ANNI KLEINO

The Imd Pathway-mediated Immune Response in *Drosophila*

ACADEMIC DISSERTATION
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To my family

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List of original communications

The thesis is based on the following original publications, which are referred to accordingly by their roman numerals:

I Valanne S, **Kleino A**, Myllymäki H, Rämet M. Iap2 is required for a sustained response in the *Drosophila* Imd pathway. *Dev Comp Immunol* 31:991-1001 (2007).

II **Kleino A**, Myllymäki H, Kallio J, Vanha-aho L-M, Oksanen K, Ulvila J, Hultmark D, Valanne S, Rämet M. Pirk is a negative regulator of the *Drosophila* Imd pathway. *J Immunol* 180:5413-5422 (2008).

III **Kleino A**[1], Valanne S[1], Ulvila J, Kallio J, Myllymäki H, Enwald H, Stöven S, Poidevin M, Ueda R, Hultmark D, Lemaitre B, Rämet M. Inhibitor of apoptosis 2 and TAK1-binding protein are components of the *Drosophila* Imd pathway. *EMBO J* 24:3423-3434 (2005).[2]

[1] Equal contribution.
[2] The publication No III was also used in the doctoral thesis of Johanna Ulvila.

Abbreviations

aa	amino acid
Ab	antibody
AMP	antimicrobial peptide
Att	Attacin
BIR	baculovirus IAP repeat
Bc	black cells
bp	base pair
bsk	basket, *Drosophila* JNK
CARD	caspase recruitment domain
Cec	Cecropin
cDNA	complementary DNA
C-terminal	carboxy-terminal
da	daughterless
DAP	diaminopimelic acid
DD	death domain
Dif	Dorsal-related immunity factor
DNA	deoxyribonucleic acid
Dnr1	Defence repressor 1
Dpt	Diptericin
Dredd	Death-related ced-3/Nedd2-like protein
Dro	Drosocin
Drs	Drosomycin
Dscam	Down syndrome cell adhesion molecule
dsRNA	double-stranded RNA
EGF	epidermal growth factor
EMS	ethane methyl sulfonate
EtOH	ethanol
FADD	Fas associated death domain
FAF1	Fas associated factor 1
FBS	fetal bovine serum
GFP	green fluorescent protein
GNBP	Gram-negative bacteria binding protein
Hep	hemipterous
His	histidine
Hsp	heat-shock protein
HMG	high mobility group
HRP	horse radish peroxidase

Iap	inhibitor of apoptosis
IκB	inhibitor of κB
IKK	IκB kinase
Imd	immune deficiency
IRC	immune responsive catalase
Ird5	immune response deficient 5
Jak	Janus kinase
JNK	Jun kinase
Jra	Jun-related antigen
kay	kayak, *Drosophila* Fos
kb	kilobase
key	kenny, *Drosophila* IKKγ
LB	Luria Bertani broth
lola	longitudinals lacking
LPS	lipopolysaccharide
luc	luciferase
MAMP	microbe-associated molecular pattern
MAPK	mitogen activated kinase
mbn-2	malignant blood neoplasm-2
Mmp1	matrix metalloproteinase 1
MPAC	maturated pro-domain of Attacin C
mRNA	messenger RNA
Mtk	Metchnikowin
MyD88	myeloid differentiation factor 88
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
nt	nucleotide
N-terminal	amino-terminal
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
Pirk	poor Imd response upon knock-in
PLB	passive lysis buffer
PO	phenoloxidase
PRR	pattern recognition receptor
Psh	Persephone
puc	puckered, the *Drosophila* Jun kinase phosphatase
qRT-PCR	quantitative reverse transcription PCR
RHD	Rel homology domain
RHIM	RIP homotypic interaction
RIP	receptor interacting protein
RING	really interesting new gene
RISC	RNA-induced silencing complex

RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
S2	Schneider-2
SCF	Skp1/Cullin/F-box
SH3	Src homology 3
shRNA	short hairpin RNA
siRNA	small interfering RNA
SODD	silencer of death domain
SPE	spätzle processing enzyme
Sr-CI	scavenger receptor class C, type I
STAT	signal transducer and activator of transcription
Tab2	Tak1-associated binding protein 2
Tak1	TGF-β -activated kinase 1
TCT	tracheal cytotoxin
TEP	thioester-containing protein
TGF	transforming growth factor
TIR	Toll/interleukin-1 receptor
th	thread, *Drosophila* gene encoding Iap1
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
Tot	Turandot
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor
UAS	upstream activating sequence
Upd-3	unpaired-3
USP	ubiquitin-specific protease
UTR	untranslated region
WntD	wnt inhibitor of Dorsal
ZnF	zinc finger

