HENNA MYLLYMÄKI

Negative Regulation of *Drosophila* Immune Response

ACADEMIC DISSERTATION
To be presented, with the permission of the Board of the Institute of Biomedical Technology of the University of Tampere, for public discussion in the Auditorium of Finn-Medi 5, Biokatu 12, Tampere, on May 24th, 2013, at 12 o’clock.

UNIVERSITY OF TAMPERE
Fearlessness blew in with the wind

Waking up to greet the sun

(Tori Amos)

To my beloved ones
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The thesis is based on these publications, which are referred to with their roman numerals.


IV. Kallio J¹, **Myllymäki H**¹, Grönholm J, Armstrong M, Vanha-aho L-M, Mäkinen L, Silvennoinen O, Valanne S, Rämet M. Eye transformer is a negative regulator of *Drosophila* JAK/STAT signaling. *FASEB J* 24: 4467-79 (2010).⁴

¹ Equal contribution.
² The publication I was also used in the doctoral thesis of Jenni Kallio.
³ The publication III was also used in the doctoral thesis of Anni Kleino.
⁴ The publication IV was also used in the doctoral thesis of Juha Grönholm and Jenni Kallio.
<table>
<thead>
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<th>Abbreviations</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMP</td>
<td>antimicrobial peptide</td>
</tr>
<tr>
<td>AP-1</td>
<td>activating protein-1</td>
</tr>
<tr>
<td>Att</td>
<td>Attacin</td>
</tr>
<tr>
<td>bsk</td>
<td>basket</td>
</tr>
<tr>
<td>Cec</td>
<td>Cecropin</td>
</tr>
<tr>
<td>cfu</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>CYLD</td>
<td>cylindromatosis</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger-associated molecular pattern</td>
</tr>
<tr>
<td>Der-2</td>
<td>Dicer-2</td>
</tr>
<tr>
<td>DAP</td>
<td>diaminopimelic acid</td>
</tr>
<tr>
<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>Deaf-1</td>
<td>Deformed epidermal autoregulatory factor-1</td>
</tr>
<tr>
<td>Dif</td>
<td>Dorsal-related immunity factor</td>
</tr>
<tr>
<td>Dnr1</td>
<td>Defence repressor 1</td>
</tr>
<tr>
<td>Dome</td>
<td>Domeless</td>
</tr>
<tr>
<td>Dpt</td>
<td>Diptericin</td>
</tr>
<tr>
<td>Dredd</td>
<td>Death-related ced-3/Nedd2-like protein</td>
</tr>
<tr>
<td>Drs</td>
<td>Drosomycin</td>
</tr>
<tr>
<td>Dscam</td>
<td>Down syndrome cell adhesion molecule</td>
</tr>
<tr>
<td>Dsp1</td>
<td>Dorsal switch protein 1</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ET</td>
<td>eye transformer</td>
</tr>
<tr>
<td>Fadd</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GNBP</td>
<td>Gram-negative bacteria binding protein</td>
</tr>
<tr>
<td>Gprk2</td>
<td>G protein-coupled receptor kinase 2</td>
</tr>
<tr>
<td>Grass</td>
<td>Gram-positive-specific serine protease</td>
</tr>
<tr>
<td>hep</td>
<td>Hemipterous</td>
</tr>
<tr>
<td>hopTum-l</td>
<td>hopTumorous-lethal</td>
</tr>
<tr>
<td>hop</td>
<td>hopscotch</td>
</tr>
<tr>
<td>Iap</td>
<td>inhibitor of apoptosis</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IkB</td>
<td>inhibitor of NF-κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>imd</td>
<td>immune deficiency</td>
</tr>
<tr>
<td>IRAK</td>
<td>interleukin-1 receptor-associated kinase</td>
</tr>
<tr>
<td>ird5</td>
<td>immune response deficient 5</td>
</tr>
<tr>
<td>JAK</td>
<td>janus tyrosine kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>e-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Jra</td>
<td>Jun-related antigen</td>
</tr>
<tr>
<td>Key</td>
<td>Kenny</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>ModSP</td>
<td>modular serine protease</td>
</tr>
<tr>
<td>Mop</td>
<td>Myopic</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>Not4</td>
<td>Cnot 4 homologue</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGN</td>
<td>peptidoglycan</td>
</tr>
<tr>
<td>PGRP</td>
<td>peptidoglycan recognition protein</td>
</tr>
<tr>
<td>pirk</td>
<td>poor Imd response upon knock-in</td>
</tr>
<tr>
<td>POSH</td>
<td>Plenty of SH3s</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition protein</td>
</tr>
<tr>
<td>PSC</td>
<td>posterior signaling center</td>
</tr>
<tr>
<td>Ptp61F</td>
<td>Protein tyrosine phosphatase 61F</td>
</tr>
<tr>
<td>puc</td>
<td>puckered</td>
</tr>
<tr>
<td>Pvr</td>
<td>PDGF- and VEGF-receptor related</td>
</tr>
<tr>
<td>Rel</td>
<td>Relish</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>RIP-1</td>
<td>receptor interacting protein 1</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>S2</td>
<td>Schneider 2</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp1/Cullin/F-box</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of Cytokine Signaling</td>
</tr>
<tr>
<td>SPE</td>
<td>Spätzle-processing enzyme</td>
</tr>
<tr>
<td>spz</td>
<td>spätzle</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-like modifier</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Tab2</td>
<td>TAK1-binding protein</td>
</tr>
<tr>
<td>Tak1</td>
<td>transforming growth factor-β-activated protein kinase 1</td>
</tr>
<tr>
<td>TEP</td>
<td>thioester-containing protein</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis receptor</td>
</tr>
<tr>
<td>Tot</td>
<td>Turandot</td>
</tr>
<tr>
<td>TRAF</td>
<td>tumor necrosis factor-associated factor</td>
</tr>
<tr>
<td>upd</td>
<td>unpaired</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>zfh1</td>
<td>zn finger homeodomain 1</td>
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</table>
Abstract

The human immune system consists of innate and adaptive immunity. Although the adaptive immune response can be highly specific against various pathogens, it takes days to get fully operational. Over those days the innate immunity is needed for limiting the infection as well as regulating the initiation of the adaptive response. Innate immune reactions are regulated by signaling cascades, which are activated by receptors that recognize microbial structures and secreted cytokine molecules. Both the Toll-like receptor (TLR) signaling cascade and the tumor necrosis factor (TNFR) signaling cascade lead to activation of the transcription factor NF-κB and expression of its numerous target genes. Another group of important regulators of inflammatory and stress responses are the Signal Transducer and Activator of Transcription (STAT) molecules, which are activated by several cytokines, such as interleukins, and the Janus kinases (JAKs). Deficiencies in the signaling cascades regulating the innate immune responses predispose people to infections, whereas too vigorous or inappropriate immune reactions cause several inflammatory and autoimmune diseases. Because the JAK/STAT signaling cascade is important in the regulation of many other biological processes as well, for example the proliferation and differentiation of blood cells, disturbances in the signaling can also lead to hematopoietic diseases and cancer.

The fruit fly *Drosophila melanogaster* has long served as an amenable and widely-used model organism in various aspects of biological research, including studying innate immunity. The usefulness of the fruit fly in this branch of research was demonstrated for example by the nomination of the Nobel Prize in Medicine in 2011 to Jules Hoffmann and Bruce Beutler for their findings showing the similarity between the human and *Drosophila* innate immune systems.

The Toll, Imd and JAK/STAT signaling cascades found in *Drosophila* closely resemble the TLR, TNFR and JAK/STAT pathways in humans. As the Toll and Imd pathways lead to production of antimicrobial peptides, they play a key role in flies’ resistance against pathogenic bacteria, fungi and yeast, whereas the JAK/STAT pathway regulates stress responses and cell-mediated immunity. Although these pathways have been intensely studied during the last decades, and it has also become clear that they need to be tightly controlled; in *Drosophila*, the molecules and mechanisms responsible for their negative regulation have remained largely elusive.
The aim of this study was to identify and characterize novel gene products that negatively regulate the *Drosophila* immune response, as well as assess their possible conservation between flies and man. In order to identify novel gene products, an RNA interference –based screen was conducted for each signaling pathway in cultured *Drosophila* cells. Thereafter the identified genes were further studied.

Two novel regulators were identified for Imd signaling: a nuclear transcription factor zfh1 and pirk, which is a target gene of Imd signaling. Overexpression of *pirk* silenced Imd pathway response also in vivo, making the flies more susceptible to bacterial infection. Pirk was shown to interact with the pathway receptor PGRP-LC and the closest downstream component imd, which suggests it might act by disturbing the function of the receptor complex. Zfh1 regulates at least some of the Imd pathway target genes also in vivo, and its human homolog ZEB1 appears to participate in regulation of TNFR signaling in human cells.

A new mechanism for JAK/STAT pathway regulation was also discovered. ET is a cell membrane protein, which resembles the *Drosophila* JAK/STAT pathway receptor Dome as well as the human IL-6 receptor and its associated protein gp130. ET was shown to negatively regulate JAK/STAT signaling both in vitro and in vivo using *ET* RNAi flies and several in vivo assays. In addition, ET interacts with the receptor Dome and the kinase hop, and thereby regulates the phosphorylation and activation of the transcription factor Stat92E. New candidates for negative regulators of *Drosophila* Toll pathway were also identified.

This study shows that the signaling cascades mediating *Drosophila* immune response are regulated by several molecules and at multiple levels, using mechanisms such as transcriptional regulation, negative feed-back loops and inhibition of receptor complexes.
Tiivistelmä


Tässä tutkimuksessa pyrittiin löytämään ja karakterisoimaan uusia banaanikärpäsen immuunipuolustusta negatiivisesti säätelyviä geenituotteita ja mekanismeja, sekä tutkimaan niiden mahdollista konservoituneisuutta kärpäsen ja ihmisen välillä. Uusien säätelymolekyylien löytämiseksi tehtiin kullekin signalointireitille genominlaajuinen RNA-häirintään (RNAi) perustuva seulonta viljellyissä banaanikärpäsen soluissa, minkä jälkeen löydettyjen geenituotteiden merkitystä kärpäsen immuunipuolustukselle tutkittiin tarkemmin.


Tutkimus osoittaa, että banaanikärpäsen immuunipuolustuksen signalointiketjujen säätelyyn osallistuu useita molekyyylejä, ja säätelyä tapahtuu monella tasolla, mukaan lukien transkriptiotason säätely, ns. negatiivisten feed-back looppien käyttö, sekä reseptorikompleksien toiminnan estäminen.
1. Introduction

Our immune systems manage to protect us by recognizing pathogenic microbes and initiating an effective response to fight them. However, in order to protect us from excessive tissue damage caused by the inflammatory responses themselves, the immune system also has to know when to terminate the response, and be sure not to attack self-molecules. At the same time, billions of commensal microbes, characterized by similar surface molecular structures as the pathogenic ones, reside within our bodies, but are left intact by the immune system. It remains largely unknown how this homeostasis is maintained. On the other hand, the consequences of losing the homeostasis by launching an immune reaction in absence of pathogenic microbes are well-known and tragic, including inflammatory autoimmune diseases, such as rheumatoid arthritis, asthma, allergy and septic shock.

Like their mammalian counterparts, the *Drosophila* immune responses are also under tight spatial and temporal control. Moreover, the *Drosophila* gut and other epithelial surfaces harbor a substantial population of commensal microbes, although with less species variation than the human gut. Thus it is clear that, like humans, *Drosophila* needs to control its immune reactions in order to avoid chronic inflammation, and can therefore provide an amenable system to elucidate the temporal and spatial control of human immune responses.

This study focused on the evolutionary conserved signaling pathways controlling innate immune responses in *Drosophila*, namely the Imd, Toll and JAK/STAT pathways. The basis was to discover new gene products that negatively regulate these pathways by carrying out genome-wide RNA interference screens in *Drosophila* cells, followed by studying their function both in molecular level and in vivo. As the *Drosophila* and human signaling pathways closely resemble each other, the underlying aim of studying the genes and mechanisms important for *Drosophila* immune defense is to elucidate the immune regulation in humans also.
2. Review of the literature

2.1. Innate immunity

Our environment constantly exposes us to potentially pathogenic microbes, but very few of them actually manage to cause clinical diseases. This is due to our immune system, which has evolved effective means of protecting our bodies from the invaders. First, epithelial surfaces such as the skin, respiratory tract mucosa and gastrointestinal mucosa provide mechanical and chemical barriers that prevent the microbes from entering the body. The microbes that do manage to cross these barriers are rapidly encountered by the cells of the immune system, which effectively recognize the foreign molecular structures (non-self) and induce a response to destroy them (Metschnikoff 1891). While staying alert for pathogenic microbes, the immune system has to be able to distinguish between them and self-molecules, as well as useful commensal microbes, and leave these intact.

The vertebrate immune system can be roughly divided into innate and adaptive immunity. The adaptive immunity utilizes complex mechanisms including somatic gene rearrangement to generate clonally diverse lymphocytes that are able to mount a specific and effective response against various pathogens and provide an immunological memory. However, the adaptive immune response is relatively slow, and therefore a more rapid means of limiting the infection is needed, which is provided by the innate immune system. Innate immunity consists of the components that are encoded in the genome, and therefore inherited, and provides the first-line host defense against invading pathogens. The main functions of the innate immune system are, as described already by Metschnikoff in 1884, rapid detection of microbes, phagocytosis and antimicrobial activity. In addition, innate immunity is required for wound healing and maintaining tissue homeostasis by phagocytosis of dead cells and debris (Modlin 2012). The detection is achieved by genome-encoded pattern recognition receptors (PRRs) that bind conserved structures found on various pathogens and damaged/infected host cells, such as components of bacterial cell wall or viral RNAs (Janeway 1989). Currently, four different classes of PRR families are known: the membrane-spanning Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and the cytoplasmic Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi & Akira 2010). The sensing of pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) induces signaling cascades that trigger and control the expression of
innate immunity effector genes as well as direct the initiation of adaptive immunity responses (Lemaitre et al, 1996; Medzhitov et al, 1997).

One of the main contributions of innate immunity in response to pathogens or tissue damage is initiation of an acute inflammation. The classic symptoms of inflammation include redness, swelling, heat, pain, and loss of tissue function, usually carried out as a rapidly terminated process that is protective and beneficial for healing of the tissues and clearing of the pathogens. At the cellular level, the inflammatory response includes events like cell death in the inflamed tissues and modifications of vascular endothelial permeability to enable recruitment of blood cells to the site of infection; these are controlled by proinflammatory cytokines such as tumor necrosis factor (TNF) and certain interleukins (ILs). In addition, other genes involved in inflammatory responses are activated, including type I interferons (IFNs), chemokines and antimicrobial proteins (Takeuchi & Akira 2010).

Defects in innate immunity mechanisms can lead to recurrent infections and increased susceptibility to certain pathogens, and can be fatal to new-born children, whose adaptive immune system has not developed yet, or to people who are immunocompromized. However, having such a great capacity to induce inflammatory reactions, innate immunity also needs control over its effector mechanisms. False alarm reactions or non-terminated responses can be as harmful to host tissue as defects, causing diseases like rheumatoid arthritis and allergies (Li & Verma 2002).

While adaptive immunity is specific to vertebrates, innate immunity is found in some form in all multicellular organisms. In higher metazoans the similarities are rather profound, and the recognition molecules and signaling cascades are highly conserved, allowing the use of model organisms such as the fruit fly (*Drosophila melanogaster*) in studying innate immunity (Hoffmann 1999; Lemaitre & Hoffmann 2007; Rämet 2012). A classic example of such fruitful use of model organisms is the discovery of *Drosophila* Toll receptor, which soon led to identification of a family of pathogen recognition receptors in humans, the Toll-like receptors (Lemaitre et al, 1996; Medzhitov et al, 1997; Poltorak et al, 1998; Rosetto et al, 1995). Moreover, also the signaling pathways triggered by ligand binding to the receptor are strikingly similar between flies and humans, both leading to activation of a nuclear factor κB (NF-κB) family transcription factor (Metzhitov et al, 1998; Wasserman 1993). Thereafter, remarkable similarities have been found in the signaling pathways controlling the immune response in the two organisms. In addition to the Toll/TLR signaling pathway, good examples of other conserved cascades are the immune
deficiency (Imd) signaling pathway in flies and Tumor necrosis factor receptor (TNFR) pathway in humans, and also the Janus Kinase and activator of transcription (JAK/STAT) signaling pathway, which is present in both species. These pathways, and the mechanisms by which they induce immune responses to deal with various pathogens and stress factors, as well as their similarities and differences in humans and flies, are discussed more thoroughly below.

2.2. NF-κB in innate immunity

After its discovery in 1986 (Sen & Baltimore 1986), NF-κB has turned out to be a major regulator of multiple cellular processes including proliferation, differentiation and cell death, together with innate and adaptive immune responses. NF-κB transcription factors regulate the expression of hundreds of target genes, and thus many common diseases, including cancer, atherosclerosis, and diabetes, are associated with deregulation of NF-κB (Vallabhapurapu & Karin 2009). Moreover, the NF-κB signaling pathways are of crucial importance in regulating innate immunity, and their impairments are associated with recurrent infections, whereas excessive and inappropriate responses can lead to many pathological conditions, including autoimmune diseases such as rheumatoid arthritis, asthma, allergy and septic shock (Li & Verma 2002).

2.2.1. NF-κB activation and regulation

There are five members in the NF-κB family of transcription factors in humans: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (also called p65), c-Rel, and RelB. All of these are characterized by presence of an N-terminal Rel homology domain (RHD), which is responsible for homo- and heterodimerization upon activation, nuclear localization, as well as for sequence-specific DNA binding (Vallabhapurapu & Karin 2009). The prototypic NF-κB in most cells is thought to be a heterodimer of RelA and p50 (Kawai & Akira 2006).

In the absence of stimulus, NF-κB proteins are sequestered in the cytoplasm by their inhibitor molecules, the IκBs. Activation of an NF-κB signaling pathway leads to phosphorylation of IκB, followed by its ubiquitination and degradation by the 26S proteasome, subsequently leading to nuclear transport of the released NF-κB proteins. In mammals, IκB is phosphorylated by the IκB kinase (IKK) complex, which consists of three major components: IKK1 (IKKa), IKK2 (IKKβ), and NF-κB essential modulator (NEMO) (IKKγ), usually existing in the complex of IKK1-IKK2 heterodimer bound to a NEMO homodimer. Activation of the IKK complex itself requires phosphorylation of IKK1 and IKK2 on two serine residues. The exact mechanism of IKK
phosphorylation remains elusive, although autophosphorylation or phosphorylation by an IKKK (e.g. TAK1, RIP1 or MEKK3) has been suggested (Liu et al, 2012).

The inhibitory protein IκB is one of the target genes of mammalian NF-κB transcription factors, which therefore creates a negative feedback loop that limits the intensity and duration of the signaling. IκBs are the major regulators of NF-κB activity, and they are thought to function by binding and inactivating NF-κB dimers in the nucleus, leading to their export back in the cytoplasm (Sun et al, 1993). In addition, a member of the Protein Inhibitor of Activated Stat (PIAS) family, which mainly regulate JAK/STAT signaling, PIAS1, is shown to prevent DNA binding by RelA. Nuclear RelA is also inhibited by at least two ubiquitin E3 ligases, COMMD1-ECS2 and PDLIM2, which induce its degradation. Ubiquitination and deubiquitination play important roles in regulating other NF-κB signaling components as well. The tumor suppressor cylindromatosis (CYLD) may remove K63-linked polyubiquitin chains from IKKγ/NEMO as well as from tumor necrosis factor-associated factor 2 (TRAF2) and thereby inhibit NF-κB activation (Vallabhapurapu & Karin 2009).

2.3. Toll-like receptor signaling

The Toll-like receptor (TLR) signaling pathway results in activation of type I interferon (IFN) and chemokines, as well as costimulatory molecules found on the surface of specialized antigen-presenting cells, the dendritic cells, which is required for initiation of adaptive immune responses, therefore showing how TLRs link innate and adaptive immunity. Moreover, TLR signaling also induces a multitude of inflammatory cytokines, including the interleukins IL-1β, IL-6, IL-12 and TNF-α (Kawai & Akira 2006). The importance of TLR-mediated microbial recognition is demonstrated by TLR-deficient mice, most of which have impaired cytokine production and increased susceptibility to certain microbes, as well as human polymorphisms causing a similar phenotype. For example, the “human disease models” include susceptibility to pyogenic bacteria caused by deficiency of interleukin-1 receptor-associated kinase 4 (IRAK4) or MyD88 (Carpenter & O’Neill 2007; Takeuchi & Akira 2010).

2.3.1. Toll-like receptors

Toll-like receptors (TLRs) comprise an important class of pattern recognition receptors, of which TLR4 was the first human Toll receptor to be found. Subsequently, nine additional members of the receptor family have been identified (Medzhitov et al, 1997; Rock et al, 1998). Most of the receptors are located on the plasma membrane, with the exception of TLR3, TLR7/8, TLR9 and
TLR 10, which are found in endolysosomes, facilitating both intra- and extracellular recognition of ligands. TLRs share a common domain structure including extracellular N-terminal leucine-rich repeats required for recognition of pathogens and a cytoplasmic Toll/IL-1R homology (TIR) domain, which is also found in the receptors of the cytokines IL-1 (as the name implies) and IL-18 and is needed for initiation of the intracellular signaling (Rock et al, 1998; Takeuchi & Akira 2010). Each TLR is specialized (either by itself or in cooperation with other TLRs) in the recognition of certain class(es) of microbial molecular structures, such as lipopolysaccharide (LPS) from Gram-negative bacteria (TLR4), single- or double-stranded RNA (ssRNA and dsRNA) from viruses (TLR7 and TLR8, and TLR3, respectively), or bacterial flagellin (TLR5). The endosomal localization of some TLRs appears to restrict their self-reactivity and thereby onset of autoimmune disease, as host RNA or DNA is usually absent from these compartments (Kawai & Akira 2006; Carpenter & O’Neill 2007).

2.3.2. Intracellular TLR signaling

Recognition and binding of the ligand by a TLR causes receptor dimerization or oligomerization, and recruitment of the cytosolic downstream signaling components. All TLRs except TLR3 require the adapter protein myeloid differentiation primary response protein 88 (MyD88) for signal transduction (Medzhitov et al, 1998). MyD88 contains a C-terminal TIR domain that interacts with the TLR TIRs, and an N-terminal death domain (DD) required for interaction with IRAKs, including IRAK1, IRAK2, IRAK4 and the negative regulator IRAK-M. IRAKs in turn are characterized by an N-terminal DD and a C-terminal Ser/Thr kinase or kinase-like domain (IRAK-M lacks the kinase activity) (Kawagoe et al, 2008; Kobayashi et al, 2002). The crystal structure of the signaling complex formed by the signaling components recruited immediately downstream of TLRs, termed Myddosome, has been recently resolved. It appears that 6 MyD88, 4 IRAK4, and 4 IRAK2 (or IRAK1) molecules bind each other via their death domains to form a hierarchically built tower-like helical oligomer, a structure which eventually brings the kinase domains of IRAKs into proximity and facilitates their phosphorylation and activation by IRAK4 (Lin et al, 2010). Phosphorylated IRAK1 or IRAK2 dissociate from the complex to interact with TRAF6, which uses the E2 ubiquitin-conjugating enzyme complex of Ubc13 and Uev1A to K63-ubiquitinate itself. TRAF6 also generates an additional unconjugated K63-ubiquitin chain, which in turn activates transforming growth factor-β-activated protein kinase 1 (TAK1). TAK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) found in a complex with the TAK1-binding proteins TAB1, TAB2 and TAB3, and is required for activation of both the IKK complex, leading to
activation of NF-κB, and the MAP kinases MKK3 and MKK6, which activate c-Jun N-terminal kinase (JNK) (Kawai & Akira 2006).

2.4. Tumor necrosis factor receptor signaling

TNF was first discovered in 1975 for its ability to lyse tumor cells, owing to its name “tumor necrosis factor” (Carswell et al, 1975). Ten years later, it was recognized that TNF plays a key role in mediating sepsis (Beutler et al, 1985), as well as in rheumatoid arthritis, which can now be treated with monoclonal anti-TNF antibodies (Elliott et al, 1994).

TNF is a proinflammatory cytokine produced by immune cells, mainly macrophages and lymphocytes. TNF exists in two forms: as a transmembrane precursor protein, and after cleavage, a mature secreted form. Both forms of TNF are biologically active and can bind two types of receptors, TNFR1 and TNFR2, which are expressed by almost all cell types. The two receptors differ by their intracellular parts: TNF-R1 has a cytoplasmic DD, whereas TNF-R2 has a TRAF-binding domain. The signaling pathways activated by the receptors are nevertheless similar, in both cases leading to activation of NF-κB and activating protein-1 (AP-1) transcription factors. In addition, TNF-R1 can activate caspases and therefore trigger apoptosis, or even necrosis via the receptor interacting proteins RIP1 and RIP3. On the other hand, the genes activated by NF-κB elicit pro-survival signals that counteract the cell death signals (Vallabhaburapu & Karin 2009).

Ligand binding to the TNF-R1 receptor triggers receptor trimerisation and translocation to lipid rafts, followed by interaction with the adaptor protein TNF-R1-associated death domain (TRADD) through death domain interactions. TRADD recruits other death domain-containing signaling molecules, such as RIP1 and the E3 ubiquitin ligase TRAF2, which is thought to K63-ubiquitinate RIP1 and recruit IKK to the receptor complex. Thus, again, ubiquitination is an important regulatory mechanism, since most TNF-R1 signaling proteins are ubiquitinated. RIP1 is inhibited by A20, which is thought to remove the K63-linked ubiquitin chain from RIP1, and add a K48-linked chain, which leads to proteasomal degradation of RIP1 (Chen 2012). The TNFR1 signaling pathway converges with the TLR signaling pathway at the Tak1/Tab2/Tab3 complex, and as with TLR signaling, also leads to activation of NF-KB transcription factor and expression of its target genes (Vallabhaburapu & Karin 2009).
2.5. JAK/STAT signaling

It was recognized more than a half century ago that many cytokines, such as interferons, interleukins and colony-stimulating factors are important for initiating innate immunity, regulating adaptive immune mechanisms and constraining immune and inflammatory responses. These cytokines bind to type I and type II cytokine receptors. 20 years ago these cytokine receptors were found to selectively associate with Janus kinases (JAKs) and signal transducer and activators of transcription (STATs) to regulate many aspects in both innate and adaptive immunity (Fu et al, 1992; O’Shea and Plenge, 2012).

In humans, there are four JAKs (JAK1, JAK3, JAK3 and TYK2) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6). According to the canonical model of JAK/STAT signaling, molecular events on the signaling pathway appear rather straightforward: ligand binding to its receptor induces receptor dimerization and activation of the receptor-associated JAKs. Activated JAKs phosphorylate themselves as well as their associated receptors, creating docking sites for STATs. STATs bind to the complex via their src homology 2 (SH2) domains and also become activated by tyrosine phosphorylation by JAKs. Activated STATs form dimers with their SH2 domains and phospho-Tyrs and translocate to the nucleus where they bind their target sequence in the promoters of pathway target genes, activating their expression.

However, in the biological context, things are somewhat less simplistic. It has been found that many of the JAKs and STATs can use multiple receptors, can form homo- and heterodimers, and for example TYK2 and STAT4 can be involved in multiple signaling pathways. Most cytokines also activate more than one STAT. STATs have thousands of genomic targets, regulate epigenetics and can act both as activators and repressors depending on the complex they recruit, as well as elicit both pro- and anti-inflammatory signals (O’Shea & Murray 2008; O’Shea & Plenge 2012).

2.5.1. Regulation and biological implications of JAK/STAT signaling

One group of the main negative regulators of JAK/STAT signaling is the Suppressor of Cytokine Signaling (SOCS) family. In mammals this family consists of eight members, each characterized by a central SH2 domain, which binds to phosphorylated tyrosine residues on JAKs and receptors, and thereby can prevent STAT association with the activated receptor complex by steric inhibition. SOCS genes are targets of JAK/STAT signaling, thereby creating a classic negative feedback loop (Starr et al, 1997).
New roles for JAK/STAT signaling in mediating differentiation of different T cell subsets are constantly emerging; and include both the “old” Th1 and Th2 subsets and the “newer” subsets such as Tregs, Th17, Th9 and the follicular helper T cells, as well as B cells (O’Shea & Plenge 2012). In addition, STATs regulate several functions in innate immunity, including activation of neutrophils and macrophages, the acute phase response, wound repair, and the promotion and inhibition of inflammation. Therefore, it is not very surprising that variations in genes coding for cytokines, their receptors, JAKs and STATs (especially pathways leading to activation of STAT3 and -4) are associated with several human diseases, including autoimmune disorders, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and Crohn’s disease. For example, polymorphisms in STAT6 and IL13 are associated with atopic dermatitis and IL6R with asthma (Casanova et al, 2012; O’Shea & Plenge 2012).

Moreover, JAK3 mutations cause the autosomal recessive form of severe combined immunodeficiency (SCID), a fatal condition characterized by impairment in T and natural killer (NK) cell development and defects in B cell function leading to extreme susceptibility to various infections. STAT5B deficiency leads to growth defects together with various autoimmune and allergic symptoms (thyroiditis, pneumonitis and severe eczema). Mutations in STAT1 cause susceptibility to mycobacterial infections, such as tuberculosis and salmonellosis, STAT3 loss-of-functions mutations cause hyper-IgE syndrome with eczema and recurrent Staphylococcal and Candida infections, whereas gain-of-functions are found in many cancers such as lymphomas. Monoclonal antibodies are used to target specific cytokines and JAKs have also become a therapeutic target as several inhibitors are being preclinically and clinically tested in order to treat rheumatoid arthritis (tofacitinib against JAK3 and JAK1), polycythemia and myelofibrosis (ruxolitinib against JAK1 and JAK2) (Casanova et al, 2012).

2.6. Drosophila as a research model

The fruit fly Drosophila melanogaster has been a favorite model organism for many researchers ever since Thomas Morgan’s pioneering work in the 1930s. In the scientist’s point of view, Drosophila has many advantages. It is relatively small and easy to maintain in lab conditions in small vials on an easily-cooked mashed potato diet; its generation time is also short (10-12 days at +25 C) and the resulting progeny is numerous. Due to extensive research use, Drosophila genetics is well-established, including release of the genomic sequence in 2000 (Adams et al, 2000), followed by genomic sequences of its related Drosophila species (Drosophila 12 Genomes
Consortium, 2007). In addition, several genetic tools have been discovered and developed, such as mutations leading to easily-observed phenotypes, balancer chromosomes and the yeast-derived UAS-GAL4 expression system (Brand and Perrimon 1993). Drosophila is also well-amenable for large-scale genetic screening by both forward and reverse genetic methods. Indeed, during the last decade, several RNAi-based screens have been carried out in vitro as well as more recently in vivo to elucidate various cellular functions in Drosophila, including those associated with immune response (see for example Bach et al, 2003; Baeg et al, 2005; Boutros et al, 2004; Cronin et al, 2009; Kleino et al, 2005; Müller et al, 2005; Rämet et al, 2002b; Rämet 2012; Valanne et al, 2012).

Despite its small size, Drosophila is an animal with many organs and structures that share functional analogies with those of humans. Due to the relatively low redundancy of its approximately 15,000 genes packed into a rather compact 180 Mb genome, the genes associated with particular functions and defects can be more easily identified; indeed, almost 80% of disease-associated genes in humans have Drosophila homologies (Reiter et al, 2001). While the biochemistry of many of the molecules coded by these genes is being thoroughly studied in human and mouse cell culture models, their in vivo roles are more difficult to determine. Therefore, a genetically tractable model system such as Drosophila can be tremendously helpful in examining these functions.

2.7. Drosophila immune system

One of the biological functions particularly well-conserved between flies and human is the innate immune system, and therefore Drosophila has become a widely-used and powerful model to study innate immunity. As mentioned, the Drosophila genome is less redundant than the mammalian one, making dissection of gene function more straightforward. Moreover, as an invertebrate Drosophila completely lacks the adaptive parts found in vertebrate immune system, and thus solely relies its pathogen defense on innate immunity. However, even the flies need a powerful immune defense system, since they feed and lay their eggs in rotting fruits, an environment constantly exposing them on potentially pathogenic bacteria and fungi. An overview of the components in the Drosophila immune system is shown in Figure 1. Besides serving as a model to clarify innate immune mechanisms in mammals, Drosophila can also provide information that helps to understand the immune reactions of other insects with more implications in human life in economical or public health aspects, such as agricultural pests and disease-spreading vectors like Anopheles mosquitos for malaria (Lemaitre & Hoffmann 2007).
Figure 1. Schematic overview of the *Drosophila* immune system. Main entry routes for pathogens are via a wounded cuticle caused by a septic injury (often by a researcher) or via gastro-intestinal or other epithelial surfaces (e.g. food-borne bacteria, yeast and fungi). Infection in the epithelial surface induces local immune response, which is mainly mediated by the Imd pathway, whereas the systemic response is initiated in the fat body, involves the Toll and the JAK/STAT pathways as well and results in secretion of effector molecules in the hemolymph. *Drosophila* blood cells originate from the lymph gland, and their types and main functions are shown schematically.

2.7.1. Local response and melanization

Similar to humans, the *Drosophila* epithelial surfaces in the trachea and digestive tract form the first physical barrier against pathogens. The epithelial cells also produce effector molecules such as antimicrobial peptides and reactive oxygen species, in response to encountered bacteria (Lemaitre & Hoffmann 2007; Tzou et al, 2000). If the cuticle is wounded, hemolymph clotting is induced, followed by melanization and epithelial cell movements (Jiravanichpaisal et al, 2006; Loof et al, 2011; Rämet et al, 2002a).
2.7.2. Cellular response

2.7.2.1. Drosophila blood cells

*Drosophila* has an open circulatory system where the hemolymph floats in the body cavity called hemocoel, where three types of hemocytes can be found: plasmatocytes, lamellocytes and crystal cells. *Drosophila* hemocytes develop in two phases. First, hematopoiesis takes place in the head mesoderm of early embryo. Second, at the late embryonic stages, hemocytes develop in a small organ found on either side of the dorsal vessel called the lymph gland. During larval development, the lymph gland grows into multilobed organ consisting of the medullary zone (MZ), the cortical zone (CZ) and the Posterior Signaling Center (PSC) (Crozatier and Meister 2007; Minakhina & Steward 2010). The latter regulates hematopoietic stem cell maintenance and development, functioning as a hematopoietic niche (Krzemien et al, 2007). In addition to signals received within the PSC, hematopoiesis is also regulated by signals from fat body and circulating hemocytes. Many of the signaling pathways that regulate *Drosophila* hematopoiesis and blood cell activation also regulate mammalian blood cell development and are involved in regulation of immune responses as well, such as the Ras/MAPK pathway, the JAK/STAT pathway, the PDGF- and VEGF-receptor related (pvr) signaling, and the Toll pathway. (Gao et al, 2009; Krzemien et al, 2007; Luo et al, 1997; Zetterwall et al, 2004).

