasmic domain of PGRP-LC were plated on X-gal plates and categorized as strong, medium or weak interactions depending on how blue the colonies turned.

toxic compound 5-fluoro deoxyuracil by the action of decarboxylase, whereas *ura3*$^{-}$ cells are resistant. Thus, yeast that spontaneously lose the *URA3* containing plasmid grow on FOA plates allowing for the selection of yeast cells that have lost the bait plasmid. The 24 clones were then re-transformed with wild-type PGRP-LC to confirm the interaction from the primary screen. In addition, the clones were separately transformed with three different RHIM mutant baits to identify clones that interact only with wild type PGRP-LC but not the RHIM mutant. And finally, irrelevant baits (IKKβ) or empty vector where used as additional negative controls. Ten clones interacted strongly with both wild type and RHIM mutant PGRP-LC and were not studied further. One clone interacted weakly even with wild type PGRP-LC and also was not pursued. For 2 clones, number 20 and 21, the retransformation failed and no useful data is available. The nine remaining clones interacted strongly with wild type PGRP-LC but not with the RHIM-like mutant baits, the irrelevant bait, or empty vector (**Table 3.2**). I was able to PCR amplify only six of these nine genes, and successfully obtained sequence from only five amplicons. I then analyzed if they function in IMD signaling, through RNAi knockdown in immune responsive S2* cells. Two of the five genes had marked phenotypes in these RNAi-based assay, and their role in IMD pathway will be discussed in detail in chapters IV and V. Thus, by performing a yeast two hybrid screen we have identified two new regulators of the IMD pathway. The other three hits from the 2-hybrid screen [Clone 4 (RfaBg) Clone 17 (Scylla) and Clone 24 (CG12935)] do not appear to affect IMD signaling, at least in these RNAi-based assays and have not been further explored (**Figure 3.3**).

Baits	LCx WT	LCx Δ172- 212	LCx Δ213- 242	LCx F218A	DmIKK	Empty vector
Clone 1	++++	++++	++++	++++	X	X
Clone 2	++++	++++	++++	++++	X	
Clone 3 (CG15678)	++++	++	++	++	X	X
Clone 4 (RfaBg)	++++	++	X	X	X	X
Clone 5	++++	++++	++++	++++	X	X
Clone 6*	++++	++	X	X	X	X
Clone 7$	++++	X	X	X	X	X
Clone 8	++++	+++	++	+++	X	X
Clone 9	++++	++++	++++	++++	X	X
Clone 10	++++	++++	++++	++++	X	X
Clone 11	++++	++++	++++	++++	X	X
Clone 12 (RYBP)	++++	++	X	X	X	X
Clone 13	++++	++++	++++	++++	X	X
Clone 14$	++++	++	X	+	X	X
Clone 15	++++	++++	++++	++++	X	X
Clone 16	++++	++++	++++	++++	X	X
Clone 17 (Scylla)	++++	++	X	X	X	X
Clone 18 $	++++	++	X	++	X	X
Clone 19	++	++	+	+	X	X
Clone 20	++++	no data	no data	no data	X	X
Clone 21	++++	no data	no data	no data	X	X
Clone 22	++++	++++	++++	++++	X	X
Clone 23	++++	++++	X	+++	X	X
Clone 24 (CG12935)	++++	++	X	+	X	X

: Clones for which no sequencing data was obtained
: Clones for which PCR amplification did not work
: Clones that do not play a role in the IMD pathway
: Clones that play a role in the IMD pathway

Table 3.2: Interaction analysis to identify clones that interact with the RHIM-like motif of PGRP-LC

24 clones that interacted strongly with the cytoplasmic domain of PGRP-LC were tested for interaction with several deletion and point mutants that alter the RHIM-like domain of PGRP-LCx. ++++, robust growth; ++, slow growth, - no growth.

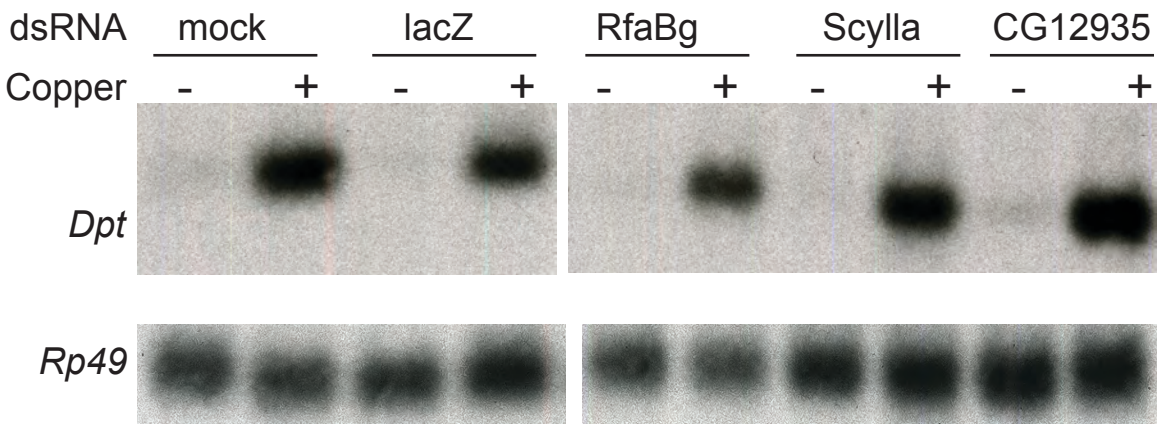


Figure 3.3: *RfaBg*, *Scylla* and *CG12935* are not regulators of IMD signaling in cells. Northern blot of *Diptericn* and *rp49* expression in S2* cells treated with lacZ dsRNA or RfaBg, Scylla or CG12935 dsRNA, and then stimulated with PGN for 6 hours.

Materials and Methods

Yeast strains, transformations and media

The bait plasmid was transformed into PJ69- 4A. All yeast transformations were done using the high efficiency LiAC method of Gietz and Woods and all media was prepared as per manufacturer's instructions (Gietz and Woods, 2002). For X-gal plates (200ml), YPAD Agar in 175 ml water was autoclaved and supplemented with 25 ml 0.7M Potassium Phosphate Buffer pH 7.0 (KPO_4) and X- gal (20mg/ ml in DMF) which was either added (50µl) on top of the plate and spread or was added (2ml) directly to the YPAD Agar before pouring the plates. And for FOA plates (200ml), -Ura-Leu Agar in 180 ml was was autoclaved and supplemented with 10 ml FOA (0.1%) and 10 ml uracil (10mM).

Yeast Two Hybrid Screen

The third instar larval library was cloned in the ACT vector and the first 290 amino acids of PGRP-LC were cloned into pGBDU (Durfee et al., 1993) (James et al., 1996). Two hybrid selection was performed as described (James et al., 1996) with modification. In brief, PGRP-LC was cloned into pGBDU. Two hybrid selection was performed as described (James et al., 1996) with modification. In brief,

Day 0

1. Inoculated 10ml of bait strain (pGBDU cyto PGRP-LCx) in -Ura medium over night at 30^{o}C at 250 rpm.Day 1
1. Thawed an aliquot of the library in 50 ml -Leu medium and grew it for 4-6 hours at 30^{o}C at 250 rpm.

2. Mixed 50 OD_{600} units of bait strain and 75 units of library in 100 YPAD (spin cells and then re-suspend in 50 ml YPAD and mix).

3. Grew the culture overnight at 30^{o}C at 150 rpm.

Day 2

1. Tittered mating on -Ura-Leu plates.

2. Pelleted 50 ml of mating and resuspended in 500 ml of -Ura-Leu media and grew it for 5-6 hours at 30^{o}C at 250 rpm.

3. Pellet the -Ura-Leu culture and resuspended in 50 ml of freezing media (8% glycerol YPAD) made 1 ml aliquots and froze it at -80^{o}C.

Day 3

1. Thaw an aliquot of the frozen culture and plated various dilution on -Ura-Leu to titre mating.

Day 4

1. Thawed aliquots of the frozen culture and plated 3 X 10^{7} colonies (3X complexity of the library) on -Ade plates at incubated them at 30oC.

Day 5

1. Observed plates for up to 10 days and picked colonies that grew on -Ade plates and re streaked on -Ade plates

PREFACE TO CHAPTER IV

This chapter was previously published in Plos Pathogen (2008):

Kamna Aggarwal, Florentina Rus, Christie Vriesema-Magnuson , Deniz Ertürk-Hasdemir, Nicholas Paquette & Neal Silverman. Rudra interrupts receptor signaling complexes to negatively regulate the IMD pathway. Plos Pathogen 2008 Aug 8, 4(8): e1000120.

Florentina Rus helped perform fly real-time PCR experiments

Christie Vriesema-Magnuson , Deniz Ertürk-Hasdemir and Nicholas Paquette provided reagents and tools for the experiments.

CHAPTER IV

Rudra interrupts receptor signaling complexes to negatively regulate the IMD pathway.

Abstract

Insects rely primarily on innate immune responses to fight pathogens. In *Drosophila,* antimicrobial peptides are key contributors to host defense. Antimicrobial peptide gene expression is regulated by the IMD and Toll pathways. Bacterial peptidoglycans trigger these pathways, through recognition by peptidoglycan recognition proteins (PGRPs). DAP-type peptidoglycan triggers the IMD pathway via PGRP-LC and PGRP-LE, while lysine-type peptidoglycan is an agonist for the Toll pathway through PGRP-SA and PGRP-SD. Recent work has shown that the intensity and duration of the immune responses initiating with these receptors is tightly regulated at multiple levels, by a series of negative regulators. Through two-hybrid screening with PGRP-LC, we identified Rudra, a new regulator of the IMD pathway, and demonstrate that it is a critical feedback inhibitor of peptidoglycan receptor signaling. Following stimulation of the IMD pathway, *rudra* expression was rapidly induced. In cells, RNAi targeting of *rudra* caused a marked up-regulation of antimicrobial peptide gene expression. *rudra* mutant flies also hyper-activated antimicrobial peptide genes and were more resistant to infection with the insect pathogen *Erwinia carotovora carotovora*. Molecularly, Rudra was found to bind and interfere with both PGRP-LC and PGRP-LE, disrupting their signaling complex. These results show that Rudra is a critical component in a negative feedback loop, whereby immune-induced gene expression rapidly produces a potent inhibitor that binds and inhibits pattern recognition receptors.

Introduction

Insects rely primarily on innate immune responses to fight pathogens. The *Drosophila* immune response has proven to be an experimentally powerful and conserved model system for the study of innate immunity (Brennan and Anderson, 2004; Cherry and Silverman, 2006; Hultmark, 2003; Lemaitre and Hoffmann, 2007). In particular, the insect immune response relies on evolutionary conserved NF-κB signaling cascades for the control of inducible antimicrobial peptide (AMP) gene transcription. This antimicrobial peptide response is critical for protection against many microbial pathogens (Lemaitre et al., 1997; Meister et al., 1997).

In *Drosophila,* two signaling pathways regulate the production of these antimicrobial peptides - the IMD and Toll pathways (Aggrawal and Silverman, 2007). The Toll pathway responds to many Gram-positive bacterial and fungal infections (Lemaitre et al., 1996), while the IMD pathway is potently activated by DAP-type peptidoglycan (PGN) from Gram-negative bacteria and certain Gram-positive bacteria (Leulier et al., 2003; Kaneko et al., 2004). Two receptors, PGRP-LC and PGRP-LE, are able to recognize DAP-type PGN at the cell surface or in the cytosol, respectively, and trigger the IMD pathway (Kaneko et al., 2006; Werner et al., 2000; Gottar et al., 2002; Ramet et al., 2001; Choe et al., 2002; Takehana et al., 2002).

Upon binding DAP-type PGN, both PGRP-LC and PGRP-LE multimerize and signal via a common motif in their N-terminal domains, known as the RHIM-like domain (Kaneko et al., 2006; Choe et al., 2005; Lim et al., 2006). The RHIM-like domain is critical for signaling by either receptor, but the mechanism(s) involved remain unclear (Kaneko et al., 2006). Genetic experiments suggest that the *imd* protein functions

immediately downstream of PGRP-LC and upstream of all other known components of the pathway (Georgel et al., 2001). IMD associates with both PGRP-LC and -LE, although the PGRP-LC RHIM-like motif is not required for this interaction (Kaneko et al., 2006). Nonetheless, the complexes formed on these receptors are likely to be critical to trigger further signal transduction.

Recent work has shown that the intensity and duration of the immune response is tightly regulated in *Drosophila*. As in mammals, over-exuberant immune responses can be detrimental, and the proper down modulation of immunity is critical for health and fecundity (Flatt et al., 2005; Lang and Mansell, 2007; Zerofsky et al., 2005). In order to keep the immune response properly modulated, the Toll and IMD pathways are controlled at multiple levels by a series of negative regulators. For example, the amidases PGRP-LB and PGRP-SC reduce the immunostimulatory activity of PGN by digesting it (Bischoff et al., 2006; Zaidman-Remy et al., 2006). Intracellularly, the IMD signaling pathway is further down–regulated by Dnr1, POSH, Caspar and the E3-ligase complex containing SkpA, dCullin and Slimb (Foley and O'Farrell, 2004; Khush et al., 2002a; Kim et al., 2006; Tsuda et al., 2006). Additionally, the JNK and Relish branches of the IMD pathway are thought to mutually inhibit each other (Park et al., 2004; Kim et al., 2007; Kim et al., 2005).

In this study, we identify and characterize a negative feedback regulator of the IMD pathway, dubbed *rudra.* Expression of *rudra* was rapidly induced following immune challenge. Moreover, in flies and cells, *rudra* is critical for controlling immune-induced gene expression. Following infection, *rudra* mutant flies hyper-activated antimicrobial peptide gene expression resulting in increased resistance to microbial infection. Using

various biochemical and genetic techniques, Rudra was found to interact with the receptors PGRP-LC and PGRP-LE and disrupt the signaling complex assembled on these receptors. Due to its ability to destroy this receptor signaling complex and inhibit immune responses, *rudra* was named for Shiva, the Indian god of destruction, who in his Rudra phase of mind causes inhibition and destruction of all life on earth.

Results

Isolation of Rudra

In order to identify potential partners and regulators of the IMD pathway receptors, a yeast two-hybrid screen was performed with the cytoplasmic domain of PGRP-LC as bait (James et al., 1996; Silverman et al., 2000). 25 strongly interacting clones were further analyzed with a set of baits that carried mutations in the RHIM-like domain of PGRP-LC (or irrelevant control baits). One clone interacted strongly with the wild-type cytoplasmic domain of PGRP-LC but weakly with the RHIM-like mutant baits (**Table 4.1**). This clone encoded amino acids 30-197 of CG15678, and will be referred to as *rudra* from hereafter.

To confirm the yeast two-hybrid results, co-immunoprecipitation experiments were performed. Using epitope tagged constructs and transient transfection in *Drosophila* S2* cells, both PGRP-LE and PGRP-LC were found to associate with Rudra (**Figure 4.2a, e**). In a heterologous system (HEK cells), similar robust associations were observed between Rudra and PGRP-LE or –LC (**Figure 4.2b,c**). The interaction between Rudra and PGRP-LE was also readily detectable, by co-immunoprecipitation, when these proteins were produced in a rabbit reticulocyte *in vitro* translation system

Baits	LCx WT	LCx Δ172-212	LCx Δ213-242	LCx F218A	DmIKK	empty vector
Prey Rudra amino acids 30- 197	++++	++	++	++	X	X

Table 4.1: Rudra interacts with cytoplasmic domain of PGRP-LC by yeast two-hybrid
The cytoplasmic domain of PGRP-LC was used as bait and Rudra was used as the prey in yeast two-hybrid assays. Rudra interacted well with the full cytoplasmic domain of PGRP-LC and the yeast cells grew robustly on Ade selection plates. However, Rudra interacted weakly with several deletion and point mutants that alter the RHIM-like domain of PGRP-LCx. ++++, robust growth; ++, slow growth, - no growth.

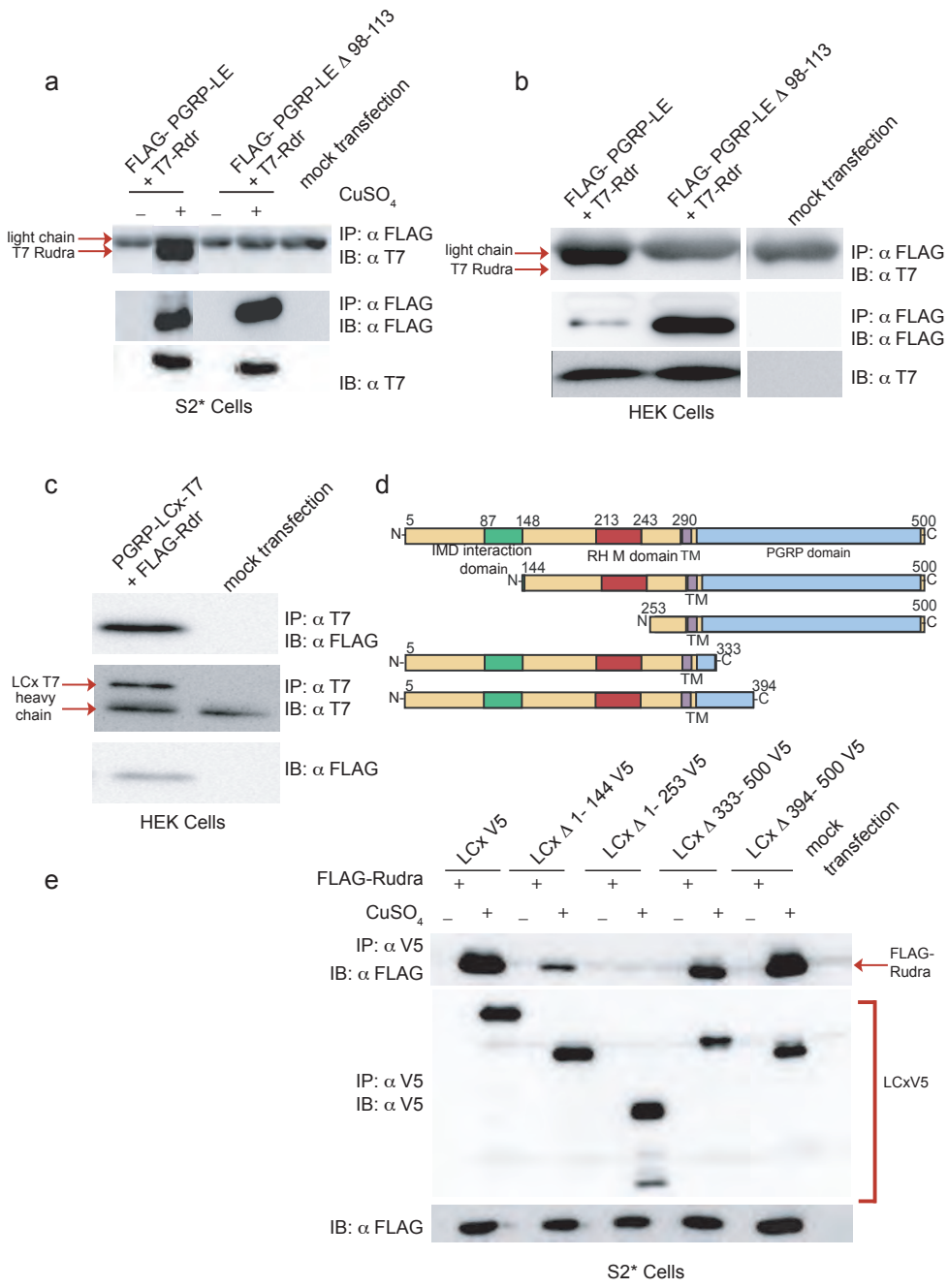


Figure 4.2: Rudra interacts with the receptors PGRP-LE and PGRP-LC
(**a,b**) Immunoprecipitation (IP) and immunoblot (IB) analysis of lysates from S2* or HEK cells transiently transfected with expression plasmids for FLAG-tagged *PGRP-LE* and/or T7-tagged *rudra.* In the *Drosophila* S2* cells, the copper inducible metallothionein promoter was used for expression and cells were treated with $CuSO_4$ or left untreated, as indicated. (**c**) Similar co-immunoprecipitation experiments from lysates of HEK cells transiently co-transfected with T7-tagged *PGRP-LCx* and FLAG-tagged *rudra* expression plasmids. (**d**) Schematic representation of the PGRP-LCx deletions mutants used in (E). (**e**) IP-IB analysis of lysates from S2* cells transiently transfected with metallothionein promoter expression plasmids encoding wild-type and deletion mutants of V5-tagged *PGRP-LCx* and FLAG-tagged *rudra,* with or without $CuSO_4$treatment, as indicated. Data are representative of at least three independent assays.

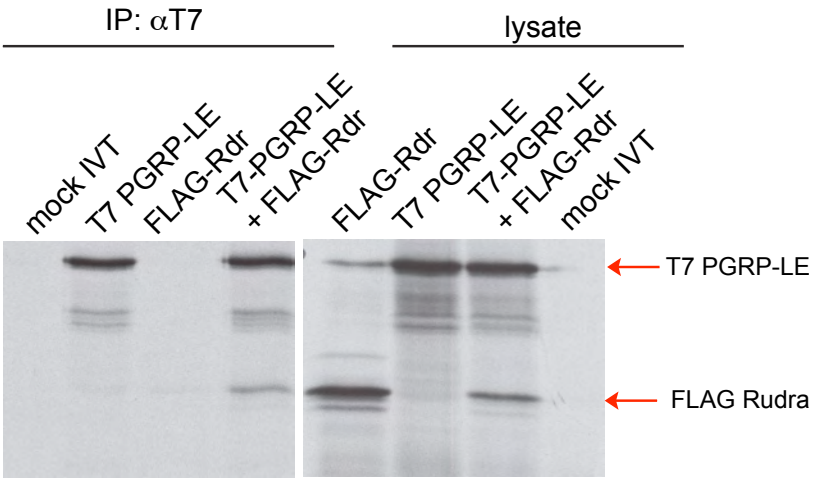


Figure 4.3: PGRP-LE and Rudra interact *in vitro*
Co-immunoprecipitation of *in vitro* co-translated PGRP-LE and Rudra. Co-immunoprecipitation was performed using anti–FLAG antibodies with ^{35}S-methionine labeled *in vitro* translated T7-Rudra and FLAG-PGRP-LE.

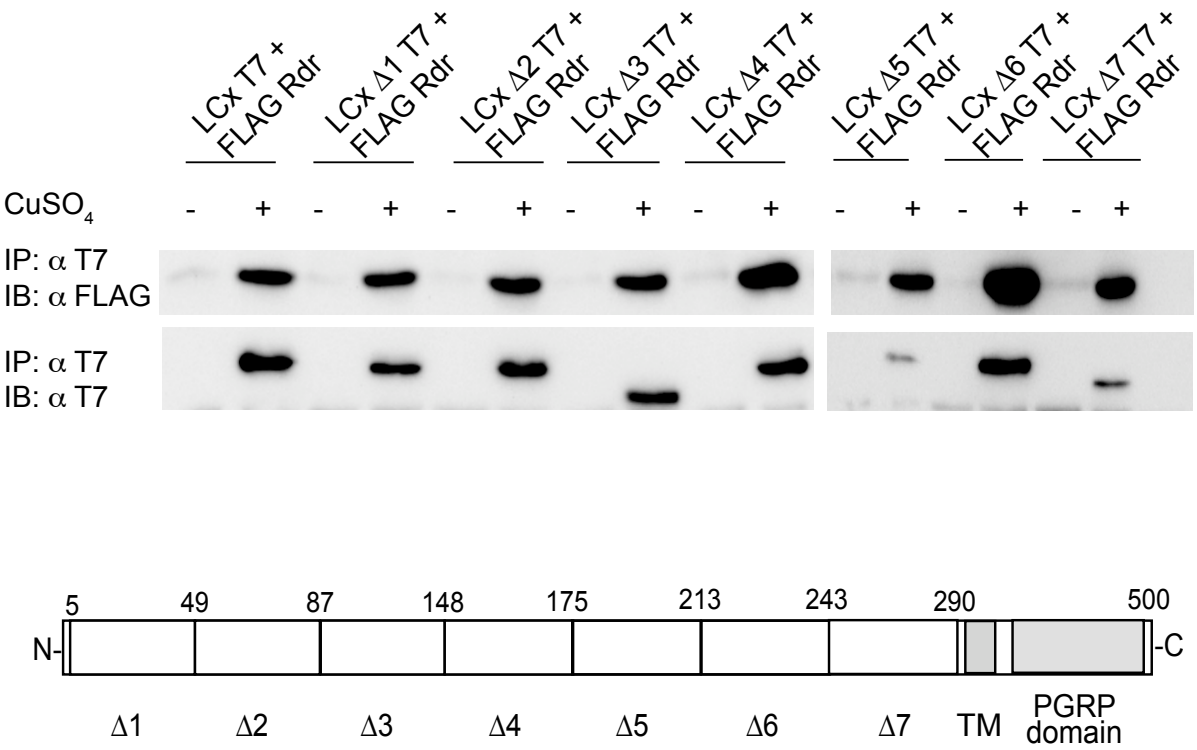


Figure 4.4: Rudra interacts with all the deletion mutants spanning the cytoplasmic domain of PGRP-LCx.
IP-IB analysis of lysates from S2* cells transiently transfected with metallothionein promoter expression plasmids encoding T7-tagged *PGRP-LCx* (wild-type and deletion mutants) and FLAG-tagged *rudra* with or without $CuSO_4$ treatment, as indicated. Lower diagram indicates the regions deleted in each mutant form of PGRP-LC.