2.7.2.2. Phagocytosis

Plasmatocytes are the most abundant of *Drosophila* hemocytes, comprising > 95 % of blood cells (Meister & Lagueux 2003). Plasmatocytes resemble mammalian monocytes/macrophages, and are involved encapsulation and AMP production, although their primary role is to carry out phagocytosis. Phagocytosis is an evolutionarily conserved process that probably originates far back to the engulfment of nutrients by unicellular organisms, thus representing an ancient defense mechanism. In multicellular organisms phagocytosis leads to engulfment of pathogens or apoptotic host cells, and is therefore crucial in both immune defense and in development, especially in holometabolous insects, which undergo dramatic tissue remodeling during their metamorphosis. The internalized particles are delivered into a phagosome, a complex membrane-coated organelle that subsequently “maturates” by fusing with lysosomes, thereby creating a hydrolytic compartment to facilitate killing of the ingested pathogen (Stuart & Ezekowitz 2008; Ulvila et al, 2011).

Phagocytosed particles are recognized and bound by cell-surface receptors, either directly, or via opsonins, which are secreted molecules that enhance phagocytosis in both insects and mammals, for
example the thioester-containing proteins (TEPs) (Stuart & Ezekowitz 2008). *Drosophila* phagocytic receptors seem to be specific to certain pathogens, and include the scavenger receptors, such as SR-C and Croquemort, the Epidermal growth factor (EGF) -like-repeat-containing receptors such as Eater and the Nimrod receptors, and the immunoglobulin superfamily member Down Syndrome Cell Adhesion Molecule (Dscam) (Kocks et al, 2005; Kurucz et al, 2007; Pearson et al, 1995; Rämet et al, 2001b; Watson et al, 2005). The importance of phagocytosis in *Drosophila* host defense is demonstrated by, for example, the *domino* mutant larvae, which lack functional phagocytes, and therefore allow the accumulation of large number of microorganisms (Braun et al, 1998), or by mutant flies lacking the phagocytic receptor Eater, which have decreased survival of bacterial infections (Kocks et al, 2005). Phagocytosis and plasmatocytes have also been implicated in initiation of the systemic AMP production by the fatbody, at least in larvae (Basset et al, 2000; Brennan et al, 2007; Charroux & Royet 2009; Defaye et al, 2009). In addition, it has been suggested that *Drosophila* might have an alternative form of adaptive immunity, including immunological memory and pathogen-specific effector molecules, possibly mediated by the numerous isoforms generated by the splice variants of Dscam (Pham et al, 2007; Watson et al, 2005).

### 2.7.2.3. Encapsulation

The second group of blood cells in *Drosophila* is the lamellocytes, which are not normally present in larvae, but are induced to differentiate by recognition of invaders too large to be phagocytosed, such as eggs laid in *Drosophila* larvae by the parasitic wasp *Leptopilina boulardi*. In such cases, these large, flattened cells attack the parasite in order to fully encapsulate it (Sorrentino et al, 2002). This is initiated by the plasmatocytes, which recognize the invader and signal to the lymph gland for lamellocyte differentiation. The mechanism controlling lamellocyte differentiation remains rather elusive, but again, there is evidence that both the Toll and JAK/STAT signaling pathways are involved, and aberrant activation of these pathways induces lamellocyte differentiation, accumulation and subsequent formation of melanotic tumors even in the absence of parasitic infection (Luo et al, 1995; Qiu et a, 1998; Sorrentino et al, 2004; Wertheim et al, 2005). Once the lamellocytes have surrounded the wasp egg, the cell capsule is melanized by crystal cells, another rare blood cell type, which induce a phenoloxidase cascade leading to synthesis of melanin and reactive oxygen species. Production of reactive oxygen species is also observed in humans as a reaction to cellular stress, but melanisation appears to be rather an insect-specific immune reaction, and no equivalent for either crystal cells or lamellocytes is found in mammals (Williams 2007).
2.7.3. Systemic response

In addition to the induction of the hemolymph cells, recognition of invading microbes also leads to the systemic production of antimicrobial peptides in the *Drosophila* organ called the fat body, which is the functional equivalent to the mammalian liver (Hoffmann et al, 1999). Antimicrobial peptides are short cationic proteins that are secreted into the hemolymph in relatively high concentrations and are thought to damage microbes by perforating their cell walls, which is another conserved mechanism in innate immunity found in all metazoans (Hultmark et al, 1982, 1983). In *Drosophila*, seven classes of AMPs are produced: Diptericins, Attacins, Drosocin, Cecropins, Defencins, Metchnikowin and Drosomycins (Asling et al, 1995; Kylsten et al, 1990; Lemaitre & Hoffmann 2007; Samakovlis et al, 1990). The set of AMPs produced is not random, but is tailored to best fight the type of the invading microbe, which is mainly controlled by two conserved NF-κB signaling cascades, the Toll and the Imd pathway (Lemaitre et al, 1995, 1996). In response to systemic infection, the *Drosophila* fat body also induces production of several other immune-related proteins, including cytokines and stress response proteins, such as the Turandot proteins, which involves activation of the JAK/STAT pathway (Brun et al, 2006; Ekengren & Hultmark 2001a). In addition to AMP production by the fat body in response to systemic infection, AMPs are produced locally on epithelial surfaces in response to air- or food-borne bacteria, which are, in fact, frequently encountered, whereas systemic infections are relatively rare events. Local production of AMPs is mainly carried out by the Imd pathway (Lemaitre & Hoffmann 2007; Ruy et al, 2010). Also hemocytes that encounter bacteria can induce Toll and Imd signaling pathways and produce AMPs, although the amount is smaller than that produced by the fat body (Irving et al, 2005).

2.7.4. Viral response

*Drosophila* is also infected by numerous viruses, which they defend against by initiating an antiviral program that includes several mechanisms found to be conserved between flies and man (Wang et al, 2010). First, the RNAi–based silencing of viral gene expression is initiated by the RNaseIII-like enzyme Dicer-2 (Dcr-2), which recognizes viral dsRNAs and RNA hairpin structures and cleaves them into 21-23 nt long virus-derived small interfering RNAs (siRNAs). siRNAs are subsequently bound by other components of the RNA-induced silencing complex (RISC), including Ago2, r2d2, Ars2, which mediate binding of one siRNA strand onto a complementary strand on viral RNA and its subsequent cleavage (Ulvila et al, 2010). The RNAi mechanism appears important in limiting viral infections in *Drosophila*, since flies with a loss-of-function mutation in the gene encoding Dcr-2 show enhanced susceptibility to infection by several RNA viruses.
(Galiana-Arnoux et al, 2006). In mammals, the RNAi pathways appear to mainly control expression of self-genes, whereas the innate immune responses to viruses are mainly carried out by the IFN response pathway, which is mainly mediated via JAK/STAT signaling (Sabin et al, 2010). Interestingly, the *Drosophila* JAK/STAT pathway has also been shown to restrict viral infections, and many virus-induced genes are dependent on the pathway (Deddouche et al, 2008; Dostert et al, 2005). Importantly, JAK/STAT pathway was shown to restrict the arbovirus Dengue in mosquitos (Souza-Neto et al, 2009). A role for the *Drosophila* NF-κB signaling pathways, the Toll and the Imd pathways, in viral restriction has also been suggested (Nakamoto et al, 2012), but the induction and efficacy of these pathways seems to be more virus-specific and might represent an indirect mechanism (Sabin et al, 2010). Finally, *Drosophila* cells are capable of restricting viral infection by inducing autophagy by inhibiting the PI3K-Akt-TOR signaling pathway (Shelly et al, 2009).

### 2.8. *Drosophila* NF-κB signaling pathways

*Drosophila* has three NF-κB family transcription factors, Dorsal-related immune factor (Dif), Dorsal and Relish (Rel), which are activated by two conserved signaling cascades, the Toll and the Imd pathway. Intracellularly, the Toll pathway resembles the mammalian TLR signaling pathway and leads to activation of Dif and/or Dorsal, while the Imd pathway is similar to the mammalian TNFR signaling and activates Relish. Both of the pathways recognize pathogens using peptidoglycan recognition proteins (PGRPs), for which the *Drosophila* genome contains 13 genes (Werner et al, 2000).

#### 2.8.1. The Toll pathway

Besides its functions in immunity, the Toll pathway is also involved in both developmental biology, and was originally identified for its importance in the dorso-ventral patterning of the embryo (Belvin & Anderson, 1996; Nüsslein-Volhard & Wieschaus, 1980; Stein et al, 1991; Steward 1989). This was later followed by the recognition of its role in immune system in defense against fungal and Gram-negative bacterial infections (Lemaitre et al, 1996; Rosetto et al, 1995; Wasserman, 1993). Loss of Toll pathway components severely impairs the *Drosomycin* response, making the flies highly susceptible to these pathogens (Lemaitre et al, 1996).

#### 2.8.1.1. *Drosophila* Toll receptors

Since the identification of Toll-1, nine Toll receptors have been identified in the *Drosophila* genome. All *Drosophila* Tolls have similar domain structure to human TLRs, with leucine-rich repeats and cystein-rich flanking motifs on the extracellular side and a Toll/IL-1R (TIR) domain in
the cytosolic side (Imler & Hoffmann 2002). However, unlike mammalian TLRs, most of the *Drosophila* Toll receptors do not seem to play major roles in immune responses (Tauszig et al, 2000). The exceptions include Toll-5, which may induce *Drosomysin* and *Metchnikowin* expression and has been shown to interact with the cytoplasmic part of Toll and Pelle (Tauszig et al, 2000); Toll-9, which can also activate *Drosomycin* possibly by using Toll pathway components (Ooi et al, 2002), and Toll-8/Tollo, which appears to downregulate Imd signaling in the airway epithelium (Akhouayri et al, 2011). In addition, Toll-7 appears to play a role in restring vesicular stomatitis virus infection by inducing antiviral autophagy independently of the canonical Toll signaling pathway (Nakamoto et al, 2012).

### 2.8.1.2. Signaling upstream of Toll receptor

*Drosophila* Toll signaling is induced by fungi and Gram-positive bacteria. Unlike mammalian TLRs, *Drosophila* Toll does not directly bind microbes, but is activated by binding of a cytokine called spätzle (spz), which needs to be activated by a proteolytic signaling cascade to remove the prodomain from masking the C terminal part (C-106) that binds the Toll receptor (Morisato & Anderson, 1994; Schneider et al, 1994; Weber et al, 2005, 2007; reviewed in Valanne et al, 2011). Four proteolytic cascades starting with recognition of microbial structures or positional cues (in embryonic development) and leading to spz activation have been discovered so far. In the dorso-ventral patterning, the cascade includes the serine proteases Nudel, Gastrulation Defective, Snake and Easter (Chasan et al, 1992; Hong & Hashimoto 1995). In microbial recognition, spz is cleaved by Spz-processing enzyme (SPE) (Jang et al, 2006). Fungi and Gram-positive bacteria can secrete virulence factors, such as the fungal PR1, which can activate the protease Persephone, which in turn activates SPE (El Chamy et al, 2008; Gottar et al, 2006). The protease cascade can also be activated by “danger signals”, such as abnormal proteolytic activity (El Chamy et al, 2008). The two remaining cascades depend on Gram-positive-specific serine protease (Grass) and its upstream molecule, modular serine protease (ModSP) (Buchon et al, 2009). This can be activated either by Lysine-type peptidoglycan of Gram-positive bacteria recognized and processed by Gram-negative binding protein (GNBP) 1 (Leulier et al, 2003; Pili-Floury et al, 2004; Wang et al, 2006), peptidoglycan-binding protein SA (PGRP-SA; S for short) and PGRP-SD (Bischoff et al, 2004; Leone et al, 2008); or β-glucan from fungi recognized by GNBP 3 (Gobert et al, 2003; Gottar et al, 2006; Kambris et al, 2006; Michel et al, 2001; see also Figure 2.).
Binding of either one or two processed spz dimers to a dimerized Toll receptor finally releases the spz prodomain and triggers a conformational change in the Toll receptors that activates downstream signaling (Gangloff et al, 2008; Weber et al, 2005).

**Figure 2. The Drosophila Toll pathway.** Recognition of Gram positive bacteria, yeast or fungi induces a protease cascade leading to cleavage of spätzle, which is then bound by the Toll receptor. Signaling leads to phosphorylation and degradation of the IκB protein Cactus, releasing Dif/Dorsal, which can then translocate to nucleus and activate their target genes. The known regulators are also shown.

### 2.8.1.3. Intracellular Toll signaling

The first downstream component of the Toll receptor is *Drosophila* MyD88 (Horng & Medzhitov 2001; Tauszig-Delamasure et al, 2002), which has recently been suggested to function as a sorting adaptor binding phosphoinositides on the plasma membrane, somewhat analogously to mammalian TIRAP (Horng et al, 2001; Marek & Kagan 2012). dMyD88 then recruits the signaling adaptor tube on the site in a signal-dependent manner by death domain interaction, since the other downstream component pelle cannot bind directly to MyD88, but needs the adaptor tube in the middle (Grosshans et al, 1994; Marek & Kagan 2012). *Drosophila* tube lacks the kinase domain
found in human IRAK-4 and in many insect orthologs of tube. Nevertheless, it appears that tube is orthologous to IRAK4 and pelle to IRAK1 (or IRAK2) (Towb et al, 2009). It was recently shown that the structure of the mammalian MyD88: IRAK4: IRAK2 complex (the “Myddosome”; see section 2.3.2.) is also applicable for Toll signaling in *Drosophila*, although the stoichiometry is different (Lin et al, 2010).

It seems likely that Toll signaling is at least initiated at the plasma membrane; however, endocytosis is also necessary for proper activation of the Toll pathway (Huang et al, 2010; Lund et al, 2010). Myopic (Mop) is required for Toll signaling upstream of Myd88 and independently of its phosphatase activity. Mop and Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), which are components of the ESCRT-0 endocytosis complex, colocalize with Toll in the endosomes (Huang et al, 2010).

Toll signaling leads to activation of Dif or Dorsal. In non-signaling conditions, the Toll pathway transcription factors are bound by the IkB protein Cactus, which needs to be phosphorylated in two N terminal sites and degraded before Dif or Dorsal are activated and can translocate into the nucleus (Fernandez et al, 2001; Geisler et al, 1992; Ip et al, 1993; Rutschmann et al, 2000a; Steward 1989). The kinase responsible of Cactus phosphorylation has long remained elusive; the *Drosophila* IkB kinases Ird5 and Kenny are only involved in Imd signaling pathway, and pelle has been the only kinase implicated in Cactus phosphorylation so far and its kinase activity is required for Cactus phosphorylation. Thus, it seems quite likely that pelle is responsible for phosphorylating Cactus (Huang et al, 2010).

**2.8.1.4. Regulation of Toll signaling**

Toll pathway activation in the fat body cells in response to infection appears to be dependent on spz produced by hemocytes, indicating that the two cell types communicate to induce an immune response (Irving et al, 2005; Shia et al, 2009). In addition, the Toll and Imd pathways can cooperate and/or act synergistically, using different dimers of the three NF-κB transcription factors to induce expression of at least part of their target genes, including *Drosomycin* (Kleino et al, 2005; Tanji et al, 2007)

Toll pathway is positively regulated by pellino, a conserved protein that interacts with pelle (Grosshans et al, 1999) and is required for normal *Drosomycin* response and resistance against Gram-positive bacteria (Haghayeghi et al, 2010). Another recently identified and conserved positive
regulator of Toll signaling is Gprk2, which is needed for proper response against both Gram positive bacteria and fungi. Gprk2 interacts with Cactus, but is not required for its degradation, and therefore is not likely to be the Cactus kinase (I; Kallio 2011, PhD thesis). Deformed epidermal autoregulatory factor-1 (Deaf-1), which functions downstream of Dif/Dorsal and binds the Metchnikowin and Drosomycin promoters, is also required for activation of Toll pathway target genes in response to both bacterial and fungal infection (Kuttenkeuler et al, 2010; Reed et al, 2008). SUMOylation may also play a role in Toll pathway regulation, since the Drosophila SUMO-activating enzyme Ubc9 was shown to down-regulate Toll signaling (Chiu et al, 2005). In addition, wispy and the friend of GATA factors u-shaped and pannier appear important for Toll pathway activation, but their exact mechanism of action, like that of Deaf-1 and Gprk2 remains to be studied (I).

Thus, even though the importance of Toll signaling in both Drosophila development and immune defense has been well-established along with the core components of the pathway, only a few positive regulators of intracellular Toll signaling have been identified so far, while negative regulation of the pathway by molecules other than Cactus remains elusive. However, it is likely that the Toll pathway also needs to be under tight control, and therefore it is likely that additional regulators, especially negative ones, are yet to be identified.

2.8.2. The Imd pathway

In contrast to the Toll pathway, Imd signaling is mainly dedicated to immune responses, in particular, mediating the humoral response against Gram-negative bacteria (Hedengren et al, 1999; Lemaitre et al, 1995). The Imd pathway is activated by meso-diaminopimelic acid (DAP)-type peptidoglycan found on Gram negative bacteria and certain Gram-positive bacteria, such as Bacillus spp (Kaneko et al, 2004; Leulier et al, 2003). DAP-type PGN is recognized and bound by two receptors, the peptidoglycan recognition proteins PGRP-LC and PGRP-LE (Choe et al, 2002; Gottar et al, 2002; Kaneko et al, 2006; Rämet et al, 2002; Takehana et al, 2002, 2004). PGRP-LC is a transmembrane protein found as three alternatively spliced isoforms, PGRP-LCa, -LCx and –LCy, of which PGRP-LCx recognizes polymeric PGN, while both PGRP-LCx and –LCa are needed for recognition of monomeric PGN fragments (called tracheal cytotoxin (TCT)) (Chang et al, 2006; Kaneko et al, 2004; Lim et al, 2006). PGRP-LE is also expressed in two forms found in the cytoplasm or as an extracellular secreted protein. The secreted version lacks the transmembrane domain, and therefore signaling ability, and is instead suggested to assist Imd signaling by binding
PGN in the hemolymph and presenting it to PGRP-LC. The full-length cytoplasmic form is thought to recognize TCT fragments that gain access into the cytoplasm, and initiate Imd pathway independently of PGRP-LC (Kaneko et al, 2006; Neyen et al, 2012; Takehana et al, 2004). In each receptor combination used, PGN binding induces receptor dimerization, multimerization or clustering and recruitment of downstream signaling molecules. The immediate signaling protein is imd, a death domain protein homologous to mammalian RIP1, which appears to mediate the two downstream arms of the signaling pathway (Choe et al, 2005; Georgel et al, 2001; Figure 3.).

First, similar to humans, the MAPKKK Tak1 is required for the activation of the IKK complex and is also needed for Relish cleavage (Lu et al, 2001; Silverman et al, 2003; Vidal et al, 2001). Tak1 is presumed to form a complex with Inhibitor of apoptosis 2 (Iap2) and Tab2, which are also required for proper Imd pathway response (Gesellchen et al, 2005; Huh et al, 2007; Kleino et al, 2005; Leulier et al, 2006; Valanne et al, 2007; Zhuang et a, 2006). Tak1 is also essential for activation of the c-Jun N-terminal kinase (JNK) pathway (Silverman et al, 2003). To activate the second signaling arm, imd recruits the DD-containing adaptor protein FAS-associated death domain protein (dFadd) and Dredd to the signaling complex via death domain interactions (Georgel et al, 2001; Leulier et al, 2002). Dredd is a caspase homologous to mammalian caspase-8, and is required for the cleavage and activation of the transcription factor Relish (Dushay et al, 1996; Leulier et al, 2000; Stöven et al, 2000, 2003).

Tak1 is therefore needed for the phosphorylation and activation of the *Drosophila* IκB kinase complex, consisting of IKKβ (also known as Immune response deficient 5 (Ird5)) and IKKγ (also known as Kenny) (Lu et al, 2001; Rutschmann et al, 2000b; Silverman et al, 2000). There are no IκBs in Imd pathway, but under normal conditions, Relish is maintained in the cytoplasm by the IκB on its own C-terminus, which masks the nuclear localization signal found in the N-terminus and inhibits dimerization by the Rel homology domain (RHD). To activate the transcription factor, the N-terminal part needs to be phosphorylated on several sites by the IKK complex, including the serine residues S28 and S29 (Silverman et al, 2000; Erturk-Hasdemir et al, 2009) and presumably cleaved by Dredd. Thereafter, while the C-terminal part remains in the cytoplasm, the active N-terminal part can translocate into the nucleus to activate transcription of target genes, including AMPs such as *Diptericins* and *Attacins* (Stöven et al, 2000, 2003). The Imd pathway signaling components are shown schematically in Figure 3.
Figure 3. The *Drosophila* Imd pathway. Imd signaling is initiated by binding of Gram-negative bacterial peptidoglycan by the receptors PGRP-LC and -LE, and leads to activation and nuclear translocation of the NF-κB transcription factor Relish. Several new regulators and details in molecular events of signaling have been discovered recently, and these are also shown schematically.

When this study was initiated, few regulators for Imd signaling had been identified and the signaling cascade was considered rather simple. However, this view has changed during recent years, as the number of identified regulators, both positive and negative, has increased significantly. It now appears that the Imd pathway is in fact delicately regulated by multiple mechanisms and molecules (reviewed by Aggarwal & Silverman 2008; Valanne et al, 2012).

2.8.2.1. Regulation of Imd signaling by PGRPs

While the Imd pathway activation depends on the receptor function of PGRP-LC and –LE, it has been shown to be downregulated by several of the other PGRP family members. These include members with amidase activity, namely PGRP-LB (Paredes et al, 2011; Zaidman-Remy et al, 2006), -SC1 and -SC2 (Bischoff et al, 2006), as well as the non-amidase PGRP-LF (Basbous et al,
PGRP-LF is a transmembrane protein that resembles PGRP-LC, but lacks the intracellular signaling domain, therefore acting as a non-signaling receptor that inhibits Ims signaling by binding PGN or PGRP-LCx-TCT complexes (Basbous et al, 2011; Maillet et al, 2008; Persson et al, 2007). The amidase PGRPs cleave PGN into smaller, less-immunogenic fragments, therefore reducing the amount of available immunostimulatory PGN (Paredes et al, 2011; Zaidman-Remy et al, 2006). In both cases, the regulators are needed for preventing constitutive Imd pathway activation in response to commensal bacteria, and the principal mechanism behind their action appears to be removal of PGN out of being bound by PGRP-LC, and allowing receptor activation only when “excess” bacteria are present, i.e. in the case of systemic or oral infection.

### 2.8.2.2. Regulation of the Imd pathway by ubiquitination

Ubiquitination has been shown to play important role in regulation of mammalian TNFR signaling. This seems to be the case with *Drosophila* Imd pathway as well, since several regulatory mechanisms involving ubiquitination have been recently discovered.

The function of Iap2 and the signaling events from imd to Tak1 have been newly clarified. Iap2 was recently shown to activate Dredd by ubiquitination (Meinander et al, 2012). Paquette and coworkers showed that Dredd cleaves imd, creating a neo-N-terminus with a binding site for Iap2, which is an E3-ligase and is associated with the E2-ubiquitin-conjugating enzymes UEV1a, Bendless (Ubc13) and Effete (Ubc5) (Zhou et al, 2005). Iap2 then K63-ubiquitinates imd, which induces activation of the downstream kinases, including Tak1, that ultimately phosphorylate Relish (Paquette et al, 2010). The ubiquitin-specific protease dUSP36/Scny negatively regulates the Imd pathway by preventing accumulation of K63-ubiquitinated imd, while promoting its K48-linked ubiquitination and subsequent degradation, thereby preventing constitutive Imd pathway activation in response to commensal microbes (Thevenon et al, 2009). Relish is also a target for ubiquitination and proteasomal degration by the Skp1/Cullin/F-box complex (Khush et al, 2002), while Tak1 is targeted for ubiquitination and degradation by the RING-finger protein Plenty of SH3s (POSH) (Tsuda et al, 2005). CYLD is a deubiquitinating enzyme that negatively regulates NF-κB signaling in both mammals and *Drosophila* (Tsichritzis et al, 2007). Dredd is inhibited by Dnr1, whereas sickie appears to be a positive regulator of Imd signaling (Foley et al, 2004). Another cytosolic protein, Caspar inhibits Relish cleavage by Dredd (Kim et al, 2006).
2.8.2.3. **JNK pathway, crossregulation and transcriptional regulators**

As in humans, the Imd pathway bifurcates at the level of Tak1 to the JNK pathway, which controls epithelial sheet movements in embryonic and pupal development, is required for controlling cytoskeletal genes and for normal dorsal closure and wound healing, and is also important in controlling immune and stress responses and apoptosis. In addition, JNK signaling seems to play a role in AMP production, at least in S2 cells, although some contrasting data exists (Boutros et al, 2002; Delaney et al, 2006; Kallio et al, 2005; Martin-Blanco et al, 2000; Rämet et al, 2002a; Silverman et al, 2003; Sluss et al, 1996).

The JNK pathway is a kinase cascade initiated by Tak1, which phosphorylates hemipterous (hep), which in turn phosphorylates dJNK (basket), which finally activates the transcription factors DJun and DFos, encoded by the genes *Jun-related antigen* (*Jra1*) and *kayak* (*kay*). *Jra1* and *Kay* form the AP-1 transcription activator, which translocates into the nucleus (Sluss et al, 1996). JNK signaling is induced rapidly after immune stimulation, and is negatively regulated by a feed-back loop involving puckered (puc), a phosphatase that targets dJNK (Dobens et al, 2001).

The signaling pathways controlling *Drosophila* immune response seem to practice some cross-regulation of each other. At least the JNK and JAK/STAT pathways are also able to negatively regulate the Imd pathway mediated response, since the dAP-1-, Stat92E-, and Dsp1-containing complexes can replace Relish at the promoters of diverse immune effector genes (Kim et al, 2005; Kim et al, 2007). It has also been reported that the *Drosophila* NF-κB transcription factors could cooperate over pathway limits, as at least Dif and Relish can function as heterodimers in vitro (Tanji et al, 2010). Nuclear Relish is positively regulated by a conserved protein called Akirin, which has been shown to mediate Relish-dependent gene expression in *Drosophila*, while its mouse homolog regulates NF-κB-dependent gene expression downstream of Toll-like, IL-1β and TNF receptors (Goto et al, 2007).

2.8.2.4 **Imd pathway and the local immune response**

The fat body is an aseptic internal tissue, which rarely encounters microbes, therefore the Imd and Toll pathways are kept silent and the systemic AMP production is “off” under normal conditions. However, the epithelial surfaces of the fly are constantly encountered by commensal microbes, and occasionally by pathogenic microbes present in the food. It has been estimated that the midgut of a normal fly living in a laboratory contains approximately 350,000 cfu of five major commensial bacteria (Ryu et al, 2008). Quite obviously, mounting a massive immune response against these...
would be very harmful for the host fly, and therefore it needs to be effectively downregulated despite the amount of PAMPs present. The Imd pathway is mainly responsible for local AMP production (including Drosomycin) on epithelial surfaces, while the Toll pathway is dispensable for epithelial immunity (Ferrandon et al, 1998; Tzou et al, 2000). Relish shows constitutive nuclear localization in the midgut epithelial cells, indicating that the Imd pathway is indeed activated by the commensal microbes. However, of the above-mentioned negative regulators, PGRP-SCs and PGRP-LB are expressed as target genes of Relish, and therefore basal activity of Relish actually maintains Imd pathway activity on a low level by providing the negative feed-back loop. In addition, more gene- and epithelial-specific means of Imd pathway regulation is provided by the homeobox protein Caudal, which binds AMP promoters and inhibits their expression (Ryu et al, 2008)

2.9. The JAK/STAT pathway in Drosophila immunity

JAK/STAT signaling is highly conserved in evolution, and due to the relative simplicity of the core JAK/STAT pathway in Drosophila, the fruit fly makes a good and widely used model to study JAK/STAT signaling in vitro as well as in vivo. The pathway has been shown to be involved in several processes in both Drosophila embryos as well as in adults, including embryonic patterning (Brown et al, 2001; Hou et al, 1996; Small et al, 1996), formation of the wing and eye (Yan et al, 1996; Bach et al, 2003), migration of border cells during oogenesis (Ghiglione et al, 2002; Silver et al, 2001), maintenance of stem cells in their niches (Kiger et al, 2001; Tulina & Matunis 2001; Wang et al, 2011), hemocyte proliferation and differentiation (Luo et al, 1995) and immune and stress responses by directly contributing in activation of infection-induced genes as well as controlling tissue repair (Boutros et al, 2002; Agaisse & Perrimon 2004)

The biochemistry of JAK/STAT signaling has been more thoroughly studied in mammalian systems, however the Drosophila signaling pathway is also assumed to function according to the canonical model, which in this case looks rather simplistic in biological context as well (shown schematically in Figure 4.). First, in Drosophila, the JAK/STAT pathway ligands consist of three cytokine-like proteins called unpaired (upd) (Harrison et al, 1998), upd2 (Gilbert et al, 2005; Hombria et al, 2005) and upd3 (Agaisse et al, 2003; Wright et al, 2011), the genes for which are clustered in the Drosophila genome and have no obvious homologs outside the Drosophila species, but share some similarity with the vertebrate leptins (Harrison et al, 1998; Castelli-Gair Hombria et al, 2005). Upd is glycosylated and associated with the extracellular matrix, which spatially limits its
activity (Harrison et al, 1998), while upd2 diffuses freely. Nevertheless, upd and upd2 seem to function somewhat redundantly, whereas upd3 expression is induced in adult hemocytes in response to bacterial challenge (Agaisse et al, 2003). The upd molecules bind to a single receptor, Domeless (Dome), which shares similarities with the mammalian cytokine class I receptors, including the fibronectin-type-III domains and the cytokine binding module (CBM) found in the extracellular side (Brown et al, 2001; Chen et al, 2002). In addition, Drosophila only has a single JAK, hopscotch (hop) (Binari and Perrimon, 1994), and one STAT transcription factor, Stat92E, which are most similar to JAK2 and STAT5, respectively (Hou et al, 1996; Yan et al, 1996). Hop and Stat92E are activated in a series of phosphorylation events induced by ligand binding and dimerization of Dome (Brown et al, 2003; Chen et al, 2002). After binding to phosphotyrosine on activated Dome with its SH2-like domain and phosphorylation on Tyr-704 by hop, Stat92E dimerizes and translocates to the nucleus where it binds its consensus sequence TTCCCGGAA on the promoters of target genes (Yan et al, 1996).

As in mammals, Drosophila Stat92E also has numerous target genes, related to developmental and other processes (Yan et al, 1996). In the context of immunity and stress response, it has been showed that septic injury, especially with Gram negative bacteria, or other stress, such as heat shock or dehydration, result in production of upd3 in hemocytes, which induces expression of the Turandot (Tot) genes in the fatbody and their accumulation in the hemolymph. Tot gene expression in the fat body also requires a functional Imd pathway, suggesting these pathways can integrate their activities (Agaisse et al, 2003; Ekengren et al, 2001; Ekengren & Hultmark 2001).

2.9.1. Regulation of Drosophila JAK/STAT signaling

Due to its involvement in various cellular processes in both Drosophila and mammals, it is clear that JAK/STAT signaling needs to be strictly controlled at different levels of the cascade. While loss-of-function alleles of the Drosophila JAK/STAT pathway components are embryonic lethal or cause varying developmental defects (e.g. Harrison et al, 1998; Yan et al, 1996; Binari & Perrimon 1994; Brown et al, 2001), hyperactivity in Drosophila hop, for example by the Tumorous-lethal (hopTum-L) allele with a substitution of glutamic acid for glycine at residue 341, results in premature differention of lamellocytes and formation melanotic tumors. The same phenotype results from overexpression of wild-type hop in the lymph glands (Harrison et al, 1995), and resembles the situation in humans where a gain-of-function mutation in human JAK2 causes myeloproliferative disorders.
Some of the known regulators of *Drosophila* JAK/STAT signaling were identified based on their homology with the mammalian counterparts. Mammalian JAK/STAT signaling is downregulated by a negative feedback loop by the SOCS proteins, by a mechanism that appears to be conserved between flies and man. The *Drosophila* genome codes three SOCS-like proteins, Socs16D, Socs36E and Socs44A. Of these, Socs44A is not a transcriptional target of Stat92E, but has nevertheless been shown to regulate JAK/STAT signaling to some extent, while no role for Socs16D in JAK/STAT regulation has been found (Rawligs et al, 2004). In contrast, Socs36E is induced by JAK/STAT signaling and thereby negatively regulates the JAK/STAT pathway via a negative feedback loop similar to that found in the human pathway. In vivo overexpression or ablation of *Socs36E* affects the JAK/STAT pathway-associated phenotypes, such as development of the eye and wing (Callus & Mathey-Prevot 2002; Karsten et al, 2002). Another conserved negative
regulator of JAK/STAT signaling is dPIAS, which interacts with activated Stat92E and could therefore inhibit its binding to target DNA, like the mammalian PIAS proteins (Betz et al, 2001).

Ken & barbie is an ortholog of the mammalian proto-oncogene B-Cell Lymphoma (BCL6), and is shown to negatively regulate a subset of JAK/STAT target genes by binding a DNA sequence that overlaps with the Stat92E binding site (Arbouzova et al, 2006). The protein tyrosine phosphatase Ptp61F was identified in two separate RNAi screens as a negative regulator of the *Drosophila* JAK/STAT pathway, its suggested function being to target activated Stat92E and/or hop for deactivation (Baeg et al, 2005; Müller et al, 2005). Stat92E activity was shown to be negatively regulated also by SUMOylation (Grönholm et al, 2010). One of the novel positive regulators identified in our screen (IV), Not4, is needed for proper Stat92E DNA binding and it’s human homologue CNOT4 also appears to regulate STAT-mediated gene responses (Grönholm et al, 2012). In addition, the bromo-domain-containing protein BRWD3 was found to regulate *Drosophila* JAK/STAT signaling positively (Müller et al, 2005). *Drosophila* JAK/STAT pathway is also known to interact with other signaling pathways, including Notch (Bach et al, 2003; Mukherjee et al, 2006) and the EGFR signaling (Callus & Mathey-Prevot 2002). Endocytosis has also been implicated in negative regulation of JAK/STAT pathway, although a positive role has also been suggested (Devergne et al, 2007; Vidal et al, 2010).

The *Drosophila* JAK/STAT pathway has been intensely screened both in vitro and in vivo with forward and reverse genetic methods, and multiple putative regulators have been identified (Bach et al, 2005; Baeg et al, 2005; Mukherjee et al, 2006; Müller et al, 2005). However, the candidate genes identified in different screens show only a limited overlap, and the functional mechanisms remain largely elusive even for many of the verified regulator genes. Therefore, it appears likely that new molecules and mechanisms that are needed for proper regulation of *Drosophila* (and mammalian) JAK/STAT signaling remain to be identified.
3. Aims of the study

*Drosophila* has proven to be an amenable model organism to study innate immunity, and the evolutionary conserved signaling cascades controlling *Drosophila* immune responses have been intensely studied during previous decades. As a result, the core components of these pathways have become relatively well known. It has also become clear that, as in mammals, also the *Drosophila* immune response is tightly regulated in order to avoid inappropriate responses that would be harmful for the host, yet the molecules and mechanisms behind the control have remained largely elusive. Therefore, the aim of this study was to identify and study novel negative regulators and regulatory mechanisms required to control the *Drosophila* immune response, as well as to study the possible conservation and human relevance of these molecules.

More specifically, the aims of this study were:

1. To identify novel negative regulators of the *Drosophila* Toll and JAK/STAT pathways by carrying out a genome-wide RNAi screen for each pathway.