(**Figure 4.3**). These data demonstrate that Rudra interacts directly with the receptors PGRP-LC and PGRP-LE.

In order to determine which domain(s) of the receptors interact with Rudra, co-immunoprecipitation assays were performed with various mutant versions of PGRP-LC or PGRP-LE. Consistent with the yeast two-hybrid data, which indicated involvement of the RHIM-like domain for interaction, a mutant form of PGRP-LE lacking the RHIM motif (Δ98-113) showed little interaction with Rudra (**Figure 4.2a,b**). Using a set of large deletions (**Figure 4.2d**), the N-terminal cytoplasmic domain of PGRP-LC was found to be essential for association with Rudra. Removal of the first 144 amino acids decreased Rudra interaction, while removal of nearly the entire cytoplasmic (Δ1-253) domain abolished interaction. The PGRP-LC extracellular domain was not involved in the interaction (**Figure 4.2e**). We then attempted to map the PGRP-LC interaction more finely with a set of mutants that span the entire cytoplasmic domain with sequential 50 amino acid deletions. However, Rudra co-immunoprecipitated with all of these deletion mutants, suggesting some redundancy in the interaction mechanism (**Figure 4.4**). The yeast two-hybrid data suggest that some of the interacting activity involves the PGRP-LC RHIM domain, while the larger deletions suggest another interaction motif likely lies in the first 144 amino acids (**Figure 4.2d,e**). Overall, we conclude that Rudra directly interacts with the signaling domains of PGRP-LC and PGRP-LE. The interaction with PGRP-LE is largely mediated by the RHIM motif while the interaction with PGRP-LC appears to involve multiple, partly redundant, mechanisms.

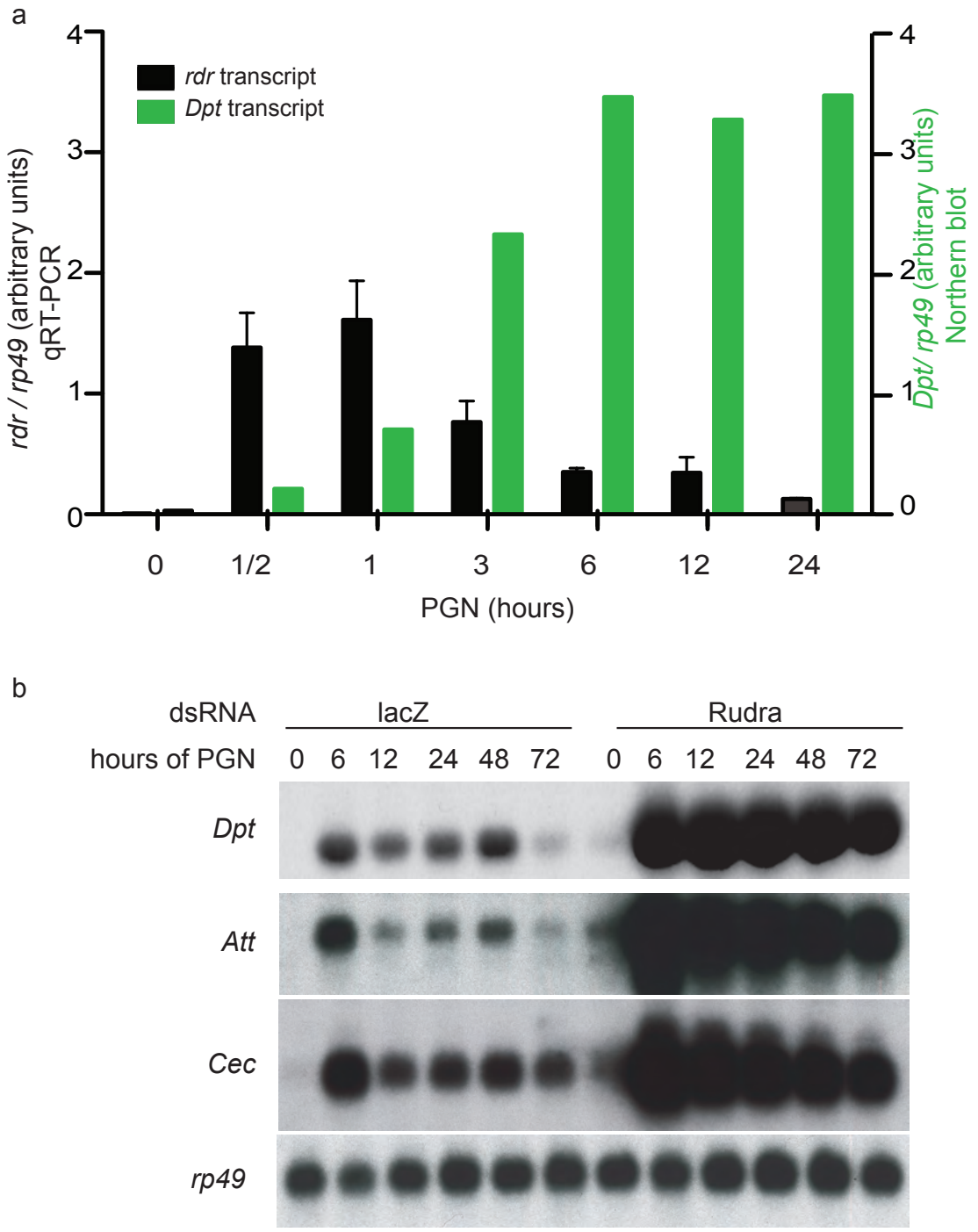


Figure 4.5: *rudra*, a negative feedback regulator of IMD signaling in cells
(a) Real-time RT-PCR analysis of *rudra* transcript from S2* cells which were stimulated with PGN for various times. *Diptericin* expression was quantified, by Northern blot, from these same cells. (**b**) Northern blot of *Dipterich, Attacin, Cecropin* and *rp49* expression in S2* cells treated with lacZ dsRNA or Rudra dsRNA, and then stimulated with PGN for various times. Data are representative of at least three independent assays. Error bars in (a) represent standard

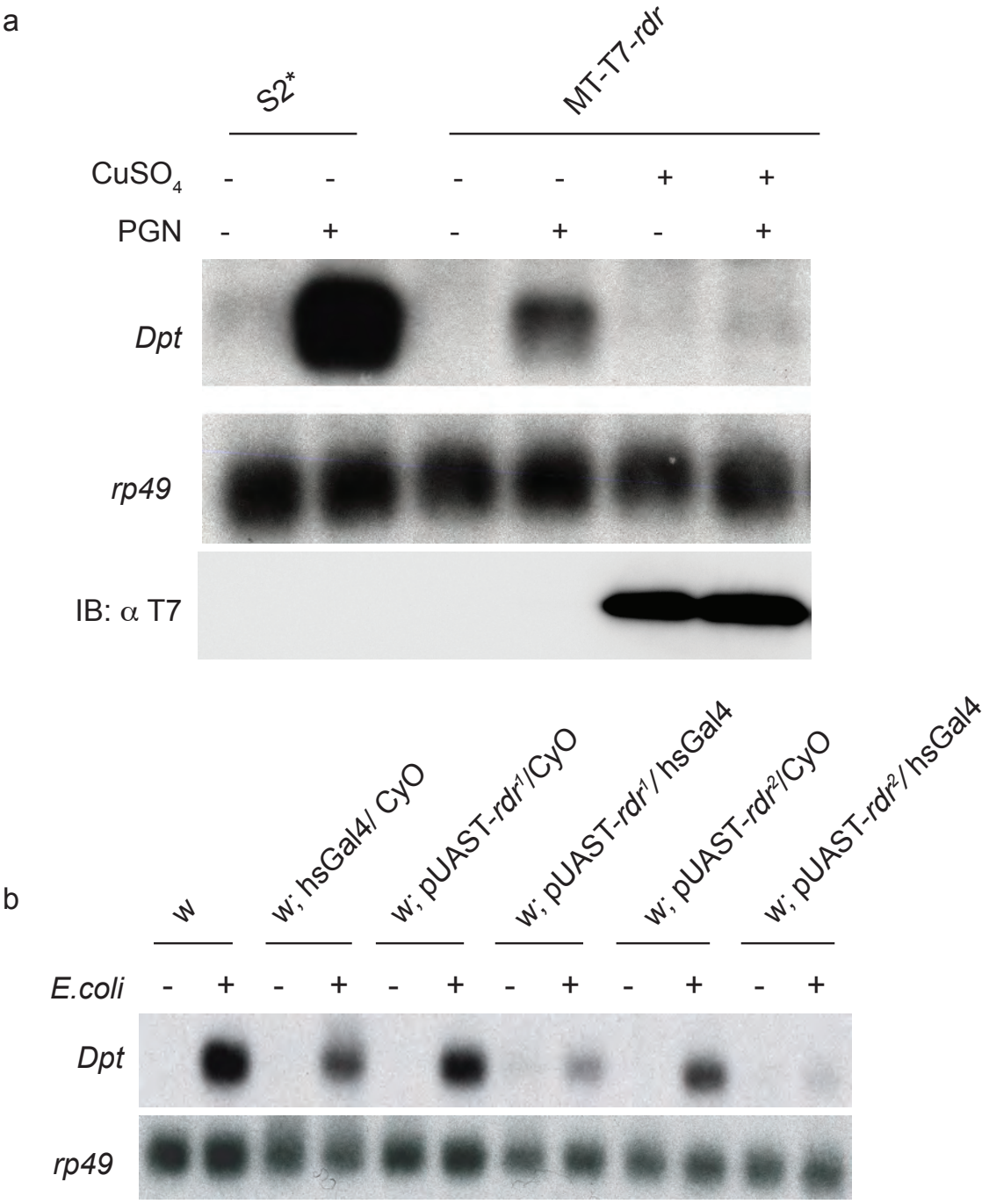


Figure 4.6: Over-expression of *rudra* blocks IMD signaling in both cells and flies.
(a) Northern blot of *Dpt* and *rp49* expression in S2* cells stably transfected with a metallothionein promoter–driven transgene expressing *rudra*. Cells were treated with $CuSO_4$ for 1.5 hours and then stimulated with PGN for 5 hours, as indicated. (**b**) Northern blot of *Diptericin* and *rp49* expression in adult flies carrying UAS promoter–driven transgenes expressing *rudra* (two independent transgenic lines). Flies were heat shocked for 1.5 hours and then RNA was isolated 8 hours after septic infection with *E.coli*. Data are representative of at least three independent assays.

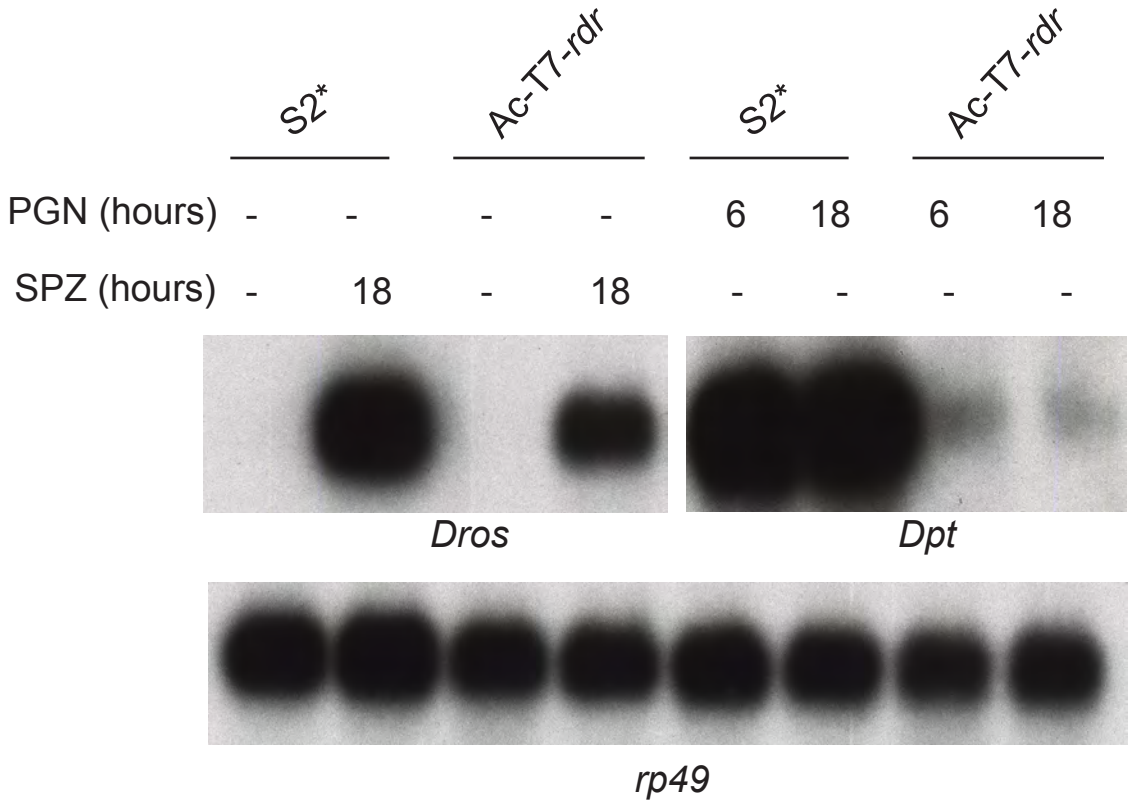


Figure 4.7: Rudra inhibits IMD signaling but not the Toll pathway
Northern blot of *Drosomycin* and *Diptericin* expression in S2* cells stimulated with SPZ-C106 or PGN, respectively, with *rp49* as a loading control. Cells expressing *rudra,* from the actin promoter, failed to respond to PGN but displayed robust SPZ-induced *Drosomycin* expression. Stimulation time as indicated.

Induction of *rudra* expression

Previous microarray studies have suggested that *rudra* is a target of the IMD signaling pathway (Park et al., 2004; Boutros et al., 2002; De Gregorio et al., 2002) [29,34,35]. In order to confirm and extend these findings, the expression of *rudra* was analyzed at various times after immune stimulation of S2* cells, by qRT-PCR. *rudra* transcript was rapidly induced, peaking in 30-60 minutes and returning to near baseline levels within 24 hours (**Figure 4.5a**). The kinetics of *rudra* expression were markedly faster and more transient than the expression of AMP genes. For example, *Diptericin* mRNA levels, as measured by Northern blotting, did not peak until 6 hours after PGN stimulation, and then remained elevated for at least 24 hours (**Figure 4.5a**). Even though the expression profiles of *rudra* and AMP genes are distinct, they both require the NF-κB factor Relish (De Gregorio et al., 2002; Kleino et al., 2005).

Rudra is a negative regulator of IMD signaling

Next, RNAi was used to characterize the function of *rudra* in the IMD pathway. S2* cells were transfected with dsRNA for *rudra*, and then stimulated with PGN for various times. As monitored by Northern blotting, antimicrobial peptide genes *Diptericin (Dpt)*, *Attacin (Att)* and *Cecropin (Cec)* were induced to markedly higher levels in cells treated with *rudra* RNAi, compared to cells transfected with a control *lacZ* dsRNA (**Figure 4.5b**). These data suggest that *rudra* is a negative regulator of IMD signaling.

To further test if *rudra* is a negative regulator of the IMD pathway, stable cell lines expressing *rudra* from a copper-inducible promoter were selected. These cell lines were treated with copper for 1.5 hours, to induce *rudra* expression, and then stimulated with PGN for 5 hours, to stimulate the IMD pathway. *rudra* over-expression potently inhibited

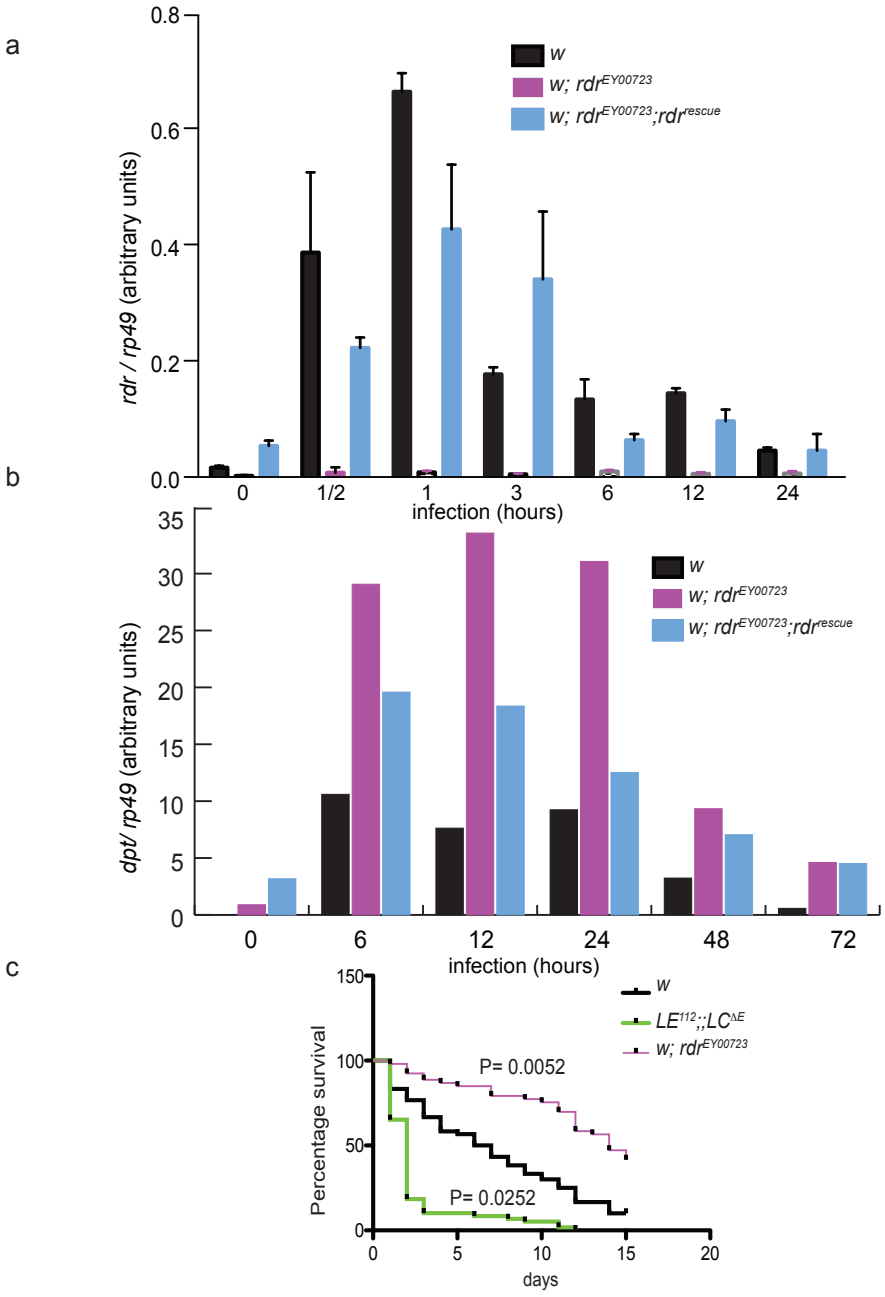


Figure 4.8: Characterization of *rudra* mutant flies

(**a**) Real-time RT-PCR analysis of *rudra* transcript from w^{1118}, $rudra^{EY00723}$, and rdr^{rescue} flies that were infected with *E.coli* for various times. (**b**) Quantified Northern blotting data of *Diptericin* and *rp49* expression in w^{1118}, $rudra^{EY00723}$ and $rudra^{rescue}$ flies following infection with *E.coli*. (**c**) Survival assays were performed following infection of w^{1118}, $rudra^{EY00723}$ and $LE^{112};LC^{\Delta E}$ flies with *E. carotovora carotovora.* Infected animals were incubated at 29°C and the number of surviving flies were counted every 24 hours. Survival data is presented in Kaplan-Meier plots and significance was analyzed by log-rank test. (a) and (b) are representative of at least 3 independent experiments, while (c) is representative of 2 independent trials, with 60 or 100 animals.

the induction of *Dpt* (**Figure 4.6a**). Also, to test if *rudra* negatively regulates the Toll pathway, stable cell lines expressing *rudra* from the actin promoter were selected. These cell lines were treated with SPZ-C106 for 18 hours to stimulate the Toll pathway. *rudra* over-expression did not robustly inhibit the induction of *Drosomycin*, as compared to its ability to inhibit PGN-induced *Diptericin* expression (**Figure 4.7**). These data demonstrate that *rudra* is potent inhibitor of the IMD pathway but has little effect on Toll signaling.

Using the UAS system and a heat shock Gal4 'driver', transgenic flies that ectopically express *rudra* were also characterized. *rudra e*xpression was induced with a 1.5 hour heat shock and then flies were challenged with *E.coli*. In two independent UAS-*rudra* lines, IMD signaling was strongly inhibited by *rudra* expression, as monitored by Northern blotting for *Dpt* induction (**Figure 4.6a**). These results are consistent with the data from cultured cells, and argue that *rudra* is a potent negative regulator of the IMD pathway *in vivo*.

In order to phenotypically characterize the loss of *rudra*, a strain carrying a P-element at position 123 in the 5' UTR of *rudra* (EY00723) was analyzed [37,38,39]. First, the level of *rudra* transcript in this strain was compared to a back-crossed *white* strain, by qRT-PCR (**Figure 4.8a**). [To generate back-crossed mutant and wild-type strains, EY00723 was crossed with the *white* strain for six generations prior to these analyses]. Similar to the cell culture data, *rudra* transcription was rapidly induced following infection in wild-type flies. Again, the induction of *rudra* expression occurs more rapidly, and is resolved more quickly, than does AMP gene expression (compare **Figure 4.8a to 4.8b**). The transposon insertion in the 5' UTR markedly inhibited *rudra*

expression, with nearly undetectable levels at all time points, demonstrating that this allele of *rudra* is a strong hypomorph. Also, a transgenic rescue strain was constructed, using a 4.5 Kbp genomic fragment (*rudra*^{rescue}). This genomic rescue construct partially restored immune-inducible expression of *rudra*, but it did not completely return to wild-type levels (**Figure 4.8a**).

Next, the immune response of wild-type, *rudra*^{EY00723} , and the *rudra*^{rescue} strains were compared. *Diptericin* expression, as monitored by Northern blotting at various times following septic *E. coli* infection, was elevated at all time points in *rudra*^{EY00723} compared to the isogenic wild-type strain (**Figure 4.8b**). The *rudra*^{rescue} transgenic line restored *Diptericin* to levels between that observed in the wild-type and *rudra* mutant flies, consistent with partially restored levels of *rudra* expression observed in this line. *rudra* heterozygotes also displayed elevated AMP gene expression (data not shown). These results, together with the data from ectopic expression, demonstrate that *rudra* is a potent negative regulator of the IMD pathway in flies, as well as in cultured cell lines.