2. To further characterize the identified molecules in order to elucidate their role in regulation of the Toll and JAK/STAT pathways.

3. To study two previously identified negative regulators of the *Drosophila* Imd pathway, zfh1 and pirk, in more detail, in order to verify their relevance in vivo and clarify their molecular mechanisms in inhibiting Imd signaling.
4. Materials and methods

4.1. dsRNA libraries and synthesis (I-IV)

In I and IV, a commercial Drosophila genome RNAi library containing a set of 13,625 PCR products with dual T7 promoter sequences (Medical Research Council (MRC) Geneservice Ltd., Cambridge, UK) was used, in addition to 2,400 dsRNAs that were transcribed from an S2 cell-derived cDNA library (Ulvila et al, 2006).

Targeted dsRNAs were synthesized from S2 cDNA by a two-step PCR reaction. In the first step, a region of approximately 1,000 bp on the gene of interest was amplified with a pair of gene specific primers. In the second reaction, the first PCR product was used as a template to amplify a 500-700 bp region on the gene of interest with primers that both contained the T7 promoter sequence (5’-GAATTAATACGACTCATATAGGGAGA-3’) in the 5’ end. The second PCR product with the T7 binding sites in each end was used as a template to synthesize the sense and antisense RNAs with the T7 MegaScript RNA polymerase (Ambion, Austin, TX). The dsRNAs were precipitated with LiCl and analyzed by gel electrophoresis and spectrophotometer.

pMT/BiP/V5-His/GFP plasmid (Invitrogen, Carlsbad, CA, USA) was used as a template for the production of the negative control GFP dsRNA, cDNA from S2 cells was used as the template for other dsRNAs.

Primers used for targeted dsRNAs were GFP, 5’-T7-GCTCGGGAGATCTCC-3’ (forward) and 5’-T7-CTAGACTCGAGCGGC-3’ (reverse); dMyD88, 5’-T7-GCTGGCCAAGCAGAAGG-3’ (forward) and 5’-T7-GGAACGAGCCAAACTTGTCC-3’ (reverse); Cactus, 5’-T7-AGCGGATGATTTG-3’ (forward) and 5’-T7-GCTTGACTGACG-3’ (reverse); imd, 5’-T7-CACAATGTGACGAGGG-3’ (forward) and 5’-T7-GCTGGACTGAC-3’ (reverse); Relish, 5’-T7-GCAAACGGACTTCGC-3’ (forward) and 5’-T7-GACAGCTACTGAC-3’ (reverse); Stat92E, 5’-T7-CTCAATGCTGCTTC-3’ (forward) and 5’-T7-GAGACACACTTGC-3’ (reverse); Socs36E, 5’-T7-GTCAGCCATCACCA-3’ (forward) and 5’-T7-TTCTGTCAGGGATC-3’ (reverse); zfh1, 5’-T7-AGAGGCTGCTGACG-3’ and 5’-T7-GCGGAATCGCAACTC-3’ (reverse). The primers used for targeted dsRNAs against the putative negative regulators of Toll and JAK/STAT pathways identified in the RNAi screen are listed in Table 1.
Table 1. Primers for targeted dsRNAs against putative negative regulators identified in the RNAi screens.

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<tr>
<th>GENE</th>
<th>FORWARD PRIMER</th>
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<td>5’-T7-GCCGACGCTTCTCC-3’</td>
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<td>Kap-a3</td>
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<tr>
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<td>5’-T7-AGCCCTACACTGTC-3’</td>
<td>5’-T7-GCCGACGCTTCTCC-3’</td>
</tr>
</tbody>
</table>

4.2. S2 cell culture (I-IV)

A macrophage-like *Drosophila* S2 cell line (Schneider, 1972) derived from late embryos was used for the RNAi screens and subsequent in vitro experiments. S2 cells were cultured in Schneider medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 25°C.

4.3. Reporter assays (I-IV)

1.0 × 10⁶ S2 cells were seeded on 24-well plates and transfected with 0.1 µg of the indicated reporter plasmid together with 0.1 µg *Actin5C*-β-galactosidase (*Act5C*-β-gal) reporter plasmid for
monitoring cell viability and transfection efficacy. Transfections were done using FuGENE transfection reagent (Roche). 1.0 µg of control and experimental dsRNAs were added in the transfection mixes. The signaling pathways were activated by co-transfecting the cells with 0.1 µg of the indicated constructs and adding heat-killed *Escherichia coli* (*E. coli*) on to the cells 24 h prior to cell lysis (in I). Protein production from the pMT/V5/HisA vector (Invitrogen/Life Technologies) was induced by addition of CuSO₄ to a final concentration of 500 µM 24 h prior to cell lysis. 72 h after transfection, the cells were harvested into 1.5 ml tubes, pelleted and lysed in 50 µl of Passive Lysis Buffer (Promega). The luciferase and β-galactosidase activities were measured from the lysates on 96-well plates using Luciferase Assay Kit (Promega) and a luminometer (Thermo Electron), and 2-Nitrophenyl-β-D-galactopyranoside (ONPG; Sigma-Aldrich) and a spectrophotometer (Thermo Electron), respectively, according to manufacturer’s recommendations.

4.4. RNA extraction and qRT-PCR (II, IV)

For quantitative RT-PCR (qRT-PCR) experiments, S2 cells were seeded on 24-well plates and treated with 3 µg of experimental or control dsRNAs, which are readily internalized by the cells. 48 h later, heat-killed *E. coli* was added to activate the Imd pathway, after which the cells were harvested at indicated time points.

Total RNAs from S2 cells, HeLa cells and flies were extracted using TRIIsure reagent (Bioline, London, U.K.) or Tri reagent (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions and subjected to quantitative RT-PCR analysis.

qRT-PCR was carried out using QuantiTect SYBR Green RT-PCR Kit (Qiagen) and an ABI7000 (Applied Biosystems) instrument and software (for S2 and HeLa cells) or the BioRad iScript™ one-step RT-PCR kit with SYBR® Green (BioRad, Hercules, CA, USA) (for flies) according to manufacturer’s instructions.

Primers used for qRT-PCR are listed in each original communication.

4.5. Cloning and constructs (II-IV)

The modified pMT/GFP/V5/His plasmid was a gift from Dr. Iivari Kleino.

*zfh1* was cloned into the EcoRI and XhoI sites of the pMT/V5/HisA vector (Invitrogen/Life Technologies) in two pieces utilizing the XhoI restriction site present in the gene. The SD06902 (DGRC, Indiana University, US) cDNA clone and S2 cell cDNA were used as templates and the
primers used were: first part, 5'-CACACAGAACATGTTGCTCTGTCTGGCGCC-3' (forward) and 5'-GGCGAGTGGAGCTGCTCG-3' (reverse); second part: 5'-CGCCTCCTCAGGCCAGCCGC-3' (forward) and 5'-CACACACTCGAGTTTCTGCTAGGGGCTTGCAATAGG-3' (reverse)

*HuZEB1* was cloned into KpnI and NotI sites of the pMT/V5-HisA vector using the cDNA from IMAGE clone 40036600 (Geneservice Ltd, Cambridge, UK) as a template, the primers used being: 5'-CACACAGGTACCATGGCGGATGGCCCC-3' (forward) and 5'-CACACAGCGGCCGACATTTGTCTT-3' (reverse)

The *pirk* overexpression construct was created by cloning the gene *CG15678* from S2 cell cDNA to the pMT/V5/His-A vector (Invitrogen). The deletion mutants PN (pirk(M1-K51)), PD (pirk(D52-V136)), and PC (pirk(V137-I197)) were created by cloning nucleotides 1-153, 154-408, and 409-591, respectively, from *pirk* cDNA to pMT/V5/His-A vector. The primers used were: PN: 5'-GCACGCTTATCCATGGGCGTCTGTA-3' (forward) and 5'-CACACACTCGAGCTTGCGTAGATG-3' (reverse); PD: 5'-CACACAGAATTCATGGATCTGGTGGGCAACTGC-3' (forward) and 5'-CACACACTCGAGCTTGCTGGCGTAGATG-3' (reverse); PC: 5'-CACACAGAATTCATGGTCAAGTCCCTGGATCTTAG-3' (forward) and 5'-GCACGCTTATCCATGGGCGTCTGTA-3' (reverse).

To obtain myc-tagged constructs of *imd* and the cytoplasmic tail of *PGRP-LC* (amino acids M1-V293), the cDNA sequences were cloned in the pMT-HisA plasmid with AgeI restriction site in the reverse primer, which cleaves off the V5 tag originally found following the multiple cloning site in the the plasmid. The myc-tag (followed by 2 stop codons) was then inserted in the plasmid by annealing the primers: 5'-CCGGTGAGGAGCAAGCTGATTCAGAGGGAGACCTGTGATGA-3' (forward) and 5'-CCGGTCATCATCAGAGTGTCCTCGAGATCAGCTCTGCTGCGTGTCGATCA-3' (reverse), treating with T4 polynucleotide kinase, and then ligating into the AgeI-digested and CIAP-treated plasmid. Myc-tagged *Iap2* and *Tak1* constructs were obtained by adding the tag at the end of the PCR product in the reverse primer, then following by PmeI-digestion, which also cleaves off the V5 tag from the pMT-HisA plasmid. The primers used for each cDNA were: *imd*: 5'-CGCGCAGGCTTATCCATGGGCGTCTGTA-3' (forward) and 5'-CCGGTCAAAAGCTCAGAGGCCAGGACCTGTGATGA-3' (reverse); *Iap2*: 5'-CACAGAATTCATGCCAGGAGCTGCTGGCGGC-3' (forward) and 5'-GCCCGG
GT TAAACTTATTACAAGT CCTCTTTCAGAAATGAGCTTTTGCTCCGAAAGGAACGTGCGCACGAATC-3’ (reverse); Tak1: 5’- CAGCGGCCGCTCATGGCCACACAGCATCGCTGGAC-3’ (forward) and 5’-

GGCCGGGTTTAAACTTATTACAAGT CCTCTTTCAGAAATGAGCTTTTGCTCCGAGTTGTG

ATGCCTGGAT C-3’ (reverse); PGRP-LC (M1-V293): 5’-

CGCGCGGTACCATGCCCCGGAGTTGG-3’ (reverse) and 5’-

CGCGGCAGGTTTTTCCAGAATGAAACGG-3’ (forward)

ET was cloned from S2 cell cDNA into KpnI and EcoRI sites of the pMT/V5-HisA vector in two fragments utilizing the KpnI restriction site found within the cDNA. The primers used were: part I: 5’-CACACACAGGTACCATGCCCGCCTGGCTGTC-3’ (forward) and 5’-

ACTCGGGGTACCTTTCGGACC-3’ (reverse); part II: 5’- CGGAAA GGTACCCCGAGTTGC-3’ (forward) and 5’- CACACAGAATTCGCGCACGGGTGGCGG-3’ (reverse).

The myc-tagged ET, hop and Dome constructs were cloned with the myc-tag added in the reverse primer sequence, followed by Pmel-digestion. The primers used were: ET: 5’-CACACACAGGTACCATGCCCGCCTGGCTGTC-3’ (forward) and 5’-

GGCCGGGTTTAAACTTATTACAAGT CCTCTTTCAGAAATGAGCTTTTGCTCCGAGTTGTG

TGCCGG-3’ (reverse); hop: 5-CACACAGAATTCATGGCCCGCCTGGCCAAACGGG-3’ (forward) and 5’- GGCCGGGTTTAAACTTATTACAAGT CCTCTTTCAGAAATGAGCTTTTGCTCCGAGTTGTG

CGGATTGTG-3’ (reverse); Dome: 5’- CACACAGGTTACCATGGTGCGCCAGGAGC-3’ (forward) and 5’-

GGCCGGGTTTAAACTTATTACAAGT CCTCTTTCAGAAATGAGCTTTTGCTCCGAGGACGTG

CCGATTGTG-3’ (reverse).

All the cloned constructs were sequenced using BigDye for the sequencing PCR reaction. For sequencing, the PCR products were precipitated with EtOH and dissolved in HiDi formamide. Protein expression from the constructs was verified by Western blot analysis, whereby S2 cells were transfected with the constructs as above, protein production was induced by adding 500 µM CuSO₄, the cells were lysed, and a volume corresponding to 25 µg of total protein was used for SDS-PAGE and immunodetection as described below.
4.6. Co-immunoprecipitation and Western blotting (III-IV)

S2 cells were seeded onto 6-well plates and transfected with 0.5 µg of the indicated C-terminally V5-tagged constructs in the pMT-V5-HisA vector together with the indicated myc-tagged constructs in the same vector. Expression of the tagged proteins was induced 48 h post-transfection by adding CuSO₄ to a final concentration of 250 µM, followed by harvesting the cells 24 h later. Two protocols for cell lysis and immunoprecipitation were used.

In III, the cells were lysed in 1% NP-40 lysis buffer (containing 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.5% Na-deoxycholate, 20 mM NaF, 1% Nonidet-P40 (Igepal CA-630; Sigma-Aldrich), and 10% glycerol), supplemented with 100 µg/ml PMSF (Sigma-Aldrich) and the Complete mini protease inhibitor cocktail (Roche Applied Sciences). Lysates were incubated on ice for 30 min, followed by centrifugation for 15 min at 16,000 x g at + 4 °C. The total protein concentration of each lysate was measured by BCA Protein Assay Kit (Pierce) with albumin standards, and a lysate volume corresponding to 1 mg of total protein for each sample was separated for co-immunoprecipitation. The lysates were first pre-cleared by adding 25 µl of a 1:1 suspension of protein G-Sepharose beads (GE Healthcare) in lysis buffer and incubating the mixture for 50 min with rotation at +4°C. The resulting supernatants were transferred to fresh tubes. 25 µl of protein G-beads were added to 1 µg of anti-c-Myc rabbit IgG antibody (Novus Biologicals) together with the pre-cleared lysates. The samples were incubated overnight with rotation at +4°C and then washed with PBS containing PMSF and protease inhibitors for 4 x 10 min.

In IV, the cells were lysed in RIPA buffer (1 X PBS, 0.5 % sodium deoxycholate, 0.1 % SDS) with 1 % NP-40 and the protease inhibitor cocktail. Co-immunoprecipitation was carried out using 30 µl Protein G Dynabeads (Invitrogen/Life Technologies) per reaction. 2 µg of anti-c-Myc rabbit IgG antibody in 200 µl of PBST (PBS + 0.02 % Tween 20 (Sigma-Aldrich)) was bound to the beads by incubating 30 min at RT with rotation. The beads were washed with PBST once and the PBST was replaced by the lysate, which was again incubated 30 min at RT with rotation. The lysate was removed and the beads were washed 4 times with PBS.

Immunoprecipitates were separated from the beads by adding 25 µl of 2 x SDS loading buffer (Laemmli sample buffer with 2-mercaptoethanol), vortexing and incubating at 95°C for 5 min, electrophoresed in NuPAGE® 10% Bis-Tris Gel (Invitrogen) for approximately 50 min on 200 V, then blotted on a nitrocellulose membrane on 25 V for 1.5 h. Proteins were detected by incubating the membrane with mouse anti-V5 and goat anti-mouse antibody horseradish peroxidase conjugate
(Invitrogen), respectively for 1.5 h. The antibodies were diluted 1:5000 in blocking buffer (PBS, 0.05 % Tween 20, 5 % milk powder). In IV, a conjugated mouse anti-V5-horseradish peroxidase antibody (Invitrogen) was used to detect proteins. Proteins were visualized by ECL Plus Western Blotting Detection System (GE Healthcare Life Sciences).

4.7. Confocal imaging (III)

S2 cells were seeded onto 24-well plates and transfected with 0.1 µg of pMT/GFP/V5/His plasmid expressing pirk as a C-terminal GFP-fusion protein. Pirk expression was induced 48 h later by adding CuSO₄ to a final concentration of 100 µM. 24 h after induction the cells were passed 1:3 to 6-well plates with a coverslip on the bottom of each well. The cells were allowed to attach for 30 mins, after which the culture medium was removed and the cells were fixed with 3.7% formaldehyde and 5% sucrose for 20 min. The coverslips were washed 3 x with PBS and mounted to objective glasses with Vectashield Mounting Medium for Fluorescence with DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories Inc.).

4.8. HeLa cell culture and transfections (II)

Human cervical cancer-derived HeLa cells were grown in DMEM plus GlutaMAX (Gibco/Life Technologies, Carlsbad, CA) with 10% FBS, 1% nonessential amino acids (Sigma-Aldrich), 100 U/ml penicillin, and 100 µg/ml streptomycin at + 37 °C with 5 % CO₂. For transfection, 6 × 10⁴ cells per well were seeded onto a 24-well plate. Twenty-four hours later, the cells were transfected with 0.1 µg NF-κB-luciferase reporter plasmid, 0.05 µg CMV-β-galactosidase reporter plasmid, and 50 pmol siRNAs (Ambion, Austin, TX) using Lipofectamine transfection reagent (Invitrogen/Life Technologies) and Opti-MEM medium (Life Technologies). GFP siRNA (Silencer GFP [eGFP], Ambion, Applied Biosystems) was used as a negative control, RelA siRNA (ID 216912) as a positive control and a mix of three independent ZEB1 siRNAs for experiments (ID’s s13883, s13884 and s13885). Forty-eight hours after transfection, NF-κB signaling was induced by adding 10 ng/ml TNF-α (Sigma-Aldrich) onto the cells. 6 h later the cells were washed with PBS and lysed in 100 µl of Passive Lysis Buffer (Promega), transferred into 1.5 ml tubes, the cell debris was then pelleted and luciferase and β-galactosidase activities were measured from the cell lysates. For qRT-PCR HeLa cells were transfected with the siRNAs, treated with TNF-α and washed with PBS as above, followed by RNA extraction and qRT-PCR analysis.
4.9. In vivo experiments

4.9.1. Drosophila maintenance and stocks (II, IV)

*Drosophila* stocks were kept on a standard mashed potato diet at 25 °C.

RNAi transgenic fly stocks used were from Vienna *Drosophila* RNAi Center: 42856 (*zfh1-IR1*) and 42857 (*zfh1-IR2*), 19756 (to target *CG14225*, hereafter referred as *ET-IR1*), 100881 (*ET-IR2*) and 43866 (*Stat92E-IR*). *Fadd-IR* and *C564-GAL4* driver flies were obtained from Prof. Bruno Lemaitre (Centre de Génétique Moléculaire, Centre National de la Recherche Scientifique, Gif-Sur-Yvette, France) and *tubulin-GeneSwitch-GAL4* flies were obtained from Prof. Howard Jacobs (Institute of Biomedical Technology, University of Tampere, Finland). *GMR-updΔ3'/FM7;CyO/Sco* and the *w;ey-GAL4* flies were from Professor Michael Boutros (German Cancer Research Center (DKFZ), Heidelberg, Germany)

*UAS-RNAi* flies were crossed over *C564-GAL4* or *tubulin-GeneSwitch-GAL4* driver flies or to *w*118 flies for controls. In flies crossed over the *tubulin-GeneSwitch-GAL4* driver, the GAL4 construct was induced by keeping 1 to 2-day-old F1 flies in vials with Mifepristone (200 μM; Sigma-Aldrich) in the food for 5 days at +29 °C prior to the infection. F1 flies crossed to *C564-GAL4* driver were also kept at +29 °C for 5 days.

For isolation of larval fat bodies, the *UAS-zfh1-IR* flies were crossed with *C564-GAL4* flies, and the parental flies were let to lay eggs for 24 hours at +25 °C, after which the vials were transferred to +29 °C. The larvae were maintained at +29 °C until they reached late L3 stage (4-5 days). The larvae were washed, and their fat bodies were dissected using sharp forceps, and kept in 20 µl of PBS on ice until RNA extraction. This was followed by RNA analysis and qRT-PCR as described above. Six biological replicates were used, each containing six fat bodies.

4.9.2. Bacterial culture and fly infections (II, IV)

For *Enterobacter cloacae* (*E. cloacae*) (strain β12) infection, week-old healthy flies were pricked with a thin tungsten needle dipped in a concentrated culture of bacteria grown overnight on LB agar plates with nalidixic acid (15 μg/ml) selection. After 4 (for Imd pathway target genes) or 16 (for JAK/STAT pathway target genes) hours the flies were snap-frozen on dry ice, homogenized in TRI Reagent using a micropestle and subjected to RNA extraction and qRT-PCR analysis. The experiments were repeated at least twice, each set containing four biological replicas, where five to six flies were used per sample.
4.9.3. Fly infection by feeding *Serratia marcescens* (IV)

*Stat92E-IR, ET-IR1* and *ET-IR2* flies crossed to the drug-inducible *tub-GeneSwitch-GAL4* driver were used in *Serratia marcescens* (*S. marcescens*) infection experiment, which was essentially performed as described previously (Nehme et al, 2007). Flies were kept in standard fly food containing a 200 µM concentration of Mifepristone (Sigma-Aldrich) for two days before infection to induce the expression of *GAL4*. *S. marcescens* (strain Db11) was cultured in LB broth at +37°C to OD600=1. The bacterial suspension was then diluted 1:10 in 50mM sucrose, and Mifepristone to a final concentration of 200 µM was added. Cotton balls were placed in the bottom of fly culture vials and moistened with 8 ml of the contaminated sucrose solution. 35-40 one-week old flies were placed in the vials, which were kept at +25°C and survival was monitored. To control that the flies died of the *S. marcescens* infection, parallel flies were placed into vials without bacteria, or Mifepristone, or both. Flies were changed to fresh infection vials every 3-4 days to prevent dehydration.

4.9.4. Eye phenotype experiments (IV)

In order to study the role of *CGI4225/ET* in regulating JAK/STAT signaling in the context of eye development, flies carrying *GMR-updΔ3’* on the X chromosome were used. These flies overexpress * upd* in the eye, which causes a marked overgrowth of the eyes, otherwise flies are healthy. *GMR-updΔ3’/FM7;CyO/Sco;+* flies were first crossed over *ET-IR1/CyO* or *Stat92E-IR* flies. The F1 flies with one copy of *GMR-updΔ3’* and one copy of *ET-IR1* or *Stat92E-IR* (and the balancer/marker *CyO* on the other copy of the second chromosome) were then crossed over eye-specific driver *w; ey-GAL4* to induce *ET* and *Stat92E* RNAi. The offspring from the first cross without the *ET* RNAi construct (e.g. flies with *CyO* on the second chromosome) were used as controls, together with *CantonS* flies. To quantify the observations more objectively a scoring system was used. Pictures of each fly’s eyes were independently evaluated by five experienced researchers from our group as a blind test and the eye phenotype were given scores 0-5, 0 representing wild type and 5 the most severe phenotype.
5. Results

5.1. RNAi screening with luciferase reporter–based assays to identify novel negative regulators of Drosophila immune response (I, IV)

In order to identify novel regulators of Drosophila immunity we set up luciferase reporter–based assays for the Toll, Imd and JAK/STAT pathways. Using these assays we carried out genome-wide RNAi screens. The assays with their controls are outlined in Figure 5. A dsRNA targeting Green Fluorescent Protein (GFP), a gene not present in the Drosophila genome, was used as a negative control to exclude effects of activating the RNAi machinery in the cells, while the positive controls were dsRNAs targeting downstream components of each pathway. In both assays, an Actin5C-β-galactosidase (Act5C-β-gal) reporter was transfected together with the luciferase reporters in order to enable monitoring of transfection efficiency, cell viability and function of the translation machinery, and thereby excluding dsRNA treatments that were affecting the cells in a more general way not specific for the signaling pathway studied.

For the Toll pathway, we used a Drosomycin-luciferase (Drs-luc) reporter to assess pathway activity and to activate the signaling pathway, we transfected S2 cells with a constitutively active form of the Toll receptor, Toll10B. dsRNA treatment targeting a pathway component downstream of Toll, namely dMyD88, completely abolished luciferase activity (Fig 5A). In addition, the Drosomycin-luciferase reporter provided a means to monitor Imd pathway activation, since in addition to being a target gene for the Toll pathway, Drosomycin is also induced by the Imd pathway in S2 cells (Kleino et al, 2005; Tanji et a, 2007). Heat-killed E. coli induced Drs-luc activity when added to S2 cells either alone or together with Toll10B, and dsRNA targeting Imd pathway components abolished this activity (Fig 5A; I Fig 1A). Therefore, we could use the Drs-luc reporter to simultaneously monitor the effect of a given dsRNA treatment on both Imd and Toll pathways, thus making a genome-wide RNAi screen less laborious (I).

The intracellular part of the JAK/STAT signaling pathway is particularly well-conserved between flies and humans. In order to identify the intracellular proteins that act by directly regulating Drosophila JAK/STAT signaling, we carried out a genome-wide RNAi screen with a luciferase assay. For this, we transfected S2 cells with a JAK/STAT-responsive TurandotM-luciferase (TotM-luc) reporter and a constitutively active mutant of the kinase hopscotch, hopTum-I, to activate JAK/STAT signaling specifically and robustly (Fig 5B; IV Fig 1A). Again, dsRNA against GFP was used as a
negative control, while dsRNA targeting the transcription factor Stat92E was used as a positive control, the latter completely blocking TotM-luc reporter activity.

In I and IV we screened a commercial Drosophila genome RNAi library consisting of 13,625 PCR products together with additional 2400 dsRNAs from an S2 cell-derived cDNA library (Ulvila et al, 2006), thus giving 16,025 sequences altogether.

Figure 5. The luciferase assays for RNAi screens to identify novel regulators of Drosophila immune response. A) Drosomycin-luciferase (Drs-luc) reporter can be used to monitor activity of both the Toll and the Imd pathway simultaneously. B) TurandotM-luciferase (TotM-luc) reporter was used in the RNAi screen for novel regulators of JAK/STAT signaling. All data are shown as means ± SD.
5.2. Results of the RNAi screens

In each screen, we obtained a list of candidate genes, whose RNAi either decreased (potential positive regulators) or increased (potential negative regulators) the luciferase reporter activity significantly and reproducibly. In each case, the candidate genes were subcloned and sequenced to confirm their identity and the primary results were verified with a second individual dsRNA specifically designed against the gene of interest to exclude possible off-target effects.

The new components and positive regulators identified are discussed in the original communications: Gprk2 for the Toll pathway (I; Kallio 2011, PhD thesis) and enok, Med27, CG31716 (Not4) and Taf1 for JAK/STAT signaling (IV; Grönholm et al, 2012; Grönholm 2012, PhD thesis; Kallio 2011, PhD thesis). In addition, putative negative regulators for each of the pathways were identified, and will be discussed below.

5.2.1. Putative negative regulators of Toll signaling

Out of the 16,025 dsRNA treatments in the original screen for regulators of the Toll and Imd pathways, 10 repeatedly increased Drs-luc response by more than 50 % without affecting cell viability, and had the same effect also with a newly designed targeted dsRNA. The assay was repeated with Toll\textsuperscript{10B} or \textit{E. coli} induction separately, as well as with \textit{E. coli} induction and an \textit{AttacinA}-luciferase (\textit{AttA}-luc) reporter. It was found out that all the newly identified genes affected Toll\textsuperscript{10B}-mediated Drs-luc activity, and none affected Drs-luc or \textit{AttA}-luc activity induced by \textit{E. coli}, indicating that all these genes represent putative novel regulators of Toll pathway. One of the dsRNAs targeted a known component of Toll signaling pathway, the \textit{Drosophila} IκB protein Cactus, which caused a 1.7-fold increase in Drs-luc activity when induced by Toll\textsuperscript{10B}. Knock-down of the other genes caused a similar effect on Drs-luc activity (1.5-2.3 –fold increase; Figure 6A). The results were also verified with an assay where the cells were transfected with an active cleaved form of the Toll pathway ligand spätzle to induce signaling (also shown in Fig 6A). This time a clearer effect was observed: knocking down Cactus increased reporter activity up to nine-fold, as did the dsRNA treatments targeting some of the other candidate genes. This is likely due to the up to 100-fold induction caused by Toll\textsuperscript{10B} in Drs-luc activity, which is difficult to raise any further, compared to the relatively lower, 7-fold induction caused by spätzle, which is more easily increased further by removing a negative regulator (see also discussion).
Figure 6. Putative negative regulators of Drosophila Toll signaling. A) In the RNAi screen, ten genes were identified whose knock-down increased Drs-luc activity compared to the GFP dsRNA treated control. One of the genes was Cactus, a known component of the Toll pathway. The dsRNA treatments were repeated with newly designed dsRNAs using both Toll\textsuperscript{10B} and its ligand spätzle (spz) for Toll pathway induction. B) In an epistasis assay, Drs-luc was induced by dsRNA targeting the I\textsuperscript{\textbeta} protein Cactus, which releases Dif/Dorsal for translocation into the nucleus. The additional Drs-luc activity resulting from knocking down the new putative Toll pathway regulators indicates they are likely to function downstream of Cactus. All data are shown as means ± SD, the relative Drs-luc values of the GFP RNAi samples induced with expression of Toll\textsuperscript{10B} or spz, or Cactus RNAi are normalized as one. N ≥ 4, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

To gain more information about the possible role of the new Toll pathway regulators identified, we did an epistasis assay, where Drs-luc was induced by dsRNA targeting the I\textsuperscript{\textbeta} protein Cactus (Fig 6B). Removing Cactus releases the transcription factors Dif/Dorsal for translocation into the nucle-
us. In this setting, knocking down *Cactus* caused an approximate 70-fold induction in *Drs*-luc expression, which was further enhanced by simultaneously knocking down the new putative Toll pathway regulators, indicating they are likely to function epistatically downstream of Cactus, for example by regulating transcription in the nucleus.

Two groups of putative nuclear regulators are indeed among the genes identified. First, several members of the *Drosophila* chromatin remodeling Brahma complex, including Brahma itself, together with Dalao, moira and osa are found in the hit list (Fig 6), suggesting chromatin modification represents a means to regulate Toll pathway mediated transcriptional activity. Another group of gene products found among the list of putative negative regulators contains members of the Integrator complex, namely omd (oocyte maintenance defects), Int6S and Int8S (Fig6), which are known to function in processing of small nuclear RNA (snRNA) 3’-ends. snRNAs are abundant in the nucleus of eukaryotic cells where they associate with proteins and form small nuclear ribonucleoprotein (snRNP) particles, which have important regulatory functions in mRNA splicing and regulation of transcription factors.

5.2.2. Putative negative regulators of JAK/STAT signaling

The RNAi screen for regulators of the *Drosophila* JAK/STAT pathway also yielded a list of putative positive and negative regulators. As with putative positive regulators (IV), the results for the putative negative regulators were verified with independent targeted dsRNAs, and the TotM-luc values obtained with the targeted dsRNAs are shown in Table 2. The presumed functions of the gene products are also listed, many of which are associated with proteolysis. The dsRNA treatments were also repeated using different reporter assays to analyze whether the genes are general regulators of JAK/STAT signaling, or more context-specific. In this case, it turned out be difficult to repeat the results or obtain conclusive data considering these genes. Figure 7. shows targeted dsRNA treatments against the genes that showed more than a 5-fold induction of TotM-luc activity in the original screen, repeated with another Stat92E-responsive reporter, 10xSTAT-luc, which contains multiplied Stat92E-binding sites from the *Socs36E* promoter. In addition, induction using both the kinase hopTum-1 and the ligand upd are shown. dsRNA targeting a known negative regulator *Socs36E* was used as a positive control, which caused a modest but significant 1.4-fold increase in hopTum-1-mediated and a more robust 3.7-fold increase in upd-mediated 10xSTAT-luc activity. However, knocking down any of the candidate regulator genes does not seem to have a conclusive effect on
Table 2. Putative negative regulators of *Drosophila* JAK/STAT signaling identified in the RNAi screen.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Function</th>
<th>TotM-luc/Act5C-β-gal mean (%) ± SD</th>
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<td>CG10772</td>
<td>Fur1</td>
<td>Proteolysis</td>
<td>2132 ± 504</td>
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</tr>
<tr>
<td>CG9423</td>
<td>Kap-α3</td>
<td>Cell cycle checkpoint</td>
<td>1963 ± 145</td>
</tr>
<tr>
<td>CG2867</td>
<td>Prat</td>
<td>Purine biosynthesis</td>
<td>917 ± 287</td>
</tr>
</tbody>
</table>

reporter activity in these assays, as many of the dsRNA treatments actually decrease hop \(^{Tum-l}\)-mediated 10xSTAT-luc activity, and the increases in upd-mediated reporter activity are rather modest as well. These data suggests they might be more specific regulators of *Turandot* gene expression, rather than general regulators of JAK/STAT signaling.

However, one of the genes that was originally identified as a potential positive regulator of JAK/STAT signaling, *CG14225* (*ET*), also caught our attention in the follow-up assays. RNAi targeting *ET* caused a strong inhibition of hop \(^{Tum-l}\)-mediated reporter activity (70-80 % reduction in both TotM-luc and 10xSTAT-luc activity, Fig 7. and IV Fig 1C). As the dsRNA treatment was repeated using upd to activate signaling instead of hop \(^{Tum-l}\), a strong phenotype was again obtained, however, an opposite one, i.e. 37-fold increase in 10xSTAT-luc activity (Fig 7; IV Fig 2). This result suggested ET might actually be a negative regulator of JAK/STAT signaling, and so this gene was subjected to further study, which is discussed in the Results section 5.5. and in IV.
Figure 7. Putative negative regulators of Drosophila JAK/STAT signaling. Targeted dsRNA treatments for the putative negative regulators were repeated using another Stat92E-responsive luciferase reporter, namely 10xSTAT-luc, and both hop\textsuperscript{Tum-I} and the pathway ligand unpaired (upd) for induction. ET dsRNA targets the gene CG14225, and curiously, causes a strong decrease in hop\textsuperscript{Tum-I}–mediated reporter activity, while increasing upd-mediated reporter activity. Data are shown as means ± SD, the 10xSTAT-luc values of the GFP RNAi samples induced with hop\textsuperscript{Tum-I} or upd are normalized as one. N • 4. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

5.3. Negative regulation of Imd signaling at the transcriptional level by zfh1 (II)

In an earlier RNAi screen for Imd pathway components and regulators, we had identified a list of candidates for novel negative regulators (Kleino et al, 2005 Table S2). dsRNA treatment against one of these genes repeatedly caused remarkably strong increase in AttA-luc activity, and therefore the gene, zfh1, was chosen for further study.

As the name implies, zfh1 codes for a transcription factor with a homeodomain and zinc finger motifs found in two clusters. It has been shown to function in regulating several developmental processes, including local cell faith determination in heart and muscles and somatic stem cell renewal (Lai et al, 1993; Leatherman & Dinardo 2008; Postigo et al, 1999), but no function in immunity has been proposed so far. The 5803 nt mRNA of zfh1 encodes a 1054 aa transcription factor with a
homeodomain and nine C$_2$-H$_2$ zinc fingers, both of which are common DNA-binding motifs (II Fig 3A). The expression of the V5-tagged \textit{zfh1} construct from a metallothionein promoter containing plasmid was verified by Western blot, and the size was as reported previously (~145 kDa for \textit{zfh1} spliceform B) (II Fig 3B) (Fortini et al., 1991).