We then asked what consequence these elevated AMP levels might have during an infection. To this end, wild-type and *rudra*^{EY00723} flies were infected with the Gram-negative pathogen *Erwinia carotovora carotovora (Ecc).* As reported previously, *Ecc* is a mildly pathogenic infection in wild-type animals, such that most flies succumb over the course ~10 days (**Figure 4.8c**) (Kim et al., 2006; Basset et al., 2000). As expected, PGRP-*LE; PGRP-LC double* mutant flies, which lack both receptors involved in detecting DAP-type PGN, were rapidly killed by this infection (P=0.0252, compared to wild-type animals). On the other hand, *rudra* mutants showed significantly improved survival compared to wild-type flies (P= 0.0052). These results show that loss of *rudra,*

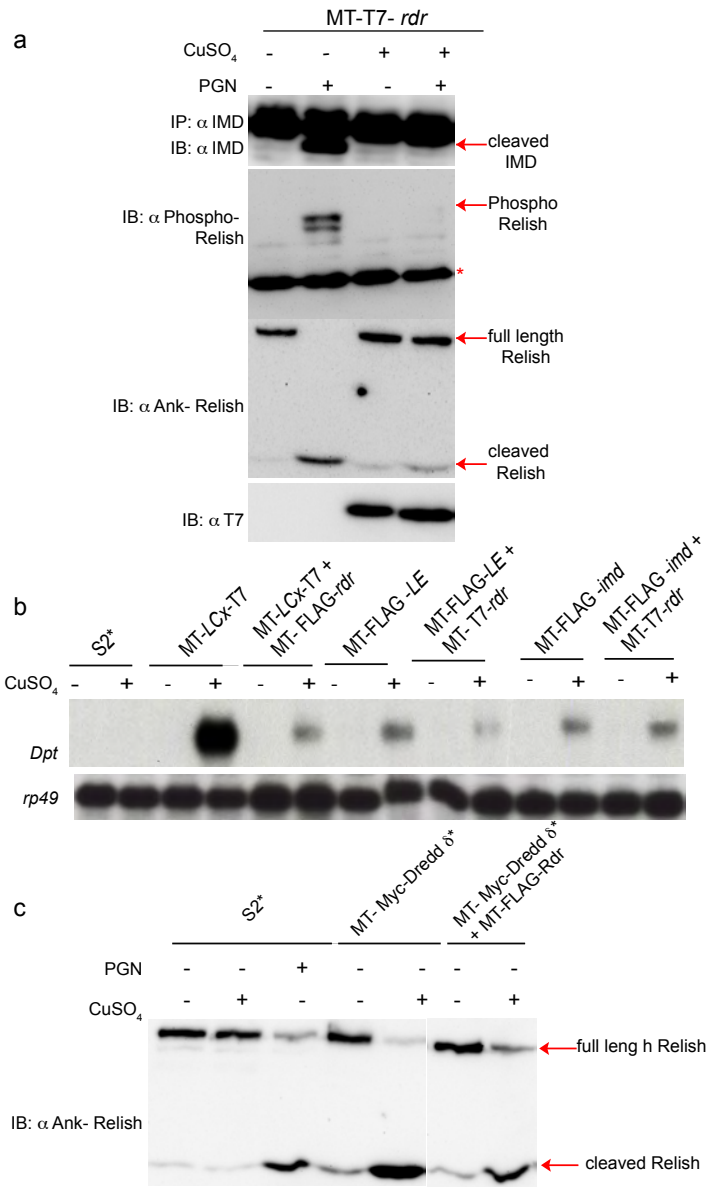


Figure 4.9: Rudra functions upstream of IMD, Dredd and Relish.
(a) Analysis of lysates from S2* cells stably transfected with a metallothionein promoter plasmid expressing T7-tagged *rudra,* with or without treatment with $CuSO_4$ and PGN, as indicated. IMD cleavage was analyzed by IP-IB (upper panel), while Relish phosphorylation and cleavage were analyzed by immunoblotting (in the middle two panels). The asterisk marks heavy chain detected by the secondary antibody. The lowest panel confirms Rudra expression with anti-T7 IB. **(b)** Northern blot of *Diptericin* and *rp49* expression levels in S2* cells stably transfected with metallothionein promoter–driven transgenes expressing *PGRP-LCx, PGRP-LE,* or *imd,* with or without concurrent expression of *rudra.* Cells were treated with $CuSO_4$ (+) or left untreated (-), and RNA was extracted after 6 hours. **(c)** Immunoblot analysis of Relish cleavage from S2* cells stably transfected with metallothionein promoter expression plasmid for *Dredd,* with or without concurrent expression of FLAG-tagged *rudra*. CuSO4 was added, for 5 hours, to induce transgene expression, as indicated. Data are representative of at least three independent assays.

and the ensuing increase in AMP levels, enhances resistance to this Gram-negative pathogen.

Rudra inhibits signaling at the receptor

We next sought to determine the molecular mechanism(s) used by Rudra to control signal transduction. Relish, the NF-κB precursor protein essential for IMD triggered gene expression, is regulated by immune-induced cleavage and phosphorylation (Stöven et al., 2000; Stöven et al., 2003; Erturk-Hasdemir et al., 2009). Rudra expression prevented both the cleavage and phosphorylation of Relish (**Figure 4.9a**). Recently, we also discovered that *imd* protein is rapidly cleaved following immune stimulation (unpublished data, N.P. and N.S) and expression of *rudra* potently inhibits this cleavage (**Figure 4.9a**). These results suggest that Rudra functions upstream of Relish activation and IMD cleavage.

AMP gene expression can be triggered by ectopically expressing certain components of the IMD pathway. In particular, over-expression of either of the receptors, *PGRP-LC* or *PGRP-LE*, or *imd* is sufficient to drive AMP gene expression. Likewise, over-expression of the caspase *Dredd* is sufficient to drive Relish cleavage. To further analyze the position that Rudra acts in the IMD pathway, it was over-expressed with these signaling components in doubly selected stable cell lines. Rudra potently inhibited signaling induced by over-expression of the receptors PGRP-LC or PGRP-LE, but had no effect on the induction of *Diptericin* expression caused by IMD over-expression (**Figure 4.9b**). Likewise, Rudra did not inhibit Relish cleavage caused by over-expressing the caspase Dredd (**Figure 4.9c**). These results suggest that Rudra functions upstream of Dredd and IMD, but downstream of the receptors, and is

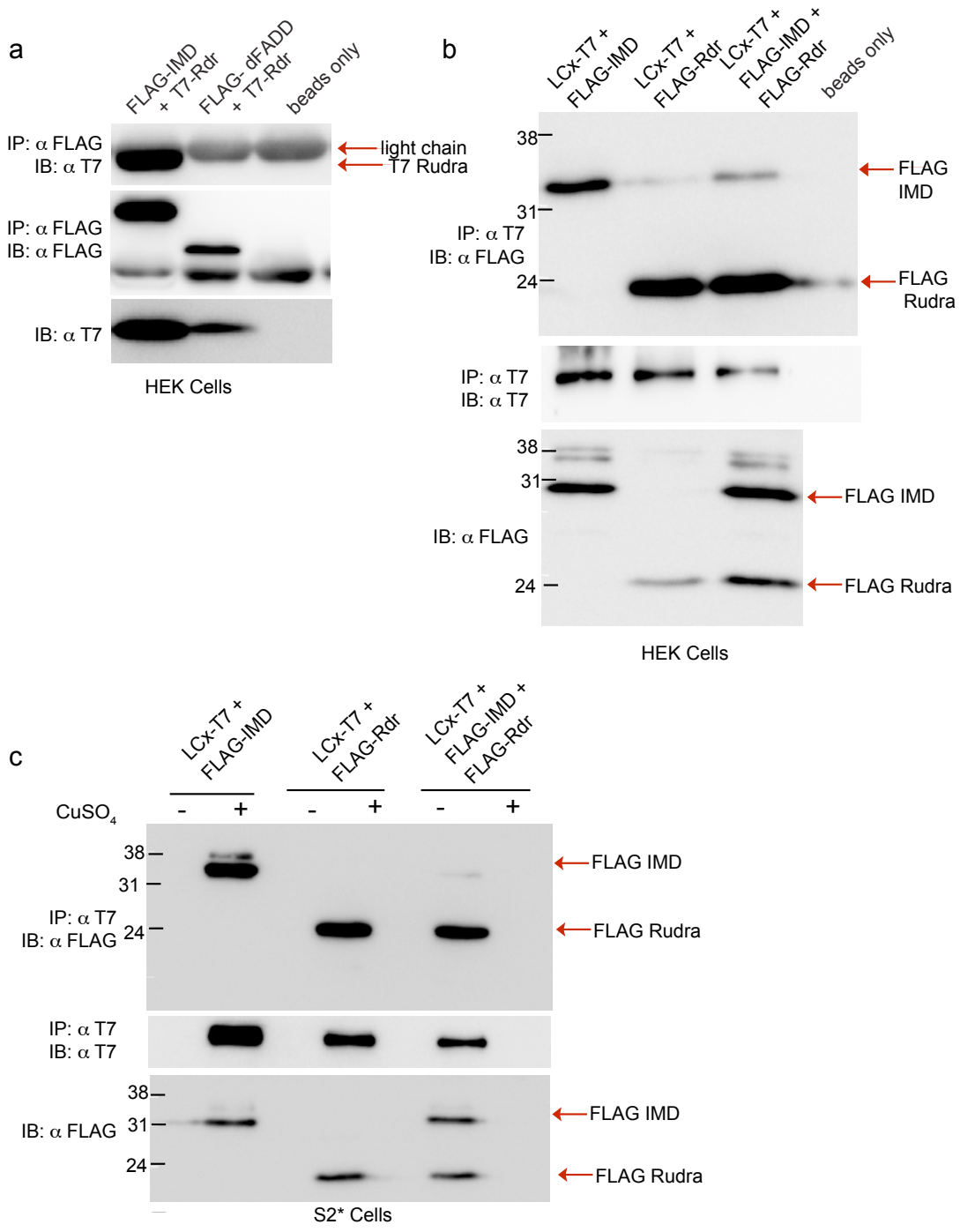


Figure 4.10: Rudra disrupts the interaction between PGRP-LCx and IMD.
(**a**) IP-IB analysis of lysates of HEK cells transiently transfected with expression plasmids for FLAG-tagged *imd* or FLAG-tagged *dFADD* and T7-tagged *rudra.* Rudra interacted with IMD but not dFADD. (**b, c**) Similar co-immunoprecipitation experiments from lysates of HEK cells (b) or S2* cells (c) simultaneously co-transfected with T7 tagged *PGRP-LCx*, FLAG tagged *IMD* and/ or FLAG-tagged *rudra*. Rudra interfered with the association between PGRP-LC and IMD. Data are representative of at least three independent assays. Data are representative of 3 independent experiments.

consistent with binding data demonstrating an association between Rudra and either PGRP-LC or PGRP-LE.

In addition to interacting with the receptors, Rudra avidly bound to IMD. The IMD association was detected by transient transfection/co-immunoprecipitation assays, in either S2* cells (data now shown) or HEK cells (**Figure 4.10a**). On the other hand, Rudra did not associate with dFADD, another factor known to interact with IMD. In all, these data argue that Rudra directly interacts with both IMD and the receptors PGRP-LC and PGRP-LE. These results suggest two possible models for the inhibition of IMD signaling by Rudra: (1) Rudra may associate with both the receptor and its signaling adaptor (IMD), holding them together in an inactive confirmation; or (2) Rudra may interact with both PGRP-LC and IMD separately, disrupting the association between the receptor and its adaptor. To probe these possibilities, co-immunoprecipitation experiments were performed with lysates from cells co-transfected with *PGRP-LC* (T7 tag), *imd* (FLAG tagged) and/or *rudra* (also FLAG tagged). In assays with just the receptor and either IMD or Rudra, PGRP-LC interacted with either the adaptor or the inhibitor, in both *Drosophila* and human cells (**Figure 4.10b,c**). However, when all three proteins were simultaneously co-expressed, PGRP-LC and Rudra still robustly co-precipitated, but the association between IMD and the receptor was markedly reduced. These data suggest that Rudra interferes with the interaction between PGRP-LC and IMD, and this disruption provides a molecular mechanism explaining how Rudra down-modulates IMD signaling at the level of the receptor, consistent with the functional and binding data presented.

Discussion

Recent work has shown that the intensity and duration of the immune response is tightly regulated in *Drosophila* (Tsuda et al., 2006; Kim et al., 2006; Foley and O'Farrell, 2004; Zaidman-Remy et al., 2006; Bischoff et al., 2004). Over-exuberant immune responses can be dangerous and the proper down modulation of immunity is important for health and fecundity (Flatt et al., 2005; Zerofsky et al., 2005). To keep the immune response properly modulated, the Toll and IMD pathways are controlled at multiple levels by multiple negative regulators. In this study, we have characterized a new negative feedback regulator of the IMD pathway. *rudra* transcript is rapidly induced following septic infection, and *rudra* mutant flies or *rudra* knockdown cells over-express antimicrobial peptides. In the case of *Erwinia carotovora carotovora* infection, this elevated level of AMP production leads to increased survival. A similar phenotype was reported for mutants lacking Caspar, which is thought to inhibit downstream signaling events (Kim et al., 2006). The results presented here, in cells and flies, demonstrate that *rudra* is a key component in a negative feedback loop that keeps the IMD pathway in check.

In addition to these loss-of-function results, over-expression of *rudra* potently blocked signaling through the IMD pathway, both in cells and in flies. Moreover, we exploited this activity to analyze which steps in the IMD pathway are inhibited by Rudra. Using various molecular assays to monitor different PGN-induced events in the IMD pathway, we found that Rudra interfered with cleavage of IMD. Signaling mediated by receptor over-expression was also inhibited by Rudra, but this was not the case for signaling induced by over-expression of downstream components. Together, these data

strongly support the notion that Rudra interferes with receptor function and is consistent with the association between Rudra and the receptors PGRP-LC or PGRP-LE.

Using assays in yeast, *Drosophila*, human cells and *in vitro,* Rudra was shown to interact directly with PGRP-LC and PGRP-LE. The interaction between PGRP-LE and Rudra required the RHIM-like domain of PGRP-LE, which is also critical for signaling by this receptor. However, the region through which PGRP-LC interacts with Rudra is less clear and likely involves multiple, partly redundant interfaces. Rudra also interacted with the *imd* protein. Moreover, Rudra interfered with the interaction between the receptor PGRP-LC and IMD, destabilizing the receptor signaling complex. From these results, we propose that Rudra is a negative feedback regulator that down modulates the IMD pathway by binding the receptors and interrupting the associations with their cognate signaling adaptor IMD. This regulatory loop is critical to properly regulate the immune response.

In agreement with the data presented here, Kleino et al. (2008) recently reported that *rudra*/CG15678 is a negative regulator of the IMD pathway, although they refer to this gene as *poor Imd response upon knock-in* (*pirk*). They showed that *rudra/ pirk* is rapidly induced following infection, similar to the data presented here, and further demonstrated that *rudra* induction is dependent on Relish, both in cells and in flies. Using reporter assays in S2 cells, they found that Pirk inhibits IMD signaling but not the Toll pathway. With transgenic RNAi fly lines, they also found that knockdown of *pirk* caused the hyper-expression of the antimicrobial peptide genes. Also, flies over-expressing Pirk blocked the activation of the IMD pathway and were more susceptible infection. These results are consistent with the data presented here, although we have

characterized a mutant allele of *rudra* and additionally show that this mutant exhibits enhanced protection against *Erwinia* infection. The data presented here also expand on the findings of Kleino et al. (2008) by showing that Rudra not only interacts with both PGRP-LC and IMD, but also that these interactions with Rudra disrupt the direct association between PGRP-LC and IMD. Kleino et al. (2008) reported that central portion of Rudra consists of two repetitive amino acid elements of unknown function and structure, which they named the Pirk domain. The Pirk domain is required for the interaction with IMD, but not with PGRP-LC. Rudra does not contain obvious homology to any other protein motifs, and no mammalian homologs are readily detected (Kleino et al., 2008).

Another recent study also showed that r*udra*/CG15678 is a negative regulator of the IMD pathway, although they refer to this gene as *PGRP-LC interacting inhibitor of IMD pathway (pims)*. Lhocine et al show that PIMS not only down-modulates the immune response following systemic infection but it is also required to suppress the IMD pathway in response to commensal bacteria. *pims* knockdown flies produce AMPs in the gut and to some extent in the fat body even in the absence of an infection. However, no AMPs are produced when the *pims* knockdown flies are grown under germ free conditions, suggesting that the commensal bacteria trigger constitutive activation of the IMD pathway when PIMS is not present. This study also shows that PIMS interacts with the receptor PGRP–LC and leads to the relocalization of the receptor from the plasma membrane to perinuclear structures (Lhocine et al., 2008). However, it is unclear if and how PIMS mediates this relocalization. More experiments need to be done to validate this data.

Recently, multiple mechanisms involved in regulating the *Drosophila* immune response have come to light. Given that it is well-established that immune activation in flies has a cost, such as reduced fecundity (Zerofsky et al., 2005; Flatt et al., 2005) and hypersensitivity to infection (Bischoff et al., 2006; Ryu et al., 2008; Kim et al., 2006; Gordon et al., 2005; Zaidman-Remy et al., 2006), it is not surprising that multiple negative regulatory circuits control the immune response. Similarly, in mammals, innate and adaptive immune responses are held in check by multiple mechanisms, in order to prevent inflammatory and autoimmune diseases while at the same time allowing an effective response to infection. Future studies will address the possible negative consequences of the lack of proper IMD regulation observed in the *rudra* mutant animals.

Materials and Methods

Reagents

Insoluble PGN from *E. coli* was purchased from Invivogen.

Fly stocks and survival experiment

rudra mutant line, EY00723, was originally isolated by the *Drosophila* Genome Project gene disruption consortium and provided by the Bloomington Drosophila Stock Center. The flies were crossed for six generations to a w^{1118} strain in order to generate back-crossed stocks. In all experiments, $rudra^{EY00723}$ mutants were compared to back-crossed w^{1118} animals. $PGRP\text{-}LE^{112}$;;$PGRP\text{-}LC^{\Delta E}$, double mutant flies were reported previously (Takehana et al., 2004). Survival experiments were performed with 60 flies at 29^{o}C, following infection by pricking in the abdomen with a microsurgery needle dipped into a concentrated pellet *of Erwinia carotovora carotovora 15* (Zaidman-Remy

et al., 2006). Surviving flies were transferred to fresh vials and counted daily, until all wild-type flies died. Kaplan-Meier plots are presented and P-values were calculated by log-rank test using GraphPad Sigma Plot.

RNA analysis and RT-PCR

Total RNA from flies or cultured cells was isolated with the TRIzol reagent (Invitrogen) as described previously (Silverman et al., 2000). Expression of *Diptericin*, *Attacin*, *Cecropin* and the control *rp49* (ribosomal protein) was analyzed by Northern blotting (Silverman et al., 2000). Northern blots were quantified with a phosphoimager (Fuji) and AMP gene expression was normalized to *rp49* levels. For qRT-PCR, RNA was DNase treated and re-extracted with phenol-chloroform. cDNA was synthesized using Superscript II (Invitrogen) and quantitative PCR analysis was performed on a DNA engine Opticon 2 cycler (MJ Research, Watertown MA) using SYBR Green (Biorad). The specificity of amplification was assessed for each sample by melting curve analysis and relative quantification was performed using a standard curve with dilutions of a standard. The quantified data was normalized to *rp49* levels. In all S2*-based cell experiments, cells were pre-treated with 1 mM 20-hydroxyecdysone for 24 to 40 hr before treatment with 500 mM CuSO4 and/ or PGN (100 ng/ ml).

RNAi experiments

dsRNA was generated and purified as reported previously (Di Nocera and Dawid, 1983). Cells were split 24 hours after transfection to 1.0 $\times 10^6$/mL and then were treated with 1 mM 20-hydroxyecdysone. After 24 hours, cells were treated (or left untreated) with PGN (100 ng/ml) for various time, as indicated.

Co-immunoprecipitation and immunoblotting assays

In vitro translation was performed following the protocol of the manufacturer (Promega). Immunoprecipitations were carried out with rabbit anti-T7 (Bethyl labs) in lysis buffer (20 mM Tris at pH 7.6, 150 mM NaCl, 2 mM EDTA, 10% Glycerol, 1% Triton X-100, 1 mM DTT, NaVO4, glycerol 2-phosphate and protease inhibitors). For immunoprecipitation from cells, Schneider S2* cells were first transfected by calcium phosphate method with appropriate expression plasmids. Cells were split 24 hours after transfection to 1.0 $\times 10^6$/mL and 24 hours later, were treated with 500 μM copper sulphate for 5 hr, when necessary, for expression from the metallothionein promoter. Immunoprecipitations were performed in lysis buffer and analyzed by SDS-PAGE followed by immunoblot analysis with anti-T7 MAb (Novagen), anti-V5 (Sigma), anti-IMD (gift of J.-M. Reichhardt) or anti-Flag (Sigma) antibodies. Stable cell lines and immunoblotting were performed as described previously (Silverman et al., 2000). The generation and characterization of phospho-specific Relish antibody will be detailed elsewhere (Erturk-Hasdemir et al., 2009).

Transgenesis and analysis of UAS-rudra and genomic rescue strains

For the UAS transgenic, the *rudra* ORF was amplified by PCR and subcloned into the *Eco*RI and *Bgl*II sites of pUAST. For genomic rescue, a BAC clone (Drosophila Resource Center (Hoskins et al., 2002)) was used as a template to amplify a 4.5 Kbp genomic fragment containing the complete *rudra* locus plus flanking sequences, which was then cloned into the *Eco*RI and *Bam*HI sites of pCaSpeR (Thummel et al., 1988). After sequence verification, standard techniques were used for P-element–mediated transformation at the MGH Drosophila transgenics facility. For immune stimulation

assays, adults (males and females in equal numbers), were infected by pricking in the abdomen with a microsurgery needle dipped into a concentrated pellet of *E. coli (1106)*, RNA was extracted 8 h later, and assayed by Northern blotting.

Stable cell lines

The *rudra* gene was cloned into pRmHa3 vector by standard methods to create constructs expressed from the metallothionein promoter. The constructs were then transfected into S2* cells in conjunction with pHs-Neo at a ratio of 50:1; stable transfectants were then selected with G418 (1 mg/ml). For double stable cell lines, the *rudra* expression plasmid was transfected into S2* cell lines that were previously selected to carry plasmids expressing either *PGRP-LC*, *PGRP-LE*, *IMD* or *Dredd.* The *rudra* plasmid was selected with a second selectable marker, either G418 (1mg/ ml) or hygromycin (20 U/ml), as appropriate.

CHAPTER V

RYBP: a new component of the IMD pathway.

Abstract

DAP–type peptidoglycan triggers the IMD pathway via PGRP–LC or PGRP–LE, while lysine–type peptidoglycan is an agonist for the Toll pathway through PGRP–SA and PGRP–SD. The receptors PGRP–LC and PGRP–LE share a short region of homology called the RHIM–like motif in their N terminus, and this motif is critical for signaling. However,the molecular mechanisms by which the RHIM–like motif functions to activate IMD signaling remain a mystery. In this chapter, I show that mutations affecting the PGRP–LC RHIM–like motif are defective in all known downstream signaling events, including IMD cleavage and ubiquitination,and, Relish cleavage and phosphorylation. However, the RHIM–like mutant receptors are capable of serving as a platform for the assembly of all known components of a receptor proximal signaling complex. These results suggest that another, unidentified component of the IMD signaling pathway may function to mediate interaction with the RHIM–like motif and I have identified a candidate interactor through a yeast two–hybrid screen (See Chapter 3). This factor is known as RYBP, it includes a highly conserved ubiquitin binding motif (NZF), and RNAi studies suggest that it is a critical component of the IMD pathway.

Introduction

Bacterial infections can trigger either the Toll or the IMD pathway, depending on the structure of the peptidoglycan (PGN) found in their cell wall. The Toll signaling pathway is most robustly triggered by lysine–type peptidoglycan, while the IMD pathway is activated by the DAP–type peptidoglycan (Kaneko et al., 2004; Leulier et al., 2003; Mengin-Lecreulx and Lemaitre, 2005; Lemaitre, 2004). In the IMD pathway, DAP–type PGN is detected by PGRP–LC and PGRP–LE (Kaneko et al., 2006; Gottar et al., 2002; Choe et al., 2002; Leulier et al., 2003; Ramet et al., 2002; Takehana et al., 2002; Takehana et al., 2004). Double mutants, lacking both PGRP–LC and LE, are unable to induce AMPs in response to most Gram–negative bacteria and are highly susceptible to these infections (Takehana et al., 2004). PGRP–LC is a type 2 transmembrane receptor, with an extracellular PGRP domain that is critical for recognizing extracellular bacteria, while PGRP–LE lacks a transmembrane domain and functions as an intracellular receptor for DAP–type PGN (Kaneko et al., 2006). Binding of polymeric/ monomeric DAP type PGN by PGRP–LC and/or PGRP–LE triggers IMD signaling pathway which leads to the activation of the NF–κB precursor protein Relish.