5.3.1. \textbf{Zfh1 inhibits Imd signaling downstream of Relish in vitro}

\textit{Zfh1} appeared to be an interesting candidate for a novel negative regulator of Imd signaling, since knocking down \textit{zfh1} dramatically increased \textit{AttacinA}-mediated luciferase activity in S2 cells (II Fig1A), but did not affect Toll pathway activity (II Fig 1D). An increase in luciferase activity was also apparent, although not as dramatic, when a \textit{CecropinB}-luciferase (\textit{CecB}-luc) reporter was used to monitor the Imd pathway activity (II Fig 1B). To further confirm these results, endogenous AMP expression was measured by qRT-PCR in S2 cells treated with \textit{zfh1} dsRNA and heat-killed \textit{E. coli}. Also in this case, \textit{zfh1} knock-down hyperactivated expression of the Imd pathway mediated AMP genes: \textit{AttacinD} (\textit{AttD}), \textit{DiptericinB} (\textit{DptB}) and \textit{CecB} (II Fig 2A-C). The efficacy of the \textit{zfh1} dsRNA treatment was confirmed by qRT-PCR (II Fig 2D). Of note, \textit{zfh1} RNAi appeared to cause some increase on the basal AMP expression on its own, without other stimulation, suggesting it could be a constitutive repressor. On the other hand, the kinetics of Imd pathway activation do not seem to be affected, as each AMP reaches its maximum expression peak at the same time point despite \textit{zfh1} knock-down.

The epistatic location of \textit{zfh1} in Imd pathway was assessed by two means. First, Imd pathway was induced in S2 cells by transfection with constructs expressing either \textit{imd} or a constitutively active \textit{Relish} (\textit{\Delta SRR}) lacking the serine-rich region on S29-S45 (Stöven et al, 2003), and \textit{zfh1} was again knocked down by RNAi (II Fig 1C). The clear increase in \textit{AttA}-luc activity obtained in this setting suggests \textit{zfh1} functions at the level of or downstream of Relish. Second, S2 cells were transfected with a construct expressing \textit{zfh1} under a metallothionein promoter (pMT-\textit{zfh1-V5}) together with an \textit{AttA}-luc reporter to monitor the Imd signaling activity and and also with constitutively active \textit{Relish} or treated with heat-killed \textit{E. coli} to activate the signaling. \textit{zfh1} overexpression blocks \textit{AttA}-luc response with both inducers, further confirming its role as a negative regulator of the Imd pathway, as well as its epistatic location at the level of or downstream of Relish (II Fig 3C). These results are consistent with the designated nuclear localization of \textit{zfh1} protein in \textit{Drosophila} embryos (Lai et al., 1991) and its binding of specific DNA sequences (Postigo et al., 1999). To confirm that the phenotype observed with \textit{zfh1} RNAi is indeed caused by a reduction in the amount of \textit{zfh1} protein,
rather than any unexpected off-target effect, we performed a rescue experiment with a dsRNA targeted against the 5' UTR region of zfh1 (the target sites of the dsRNAs on zfh1 mRNA are shown in III Fig 3A). This dsRNA treatment caused a similar hyperactivation in Imd pathway activity upon E. coli stimulation, as the dsRNA targeted against the coding region of zfh1, and the phenotype was almost completely rescued by ectopic expression of zfh1.

5.3.2. Effect of zfh1 on AMP expression in vivo

Next we wanted to assess zfh1’s role in Drosophila immune defense in vivo. For this, we crossed parental strains containing a UAS element followed by a sequence coding for a hairpin loop targeted against the zfh1 sequence (zfh1-IR) and a driver line having the transcriptional activator protein GAL4 controlled by the C564 promoter, thereby resulting in offspring flies that had the zfh1 RNAi directed to the fat body. Two UAS-zfh1 RNAi lines and a UAS-Fadd RNAi line (as a positive control) were crossed to the C564-GAL4 lines. For additional controls, the RNAi lines and the driver line were crossed to wild-type w1118 flies. Week-old F1 flies were infected with the gram-negative bacterium E. cloacae and total RNAs were isolated after four hours. qRT-PCR analysis was used to measure the expression level of the Imd pathway target genes CecB and DptB (II Fig 5A-B). While knocking down the Imd pathway component Fadd in the fat body severely impairs the induction of both CecB and DptB upon infection, zfh1 RNAi increases CecB induction by approximately two-fold, whereas no significant effect on DptB expression was observed. Moreover, no CecB or DptB expression was seen in uninfected zfh1 RNAi flies, indicating that, in contrast to S2 cells, zfh1 does not seem to affect the basal expression of AMPs in vivo (II Fig 5A-B). Thus, the role of zfh1 as a universal negative regulator of the Imd pathway in vivo could not be verified using this traditional UAS-GAL4 system. To exclude inefficient knock-down of the target gene as the reason for weak phenotypes observed, zfh1 expression level in the knock-down flies was assessed. For this, RNA from dissected fat bodies from UAS-zfh1-IR/C564-GAL4 L3 larvae was used as a template for qRT-PCR. As shown in II Fig 5C, C564-GAL4-driven zfh1 RNAi causes a substantial decrease in zfh1 expression level in larval fat body compared to w1118 –crossed controls, indicating the knock-down is relatively efficient at least in this setting.

Different genetic backgrounds of fly strains can cause ambiguity in Drosophila in vivo experiments; in addition, knocking down a gene can lead to developmental defects in embryonic or larval stages, causing misleading results. To avoid these biases, we used another UAS-GAL4 application with an inducible ubiquitous tubulin-GeneSwitch-GAL4 (tub-GeneSwitch-GAL4) driver. Using this
system, the UAS constructs remain inactive until GAL4 is induced by adding the drug Mifepristone into adult flies’ food. Also in this setting, Fadd RNAi abolished CecB and DptB induction, and again, zfh1 RNAi caused a significant hyperactivation of CecB expression in response to E. cloacae infection, however the effect on DptB expression was less clear (II Fig 5D-E). AttB and AttD induction was also monitored by qRT-PCR, but similarly to DptB, no conclusive phenotype was observed in zfh1 RNAi flies with either C564-GAL4 or tub-GeneSwitch-GAL4 (data not shown). Hence, knocking down zfh1 in vivo appears to hyperactivate CecB induction upon infection, but no consistent increases in expression levels of other Imd pathway induced AMPs can be observed.

5.3.3. ZEB1 regulates NF-κB activity in HeLa cells

Zfh1 has a human homologue, ZEB1, which is also a zinc finger and homeodomain containing transcription factor. ZEB1 and zfh1 share similar domain structure, have sequence similarity especially in the zinc fingers (30-80 %) and the homeodomain and are shown to have similar DNA binding and transcription repression properties (II Fig 3A; Fortini et al., 1991; Postigo and Dean, 1997; Postigo et al., 1999). Since the Drosophila Imd pathway closely resembles the mammalian TNFR signaling pathway, we wanted to study if the two proteins might also share a conserved function. First, we overexpressed human ZEB1 in S2 cells to see if it affected Imd signaling activity. The zfh1 RNAi phenotype caused by dsRNA targeting the 5’ UTR region was indeed partially rescued by ectopic expression of ZEB1 (II Fig 3E). Next, we studied the role of ZEB1 in human HeLa cells, which were transfected with a NF-κB-luciferase (NF-κB-luc) reporter, together with CMV-β-galactosidase (CMV-β-gal) plasmid to monitor cell viability, and a three-siRNA mix to knock down ZEB1 or the control siRNAs (targeting GFP as the negative control and RelA as the positive control). TNFR signaling was induced by adding TNF-α to the cells (II Fig 4A). Interestingly, instead of hyperactivating TNFR signaling, ZEB1 knock-down strongly decreased NF-κB-luc activity in this setting. Similar results were obtained when endogenous interleukin-1β (IL-1β) expression was analyzed by qRT-PCR in HeLa cells transfected with the siRNAs and stimulated with TNF-α (II Fig 4B). ZEB1 RNAi appeared to be effective, as a decrease of around 80 % in the expression level of ZEB1 was obtained compared to controls (II Fig 4C). Hence, ZEB1 appears to have a role in regulating TNFR signaling in human HeLa cells, but with an impact opposite to that observed in Drosophila cells.
5.4. Regulation of the Imd pathway via negative feed-back loop by pirk (III)

5.4.1. Pirk suppresses the Imd pathway response in vitro and in vivo

One of the target genes of mammalian NF-κB signaling codes for the NF-κB inhibitory protein IκB, which thus creates a negative feed-back loop to control signaling activity. In Drosophila, the inhibitory domain of Relish is encoded by the same gene, thus a feed-back regulation similar to mammals is unlikely to occur. However, if other regulatory proteins acting via a negative feed-back loop should exist, their gene expression should be induced via the Imd pathway upon microbial challenge. Indeed, a microarray analysis in S2 cells revealed a previously uncharacterized gene, CG15678/pirk, that is induced rapidly, peaking at 1 hour after induction with heat-killed E. coli (Kallio et al, 2005; Rämet et al, 2002; Valanne et al, 2007). The induction of pirk expression was also dependent on Relish both in vitro and in vivo, indicating that pirk might in fact be a novel negative feed-back regulator of Imd signaling (III Fig. 1A-B).

![Figure 8. Pirk negatively regulates the Imd pathway.](image)

**Figure 8. Pirk negatively regulates the Imd pathway.** Knocking down pirk in S2 cells hyperactivates AttacinA-luciferase (AttA-luc) expression in response to heat-killed E. coli. Data are shown as means ± SD, ** p ≤ 0.01, *** p ≤ 0.001.

We next studied pirk’s role in Imd pathway regulation using the AttA-luc reporter-based assay in S2 cells. The cells were treated with pirk dsRNA, or GFP dsRNA as a negative control. 48 h later, heat-killed E. coli was added onto cells to activate Imd pathway, and luciferase activity was measured at 1, 4, 8 and 24 h after induction. pirk RNAi hyperactivated Imd pathway response in all time points studied, indicating pirk negatively regulates Imd pathway mediated AMP expression in S2 cells (Fig 8; III Fig 2A; Kallio et al, 2005). A similar effect was seen in expression of
endogenous AMPs both in S2 cells and in flies where *pirk* expression was silenced by RNAi in fat body and lymph glands (III Fig 2C-D; III Fig 3; Kleino 2010, PhD thesis). Furthermore, ectopic expression of *pirk* abolished Imd pathway activation both in S2 cells (III Fig 2E) and in vivo – hence the name: *poor imd response upon knock-in* (III Fig 4).

Figure 9. Pirk interacts with the Imd pathway receptor complex. A) Pirk protein contains a central region that is conserved among the *Drosophila* species, named the pirk domain (PD). B) The pirk domain and the C-terminal region of pirk are capable of inhibiting the Imd pathway, while the N-terminal part appears to be dispensible. C and D) The pirk domain co-immunoprecipitates with imd, while the C-terminal part of pirk co-immunoprecipitates more strongly with the cytoplasmic tail of PGRP-LC. In B) data are shown as means ± SD, *** p ≤ 0.001.

5.4.2. Pirk interacts with the Imd pathway receptor complex

*Pirk* encodes a 197-aa protein with no recognizable signal sequence, suggesting it to be an intracellular protein. We visualized the cellular localization of pirk protein via confocal microscopy by expressing a GFP-pirk fusion protein in S2 cells. GFP-pirk was seen in the cytoplasm outside of the nucleus (III Fig 6A). To study the molecular mechanism of pirk’s function we first wanted to find out its interaction partners. We therefore carried out co-immunoprecipitation assays in S2 cells transfected with a V5-tagged pirk construct together with myc-tagged constructs expressing known
Imd pathway components PGRP-LC, imd, lap2 and Tak1. Pirk co-immunoprecipitated with imd and the cytoplasmic tail of the receptor PGRP-LC (III Fig 6D), suggesting protein interactions with these components.

To get a closer look at pirk’s function, its protein sequence was analysed more thoroughly. Pirk has no characterized domain structure or homologues, but a sequence search among the holometabolous insect genomes revealed putative orthologues, which share a conserved central domain (named the pirk domain) with repetitive structure, while the N- and C-terminal regions vary among insect species (Fig 9A; III Fig 5). Based on the discovered protein structure of pirk, we generated deletion constructs of the protein (Fig 9; III Fig 5A), and studied their interactions with imd and the cytoplasmic tail of PGRP-LC, and whether overexpression of the deletion mutants have an effect on Imd pathway activity in the AttA-luc assay. The N-terminal part (PN; consisting of amino acids M1-K51) showed no interaction with either imd or PGRP-LC (data not shown), and accordingly, its ectopic expression had no effect on Imd pathway activity in S2 cells (Fig 9B; III Fig 7C). Instead, the central part, the pirk domain (PD; amino acids D52-V13) co-immunoprecipitated with imd, whereas the C-terminal part (PC; amino acids V137-I197) co-immunoprecipitated with the cytoplasmic tail of PGRP-LC (Fig 9C-D; III Fig 7A-B). Ectopic expression of both of these constructs blocked Imd pathway response in S2 cells (Fig 9B; III Fig 7C). Thus, while the N-terminal region of pirk appears to be dispensable, pirk’s function in Imd pathway regulation is mediated by the pirk domain and the C-terminal part, which interact with imd and PGRP-LC, respectively, and possibly interfere with the function of the receptor complex.

5.5. ET as a negative regulator of JAK/STAT signaling (IV)

5.5.1. ET downregulates JAK/STAT signaling at the receptor level

ET was originally identified as a potential positive regulator of JAK/STAT signaling, as ET RNAi decreased hopTum-l-induced TotM-luc and 10xSTAT-luc reporter activity by up to 80%. However, when the same assay was repeated using the pathway ligand upd for induction, an opposite result was obtained; ET RNAi actually dramatically increased reporter activity (Fig 7; IV Fig 3B-C). Correspondingly, ectopic expression of ET in S2 cells abolished upd-induced 10xSTAT-luc activity almost completely (Fig 10B), while hopTum-l-induced reporter activity was slightly enhanced (IV Fig 3E), indicating ET could actually be a negative regulator of JAK/STAT signaling.
Figure 10. ET as a negative regulator of Drosophila JAK/STAT signaling. A) ET codes for a transmembrane protein resembling Dome with the extracellular cytokine-binding module (CBM), but lacking the fibronectin-type III domain (FnIII) and the intracellular STAT-binding site. B) Overexpression of ET blocks upd-induced JAK/STAT pathway activity in S2 cells. C) ET co-immunoprecipitates with hop and Dome. In B), the data are shown as means ± SD, n ≥ 4, *** p ≤ 0.001.

The ET gene is located next to Dome in the Drosophila genome, and the gene product is a type-I transmembrane protein sharing similarities with Dome; they both contain an extracellular cytokine-binding module, although ET lacks the three extracellular fibronectin domains and the intracellular STAT-binding motif found in Dome (Arbouzova and Zeidler, 2006; Fig 10A; IV Fig 3A). Moreover, while Dome shares similarities with members of the mammalian interleukin-6 receptor family, ET’s closest human homologue is the signal transducing protein gp130, which associates with lig-and-bound IL-6 receptors and is needed for the activation of downstream signaling components (Taga & Kishimoto, 1997). We hypothesized that the curious results obtained for ET in the luciferase assays with different activators might be due to its presumed location on the plasma membrane,
epistatically between upd and hop, and were next interested to study if ET is involved in protein interactions with the *Drosophila* JAK/STAT pathway components. For this, S2 cells were transfected with myc-tagged ET together with V5-tagged JAK/STAT pathway components to see if they co-immunoprecipitate. Indeed, ET appears to co-immunoprecipitate with both Dome and hop, which also co-immunoprecipitate with each other, suggesting ET, Dome and hop function as a complex to mediate JAK/STAT signaling (Fig 10C; IV Fig 4B). To gain insight in how ET might function to negatively regulate JAK/STAT pathway, we first analyzed if ET affects receptor dimerization. S2 cells were transfected with both a myc-tagged and a V5-tagged Dome construct, which co-immunoprecipitate with each other. Overexpressing (or knocking down) ET did not affect this co-immunoprecipitation. The interaction seen between hop-V5 and Dome-myc was not affected by overexpression or knock-down of ET either (IV Fig 5A), suggesting ET does not function by disrupting Dome dimerization or Dome-hop interaction, at least not to the extent that could be seen in a co-IP assay. However, knocking down ET did cause hyperphosphorylation of Stat92E, further confirming it functions in the complex with the upstream pathway components Dome and hop and directly regulates the pathway activity (IV Fig 5B; Grönholm 2012, PhD Thesis).

### 5.5.2. ET’s role as a JAK/STAT pathway negative regulator in vivo

To gain insight into ET’s role as a regulator of JAK/STAT signaling in vivo, the UAS-GAL4 system was again used. A fly line carrying UAS-RNAi construct targeting ET (ET-IR1) was crossed to a ubiquitous inducible *tubulin-GeneSwitch-GAL4* driver, while a Stat92E RNAi line served as a control. To study ET’s effect on the JAK/STAT pathway activity, the *Turandot A* and *M* genes were used as the readout, since these genes are known to be induced JAK/STAT pathway -dependently in the fat body in response to stress (Agaisse et al, 2003; Ekengren et al, 2001a, b; Brun et al, 2006). The stress in this case was caused by infecting the flies with *E. cloacae*. When the RNAi was induced by adding Mifepristone into flies’ food, Stat92E RNAi flies lost the induction of both *TotA* and *TotM*, compared to the same flies on a normal diet. The opposite occurs with ET RNAi flies; inducing the RNAi hyperactivated the expression of *TotA* and *TotM* (IV Fig 6C-D), indicating that ET also negatively regulates the *Tot* stress gene response genes in vivo.

Besides its function in regulating immune and stress responses, the JAK/STAT pathway is shown to be important in regulation of cell proliferation and differentiation both during development and in adult flies. ET’s function in the regulation of these processes was assessed by two assays. First, it
Figure 11. Biological effect of ET RNAi in Drosophila in vivo. A) Knocking down ET enhances survival in an intestinal infection model. ET-IR2/tub-GeneSwitch-GAL4 flies with the RNAi induced with Mifepristone survive better from intestinal infection caused by S. marcescens, whereas flies with induced Stat92E RNAi die faster. B) ET RNAi enhances eye overgrowth caused by ectopic expression of upd in the developing eye. Stat92E RNAi causes a phenotype with small eyes despite the upd overexpression.

has been shown that the JAK/STAT pathway is activated in the Drosophila midgut intestinal stem cells in response to intestinal bacterial infection by upd molecules produced by the intestinal enterocytes, and that the signaling activity and subsequent cell division is needed for regeneration of the
damaged gut epithelium (Cronin et al, 2009; Jiang et al, 2009). We hypothesized this could be assessed by using an intestinal infection assay with *Serratia marcescens*, which is an entomopathogenic bacterium that can infect *Drosophila* through the digestive tract and cause disruption of the gut morphology and eventually death in a few days (Nehme et al, 2007). To study ET’s role in resistance to *S. marcescens* infection, the *tubulin-GeneSwitch-GAL4* driver together with two ET RNAi lines was used, while the *Stat92E* RNAi line, wild type flies and *Eater* mutant flies lacking an important phagocytic receptor and known to be susceptible to *S. marcescens* (Kocks et al, 2005) served as controls. Flies were fed with food contaminated by *S. marcescens* and with or without Mifepristone to induce the RNAi or leave it uninduced, respectively. Flies with induced *Stat92E* RNAi were more susceptible to *S. marcescens* infection - dying faster than the flies with uninduced RNAi or wild-type flies, whereas ET RNAi flies appeared to be more resistant to *S. marcescens* and survive better (Fig 11A; IV Fig 7A-B). As the previous reports, this result suggests that JAK/STAT signaling is important in survival of the flies from intestinal infection. The enhanced survival of ET RNAi flies might be due to improved stem cell renewal and gut wall repair caused by hyperactivated JAK/STAT signaling (Jiang et al, 2009), although verifying this would require more experiments.

Second, the JAK/STAT pathway is known to have several functions in regulating developmentary processes, such as development of the *Drosophila* eye imaginal discs. This is demonstrated by experiments where *upd* is overexpressed in the developing eyes, resulting in overgrown eyes in the adult fly (Zeidler et al, 1999; Bach et al, 2003). The overgrowth phenotype has been shown to be modified by overexpression or removal of the downstream JAK/STAT pathway components (Bach et al, 2003; Müller et al, 2005; Arbouzova et al, 2006; Mukherjee et al, 2006). To test whether ET has an effect on eye development, the *GMR-updA3)* flies were crossed so that they also expressed the ET RNAi construct under an eye-specific driver (*ey-GAL4*). The results are summarized in Figure 11B, which shows that the eye overgrowth phenotype caused by *GMR-updA3)* is fully rescued in flies expressing *Stat92E* RNAi under *ey-GAL4* driver – in fact, these flies have smaller eyes than wild-type flies. In contrast, the ET RNAi construct makes the eye overgrowth phenotype even worse, and thus we decided to name the gene *eye transformer* (Fig 11; IV Fig 7C). The observations were also quantified by researchers from our group in a blind test, where the eye phenotypes were evaluated and given scores from 0 to 5 according to their severity - 0 representing wild-type eye and 5 the most severe phenotype (IV Fig 7D).
6. Discussion

During the last decade, the signaling pathways regulating the Drosophila immune response have been under intense investigation. In addition to traditional genetic approaches, RNAi screens and biochemical studies have succeeded in identifying new components and regulators of the Drosophila Toll, Imd and JAK/STAT signaling cascades (see for example Aggarwal et al, 2008; Baeg et al, 2005; Choe et al, 2002; Gesellchen et al, 2005; Gottar et al, 2002; Grönholm et al, 2012; Kleino et al, 2005; Müller et al, 2005; Rämet et al, 2002; Valanne et al, 2011, 2012). It has become evident that, like in humans, the Drosophila immune response is also tightly regulated. Induction of a rapid and effective immune response against invading pathogens is crucial for a multicellular animal; however, proper silencing and control of said response is just as important. Moreover, there are bacteria and fungi present in many tissues, such as the gut, as useful commensals rather than pathogens, and they therefore need to be tolerated by the immune system. As such, the signaling pathways responsible for initiating the immune response are regulated on multiple levels. Importantly, many of these regulatory mechanisms are conserved from flies to man.

6.1. RNAi screens to find novel molecules regulating Drosophila immune response

Andrew Fire and Craig C Mello’s experiments in the nematode Caenorhabditis elegans set the basis for understanding the RNA interference machinery and its power in specific silencing of target genes (Fire et al, 1998). Soon it was discovered that introduction of double-stranded RNA silences the gene with the corresponding sequence also in Drosophila cells (Hammond et al, 2000). Since then, RNA interference has been a popular and powerful method to manipulate gene expression in Drosophila, and many large-scale screens have been carried out to identify gene products involved in various biological processes (Bach et al, 2003; Baeg et al, 2005; Boutros et al, 2004; Cronin et al, 2009; Kleino et al, 2005; Müller et al, 2005; Rämet et al, 2002). RNAi-based screening appears to be suitable for identifying and characterizing previously unknown components of many signaling pathways, therefore it is not surprising that RNAi has been the method of choice also for many studies elucidating those involved in Drosophila immunity.

6.1.1. General aspects

Drosophila S2 cells are especially amenable for large-scale RNAi screening because the dsRNA can be introduced in long fragments of 500-1000 bp, which are easily synthesized, and then internalized in the cells by scavenger-receptor mediated endocytosis (Saleh et al, 2006; Ulvila et al,
After internalization, the dsRNAs are diced into 21-23 bp fragments by the cell’s RNAi machinery. Although off-target effects cannot be completely excluded, in most cases the RNAi silencing appears to be very specific to the target gene, possibly due to the low concentration of each short dsRNA fragment (Kleino et al, 2005; Valanne et al, 2011). Since the efficacy of the gene knock-down is not monitored while screening, false negatives due to poor silencing remain a possible drawback. Nevertheless, validation of the observed RNAi phenotype by using at least one independent dsRNA targeting a different site of the gene (e.g. different exons or the UTR regions, if available) is easily done, and should be a standard procedure. Moreover, the availability of genome-wide collections of Drosophila UAS-RNAi lines facilitates easy in vivo validation of the phenotypes observed in vitro, or even carrying out the whole screen in vivo (Dietzl et al, 2007; Cronin et al, 2009).

In addition to RNAi silencing itself, designing the read-out of the screen is of crucial importance. This usually includes choosing a means for pathway activation as well as a reporter system to monitor effects of gene silencing for the given pathway. Quite often there will be balancing between specificity and robustness (to avoid false positive effects) and sensitivity (to avoid false negative results, e.g. to be able to recognize even the more modest effects). In addition, internal control for cell viability, such as the Actin5C-β-galactosidase reporter used in the screens of this study, is highly important to exclude genes whose knock-down affects cell function in a more general way not related to the signaling pathway studied (Valanne et al, 2011).

In general, the findings of the RNAi screens carried out for identifying novel regulators of Drosophila Toll, Imd and JAK/STAT pathways show rather varying degrees of overlap. However, in several cases the most important findings of a screen have been identified and more thoroughly studied by more than one group, while the role and importance of the rest of the genes on the hit lists often remains to be confirmed. The results of the RNAi screens that aimed to identify genes important for Drosophila immune response, including the ones used in this study, and the negative regulators identified, will be discussed in more detail below (see also Fisher et al, 2012, 2013; Valanne et al, 2011).

### 6.1.2. RNAi screen to identify regulators of the Toll pathway

Activation of the Toll pathway requires recognition of Gram positive or fungal microorganisms, followed by a protease signaling cascade. However, these proteins are secreted and function in a non-cell-autonomous manner, and therefore require an in vivo system for proper activation.
(Kambris et al, 2006). Thus, for an in vitro RNAi screen, a more downstream activator needs to be used, for example the constitutively active Toll$_{10B}$ used for screening, or the mature form of the Toll ligand, spätzle used for verifying the primary results in I of this study. In addition, a hEGFR-Toll fusion protein has been used (Kuttenkeuler et al, 2010).

It is apparent that the means of pathway activation can affect the results of an RNAi screen. Ectopic expression of Toll$_{10B}$ induces Toll signaling at a very high level; expression of spätzle causes a more moderate induction, likely resembling the physiological situation more. This probably explains the difference in the fold increase of hyperactivation caused by knocking down Cactus and the other putative negative regulators of Toll signaling. In our original screen also E. coli was added onto the cells to induce even higher Drosomycin response, which may explain the relatively low number of candidate negative regulators identified. Furthermore, Toll$_{10B}$ is a constitutively active mutant form of the pathway receptor, and by overexpressing this molecule one presumably by-passes the biological requirement for the ligand and the ligand-receptor interaction for signaling, which might otherwise be limiting factors in signaling activity. This kind of constant input might also interfere with some of the naturally occurring inhibitory mechanisms, causing them to function less effectively. In addition, despite the Drs-luc reporter being a somewhat artificial system to monitor pathway activity, it too has a “maximum capacity” for promoter activity and subsequent luciferase production. It appears that the overexpression of Toll$_{10B}$ alone pushes pathway activity rather close to the “full-speed” Drs-luc expression, whereas spz overexpression causes more moderate activation. This in turn leaves more room for boosting the luciferase expression further, for example by removing “brakes” such as Cactus, and could therefore be more suitable means for pathway induction for a screen aimed at identifying negative regulators for Toll signaling. On the other hand, such an assay might not be as efficient for identifying positive regulators, since observing a decrease in an induction level that is already low might be difficult. In addition, even in identification of putative negative regulators, specificity issues might arise, since false positives or genes that affect the pathway studied only indirectly, but are nevertheless able to cause variation in the reporter activity, might confuse the results. Whatever the induction method chosen for an RNAi screen, the cut-off limits should always be considered carefully in a case-specific manner to yield a reasonable number of “primary” candidate genes, and those results need to be verified with independent dsRNAs and using another induction method and/or reporter gene.
6.2.3. RNAi screening for novel regulators of the Drosophila JAK/STAT pathway

In our RNAi screen for negative regulators of Drosophila JAK/STAT pathway, the primary results with the TotM-luc reporter appeared not to be reproducible with the other reporters, indicating the candidate genes might be context-dependent negative regulators, but not general ones (Fig 7). The same phenomenon was observed when the candidate genes for positive regulators were studied (IV Figs 1 and 2). These genes were discarded from further studies, since we specifically wanted to identify novel general regulators of JAK/STAT signaling. However, the inability to of these genes to produce an RNAi phenotype with all the JAK/STAT reporters tested does not mean they cannot be biologically significant regulators of the pathway. Ken & Barbie is an example of such a selective regulator that only affects a subset of Stat92E target genes (Arbouzova et al, 2006; Baeg et al, 2005).

The Drosophila JAK/STAT pathway has been subjected to RNAi screening by other groups too, but there is a surprisingly small overlap between the findings of the screens (IV, Baeg et al, 2005; Müller et al, 2005). This has already prompted further analysis and follow-up screening by Müller and coworkers and Fisher and coworkers (Müller et al, 2008; Fisher et al, 2012). One aspect explaining the differences in the results is of course the basic screen settings: the cell line used, the reporter and the method of activation used. In contrast to our screen, Müller et al (2005) used the 6x2xDraf-luc reporter, upd-induction and the hemocyte-like Kc167 cells in their RNAi screen, and found 67 putative positive regulators and 24 putative negative regulators. Baeg et al (2005), on the other hand, used the 10xSTAT-luc reporter together with a Schneider 2 cell line derivative (S2-NP), which exhibits robust endogenous JAK/STAT activity due to endogenous upd2 expression, and identified 29 putative positive regulators and 92 putative negative regulators. In comparison to our list of four positive and one negative identified regulators (after confirming the primary results), the number of genes affecting JAK/STAT signaling identified by the two other screens seems rather large. The only gene found in all three screens, enok, was identified as a negative regulator in both the Müller et al and the Baeg et al screens. Whilst being consistent with our result with the 10xSTAT-luc reporter and upd-induction, we also found that the phenotype is different with other reporter/inducer settings. Both the Müller and Baeg screens identified the tyrosine phosphatase Ptp61F as a negative regulator of JAK/STAT signaling, whereas the gene was not identified in our screen. This may be due to the use of hopTum-l as pathway inducer in our screen, since Ptp61F is suggested to target hop for dephosphorylation. The lack of gene products acting at the receptor level
(except for ET) might also partly explain the lower number of JAK/STAT pathway regulators identified in our screen compared to the other two screens.

6.2. What do we know about negative regulation of the Toll pathway?

Although it seems likely that the core components of *Drosophila* Toll signaling pathway were already identified before the RNAi screening era, some novel regulators have been identified in the more recent RNAi screens. The positive regulators include Deaf-1, Myopic, Gprk2, ush and pnr, which are discussed in the Review of Literature (Reed et al, 2008; Kuttenkeuler et al, 2010; Huang et al, 2010; I). In addition, clues elucidating negative regulation of *Drosophila* Toll signaling have emerged.

Several members of the Brahma complex (Brahma, moira, dalao, osa) were identified in our screen (I) as potential negative regulators of *Drosophila* Toll pathway. Brahma and dalao were also identified as potential negative regulators of Toll signaling by Kuttenkeuler et al (2010). The *Drosophila* Brahma complexes are orthologues of the yeast SWI/SNF chromatin remodeling complex, and they use the energy of ATP hydrolysis to change the packaging state of chromatin by moving or restructuring nucleosomes, enabling proper exposition of the DNA elements that control gene expression (Clapier & Cairns 2009). Brahma is the catalytic subunit with the ATPase activity, whereas dalao, moira and osa are non-catalytic regulatory components (osa is only found in BAP complexes). Although the Brahma complexes are usually implied in facilitating gene expression, there is also evidence of their involvement in repressing gene expression. For example, the osa containing Brahma complex can repress wingless (wg) target genes presumably by maintaining the chromatin at the promoters of wg-responsive genes in a repressive conformation, thereby prohibiting the association of other transcription factors with their binding sites. In turn this would prevent the recruitment of components of the basal transcription machinery, only giving way to activating transcription factors once they accumulate in the nucleus in large enough number (e.g. when signaling is highly activated). Therefore, loss of function of osa or other components of the Brahma complex induce ectopic expression of Wg target genes in flies (Collins & Treisman 2000).

It is difficult to draw conclusions about the mechanism the Brahma complex could use to downregulate *Drosophila* Toll signaling based on the limited experimental data we currently have, however we did find out that knocking down the Brahma complex components in S2 cells also causes subtle activation of Drs-luc even without Toll pathway induction (from the 2.7-fold
induction by knock-out of moira to the 5.5-fold induction by osa RNAi), suggesting that these proteins might be involved in repressing the Drs promoter activity in a constitutive manner.

The nuclear protein required for Imd pathway target gene expression, Akirin is shown to interact with components of the Brahma complex during embryonic myogenesis, likely functioning as an accessory protein in the complex (Nowak et al, 2012). However, dsRNA targeting Akirin had no effect on Toll pathway mediated Drs-luc activity (Goto et al, 2008), and it might therefore be more specific for promoting transcription of Imd pathway target genes. The precise role of the Brahma complex, and its possible interplay with Akirin in regulation of Drosophila Toll and/or Imd pathway remains to be studied.

6.3. Zfh1 as a negative regulator of Imd signaling
Mammals use several regulatory mechanisms and molecules to control NF-κB target gene expression on the transcriptional level, including NF-κB coactivator and repressor proteins such as the CBP/p300 complex (Li and Verna, 2002). Therefore it is likely that such regulation mechanisms exist in Drosophila as well.

Among the genes identified as potential negative regulators of Imd signaling in our earlier RNAi screen (Kleino et al, 2005) was zfh1, with its knock-out having an extreme effect on AttA-luc activity. As the result was reproducible with the CecB-luc reporter as well and no significant effect on Toll signaling or cell viability was observed upon knock-down in S2 cells, we decided to study this gene more thoroughly. Furthermore, knocking down zfh1 also increased expression of endogenous AMPs in S2 cells in response to E. coli, whereas its overexpression blocked Imd pathway activation downstream or at the level of Relish.

However, the exact mechanism of zfh1’s action in the immune context remains elusive, partly due to the domain composition of zfh1, which enables several possible mechanisms of regulation. Zfh1 is a transcriptional repressor that contains two zinc finger clusters and a homeodomain. It binds E boxes on target gene promoter regions, but both zinc fingers and homeoboxes can bind DNA and regulate gene expression, possibly on different sites. Zinc fingers can also bind RNA or proteins. Since zfh1 RNAi alone causes activation of Imd pathway mediated AMPs, it could be a constitutive repressor. This could be accomplished by direct binding to and displacement or repression of Relish from the AMP promoter, since zfh1 clearly functions downstream or parallel to Relish in the Imd pathway regulation (Fig 12). Also the dramatic effect that both zfh1 knock-down and
overexpression have on Relish-dependent AMP expression in S2 cells makes a direct effector mechanism a tempting hypothesis. However, no interaction between zfh1 and Relish was observed in a co-immunoprecipitation assay (data not shown). Thus, we cannot exclude the possibility that zfh1 regulates Imd signaling in a more indirect way, such as inducing a repressor or repressing an activator for Imd pathway or mediating cross talk with another signaling pathway.

Intriguingly, we also found out that the human homologue of zfh1, ZEB1, regulates TNF-α-mediated NF-κB signaling in HeLa cells, although our results with ZEB1 siRNA suggest a positive regulation of NF-κB by ZEB1 in this context. However, several regulatory mechanisms have been proposed for ZEB1, facilitated by its domain structure similar to that of zfh1’s, which allows interaction with both DNA and coactivator and corepressor proteins. Thus, the repressor or activator status of ZEB1 can be determined by the expression level of the co-regulator, or can involve cell-type specific differences in post-translational modifications, or depend on the DNA context (Vandewalle et al, 2009). It is possible that the choice of cell-type affected the results in this study. Although HeLa cells are a widely used model for molecular biology studies, they are essentially malignant, and therefore might provide an altered environment where some proteins may exhibit changed functions. Thus it would be interesting to see if ZEB1 also has the same effect on TNFR signaling in another, non-cancerous cell type, especially since in Drosophila S2 cells ectopic expression of ZEB1 did partially rescue the RNAi phenotype, suggesting it is able to negatively regulate Imd pathway target genes in these cells.