PGRP–LC and PGRP–LE detect DAP–PGN through their C–terminal PGN–binding PGRP–domain and transduce signals through their extended N–terminal domains (Choe et al., 2005; Kaneko et al., 2006). PGRP–LC and –LE share approximately 50% homology in the their C–terminal PGRP domain but in their N–termini only a short stretch of about 20 amino acids displays any homology between these two receptors. In both receptors, deletion or mutation within this conserved domain abrogates signaling (Kaneko et al., 2006). This conserved signaling motif has

weak homology to the RHIM domain found in the mammalian RIP1 and TRIF proteins. In these mammalian NF–κB signaling proteins, the RHIM domain mediates a homotypic interaction between them. Given the weak similarity to the mammalian RHIM domain, we refer to this conserved signaling motif in PGRP–LC and PGRP–LE as the RHIM–like domain. The molecular mechanism by which the RHIM–like domains in PGRP–LC and–LE function is unclear. One factor that interacts with both PGRP–LC and –LE is IMD, a death domain containing protein with significant similarity to the death domain of RIP1 (Aggarwal et al., 2008; Georgel et al., 2001; Choe et al., 2005; Kaneko et al., 2006). In the case of PGRP–LE, IMD binding requires the RHIM–like domain and this is a possible explanation for the RHIM–like domains function (data not shown). However the association between IMD and PGRP–LC maps to another region, not the RHIM–like motif. Curiously, the IMD interacting region of PGRP–LC does not appear to be critical for signaling; it is only the RHIM–like domain that is essential. Together, these data suggest that the RHIM–like domain has a function beyond that of IMD recruitment and that direct IMD recruitment to these receptors may not always be necessary for signaling.

In this study we show that PGRP–LCx RHIM mutant are unable to activate any of the known events downstream of the receptor. We also show that a receptor proximal complex can be assembled on PGRP–LC but is not markedly influenced by the RHIM–like motif. In addition, I have also characterized a new component of the IMD pathway, known as RYBP, which may function through the RHIM–like motif.

RYBP, is an ancronym for Ring and YY1 binding protein (Garcia et al., 1999) and is also referred to as DED–associated Factor (DEDAF) (Zheng et al., 2001) or YEAF1

(Sawa et al., 2002). RYBP belongs to the RYBP/ YAF–2 family of small, basic, NZF–domain containing ubiquitin binding protein and is highly conserved from invertebrates to mammals (Arrigoni et al., 2006). RYBP was first identified in a yeast two hybrid screen as an interacting partner of Polycomb group (PcG) of proteins. RYBP and YAF–2 share about 78% homology in their NZF domain and are less similar in there C terminal domain. RYBP interacts with the PcG proteins Ring 1A, Ring 1B and M33, these are all components of the PRC1 complex. The PRC1 multi–protein complex functions as transcriptional repressors (Simon and Kingston, 2009). RYBP and YAF2 both interact with the transcription factors YY1, E2F6 and hGABP/ E4TF1 and are proposed to function as an adaptor protein/ cofactor that recruits PcG. Though RYBP and YAF2 are structurally similar they are functionally distinct. RYBP mediates transcriptional repression and in contrast YAF2 activates transcription. (Sawa et al., 2002; Schlisio et al., 2002). Available data also suggests that RYBP binds ubiquitnated H2A and this ubiquitnated H2A is important for PcG mediated gene silencing (Arrigoni et al., 2006).

In addition to its role in transcriptional repression RYBP is also implicated in promoting apoptosis. RYBP interacts with various death effector domain (DED) containing proteins like DED–containing DNA–binding protein (DEDD), FADD and pro–caspase 8 and 10 (Zheng et al., 2001). DEDD is a nucleolar protein and it promotes apoptosis both in the nucleolus and in the cytoplasm. RYBP promotes the formation of the death inducing signaling complex composed of Fas, FADD and procaspase 8 in 293T cells and it also enhances Fas and caspase 10 DED–mediated apoptosis in lymphoma cell lines. In contrast to RYBP data in zebrafish suggests that YAF2 inhibits

apoptosis and functions as a survival factor during early zebrafish development and embryogenesis (Stanton et al., 2006). RYBP has also been shown to interact with the viral proteins Apoptin and Hippi and both these proteins are also pro–apoptotic (Stanton et al., 2007; Danen-van Oorschot et al., 2004).

RYBP deficient mice die around embryonic day (E) 5.5– 6.0 and lack the normal apoptotic responses that accompany implantation. And RYBP heterozygous null mice exhibit an ex encephalic phenotype because of disrupted neural tube closure (Pirity et al., 2007). The neural phenotypes observed with RYBP heterozygous mice are similar to those observed with *caspase 3*, *9* or *apaf1* heterozygous null mice (Lossi and Merighi, 2003).

The *Drosophila* RYBP is a 150 amino acid protein and is expressed maternally and ubiquitously throughout development (Bejarano et al., 2005). dRYBP protein behaves like a *Polycomb* dependent transcriptional repressor throughout development. Over–expressed dRYBP produces homeotic like phenotypes that can be repressed by mutations in PcG/ trxG genes. However, dRYBP loss–of–function mutant phenotypes are variable both in their expression and penetrance. dRYBP mutation results in many defects including, progressive lethality during development, defects in morphogenesis, reduced size of wing and cell differentiation defects (Gonzalez et al., 2008). These findings have been interpreted to suggest that dRYBP functions together with the PcG proteins. Whether this model is correct or not is not yet clear, and certainly the molecular function of RYBP is very opaque. One obvious possibility, due to its highly conserved NZF domain, is that RYBP functions through ubiquitin binding.

Like the mammalian RYBP *Drosophila* protein is also suggested to have pro–apoptotic phenotype. Over–expression of dRYBP induces apoptosis in the imaginal discs and this death depends on the pro– apoptotic genes *reaper, hid and grim*. Also, dRYBP induced apoptosis is dependent on FADD and *DREDD* which are not components of the canonical apoptosis pathway. However, no apoptosis related phenotype is observed with *dRYBP* mutant flies (Gonzalez and Busturia, 2009). The role of *DREDD* and *FADD* in the RYBP–induced death suggested to us that the IMD pathway may be involved. In fact, hyper–activation of the IMD pathway is known to induce cell death, probably through the JNK pathway (Georgel et al., 2001). In our present study, we identified a new component of the IMD pathway called dRYBP through a yeast two hybrid screen. We show that dRYBP is critical for IMD signaling in S2* cells; knockdown of dRYBP abrogates IMD signaling. Also, dRYBP interacts with the receptor PGRP–LC through the IMD interaction domain and the RHIM– like domain.

Results

PGRP–LCx RHIM mutants fail to activate any downstream event

Various PGR–LCx over–expressing cell lines were used to determine the molecular mechanism(s) that are not initiated by the PGRP–LC RHIM like mutants. Over–expression of wild– type PGRP–LCx is sufficient to activate IMD pathway, as assayed by AMP induction, while over–expressed PGRP–LCx RHIM mutants are inactive (Kaneko et al., 2006)(Chapter 2). The PGRP–LC RHIM–like mutants must fail to initiate some events in the IMD pathway that results in failure of induction of AMPs like *Diptericin*. Therefore, series of biochemical assays were done to determine the events of IMD pathway that fail to initiate when the PGRP–LC RHIM–like mutants are

over–expressed. Recently, work by our lab shows that *imd* protein is rapidly cleaved following immune stimulation (Paquette et al., 2010), and the data in **Figure 5.1a** shows similar result that over–expression of wild type PGRP–LC leads to the cleavage of IMD. However, the , PGRP–LC RHIM–like mutants fail to cleave IMD (**Figure 5.1a**). Relish, the NF–κB precursor protein essential for IMD triggered gene expression, is regulated by immune–induced cleavage and phosphorylation (Stöven et al., 2000; Stöven et al., 2003; Erturk-Hasdemir et al., 2009) and over–expressed wild type PGRP–LC similarly drives Relish cleavage and phosphorylation. On the other hand, PGRP–LC RHIM–like mutants fail to cleave and phosphorylate Relish (**Figure 5.1a**). Together, these results indicate that PGRP–LC RHIM–like mutants possibly fail to activate all molecular events downstream of the receptor, suggesting they are deficient in one or more essential signaling activities that must occur immediately downstream of the receptor.

In transient transfection/co–immunoprecipitation assays, IMD interacts with both PGRP–LC and –LE. In the case of PGRP–LE, IMD binding requires the RHIM–like domain (data not shown), while the association between over–expressed IMD and PGRP–LC is not mediated by the RHIM–like domain but maps to another distinct region (Kaneko et al., 2006) (Chapter 2). I next sought to examine the association of PGRP–LC, wild type and RHIM–like mutant, with endogenously expressed IMD. Note, I have access to an excellent IMD antisera that easily detects endogenous IMD, but no useful PGRP–LC antibody is available. Therefore, I used stably transfected cell lines that express various PGRP–LC genes, wild type and mutant, from the copper–inducible metallothionein promoter. The analysis of the interaction of endogenous IMD and PGRP–LC revealed a different finding, as compared to the above mentioned studies

with transient transfection, over–expression of both PGRP–LC and IMD. In particular, we find that IMD interacts robustly with wild type PGRP–LC but weakly with the RHIM–like mutants. Also the PGRP–LC lacking the IMD interaction domain interacts weakly with IMD **(Figure 5.1a)**. This result suggest that the transient transfection experiments may be misleading, and that the RHIM–like motif may have an important role recruiting/ binding IMD. In addition the IMD interaction domain may also have a role in IMD interaction.

Signaling pathways often rely on the transient assembly of large multiprotein signaling complexes on the cytosolic domains of activated receptors–so called receptor proximal signaling complexes (Micheau and Tschopp, 2003). The assembly of these signaling hubs relies on post translational modification and one such modification is ubiquitination. Ubiquitination plays an important role in mammalian TNF–R1 and TLR signaling (Bianchi and Meier, 2009; Haas et al., 2009). Recent work by our lab shows that IMD is K63–ubiquitinated in a signal–dependent manner and this is important for signaling by the IMD pathway. IMD is also ubiquitnated when the IMD pathway is activated by over–expressing the receptor PGRP–LCx but the PGRP–LC RHIM–like mutants fail to ubiquitinate IMD (**Figure 5.1b**). Surprisingly, the ∆3 mutant also failed to drive robust IMD ubiquitination. This is perhaps expected, because ∆3 interacts poorly (or not at all) with IMD, however it is capable of inducing AMP gene expression.

To date, receptor associated polyubiquitin has not been examined.. For this, I performed an immunoprecipitation of PGRP–LC from stable cell lines over expressing the receptor both the wild type and RHIM–like mutant and probed for ubiquitination. PGRP–LC co–precepitated as a highly ubiquitinated protein and RHIM–like mutants

(Δ5, Δ6, F218A) failed to coprecipitate with polyubiquitin. On the other hand, Δ3 mutant, which lacks the IMD–interaction domain precipitated a robust polyubiquitin signal, even more robust then wild type. Another deletion mutant, Δ1, with strong signaling activity and no known deficiencies, was also associated with a robust ubiquitin signal. This data suggests that either the receptor itself gets ubiquitinated or it associates with a protein that is ubiquitinated. However, this ubiquitinated protein cannot be IMD because no IMD ubiquitination is observed in stable cells over–expressing PGRP–LC Δ3 Thus, the receptor associated ubiquitin most closely correlates with the signaling activity, or lack thereof, observed in various PGRP–LC forms and may be key to understanding the molecular mechanisms underlying receptor–mediated signal transduction.

A receptor proximal complex is assembled when components of the IMD pathway are over–expressed

In the TNFR–1 signaling pathway it is well established that a receptor proximal complex(es) is assembled following ligand binding, and this complex is critical for signaling (Micheau and Tschopp, 2003). To test if a receptor proximal complex is also assembled in the case of the IMD pathway co–immunoprecipitation experiments were performed. In this experiment, wild–type receptor PGRP–LCx was co– transiently transfected with various components of a putative receptor proximal complex – IMD, FADD and DREDD. PGRP–LCx interacted with IMD and FADD and a very weak interaction was observed with DREDD. The interaction of PGRP–LCx with DREDD was weak even when IMD or FADD were included in the transient co–transfection. However the interaction between DREDD and PGRP–LCx was markedly increased when both IMD and FADD were included (**Figure 5.2a**). This data suggests that a receptor

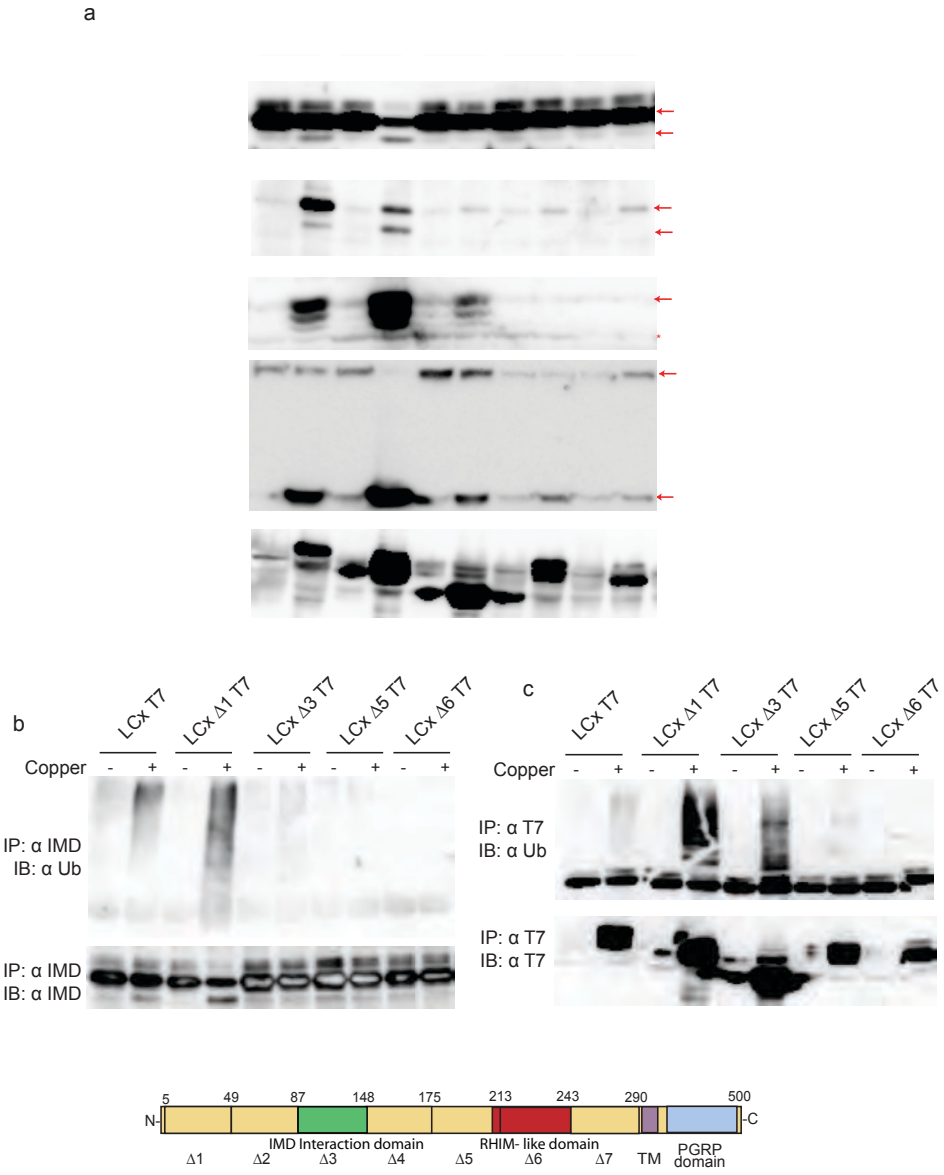


Figure 5.1: PGRP–LCx RHIM mutants fail to activate any downstream event
(a) Analysis of lysates from S2* cells stably transfected with a metallothionein promoter plasmid expressing T7–tagged wild–type or mutant *PGRP–LC,* with or without treatment with $CuSO_4$ as indicated. IMD cleavage, Relish phosphorylation and cleavage were analyzed by immunoblotting. The asterisk marks heavy chain detected by the secondary antibody. The lowest panel confirms PGRP–LCx expression with anti–T7 IP– IB. IMD interaction with PGRP–LC was monitored by IP–IB analysis. (**b**) IP– IB analysis of the ubiquitination status of endogenous IMD from S2* cells stably transfected with metallothionein promoter expression plasmids encoding T7–tagged *PGRP–LCx* (wild–type and deletion mutants) with or without $CuSO_4$ treatment, as indicated. (**c**) IP– IB analysis of the ubiquitination status of immunoprecipitated receptor from S2* cells stably transfected with metallothionein promoter expression plasmids encoding T7–tagged *PGRP–LCx* (wild–type and deletion mutants) with or without $CuSO_4$ treatment, as indicated. Lower diagram indicates the regions deleted in each mutant form of PGRP–LC.

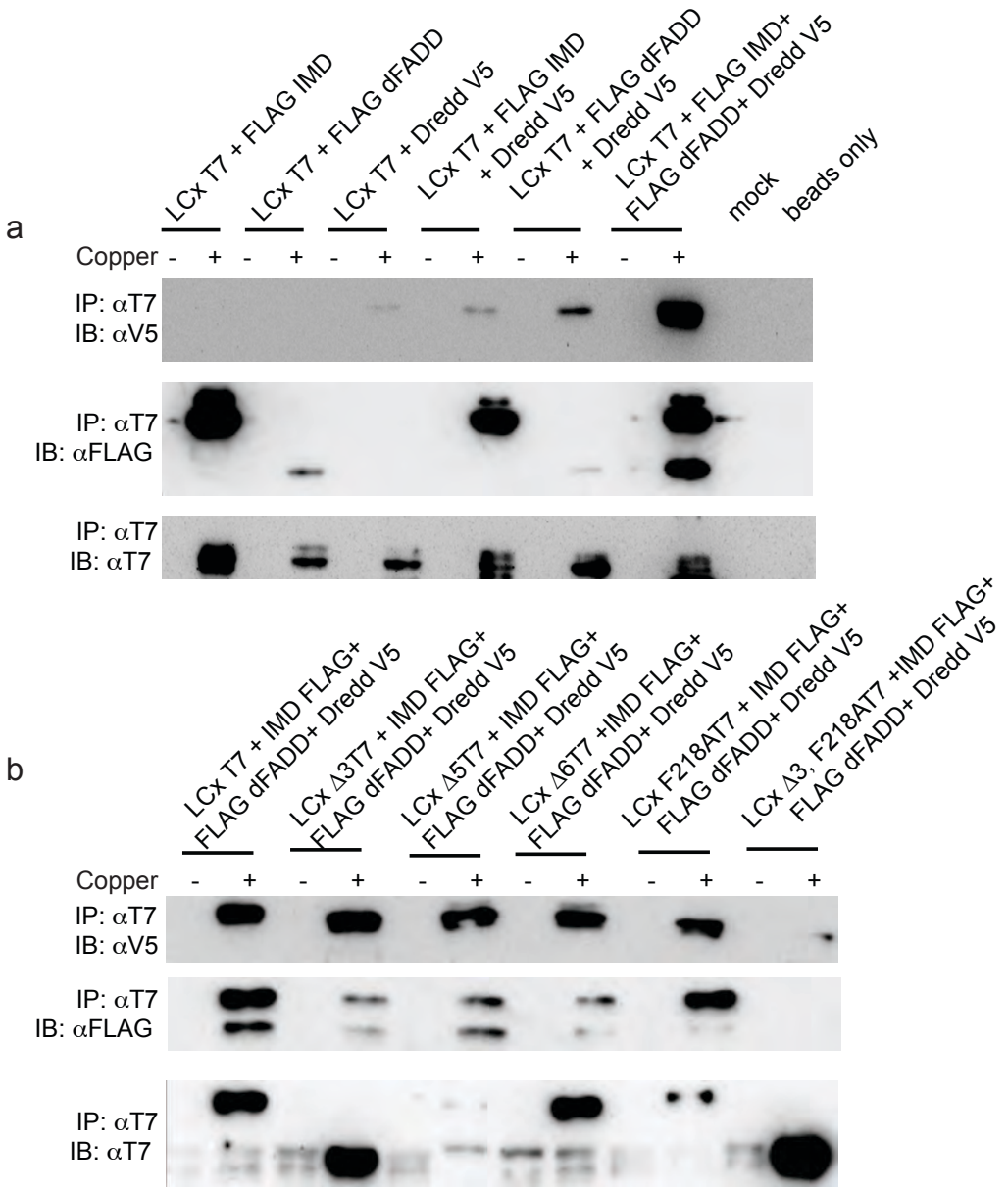


Figure 5.2: A receptor proximal complex is assembled when components of the IMD pathway are over– expressed.
(a) IP–IB analysis of lysates of S2* cells transiently transfected with metallothionein promoter plasmid expressing T7–tagged *PGRP–LCx*, FLAG–tagged *imd*, FLAG–tagged *dFADD* and V5 tagged *DREDD*. DREDD is recruited to the receptor PGRP–LC in a FADD and IMD dependent manner.
(b) Similar co–immunoprecipitation experiments from lysates of S2* cells transiently transfected with metallothionein promoter plasmid expressing T7–tagged *PGRP–LCx* or various PGRP–LCx mutants. DREDD is recruited normally even to the PGRP–LC RHIM–like mutants.

proximal complex, including the caspase–8 like DREDD, is assembled when the receptor and all the known components of the receptor proximal complex are all expressed together. More experiments are required to establish if a similar receptor proximal complex is assembled under physiological conditions. Surprisingly, receptors lacking the RHIM–like motif were able to productively assemble this receptor proximal complex, at least to the level I was able to monitor in these rather crude assays. Likewise, the Δ3 mutant, which fails to interact with IMD in a binary co–transfection assays, was also able to assemble this receptor proximal complex. Only double mutants, which lacked both the RHIM–like motif and Δ3 region, failed in this receptor proximal complex assay (**Figure 5.2b**).

RYBP interacts with the receptor PGRP–LC and it is a new component of the IMD pathway

Given that the RHIM–like mutants displayed nearly normal assembly of this receptor proximal complex and the receptor–associated ubiquitination is altered, the RHIM–like motif may function through an unidentified protein that is involved in this ubiquitination event. In order to identify potential partners and regulators of the IMD pathway receptors, a yeast two–hybrid screen was performed with the cytoplasmic domain of PGRP–LC as bait (Chapter 3). The 24 most strongly interacting clones were further analyzed with a set of baits that carried mutations in the RHIM–like domain of PGRP–LC (or irrelevant control baits), as detailed in Chapter 3. One clone interacted strongly with the wild–type cytoplasmic domain of PGRP–LC but very weakly with the RHIM–like mutant baits (**Table 3.1**). This clone encoded for a gene called *RYBP*.