Zfh1 is shown to participate in multiple developmental processes, including local cell faith and positioning in mesodermally derived tissues such as heart and muscles as well as somatic stem cell renewal (e.g. Lai et al, 1993; Postigo et al, 1999; Leatherman and Dinardo 2008), which makes in vivo experiments challenging. The UAS-zfh1 fly line is available, but ectopic expression of zfh1 in the fat body is lethal at embryonic or larval states, and even with the inducible tub-GeneSwitch-GAL4 driver only part of the offspring eclose successfully (perhaps due to leakiness of the driver) and die within a few days after replacement to Mifepristone-containing food. We therefore reasoned that the possible abnormalities in these flies’ immune response could also result from developmental defects or tissue damage not related to Imd signaling, and could not be considered reliable. In contrast, neither C564-GAL4 nor tub-GeneSwitch-GAL4 driven UAS-zfh1 RNAi reduced flies’ viability or caused obvious phenotypic changes, and even though the possibility of subtle developmental defects cannot be excluded, the flies’ ability to mount normal (or elevated) immune
responses indicates functional fat body tissue. However, zfh1 could also be a more important repressor of Imd pathway mediated AMPs in a tissue other than fatbody, and therefore the phenotype would not be clearly revealed in a systemic infection model. This kind of repression mechanism is used by Caudal, which inhibits constitutive activation of Relish-dependent AMPs by commensal microbiota in the fly intestine (Ryu et al., 2008). On the other hand, the extremely strong knock-down phenotype observed in S2 cells could partly be due to enriched expression of zfh1 in these cells (FlyAtlas CG1322-RB; Chintapalli et al., 2007), perhaps making it a more prominent regulator of Imd signaling in S2 cells than in other cell types.

Figure 12. Zfh1 and pirk as negative regulators of the Drosophila Imd pathway. Pirk is induced as a target gene of the Imd (or Ras/MAPK) signaling pathway, and therefore forms a negative feed-back loop to inhibit signaling at the level of the receptor complex, while zfh1 downregulates the Imd pathway in the nucleus, at the level or downstream of Relish.
6.4. Pirk as a negative regulator of the Imd pathway

One of the main regulatory mechanisms used by TNFR signaling is a negative feedback loop via resynthesis of the inhibitor protein IkB as a target gene of the pathway, enabling restriction of NF-kB transcriptional activity. Since the Drosophila Imd pathway transcription factor Relish has the inhibitory domain in its C-terminus, this kind of negative feedback-loop cannot be a regulatory mechanism used by Relish, even though it is a target gene of Imd pathway (Dushay et al, 1996). Thus, we hypothesized Drosophila could use another negative feed-back loop mechanism to limit the duration of Imd signaling, which should be characterized by a gene that is effectively, rapidly and specifically induced in response to an activated signaling pathway. We discovered the gene CG15678, or pirk fills the criteria both in vitro and in vivo (Boutros et al, 2002; Kallio et al, 2005; Park et al, 2004; Rämet et al, 2002; Valanne et al, 2007).

Co-immunoprecipitation assays revealed pirk interacts with Imd and the cytoplasmic tail of PGRP-LC, supporting the other experimental data about pirk’s epistatic location among the Imd pathway components (Fig 12). Moreover, even though no clear vertebrate homologs were found in tblastn search, likely orthologs were identified in the 18 sequenced holometabolous insects. Based on the tblastn search, a conserved region, which we named the pirk domain, is found in the middle of the 197 amino acid protein, while the N-terminal and C-terminal regions are variable between different insect orders. Further experiments with deletion constructs showed that the pirk domain co-immunoprecipitated with imd, the C-terminal part co-immunoprecipitated with the cytoplasmic tail of PGRP-LC, and these constructs were able to inhibit Imd pathway activity in S2 cells. In contrast, the N-terminal part of pirk did not co-immunoprecipitate with either imd or PGRP-LC and was dispensable for inhibitory function. Unfortunately, the lack of conservation between pirk and known mammalian proteins prevents sequence homology-based speculations of pirk’s exact mechanism of action, and determining whether any structurally or functionally related proteins exist outside the insect phyla would most likely require solving the three-dimensional structure of pirk. For determining its molecular function a structure of pirk bound to the imd-PGRP-LC receptor complex would probably be informative, along with identification of the domain of imd required for the interaction. The lack of conservation between pirk and mammalian proteins may be in part explained by the difference between the receptors for Imd pathway and TNFR pathway, even though the more downstream components are better conserved between Drosophila pathway and mammals. With regards to the evolutionary origin of the pirk gene itself, it can be speculated that it was
somehow adopted by the holometabolous insects in the relatively recent past, or that it perhaps represents more ancient form of an inhibitory feed-back loop, which was later replaced by IκB.

Demonstration of how intensely the *Drosophila* Imd pathway has been studied over the last years was given by two research papers published only a couple of months after III, describing a new regulator of the *Drosophila* Imd pathway, encoded by the gene *CG15678* (Aggarwal et al, 2008; Lhocine et al, 2008). Aggarwal and coworkers identified the protein they named rudra in a yeast two-hybrid screen for strong interactors with the RHIM-like motif found on the cytoplasmic tail of PGRP-LC (interestingly, their rudra clone contained the amino acids 30-197, supporting the dispensability of the N-terminal region of pirk/rudra). In their paper Aggarwal and coworkers also provide more mechanistic information about pirk/rudra’s molecular function by showing that pirk/rudra directly disrupts the PGRP-LC/imd protein complex by binding to the cytoplasmic tail of PGRP-LC, thereby inhibiting its interaction with imd and also the subsequent cleavage of imd needed for downstream signaling (Paquette et al, 2010). They also showed that rudra interacts with the RHIM-like domain of PGRP-LE (Aggarwal et al, 2008; Fig 12).

In vivo experiments show that *pirk* overexpressing flies were more susceptible to *E. cloacae* infection (III), whereas *rudra* mutant flies were more resistant to *Erwinia carotovora carotovora* (*Ecc*) infection (Aggarwal et al, 2008). This may seem slightly counterintuitive: what use is a gene product that results in less effective immune response against pathogenic bacteria or makes the flies susceptible to non-pathogenic ones? However, in addition to the “fighting the invading pathogens” aspect, immune systems need to be able to maintain self-tolerance to commensal microbiota, and this aspect of pirk’s function was assessed by a third research group.

Lhocine and coworkers named *CG15678* as *PGRP-LC-interacting inhibitor of Imd signaling* (*pims*) (Lhocine et al, 2008). They found four putative Relish binding sites within 1.2 kb of the *pims/pirk* transcription starting site, supporting the Imd pathway –dependent expression of the gene. *Pims* was highly induced also after oral infection with *Ecc*, especially in the gut, and basal *pims* expression was detected in the gut of conventionally reared wild type flies, but not in germ-free animals. This suggests that *pims* expression in the gut is driven by the exposure to commensal microbiota, resembling the induction of *PGRP-SC1* and *PGRP-LB*, two previously characterized negative regulators of the Imd pathway (Ryu et al, 2010). *Pims* homozygous mutant flies have a reduced life span, and increased basal *Dipt* expression especially in the gut, suggesting that pims could act as a safety mechanism that protects the gut from constitutive commensal microbiota-mediated activation
of AMPs. Moreover, after being fed with Ecc-contaminated food, pims mutants and pims RNAi flies also had significantly induced Dpt expression in the fat body, indicating a systemic immune response, whereas wild type flies did not mount an immune response against this non-pathogenic bacterium. Lhocine et al also report that coexpression of pims with PGRP-LCx led to a significant reduction of PGRP-LCx protein levels by changing the subcellular localization and surface availability of PGRP-LCx, suggesting that pims either triggers the internalization of PGRP-LCx or, alternatively, prevents PGRP-LCx from reaching the plasma membrane. Thus, pims contributes to the threshold of the Imd pathway mediated immune response, thereby establishing immune tolerance and development of host-commensal interactions (Lhocine et al, 2008).

Pirk’s function appears specific to the Imd pathway, as it has no effect on Toll signaling. However, it was recently shown that pirk is also involved in mediating cross talk between signaling pathways. Ragab and coworkers showed that pirk expression is also induced by the Ras/MAPK pathway, which therefore down-regulates the Imd pathway (Ragab et al, 2011). Several components of this pathway were identified in our earlier RNAi screen (Kleino et al, 2005) as potential negative regulators of Imd pathway as well.

6.5. Negative regulation of the JAK/STAT pathway by ET

Identification of ET as a negative regulator of Drosophila JAK/STAT pathway was a slightly curious process, as the gene was originally found among the potential positive regulators in the screen where ectopic hopTum-1 was used for JAK/STAT pathway activation. However, when the primary screen results were confirmed using different Stat92E-responsive reporters, and more importantly, the pathway ligand upd as the pathway activator, it appeared that ET is in fact a negative regulator. The opposite phenotype observed in the different assays could be due to several reasons. Protein sequence analysis as well as the co-IP assays showing interaction with Dome indicated that ET is located on the cell membrane, and is therefore epistatically between upd and hop (shown schematically in Figure 13.). The hopTum-1 mutation or ectopic expression of the mutant hop causes hyperactivation of JAK/STAT signaling that results in melanotic tumors in vivo (Harrison et al, 1995), and is therefore likely to be somewhat pathological even in cultured cells. It is plausible that such a strong input of signal activation by a gain-of-function mutant of a signaling component will be able to disrupt normal negative regulatory mechanisms, especially if located epistatically “downstream” of the regulator, whereas JAK/STAT pathway activation by ectopic upd resembles the physiological situation more closely. Moreover, even though hop and Stat92E were
among the positive regulators identified in the original screen, Dome was not. Similar to ET, the knock-down phenotype of Dome appeared rather curious: when upd was used to activate signaling, Dome RNAi abolished reporter activity, as expected. However, when hop
Tum-1 was overexpressed and Dome knocked down, the reporter activity was doubled, compared to the GFP dsRNA treated control. These results suggest that hop
Tum-1 is able to function somewhat independently of the upstream receptor (see also Harrison et al, 1995), or might even use ET’s short cytoplasmic tail as its docking site. It would be interesting to study ET’s regulatory role more thoroughly in vivo, for example, to see whether the same effect is observed in hop
Tum-1 genetic background as in S2 cells. In addition, it would be interesting to study if something similar is observed in human cells with regards to JAK2 mutations and the gp130/IL-6 receptor complex. This might provide useful information regarding the pathology of the gain-of-function allele(s) of hop, and also help to elucidate the mechanisms behind mutations in human JAKs that are associated with malignancies.

We showed that ET interacts with both Dome and hop, but does not seem to disrupt the association of hop with Dome. However, assays involving overexpression of tagged proteins might not reveal subtle changes in protein interactions, and therefore the exact mechanism of ET’s function cannot be deduced by these results. Since ET’s cytoplasmic tail is much shorter than that of Dome’s and lacks the Stat92E-binding site, it seems plausible that ET inhibits intracellular signaling, which would resemble the mechanism of Imd pathway inhibition by PGRP-LF (Basbous et al, 2011; Mailet et al, 2008; Persson et al, 2007). However, as we were unable to perform co-IP assays showing interactions for upd, the function of the cytokine-binding modules found on ET’s extracellular part remains elusive, as well as the exact nature of the interaction of ET and Dome and the composition and stoichiometry of the complex formed.

ET appears to be conserved between flies and man as both Dome and ET show homology with mammalian IL-6 receptor family members and the signal transducer gp130. In mammals gp130, which is the closest homologue to ET, also forms complexes with several cytokine receptors that have bound their ligands. gp130 is constitutively associated with JAKs on its cytoplasmic domain, and is required for mediating the activation of the signal to downstream signaling molecules, a function opposite to that of ET. On the other hand, the soluble form of gp130 is able to sequester ligands and therefore inhibiting signaling (Narazaki et al, 1993). Overexpression of a Dome construct lacking the intracellular signaling domain also acts as a signaling antagonist (Brown et al, 2001). Nevertheless, the conservation of the components in the signaling complex between the
species is apparent and an interesting phenomenon, even though their biological roles might have changed.

![Figure 13. ET as a negative regulator of the Drosophila JAK/STAT pathway.](image)

**Figure 13. ET as a negative regulator of the Drosophila JAK/STAT pathway.** ET is a transmembrane protein that interacts with Dome and hop, and thereby antagonizes the function of the JAK/STAT pathway receptor complex.

More confidence of ET’s importance as a JAK/STAT pathway regulator is provided by a notable research paper published soon after ours by Makki and coworkers, describing the very same gene, which they call *latran (lat)*, as a negative regulator of *Drosophila* JAK/STAT pathway (Makki et al, 2010). Makki and coworkers focused on studying ET/latran’s role in regulating hemocyte homeostasis in the posterior signaling center (PSC) of larval lymph glands. They have previously shown that the PSC non-cell-autonomously maintains JAK/STAT signaling in prohemocytes, thereby keeping them in a non-differentiating state (Krzemién et al, 2007). In the current paper they showed that ET/latran is required to switch off JAK/STAT signaling in order to induce lamellocyte differentiation, which is needed for example in the case of attack by parasitic wasp eggs. Dome and ET/latran were shown to interact in vivo and it was also found that *ey-GAL4* driven expression of *ET/lat* resulted in small eyes in adult flies. Additionally, *ET/lat* expression was shown to be upregulated in response to wasp parasitization, whereas expression of *Dome* and *upd3* was downregulated, the former probably being a result of decreased JAK/STAT signaling activity since Dome is a target gene for the pathway. Thus, even though Dome and *ET/lat* are located adjacent to
each other in the *Drosophila* genome, they do not seem to share transcriptional control. Notably, we also noticed high induction of *ET* expression in larvae in response to parasitic wasp eggs (data not shown).

A slight discrepancy is seen when comparing the figure 3A in IV and figure 1A in Makki and coworker’s paper. The gene coding for *ET/lat* contains three exons, the first of which is only 88 nucleotides. Makki et al used RACE-PCR to define the translation initiation site on the second exon, resulting in a protein of 645 amino acids instead of the 713 aa predicted if translation was initiated from the methionine site on the first exon. Lack of the first exon in the final transcript would be corresponding with our observation that a dsRNA targeted exclusively against the first exon did not produce any phenotype in S2 cell reporter assays, in contrast to the three other dsRNAs against the second and third exons. Our expression construct contained the first exon sequence as well, but it is possible that this might have been spliced out before translation. Nevertheless, the construct apparently produced a functional protein with the tag in the C-terminus.

Taken together, our results along with those of Makki and coworkers give a rather conclusive picture of ET as a negative regulator of the *Drosophila* JAK/STAT pathway both in vitro and in vivo, including several tissues in both larvae and adult flies. Therefore, it would be interesting study ET’s role more thoroughly in vivo, especially in relation to malignancies caused by the *hop*<sup>Tum-1</sup> mutation, as well as in vitro in the biochemistry of the receptor complex, which could also hopefully elucidate some aspects of the function and regulation of mammalian JAK/STAT pathway receptor complexes.
7. Conclusions and future perspectives

*Drosophila* has proven to be a feasible model to study innate immunity, in particular, the conserved signaling pathways involved in mediating innate immune responses. It has become obvious that despite the superficial simplicity of the *Drosophila* versions of these pathways due to reduced redundancy of the core components, several regulators are required for their proper control. Moreover, as in humans, spatial and temporal control of the *Drosophila* immune responses by negative regulators is highly important, and several novel negative regulators and regulatory mechanisms have been identified during recent years. It has become apparent that the *Drosophila* immune signaling pathways are under tight control at multiple levels, which is further demonstrated by the novel negative regulators discovered for the *Drosophila* Toll, Imd and JAK/STAT pathways.

The findings of the RNAi screens included in this thesis, as well as other screens have already led to interesting follow-up studies further elucidating the regulatory mechanisms of the *Drosophila* immune responses. In particular, the signaling events in the Imd pathway have been intensely studied since the discovery of Iap2, Tab2 and pirk, and now appear to be rather well-defined in all their complexity. For example, several ubiquitination events are required, implying that this mechanism is important in regulation of both the *Drosophila* Imd signaling and the mammalian TNFR signaling.

There is some evidence of cross-regulation between the signaling pathways controlling the *Drosophila* immune responses, and therefore it would be interesting to study this phenomenon in more detail both in vitro and in vivo; *pirk* being a target gene for the Ras/MAPK pathway is an exciting example. It is also likely that the signaling pathways are regulated tissue-specifically, and that they can mediate communication between the *Drosophila* tissues, such as the hemocytes and fat body by inducing signaling molecules that act non-cell-autonomously. Better understanding of these aspects would help to build a more comprehensive molecular-level picture of regulation of the immune components in the whole organism level. In addition, huge amount of data considering different aspects of the *Drosophila* immunity has been produced by the several RNAi screens (as well as other methods), and therefore a systems biological study combining the results would likely be informative in composing the bigger picture and make the wealth of data more easily accessible (Shamu et al, 2012). Several data sources are already available: FlyBase (http://www.flybase.org) is a favorite site for any *Drosophila* researcher studying a new (or old) exciting gene, and a protein interaction map is also available. In addition, more specifically related to RNAi screening data, the
newly updated GenomeRNAi database is a good example of a resource that provides useful information to the whole research community, and should enable meta-analysis of existing information as well as comparison of new data (http://www.genomernai.org; Schmidt et al, 2013). However, one should bear in mind that the systems biological analyses can only be as good as the data input, and therefore one should pay attention to the data source before drawing too far-fetched conclusions. For example, in the case of RNAi screens, it is important to recognize that the screening conditions can have a substantial effect on the hit list obtained –fortunately, these aspects can also be assessed via GenomeRNAi.

In this study, we have identified novel regulators for the signaling pathways mediating the Drosophila immune response, and shown that these pathways are regulated at multiple levels. Brahma complex is a potential negative regulator of the Toll pathway at the chromatin level, and zfhl is a negative regulator of the Imd pathway at the transcriptional level. In addition, we identified a second Imd pathway negative regulator, named pirk, which is rapidly induced by a negative feedback loop, and functions at the level of PGRP-LC and imd, possibly by disrupting the function of the receptor complex. Finally, we discovered a novel negative regulator of the Drosophila JAK/STAT pathway, called ET, which is a transmembrane protein that resembles and interacts with Dome, thereby inhibiting the signaling at the receptor level.
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Henna Myllymäki
9. References


Huh JR, Foe I, Muro I, Chen CH, Seol JH, Yoo SJ, Guo M, Park JM, Hay BA (2007) The *Drosophila* inhibitor of apoptosis (IAP) DIAP2 is dispensable for cell survival, required for the...
innate immune response to gram-negative bacterial infection, and can be negatively regulated by the reaper/hid/grim family of IAP-binding apoptosis inducers. J Biol Chem 282: 2056-2068.


Postigo AA, Dean DC (1997) ZEB, a vertebrate homolog of Drosophila Zfh-1, is a negative regulator of muscle differentiation. EMBO J 16: 3935-3943.


10. Original communications
Genome-Wide RNA Interference in Drosophila Cells Identifies G Protein-Coupled Receptor Kinase 2 as a Conserved Regulator of NF-κB Signaling

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Because NF-κB signaling pathways are highly conserved in evolution, the fruit fly Drosophila melanogaster provides a good model to study these cascades. We carried out an RNA interference (RNAi)-based genome-wide in vitro reporter assay screen in Drosophila for components of NF-κB pathways. We analyzed 16,025 dsRNA-treatments and identified 10 novel NF-κB regulators. Of these, nine dsRNA-treatments affect primarily the Toll pathway. G protein-coupled receptor kinase (Gprk2), CG15737/Toll pathway activation mediating protein, and u-shaped were required for normal Drosomycin response in vivo. Interaction studies revealed that Gprk2 interacts with the Drosophila IκB homolog Cactus, but is not required in Cactus degradation, indicating a novel mechanism for NF-κB regulation. Morpholino silencing of the zebrafish ortholog of Gprk2 in fish embryos caused impaired cytokine expression after Escherichia coli infection, indicating a conserved role in NF-κB signaling. Moreover, small interfering RNA silencing of the human ortholog GRK5 in HeLa cells impaired NF-κB reporter activity. Gprk2 RNAi flies are susceptible to infection with Enterococcus faecalis and Gprk2 RNAi rescues Tollr-induced blood cell activation in Drosophila larvae in vivo. We conclude that Gprk2/GRK5 has an evolutionarily conserved role in regulating NF-κB signaling. The Journal of Immunology, 2010, 184: 6188–6198.

Nuclear factor-κB signaling is involved in a variety of cellular processes, including control of both the innate and adaptive immune systems. The NF-κB/Rel family of transcription factors consists of five members in humans. These proteins control the expression of hundreds of target genes, including various cytokines and chemokines, in a tightly regulated manner (1). In mammals, immune-related NF-κB activation mainly occurs via two signaling pathways, the TNFR pathway and the TLR pathway. NF-κB signaling pathways are highly conserved in evolution, and therefore, similar signaling cascades are found in lower eukaryotes, such as the fruit fly Drosophila melanogaster. Drosophila systemic immune response is largely mediated by two NF-κB signaling cascades, the Toll and the immune deficiency (Imd) pathway, which closely resemble mammalian TLR and TNFR signaling cascades, respectively (2). Both signaling cascades lead to activation and nuclear localization of Drosophila NF-κB family protein and expression of a distinct but overlapping set of antimicrobial peptide genes (3–5). Thus, lacking adaptive immunity, the fruit fly makes a useful and simpler model to study the signaling cascades and their involvement in innate immune responses.

The Drosophila Imd pathway is activated in response to Gram-negative bacterial infection. After ligand binding to the receptor peptidoglycan recognition protein (PGPR)-LC (6–8), the signal proceeds via downstream components, which include the death-domain protein Imd (9), the MAPK kinase kinase TGF–β–activated kinase 1 (Tak1) (10), and a Drosophila homolog of Fas-associated death domain protein (FADD) (11). The signaling leads to activation of Drosophila IκB kinases Kenny and immune response deficient 5 (12), which phosphorylate the inhibitory domain of the NF-κB family transcription factor Relish (13), resulting in Relish cleavage by the caspase Dredd (14–16). Subsequently, the activated N-terminal 68-kDa Relish is translocated into the nucleus, where it activates transcription of antimicrobial peptide genes. In addition, inhibitor of apoptosis 2 and TGF–β–activated kinase 1-associated binding protein 2 (Tab2) are shown to play a key part in the regulation of Relish activity (17–20).

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Abbreviations used in this paper: Ac5C–β–gal, Actin SC–β–galactosidase; CaM, Calmodulin; DiF, Dorsal-related immunity factor; EGF, epidermal growth factor; EGFR, EGF receptor; EXT, extension to kinase domain; FADD, Fas-associated death domain protein; Gprk, G protein-coupled receptor kinase; GRK, G protein-coupled receptor kinase; hmt4, hemolymph4; IM, immune-induced molecule; Imd, immune deficiency; kinase, kinase domain; MED25, mediator complex subunit 25; MRC, Medical Research Council; NIG-FLY, Fly Stocks of the National Institute of Genetics; PGRP, peptidoglycan recognition protein; qRT-PCR, quantitative RT-PCR; RGS, regulator of G protein signaling; RNAi, RNA interference; siRNA, small interfering RNA; Tab2, TGF–β–activated kinase 1-associated binding protein 2; Tak1, TGF–β–activated kinase 1; UAS, upstream activating sequence; VDRC, Vienna Drosophila RNAi Center.

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The Toll pathway is activated by Gram-positive bacteria and fungi (3) recognized by several pattern recognition receptors (2) including PGRP-SA (21), leading to proteolytic cleavage and activation of the cytokine Spätzle and its binding to the Toll receptor (22, 23). Intracellular components of Toll pathway include the death domain proteins Drosophila MyD88 (24), Tube, and Pelle (3). Finally, the signaling leads to degradation of the NF-κB inhibitory protein Cactus and nuclear localization of Dorsal-related immunity factor (Dif) and/or Dorsal (25). It has also been shown that Toll signaling is involved in the activation of the cellular immune system (26, 27). It is likely that components yet to be found are involved in regulating the cascade.

To identify novel gene products involved in Drosophila NF-κB signaling, we carried out a genome-wide screen for 16,025 dsRNAs using a Drosomycin luciferase reporter-based assay that enables us to monitor both the Toll and Imd pathways. We identified 10 novel NF-κB regulators, of which 9 act primarily on the Toll pathway. Furthermore, we identified G protein-coupled receptor kinase (Gprk2)/GRKS as an evolutionary conserved regulator of NF-κB signaling.

Materials and Methods

Drosophila dsRNA libraries and synthesis of targeted dsRNAs

The dsRNAs used in the RNA interference (RNAi) screen (16,025) were produced from a commercial Drosophila genome RNAi library consisting of a set of 13,625 PCR products with dual T7 promoter sequences (Medical Research Council [MRC] Geneservice, Cambridge, U.K.) and from in-house–made S2 cell-derived cDNA library (2,400). dsRNAs were synthesized from PCR product or plasmid templates with the T7 MegaScript RNA polymerase kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Concentration of each dsRNA was measured using PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR) or NanoDrop (Thermo Fisher Scientific, Waltham, MA). Targeted dsRNAs were produced using cDNA from S2 cells as a template in a two-step PCR with nested primers (Supplemental Table I), the second primers containing a T7 promoter sequence (5′-TAA-TACGACTCACTATAGGG-3′) at the 5′ end. pMT/Bip/V5/His/GFP plasmid (Invitrogen/Life Technologies, Carlsbad, CA) was used as a template for the production of the negative control GFP dsRNA.

S2 cell treatments and reporter assays

S2 cell culture, transfections, dsRNA treatments, and reporter assays for the Toll and Imd pathways were performed essentially as previously described (17, 28). For the genome-wide screen, S2 cells were transfected with 0.1 μg Drosomycin luciferase (29) and 0.1 μg Actin 5C-β-galactosidase (Act5C-β-gal) reporter plasmids. In addition, the cells were treated with 0.5 μg dsRNA. To screen both Toll and Imd pathways simultaneously, the Toll pathway was first activated by transfecting S2 cells with 0.1 μg Toll166 construct. Forty-eight hours posttransfection and 24 h prior to measurements, the Imd pathway was activated by adding heat-killed Escherichia coli.

S2 cell transfections for quantitative RT-PCR

For quantitative RT-PCR (qRT-PCR) experiments, S2 cells were seeded on 24-well plates and transfected with 0.1 μg Toll166 construct and 0.5 μg dsRNA. Seventy-two hours later, cells were harvested and lysed in TRSure reagent (Bioline, London, U.K.) by pipetting up and down 10 times. Total RNAs were extracted according to the manufacturer’s instructions and RNAs subjected to quantitative RT-PCR analysis as detailed below.

HeLa cell culture and transfections

HeLa cells were grown in DMEM plus Glutamax ( Gibco/Life Technologies, Carlsbad, CA) with 10% FBS, 1% nonessential amino acids (Sigma–Aldrich, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin. For transfection, 6 × 10⁴ cells per well were seeded onto 24-well plate. Twenty-four hours later, the cells were transfected with 0.1 μg NF-κB luciferase, 0.05 μg CMV-β-galactosidase reporter plasmid, and 50 pmol small interfering RNAs (siRNAs) (Ambion, Austin, TX) using Lipofectamine transfection reagent (Invitrogen/Life Technologies) and Opti-MEM medium (Life Technologies). siRNAs used were as follows: GFP siRNA (Silencer GFP [sc-39212], negative control), GRK2 siRNA (catalog number AM16704), ID 110898, and RelA (catalog number AM16704; ID 216912, positive control). Forty-eight hours posttransfection, NF-κB signaling was induced with 10 ng/ml TNF-α (Sigma–Aldrich), and 6 h later, luciferase and β-galactosidase activities were measured from the cell lysates.

Immunohistochemistry with Gprk2/GRKS constructs

To create a Gprk2-GFP fusion protein, the full-length cDNA for Drosophila Gprk2 gene was amplified by PCR and cloned into the KpnI site of the modified Drosophila expression vector pMT/GFP/V5/His, a kind gift from Dr. I. Kleino (University of Helsinki, Helsinki, Finland). S2 cells were transfected with the Gprk2-GFP fusion construct essentially as described previously (30). Overexpressed Gprk2-GFP fusion in S2 cells was precipitated with 350 μl M Protein for 36 h. For Heliothis zea, S2 cells were seeded onto coverslips on six-well plates, and 24 h later, the cells were transfected with 0.1 μg GRK5-GFP construct. Thirty-six hours later, the coverslips were mounted to slides with Vectashield mounting medium for fluorescence with 4′,6-Diamidino-2-Phenylindole (Vector Laboratories, Burlingame, CA). Cells were imaged with an Olympus IX70 confocal microscope (Olympus, Tokyo, Japan) and analyzed with Andor iQ software (Andor Technology, Belfast, U.K.).

Zebrafish maintenance and morphology silencing

AB wild-type zebrafish strain was maintained according to standard protocols (31). Translation-blocking morpholinos targeting zebrafish GRK5 (ZDB-GENE:009029-1198) (5′-GCCACCATATACTTCTTCACTTATT-3′) and MyD88 (ZDB-GENE:040219-3) (5′-GGCTCTATACATTGTTGATGC-3′), as well as a mismatch-GRK5 control morpholino (5′-GCCACCATTATTGTCAATGTGATT-3′), were obtained from Gene Tools (Philomor, OR). A total of 1 nl 25 μM morpholinos in 0.2 M KCl was injected into the yolk sacs of AB wild-type embryos at the 1-2 cell stage.

Zebrafish infections

For infection experiments, E. coli was grown in Luria–Bertani broth until OD of 0.3 at 600 nm. Bacterial cells were pelleted by centrifugation (10,000 × g, 5 min), washed with 0.2 M KCl, pelleted, and diluted 1:2 in 0.2 M KCl. Prior to infection, 70 kDa rhodamine dextran tracer was added to the bacterial suspension. Morpholino-injected zebrafish larvae were manually dechorionated at 24–28 h postfertilization, after which 1 nl prepared E. coli suspension was injected into the yolk. Before and after the infections with each needle, one injection plate was plated for checking the bacterial quantity. Infected larvae were kept at 28°C for 2–24 h postinfection, after which they were snap-frozen for total RNA extraction. Total RNAs were extracted according to standard procedures.

Coominoprecipitation

S2 cells were transfected with Gprk2-V5 full-length or deletion constructs and Cactus-myc constructs in pMT/V5/HisA vector (Invitrogen/Life Technologies) and coimmunoprecipitated, separated, transferred on the membrane, and detected essentially as described (30).

Stable S2 epidermal growth factor receptor–Toll cells, Western blotting, and quantification

S2 cells with stable integration of a chimeric epidermal growth factor receptor (EGFR)-Toll construct were made according to Ref. 32, and the response to Toll signaling by EGFR was verified with Drosomycin luciferase construct. Stable S2/EGFR-Toll cells were grown in six-well culture dishes and treated with 15 μg dsRNA in a total volume of 3 ml medium for 4 d. Induction of the Toll pathway was done by addition of EGFR (0.5 μg/ml) (Molecular Probes) for 30 min. Cytoplasmic extracts of S2/EGFR/Toll cells were separated by electrophoresis, transferred to a Hybrid-Membrane (GE Healthcare Life Sciences), and blocked. Cactus protein on the membrane was detected with polyclonal rabbit anti-Cactus Ab and HRP-linked donkey anti-rabbit IgG (GE Healthcare Life Sciences, Uppsala, Sweden). Rabbit polyclonal anti-GM130 Ab (Abcam, Cambridge, MA) targeting a Drosophila Golgi protein GM130 was used as a loading control. Band quantifications were done with Adobe Photoshop 7 software (Adobe Systems, San Jose, CA) as follows: to obtain the absolute intensity, the mean value of each band was multiplied by the pixel value. The relative intensity was calculated by normalizing absolute intensities with the absolute intensity of the negative control, which was set to the value of 1. Quantifications were carried out on three separate Western blots.

Fly stocks and maintenance

Drosophila stocks were kept on a standard mashed potato diet at room temperature or at 25°C. C564-GAL4 flies express GAL4 in the adult fatbody; the P[UAS-Toll166]; 1 stock carries a Toll166 insert on the X chromosomal lineage.

Downloaded from http://jimmunol.org at Temple University Library on April 13, 2019.
chromosome and the hemocytin\textsuperscript{a} (im3)-GAL4, UAS-GFP stock constitutively expresses GFP in the majority of blood cells (33). The upstream activating sequence (UAS)-RNAi fly stocks listed in Supplemental Table I were obtained from the Vienna Drosophila RNAi Center [VDRC, Vienna, Austria (34)] or the Kyoto Fly Stocks of the National Institute of Genetics (NG-FLY) [Drosophila Genetic Resource Center, Kyoto Institute of Technology, Kyoto, Japan]. The C564-GAL4 flies were crossed with UAS-RNAi flies, and the adult flies carrying one copy of the UAS-RNAi construct and one copy of the GAL4 driver were used in infections. UAS-RNAi flies crossed to w\textsuperscript{1188} were used as controls in infection experiments.

Fly infection and RNA extraction

To produce induced \textit{Drosomycin} expression via septic injury, flies were pricked with a thin tungsten needle previously dipped in a concentrated culture of \textit{Micrococcus luteus} and grown at 25°C. Twenty-four hours later, five flies per sample were collected and snap-frozen in dry ice. Alternatively, expression of Toll pathway target genes was induced by natural fungal infection with \textit{Beauveria bassiana} at 29°C for 48 h as previously described (35), after which the flies were collected as mentioned above. Total RNAs were extracted according to standard procedures and RNAs subjected to qRT-PCR analysis as detailed below.

qRT-PCR

Extracted total RNAs from S2 cells, zebrafish embryos, or flies were used in qRT-PCR experiments. qRT-PCR for expression levels of chosen genes was carried out from dilutions of the extracted RNAs using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) and the ABI7000 instrument (Applied Biosystems, Foster City, CA). Primers and sizes of PCR products are listed in Supplemental Table II.

Fly survival experiments

To assess Toll pathway-mediated immunity, flies were first immunized by pricking them with \textit{M. luteus} as described above (\textit{M. luteus} infection activates the Toll pathway). Twenty-four hours later, the flies were infected with \textit{Enterococcus faecalis} by pricking as above. The infected flies were kept at room temperature, and their survival was monitored for 24 h. For \textit{Imd} pathway-mediated immunity, flies were pricked with a thin tungsten needle previously dipped in a concentrated culture of \textit{Enterobacter cloacae} (a Gram-negative bacterium), and their survival was monitored for 48 h.