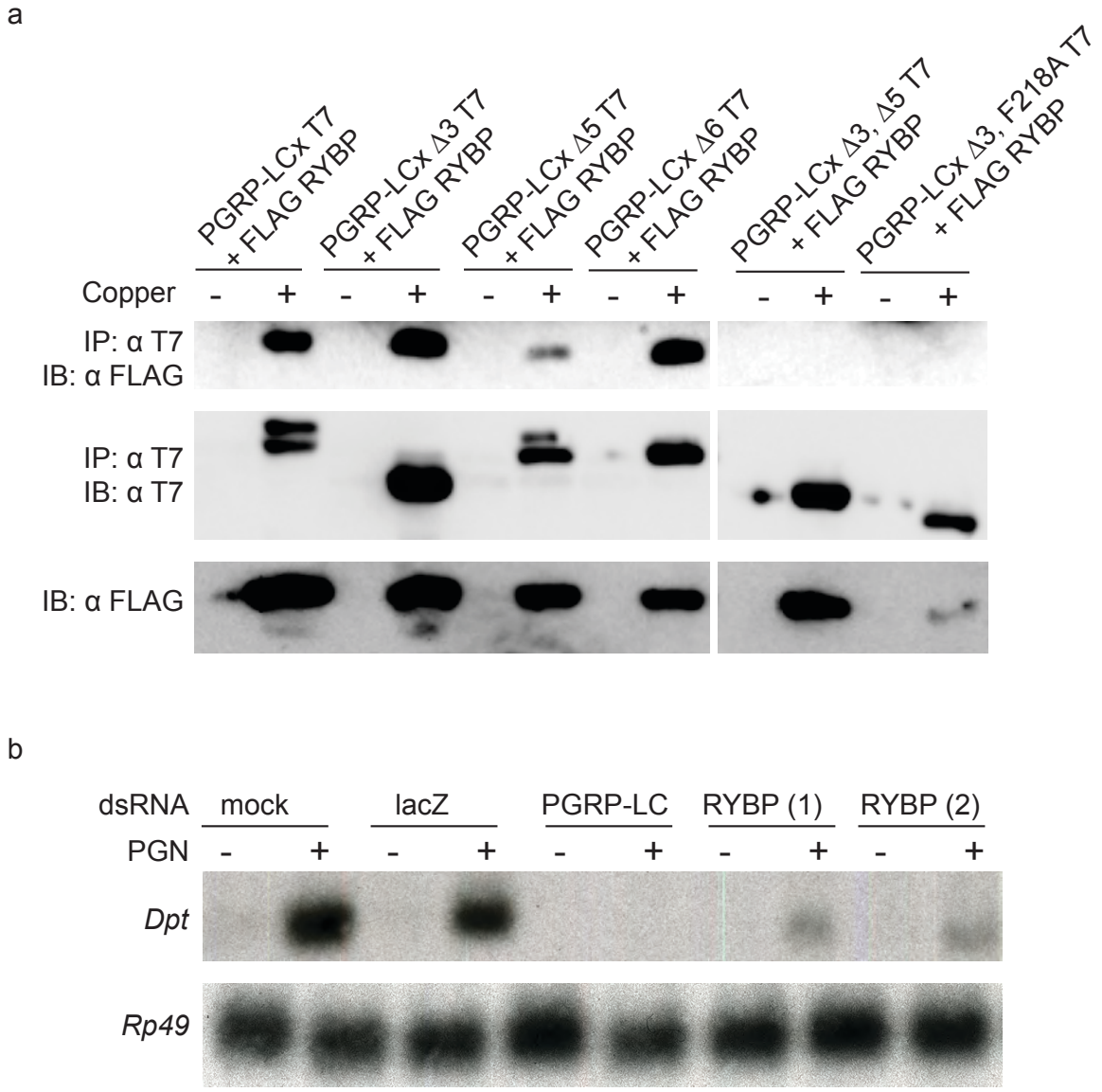


Figure 5.3: RYBP interacts with the receptor PGRP–LC and it is a new component of the IMD pathway
(a) IP–IB analysis of lysates of S2* cells transiently transfected with metallothionein promoter plasmid expressing T7–tagged *PGRP–LCx* (wildtype and mutant), and FLAG–tagged *RYBP*. RYBP fails to interact with the receptor that lacks both the IMD interaction domain and RHIM–like domain. **(b)** Northern blot of *Diptericn* and *rp49* expression in S2* cells treated with lacZ dsRNA or two different Rudra dsRNA, and then stimulated with PGN for 6 hours.

To confirm the yeast two–hybrid results, co–immunoprecipitation experiments were performed. Using epitope tagged constructs and transient transfection in *Drosophila* S2* cells, both PGRP–LC and PGRP–LE were found to associate with RYBP (**Figure 5.3a and data not shown**). In order to determine which domain(s) of the receptors are required for interaction with RYBP, co–immunoprecipitation assays were performed with various mutant versions of PGRP–LC. Removal of the IMD interaction domain (Δ3) or the RHIM–like domain (Δ5, Δ6) alone did not abrogate the interaction of RYBP with the receptor PGRP–LC. However, when both the IMD interaction domain and the RHIM–like domain were mutated (Δ3Δ6, Δ3F218A) RYBP failed to interact with the receptor (**Figure 5.3a**). Overall, we conclude that RYBP interacts with the receptors PGRP–LC and PGRP–LE. The interaction with PGRP–LC is mediated by the IMD interaction domain and the RHIM motif, in a redundant manner. These results may suggest that RYBP interacts with two different motifs of PGRP–LC, or it may indicate that RYBP interacts by direct and indirect mechanisms.

Next, RNAi was used to characterize the function of *RYBP* in the IMD pathway. S2* cells were transfected with two different dsRNA for *RYBP*, and then stimulated with PGN for 6 hours. Knockdown of *RYBP* by RNAi markedly inhibited Diptericin induction almost to the level seen with knockdown of various components of the IMD pathway like PGRP–LC (**Figure 5.3b**). These data suggest that RYBP is a new component of the IMD pathway and is important for signaling in S2* cells.

Discussions

In the last few years we have advanced our understanding about the molecular events that take place in the cytoplasm when the IMD pathway is activated (Aggarwal

and Silverman, 2008; Lemaitre and Hoffmann, 2007; Paquette et al 2010). However, we still lack a clear understanding of the events that take place at the receptor. Our earlier work showed that the receptors PGRP–LC and PGRP–LE share a short region of homology called the RHIM– like motif in there N terminal domains, and this motif is essential for signaling, in cells and in animals. However, it is unclear how this RHIM–like motif functions to support signaling (Kaneko et al., 2006). In this study, we now show that the RHIM–motif seems to be critical for all previously characterized molecular events downstream of PGRP–LC, including IMD cleavage, ubiquitination and Relish activation consistent with the severe defects observed at the level of AMP gene induction.

However, the role of the RHIM–like motif in the association of the receptor with various downstream components is less clear. Based on over–expression (transient transfection co–immunoprecipitation) assays, the RHIM–like motif is not critical for the association of PGRP–LC and IMD. Instead this association involves the 'IMD interaction domain', defined by Δ3. The RHIM–like motif is also not essential for the formation of a receptor proximal complex containing PGRP–LC, IMD, FADD, and DREDD. On the other hand, the endogenously expressed IMD appears to interact much more weakly with the RHIM–deficient PGRP–LCs. This lack of interaction with endogenous IMD is also observed with the Δ3 mutant, and together, this may suggest redundant mechanisms are involved in recruiting IMD to PGRP–LC. The weak association of endogenous IMD and the RHIM–mutant receptors is consistent with the greatly diminished signaling capacity of these mutants. Interestingly, I also discovered that PGRP–LC may be ubiquitinated and this modification correlates with the presence

of the RHIM–like motif. Clearly, the receptor ubiquitination data presented here does not differentiate between direction conjugation of ubiquitin to PGRP–LC and the association with another ubiquitinated protein. However, if the later is the case, it does not appear to IMD that is responsible for the ubiquitin signal associating with PGRP–LC. Further experiments are necessary to discriminate between these possibilities. Also, given the lack of antibody reagents, I have so far been able to examine only over–expressed PGRP–LC for this ubiquitin modification/association.

Most notable is the fact that this receptor–associated ubiquitin correlates best with the signaling activities of various PGRP–LC mutants. While the Δ3 receptor signals near normal levels in cells and in flies, even in response to infection, it showed defects in IMD association, IMD cleavage, IMD ubiquitination, and Relish ubiquitination. Sometimes these defects were not as severe as that observed with the RHIM–like mutant receptors, but clearly this mutant has some defects in inducing all these molecular events. On the other, receptor associated ubiquitin was more robust with the Δ3 receptor while it was completely abrogated with the RHIM–like mutants, Δ5, Δ6 and F218A. This robust receptor associated ubiquitination may explain the ability of Δ3 to support signaling, while the RHIM mutants are inactive. We hypothesize that this receptor associated ubiquitin may provide a platform for the signaling complex to be assembled and trigger sufficient signaling to activate AMP gene expression. In the case of the RHIM–like mutant receptors, no ubiquitination is observed–neither associated with IMD nor associated with the receptor and PGRP–LC RHIM mutant is unable to signal. Further support for this hypothesis is provided by the finding that even though PGRP–LC Δ3 does not bind IMD, cleaved and phosphorylated Relish can still be

detected albeit at levels lower then observed wtih wild– type PGRP–LC. However, more experiments need to be done to explore this possibility. .

Additionally a new component of the IMD pathway called RYBP was also identified. RYBP interacts with both the receptors PGRP–LE and PGRP–LC and its interaction with PGRP–LC is mediated through the IMD interaction domain and the RHIM– like motif, in a redundant manner. This redundancy may be because RYBP interacts with two different motifs of PGRP–LC, or it may indicate that RYBP interacts by direct and indirect mechanisms. One possibility is that RYBP interacts with another component of the IMD pathway which in turn interacts with the receptor. Mammalian RYBP is known to interact with proteins containing a DED domain. Thus,RYBP might interact with FADD and DREDD as they both contain a DED domain and preliminary data suggests that RYBP interacts with DREDD. Knock–down of RYBP in S2* cells inhibits IMD signaling. RYBP is a new regulator of the IMD pathway and it may do so by interacting with the RHIM domain of PGRP–LC. The presence of a NZF domain in the N terminal of RYBP suggests that it is a ubiquitin binding receptor. A better understanding about the role of RYBP in IMD signaling might provide some insights into the receptor proximal events.

Methods

RNA analysis

Total RNA from flies or cultured cells was isolated with the TRIzol reagent (Invitrogen) as described previously (Silverman et al., 2000). Expression of *Diptericin* and the control *rp49* (ribosomal protein) was analyzed by Northern blotting (Silverman et al., 2000). In all S2*–based cell experiments, cells were pre–treated with 1 mM 20–

hydroxyecdysone for 24 to 40 hr before treatment with 500 mM CuSO4 and/ or PGN (100 ng/ ml).

RNAi experiments

dsRNA was generated and purified as reported previously (Di Nocera and Dawid, 1983). Cells were split 24 hours after transfection to 1.0 ×10^6/mL and then were treated with 1 mM 20–hydroxyecdysone. After 24 hours, cells were treated (or left untreated) with PGN (100 ng/ml) for approximately 6 hours.

Co–immunoprecipitation and immunoblotting assays

Immunoprecipitations were carried out with rabbit anti–T7 (Bethyl labs) in lysis buffer (20 mM Tris at pH 7.6, 150 mM NaCl, 2 mM EDTA, 10% Glycerol, 1% Triton X–100, 1 mM DTT, NaVO4, glycerol 2–phosphate and protease inhibitors). For immunoprecipitation from cells, Schneider S2* cells were first transfected by calcium phosphate method with appropriate expression plasmids. Cells were split 24 hours after transfection to 1.0 ×10^6/mL and 24 hours later, were treated with 500 µM copper sulphate for 5 hr, when necessary, for expression from the metallothionein promoter. Immunoprecipitations were performed in lysis buffer and analyzed by SDS–PAGE followed by immunoblot analysis with anti–T7 MAb (Novagen), anti–IMD (gift of J.–M. Reichhardt), anti ankyrin, anti V5 (Sigma) or anti–Flag (Sigma) antibodies. Stable cell lines and immunoblotting were performed as described previously (Silverman et al., 2000).

CHAPTER VI

DISCUSSION:

Speculations on the regulation of IMD pathway via the RHIM–like motif and the connection with ubiquitination

Overview

This thesis research has focused on understanding the molecular mechanisms of innate immune signal transduction in the *Drosophila* IMD pathway. This work demonstrates that receptors PGRP–LC and –LE both recognize DAP type PGN and full length PGRP–LE acts as an intracellular receptor for monomeric PGN. Microarray data suggests that PGRP-LE is expressed in all barrier and immune competent tissues in larvae and adults suggesting that it is critical for recognition of intracellular DAP type PGN (Chintapalli et al., 2007). Additionally, we show that PGRP–LC and PGRP–LE signal through a RHIM–like motif (Chapter 2). In this work, we also characterize two new members of the IMD pathway. RYBP is a new component of the IMD pathway and Rudra is a feedback negative regulator of the IMD pathway (Chapter 4 and 5).

As a whole, the work presented in this thesis adds more detail to the molecular events that take place proximal to the receptor (**Figure 6.1**). Binding of PGN by the receptors PGRP–LC and LE activates downstream signaling. I hypothesize that PGN–induced receptor clustering or multimerization then activates downstream signaling. The RHIM–like motif of these two receptors is very important for this activation. No downstream signaling events are initiated when the RHIM–like motif is mutated or deleted. However, the RHIM–like mutant receptors are capable of serving as a platform for the assembly of all known components of a receptor proximal signaling complex. These results suggest that another, unidentified component of the IMD signaling pathway may function to mediate the function of , and perhaps interact with, the RHIM–like motif.

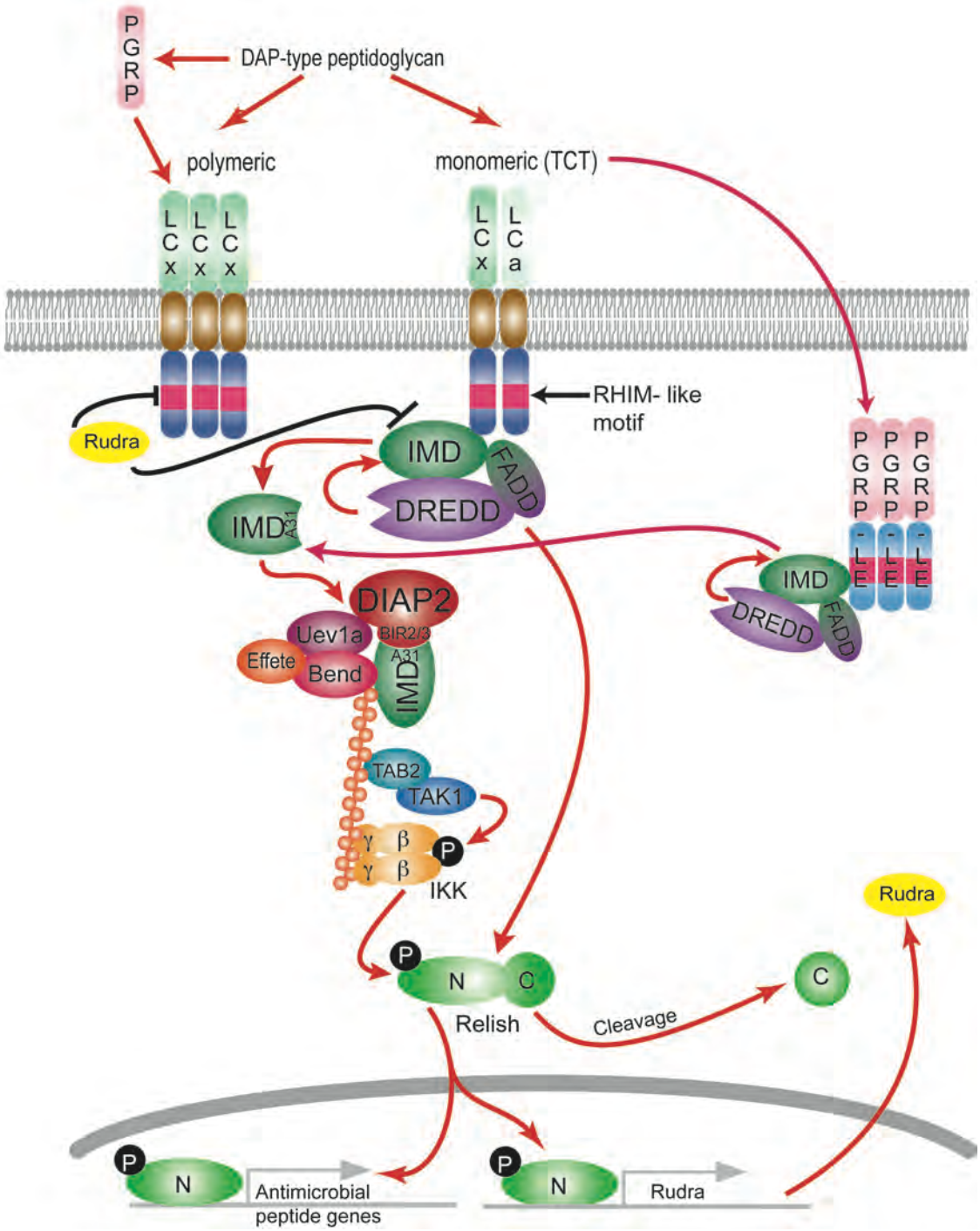


Figure 6.1 Comprehensive IMD signaling pathway model
A comprehensive model of IMD pathway signaling as described in the text.

A yeast two–hybrid screen was performed to identify proteins that might interact with the receptor PGRP–LC through its RHIM–like domain (Chapter 3). With this approach, two new components of the IMD pathway were identified. The first component I characterized is called Rudra and it is a critical feedback inhibitor of peptidoglycan receptor signaling. The other factor is known as RYBP, it includes a highly conserved ubiquitin binding motif (NZF), and RNAi studies suggest it is a critical component of the IMD pathway. Further characterization of this protein might shed light on how the RHIM–like motif of PGRP–LC regulates the IMD pathway.

Receptor activation leads to the cleavage of IMD. Cleavage of IMD allows it to interact with the E3 ligase DIAP2. In concert with the E2 conjugating enzymes, Uev1a, Bendless and Effete, IMD is then K63–polyubiquitinated. In a manner similar to that seen in mammalian NF–κB signaling, it is then proposed that this K63–polyubiquitin chain acts as a scaffold for recruitment and activation of the downstream kinase TAK1. TAK1 then activates two different arms of the IMD pathway: the JNK and the NF–κB arms (Takaesu et al., 2001; Jiang et al., 2002; Wang et al., 2001; Sakurai et al., 2000; Kishimoto et al., 2000; Xia et al., 2009).

Recent work from our lab has exemplified the importance of ubiquitination in regulation of the IMD pathway, hence I provide a small overview of ubiquitination before proceeding to discuss my work (Paquette et al., 2010).

Ubiquitination

Ubiquitin is a highly conserved 76 amino acid polypeptide and it is best characterized for its role in protein degradation. However, a recent body of literature provides ample

evidence that ubiquitination is a critical regulator of cellular functions like chromatin dynamics, membrane trafficking, DNA repair, and protein kinase activation, independent of its role in protein turnover (Chen and Sun, 2009; Pickart, 2004; Bianchi and Meier, 2009; Hershko, 1983).

The ubiquitin molecule itself has 7 lysine residues that can act as acceptor sites for the conjugation with additional ubiquitin molecules leading to ubiquitin polymers. The two most well studied ubiquitin polymers are K48 and K63. In addition, recently it has been demonstrated that ubiquitin can also form linear chains, where one ubiquitin is conjugated to the next in a classic peptide–bond connecting the N–termini of one ubiquitin to the C–terminus of another (Tokunaga et al., 2009; Xu et al., 2009b; Hoeller et al., 2006). K48–linked polyubiquitin chains promote protein degradation, by directing polyubiquitinated proteins to the proteasome. On the other hand, K63–linked polyubiquitin chains are not linked to proteasomal degradation and is thought to function by serving as a scaffold in various biological contexts. Recent work by Xu et al. provides evidence for the existence of free K63 chains that are not conjugated to a target protein and these unanchored chains are also serve as a scaffold in TLR signaling (Xia et al., 2009). Similarly linear chains are also thought to function as scaffold for the regulation IL-1 and TNF signaling (Rahighi et al., 2009) (Haas et al., 2009).

The conjugation of ubiquitin is a three step process. The first step involves the ubiquitin activating enzyme (E1) that binds to the processed ubiquitin via a thioester bond between the active cysteine of the E1 and the C–terminal glycine of the ubiquitin moiety. This ‘activated’ ubiquitin is then transferred to a ubiquitin conjugating enzyme (E2) via the formation of another thioester bond. Lastly the ubiquitin protein ligase (E3)

transfers the ubiquitin molecule from E2 to the lysine residue of the substrate. The E3 determines the substrate specificity, through its protein–target binding activity. However, it is usually the E2 that determines what kind of ubiquitin chains will be added (Jin et al., 2008; Kirkpatrick et al., 2006; Hofmann and Pickart, 1999; Windheim et al., 2008; Ye and Rape, 2009).

Ubiquitin receptors recognize ubiquitinated proteins and recruit/ link them to downstream processes (Hoeller et al., 2006; Dikic et al., 2009). All ubiquitin receptors have a small ubiquitin binding domain (UBD) which often detect distinct ubiquitin linkages. For example, TAB2 contains a zinc finger type UBD called an NZF that specifically binds K63 polyubiquitin chains (Kulathu et al., 2009; Sato et al., 2009). In contrast NEMO contains two UBD domains, a coiled–coil UBD called UBAN that specifically binds linear polyubiquitin chains and a zing finger type UBD that binds K63 polyubiquitin chains (Laplantine et al., 2009; Lo et al., 2009; Komander et al., 2009; Yoshikawa et al., 2009).

Ubiquitination Regulates Innate Immune Signaling

One of the pathways most well studied in the context of polyubiquitination is the TNF signaling pathway. The most popular model of TNF signaling is that the TNF receptor upon activation leads to the recruitment of the adaptor protein TRADD, the E3 ligases TRAF2, TRAF5, cIAP1 and cIAP2 and the kinase RIP1 (Micheau and Tschopp, 2003). RIP1, once recruited to the receptor, is K63–polyubiquitinated. by the E2 enzymes Ubc13/ Uev1a and the E3 ligase TRAF2 (Ea et al., 2006; Li et al., 2006; Wertz et al., 2004; Newton et al., 2008). The Ub chains on RIP1 then recruit TAK1 via the Ub receptor TAB2/ TAB3 and IKK complex via the Ub receptor NEMO. The UBD domains

of both TAB2 and NEMO bind K63 polyubiquitin. Once recruited to the scaffold, TAK1 activates IKKβ which in turn phosphorylates IκB and targets it for K48 –linked polyubiquitination and proteasome–mediated degradation (Xu et al., 2009a)(Rothwarf et al., 1998; Mercurio et al., 1997; Zandi et al., 1997; Hacker and Karin, 2006; Chen et al., 1996). Degradation of IκB liberates NF–κB and leads to its translocation into the nucleus and activation of target gene transcription .

Although the above mentioned model is a widely accepted model for TNF signaling, recent data challenges this model and suggest a different scenario. Data from various groups suggests that that TRAF2 may play only a minor role as the E3 ligase for RIP1. Instead, TRAF2 seems to play a critical role as an adaptor linking cIAP1/2 to the TNFR complex, and these, in turn, functions as the E3 ligase in the TNF–induced RIP1 K63–polyubiquitination reaction (Wu et al., 2007; Bertrand et al., 2008). cIAPs can bind to several different E2s like Ubc13/ Uev1 and UbcH5 and hence it is unclear which kind of polyubiquitin chains it synthesizes (Yang and Du, 2004; Varfolomeev et al., 2008). Also, results from several groups suggest that K63 polyubiquitin chains generated by Ubc13 are not required for TNF signaling, instead polyubiquitin chains generated by Ubc5 are required for IKK activation (Rahighi et al., 2009; Tokunaga et al., 2009; Xu et al., 2009a). The identity of these non–K63 polyubiquitin chains is unclear, however linear ubiquitin chains are the leading candidate.

Several pieces of data support the possibility that linear chains are critical for TNF signaling. First, NEMO binds linear chains with much higher efficiency as compared to K63 polyubiquitin chains (Rahighi et al., 2009). Secondly, NEMO mutants

that fail to bind linear Ub are unable to complement NEMO knockout cells. Finally, NEMO also gets conjugated with linear polyubiquitin. The only E3 ligase known to generate linear chains is LUBAC (Linear Ub Assembly Complex) and it is composed of two RING finger proteins: HOIL–1L (heme–oxidized iron–regulatory protein 2 ubiquitin ligase 1) and HOIP (HOIL–1L–interacting protein) (Kirisako et al., 2006). LUBAC can interact with several different E2 enzymes like UbcH5, E2–25K and UbcH10 to generates linear chains. This data suggests that the E3 ligase rather than the E2 enzyme determines linear polyubuqiutin chain synthesis (Kirisako et al., 2006; Haas et al., 2009).

A new working model for TNF–R1 suggests that the activated receptor recruits the adaptors TRADD and TRAF2 which in turn recruit the E3 ligase cIAP1/2. cIAP then K63 ubiquitnates itself and RIP1 and maybe other components of this complex. The K63 polyubiquitination leads to the recruitment of TAK1/TAB2/TAB3, NEMO/IKKα/IKKβ. LUBAC is also recruited to this scaffold through the UBD (NZF type) of HOIP. Once recruited, LUBAC adds linear ubiquitin chains to NEMO and maybe other components (Haas et al., 2009). These linear chains significantly stabilize the complex, which in turn leads to the activation of the downstream events.

The role of ubiquitin in TNF signaling is complicated, but IL–1β signaling may provide a more simple example of the role of ubiquitin in NF–κB signaling. Stimulation of cells with IL–1 leads to the recruitment of IRAK1 and IRAK4 to the IL–1R complex, which in turn recruit TRAF6. TRAF6 is an E3 ligase, which in conjunction with the E2 enzymes Uev1a/ Ubc13, mediates K63 polyubiquitination of target proteins including NEMO, IRAK and TRAF6 itself. The K63 polyubiquitin chains provides a scaffold for the

recruitment of the Ub receptor TAB2/ TAB3 which lead to the recruitment and auto phosphorylation of TAK1. IKKα/ IKKβ are also recruited to the scaffold via the ubiquitin receptor NEMO. Possibly the proximity of IKKβ and TAK1 allows TAK1 to phosphorylate and activate IKK. TAK1 also phosphorylates MKK4/7 which in turn activate the JNK and p38 kinase cascade (Chen, 2005; Rowe et al., 2006). Unlike the TNFR pathway, K63 ubiquitin chains are essential for IL–1R signaling (Xu et al., 2009a).

The simple view of IL–1 signaling may not hold for very long as recent reports suggest that other kinds of ubiquitin chains also regulate this pathway. Available data suggests that NEMO gets conjugated to linear ubiquitin and this is important for its ability to activate NF–κB and not the MAPK pathway (Tokunaga et al., 2009). The role of linear chains in IL–1 signaling is controversial as recent work by Xu et al shows that only K63 polyubiquitination is essential for IKK activation. Interestingly work by Chen and colleagues also suggests that unanchored K63 polyubiquitin chains, which are not conjugated to any target protein, are enough to activate TAK1 and NF–κB signaling. Recruitment of TAK1 to free K63 polyubiquitin through TAB2 leads to its autophosphorylation and hence activation.

Though a lot is known about the role of polyubiquitination in the regulation of NF–κB signaling a number of questions still remain. First, it is still unclear which kind of linkages are involved in the regulation of TNF and IL–1 signaling. The role of K63, linear and mixed linkages in each pathway is unclear. Second, what are the components of the two pathways that are ubiquitinated, which specific conjugation targets, if any, are critical for signaling?. Following stimulation, TNFR–1 and TRADD migrate as higher weight proteins and it is speculated that they get also ubiquitinated,

but no one has tested this hypothesis directly. Third it is unclear what kind of polyubiquitin chains cIAP synthesizes under physiological conditions as it has the ability to interact with different E2s. Fourth the specificity of all the ubiquitin receptors is unclear. TAB2 specifically binds K63 polyubiquitin chains but the specificity of NEMO is still unresolved. Additionally recent work by some groups suggest that K63–polyubiquitination is not important for TNF signaling. Therefore, it is unclear how TAB2 functions as a ubiquitin receptor in TNF signaling.

Analysis of the role of ubiquitination in a less complex model system like *Drosophila* may help address some of the above questions. The IMD signaling pathway provides an ideal platform for these studies. Even though the receptor PGRP–LC is not homologous to TLRs, other components of the IMD pathway are conserved. Also, the signaling components downstream of the receptor PGRP–LC share homology to both the MyD88–independent TLR signaling pathway and TNFR pathway.

Regulation of IMD signaling via the RHIM–like motif

PGRP–LC and PGRP–LE detect DAP–PGN through their C–terminal PGN–binding PGRP domain and transduce signaling through their extended N–terminal domains (Choe et al., 2005; Kaneko et al., 2006). PGRP–LC and LE share approximately 50% homology in the their C–terminal PGRP domain but there is only a short stretch of about 20 amino acids that is homologous in the N–terminal domains of PGRP–LC and –LE. In both receptors, deletion or mutation within this conserved domain abrogates signaling (Figure 2.7). This conserved signaling motif shows weak homology to the RHIM domain found in the mammalian RIP and TRIF proteins, which

mediates homotypic interactions between them. The molecular mechanism by which the RHIM–like domains in PGRP–LC function is unclear.

The receptor PGRP–LC interacts with the downstream protein IMD. However, the interaction between IMD and PGRP–LC, which is detected in transfection experiments, does not map to the RHIM–like domain but maps to another distinct region (Δ3) **(**Figure 2.9a). Our data suggests that the IMD interaction domain of PGRP–LC is not important for signaling as flies expressing PGRP–LCΔ3 are able support *E.coli* infection–induced *diptericin* (Figure 2.9c). This data suggest that the RHIM–like domain has a function beyond the recruitment of IMD and that direct IMD recruitment to these receptors may not always be necessary for signaling. However, a caveat of the transient transfection/co–immunoprecipitation experiment is that both the receptor and IMD are over–expressed and sometimes over–expression data does not reflect the actual interactions that occur under physiological conditions. Therefore, to get a more physiologically relevant picture, I analyzed the interaction of over–expressed wild type and RHIM–mutant receptor with endogenous IMD. We had to rely on stable cell lines over–expressing PGRP–LC because of the lack of a good PGRP–LC antibody, and, of course, the RHIM–mutant receptors must be expressed exogenously. However, we have an excellent antibody to detect endogenous IMD. I found that endogenous IMD interacts robustly with wild–type PGRP–LC, but weakly with the RHIM–like mutants (Figure 5.1a). Also the PGRP–LC lacking the IMD interaction domain also interacts weakly with IMD. This data differs from the transient transfection/ co–immunoprecipitation experiments (Figure 5.1a) and suggests that the RHIM–like motif may play a significant role in recruiting IMD.

In this study, moving back to the cotransfection/coimmuniprecipitaiton assays, I also show that a receptor proximal complex assembles on PGRP–LC. In particular, DREDD is recruited to the receptor in a FADD–and IMD–dependent manner (Figure 5.2a). My data suggests that the RHIM–like mutant of PGRP–LCx is not defective in assembly of this receptor proximal complex **(**Figure 5.2b). However, it is important to note that the biggest caveat of this experiment is that the analysis is based on over–expression of various components of the IMD pathway. In order to analyze the actual receptor proximal events, we are in the process of making antibodies against all the components of the IMD pathway.

Various biochemical assays were carried out to determine the molecular events that fail to initiate when PGRP–LC RHIM–like mutant receptors are over–expressed in S2* cells (Note, receptor over–expression is sufficient to drive signalign). Analysis of downstream signaling events following receptor over–expression suggests that PGRP–LC RHIM–like mutants fail to activate all the downstream molecular events including IMD cleavage, IMD ubiquitination, Relish cleavage and Relish phosphorylation (Figure 5.1a). This data again suggests that the RHIM–like motif is critical for signaling. Interestingly, when cell lines over–expressing PGRP–LC lacking the IMD interaction domain (∆3) were analyzed no cleaved or ubiquitinated IMD was detectable but low levels of cleaved and phosphorylated Relish could be observed. This data suggests that PGRP–LC ∆3 might be a hypomorph. To test if PGRP–LC∆3 is a hypomorph, flies expressing PGRP–LC∆3 will be infected with *E.coli* and then a time course of *diptericin* induction will be done. It is unclear how PGRP–LC∆3 can cleave and phosphorylate Relish. One possibility is that IMD is cleaved and ubiquitinated at low, nearly

undetectable, levels but this is enough for Relish cleavage and phosphorylation. Another possibility is that over–expressed PGRP–LCΔ3 can somehow bypass the requirement for cleaved and ubiquitinated IMD, directly leading to the cleavage and phosphorylation of Relish. If this hypothesis is correct, knockdown of IMD in cells over–expressing PGRP–LCΔ3 will not effect induction of AMPs genes.

Analysis of the receptor associated ubiquitination showed that PGRP–LC RHIM–like mutants failed to associate with any poly ubiquitin signal whereas wild–type and PGRP–LCΔ3 precipitated a robust ubiquitination signal (Figure 5.1c). This data suggests that the either the receptor itself is ubiquitinated or it associates with a protein that is ubiquitinated. However, this ubiquitinated protein cannot be IMD because no IMD ubiquitination is observed in stable cells over–expressing PGRP–LCΔ3 (Figure 5.1b). Also, the receptor PGRP–LC associates with polyubiquitin signal even after *imd* has been knocked by RNAi. The receptor associated ubiquitination most closely reflects the phenotype observed with AMP induction when the various PGRP–LC mutants are over–expressed (Figure 2.7a) Therefore, a better understanding of these receptor associated ubiquitination events may provide an insight into the role of the RHIM–like motif. One hypothesis that may explain why PGRP–LCΔ3 induces AMPs but the RHIM mutants fail do so, is that under wild–type conditions ubiquitinated IMD provides a platform for the assembly of a receptor proximal complex, however, in a situation where IMD cannot interact with the receptor (like PGRP–LCΔ3) another component of the IMD pathway that also gets ubiquitinated provides the platform for the assembly of the signaling complex. And in the case of the RHIM–like mutant, no molecular events down stream of the receptor are activated because no component of

the IMD pathway can be ubiquitinated and/or the ubiquitinated components fail to interact with the receptor.

Analysis of receptor associated ubiquitination raises many important questions. First what is the target protein that is getting detected in the receptor associated ubiquitination assays? Second, what kind of ubiquitin chains are conjugated to this target protein and what are the E2 and E3 enzymes involved?

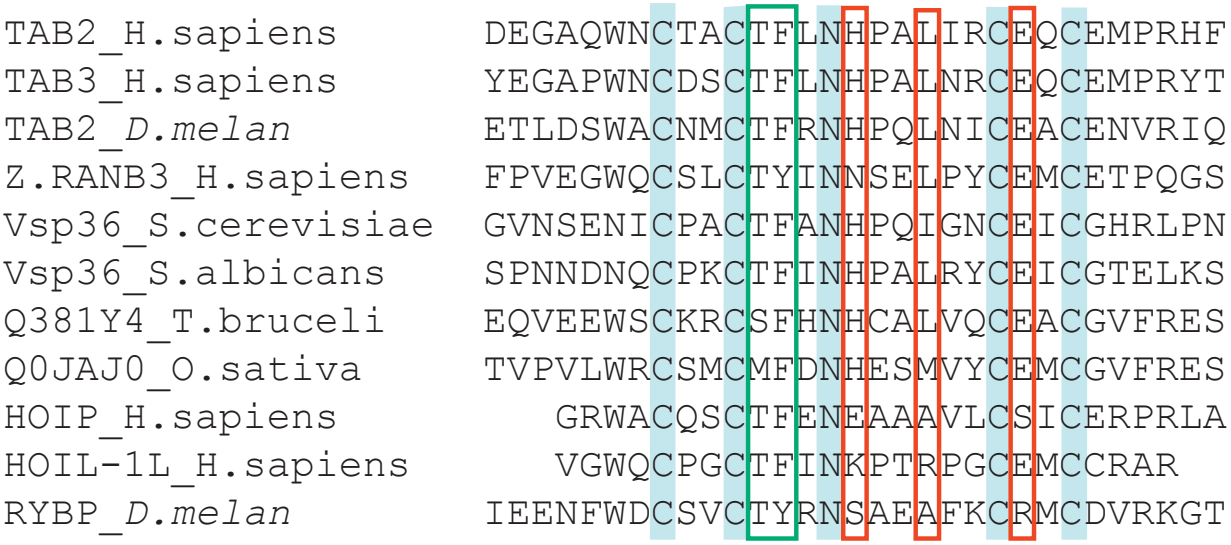
RYBP is a new component of the IMD pathway

I performed a yeast two–hybrid screen to identify new components of the IMD pathway that interact with the receptor PGRP–LC via the RHIM–like domain (Chapter 3). One protein that interacted with the receptor through its RHIM–like motif was RYBP (Figure 3.2). RYBP is a 150 amino acid protein and contains an NZF domain in its N terminus. Knock–down of RYBP in S2 cells inhibits signaling via the IMD pathway (Figure 5.3b). Additionally, analysis of the interaction of RYBP with PGRP–LC in S2 cells, showed that this interaction is mediated through both IMD interaction domain and the RHIM–like motif of PGRP–LC in a redundant manner (Figure 5.3a) [This is not the case in yeast, where the two–hybrid data suggest the PGRP–LC/RYBP interaction is strictly dependent on the RHIM–like motif]. This data suggests that RYBP may either mediate its interaction with the receptor through multiple domains, or that RYBP may interact directly with PGRP–LC via the RHIM–like motif and indirectly, through other components of the pathway, with the IMD interaction domain. Interaction studies need to be done in an another heterologous system to tease apart direct interactions from indirect ones. In mammals RYBP interacts with all death effector domain (DED) containing proteins, such as caspase 8, 10, FADD and DEDD (Zheng et al., 2001).

Therefore, it will not be surprising if we find that RYBP interacts with DREDD and/or FADD , because both proteins have a DED like domain. Indeed preliminary data suggests that RYBP interacts with DREDD.

The presence of the NZF domain in the N terminal of RYBP raises the possibility that it is a ubiquitin receptor like TAB2 (which also has a NZF domain in its C terminus) (Bejarano et al., 2005; Arrigoni et al., 2006). TAB2 specifically recognizes K63 linked polyubiquitin chains and this specificity is determined by residues that mediate interaction with the distal and proximal ubiquitin (Komander et al., 2009; Kulathu et al., 2009; Sato et al., 2009). Residues that allow TAB2 to specifically bind K63 polyubiquitin are not conserved in the NZF domains of HOIL–1L and HOIP (components of the E3 ligase LUBAC). HOIL–1 does not bind K48 Ub, but it binds linear Ub and K63 Ub chains and HOIP preferentially binds K63 chains (Haas et al., 2009). Like the HOIL–1 and HOIP NZF domains, the residues that mediate interaction with distal and proximal ubiquitins in K63 chains are not conserved in RYBP, hence it is possible that RYBP can interact with different polyubiquitin chains (**Figure 6.2**).

Work in our lab suggests that ubiquitination plays an important role in the regulation of IMD pathway, however, the exact mechanisms are unclear. One possible scenario, suggested from this work is that following ligand stimulation the receptor gets ubiquitinated (in addition to the ubiquitination of IMD). These chains could be linear, K63, mixed or other. The ubiquitinated receptor may then be detected by the ubiquitin receptor RYBP. RYBP interacts with the receptor transiently through the polyubiquitin chains on the receptor but then forms more stable interactions through the RHIM–like motif. Another possibility is that the interaction of RYBP with the polyubiquitin chains



6.2: Sequence alignment of NZF domains containing proteins

Conserved residues are shown with blue background. Green and red boxes denote residues at the distal and proximal ubiquitin binding sites, respectively.

and the RHIM–like motif of PGRP–LC are important for the assembly of a stable receptor proximal complex. RYBP in turn recruits DREDD to the receptor. IMD maybe interacts with the receptor PGRP–LC even before it is activated or IMD may be gets recruited to the receptor following stimulation. FADD is an adaptor protein, interacting with both IMD and DREDD, and it helps stabilize the receptor proximal complex. The recruitment of DREDD to the receptor somehow activates its proteolytic activity. The proximity between DREDD and IMD then allows DREDD to cleave IMD and lead to its ubiquitination. K63 polyubiquitinated IMD then acts as a platform to recruit downstream signaling complexes and lead to the activation of NF–κB and JNK signaling (**Figure 6.3**)

Recent work highlights the complexity of ubiquitination and its important role in the regulation of signaling pathways in mammals and *Drosophila*. It will be exciting to learn in greater detail how ubiquitination regulates the IMD pathway and how this compares to mammalian NF–κB signalign pathways. It will also be interesting to see how similar or different the regulation of IMD pathway is in comparison to TNF and TLR signaling. Homologs of signaling pathway components involved in *Drosophila* innate immunity have been identified in humans and other organisms. Lacking an adaptive immune response, *Drosophila* serves as an important model system for further understanding of innate immunity and host/pathogen interactions. To that end, this research sheds light on the regions of the receptor that are important for immune signaling and it begins to illuminate the receptor proximal events that may play a role in insect NF–κB signaling. A better understanding of the signaling events in the IMD pathway may help shed light on some of the prominent questions in the mammalian innate immune signaling pathways.

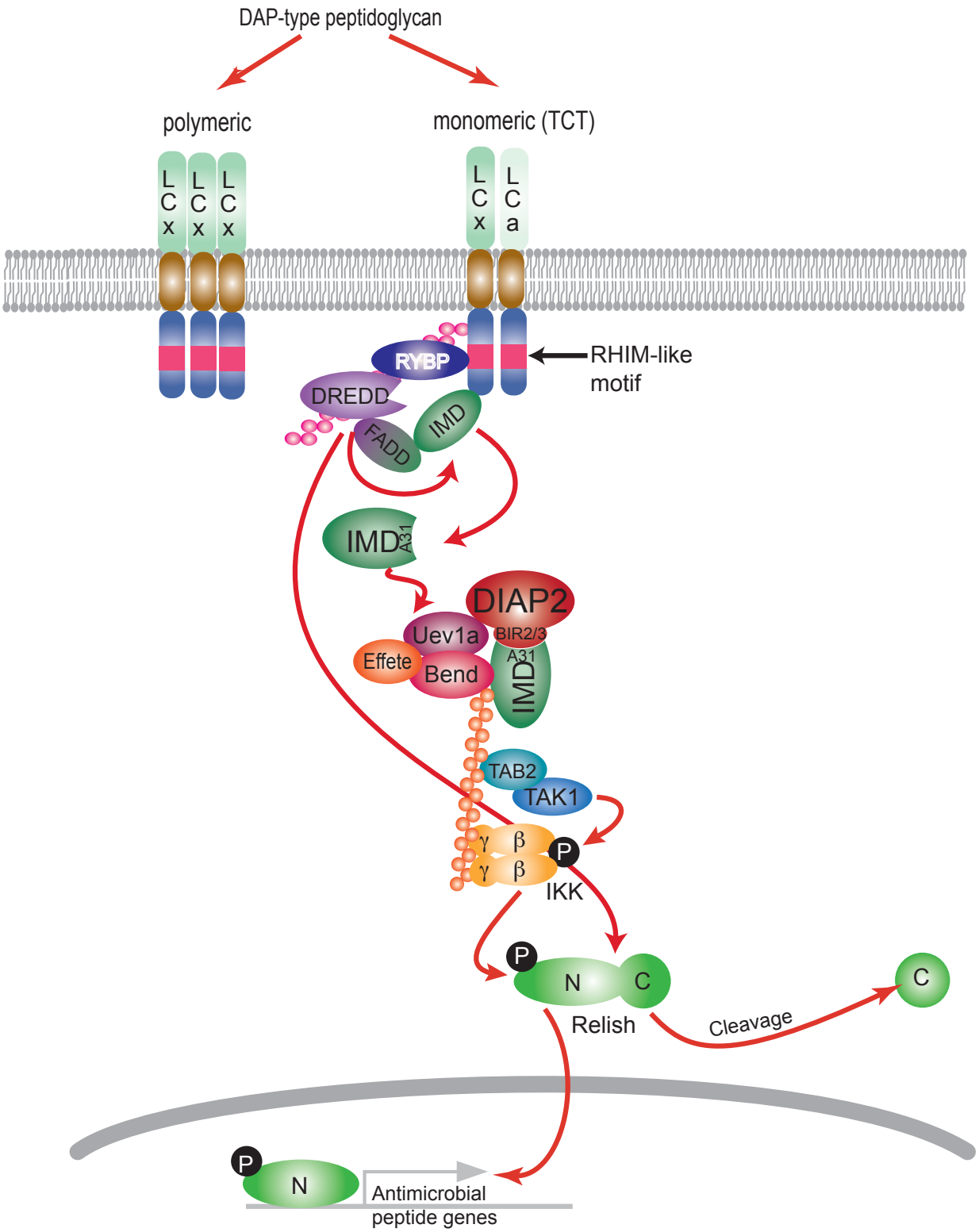


Figure 6.3 Proposed model of IMD signaling pathway
A comprehensive model of the proposed IMD pathway signaling as described in the text.

BIBLIOGRAPHY

Agaisse, H., Petersen, U.M., Boutros, M., Mathey-Prevot, B., and Perrimon, N. (2003). Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury. Dev Cell **5**: 441–450.

Aggarwal, K. et al. (2008). Rudra interrupts receptor signaling complexes to negatively regulate the IMD pathway. PLoS Pathog **4**: e1000120.

Aggarwal, K., and Silverman, N. (2008). Positive and negative regulation of the Drosophila immune response. BMB Rep **41**: 267–277.

Aggrawal, K., and Silverman, N. (2007). Peptidoglycan recognition in Drosophila. Biochem Soc Trans **35**: 1496–1500.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell **124**: 783–801.

Alexander, W.S. (2002). Suppressors of cytokine signalling (SOCS) in the immune system. Nat Rev Immunol **2**: 410–416.

Arrigoni, R. et al. (2006). The Polycomb-associated protein Rybp is a ubiquitin binding protein. FEBS Lett **580**: 6233–6241.

Basset, A. et al. (2000). The phytopathogenic bacteria Erwinia carotovora infects Drosophila and activates an immune response. Proc Natl Acad Sci U S A **97**: 3376–3381.

Bejarano, F., Gonzalez, I., Vidal, M., and Busturia, A. (2005). The Drosophila RYBP gene functions as a Polycomb-dependent transcriptional repressor. Mech Dev **122**: 1118–1129.

Belvin, M.P., Jin, Y., and Anderson, K.V. (1995). Cactus protein degradation mediates Drosophila dorsal-ventral signaling. Genes Dev **9**: 783–793.

Bergmann, A. et al. (1996). A gradient of cytoplasmic Cactus degradation establishes the nuclear localization gradient of the dorsal morphogen in Drosophila. Mech Dev **60**: 109–123.

Bertrand, M.J. et al. (2009). Cellular inhibitors of apoptosis cIAP1 and cIAP2 are required for innate immunity signaling by the pattern recognition receptors NOD1 and NOD2. Immunity **30**: 789–801.

Bertrand, M.J. et al. (2008). cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Mol Cell **30**: 689–700.

Beutler, B.A. (2009). TLRs and innate immunity. Blood **113**: 1399–1407.

Bianchi, K., and Meier, P. (2009). A tangled web of ubiquitin chains: breaking news in TNF-R1 signaling. Mol Cell **36**: 736–742.

Bischoff, V. et al. (2006). Downregulation of the Drosophila immune response by peptidoglycan-recognition proteins SC1 and SC2. PLoS Pathog **2**: e14.

Bischoff, V. et al. (2004). Function of the Drosophila pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria. Nat Immunol **5**: 1175–1180.

Boone, D.L. et al. (2004). The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nat Immunol **5**: 1052–1060.

Boutros, M., Agaisse, H., and Perrimon, N. (2002). Sequential Activation of Signaling Pathways during Innate Immune Responses in Drosophila. Dev Cell **3**: 711–722.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118**: 401–415.
Brennan, C.A., and Anderson, K.V. (2004). Drosophila: the genetics of innate immune recognitioncgchech and response. Annu Rev Immunol **22**: 457–483.
Brint, E.K. et al. (2004). ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. Nat Immunol **5**: 373–379.
Buchon, N. et al. (2009). A single modular serine protease integrates signals from pattern-recognition receptors upstream of the Drosophila Toll pathway. Proc Natl Acad Sci U S A **106**: 12442–12447.
Bunch, T.A., Grinblat, Y., and Goldstein, L.S. (1988). Characterization and use of the Drosophila metallothionein promoter in cultured Drosophila melanogaster cells. Nucleic Acids Res **16**: 1043–1061.
Burns, K. et al. (2000). Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. Nat Cell Biol **2**: 346–351.
Burns, K. et al. (2003). Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. J Exp Med **197**: 263–268.
Burns, K. et al. (1998). MyD88, an adapter protein involved in interleukin-1 signaling. J Biol Chem **273**: 12203–12209.
Cao, Z., Henzel, W.J., and Gao, X. (1996a). IRAK: a kinase associated with the interleukin-1 receptor. Science **271**: 1128–1131.
Cao, Z., Xiong, J., Takeuchi, M., Kurama, T., and Goeddel, D.V. (1996b). TRAF6 is a signal transducer for interleukin-1. Nature **383**: 443–446.
Carty, M. et al. (2006). The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. Nat Immunol **7**: 1074–1081.
Cha, G.H. et al. (2003). Discrete functions of TRAF1 and TRAF2 in Drosophila melanogaster mediated by c-Jun N-terminal kinase and NF-kappaB-dependent signaling pathways. Mol Cell Biol **23**: 7982–7991.
Chang, C.I., Chelliah, Y., Borek, D., Mengin-Lecreulx, D., and Deisenhofer, J. (2006). Structure of Trachael Cytotoxin in Complex with a Heterodimeric Pattern-Recognition Receptor. Science **311**: 1761–1764.
Chang, C.I. et al. (2005). Structure of the ectodomain of Drosophila peptidoglycan-recognition protein LCa suggests a molecular mechanism for pattern recognition. Proc Natl Acad Sci U S A **102**: 10279–10284.
Chang, C.I. et al. (2004). A Drosophila pattern recognition receptor contains a peptidoglycan docking groove and unusual l,d-carboxypeptidase activity. PLoS Biol **2**: E277.
Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. Nature **410**: 37–40.
Chen, W., White, M.A., and Cobb, M.H. (2002). Stimulus-specific requirements for MAP3 kinases in activating the JNK pathway. J Biol Chem **277**: 49105–49110.
Chen, Z.J. (2005). Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol **7**: 758–765.
Chen, Z.J., Parent, L., and Maniatis, T. (1996). Site-specific phosphorylation of IkBa by a novel ubiquitination-dependent protein kinase activity. Cell **84**: 853–862.