Fly larvae in vivo experiments

To assess the distribution of blood cells in \textit{Drosophila} larvae in vivo, parental crosses were kept for 2 d at 29°C in stained mashed potato food, which permits the staging of larval progeny as described previously (27). The experiment was blinded by assigning arbitrary numbers to the fly bottles. Collected larvae were gently washed and embedded with the dorsal side up in 50% chilled glycerol between a glass slide and a coverslip. For immobilization, slides were kept at −20°C for 18 min before examining under UV light on an Axiosplan microscope (Carl Zeiss, Jena, Germany). Digital pictures were taken with a Hamamatsu C9100-02 camera (Hamamatsu Photonics, K.K., Hamamatsu City, Japan), controlled by the Openlab program (Improvision, Coventry, U.K.). For each cross, 20 F1 progeny larvae were graded for the percentage of their segments showing a band formed by islets of sessile hemocytes under the epidermis. In the grading system, grade 1 larvae showed sessile hemocyte bands in 100% of the segments. Larvae receiving grade 2 or 3 showed bands in <75 or 50% of their segments, respectively. Larvae showing no bands or bands only in the most posterior 25% of their segments received grade 4. All crosses were repeated three times and the average grades of three independent experiments calculated.

Data analysis

Statistical analyses of reporter assays, qRT-PCR, and Western blot band quantification were carried out using one-way ANOVA. Statistical analysis of fly larvae in vivo experiments was performed using one-way ANOVA and Bonferroni as post hoc method. Statistical analysis of fly survival experiments was carried out using the log-rank (Mantel-Cox) test; \( p < 0.05 \) was considered to be statistically significant.

Results

Drosomycin expression is controlled by both the Toll and Imd pathways in \textit{Drosophila} S2 cells

\textit{Drosomycin} promoter-driven luciferase activity has been used to monitor the \textit{Drosophila} Toll pathway activity (29). However, we have observed earlier (17) that in S2 cells, RNAi targeting components of the Imd pathway decreased the \textit{Drosomycin luciferase} expression induced by the constitutively active form of Toll (Toll\textsuperscript{106}). To investigate the respective roles of the Toll and Imd pathways to the regulation of \textit{Drosomycin}, we analyzed the \textit{Drosomycin luciferase} activity in S2 cells activated with both Toll\textsuperscript{106} overexpression and heat-killed \textit{E. coli}. As shown in Fig. 1A, \textit{Drosomycin} expression was induced by Toll\textsuperscript{106}, and this induction was further enhanced if the Imd pathway was also activated by \textit{E. coli}. This induction, stimulated by both pathways, can be drastically decreased by silencing known components of the Toll pathway, namely Toll, MyD88, or \textit{dorsal}. Imd pathway components, namely PGRP-LC, Imd, Tub2, and Relish, are also required for normal induction in this assay. If both pathways are silenced by targeting both Relish and MyD88, \textit{Drosomycin} activation is totally blocked (Fig. 1A). These results are in line with previous studies indicating that \textit{Drosomycin} expression can be induced by both the Toll and Imd pathways in vivo and in vitro (5, 36). These results also show that \textit{Drosomycin luciferase} activity can be used in screening for components of both the Toll and Imd signaling cascades in \textit{Drosophila} S2 cells.

Genome-wide analysis of the Toll and Imd pathways in \textit{Drosophila} S2 cells

To identify gene products required for signaling via the Toll and Imd pathways, we examined the effect of 16, 025 dsRNA treatments for \textit{Drosomycin luciferase} reporter activity in response to induction with Toll\textsuperscript{106} and \textit{E. coli} in \textit{Drosophila} S2 cells. The dsRNA collection was obtained by transcribing PCR products from the commercial \textit{Drosophila} genome-wide library (MRC Geneservice) to dsRNAs (13,607) and transcribing dsRNAs (2,418) from S2 cell-derived cDNA library (37).

\textit{Drosophila} S2 cells were transfected with \textit{Drosomycin luciferase} and Act5C−β-gal reporter constructs, Toll\textsuperscript{106}, and dsRNAs. dsRNAs targeting MyD88 and Relish were used as positive controls and GFP as a negative control in each experiment. \textit{E. coli} was added 24 h prior to luciferase and β-galactosidase measurements. Out of 16,025 dsRNA treatments, 23 repeatedly decreased the \textit{Drosomycin luciferase} reporter activity >50% without considerably affecting the cell viability as measured by Act5C−β-gal reporter activity (Fig. 1B). Corresponding templates were sequenced and targeted PCR primers for dsRNA synthesis designed to confirm that the effect had been due to dsRNA according to the library data and not due to contaminating dsRNAs. As shown in Fig. 1C, five dsRNA treatments representing known components of the Toll pathway (\textit{Toll, MyD88, tube, pelle, and dorsal}), and eight dsRNA treatments representing known Imd pathway components (Relish, kenp, FADD, \textit{Tak1, imd, Tab2, Imd, and inhibitor of apoptosis 2}) were identified, indicating that our screen effectively found components of both the \textit{Drosophila} NF-κB signaling pathways. Importantly, 10 novel regulators of NF-κB signaling were identified (Fig. 1C). Corresponding genes were subjected to further studies.

Nine of the identified regulators are required for signaling via the Toll pathway

Our RNAi screen effectively identified components of both the Toll and Imd signaling cascades. To find out which pathway is affected by these regulators, we carried out separate assays for the Toll and Imd pathways in S2 cells with targeted dsRNAs. Imd pathway activity was measured with a reporter assay in which \textit{Attacin A}-driven luciferase (\textit{AttA-luc}) construct, Act5C−β-gal, and dsRNAs were transfected into S2 cells, cells treated with \textit{E. coli}, and reporter activities measured (Fig. 2A). Out of the 10 novel candidate genes, mediator complex subunit 25 (\textit{MED25}) RNAi was shown to affect Imd pathway at the same level as RNAi to known Imd pathway...
FIGURE 1. Genome-wide RNAi screen to identify genes required for NF-κB signaling in Drosophila S2 cells. A, Drosomycin luciferase reporter activity is regulated by both the Toll and Imd pathways in S2 cells. S2 cells were transfected with Drosomycin luciferase and Act5C–β-gal reporters. Toll pathway was induced by overexpression of Toll106 and the Imd pathway by heat-killed E. coli treatment for 24 h. RNAi targeting known components of the Toll pathway (Toll, MyD88, or dorsal) or components of the Imd pathway (Tab2, PGRP-LC, imd, or Relish) caused a strong reduction in the Drosomycin luciferase/Act5C–β-gal activity. Relish + MyD88 RNAi completely blocked the relative Drosomycin activation. Data are shown as mean ± SD; n ≥ 3. B, Results of the genome-wide RNAi screen of the Drosophila NF-κB signaling. The 16,025 independent dsRNA treatments were analyzed for effect on Drosomycin luciferase reporter activity induced via both the Toll and Imd signaling pathways. S2 cells were transfected with Drosomycin luciferase and Act5C–β-gal reporters, Toll106, and dsRNAs and treated with heat-killed E. coli. Luciferase and β-galactosidase values were plotted on a log-scale. Negative control samples (GFP RNAi) are illustrated with light blue crosses. Toll pathway positive controls (MyD88 RNAi) are shown in purple, and Imd pathway positive controls (Relish RNAi) are shown in yellow. Samples with values in the top left corner of the plot include the most potential regulators of NF-κB signaling in Drosophila S2 cells. C, Targeted dsRNA treatments of potential regulators of the Drosophila NF-κB signaling confirm 23 dsRNA treatments that decrease Drosomycin luciferase reporter activity by >40%. Drosomycin luciferase and Act5C–β-gal values of induced GFP dsRNA-treated cells were set to 1. Data are shown as mean ± SD; n ≥ 3. Statistics refer to Drosomycin luciferase values.
FIGURE 2. MED25 affects mainly the Imd signaling cascade. U-shaped affects both Toll and Imd signaling, and the other eight identified novel NF-κB regulators affect mainly Toll signaling. Data are shown as mean ± SD; n = 3. In each panel, statistics refer to white bars. A, For Imd pathway, S2 cells were transfected with AnTa-luc reporter, Act5C-B-gal reporter, and indicated dsRNAs and induced with heat-killed E. coli. In addition to known Imd pathway components (kenny, Tab2, Imd, Tak1, FADD, and Relish), MED25 RNAi decreased the AnTa-luc reporter activity compared to the known components. u-shaped RNAi also significantly affected the AnTa-luc reporter activity. B, For Toll pathway activity, S2 cells were transfected with Toll56, Drosomycin luciferase reporter, Act5C-B-gal reporter, and indicated dsRNAs. Of the novel regulators, pannier, CG4325, CG32133, u-shaped, CG15737, achaete, Gprk2, CG31660, and Spt6 dsRNAs considerably decreased the Drosomycin luciferase reporter activity. Also, most of the Imd pathway components' regulators (kenny, Tab2, Imd, Tak1, MED25, and Relish) affected the Drosomycin luciferase activity. C, Drosomycin expression is inhibited by dsRNA treatments targeting all of the known tested components of Toll pathway and 10 dsRNAs identified from our collections. Endogenous Drosomycin expression in Toll56-transfected dsRNA-treated S2 cells was analyzed using qRT-PCR and normalized to Act5C expression values. D, Schematic representation of the Drosophila Toll pathway. E, Epistasis analysis of the identified regulators of Toll signaling. Drosomycin luciferase expression was induced by cactus RNAi. Ten dsRNA treatments (dorsal, pannier, CG15737, Relish, Sp6, Gprk2, CG4325, u-shaped, CG32133, and achaete) blocked this induction and therefore appear to act downstream or independently of Cactus. Five dsRNA treatments (Toll, tube, pelle, CG31660, and MyD88) did not considerably affect this induction, indicating that these gene products act upstream of Cactus.
components (21% of pathway activity left; Fig. 2A). Also, u-shaped RNAi decreased significantly the Imd pathway activity (48% of activity left). Sptr6, CG31660, and CG15737 RNAi had little effect on Imd pathway activity, whereas RNAi targeting achaete, Gprk2, pannier, CG4325, and CG32133 resulted in hyperactivation of the pathway (Fig. 2A, on the right with a separate scale).

Out of the 10 novel candidate genes, 9 dsRNA treatments decreased the Drosomycin luciferase activity of Toll-induiced S2 cells >60% (Fig. 2B). The Drosomycin luciferase activity of S2 cells treated with these dsRNAs was also reduced by at least 50% when the Toll pathway was activated by overexpression of the cleaved, active Spitz ligand (Spz, data not shown). MED25 dsRNA treatment did not have as strong an effect on Toll pathway alone (Fig. 2B), so it was omitted from further analyses.

To ensure that our results were not due to an artifact related to the use of a reporter construct, we analyzed the relative endogenous Drosomycin expression levels of Toll-induced S2 cells by qRT-PCR. In this assay, Toll transfection to S2 cells induced the relative endogenous Drosomycin expression (Drosomycin/Act5C) in S2 cells ~25-fold (Fig. 2C). Treating cells with dsRNAs targeting the known components of the Toll pathway, Toll, MyDD88, tube, pelle, or dorsal, decreased the pathway activity by >55%. Similarly, RNAi targeting all of the nine novel Toll pathway regulators identified in the reporter assay, namely u-shaped, pannier, CG4325, Gprk2, CG15737, CG32133, CG31660, achaete, and Sptr6 caused a statistically significant reduction in endogenous Drosomycin expression. Of note, RNAi targeting Relish, the NF-κB factor in the Imd pathway, also caused a statistically significant reduction in Toll-induced Drosomycin expression.

To gain more insight into the mechanism of how the novel regulators are functioning on the Toll pathway, we silenced cactus, the Drosophila homolog of human ISK, by RNAi (Fig. 2D, 2E). Silencing cactus results in Def/Dorsal translocation into the nucleus (Fig. 2D) and >40-fold induction of the Toll pathway in a Drosomycin luciferase reporter assay (Fig. 2E). RNAi targeting known components of the pathway upstream of Cactus, namely Toll, tube, pelle, and MyDD88 have no or very little effect on Cactus RNAi-induced Drosomycin luciferase activity. Conversely, RNAi targeting dorsal, the Drosophila NF-κB homolog in the Toll pathway (downstream of Cactus), blocks this induction completely (Fig. 2E). Results indicate that CG31660 appears to act upstream of Cactus, whereas pannier, CG15737, Sptr6, Gprk2, CG4325, u-shaped, CG32133, and achaete appear to act downstream or independently of Cactus. Relish also acts downstream of Cactus.

Gprk2, CG15737/Toll pathway activation mediating protein, and u-shaped RNAi flies have reduced Drosomycin expression in Drosophila in vivo

To investigate whether the identified nine genes are important for the Toll pathway signaling in vivo, we carried out in vivo RNAi experiments with fly lines carrying UAS-RNAi constructs targeting these genes. RNAi flies (Supplemental Table I) were crossed with the C56-GAL4 driver line, which drives expression of the dsRNA in the fatbody. Fly strains without the driver (i.e., RNAi strains over w¹¹¹B flies) were used as controls. The Toll pathway was activated by M. luteus septic injury for 24 h, after which total RNA was isolated. Relative Drosomycin expression in RNA samples was measured by qRT-PCR (Fig. 3). MyDD88 RNAi crossed with C56-GAL4 flies were used as positive controls (Fig. 3A). Two Gprk2 RNAi fly lines, namely Gprk2 R-1 and Gprk2 R-3, crossed with C56-GAL4 showed a significant decrease in Drosomycin expression (Fig. 3B, 3C).

Moreover, the fly line expressing the CG15737 RNAi construct showed a statistically significant reduction in Drosomycin expression compared with control flies, so we decided to name the CG15737 gene TAMP (Fig. 3D). Also, flies expressing the u-shaped RNAi construct showed a statistically significant reduction in Drosomycin expression compared with control flies (Fig. 3E). In vivo RNAi targeting other identified Toll pathway candidate genes did not significantly decrease Drosomycin expression (Supplemental Fig. 1).

Drosophila Gprk2 is homologous to GRK5 from other organisms

Because of the strong phenotype obtained in both in vitro and in vivo Drosophila RNAi assays and its evolutionary conservation, we decided to subject Gprk2 to further studies. Drosophila Gprk2 (CG17998) is well conserved and has high sequence similarity with human, mouse, and zebrafish GRK5 (Supplemental Fig. 2). Gprk2 codes for a 714-aa protein that has three known domain structures: a regulator of G protein signaling (RGS) domain, a serine/threonine protein kinase catalytic domain, and an extension to kinase domain (Fig. 4A). It belongs to a protein family, the members of which are multifunctional, GTPase-activating proteins (38). When the Gprk2-GFP construct was overexpressed in Drosophila S2 cells, it was shown that Gprk2 is localized on the cell membrane or cytoplasm (Supplemental Fig. 3A). Similarly, the human GRK5-GFP construct was located on the cell membrane or cytoplasm when overexpressed in HeLa cells (Supplemental Fig. 3B).

Gprk2/GRK5 has an evolutionarily conserved role in NF-κB signaling

To investigate whether Gprk2/GRK5 has an evolutionarily conserved role in NF-κB signaling, we examined the role of GRK5 in NF-κB signaling in human HeLa cells in vitro. HeLa cells were transfected with NF-κB luciferase and CMV-β-galactosidase reporters and GRK5 or control siRNAs. Six hours prior to measurements, NF-κB signaling was induced with TNF-α. When HeLa cells are treated with GRK5 siRNA, the relative NF-κB-luc activity is reduced >60% (Fig. 4B). This indicates that GRK5 is an important regulator of human NF-κB signaling in vitro.

To study the role of Gprk2/GRK5 for vertebrate innate immune response in vivo, we silenced the zebrafish GRK5 in embryos with a translation-blocking morpholino. E. coli was injected into GRK5 morphant zebrafish larvae at 48 h postfertilization and proinflammatory cytokote levels were monitored 18 h postinjection. TNF-α mRNA expression was induced ~600-fold and IL-1β 300-fold (data not shown). In larvae lacking GRK5, the relative TNF-α expression (Fig. 4C) and IL-1β (Fig. 4D) was significantly reduced from that of control morpholino-treated larvae. Blocking the translation of MyD88 also resulted in reduction of TNF-α and IL-1β expression levels (Fig. 4C, 4D). These results indicate that GRK5 is essential for NF-κB signaling in vertebrate immune system in vivo.

Gprk2 interacts with Cactus but is not required for its degradation upon signaling

Because Gprk2 acts at the level or downstream Cactus in the cactus dsRNA epistasis experiment (Fig. 2E), and because of reports of mammalian GRK5 interaction with members of the ISK family (39–41), we decided to investigate the interaction of Gprk2 with Cactus and Dorsal. V5-tagged full-length Gprk2 and deletion constructs were communoprecipitated with myc-tagged Cactus and Dorsal in S2 cells. The full-length Gprk2, Calmodulin (CaM) binding-site deletion (ΔCaM1), and RGS-domain deletion (ΔRGS) constructs interact with Cactus protein, indicating that RGS and CaM1 domains are not needed in Gprk2 and Cactus interaction. In the kinase deletion (Δkinase) construct, this interaction is virtually not detectable anymore, which suggests that the kinase domain is important for the interaction, or that the protein, lacking a large domain, is not correctly folded anymore, resulting in loss of the interaction (Fig. 5A,
FIGURE 3. RNAi targeting Gprk2, CG15737/TAMP, and u-shaped reduces Drosomycin expression in Drosophila in vivo. Fly lines containing indicated UAS-RNAi constructs crossed over the C564-GAL4-driver flies, and controls were infected with M. litterae by pricking and collected 24 h later. Total RNAs were extracted and Drosomycin expression levels measured by qRT-PCR. Results were normalized to Act5C expression values. In each experiment, the relative Drosomycin expression value of the control flies was set to 1. A, MyD88 RNAi flies were used as a positive control. Gprk2 (B, C, TAMP (D), and u-shaped (E) RNAi flies show impaired Drosomycin expression (p < 0.05) compared with controls without the driver. Data are shown as mean ± SD.

Supplemental Fig. 4). V5-tagged Gprk2 proteins did not coimmunoprecipitate with Dorsal-myc (data not shown).

To investigate the functional significance of Cactus-Gprk2 interaction, we used an established EGFR-Toll pathway induction system (32) to monitor Cactus degradation. S2 cells expressing a chimeric EGFR-Toll construct were treated with GFP, Gprk2, and MyD88 dsRNAs and Cactus degradation was monitored on a Western blot (Fig. 5B). Also, a loading control was carried out with anti-GM130 Ab (Abcam) targeting a Drosophila Golgi protein (Fig. 5B). Cactus band intensities were quantified from three separate Western blots with Adobe Photoshop 7 software (Adobe Systems) and normalized to the loading control GM130 band intensities (Fig. 5C). Gprk2 RNAi did not affect degradation of Cactus. Furthermore, we carried out kinase experiments with coimmunoprecipitated Cactus and Gprk2, but were not able to show Gprk2-mediated phosphorylation of the Cactus protein (data not shown). We conclude that Gprk2 interacts directly or indirectly with Cactus, but is not required for Cactus degradation upon signaling.

Gprk2 RNAi flies infected with B. bassiana have reduced expression of the Toll pathway target genes in Drosophila in vivo

To investigate the role of Gprk2 on Toll pathway-mediated immunity in vivo, we carried out an experiment in which Gprk2 RNAi flies and controls were subjected to natural fungal infection with an insect pathogen B. bassiana at +29°C for 48 h, after which total RNAs were isolated. RNAs from noninfected flies were isolated as a control for the infection. Expression of Toll pathway target genes, namely Drosomycin, IM1, and IM2 was measured by qRT-PCR (Fig. 6A–C, respectively). Results were normalized to Act5C.
expression values. Both Gprk2 R-1 and Gprk2 R-3 RNAi lines crossed with the driver C564-GAL4 showed a reduced expression of Toll pathway target genes. w¹¹¹⁸ flies over the C564-GAL4 driver and Myd88 RNAi flies over the C564-GAL4 driver were used as negative and positive controls, respectively. These results indicate that in Gprk2-silenced flies, Toll pathway-induced genes are poorly activated after fungal infection.

Gprk2 RNAi flies are susceptible to infection with Gram-positive bacteria E. faecalis

To examine whether the effect of Gprk2 silencing on Toll pathway is sufficient to impair the fly’s survival, we used septic injury with Gram-positive bacteria. The Toll pathway-mediated immune response was first induced by prickling flies with a needle dipped into a culture of M. luteus. M. luteus infection activates the Toll pathway response including Drosomycin expression. Twenty-four hours later, the flies were infected with E. faecalis by prickling as above. Both Gprk2 RNAi lines crossed with the C564-GAL4 driver show a statistically significant reduction in survival compared with the control line without the driver (Fig. 7A, 7B). When infected with the Gram-negative bacterium E. cloacae, there was no difference between the Gprk2 RNAi flies and controls (Supplemental Fig. 5A, 5B). In conclusion, Gprk2 is needed for normal defense against Gram-positive bacteria E. faecalis.

Gprk2 RNAi construct can rescue UAS-Toll¹⁰b blood cell activation in Drosophila larvae in vivo

To examine if Gprk2 RNAi can inhibit blood cell activation caused by a constitutively activated Toll pathway in vivo, transgenic RNAi fly lines of Gprk2 R-3, and Myd88 as a control, were combined with blood cell-specific hmt³-GAL4, UAS-GFP driver. Males originating from these stocks were crossed to females of the UAS-Toll¹⁰b

**FIGURE 5.** Gprk2 interacts with Cactus, but is not required for Cactus degradation upon signaling. A, The full-length Gprk2, CaM binding site deletion (ΔCaM1) and RGS-domain deletion (ΔRGS) constructs interact with Cactus protein. In the kinase deletion (Δkinase) construct, this interaction is virtually nondetectable. Protein expression was induced with CuSO₄ (250 μM). Proteins were communoprecipitated with anti-myC Ab and detected with anti-V5 Ab. Expression of pMT-Gprk2-V5 and pMT-Cactus-myc constructs in S2 cells induced with CuSO₄ (250 μM) detected with anti-V5 and anti-myc Abs, respectively. B, Gprk2 RNAi does not affect Cactus degradation upon signaling. S2 cells expressing a chimeric EGFR-Toll construct were treated with GFP, Gprk2, and Myd88 dsRNAs. Toll pathway was activated with EGF, and Cactus protein in cytoplasmic extracts was detected by SDS-PAGE and Western blotting with anti-Cactus Ab. Gel loading was controlled using anti-GM130 Ab targeting a Drosophila Golgi protein. C, Cactus band intensities were quantified from three separate Western blots and normalized to the loading control GM130 band intensities. Data are shown as mean ± SD; n = 3.
Drosophila Gprk2 IN NF-κB SIGNALING

with which we were able to monitor both the Toll and the Imd pathway activities simultaneously. Based on careful setup of the assay, biologically meaningful cutoffs and assessment of the general well-being of the cells using Act–5C–β-gal reporter, we obtained a sensible hit list of 23 genes. These included 5 known components of the Toll signaling pathway, 8 known components of the Imd pathway, and 10 previously uncharacterized novel regulators of Drosophila NF-κB signaling. Noteworthy, only one new regulator (MED25) strongly affected the Imd pathway, whereas there were nine dsRNA treatments that primarily decreased the Toll pathway activity. This is in accordance with the notion that several RNAi screens have already been carried out for the Imd pathway, whereas the Toll pathway is less thoroughly studied using RNAi. The results related to Toll pathway activity were further confirmed by secondary (Sp³^-based) and tertiary (qRT-PCR for endogenous Drosomycin) assays. After these confirmation and validation steps, we ended up with a solid hit list of nine novel modifiers of the Toll signaling pathway, namely u-shaped, pannier, achaete, TAM, CG3425, CG32133, CG31660, Sp²6, and Gprk2.

Our screen failed to identify one known positive regulator of the Toll pathway downstream of Toll receptor (Dif) and two components of the Imd pathway (PGRP-LC and Dredd). Targeted RNAi for Dif did not decrease the Drosomycin luciferase reporter activity either when induced by Toll¹⁰⁴ or Toll¹⁰⁸ together with E. coli. This suggests that in S2 cells, dorsal has a more important role in Toll pathway-mediated signaling than Dif. As for PGRP-LC and Dredd, we conclude that these two were not successfully targeted by our dsRNA libraries. In fact, there were several instances in which there was more than one PCR product, occasionally none of them corresponding to the indicated gene, in a single well of the MRC Gene-service PCR product library. Therefore, we found it imperative to TA-clone and sequence every PCR template corresponding to interesting RNAi phenotypes, to design gene-specific primers, and to carry out independent RNAi with targeted dsRNAs. If there were multiple PCR products, all corresponding targeted dsRNAs were tested to identify the one that caused the observed phenotype.

U-shaped is the Drosophila Friend of GATA homolog with a known important role in hemocytes. U-shaped has been shown to interact with and negatively regulate pannier, a Drosophila GATA transcription factor (42). U-shaped and pannier together with achaete (and scute) also regulate the bristle formation in Drosophila (43). This suggests that u-shaped, pannier, and achaete may act together in the process of Toll pathway regulation. TAMP (CG31557) encodes a protein with an N-terminal domain homologous to poly (A) polymerase proteins. CG3425 is a small protein with a RING finger domain, which is likely to be a E3 ubiquitin-protein ligase activity and is often involved in mediating protein-protein interactions. CG32133 is a large protein with postulated molecular functions in transcription factor binding, but the biological processes it mediates are unknown. Drosophila Sp²6 has homology to Saccharomyces cerevisiae Sp²6p, which has been implicated in transcription initiation and maintaining normal chromatin structure during transcription elongation (44). CG31660 bears homology to human G protein-coupled receptor 158, and it contains a domain typical for a metabolotropic glutamate family. Metabotropic glutamate receptors are coupled to G proteins and stimulate the inositol phosphate/Ca²⁺ intracellular signaling pathway (45). It is likely that understanding the exact molecular functions of these genes will reveal novel levels and means to delicately control NF-κB pathway-mediated immune response.

Drosophila RNAi fly collections, namely the VDRC (Vienna, Austria) and NIG-FLY (Kyoto, Japan), provide a tool to study the importance of a selected gene product to a chosen function in the whole organism scale. Crossing RNAi flies with an appropriate

**FIGURE 6.** Gprk2 RNAi flies have impaired expression of Toll pathway target genes when subjected to B. bassiana natural fungal infection. Gprk2 RNAi flies crossed with C564-GAL4 driver and controls were infected with B. bassiana, incubated for 48 h at 29°C, and collected. Total RNAs extracted from flies were subjected to qRT-PCR analysis. The relative Drosomycin, IM1, or IM2 value of the control flies (w¹¹¹⁸/C564) was set to 1. A. Relative Drosomycin expression. Relative IM1 expression (B) and relative IM2 expression (C) in infected flies. Data are shown as mean ± SD, n = 3.

Discussion

Large-scale in vitro RNAi screening has become a commonly used method to identify gene products involved in numerous cellular processes. In this study, we used a luciferase-based reporter assay,
**FIGURE 7.** Gprk2 RNAi flies are susceptible to infection with *E. faecalis*, and Gprk2 RNAi rescues Toll-induced blood cell activation in *Drosophila* larvae in vivo. A and B, The Toll pathway was activated by pricking flies with *M. luteus*, and 24 h later, the flies were pricked with *E. faecalis*. The survival of the flies was monitored for 24 h. MyD88 RNAi flies were used as a positive control. Both Gprk2 RNAi lines (Gprk2 R-1 and Gprk2 R-3) crossed over the C564-GAL4 driver show a statistically significant reduction in survival compared with controls. Gprk2 R-1 × C564-GAL4 (n = 132); Gprk2 R-1 control without the driver (n = 117); Gprk2 R-2 × C564-GAL4 (n = 105); Gprk2 R-3 control without the driver (n = 99). C, Sessile hemocyte banding pattern (i), lost upon constitutive activation of the Toll signaling pathway in blood cells (ii), could be rescued by Gprk2 (iii) or MyD88 RNAi (iv). D, The average grades of three independent crosses, n = 20 larvae per cross, ± SEM. Grade 1, sessile hemocyte bands in 100% of segments; grade 2, bands in <75% of segments; grade 3, bands in <50% of segments; and grade 4, no bands or bands only in the most posterior 25% of segments.

GAL4 driver fly line results in silencing of the targeted gene in the chosen tissue in the progeny. However, it is recognized that as much as 35–40% of fly lines may give a false-negative result, which may be due to multiple reasons related to RNAi, driver GAL4 strain, and/or the assay chosen (34). In our in vivo infection assays, we found a phenotype with a statistical difference to controls in 5 out of 11 strains (Fig. 3, Supplemental Fig. 1). In addition, two other strains (Spr6 and *pannier* RNAi strains; Supplemental Fig. 1) showed a similar but nonsignificant trend. Therefore, our results are in line with the estimates presented by Dietzl and coworkers (34). Of note, the false-positive rate is estimated to be <2%, which means that it is very likely the reduction in *Dromysomyacin* expression in our driver-induced strains is due to silencing of the gene in question (34).

Importantly, we identified a novel, evolutionarily conserved regulator of NF-κB signaling, Gprk2, in our screen in S2 cells. Gprk2 is very well conserved and has high sequence similarity at the amino acid level with vertebrate GRK5. GRKs are known to phosphorylate G proteins, thus causing receptor desensitization and switching off of the G protein-coupled receptor signaling pathway (38). In addition to G proteins, GRKs are known to phosphorylate various other substrates and to modulate cellular responses in a phosphorylation-independent manner (46). Although mostly membrane-bound, GRK5 has been shown to contain a functional nuclear localization sequence (47), and as function as a histone deacetylase kinase in the nucleus of cardiomyocytes has been reported (48).

Recently, there have been implications as to the involvement of the human GRK5 to NF-κB-mediated immunity: in a recent report, the human GRK5 has been shown to participate in TNF-α-induced NF-κB signaling via direct interaction with and phosphorylation of IκBα (39). Also, effects on LPS-induced ERK1/2 signaling (40) and NF-κB transcriptional activity (41) have been proposed. In *Drosophila*, Gprk2 has been shown to regulate hedgehog signaling (49), but no involvement in innate immunity has previously been reported. In this study, we have shown that Gprk2 is an evolutionarily conserved regulator of innate immune signaling. Furthermore, we were able to show that Gprk2 is required for normal microbial resistance in vivo. Interestingly, although Gprk2 physically interacts with Cactus, it is not required for signal-induced Cactus degradation. It will be of great interest in the future to investigate the exact role of the Gprk2-Cactus interactions.

NF-κB signaling is of paramount importance for regulating immune response both in flies and vertebrates. The power of the *Drosophila* model includes the possibility of combining large-scale RNAi screening with sophisticated in vivo tools. In this study, we carried out a genome-wide RNAi screen in cultured *Drosophila* cells and identified 10 novel regulators of *Drosophila* NF-κB signaling. The evolutionarily conserved role for Gprk2/GRK5 in NF-κB pathway activation was shown using human HeLa cells in vitro and zebrafish embryos in vivo. Finally, the importance of Gprk2 for *Drosophila* NF-κB signaling was demonstrated both in vitro and in vivo.
FIGURE S1 Spt6, pannier, achaete, CG4325, CG31660 and CG32133 RNAi fly lines crossed over C564-GAL4-driver do not show altered Drs expression levels after M. luteus infection compared to controls as measured by qRT-PCR. Results were normalized to Act5C expression values. Data are shown as mean ± s.d., N ≥ 3.
FIGURE S2 Drosophila Gprk2 is homologous to GRK5 from other organisms. A comparison of amino acid sequences of Gprk2/GRK5 homologs from human, mouse, zebrafish and Drosophila. The RGS domain is shown in a gray background, the kinase domain bold and in a gray background and the extension to kinase domain italicized and in a gray background. * = identical amino-acid; : = conserved substitutions have been observed; . = semi-conserved substitutions have been observed.
FIGURE S3 Gprk2/GRK5 is localized on the cell membrane or cytoplasm. A, Gprk2 is localized on the cell membrane/cytoplasm in S2 cells. S2 cells were transfected with a Gprk2-GFP construct and examined 48 h later by confocal microscopy. B, GRK5 is localized on the cell membrane/cytoplasm in HeLa cells. HeLa cells were transfected with a GRK5-GFP construct and examined 36 h later by confocal microscopy.
FIGURE S4 The full length Gprk2, Calmodulin binding-site deletion (ΔCaM1) and RGS-domain deletion (ΔRGS) constructs interact with Cactus protein. In the kinase deletion (Δkinase) construct this interaction is virtually nondetectable. Protein expression was induced with CuSO₄ (250μM). Proteins were co-immunoprecipitated with anti-V5 antibody and detected with anti-myc antibody.
FIGURE S5 Both A, Gprk2 R-1 and B, Gprk2 R-3 RNAi fly lines show no difference in survival from *E. cloaca* septic injury between driver-induced and control flies. 

*Gprk2 R-1 x C564-GAL4* (N = 40); *Gprk2 R-1*, no driver (N = 20); *Gprk2 R-3 x C564-GAL4* (N = 40); *Gprk2 R-3*, no driver (N = 35).
### Supplemental Tables

**Table SI** RNAi fly stocks.

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Table SII PCR-primers.

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T7 + GGA CCC TCT GTT ATC CGG 

pannier
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T7 + CTG TGC GAT GTG TAG G 

u-shaped (I)  
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GT CTG GTG CAT GCG CAT GTG 

u-shaped (II)  
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T7 + GCT GTA GGA GCA CTG G 

Gprk2 (I)  
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AGA GCT CTC AAA CTC CC 

Gprk2 (II)  
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T7 + CGT TCA CAC ACT GGG C 

Spt6 (I)  
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Spt6 (II)  
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T7 + GCT GTA CGC ATT CCG 

MED25 (I)  
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MED25 (II)  
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T7 + CCG TCG GCC TTC ATG A 

cactus (I)  
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cactus (II)  
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T7 + GAC TGC AGC TGC AGC 

qRT-PCR primers

Drosophila qRT-PCR primers:

Des  
ATG ATG CAG ATC AAC TAC TCG TAC AAG TAC AAG TAC
GCA TCC TGC CCA CCA GC
210

ActSC  
CGA AGA AGT TGC TGC TCT GG
AGA ACG ATA CCG GTG GTA CG
453

IM1  
CTC GGT CTC TCG GTG GTG GCC
CCG TGG ACA TCG CAC ACC C
95

IM2  
CTT CTC AGT CGT CAC CGT CC
TCC ACC GTG CAC ATT GCA G
124

Zebrafish qRT-PCR primers:

TNF-α  
GGG CAA TCA ACA AGA TGG AAG
GCA GCT GAT GTG CAA AGA CAC
250

IL-1β  
TGG ACT TCG CAC CAC AAA ATG
GTT CAC TCC ACG CTC TGG GTG T
124

β-Actin∗  
ATG GAT GAG GAA ATC GCT G
ATG CCA ACC ATC ACT CCC TG
130

Transcription factor zfhl downregulates Drosophila Imd pathway

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\textbf{A B S T R A C T}

The fruit fly Drosophila melanogaster has a powerful innate immune system, which culminates on the synthesis of potent antimicrobial peptides (AMPs). This is mainly controlled by two conserved signaling cascades, the Toll and the immune deficiency (Imd) pathways. Like in humans, Drosophila immune responses need to be under tight control at multiple levels to avoid harmful inflammation. We have identified the transcription factor Zn finger homeodomain 1 (zfhl) as a negative regulator of Drosophila Imd signaling. Knocking down zfhl in Drosophila S2 cells hyperactivates Imd pathway-mediated AMP expression, whereas forced zfhl expression blocks Imd pathway response downstream of, or parallel to, the Imd pathway transcription factor Relish. In vivo zfhl RNAi hyperactivates CecropinB induction upon gram-negative bacterial infection. We conclude that zfhl is an important regulator of the immune response in Drosophila.