Chen, Z.J., and Sun, L.J. (2009). Nonproteolytic functions of ubiquitin in cell signaling. Mol Cell **33**: 275–286.
Cherry, S., and Silverman, N. (2006). Host-pathogen interactions in drosophila: new tricks from an old friend. Nat Immunol **7**: 911–917.
Cheung, P.C., Nebreda, A.R., and Cohen, P. (2004). TAB3, a new binding partner of the protein kinase TAK1. Biochem J **378**: 27–34.
Chintapalli, V.R., Wang, J., and Dow, J.A. (2007). Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat Genet **39**: 715–720.
Cho, J.H. et al. (2005). Human peptidoglycan recognition protein S is an effector of neutrophil-mediated innate immunity. Blood **106**: 2551–2558.
Choe, K.M., Lee, H., and Anderson, K.V. (2005). Drosophila peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor. Proc Natl Acad Sci U S A **102**: 1122–1126.
Choe, K.M., Werner, T., Stöven, S., Hultmark, D., and Anderson, K.V. (2002). Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in Drosophila. Science **296**: 359–362.
Chu, K., Niu, X., and Williams, L.T. (1995). A Fas-associated protein factor, FAF1, potentiates Fas-mediated apoptosis. Proc Natl Acad Sci U S A **92**: 11894–11898.
Cookson, B.T., Cho, H.L., Herwaldt, L.A., and Goldman, W.E. (1989a). Biological activities and chemical composition of purified tracheal cytotoxin of Bordetella pertussis. Infect Immun **57**: 2223–2229.
Cookson, B.T., Tyler, A.N., and Goldman, W.E. (1989b). Primary structure of the peptidoglycan-derived tracheal cytotoxin of Bordetella pertussis. Biochemistry **28**: 1744–1749.
Cox, C.R., and Gilmore, M.S. (2007). Native microbial colonization of Drosophila melanogaster and its use as a model of Enterococcus faecalis pathogenesis. Infect Immun **75**: 1565–1576.
Cusson-Hermance, N., Khurana, S., Lee, T.H., Fitzgerald, K.A., and Kelliher, M.A. (2005). Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. J Biol Chem **280**: 36560–36566.
Danen-van Oorschot, A.A. et al. (2004). Human death effector domain-associated factor interacts with the viral apoptosis agonist Apoptin and exerts tumor-preferential cell killing. Cell Death Differ **11**: 564–573.
Davis, R.J. (1999). Signal transduction by the c-Jun N-terminal kinase. Biochem Soc Symp **64**: 1–12.
De Gregorio, E. et al. (2002). An immune-responsive Serpin regulates the melanization cascade in Drosophila. Dev Cell **3**: 581–592.
De Gregorio, E., Spellman, P.T., Rubin, G.M., and Lemaitre, B. (2001). Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proc Natl Acad Sci U S A **98**: 12590–12595.
De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M., and Lemaitre, B. (2002). The Toll and Imd pathways are the major regulators of the immune response in Drosophila. Embo J **21**: 2568–2579.
Delaney, J.R., and Mlodzik, M. (2006). TGF-beta activated kinase-1: new insights into the diverse roles of TAK1 in development and immunity. Cell Cycle **5**: 2852–2855.

Deng, L. et al. (2000). Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell **103**: 351–361.
Di Nocera, P.P., and Dawid, I.B. (1983). Transient expression of genes introduced into cultured cells of Drosophila. Proc Natl Acad Sci U S A **80**: 7095–7098.
Diehl, G.E. et al. (2004). TRAIL-R as a negative regulator of innate immune cell responses. Immunity **21**: 877–889.
Dikic, I., Wakatsuki, S., and Walters, K.J. (2009). Ubiquitin-binding domains - from structures to functions. Nat Rev Mol Cell Biol **10**: 659–671.
Dinarello, C.A. (2002). The IL-1 family and inflammatory diseases. Clin Exp Rheumatol **20**: S1–13.
Dinarello, C.A. (2005). Interleukin-1beta. Crit Care Med **33**: S460–2.
Divanovic, S. et al. (2005a). Inhibition of TLR-4/MD-2 signaling by RP105/MD-1. J Endotoxin Res **11**: 363–368.
Divanovic, S. et al. (2005b). Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105. Nat Immunol **6**: 571–578.
Drier, E.A., and Steward, R. (1997). The dorsoventral signal transduction pathway and the Rel-like transcription factors in Drosophila. Semin Cancer Biol **8**: 83–92.
Dunne, A., and O'Neill, L.A. (2003). The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. Sci STKE **2003**: re3.
Durfee, T. et al. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes & Dev. **7**: 555–569.
Dushay, M.S., Åsling, B., and Hultmark, D. (1996). Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defense of Drosophila. Proc. Natl. Acad. Sci. USA **93**: 10343–10347.
Dziarski, R., Platt, K.A., Gelius, E., Steiner, H., and Gupta, D. (2003). Defect in neutrophil killing and increased susceptibility to infection with non-pathogenic Gram-positive bacteria in peptidoglycan recognition protein-S (PGRP-S)-deficient mice. Blood
Ea, C.K., Deng, L., Xia, Z.P., Pineda, G., and Chen, Z.J. (2006). Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell **22**: 245–257.
El Chamy, L., Leclerc, V., Caldelari, I., and Reichhart, J.M. (2008). Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways 'upstream' of Toll. Nat Immunol **9**: 1165–1170.
Erturk-Hasdemir, D. et al. (2009). Two roles for the Drosophila IKK complex in the activation of Relish and the induction of antimicrobial peptide genes. Proc Natl Acad Sci U S A **106**: 9779–9784.
Fernandez, N.Q., Grosshans, J., Goltz, J.S., and Stein, D. (2001). Separable and redundant regulatory determinants in Cactus mediate its dorsal group dependent degradation. Development **128**: 2963–2974.
Ferrandon, D. et al. (1998). A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. Embo J **17**: 1217–1227.
Filipe, S.R., Tomasz, A., and Ligoxygakis, P. (2005). Requirements of peptidoglycan structure that allow detection by the Drosophila Toll pathway. EMBO Rep **6**: 327–333.

Fitzgerald, K.A. et al. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol **4**: 491–496.
Fitzgerald, K.A. et al. (2001). Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature **413**: 78–83.
Fitzgerald, K.A. et al. (2003). LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med **198**: 1043–1055.
Flatt, T., Tu, M.P., and Tatar, M. (2005). Hormonal pleiotropy and the juvenile hormone regulation of Drosophila development and life history. Bioessays **27**: 999–1010.
Foley, E., and O'Farrell, P.H. (2004). Functional dissection of an innate immune response by a genome-wide RNAi screen. PLoS Biol **2**: E203.
Franchi, L., Warner, N., Viani, K., and Nunez, G. (2009). Function of Nod-like receptors in microbial recognition and host defense. Immunol Rev **227**: 106–128.
Fritz, J.H. et al. (2007). Nod1-mediated innate immune recognition of peptidoglycan contributes to the onset of adaptive immunity. Immunity **26**: 445–459.
Ganguly, A., Jiang, J., and Ip, Y.T. (2005). Drosophila WntD is a target and an inhibitor of the Dorsal/Twist/Snail network in the gastrulating embryo. Development **132**: 3419–3429.
Garcia, E., Marcos-Gutierrez, C., del Mar Lorente, M., Moreno, J.C., and Vidal, M. (1999). RYBP, a new repressor protein that interacts with components of the mammalian Polycomb complex, and with the transcription factor YY1. EMBO J **18**: 3404–3418.
Garlanda, C. et al. (2004). Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. Proc Natl Acad Sci U S A **101**: 3522–3526.
Garver, L.S., Wu, J., and Wu, L.P. (2006). The peptidoglycan recognition protein PGRP-SC1a is essential for Toll signaling and phagocytosis of Staphylococcus aureus in Drosophila. Proc Natl Acad Sci U S A **103**: 660–665.
Gelius, E., Persson, C., Karlsson, J., and Steiner, H. (2003). A mammalian peptidoglycan recognition protein with N-acetylmuramoyl-L-alanine amidase activity. Biochem Biophys Res Commun **306**: 988–994.
Georgel, P. et al. (2001). Drosophila immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis. Dev Cell **1**: 503–514.
Gietz, R.D., and Woods, R.A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol **350**: 87–96.
Gilchrist, M. et al. (2006). Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. Nature **441**: 173–178.
Gillespie, S.K., and Wasserman, S.A. (1994). Dorsal, a Drosophila Rel-like protein, is phosphorylated upon activation of the transmembrane protein Toll. Mol Cell Biol **14**: 3559–3568.
Gioannini, T.L. et al. (2004). Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. Proc Natl Acad Sci U S A **101**: 4186–4191.
Gobert, V. et al. (2003). Dual activation of the Drosophila Toll pathway by two pattern recognition receptors. Science **302**: 2126–2130.

Goldman, W.E., Klapper, D.G., and Baseman, J.B. (1982). Detection, isolation, and analysis of a released Bordetella pertussis product toxic to cultured tracheal cells. Infect Immun **36**: 782–794.
Gonzalez, I., Aparicio, R., and Busturia, A. (2008). Functional characterization of the dRYBP gene in Drosophila. Genetics **179**: 1373–1388.
Gonzalez, I., and Busturia, A. (2009). High levels of dRYBP induce apoptosis in Drosophila imaginal cells through the activation of reaper and the requirement of trithorax, dredd and dFADD. Cell Res **19**: 747–757.
Gordon, M.D., Dionne, M.S., Schneider, D.S., and Nusse, R. (2005). WntD is a feedback inhibitor of Dorsal/NF-kappaB in Drosophila development and immunity. Nature **437**: 746–749.
Gottar, M. et al. (2006). Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. Cell **127**: 1425–1437.
Gottar, M. et al. (2002). The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature **416**: 640–644.
Guan, R., and Mariuzza, R.A. (2007). Peptidoglycan recognition proteins of the innate immune system. Trends Microbiol **15**: 127–134.
Guntermann, S., Primrose, D.A., and Foley, E. (2009). Dnr1-dependent regulation of the Drosophila immune deficiency signaling pathway. Dev Comp Immunol **33**: 127–134.
Ha, E.M. et al. (2009a). Regulation of DUOX by the Galphaq-phospholipase Cbeta-Ca2+ pathway in Drosophila gut immunity. Dev Cell **16**: 386–397.
Ha, E.M. et al. (2009b). Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in drosophila gut. Nat Immunol **10**: 949–957.
Ha, E.M., Oh, C.T., Bae, Y.S., and Lee, W.J. (2005a). A direct role for dual oxidase in Drosophila gut immunity. Science **310**: 847–850.
Ha, E.M. et al. (2005b). An antioxidant system required for host protection against gut infection in Drosophila. Dev Cell **8**: 125–132.
Haas, T.L. et al. (2009). Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. Mol Cell **36**: 831–844.
Hacker, H., and Karin, M. (2006). Regulation and function of IKK and IKK-related kinases. Sci STKE **2006**: re13.
Hardy, M.P., and O'Neill, L.A. (2004). The murine IRAK2 gene encodes four alternatively spliced isoforms, two of which are inhibitory. J Biol Chem **279**: 27699–27708.
Harrison, D.A., Binari, R., Nahreini, T.S., Gilman, M., and Perrimon, N. (1995). Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. Embo J **14**: 2857–2865.
Hasegawa, M. et al. (2008). A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation. EMBO J **27**: 373–383.
Hedengren, M. et al. (1999). Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. Mol. Cell **4**: 827–837.
Hershko, A. (1983). Ubiquitin: roles in protein modification and breakdown. Cell **34**: 11–12.

Hoeller, D., Hecker, C.M., and Dikic, I. (2006). Ubiquitin and ubiquitin-like proteins in cancer pathogenesis. Nat Rev Cancer **6**: 776–788.
Hoffmann, A., and Baltimore, D. (2006). Circuitry of nuclear factor kappaB signaling. Immunol Rev **210**: 171–186.
Hoffmann, A., Neumann, P., Schierhorn, A., and Stubbs, M.T. (2008). Crystallization of Spatzle, a cystine-knot protein involved in embryonic development and innate immunity in Drosophila melanogaster. Acta Crystallogr Sect F Struct Biol Cryst Commun **64**: 707–710.
Hoffmann, J.A., and Reichhart, J.M. (2002). Drosophila innate immunity: an evolutionary perspective. Nat Immunol **3**: 121–126.
Hofmann, R.M., and Pickart, C.M. (1999). Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell **96**: 645–653.
Holland, P.M., Suzanne, M., Campbell, J.S., Noselli, S., and Cooper, J.A. (1997). MKK7 is a stress-activated mitogen-activated protein kinase kinase functionally related to hemipterous. J Biol Chem **272**: 24994–24998.
Horng, T., and Medzhitov, R. (2001). Drosophila MyD88 is an adapter in the Toll signaling pathway. Proc Natl Acad Sci U S A **98**: 12654–12658.
Hoskins, R.A. et al. (2002). Heterochromatic sequences in a Drosophila whole-genome shotgun assembly. Genome Biol **3**: RESEARCH0085.
Hu, X., Yagi, Y., Tanji, T., Zhou, S., and Ip, Y.T. (2004). Multimerization and interaction of Toll and Spatzle in Drosophila. Proc Natl Acad Sci U S A **101**: 9369–9374.
Hultmark, D. (2003). Drosophila immunity: paths and patterns. Curr Opin Immunol **15**: 12–19.
Inohara, N. et al. (1999). Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. J Biol Chem **274**: 14560–1457.
Inohara, N. et al. (2003). Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J Biol Chem **278**: 5509–5512.
Irving, P. et al. (2001). A genome-wide analysis of immune responses in Drosophila. Proc Natl Acad Sci U S A **98**: 15119–15124.
Ishii, K.J. et al. (2008). TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature **451**: 725–729.
Ishitani, T. et al. (2003). Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling. EMBO J **22**: 6277–6288.
Isoda, K., and Nusslein-Volhard, C. (1994). Disulfide cross-linking in crude embryonic lysates reveals three complexes of the Drosophila morphogen dorsal and its inhibitor cactus. Proc Natl Acad Sci U S A **91**: 5350–5354.
Ito, K., Awano, W., Suzuki, K., Hiromi, Y., and Yamamoto, D. (1997). The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. Development **124**: 761–771.
Iwaki, D. et al. (2002). The extracellular toll-like receptor 2 domain directly binds peptidoglycan derived from Staphylococcus aureus. J Biol Chem **277**: 24315–24320.
Iwami, K.I. et al. (2000). Cutting edge: naturally occurring soluble form of mouse Toll-like receptor 4 inhibits lipopolysaccharide signaling. J Immunol **165**: 6682–6686.
James, P., Halladay, J., and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics **144**: 1425–1436.

Janeway, C.A.J., and Medzhitov, R. (2002). Innate immune recognition. Annu Rev Immunol **20**: 197–216.
Jang, I.H. et al. (2006). A Spatzle-processing enzyme required for toll signaling activation in Drosophila innate immunity. Dev Cell **10**: 45–55.
Janssens, S., Burns, K., Tschopp, J., and Beyaert, R. (2002). Regulation of interleukin-1- and lipopolysaccharide-induced NF-kappaB activation by alternative splicing of MyD88. Curr Biol **12**: 467–471.
Jiang, Z., Ninomiya-Tsuji, J., Qian, Y., Matsumoto, K., and Li, X. (2002). Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. Mol Cell Biol **22**: 7158–7167.
Jin, L., Williamson, A., Banerjee, S., Philipp, I., and Rape, M. (2008). Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. Cell **133**: 653–665.
Kambris, Z. et al. (2006). Drosophila immunity: a large-scale in vivo RNAi screen identifies five serine proteases required for Toll activation. Curr Biol **16**: 808–813.
Kanayama, A. et al. (2004). TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Mol Cell **15**: 535–548.
Kaneko, T. et al. (2004). Monomeric and Polymeric Gram-Negative Peptidoglycan but Not Purified LPS Stimulate the Drosophila IMD Pathway. Immunity **20**: 637–649.
Kaneko, T., Golenbock, D., and Silverman, N. (2005). Peptidoglycan recognition by the Drosophila Imd pathway. J Endotoxin Res **11**: 383–389.
Kaneko, T., and Silverman, N. (2005). Bacterial recognition and signalling by the Drosophila IMD pathway. Cell Microbiol **7**: 461–469.
Kaneko, T. et al. (2006). PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. Nat Immunol **7**: 715–723.
Kang, D., Liu, G., Lundstrom, A., Gelius, E., and Steiner, H. (1998). A peptidoglycan recognition protein in innate immunity conserved from insects to humans. Proc Natl Acad Sci U S A **95**: 10078–10082.
Kawagoe, T. et al. (2009). TANK is a negative regulator of Toll-like receptor signaling and is critical for the prevention of autoimmune nephritis. Nat Immunol **10**: 965–972.
Kawauchi, J. et al. (2002). Transcriptional repressor activating transcription factor 3 protects human umbilical vein endothelial cells from tumor necrosis factor-alpha-induced apoptosis through down-regulation of p53 transcription. J Biol Chem **277**: 39025–39034.
Kayagaki, N. et al. (2007). DUBA: a deubiquitinase that regulates type I interferon production. Science **318**: 1628–1632.
Khush, R.S., Cornwell, W.D., Uram, J.N., and Lemaitre, B. (2002a). A ubiquitin-proteasome pathway represses the Drosophila immune deficiency signaling cascade. Curr Biol **12**: 1728–1737.
Khush, R.S., Leulier, F., and Lemaitre, B. (2002b). Immunology. Pathogen surveillance--the flies have it. Science **296**: 273–275.
Kim, L.K. et al. (2007). Down-regulation of NF-kappaB target genes by the AP-1 and STAT complex during the innate immune response in Drosophila. PLoS Biol **5**: e238.

Kim, M., Lee, J.H., Lee, S.Y., Kim, E., and Chung, J. (2006). Caspar, a suppressor of antibacterial immunity in Drosophila. Proc Natl Acad Sci U S A **103**: 16358–16363.
Kim, M.S., Byun, M., and Oh, B.H. (2003). Crystal structure of peptidoglycan recognition protein LB from Drosophila melanogaster. Nat Immunol **4**: 787–793.
Kim, T. et al. (2005). Downregulation of lipopolysaccharide response in Drosophila by negative crosstalk between the AP1 and NF-kappaB signaling modules. Nat Immunol **6**: 211–218.
Kim, Y.-S. et al. (2000). Lipopolysaccharide-activated kinase, an essential component for the induction of the antimicrobial peptide genes in *Drosophila melanogaster* cells. J. Biol. Chem. **275**: 2071–2079.
Kinjyo, I. et al. (2002). SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity **17**: 583–591.
Kirisako, T. et al. (2006). A ubiquitin ligase complex assembles linear polyubiquitin chains. EMBO J **25**: 4877–4887.
Kirkpatrick, D.S. et al. (2006). Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. Nat Cell Biol **8**: 700–710.
Kishida, S., Sanjo, H., Akira, S., Matsumoto, K., and Ninomiya-Tsuji, J. (2005). TAK1-binding protein 2 facilitates ubiquitination of TRAF6 and assembly of TRAF6 with IKK in the IL-1 signaling pathway. Genes Cells **10**: 447–454.
Kishimoto, K., Matsumoto, K., and Ninomiya-Tsuji, J. (2000). TAK1 mitogen-activated protein kinase kinase kinase is activated by autophosphorylation within its activation loop. J Biol Chem **275**: 7359–7364.
Kleino, A. et al. (2008). Pirk is a negative regulator of the Drosophila Imd pathway. J Immunol **180**: 5413–5422.
Kleino, A. et al. (2005). Inhibitor of apoptosis 2 and TAK1-binding protein are components of the Drosophila Imd pathway. Embo J **24**: 3423–3434.
Kobayashi, K. et al. (2002). IRAK-M is a negative regulator of Toll-like receptor signaling. Cell **110**: 191–202.
Komander, D. et al. (2009). Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. EMBO Rep **10**: 466–473.
Koropatnick, T.A. et al. (2004). Microbial factor-mediated development in a host-bacterial mutualism. Science **306**: 1186–1188.
Krikos, A., Laherty, C.D., and Dixit, V.M. (1992). Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements. J Biol Chem **267**: 17971–17976.
Kulathu, Y., Akutsu, M., Bremm, A., Hofmann, K., and Komander, D. (2009). Two-sided ubiquitin binding explains specificity of the TAB2 NZF domain. Nat Struct Mol Biol **16**: 1328–1330.
Lamothe, B. et al. (2007). Site-specific Lys-63-linked tumor necrosis factor receptor-associated factor 6 auto-ubiquitination is a critical determinant of I kappa B kinase activation. J Biol Chem **282**: 4102–4112.
Lang, T., and Mansell, A. (2007). The negative regulation of Toll-like receptor and associated pathways. Immunol Cell Biol **85**: 425–434.
Laplantine, E. et al. (2009). NEMO specifically recognizes K63-linked poly-ubiquitin chains through a new bipartite ubiquitin-binding domain. EMBO J **28**: 2885–2895.

Latz, E. et al. (2004). TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol **5**: 190–198.
LeBouder, E. et al. (2003). Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. J Immunol **171**: 6680–6689.
Lemaitre, B. (2004). The road to Toll. Nat Rev Immunol **4**: 521–527.
Lemaitre, B., and Hoffmann, J. (2007). The Host Defense of Drosophila melanogaster. Annu Rev Immunol **25**: 697–743.
Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell **86**: 973–983.
Lemaitre, B., Reichhart, J.M., and Hoffmann, J.A. (1997). Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc. Natl. Acad. Sci. USA **94**: 14614–14619.
Lengyel, J.A., and Iwaki, D.D. (2002). It takes guts: the Drosophila hindgut as a model system for organogenesis. Dev Biol **243**: 1–19.
Leone, P. et al. (2008). Crystal structure of Drosophila PGRP-SD suggests binding to DAP-type but not lysine-type peptidoglycan. Mol Immunol **45**: 2521–2530.
Leulier, F. et al. (2003). The Drosophila immune system detects bacteria through specific peptidoglycan recognition. Nat Immunol **4**: 478–484.
Leulier, F., Rodriguez, A., Khush, R.S., Abrams, J.M., and Lemaitre, B. (2000). The *Drosophila* caspase Dredd is required to resist Gram-negative bacterial infection. EMBO Reports **1**: 353–358.
Lhocine, N. et al. (2008). PIMS modulates immune tolerance by negatively regulating Drosophila innate immune signaling. Cell Host Microbe **4**: 147–158.
Li, H., Kobayashi, M., Blonska, M., You, Y., and Lin, X. (2006). Ubiquitination of RIP is required for tumor necrosis factor alpha-induced NF-kappaB activation. J Biol Chem **281**: 13636–13643.
Liehl, P., Blight, M., Vodovar, N., Boccard, F., and Lemaitre, B. (2006). Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS Pathog **2**: e56.
Liew, F.Y., Xu, D., Brint, E.K., and O'Neill, L.A. (2005). Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol **5**: 446–458.
Lim, J.H. et al. (2006). Structural Basis for Preferential Recognition of Diaminopimelic Acid-type Peptidoglycan by a Subset of Peptidoglycan Recognition Proteins. J Biol Chem **281**: 8286–8295.
Liu, C., Gelius, E., Liu, G., Steiner, H., and Dziarski, R. (2000). Mammalian peptidoglycan recognition protein binds peptidoglycan with high affinity, is expressed in neutrophils, and inhibits bacterial growth. J Biol Chem **275**: 24490–24499.
Liu, Z.P., Galindo, R.L., and Wasserman, S.A. (1997). A role for CKII phosphorylation of the cactus PEST domain in dorsoventral patterning of the Drosophila embryo. Genes Dev **11**: 3413–3422.
Lo, D. et al. (2003). Peptidoglycan recognition protein expression in mouse Peyer's Patch follicle associated epithelium suggests functional specialization. Cell Immunol **224**: 8–16.

Lo, Y.C. et al. (2009). Structural basis for recognition of diubiquitins by NEMO. Mol Cell **33**: 602–615.
Lossi, L., and Merighi, A. (2003). In vivo cellular and molecular mechanisms of neuronal apoptosis in the mammalian CNS. Prog Neurobiol **69**: 287–312.
Lu, X. et al. (2006). Peptidoglycan recognition proteins are a new class of human bactericidal proteins. J Biol Chem **281**: 5895–5907.
Lu, Y., Wu, L.P., and Anderson, K.V. (2001). The antibacterial arm of the drosophila innate immune response requires an IkappaB kinase. Genes Dev **15**: 104–10.
Maillet, F., Bischoff, V., Vignal, C., Hoffmann, J., and Royet, J. (2008). The Drosophila peptidoglycan recognition protein PGRP-LF blocks PGRP-LC and IMD/JNK pathway activation. Cell Host Microbe **3**: 293–303.
Manfruelli, P., Reichhart, J.M., Steward, R., Hoffmann, J.A., and Lemaitre, B. (1999). A mosaic analysis in Drosophila fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. EMBO J **18**: 3380–3391.
Mansell, A. et al. (2006). Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. Nat Immunol **7**: 148–155.
Martinon, F., and Tschopp, J. (2005). NLRs join TLRs as innate sensors of pathogens. Trends Immunol **26**: 447–454.
Mathur, P. et al. (2004). Murine peptidoglycan recognition proteins PglyrpIalpha and PglyrpIbeta are encoded in the epidermal differentiation complex and are expressed in epidermal and hematopoietic tissues. Genomics **83**: 1151–1163.
Matova, N., and Anderson, K.V. (2006). Rel/NF-kappaB double mutants reveal that cellular immunity is central to Drosophila host defense. Proc Natl Acad Sci U S A **103**: 16424–16429.
Mayr, B.M., Canettieri, G., and Montminy, M.R. (2001). Distinct effects of cAMP and mitogenic signals on CREB-binding protein recruitment impart specificity to target gene activation via CREB. Proc Natl Acad Sci U S A **98**: 10936–10941.
McGettigan, J. et al. (2005). Insect renal tubules constitute a cell-autonomous immune system that protects the organism against bacterial infection. Insect Biochem Mol Biol **35**: 741–754.
Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. Nature **449**: 819–826.
Meister, M., Lemaitre, B., and Hoffmann, J.A. (1997). Antimicrobial peptide defense in Drosophila. Bioessays **19**: 1019–1026.
Mellroth, P. et al. (2005). Ligand-induced dimerization of Drosophila peptidoglycan recognition proteins in vitro. Proc Natl Acad Sci U S A **102**: 6455–6460.
Mellroth, P., Karlsson, J., and Steiner, H. (2003). A scavenger function for a Drosophila peptidoglycan recognition protein. J Biol Chem **278**: 7059–7064.
Melly, M.A., McGee, Z.A., and Rosenthal, R.S. (1984). Ability of monomeric peptidoglycan fragments from Neisseria gonorrhoeae to damage human fallopian-tube mucosa. J Infect Dis **149**: 378–386.
Mengin-Lecreulx, D., and Lemaitre, B. (2005). Structure and metabolism of peptidoglycan and molecular requirements allowing its detection by the Drosophila innate immune system. J Endotoxin Res **11**: 105–111.
Mercurio, F. et al. (1997). IKK-1 and IKK-2: cytokine-activated IkB kinases essential for NF- kB activation. Science **278**: 860–866.

Meylan, E. et al. (2005). Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature **437**: 1167–1172.
Meylan, E. et al. (2004). RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol **5**: 503–507.
Meylan, E., Tschopp, J., and Karin, M. (2006). Intracellular pattern recognition receptors in the host response. Nature **442**: 39–44.
Micheau, O., and Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell **114**: 181–190.
Michel, T., Reichhart, J.M., Hoffmann, J.A., and Royet, J. (2001). Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature **414**: 756–759.
Mishima, Y. et al. (2009). The N-terminal domain of Drosophila Gram-negative binding protein 3 (GNBP3) defines a novel family of fungal pattern recognition receptors. J Biol Chem **284**: 28687–28697.
Muzio, M., Ni, J., Feng, P., and Dixit, V.M. (1997). IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science **278**: 1612–165.
Naka, T. et al. (1998). Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice. Proc Natl Acad Sci U S A **95**: 15577–15582.
Nakagawa, R. et al. (2002). SOCS-1 participates in negative regulation of LPS responses. Immunity **17**: 677–687.
Newton, K. et al. (2008). Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. Cell **134**: 668–678.
Nicolas, E., Reichhart, J.M., Hoffmann, J.A., and Lemaitre, B. (1998). In vivo regulation of the IkappaB homologue cactus during the immune response of Drosophila. J Biol Chem **273**: 10463–10469.
Nishiya, T., Kajita, E., Miwa, S., and Defranco, A.L. (2005). TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. J Biol Chem **280**: 37107–37117.
O'Neill, L.A. (2003). The role of MyD88-like adapters in Toll-like receptor signal transduction. Biochem Soc Trans **31**: 643–647.
Ochiai, M., and Ashida, M. (1999). A pattern recognition protein for peptidoglycan. Cloning the cDNA and the gene of the silkworm, Bombyx mori. J Biol Chem **274**: 11854–11858.
Oganesyan, G. et al. (2006). Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature **439**: 208–211.
Ogura, Y. et al. (2001). Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF{kappa}B. J Biol Chem **276**: 4812–4818.
Opipari, A.W.J., Boguski, M.S., and Dixit, V.M. (1990). The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. J Biol Chem **265**: 14705–14708.
Paquette, N. et al. (2010). Caspase-mediated cleavage, IAP binding, and ubiquitination: linking three mechanisms crucial for Drosophila NF-kappaB signaling. Mol Cell **37**: 172–182.
Park, J.M. et al. (2004). Targeting of TAK1 by the NF-kappa B protein Relish regulates the JNK-mediated immune response in Drosophila. Genes Dev **18**: 584–594.

Park, J.W. et al. (2007). Clustering of peptidoglycan recognition protein-SA is required for sensing lysine-type peptidoglycan in insects. Proc Natl Acad Sci U S A **104**: 6602–6607.
Park, J.M., and Gambhir, S.S. (2005). Multimodality Radionuclide, Fluorescence, and Bioluminescence Small-Animal Imaging. IEEE **93**: 771–.
Park, M.Y., Jang, H.D., Lee, S.Y., Lee, K.J., and Kim, E. (2004). Fas-associated factor-1 inhibits nuclear factor-kappaB (NF-kappaB) activity by interfering with nuclear translocation of the RelA (p65) subunit of NF-kappaB. J Biol Chem **279**: 2544–2549.
Pasare, C., and Medzhitov, R. (2004). Toll-like receptors and acquired immunity. Semin Immunol **16**: 23–26.
Perkins, N.D. et al. (1997). Regulation of NF-kappaB by cyclin-dependent kinases associated with the p300 coactivator. Science **275**: 523–527.
Persson, C., Oldenvi, S., and Steiner, H. (2007). Peptidoglycan recognition protein LF: a negative regulator of Drosophila immunity. Insect Biochem Mol Biol **37**: 1309–1316.
Pickart, C.M. (2004). Back to the future with ubiquitin. Cell **116**: 181–190.
Pirity, M.K. et al. (2007). Rybp, a polycomb complex-associated protein, is required for mouse eye development. BMC Dev Biol **7**: 39.
Qin, J., Jiang, Z., Qian, Y., Casanova, J.L., and Li, X. (2004). IRAK4 kinase activity is redundant for interleukin-1 (IL-1) receptor-associated kinase phosphorylation and IL-1 responsiveness. J Biol Chem **279**: 26748–26753.
Qiu, P., Pan, P.C., and Govind, S. (1998). A role for the Drosophila Toll/Cactus pathway in larval hematopoiesis. Development **125**: 1909–1920.
Qureshi, S.T. et al. (1999). Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med **189**: 615–625.
Rahighi, S. et al. (2009). Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation. Cell **136**: 1098–1109.
Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., and Ezekowitz, R.A. (2002). Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli. Nature **416**: 644–648.
Ramet, M. et al. (2001). Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity **15**: 1027–1038.
Reach, M. et al. (1996). A gradient of cactus protein degradation establishes dorsoventral polarity in the Drosophila embryo. Dev Biol **180**: 353–364.
Rebsamen, M. et al. (2009). DAI/ZBP1 recruits RIP1 and RIP3 through RIP homotypic interaction motifs to activate NF-kappaB. EMBO Rep **10**: 916–922.
Regamey, A. et al. (2003). The tumor suppressor CYLD interacts with TRIP and regulates negatively nuclear factor kappaB activation by tumor necrosis factor. J Exp Med **198**: 1959–1964.
Ren, C., Webster, P., Finkel, S.E., and Tower, J. (2007). Increased internal and external bacterial load during Drosophila aging without life-span trade-off. Cell Metab **6**: 144–152.
Rosenthal, R.S. (1979). Release of soluble peptidoglycan from growing gonococci: hexaminidase and amidase activities. Infect Immun **24**: 869–878.
Rothwarf, D.M., Zandi, E., Natoli, G., and Karin, M. (1998). IKKg is an essential regulatory subunit of the IkB kinase complex. Nature **395**: 297–300.

Rowe, D.C. et al. (2006). The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. Proc Natl Acad Sci U S A **103**: 6299–6304.
Royet, J., and Dziarski, R. (2007). Peptidoglycan recognition proteins: pleiotropic sensors and effectors of antimicrobial defences. Nat Rev Microbiol **5**: 264–277.
Rutschmann, S. et al. (2000). Role of Drosophila IKK gamma in a toll-independent antibacterial immune response. Nat Immunol **1**: 342–347.
Ryu, J.H. et al. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. Science **319**: 777–782.
Ryu, S.W. et al. (2003). Fas-associated factor 1, FAF1, is a member of Fas death-inducing signaling complex. J Biol Chem **278**: 24003–24010.
Saha, S. et al. (2009). PGLYRP-2 and Nod2 are both required for peptidoglycan-induced arthritis and local inflammation. Cell Host Microbe **5**: 137–150.
Sakurai, H., Miyoshi, H., Mizukami, J., and Sugita, T. (2000). Phosphorylation-dependent activation of TAK1 mitogen-activated protein kinase kinase kinase by TAB1. FEBS Lett **474**: 141–145.
Samakovlis, C., Åsling, B., Boman, H.G., Gateff, E., and Hultmark, D. (1992). In vitro induction of cecropin genes--an immune response in a Drosophila blood cell line. Biochem. Biophys. Res. Commun. **188**: 1169–1175.
Samakovlis, C., Kimbrell, D.A., Kylsten, P., Engstrom, A., and Hultmark, D. (1990). The immune response in Drosophila: pattern of cecropin expression and biological activity. Embo J **9**: 2969–2976.
Santamaria, P., and Nusslein-Volhard, C. (1983). Partial rescue of dorsal, a maternal effect mutation affecting the dorso-ventral pattern of the Drosophila embryo, by the injection of wild-type cytoplasm. Embo J **2**: 1695–1699.
Sato, S. et al. (2003). Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J Immunol **171**: 4304–4310.
Sato, Y., Yoshikawa, A., Yamashita, M., Yamagata, A., and Fukai, S. (2009). Structural basis for specific recognition of Lys 63-linked polyubiquitin chains by NZF domains of TAB2 and TAB3. EMBO J **28**: 3903–3909.
Sawa, C. et al. (2002). YEAF1/RYBP and YAF-2 are functionally distinct members of a cofactor family for the YY1 and E4TF1/hGABP transcription factors. J Biol Chem **277**: 22484–22490.
Schilling, D., Thomas, K., Nixdorff, K., Vogel, S.N., and Fenton, M.J. (2002). Toll-like receptor 4 and Toll-IL-1 receptor domain-containing adapter protein (TIRAP)/myeloid differentiation protein 88 adapter-like (Mal) contribute to maximal IL-6 expression in macrophages. J Immunol **169**: 5874–5880.
Schleifer, K.H., and Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev **36**: 407–477.
Schlisio, S., Halperin, T., Vidal, M., and Nevins, J.R. (2002). Interaction of YY1 with E2Fs, mediated by RYBP, provides a mechanism for specificity of E2F function. EMBO J **21**: 5775–5786.

Schmitz, J. et al. (2005). IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity **23**: 479–490.
Senger, K. et al. (2004). Immunity regulatory DNAs share common organizational features in Drosophila. Mol Cell **13**: 19–32.
Sharma, S. et al. (2003). Triggering the interferon antiviral response through an IKK-related pathway. Science **300**: 1148–1151.
Shen, B., Liu, H., Skolnik, E.Y., and Manley, J.L. (2001). Physical and functional interactions between Drosophila TRAF2 and Pelle kinase contribute to Dorsal activation. Proc Natl Acad Sci U S A **98**: 8596–8601.
Shim, J.H. et al. (2005). TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev **19**: 2668–2681.
Silverman, N. et al. (2003). Immune activation of NF-kappaB and JNK requires Drosophila TAK1. J Biol Chem **278**: 48928–48934.
Silverman, N. et al. (2000). A Drosophila IkappaB kinase complex required for Relish cleavage and antibacterial immunity. Genes Dev **14**: 2461–271.
Simon, J.A., and Kingston, R.E. (2009). Mechanisms of polycomb gene silencing: knowns and unknowns. Nat Rev Mol Cell Biol **10**: 697–708.
Sluss, H.K. et al. (1996). A JNK signal transduction pathway that mediates morphogenesis and an immune response in Drosophila. Genes Dev **10**: 2745–2758.
Spencer, E., Jiang, J., and Chen, Z.J. (1999). Signal-induced ubiquitination of IkBa by the F-box protein Slimb/bTrCP. Genes & Dev. **13**: 284–294.
Stanton, S.E., Blanck, J.K., Locker, J., and Schreiber-Agus, N. (2007). Rybp interacts with Hippi and enhances Hippi-mediated apoptosis. Apoptosis **12**: 2197–2206.
Stanton, S.E., McReynolds, L.J., Evans, T., and Schreiber-Agus, N. (2006). Yaf2 inhibits caspase 8-mediated apoptosis and regulates cell survival during zebrafish embryogenesis. J Biol Chem **281**: 28782–28793.
Starr, R. et al. (1998). Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. Proc Natl Acad Sci U S A **95**: 14395–14399.
Stenbak, C.R. et al. (2004). Peptidoglycan molecular requirements allowing detection by the Drosophila immune deficiency pathway. J Immunol **173**: 7339–7348.
Stöven, S., Ando, I., Kadalayil, L., Engström, Y., and Hultmark, D. (2000). Activation of the *Drosophila* NF-kB factor Relish by rapid endoproteolytic cleavage. EMBO Rep **1**: 347–352.
Stöven, S. et al. (2003). Caspase-mediated processing of the Drosophila NF-{kappa}B factor Relish. Proc Natl Acad Sci U S A
Strober, W., Murray, P.J., Kitani, A., and Watanabe, T. (2006). Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol **6**: 9–20.
Sun, H., Bristow, B.N., Qu, G., and Wasserman, S.A. (2002a). A heterotrimeric death domain complex in Toll signaling. Proc Natl Acad Sci U S A **99**: 12871–12876.
Sun, X., Yin, J., Starovasnik, M.A., Fairbrother, W.J., and Dixit, V.M. (2002b). Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3. J Biol Chem **277**: 9505–9511.
Suzuki, N., Suzuki, S., and Yeh, W.C. (2002). IRAK-4 as the central TIR signaling mediator in innate immunity. Trends Immunol **23**: 503–506.

Swaminathan, C.P. et al. (2006). Dual strategies for peptidoglycan discrimination by peptidoglycan recognition proteins (PGRPs). Proc Natl Acad Sci U S A **103**: 684–689.
Takaesu, G. et al. (2000). TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. Mol Cell **5**: 649–658.
Takaesu, G. et al. (2001). Interleukin-1 (IL-1) receptor-associated kinase leads to activation of TAK1 by inducing TAB2 translocation in the IL-1 signaling pathway. Mol Cell Biol **21**: 2475–284.
Takehana, A. et al. (2002). Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in Drosophila larvae. Proc Natl Acad Sci U S A **99**: 13705–13710.
Takehana, A. et al. (2004). Peptidoglycan Recognition Protein (PGRP)-LE and PGRP-LC act synergistically in *Drosophila* immunity. Embo J **23**: 4690–4700.
Tanji, T., Hu, X., Weber, A.N., and Ip, Y.T. (2007). Toll and IMD pathways synergistically activate innate immune response in Drosophila. Mol Cell Biol
Tauszig-Delamasure, S., Bilak, H., Capovilla, M., Hoffmann, J.A., and Imler, J.L. (2002). Drosophila MyD88 is required for the response to fungal and Gram-positive bacterial infections. Nat Immunol **3**: 91–97.
Thevenon, D. et al. (2009). The Drosophila ubiquitin-specific protease dUSP36/Scny targets IMD to prevent constitutive immune signaling. Cell Host Microbe **6**: 309–320.
Thomassen, E., Renshaw, B.R., and Sims, J.E. (1999). Identification and characterization of SIGIRR, a molecule representing a novel subtype of the IL-1R superfamily. Cytokine **11**: 389–399.
Thummel, C.S., Boulet, A.M., and Lipshitz, H.D. (1988). Vectors for Drosophila P-element-mediated transformation and tissue culture transfection. Gene **74**: 445–456.
Tokunaga, F. et al. (2009). Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. Nat Cell Biol **11**: 123–132.
Towb, P., Galindo, R.L., and Wasserman, S.A. (1998). Recruitment of Tube and Pelle to signaling sites at the surface of the Drosophila embryo. Development **125**: 2443–250.
Trompouki, E. et al. (2003). CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature **424**: 793–796.
Tsuda, M., Seong, K.H., and Aigaki, T. (2006). POSH, a scaffold protein for JNK signaling, binds to ALG-2 and ALIX in Drosophila. FEBS Lett **580**: 3296–3300.
Tydell, C.C., Yount, N., Tran, D., Yuan, J., and Selsted, M.E. (2002). Isolation, characterization, and antimicrobial properties of bovine oligosaccharide-binding protein. A microbicidal granule protein of eosinophils and neutrophils. J Biol Chem **277**: 19658–19664.
Tzou, P. et al. (2000). Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity **13**: 737–748.
Tzou, P., Reichhart, J.M., and Lemaitre, B. (2002). Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient Drosophila mutants. Proc Natl Acad Sci U S A **99**: 2152–2157.
Uehara, A. et al. (2006). Meso-diaminopimelic acid and meso-lanthionine, amino acids specific to bacterial peptidoglycans, activate human epithelial cells through NOD1. J Immunol **177**: 1796–1804.

Varfolomeev, E. et al. (2008). c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. J Biol Chem **283**: 24295–24299.
Viala, J. et al. (2004). Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immunol **5**: 1166–1174.
Vidal, S. et al. (2001). Mutations in the Drosophila dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses. Genes Dev **15**: 1900–1912.
Visintin, A., Latz, E., Monks, B.G., Espevik, T., and Golenbock, D.T. (2003). Lysines 128 and 132 enable LPS binding to MD-2, leading to Toll-like receptor 4 aggregation and signal transduction. J Biol Chem **278**: 48313–48320.
Wald, D. et al. (2003). SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. Nat Immunol **4**: 920–927.
Wang, C. et al. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature **412**: 346–351.
Wang, J., Hu, Y., Deng, W.W., and Sun, B. (2009). Negative regulation of Toll-like receptor signaling pathway. Microbes Infect **11**: 321–327.
Wang, L. et al. (2008). Peptidoglycan recognition protein-SD provides versatility of receptor formation in Drosophila immunity. Proc Natl Acad Sci U S A **105**: 11881–11886.
Wang, L. et al. (2006). Sensing of Gram-positive bacteria in Drosophila: GNBP1 is needed to process and present peptidoglycan to PGRP-SA. Embo J **25**: 5005–5014.
Wang, Z.M. et al. (2003). Human peptidoglycan recognition protein-L is an N-acetylmuramoyl-L-alanine amidase. J Biol Chem **278**: 49044–49052.
Weber, A.N. et al. (2003). Binding of the Drosophila cytokine Spatzle to Toll is direct and establishes signaling. Nat Immunol **4**: 794–800.
Werner, T., Borge-Renberg, K., Mellroth, P., Steiner, H., and Hultmark, D. (2003). Functional diversity of the Drosophila PGRP-LC gene cluster in the response to lipopolysaccharide and peptidoglycan. J Biol Chem **278**: 26319–26322.
Werner, T. et al. (2000). A family of peptidoglycan recognition proteins in the fruit fly Drosophila melanogaster. Proc Natl Acad Sci U S A **97**: 13772–13777.
Wertz, I.E. et al. (2004). De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature **430**: 694–699.
Wesche, H. et al. (1999). IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase (IRAK) familyh. J Biol Chem **274**: 19403–19410.
Wesche, H., Henzel, W.J., Shillinglaw, W., Li, S., and Cao, Z. (1997). MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity **7**: 837–847.
Windheim, M., Peggie, M., and Cohen, P. (2008). Two different classes of E2 ubiquitin-conjugating enzymes are required for the mono-ubiquitination of proteins and elongation by polyubiquitin chains with a specific topology. Biochem J **409**: 723–729.
Wooff, J., Pastushok, L., Hanna, M., Fu, Y., and Xiao, W. (2004). The TRAF6 RING finger domain mediates physical interaction with Ubc13. FEBS Lett **566**: 229–233.
Wu, H., Tschopp, J., and Lin, S.C. (2007). Smac mimetics and TNFalpha: a dangerous liaison? Cell **131**: 655–658.
Wu, L.P., and Anderson, K.V. (1998). Regulated nuclear import of Rel proteins in the Drosophila immune response. Nature **392**: 93–97.

Wu, L.P., Choe, K.M., Lu, Y., and Anderson, K.V. (2001). Drosophila immunity: genes on the third chromosome required for the response to bacterial infection. Genetics **159**: 189–199.
Xia, Z.P. et al. (2009). Direct activation of protein kinases by unanchored polyubiquitin chains. Nature **461**: 114–119.
Xu, M., Skaug, B., Zeng, W., and Chen, Z.J. (2009a). A ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK activation by TNFalpha and IL-1beta. Mol Cell **36**: 302–314.
Xu, P. et al. (2009b). Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. Cell **137**: 133–145.
Yajima, M. et al. (2003). A newly established in vitro culture using transgenic Drosophila reveals functional coupling between the phospholipase A2-generated fatty acid cascade and lipopolysaccharide-dependent activation of the immune deficiency (imd) pathway in insect immunity. Biochem J **371**: 205–210.
Yamamoto, M. et al. (2002). Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature **420**: 324–329.
Yamamoto, M. et al. (2003a). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol **4**: 1144–1150.
Yamamoto, M. et al. (2003b). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science **301**: 640–643.
Yamamoto, M. et al. (2002). Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J Immunol **169**: 6668–6672.
Yang, Q.H., and Du, C. (2004). Smac/DIABLO selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP and livin in HeLa cells. J Biol Chem **279**: 16963–16970.
Yano, T. et al. (2008). Autophagic control of listeria through intracellular innate immune recognition in drosophila. Nat Immunol **9**: 908–916.
Ye, Y., and Rape, M. (2009). Building ubiquitin chains: E2 enzymes at work. Nat Rev Mol Cell Biol **10**: 755–764.
Yoshida, H., Jono, H., Kai, H., and Li, J.D. (2005). The tumor suppressor cylindromatosis (CYLD) acts as a negative regulator for toll-like receptor 2 signaling via negative cross-talk with TRAF6 AND TRAF7. J Biol Chem **280**: 41111–41121.
Yoshida, H., Kinoshita, K., and Ashida, M. (1996). Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, Bombyx mori. J Biol Chem **271**: 13854–13860.
Yoshikawa, A. et al. (2009). Crystal structure of the NEMO ubiquitin-binding domain in complex with Lys 63-linked di-ubiquitin. FEBS Lett **583**: 3317–3322.
Zaidman-Remy, A. et al. (2006). The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection. Immunity **24**: 463–473.
Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M., and Karin, M. (1997). The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell **91**: 243–252.
Zerofsky, M., Harel, E., Silverman, N., and Tatar, M. (2005). Aging of the innate immune response in Drosophila melanogaster. Aging Cell **4**: 103–108.
Zettervall, C.J. et al. (2004). A directed screen for genes involved in Drosophila blood cell activation. Proc Natl Acad Sci U S A **101**: 14192–14197.

Zhang, Z. et al. (2005). The Vaccinia Virus N1L Protein Influences Cytokine Secretion in Vitro after Infection. Ann N Y Acad Sci **1056**: 69–86.
Zheng, L., Schickling, O., Peter, M.E., and Lenardo, M.J. (2001). The death effector domain-associated factor plays distinct regulatory roles in the nucleus and cytoplasm. J Biol Chem **276**: 31945–31952.
Zhou, R. et al. (2005). The role of ubiquitnation in Drosophila innate immunity. J Biol Chem