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1. Introduction

Innate immunity provides the first-line host defense against invading microorganisms. Due to the conservation of the mechanisms underlying the innate immune responses, the fruit fly Drosophila melanogaster has become a widely-used and powerful model to study innate immunity (Rämet, 2012). The Drosophila immune response can be roughly divided into three parts: local, cellular and systemic response (Lemaitre and Hoffmann, 2007). The systemic response culminates on the secretion of antimicrobial peptides (AMPs) by the fat body. Synthesis of AMPs is mainly controlled by two conserved NF-kB signaling cascades, the Toll and the Imd pathway (Dushay et al., 1996; Lemaitre et al., 1996; Valanne et al., 2011, 2012). While the Toll pathway contributes to both immunity and developmental processes (Lemaitre et al., 1996; Valanne et al., 2011), Imd signaling is only involved in regulating immune responses. More specifically, the Imd pathway mediates the response against bacteria (Lemaitre et al., 1995) which are recognized by the receptor peptidoglycan-binding protein LC (PGRP-LC) (Choe et al., 2002; Gottar et al., 2002; Rämet et al., 2002) or by PGRP-LE (Kanelo et al., 2006; Lim et al., 2006; Takehana et al., 2004). Binding of DAP-type peptidoglycan (PGN) to the receptor triggers receptor dimerization and recruitment of downstream signaling components Imd, FAS-associated death domain protein (dfADD) and death-related Ced-3/Nedd2-like protein (Dredd) to the signaling complex via death domain interactions (Georgel et al., 2001; Leulier et al., 2000, 2002). Dredd is required for the cleavage and activation of both Imd and the transcription factor Relish (Stöven et al., 2003; Paquette et al., 2010). The Drosophila homolog of the TGF-β activated kinase 1 (Tak1; Vidal et al., 2001; Silverman et al., 2003) and its associated protein Tak1-binding protein 2 (Tab2) are required for the activation of the IκB kinase (IKK) complex (Kleino et al., 2005; Gesellchen et al., 2005, 2007; Zhuang et al., 2006), which consists of two subunits, immune response deficient 5 (Ird5) and Kenny, and is needed for Relish cleavage (Rutshcmann et al., 2000; Silverman et al., 2000). After phosphorylation of several sites (Silverman et al., 2000; Ertürk-Hasdemir et al., 2009) and proteolytic cleavage of the inhibitory C-terminal part, the active N-terminal part of Relish translocates into the nucleus to activate target gene transcription (Stöven et al., 2000, 2003). In addition, induction of the Imd pathway requires Drosophila inhibitor of apoptosis 2 (Iap2) (Kleino et al., 2005; Gesellchen et al., 2005; Leulier et al., 2006; Huh et al., 2007; Valanne et al., 2007; Paquette et al., 2010), which is likely to mediate Imd ubiquitination, together with the K63-ubiquitinating enzymes Uev1/Bend/Ubc5 (Huh et al., 2007; Paquette et al., 2010). The positive regulator sickie and the nuclear factor Akirin are also required for full Imd response (Foley and O’Farrell, 2004; Goto et al., 2008).

As an uncontrolled immune response can be harmful for the host tissues, tight regulation of the NF-kB signaling pathways is crucial in both mammals and in Drosophila to avoid chronic inflammation (Li and Verma, 2002; Vallabhapurapu and Karin, 2009). Several negative regulators and regulation mechanisms of Imd pathway have been identified in recent years. PGRP-LF inhibits the Imd pathway at the receptor level by directly interacting with...
2. Materials and methods

2.1. S2 cell culture and transfections

Targeted dsRNAs were synthesized from S2 cDNA essentially as described in Valanne et al. (2007). All the primers had a T7 binding sequence (5'-GAATATATATTATATATGAGA-3') on the 5' end. Primers used for targeted dsRNAs were: zfh1, 5'-T7-AGA- GGCGTCTGCACAC-3' (forward) and 5'-T7- AACTCATCCTATGGAGA-3' (reverse); zfh1 5'UTR, 5'-T7-ACGGCACACCCGCACG-3' (forward) and 5'-T7-CTTGGATCTGGATAGT-3' (reverse); the primers for Green Fluorescent Protein (GFP), Relish, DmyD88 and idm dsRNAs were as in Kleino et al. (2005).

Drosophila S2 cells were cultured in Schneider medium (Sigma-Aldrich, St. Louis, MO, US) with 10% FBS, 100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin at 25 °C. Transfections and dsRNA treatments were performed essentially as described in Kleino et al. (2005). 1.0 × 10^5 S2 cells were seeded on 24-well plates and transfected with 0.1 \( \mu \)g of the indicated reporter plasmid, together with 5'GG-ACACVATTCTCGAGTTTCTGG-3' (reverse); IL-1, 5'-CGACACTGATGACCGAACC-3' (forward) and 5'-CAACACCATGAGACACCCGCC-3' (reverse); ZEB1, 5'- CCAACCTAGTACGCTGGC-3' (forward) and 5'-CAGAATCTCTACTG-3' (reverse); GAPDH, 5'-TTGCCGCGAGCCAGCAGAAATG-3' (forward); Act5C, AtbB, CecB and DptB primers were as in Kleino et al. (2008).

2.2. HeLa cell culture and transfections

HeLa cells were cultured and transfected as in Valanne et al. (2010). For siRNA silencing experiments, GFP siRNA (Silencer GFP [eGFP]; catalog number AM4626, Ambion, Life Technologies, Carlsbad, CA, US) was used as a negative control, RelA siRNA (catalog number AM16704; ID 216912) as a positive control and for silencing ZEB1, a mixture of three independent siRNAs were used (IDs s13883, s13884 and s13885).

2.3. RNA extraction and qRT-PCR

Total RNAs from S2 cells, HeLa cells, adult flies and larvae were extracted using TRIzol reagent (Bioline, London, UK) or Tri reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. RNAs were then subjected to quantitative RT-PCR analysis essentially as in Kalio et al. (2010) and in Vanha-aho et al. (2012) using QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) and an ABI7000 (Applied Biosystems, Life Technologies, Carlsbad, CA, US) instrument and software (for S2 and HeLa cells) or the BioRad iScript™ one-step RT-PCR kit with SYBR® Green (BioRad, Hercules, CA, USA) (for flies) according to the manufacturer's instructions.

Primers used for qRT-PCR were: Attd, 5'-GAGATATCGGCGGCTGACGCACTAGTAACG-3' (reverse); zfh1, 5'-AGATGATGGGCGAGGAGGAGACCC-3' (forward) and 5'-CCTGAGCTGAACTCTG-3' (reverse); IIL-1, 5'-CAGACACTGATGACCGAACC-3' (forward) and 5'-CAACACCATGAGACACCCGCC-3' (reverse); ZEB1, 5'-CTTGGATCTGGATAGT-3' (forward) and 5'-GGTACCATGCAACAGAAGG-3' (reverse); GAPDH, 5'-TTGCCGCGAGCCAGCAGAAATG-3' (forward); Act5C, AtbB, CecB and DptB primers were as in Kleino et al. (2008).

2.4. Cloning and constructs

zfh1 was cloned into the EcoRI and XhoI sites of pMT/V5/HisA vector (Invitrogen/Life Technologies) in two pieces utilizing the XhoI site present in the gene. SD06902 (DGRC, Indiana University, US) CDNA clone and S2 cell CDNA were used as templates, and the primers used were: first part, 5'-CAACACAGAACAAAAACGGCGACACAC-3' (forward) and 5'-GGCAGATGGGAGGAGTCTCAGC-3' (reverse); second part, 5'-CCGCTCTCTGCGAGCGACACAGC-3' (forward) and 5'-CAACACACTGATGACCGAACC-3' (reverse), resulting in a construct with a V5 tag in the 5' end.

huzEB1 was cloned into KpnI and NotI sites of pMT/V5/HisA vector using the cDNA from IMAGE clone #40036600 (Geneservice Ltd., Cambridge, UK) as a template, and the primers used were:...
5'-CACACAGGTACCAGGGATGGGCCC-3' (forward) and 5'-CAC-ACACGGGCCCAGCTTCTTCTG-3' (reverse). No tag is expressed due to a frameshift caused by vector cleavage using NotI.

2.5. Fly maintenance and stocks

Drosophila stocks were kept on a standard mashed potato diet at 25 °C. zfh1 RNAi transgenic fly stocks from Vienna Drosophila RNAi Center were used: 42856 (UAS-zfh1-IR1) and 42857 (UAS-zfh1-IR2), UAS-Fadd-IR and C564-GAL4 driver flies were obtained from Prof. Bruno Lemaitre (Global Health Institute, School of Life Science, Ecole Polytechnique Federale de Lausanne (EPFL), 1015 Lausanne, Switzerland) and tubulin-GeneSwitch-GAL4 flies were obtained from Prof. Howard Jacobs (Institute of Biomedical Technology, University of Tampere, Finland).

UAS-RNAi flies were crossed over C564-GAL4 or tubulin-GeneSwitch-GAL4 driver flies or to w1118 flies for controls. In flies crossed over the tubulin-GeneSwitch-GAL4 driver, the GAL4 expression was induced by keeping 1–2-day-old F1 flies in vials with Mepifrone (Sigma–Aldrich, St. Louis, MO, US) (200 μM) in the food for 5 days at +29 °C prior to the infection. F1 flies crossed to C564-GAL4 driver were also kept at +29 °C for 5 days. For dissection of fat bodies, the flies were allowed to lay eggs at room temperature for 24 h, after which the vials were transferred to +29 °C, until the larvae reached the L3 stage (4–5 days). Six larvae per RNA extraction were washed and their fat bodies were dissected using sharp forceps and collected in 20 μl of PBS.

2.6. Bacterial culture and fly infections

For Enterobacter cloacae (strain β12) infection, week-old healthy male flies were pricked with a thin tungsten needle dipped in a concentrated culture of bacteria grown overnight on nalidixic acid (15 μg/ml) selection. After 4 h the flies were snap-frozen on dry ice and subjected to RNA extraction and qRT-PCR analysis. The experiment was repeated twice, each set containing four biological replicates, where six flies were used per sample.

2.7. Statistical analysis

Statistical significance was assessed by two-tailed Student’s t-test or Two-way ANOVA with a confidence interval of 5%.

3. Results

3.1. RNAi silencing of zfh1 hyperactivates Imd pathway target gene expression in S2 cells

To evaluate the importance of zfh1 expression for the Imd pathway activity in Drosophila S2 cells, target gene promoter-driven luciferase activities were measured upon activation with heat-killed E. coli. As shown in Fig. 1A, RNAi silencing of zfh1 caused dramatic increase in AttacinA-mediated luciferase activity (AttA-luc). Similar result was observed with another Imd pathway gene driven luciferase reporter, namely CecropinB-luc (CecB-luc) (Fig. 1B), although in this case the effect of RNAi on reporter activity was less dramatic. Notably, RNAi silencing of zfh1 enhanced AttA-luc expression even without induction of Imd signaling with heat-killed E. coli, suggesting that zfh1 is a constitutive repressor of Imd signaling.

We next investigated whether zfh1 RNAi affects AttA-luc activity triggered by other inducers than E. coli. For this, S2 cells were transfected with plasmids expressing Imd or the constitutively active form of Relish (ΔSSR) which lacks the serine-rich region on S29-S45 (Stöven et al., 2003), together with zfh1 or control dsRNAs (Fig. 1C). Both plasmids cause an induction in the Imd pathway target gene expression in S2 cells, allowing epistasis analysis of zfh1 in Imd signaling. As shown in Fig. 1C, zfh1 dsRNA treatment caused a clear increase in AttA-luc activity in both cases, indicating that zfh1 functions downstream of both Imd and Relish. These results are consistent with the reported nuclear localization of zfh1 protein in Drosophila embryos (Lai et al., 1991) and its binding of specific DNA sequences (Postigo et al., 1999).

Zfh1 does not appear to affect gene expression or reporter plasmid activity in S2 cells in general, since zfh1 RNAi had no effect on Toll signaling activity, as measured by Drosomycin-luciferase (Drs-luc) reporter activity upon induction with constitutively active form of the Toll receptor, Toll1108 (Fig. 1D). In addition to the luciferase reporter plasmids, S2 cells were transfected with another reporter where β-galactosidase is expressed under the promoter of Actin5C, a house-keeping gene constitutively expressed in living cells. This provides a simple means to monitor effects of experimental treatments on cell viability (Kleino et al., 2005). As shown in Supplementary Fig. 1, RNAi targeting of zfh1 does not decrease Act5C-β-gal activity compared to GFP dsRNA treated control, and is therefore not likely to affect cell viability under these conditions.

To further validate the importance of zfh1 for the Imd pathway target gene expression, we ran qRT-PCR on the endogenous AMPs in S2 cells treated with heat killed E. coli and either zfh1 dsRNA, or GFP dsRNA as a negative control (Fig. 2). Again, zfh1 RNAi caused increased expression of all AMPs tested: AttacinD (AttD), DipterincB (DptB) and CecropinB (CecB) (Fig. 2A–C). zfh1 RNAi appears to only affect the magnitude of AMP induction, but not the kinetics, since the increase in each time point is relative to the normal expression level, and the maximum expression was observed at the same time point as in controls, i.e. at 24 h for AttD and DptB and at 4 h for CecB. Of note, similarly to AttA-luc reporter activation, zfh1 RNAi induces AMP expression in S2 cells without exposure to heat-killed E. coli, again pointing to a role as a constitutive repressor (Fig. 2A–C, left panels). The efficacy of the zfh1 dsRNA treatment was confirmed by qRT-PCR (Fig. 2D).

3.2. Forced zfh1 expression suppresses Imd pathway target gene expression in S2 cells

Based on RNAi results, zfh1 is an important negative regulator of the AMP gene expression in Drosophila cells. Next, we tested whether overexpression of zfh1 affects the Imd pathway activity in S2 cells. zfh1 encodes a 1054 aa transcription factor with a homeodomain and nine C2–H2 zinc fingers, both of which are common DNA-binding motifs (Fig. 3A). The expression of the V5-tagged zfh1 construct from a metallothionein promoter containing plasmid was verified by Western blot, and the size was as reported previously (~145 kDa for zfh1 spliceform B) (Fig. 3B) (Fortini et al., 1991). Thereafter, zfh1 was ectopically expressed in S2 cells, the AttA-luc reporter was used as a read out for the Imd pathway activity, and Imd signaling was induced, either by adding heat-killed E. coli onto cells, or by transfecting the cells with wild-type Relish or with the constitutively active form of Relish (ΔSSR) (Stöven et al., 2003). As shown in Fig. 3C, overexpression of zfh1 effectively reduces AttA-luc activity, further indicating that zfh1 is a negative regulator of the AMP gene expression in Drosophila S2 cells, and that zfh1 functions at the level or downstream of Relish.

We next used zfh1 construct to confirm that the phenotype observed with zfh1 RNAi is indeed caused by a reduction in the amount of zfh1 protein, rather than any unexpected off-target effect. To this end, we performed a rescue experiment with a dsRNA targeted against the 5’ UTR region of zfh1 (the target sites of the dsRNAs on zfh1 mRNA are shown in Fig. 3A). As shown in Fig. 3D, this dsRNA treatment caused a similar hyperactivation in Imd pathway activity upon E. coli stimulation, as the dsRNA targeted against the coding region of zfh1, and the phenotype
was almost completely rescued by ectopic expression of zfh1. Furthermore, the zfh1 RNAi phenotype was also partially rescued by ectopic expression of human ZEB1 (Fig. 3D), which is a homologue of zfh1 sharing similar domain structure and DNA binding and transcription repression properties (Fig. 3A; Fortini et al., 1991; Postigo and Dean, 1997; Postigo et al., 1999).

3.3. ZEB1 regulates NF-κB activity in HeLa cells

Since the Drosophila Imd pathway closely resembles the mammalian tumor necrosis factor receptor (TNFR) signaling pathway, and the human homologue of zfh1 affected Imd pathway activity, we next studied the role of ZEB1 in human cells to see if Zfh1/ ZEB1 has an evolutionary conserved role in NF-κB signaling. For this, we used HeLa cells, which were transfected with NF-κB-luciferase (NF-κB-luc) reporter, together with CMV-β-galactosidase (CMV-β-gal) plasmid, and ZEB1 or the control siRNAs (GFP siRNA as the negative control and RelA siRNA as the positive control). For ZEB1 knock-down, a mix of three siRNAs targeting different sites of the gene was used. TNFR signaling was induced by adding TNF-α onto the cells (Fig. 4A). Interestingly, instead of hyperactivating TNF signaling, ZEB1 siRNAs strongly decreased NF-κB-luc activity in this setting. Similar results were obtained when endogenous interleukin-1β (IL-1β) expression was analyzed by qRT-PCR in HeLa cells transfected with the siRNAs and stimulated with TNF-α (Fig. 4B). The mix of three siRNAs against ZEB1 was effective, as a decrease of around 80% in the expression level of ZEB1 was obtained compared to controls (Fig. 4C). Of note, ZEB1 siRNAs did not affect β-galactosidase activity, indicating normal cell viability. To exclude the possibility of off-target effects, the siRNAs targeting ZEB1 were also tested individually and in pairs for their effect on NF-κB-luciferase activity, and similar results were obtained (Fig. S2). In conclusion, ZEB1 appears to have a role in regulating TNF signaling in human HeLa cells, but with an impact opposite to that observed in Drosophila cells.

3.4. Effect of zfh1 on AMP expression in vivo

To assess the role of zfh1 in Drosophila immune defense in vivo, we used UAS-GAL4 system (Brand and Perrimon, 1993) for in vivo RNAi. Two UAS-zfh1 RNAi lines and a UAS-Fadd RNAi line (as a positive control) were first crossed to C564-GAL4 driver to direct RNAi to the fat body; the primary organ responsible for AMP synthesis upon septic infection. As additional controls, the RNAi lines and the driver line were crossed to wild-type w1118 flies. Week-old F1 flies were infected with gram-negative bacterium E. cloacae and total RNAs were isolated after four hours. qRT-PCR analysis was used to measure the expression level of the Imd pathway target genes CecB and DptB (Fig. 5A and B). As shown in Fig. 5A and B, Fadd RNAi in the fat body severely impaired the induction of both CecB and DptB upon infection. Zfh1 RNAi increased CecB induction by approximately twofold, whereas the effect on DptB expression was not significant. Moreover, CecB and DptB expression was non-existent in uninfected flies of all genotypes, indicating that, in contrast to S2 cells, zfh1 does not affect the basal expression of AMPs in vivo (Fig. 5A and B). Thus, the role of zfh1 as a universal negative regulator of the Imd pathway in vivo was not confirmed using this traditional UAS-GAL4 system. Since inefficient knock-down of the target gene could explain weak phenotypes observed, we monitored zfh1 expression in the knock-down flies. For this, zfh1 RNAi strains were crossed to C564-GAL4 driver line and dissected fat bodies from the resulting progeny L3 larvae were used as a template for qRT-PCR. As shown in Fig. 5C, C564-GAL4-driven zfh1 RNAi caused a clear decrease in zfh1 expression level in larval fat body compared to w1118 – crossed controls, indicating the knock-down was relatively efficient at least in this setting. Of note, zfh1-IR/

![Fig. 1. RNAi targeting zfh1 hyperactivates Imd pathway response, but does not affect Toll signaling pathway activity in Drosophila S2 cells. (A) zfh1 RNAi strongly enhances Imd pathway induction in response to heat-killed E. coli as measured by AttacinA-luciferase (AttA-luc) as well as (B) CecropinB-luciferase (CecB-luc) activity compared to GFP dsRNA treated controls in S2 cells. (C) zfh1 functions downstream of Imd and Relish in S2 cells. For epistatic analysis, S2 cells were transfected with AttA-luc and Act5C-β-gal reporters, zfh1 and control dsRNAs and Imd or constitutively active Relish (sSRR) plasmids to induce Imd pathway. (D) zfh1 RNAi does not affect Tollβ-mediated Drosomycin-luc (Drs-luc) activity. In (A), (B) and (C), the fold inductions of AttA-luc/Act5C-β-gal and CecB-luc/Act5C-β-gal values and the relative Act5C-β-gal values are calculated by setting the value of the GFP dsRNA treated sample with no pathway induction as one, in (D) the Drs-luc/Act5C-β-gal value of GFP dsRNA treated sample with Tollβ induction is set to one. Data are shown as mean ±/− standard deviation, N > 6. *p < 0.05; **p < 0.005; ***p < 0.001 (Student’s t-test).](image)
C564-GAL4 flies were viable and had no visible abnormalities in either adult or larval stages.

In order to avoid bias caused by genetic background and possible developmental defects caused by the RNAi knock-down, we crossed the RNAi lines to an inducible ubiquitous tubulin-GeneSwitch-GAL4 (tub-GeneSwitch-GAL4) driver. Using this system, the UAS constructs remained inactive (“uninduced” samples in Fig. 5D and E) until GAL4 is induced by adding the drug Mifepristone into flies’ food (“RNAi induced” samples in Fig. 5D and E). Similar to results obtained with C564-GAL4, in the tub-GeneSwitch-GAL4 system Fadd RNAi also abolished CecB and DptB induction. zfh1 RNAi caused a significant hyperactivation of CecB expression in response to

**Fig. 2.** RNAi targeting zfh1 hyperactivates endogenous Imd pathway target genes in Drosophila S2 cells. Endogenous (A) AttacinD (AttD), (B) DipterinB (DptB) and (C) CecropinB (CecB) induction is enhanced by zfh1 RNAi in S2 cells stimulated with heat-killed E. coli for indicated times. (D) dsRNA targeted against zfh1 effectively blocks zfh1 expression. S2 cells were treated with zfh1 or GFP dsRNAs and Imd signaling was induced by adding heat-killed E. coli. Cells were harvested at indicated time points, and the total RNA isolated was used as a template for qRT-PCR. The fold induction of AMP/Act5C expression levels were calculated by setting the value of the GFP dsRNA treated 0 h sample as one. The relative values for each AMP the 0 h time points are also shown separately on the left panels of each graph (note the different scale). Data are shown as mean +/- standard deviation, N = 4, shown is a representative experiment out of two repeats. *p < 0.05; **p < 0.005; ***p < 0.001 (Two-way ANOVA (A–C), Student’s t-test (D)).
E. cloacae infection, but again, no clear effect on DptB expression was observed. AttB and AttD induction was also monitored by qRT-PCR, but as for DptB, no conclusive phenotype was observed in zfh1 RNAi flies with either C564-GAL4 or tub-GeneSwitch-GAL4 (data not shown). Hence, knocking down zfh1 in vivo hyperactivated CecB induction upon infection, but no consistent increases in expression levels of other Imd pathway induced AMPs were observed.

4. Discussion

During the last decade, the signaling pathways regulating the Drosophila immune response have been under intense investigation (reviewed in Valanne et al., 2011; Valanne et al., 2012). In addition to traditional genetic approaches, RNAi screens and biochemical studies have succeeded in identifying new components and regulators of the Drosophila Toll, Imd and JAK/STAT signaling cascades (Aggarwal et al., 2008; Choe et al., 2002; Gesellchen et al., 2005; Gottar et al., 2002; Grönholt et al., 2012; Kallio et al., 2010; Kleino et al., 2005, 2008; Rämet et al., 2002; Valanne et al., 2010). It has become evident that, like in humans, the Drosophila immune response is also tightly regulated. Induction of a rapid and effective immune response against invading pathogens is crucial for a multicellular animal; however, proper silencing and control of said response is just as important. Moreover, there are bacteria and fungi present in many tissues such as the gut, not as pathogens but as useful commensals, and they therefore need to be tolerated by the immune system. As such, the signaling pathways responsible for initiating the immune response are regulated on multiple levels. Importantly, many of these regulatory mechanisms are conserved from flies to man.

The Drosophila Imd pathway shares many similarities with mammalian TNFR signaling. Both pathways use NF-κB family transcription factors, which are inhibited by masking their nuclear localization signals by ankyrin repeat-containing inhibitory proteins (Cactus for Drosophila Toll pathway transcription factors Dif and Dorsal and inhibitors of NF-κB (IκB) for mammalian RelA, c-Rel and RelB), or by an inhibitory domain included in the protein itself (Drosophila Relish and mammalian p100/p52 and p105/p50). Upon signaling pathway activation, the inhibitory protein is degraded or the inhibitory domain cleaved, releasing the activated transcription factors for dimerization and translocation into the nucleus (Stöven et al., 2000; Vallabhapurapu and Karin 2009).
ZEB1 blocks ZEB1 regulates TNFR signaling in human cells. (A) siRNA knock-down of ZEB1 blocks NF-κB-luciferase (NF-κB-luc) and (B) endogenous interleukin-1β (IL-1β) induction in response to TNF-α stimulation in HeLa cells. (C) ZEB1 siRNAs efficiently block ZEB1 expression in HeLa cells. HeLa cells were transfected with NF-κB-luciferase and CMV-β-galactosidase reporter plasmids together with mixture of three ZEB1 siRNAs or negative and positive control siRNAs (GFP and RelA, respectively) (A), or with the siRNAs only (B and C), and treated with TNF-α for 6 h prior to cell lysis and measurement of luciferase activity (A) or RNA extraction and qRT-PCR analysis (B and C). Data are shown as mean ± standard deviation, N = 6 for (A) and N = 4 for (B and C). *p < 0.05; **p < 0.005; ***p < 0.001 (Student’s t-test).

Fig. 4. ZEB1 regulates TNFR signaling in human cells. (A) siRNA knock-down of ZEB1 blocks NF-κB-luciferase (NF-κB-luc) and (B) endogenous interleukin-1β (IL-1β) induction in response to TNF-α stimulation in HeLa cells. (C) ZEB1 siRNAs efficiently block ZEB1 expression in HeLa cells. HeLa cells were transfected with NF-κB-luciferase and CMV-β-galactosidase reporter plasmids together with mixture of three ZEB1 siRNAs or negative and positive control siRNAs (GFP and RelA, respectively) (A), or with the siRNAs only (B and C), and treated with TNF-α for 6 h prior to cell lysis and measurement of luciferase activity (A) or RNA extraction and qRT-PCR analysis (B and C). Data are shown as mean ± standard deviation, N = 6 for (A) and N = 4 for (B and C). *p < 0.05; **p < 0.005; ***p < 0.001 (Student’s t-test).

NF-κB is a target gene of NF-κB itself, and is thus resynthesized upon NF-κB activation and inhibits subsequent transcriptional NF-κB activity, therefore forming a negative feedback-loop. Imd pathway transcription factor Relish has the inhibitory domain in its C-terminus, and therefore this kind of negative feedback-loop cannot be utilized in Drosophila, even though Relish is a target gene of the Imd pathway (Dushay et al., 1996). Instead, the Imd pathway is regulated by another negative feed-back mechanism involving pirk (Kallio et al., 2005; Kleino et al., 2008; Aggarwal et al., 2008; Lhocine et al., 2008). Pirk is rapidly induced by Imd signaling and limits the intensity and duration of the Imd pathway by interfering the function of the PGRP-LC/Imd receptor complex.

Here we describe another negative regulatory mechanism involving zfh1, which controls Imd signaling at the transcriptional level, having an especially prominent role in S2 cells, and a lesser one in vivo. Even though zfh1 functions at the level of or downstream of Relish, the exact mechanism of its action in an immunity context remains elusive. Zfh1 is a transcriptional repressor that contains two zinc finger clusters and a homeodomain. It binds E boxes on target gene promoter regions, but both zinc fingers and homeodomains can bind DNA and regulate gene expression, possibly on different sites. Zinc fingers can also bind RNA or proteins. Therefore, there are many possible mechanisms whereby zfh1 can repress Imd signaling. Since zfh1 RNAi alone causes activation of Imd pathway mediated AMPs, it appears as a constitutive repressor of the pathway. This could be accomplished by direct binding to a target gene promoter, perhaps in this way displacing or inhibiting Relish. The dramatic effect of zfh1 knock-down on Relish-dependent AMP expression in S2 cells makes a direct effector mechanism a tempting hypothesis; however no interaction between zfh1 and Relish was observed in co-immunoprecipitation assays (data not shown). Furthermore, deletion of single putative binding sites for zfh1 found on the promoter region of the Atg-luc reporter construct failed to rescue the observed zfh1 RNAi phenotype (Supplementary Fig. 3). Thus, we cannot exclude the possibility that zfh1 regulates Imd signaling in a more indirect way, such as inducing a repressor or repressing an activator of the Imd pathway. Mediating crosstalk with one or multiple other signaling pathway(s) is another possible mechanism of action for zfh1, especially since it has been implicated in direct or genetic interactions with several pathways, including bone morphogenetic protein (BMP), Jun N-terminal kinase (JNK) and janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathways (Frandsen et al., 2008; Leatherman and Dinardo, 2008; Ohayon et al., 2009).

Intriguingly, we found out that also the human homologue of zfh1, ZEB1, regulates TNF-α-mediated NF-κB signaling in HeLa cells, although our results with ZEB1 siRNA suggest a positive regulation of NF-κB by ZEB1 in this context. However, several regulatory mechanisms have been proposed for ZEB1, facilitated by its domain structure, which allows interaction with both DNA and coactivator and corepressor proteins. Thus, the repressor or activator status of ZEB1 can be determined by the expression level of the co-regulator, which can involve cell-type specific differences in post-translational modifications, or depend on the DNA context (Vandewalle et al., 2009).

Zfh1 is shown to participate in multiple developmental processes, including local cell fate and positioning in mesodermally derived tissues such as heart and muscles as well as somatic stem cell renewal (e.g. Lai et al., 1991; Lai et al., 1993; Leatherman and Dinardo 2008; Postigo et al., 1999), making in vivo experiments challenging. UAS-zfh1 fly line is available, but ectopic expression of zfh1 in the fat body is lethal at embryonic or larval states, and even with tub-GeneSwitch-GAL4 driver only part of the offspring eclose successfully (perhaps due to leakiness of the driver) and die within a few days after replacement to Mifepristone-containing food. We therefore reasoned that the possible abnormalities in these flies’ immune response could as well result from developmental defects or tissue damage not related to Imd signaling, and could not be considered reliable. In contrast, neither C564-GAL4 nor tub-GeneSwitch-GAL4 driven zfh1 RNAi reduced flies’ viability or caused obvious phenotypic changes, and even though the
possibility of subtle developmental defects cannot be excluded, the flies’ ability to mount a normal (or elevated) immune response indicates functional fat body tissue. C564-GAL4-driven zfh1 RNAi appears to result in considerable decrease in zfh1 expression at least in larval fat bodies, but we cannot completely exclude the possibility that the remaining zfh1 molecules are able to adequately regulate the Imd response in vivo.

Alternatively, zfh1 could also be a more important repressor of Imd pathway mediated AMPs in a tissue other than fat body, and therefore the phenotype would not be clearly revealed in a systemic infection model. This kind of repression mechanism has been described for Caudal, which inhibits constitutive activation of Relish-dependent AMPs by commensal microbiota in the fly intestine (Ryu et al., 2008). On the other hand, the extremely strong knock-down phenotype observed in S2 cells could partly be due to enriched expression of zfh1 in these cells (FlyAtlas: CG1322-RB; Chintapalli et al., 2007), perhaps making it more prominent regulator of Imd signaling in S2 cells than in other cell types. Since S2 cells are derived from hemocytes, it is also possible that zfh1 has a more important role in regulating AMP expression in hemocytes than fat body cells. Knocking down zfh1 did not have an effect on viability or morphology of S2 cells, indicating that it is unlikely to cause a fundamental change in the differentiation stage or other properties of S2 cells that would make them more prone to produce antimicrobial peptides. In addition, the Toll pathway mediated Drosomycin expression was unaffected in zfh1 knock-down cells.

5. Conclusion

We have identified zfh1 as a novel negative regulator of the Drosophila Imd signaling. In S2 cells, zfh1 acts on transcriptional level. The human homologue of zfh1, ZEB1, regulates positively TNFR signaling in HeLa cells. The molecular mechanism behind the regulation of the Imd and TNFR signaling by these transcription factors remains to be studied.

Competing interests

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2012.10.007.

References


Supplementary data

Supplementary Figures

**Supplementary Fig 1.** RNAi targeting zfh1 does not decrease cell viability. In each of the luciferase assay-based experiments, S2 cells were also transfected with Actin5C-β-galactosidase reporter to enable monitoring of cell viability. Data are shown as mean +/- standard deviation, N > 6. *p < 0.05; **p < 0.005; ***p < 0.001 (Student's t-test).

**Supplementary Fig 2.** siRNA knock-down of ZEB1 blocks NF-κB-luciferase (NF-κB-luc) induction in response to TNF-α stimulation in HeLa cells. Three ZEB1 siRNAs were tested for their effect on TNF-α-mediated NF-κB-luciferase activity individually and in pairs. HeLa cells were transfected with NF-κB-luciferase and CMV-β-galactosidase reporter plamids together with indicated combinations of ZEB1 siRNAs or negative and positive control siRNAs (GFP and RelA, respectively) and treated with TNF-α for 6 h prior to cell lysis and measurement of luciferase activity. ZEB1 siRNA IDs are (1) s13883; (2) s13884 and (3) s13885. Data are shown as mean +/- standard deviation, N > 6. *p < 0.05; **p < 0.005; ***p < 0.001 (Student’s t-test).
Supplementary Fig 3. Deletion of single putative binding sites for zfh1 found on the promoter region of the AttacinA-luciferase reporter construct failed to rescue the observed zfh1 RNAi phenotype. Four E box sequences (marked Δ1–4) found on AttA promoter region were deleted or mutated. None of the deletions had an effect on either AttA-luc induction in response to constitutively active Relish (ΔSRR) or AttA-luc hyperactivation in response to treatment with zfh1 dsRNA. FL = the full-length, 2416 nt AttA promoter; Δ1 = the 1210 nt minimum promoter, where E box 1 has been deleted; Δ2–4 = 1210 nt promoters where E box sequences 2–4 have been mutated. Data are shown as mean +/- standard deviation, N = 4. *p < 0.05; **p < 0.005; ***p < 0.001 (Student’s t-test).

Supplementary Materials and Methods

AttA-luc constructs and site-directed mutagenesis
The 1210 nt minimum promoter for AttA-luc was cloned from the plasmid containing the full-length 2416 nt promoter sequence according to Busse et al., 2007. The primers used were: 5’-CACACACAGCTAGCAGATGTGTGCATACCGCG-3’ (forward) and 5’-CACACACAGCTAGCATGTTGCTGAACTG-3’ (reverse). Four putative zfh1 binding sites (E box sequences) were chosen for deletions based on Postigo et al., 1999 and Brody et al., 2008; and the sites were: site 1 at -1219 CACCTG; site 2 at -824 nt CAGATG; site 3 at -371 CAAGTG and site 4 at -77 nt CAGATG. Site 1 was deleted in the 1210 nt minimum promoter, and the other three sites were deleted by site-directed mutagenesis, which was carried out according to Zheng et al., 2004. The primers used were: Δ2: 5’-GCGAAATAACCAGGGCTGCCAGATAATCATAATGGGG-3’ (forward) and 5’-GATTTCAGCCCTCAGGGTATTTTCGCTAGAATTTAAGAAGAAG-3’ (reverse); Δ3: 5’-
GCCTGACATCCAGGAGAAATACGATAGAGAATCCC-3’ (forward) and 5’-CGTATTTCTCCTGGATGTCAGGCTTTATCAATACG-3’ (reverse); Δ4: 5’-GATCGGCAATCCGAGGAATCATGTCAATCATCAG-3’ (forward) and 5’-GACATGATTCCCGATGGGATCTCGGCTGGATG-3’ (reverse). S2 cell transfections, dsRNA treatments and luciferase measurements were carried out as described in the text.

Supplementary References


Pirk Is a Negative Regulator of the *Drosophila* Imd Pathway

Anni Kleino, Henna Myllymäki, Jenni Kallio, Leena-Maija Vanha-aho, Kaisa Oksanen, Johanna Ulvila, Dan Hultmark, Susanna Valanne, and Mika Rämet

NF-κB transcription factors are involved in evolutionarily conserved signaling pathways controlling multiple cellular processes including apoptosis and immune and inflammatory responses. Immune response of the fruit fly *Drosophila melanogaster* to Gram-negative bacteria is primarily mediated via the Imd (immune deficiency) pathway, which closely resembles the mammalian TNFR signaling pathway. Instead of cytokines, the main outcome of Imd signaling is the production of antimicrobial peptides. The pathway activity is delicately regulated. Although many of the Imd pathway components are known, the mechanisms of negative regulation are more elusive. In this study we report that a previously uncharacterized gene, *pirk*, is highly induced upon Gram-negative bacterial infection in *Drosophila* in vitro and in vivo. *pirk* encodes a cytoplasmic protein that coimmunoprecipitates with Imd and the cytoplasmic tail of peptidoglycan recognition protein LC (PGRP-LC). RNA interference-mediated down-regulation of Pirk caused Imd pathway hyperactivation upon infection with Gram-negative bacteria, while overexpression of *pirk* reduced the Imd pathway response both in vitro and in vivo. Furthermore, *pirk*-overexpressing flies were more susceptible to Gram-negative bacterial infection than wild-type flies. We conclude that *pirk* is a negative regulator of the Imd pathway. *The Journal of Immunology*, 2008, 180: 5413–5422.

Signaling mediated by members of the TNF superfamily of cytokines is involved in many cellular processes in both normal and diseased stages. Besides having anticancer potential, TNF is of crucial importance in regulating the innate and adaptive immune systems and in initiating host responses against microbial infections. Impaired TNF response is associated with recurrent bacterial infections. In contrast, excessive and inappropriate TNF signaling can lead to many pathological stages such as septic shock, rheumatoid arthritis, allergy, and asthma (1, 2).

The *Drosophila* Imd (immune deficiency) signaling pathway closely resembles the TNF signaling pathway; both share several homologous components and lead to nuclear localization of an NF-κB family transcription factor (3–5). *Drosophila* Imd signaling is triggered by recognition of Gram-negative bacteria by the receptor peptidoglycan recognition protein (PGRP)7 LC (6–8). The signal proceeds via Imd, a homologue to mammalian RIP (receptor interacting protein) (9, 10), the adaptor protein dFADD (FAS-associated death domain protein) (11), the *Drosophila* IKK (IκB kinase) complex Kenny/IrId5 (immune response deficient 5), which phosphorylates the NF-κB transcription factor Relish (12), and the caspase Dredd (death-related Ced-3/Nedd2-like protein), which is thought to cleave the C-terminal inhibitory domain of Relish (13–15). In addition, *Drosophila* Tak1 (TGF-β-activated kinase) (16), Tab2 (Tak1 binding protein 2), and Iap2 (inhibitor of apoptosis protein 2) (17–20) are required for signal transduction via the Imd pathway. Finally, the activated N-terminal part of Relish translocates to the nucleus where it binds DNA and activates the transcription of several target genes, including antimicrobial peptides, which effectively target and kill bacteria. Imd signaling is crucial for the fly’s immune defense, especially against Gram-negative bacteria.

Several genome-wide screens have identified components and positive regulators of Imd signaling (17, 18, 21). Overall, the components of Imd signaling are relatively well known and the signaling itself appears to be tightly regulated to avoid unwanted and prolonged responses (22). The duration of mammalian TNF signaling is regulated by a negative feedback loop because the activated NF-κB induces, among other target genes, the expression of its inhibitory protein IκB, which in turn can bind to NF-κB and displace it from its DNA binding sites (2). To date, the negative regulators of Imd signaling are less well known. Secreted PGRPs PGRP-LB, PGRP-SC1, and PGRP-SC2 have been reported to suppress Imd pathway response possibly by enzymatic degradation of peptidoglycan, which otherwise would trigger the pathway activation (23, 24). The membrane-bound PGRP-LF has also been reported to be immune suppressive, although the mechanism remains elusive (25). Other suggested mechanisms of down-regulation target the transcription factor Relish by proteasomal degradation (26) or by preventing its cleavage and nuclear translocation (27) or transcriptional activity by formation of a repressosome complex (28). In this study we have identified a previously uncharacterized gene called *pirk* (poor Imd response upon knock-in) among genes induced upon Gram-negative bacterial challenge. Pirk mediates Imd pathway inhibition at the level of Imd and PGRP-LC.

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9 Abbreviations used in this paper: PGRP, peptidoglycan recognition protein; AtIII, Attacin B; CecB, Cecropin B; DtpB, Diptericin B; Iap2, inhibitor of apoptosis protein 2; Imd, immune deficiency; luc, luciferase construct; Pirk, poor Imd response upon knock-in; Puc, Puckered; RNAi, RNA interference; qRT-PCR, quantitative RT-PCR; Tak1, TGF-β-activated kinase; TorM, TurnandoM.

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Materials and Methods

**Genome-wide analysis of mRNA levels in S2 cells by oligonucleotide microarrays.**

dsRNA treatments, Imd pathway induction, total RNA extractions, and oligonucleotide microarrays were performed as previously reported (20).

**Cell culture and transfections.**

S2 cells, which are *Drosophila* hemocyte-like cells, were maintained in Schneider’s insect cell culture medium (Sigma-Aldrich) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 25°C. All transfections were performed with FuGENE transfection reagent (Roche Applied Science) according to the manufacturer’s instructions.

**dsRNA treatments and luciferase assays.**

dsRNA treatments and luciferase assays for monitoring Imd and Toll pathway activities were performed as described earlier (20, 17, 29). To monitor Jak/STAT pathway activity, the cells were transfected with *TotM* (Turan-dotM)-luciferase (-lac) reporter plasmid and constitutively active hpg-scotch, which activates Jak/STAT signaling. Cell viability was assessed using *Actin5C*-galactosidase reporter transfections were performed on 24-well plates and 1 μg of dsRNA was used for each dsRNA treatment.

The *pirk* overexpression construct was created by cloning CG15078 from S2 cell cDNA to pMTV5/His-A vector (Invitrogen Life Technologies). Deletion mutants *Pirk*ΔN51, *Pirk*-C152, and *Pirk*-C152;153 were created by cloning nucleotides 1–153, 154–408, and 409–591, respectively, from *pirk* cDNA to pMTV5/His-A vector.

**Drosophila stocks.**

*C56A-GAL4* driver flies were obtained from Prof. B. Lemaire (Centre de Génétique Moléculaire, Centre National de la Recherche Scientifique, Gif-Sur-Yvette, France) and CG15078 RNAi flies were from Prof. R. Ueda (National Institute of Genetics, Mishima, Shizuoka, Japan). The *pirk* cDNA was ligated with a V5 epitope tag and cloned into a PUAST plasmid (30). Flies overexpressing *pirk* were generated by microinjecting puAST-pirk-V5 construct to w1118 background in the Umeâšý Fire and Worm Transgene Facility, Umeå, Sweden. Independent UAS-pirk-V5 transgenic fly lines used in experiments were referred to as UAS-pirk222, UAS-pirk333, UAS-pirk552, and UAS-pirk724, having the insertion in the third, X, third, and second chromosomes, respectively.

**Infection and RNA extraction from flies.**

Flies were infected by prickimg them with a tungsten needle dipped into a concentrated culture of *E. coli* [0, 1, 4, or 8 h before RNA extraction]. Triplicates of five flies (three males and two females) were snap frozen in dry ice and homogenized in TRIzol reagent (Invitrogen Life Technologies), and the total RNAs were extracted according to the manufacturer’s instructions. For survival assay, 50 flies of each line were infected as described above and their survival was monitored for 48 h at 25°C.

**Protein extraction from flies and Western blotting.**

To extract total proteins, 10 flies of each line were anesthetized, snap frozen in dry ice and homogenized in 80 μl of lysis buffer (10 mM Tris (pH 8.0), 140 mM NaCl, 1% Triton X-100, and Complete mini protease inhibitor cocktail (Roche Applied Science)). Fly extracts were incubated on ice for 45 min, centrifuged at 16,000 g for 15 min, and the protein concentration of the cleared lysates was measured using a BCA protein assay kit (Pierce). Forty micrograms of each lysate was electrophoresed in NuPAGE 10% Bis-Tris gel (Invitrogen Life Technologies), blotted on a nitrocellulose membrane, and detected by Western blotting using mouse anti-V5 primary Ab (Invitrogen Life Technologies) and goat anti-mouse Ab Horseradish Peroxidase (Molecular Probes) together with ECL Plus Western blotting detection system (GE Healthcare).

**Quantitative real-time PCR.**

Quantitative RT-PCR (qRT-PCR) was performed by either a LightCycler (Roche) or an ABI7000 (Applied Biosystems) instrument using the Quan-titect SYBR Green RT-PCR kit (Qiagen) according to the manufacturer’s instructions. Results were analyzed with the LightCycler version 3.5 software or ABR 7000 System SDS software version 1.2.3. Primers contained the following sequences: *Actin5C* (A3C5), 5′-CCAGAAGTTGTCTGGTCTG CTGG-3′ (forward) and 5′-AGACAGATCCCGTGTCGTCG-3′ (reverse); *Atb* (5′-CGTGGTCCCAACCCAGGACCC-3′ (forward) and 5′-CTCGGTCAATCCACCTC-3′ (reverse); Cede, (5′-TGTGGGACACTCA

**FIGURE 1.** *pirk* is strongly and rapidly induced after Gram-negative bacterial infection in a Relish-dependent manner in vitro and in vivo. A, Relative gene induction profiles of the genes induced >5-fold upon Gram-negative bacteria infection, 11 in total. To induce the Imd pathway, *Drosophila* S2 cells were treated with heat-killed *E. coli* for 0.5, 1, 4, 24, and 32 h. Thereafter the total RNAs were extracted and the expression level of 18,500 transcripts was measured using oligonucleotide microarrays. One hundred percent (100%) indicates the maximum induction of each gene. B, *pirk* induction is Relish-dependent in vitro. S2 cells were treated with either control (GFP) or Relish dsRNA 48 h before Imd pathway activation with heat-killed *E. coli*. Total RNAs were extracted at the 4-h time point and used in oligonucleotide microarrays. *C. pirk* induction is Relish-dependent in vivo. Wild-type (Canton S) and Relish220 flies were infected by *E. cloacae* and the expression level of *pirk* was quantified by qRT-PCR at 0, 1, 4, and 8 h after infection. Error bars represent the SD; n = 3.

**TCCTGCG-3′ (forward) and 5′-TCCAGGAGCCTGATTAGTA-3′ (reverse); Dph, 5′-GACTGCTTTGCTCCCTC-3′ and 5′-CTCTGGAGTATACTCTCC-3′ (reverse); pirk, 5′-CGATGAGCTGCTCCAC-3′ (forward) and 5′-TGTGGCCAGGTAGTACC-3′ (reverse); and puc, 5′-GACGCGGAGCCGTTAGTGC-3′ (forward) and 5′-GGCGTTGAGTATGAGCT-3′ (reverse).**

**Confocal microscopy.**

S2 cells were seeded onto 24-well plates and transfected with 0.1 μg of a modified pMTV5/GFP/V5/His plasmid (a gift from Dr. I. Klein) expressing *Pirk* as C-terminal GFP fusion protein. Pirk expression was induced 24 h later by adding CuSO4 to a final concentration of 100 μM. Forty-eight hours after induction the cells were passaged in 1:3 to 6-well plates with a coverslip on the bottom of each well. The cells were allowed to attach for 30 min after which the culture medium was removed and the cells were fixed with 3.7% formaldehyde and 5% sucrose for 20 min. The coverslips were washed three times with PBS and mounted to objective glasses with Vectashield mounting medium for fluorescence with 4′,6-diamidino-2-phenylindole (Vector Laboratories).

**Comunntropcrification and Western blotting.**

S2 cells were seeded onto 6-well plates and transfected with 0.5 μg of C-terminal V5-tagged full-length *pirk* construct or deletion construct in pMTV5/HisA vector (Invitrogen Life Technologies) and 0.5 μg of myc-tagged *imd*, *iap*, and *Tak1* or the cytoplasmic tail of PGRP-L1*23* in the same vector of which the V5 tag was replaced by myc tag. Expression of the tagged proteins was induced 24 h post-transfection by adding CuSO4 to a final concentration of 250 μM. Cells were harvested 48 h later and lysed in 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 50 mM

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Pirk IS AN Imd PATHWAY NEGATIVE REGULATOR

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FIGURE 2. Pirk is a negative regulator of the *Drosophila* Imd pathway in vitro. A. Knocking down pirk by RNAi increases Imd pathway-mediated AttA response at 4, 8 and 24 h compared with GFP controls. S2 cells were transfected with AttA-luc reporter plasmid and treated with dsRNAs, and the Imd pathway was induced 48 h later by heat-killed *E. coli*. GFP dsRNA was used as a negative control and Relish dsRNA as a positive control. B-D, Imd pathway mediated induction of antimicrobial peptide genes is increased in pirk RNAi knock-down cells. S2 cells were treated with pirk dsRNA 48 h before adding heat-killed *E. coli*, and total RNAs were extracted at 0, 1, 4, and 24 h after induction. The levels of *pirk* (*B*), *AttA* (*C*), and *DopB* (*D*) transcripts were analyzed by qRT-PCR and normalized to *Actin-5C* transcript levels. E. In vitro overexpression of pirk suppresses Imd pathway activation in S2 cells. S2 cells were transfected either with an empty vector or a pirk overexpression construct together with AttA-luc reporter. Imd pathway was activated by heat-killed *E. coli*. F and G, Pirk does not affect Toll pathway activity but decreases Jak/STAT-mediated *TolM* response. S2 cells were transfected with Drosomycin-luc (*F*) or *TolM-luc* (*G*) reporter plasmids for *Toll* and Jak/STAT pathway, respectively, and treated with dsRNAs. Luciferase activity was measured 72 h posttransfection. *Drosophila* M*yD88* (d*MyD88*) dsRNA was used as a positive control for Toll pathway and STAT dsRNA for Jak/STAT pathway. hop, hopscotch.

NaCl, 0.5% sodium deoxycholate, 20 mM NaF, 1% Nonidet-P40, 10% glycerol, 100 μg/ml PMSF, and Complete mini protease inhibitor cocktail (Roche Applied Science)). Lysate volume corresponding to 1 mg of total protein for each sample (as measured by BCA protein assay kit; Pierce) was first precleared by adding 25 μl of a 1:1 suspension of protein G-Sepharose (GE Healthcare) in lysis buffer and incubating the mixture for 50 min with rotation at 4°C. The resulting supernatants were transferred to fresh tubes. Twenty-five microliters of protein G-beads were added to 1 μg of anti-c-Myc rabbit IgG Ab. The samples were incubated overnight with rotation at 4°C and then washed with PBS containing PMSF and protease inhibitors for 4 × 10 min. Immunoprecipitates were separated from the beads by adding 25 μl of 2 × SDS loading buffer, vortexing, and incubating at 95°C for 5 min, then electrophoresing in NuPAGE 10% Bis-Tris gel (Invitrogen Life Technologies), and blotting on nitrocellulose membrane. Proteins were detected by 1/5000 diluted mouse anti-V5 or rabbit anti-c-Myc primary Ab and by goat anti-mouse or anti-rabbit Ab HRP conjugates (Invitrogen Life Technologies), respectively, and visualized by ECL Plus Western blotting detection system (GE Healthcare).

**Statistical analysis**

Statistical analysis of results was conducted using one-way ANOVA and, for survival experiments, logrank analysis. *p* < 0.05 was considered significant.

**Results**

Pirk is induced upon Gram-negative bacterial infection in vivo and in vitro in Relish-dependent manner

Antimicrobial response of *Drosophila* is delicately regulated and includes a precise temporal control. *Drosophila* Imd pathway transcription factor Relish contains both NF-κB and IκB homologous parts and is activated when the C-terminal IκB part is cleaved. We hypothesized that inhibition of the Imd signaling is mediated by a negative feedback loop. In theory, a putative negative regulator
should be induced upon microbial challenge via the Imd pathway. Previously, we have identified all genes that are induced upon Gram-negative bacterial challenge via the Imd pathway in S2 cells (29). In this study, RNAi targeting a gene of unknown function, CG15678, enhanced Imd pathway activity in vitro. To further examine the role and function of this gene, which we call pirk, we first monitored the expression kinetics of the genes induced upon heat-killed Escherichia coli treatment. According to microarray analysis, 11 genes were expressed >5-fold in 4 h in S2 cells (20). Fig. 1A shows the relative induction profiles of these genes. Most of them were steadily induced and reached their maximum induction at 24 h after bacterial challenge. Antimicrobial peptide gene CecB (Cecropin B) was an exception, reaching its maximum at 4 h. However, only pirk was rapidly induced, reaching its maximum already at 1 h postinfection and then decreasing.

To examine whether this induction is Relish-dependent, we treated S2 cells with either control (GFP) or Relish dsRNA 48 h before inducing the Imd pathway activation by E. coli and analyzed pirk expression levels at 4 h by oligonucleotide microarrays. Fig. 1B shows that pirk expression levels were abolished after

**FIGURE 3.** pirk in vivo RNAi increases Imd pathway activity. Two independent pirk in vivo RNAi fly lines were crossed to C564-GAL4 driver, and the offspring was infected with E. cloacae. Total RNAs from triplicate samples were extracted at 0, 1, 4, and 8 h after infection, and expression levels of three Imd pathway-mediated antimicrobial peptide genes, AtIIB (A), CecB (B), and DptB (C), were analyzed by qRT-PCR. w1118 was used as a wild-type control. Error bars represent SD, n = 3. Asterisks indicate statistical significance: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**FIGURE 4.** Overexpression of pirk suppresses Imd pathway activation in vivo. A, UAS-pirk-V5 transgenic fly lines express Pirk-V5 protein. Two independent lines, UAS-pirk221 and UAS-pirk432, were crossed over w1118 or C564-GAL4 driver flies, and Pirk-V5 expression of the offspring was analyzed from whole fly extracts by Western blot. B–D, Two independent UAS-pirk-V5 fly lines, UAS-pirk221 and UAS-pirk432, were crossed to C564-GAL4 driver (induced) and to w1118 (uninduced). The Imd pathway was activated with E. cloacae, and RNAs were extracted at 0 and 4 h after infection. Expression levels of AtIIB (B), CecB (C), and DptB (D) were analyzed by qRT-PCR. Error bars represent SD, n = 3. Asterisks indicate statistical significance: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Relish RNAi. To confirm this in vivo, we infected wild-type (Canton S) and Relish null mutant (Relish720) flies by septic infection with E. cloacae and monitored pirk expression levels by qRT-PCR at 0, 1, 4, and 8 h after bacterial challenge. pirk was highly induced in Canton S but not detected in Relish720 flies (Fig. 1C). These results indicate that pirk expression is Relish-dependent both in vitro and in vivo.
Pirk specifically suppresses Imd pathway activity in vitro

To study the role of Pirk in Imd pathway regulation, we used an Attacin A-luc reporter-based assay in S2 cells treated either with control (GFP dsRNA as negative and Relish dsRNA as positive control) or pirk dsRNA, together with heat-killed E. coli to activate the Imd response. Fig. 2A shows Attacin A-luc activity representing Imd pathway activation measured at 1, 4, 8, and 24 h after induction with heat-killed E. coli. Attacin A-luc activity in pirk dsRNA-treated cells was increased at all time points studied. The results were similar with Cecropin AI-luc reporter (data not shown). We also monitored expression levels of Imd pathway-dependent antimicrobial peptide genes in S2 cells treated either with control (GFP) or pirk dsRNA by quantitative RT-PCR. Again, the Imd pathway was activated by adding heat-killed E. coli to the cells. As expected, pirk expression in pirk RNAi knock-down cells was strongly decreased, indicating the effectiveness of the dsRNA treatment (Fig. 2B). Fig. 2, C and D show the relative expression levels of AntB (Attacin B) and DptB (Diptericin A), which were significantly higher in pirk dsRNA-treated cells compared with the GFP dsRNA-treated controls. In conclusion, pirk RNAi increases Imd pathway activation in vitro.

Next, we tested whether in vitro overexpression of pirk affects Imd pathway activity. S2 cells were transfected with luciferase reporter construct together with either empty or pirk overexpressing pMT vector, and the Imd pathway was activated by adding heat-killed E. coli to the cells. Relative Attacin A-luc reporter activities are shown in Fig. 2E. pirk overexpression reduced Imd pathway activation by >70%, indicating that Pirk suppresses Imd signaling in S2 cells.

To study whether Pirk action is Imd pathway specific, we tested the effect of pirk RNAi on activation of the Toll (Fig. 2F) and Jak/STAT (Fig. 2G) pathways. For analyzing the Toll pathway, S2 cells were transfected with Drosomycin-luciferase (Dros-luc) reporter and, to activate the pathway, constitutively active Toll10B together with GFP (negative control), dMyd88 (positive control), or pirk dsRNA. For Jak/STAT pathway, transfections were conducted with Toll-luc reporter together with GFP (negative control), STAT (positive control), and pirk dsRNAs, and the pathway was activated by overexpressing the Janus kinase hopscotch. pirk RNAi did not affect the activity of the Toll pathway. Similar results were obtained by activating the Toll pathway with an active form of the Spätzle ligand (data not shown). However, pirk RNAi significantly reduced Jak/STAT pathway activity. These results indicate that Pirk is not a general inhibitor of signaling cascades in S2 cells.

Pirk suppresses Imd pathway activity in vivo

To examine the effect of Pirk on Imd pathway activation in vivo, we first monitored Imd pathway-mediated, antimicrobial peptide gene expression levels in pirk in vivo in RNAi flies by qRT-PCR. Two independent UAS-RNAi lines, CG15678 R1 and -R2 were crossed to C564-GAL4 driver (31) to target pirk-RNAi construct expression to hemocytes and fat body, and Imd pathway activation in a week-old offspring was induced by septic injury with E. cloacae. w1118 flies were used as a control. Like in vitro (Fig. 2, C and D), in vivo RNAi of pirk significantly increased AttB, CecB, and DptB expression levels (Fig. 3, A–C, respectively) at the 4- and 8-h time points after Gram-negative bacterial infection.

Subsequently, we wanted to investigate whether overexpression of pirk in vivo was sufficient to reduce Imd pathway activation upon E. cloacae infection. Therefore, we generated pirk-V5-overexpressing flies using the UAS-GAL4 system (30). UAS-pirk-V5 flies from two independent lines were crossed over a C564-GAL4 driver. To ascertain that the transgenic pirk was translated to protein, we homogenized both uninduced and C564-GAL4-induced pirk transgenic flies in lysis buffer and analyzed the lysates by Western blotting using anti-V5 Ab (Fig. 4A). To study the Imd pathway response, the 1-wk-old offspring were exposed to E. cloacae by septic injury. AttB, CecB, and DptB expression levels were analyzed by qRT-PCR at 0 and 4 h postinfection (Fig. 4B-D, respectively). AttB, CecB, and DptB were highly expressed at 4 h postinfection in control flies. The expression levels were significantly lower in pirk-overexpressing flies compared with uninduced controls. This indicates that Pirk suppresses the Imd pathway activity in vivo.

Pirk is a cytoplasmic protein interacting with Imd and cytoplasmic part of PGRP-LC

Pirk is a 197-aa protein with no recognizable signal sequence or previously characterized domain structure. No Pirk homologues have been described from other species, but a blastn search of the assembled genomic sequences on the Flybase web site (flybase.bio.indiana.edu) identified likely orthologs in 18 sequenced holometabolous insect genomes. They all share a central conserved domain, but the N-terminal and C-terminal regions differ between different insect orders. The central domain, which we call the Pirk domain, shows a weakly conserved repetitive structure with three copies of an 18-aa repeat and two copies of a possibly related 12-aa repeat, referred to as A1–3 and B1–2, respectively (Fig. 5). In all cases the open reading frames were uninterrupted by introns. Additional exons may exist, but cDNA and expressed sequence tag sequences support the absence of introns in Drosophila melanogaster, Aedes aegypti, and Bombyx mori. The four different B. mori sequences probably include allelic variants of two to three paralogous genes. Curiously, we found no homologue in the Anopheles genome.

Pirk has no recognizable signal sequence and is thus expected to be an intracellular protein. To test this, we visualized the cellular localization of an expressed GFP-Pirk fusion protein using confocal microscopy. GFP-Pirk was detected as green dots in the cytoplasm outside the blue 4',6-diamidino-2-phenylindole (DAPI)-stained nucleus, as shown in Fig. 6A. Treatment of GFP-Pirk-expressing S2 cells with E. coli did not affect Pirk’s localization (data not shown). Pirk was also detected in cell extracts but not in the cell culture medium by Western blotting (data not shown). These results imply that Pirk is a cytoplasmic protein.

To study the mechanism of Pirk-mediated inhibition of Imd signaling, we conducted epistasis analysis using the active form of Relish. S2 cells were transfected with Rel ΔS29–45 and CuSO4-induced pirk construct together with Attacin A-luc reporter construct to see whether overexpression of Pirk affected Relish-induced Imd pathway activity (Attacin A-luc expression). No effect was detected, indicating that Pirk is located upstream of Relish in the Imd signaling cascade (Fig. 6B).

The Drosophila Imd pathway also triggers an immediate response via JNK pathway, because the JNK pathway branches from the Imd signaling cascade at the level of Tak1 (22, 41). To find out whether Pirk suppresses the Imd pathway activity upstream of Tak1, we analyzed the effect of pirk RNAi on JNK pathway activity. This was performed by measuring the expression levels of a JNK pathway target gene, puc (Pucker), by qRT-PCR. pirk RNAi increased puc expression in S2 cells (Fig. 6C), indicating that Pirk negatively regulates the JNK pathway-mediated response as well. This suggests that Pirk suppresses the Imd pathway activity at the level of or upstream of Tak1.

To determine the mechanism for Pirk-mediated Imd pathway suppression, we communoprecipitated V5-tagged Pirk with the
Myc-tagged Imd pathway components lap2, Tak1, Imd, or the cytoplasmic tail of PGRP-LC (LC(M1–V293)) in S2 cells. As shown in Fig. 6D, Pirk coimmunoprecipitated with Imd and the cytoplasmic part of PGRP-LC. In addition, a faint band was visible in lap2 samples, but not in Tak1 samples. These results imply that Pirk is likely to interact with Imd and the cytoplasmic tail of PGRP-LC and either directly or indirectly with lap2, but not with Tak1. Therefore, it is likely that Pirk suppresses Drosophila Imd signaling by interacting directly with Imd and the cytoplasmic part of PGRP-LC.

To gain more mechanistic insight into the interactions between Pirk, Imd, and the cytoplasmic tail of PGRP-LC, we created three Pirk deletion constructs based on the predicted protein structure described in Fig. 5A and tested whether they coimmunoprecipitated with Imd and PGRP-LC. At first, the expression of these deletion mutants was confirmed by Western blotting (data not shown). The N-terminal part of Pirk, Pirk(M1–K51), coimmunoprecipitated neither with Imd nor the PGRP-LC cytoplasmic tail (data not shown). Pirk(D52–V136) encoding the Pirk domain coimmunoprecipitated with Imd (Fig. 7A), whereas the C-terminal part, Pirk(V137–4197), coimmunoprecipitated with cytoplasmic part of PGRP-LC (Fig. 7B). In addition, a weak band was detected when Pirk(D52–V136) coimmunoprecipitated with the cytoplasmic part of PGRP-LC and when Pirk(V137–4197) coimmunoprecipitated with Imd. To test whether these deletion constructs inhibited the inhibitory action of Pirk, we monitored Imd pathway activity in S2 cells overexpressing Pirk or Pirk deletion mutants (Pirk(M1–K51), Pirk(D52–V136), and Pirk(V137–4197)) by luciferase assay (Fig. 7C). The N-terminal part of Pirk had no effect on Imd pathway activity. However, both the Pirk domain and the C-terminal part of Pirk abolished Imd pathway activation. These results implicate that Pirk interacts with Imd primarily via the Pirk domain and with the cytoplasmic tail of PGRP-LC via the C-terminal domain. Each of these domains is capable of suppressing the Imd pathway activation.

**Discussion**

A malfunctioning immune system can cause severe damage to the target tissue. NF-κB signaling is of paramount importance in regulating the immune response and thus must be delicately controlled. In mammals, NF-κB activation is regulated by posttranslational modifications of NF-κB and other pathway proteins. The primary NF-κB inhibitors, however, are 1κB proteins, which are expressed in a tissue-specific manner and have different affinities to individual NF-κB protein complexes. The Drosophila 1κB homologue Cactus (42) inhibits Toll pathway by binding the NF-κB proteins Dif (Dorsal-related immunity factor) and Dorsal. However, the Imd pathway transcription factor Relish is inactive until it is cleaved. Still, signaling events leading to Relish activation must be carefully regulated, which implies that there are other 1κB-independent regulatory mechanisms.

In Drosophila, Imd pathway activation is triggered by diaminopimelic acid (DAP)-type peptidoglycan (43), which binds to the receptor PGRP-LC (44). Recent studies implicate that one way of regulating the Imd pathway activity is to dampen the initial stimulus. The extracellular proteins PGRP-LB, PGRP-SC1, and PGRP-SC2 are amidases, which are thought to degrade peptidoglycan and thus act as detoxifying proteins (23, 24). This type of regulation may be especially important when the fly, during all developmental stages, has to adapt to the presence of commensal bacteria in the gut without evoking massive production of antimicrobial peptides or activating apoptosis pathways. Besides the extracellular PGRPs, the membrane-bound PGRP-LF has also been reported to play an immune-suppressing role (45, 25), but the mechanism of this action is still unknown. Another suggested regulatory mechanism of the Imd pathway is proteasomal degradation of either Relish or proteins involved in Relish activation in an SCF (Skp, Cullin, F-box) complex-dependent manner (26). Others have reported that Caspar, the *Drosophila* FAF1 (Fas associated factor 1) homologue, would suppress Imd pathway activation by preventing Relish cleavage (27). Furthermore, a recent report implies that the active form of Relish, REL-68, can be displaced from the promoter region by a repressor complex formed by JNK and the JAK/STAT pathway transcription factors DAP-1 and Stat92E together with a high mobility group protein Dsg1 (Dorsal switch protein 1), consequently leading to termination of transcription (28).

In this study we report a novel negative regulatory protein of the Imd pathway that acts on a different level than the previously characterized negative regulators. Pirk is rapidly induced upon the activation of Imd signaling and is likely to mediate its action via direct interaction with Imd and the cytoplasmic part of PGRP-LC. Whereas all the known intracellular components of the Imd pathway are conserved from flies to man, the Pirk sequence is not that highly conserved. We were unable to identify any clear homologues outside the holometabolous insects. There is a putative domain structure (Fig. 5), referred to as the Pirk domain, at the central part of the protein that may be present in other species.
FIGURE 6. Pirk is a cytoplasmic protein that coimmunoprecipitates with Imd and the cytoplasmic part of PGRP-LC. A, Pirk is localized in the cytoplasm. S2 cells were transfected with GFP-Pirk fusion protein and GFP-Pirk expression was thereafter analyzed using confocal microscopy. B, Pirk is located upstream of Relish in Imd signaling. Pirk overexpression has no effect on Relish-induced Imd pathway activity. The Imd pathway was activated by overexpressing the active form of Relish (RεΔ29–45), and pirk was expressed under a CuSO₄-responsive promoter. Pathway activity was measured using AtxA-luc reporter. C, Pirk negatively regulates JNK pathway. Knocking down pirk increases JNK pathway-mediated expression of puc. S2 cells were treated with pirk dsRNA, JNK signaling was activated with heat-killed E. coli, and puc expression levels were measured by qRT-PCR at the indicated time points. D, Pirk coimmunoprecipitates with Imd and the cytoplasmic tail of PGRP-LC. S2 cells were transfected with myc-tagged constructs of imd, lap2, Tak1, or the cytoplasmic tail of PGRP-LC (LC(351–493)) and V5-tagged pirk. Immunoprecipitation (IP) was done with anti-Myc (α myc) Ab and immunoblotting (IB) with anti-V5 (α V5) Ab. Asterisk at the uppermost panel indicates a non-specific band.

Determining whether any functionally or structurally related proteins exist in mammals may require solving of the three-dimensional structure of Pirk. Of note, the receptors of Imd and TNFR signaling pathways are not conserved. Therefore, the interaction of Pirk with PGRP-LC may explain why Pirk is not highly conserved from insects to mammals.

FIGURE 7. Inhibitory action of Pirk is mediated by the Pirk domain and the C-terminal region of Pirk. A and B, Pirk domain coimmunoprecipitates with Imd and C-terminal region of Pirk with cytoplasmic tail of PGRP-LC. In addition, a faint signal is seen when the Pirk domain is immunoprecipitated with PGRP-LC cytoplasmic tail and the C-terminal region of Pirk is immunoprecipitated with Imd. S2 cells were transfected either with V5-tagged Pirk domain encoding PirkD352-V436 construct (A) or Pirk C-terminal region encoding PirkV157–4197 construct (B), and myc-tagged imd or myc-tagged cytoplasmic part of PGRP-LC (LC(351–493)). Immunoprecipitation (IP) was performed using anti-Myc (α myc) Ab, and immunoblotting (IB) with anti-V5 (α V5) V5 Ab. C, Pirk domain and the Pirk C-terminal region strongly inhibit Imd signaling. S2 cells were transfected either with an empty vector, full-length pirk, or deletion constructs (PirkΔK31, PirkΔD352-V436, and PirkΔV157–4197) together with an AtxA-luc reporter. Imd pathway was activated by heat-killed E. coli for 24 h.

FIGURE 8. Pirk-overexpressing flies are susceptible to E. cloacae infection. Three independent UAS-pirk-V5 lines (UAS-pirk224, UAS-pirk265, and UAS-pirk42) were crossed over C564-GAL4 (induced) or w1118 (un-induced), and the survival of the offspring was monitored after septic injury by E. cloacae. C564-GAL4 over w1118 and Relish220 flies were used as negative and positive controls, respectively.
FIGURE 9. Schematic representation of Imd pathway regulation. Expression of *pik* is rapidly activated Relish dependently after infection by Gram-negative bacteria. We hypothesize that Pirk down-regulates the Imd pathway and the cytoplasmatic part of PGRP-PC via the C-terminal region. Curiously, the domain mediating the interaction between PGRP-PC and Imd is not required for signaling (10, 46), which raises a question of whether there still are unidentified components or adaptor proteins essential for signal transduction. Furthermore, the domain of Imd, which mediates interaction with the Pirk domain, is currently not known. Whether a similar regulatory mechanism is applied in mammalian TNFR signaling remains to be elucidated.

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Disclosures
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References
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