Abstract

Innate immunity is the first line of defense against microbes and it is indispensable in preventing infections as well as in the development and regulation of the adaptive immune system. Innate immunity is based on the ability of genome-encoded proteins to recognize and bind microbial surface structures, which is followed by the activation of the innate immune response via various cell signaling pathways.

Tumor necrosis factor receptor (TNFR) signaling and cytokine release are strictly regulated and essential for a normal immune response. However, in certain diseases, like infections and autoimmune diseases, cytokines are produced in excess, which prolongs the inflammation and causes tissue damage. Clinical medicine is trying to prevent cytokine overproduction by suppressing TNFR signaling. However, this is challenging, since despite the extensive research carried out in this field in recent years, the mechanisms and regulation of TNFR signaling are not thoroughly understood.

The pathways of innate immunity signaling are evolutionarily conserved from insects to humans. Unlike mammals, insects have no adaptive immunity, and hence they are completely dependent on their innate immune response. For these reasons the fruit fly (*Drosophila melanogaster*) is a suitable model organism for studying innate immunity. In *Drosophila*, the immune response to Gram-negative bacteria is mediated mainly via the Imd (immune deficiency) signaling pathway, whose intracellular parts resemble the mammalian TNFR signaling pathway. The aim of this research project was to identify and molecularly characterize the components of the Imd pathway and regulatory proteins using a large-scale RNA interference (RNAi) screen. The function and importance of three of the identified regulators, namely Tak1-associated binding protein 2 (Tab2), Inhibitor of apoptosis 2 (Iap2), and Poor Imd response upon knock-in (Pirk), were then further assessed. Using *Drosophila* S2 cells we showed that Tab2 is essential for both the early and sustained immune responses, while Iap2 mainly regulates the sustained immune response. In addition, we discovered that when Iap2 was removed from fruit flies by *in vivo* RNAi the flies became susceptible to Gram-negative bacterial infections.

Pirk was a previously unknown protein that we demonstrated could suppress the Imd pathway activity both in S2 cells and in flies. The inhibitory action of Pirk was shown to be efficient enough to sensitize the flies to Gram-negative bacterial infections. We found that Pirk interacts with the receptor of the Imd pathway, PGRP-LC (Peptidoglycan recognition protein LC), and the down-

stream component IMD. However, the elucidation of the exact mechanism of Pirk action requires further studies.

The present study demonstrates that Imd signaling is strictly regulated and more complex than was previously thought. *Drosophila* as a model organism provides tools to efficiently study innate immunity. In addition, the results gained from this research in flies can provide new perspectives and may help understand also the mechanisms of signaling in the mammalian innate immune system.

Tiivistelmä

Synnynnäinen immuniteetti on elimistön ensimmäinen puolustusjärjestelmä taudinaiheuttajia vastaan. Se on välttämätön niin infektioiden ehkäisyssä kuin hankitun immuunivasteen kehityksessä ja säätelyssä. Synnynnäinen immuniteetti perustuu siihen, että perimän koodaamat proteiinit kykenevät tunnistamaan erilaisia mikrobien pintarakenteita ja välittämään tästä tiedon soluille signalointireittejä pitkin. TNFR (tuumorinekroositekijäreseptori) -signalointireitti ja sen käynnistämä sytokiinieritys ovat tarkkaan säädeltyjä ja välttämättömiä normaalille immuunivasteelle. Kuitenkin tietyissä sairauksissa, kuten infektioissa ja autoimmuunitaudeissa, liian voimakas sytokiinituotanto ylläpitää tulehdusta ja aiheuttaa kudostuhoa. Liiallista sytokiinien tuotantoa voidaan estää TNFR-signalointia hillitsemällä. Tämä on kuitenkin haastavaa, sillä huolimatta alalla viime vuosien aikana tehdystä tutkimuksesta, signalointimekanismeja ja niiden säätelyä ei vielä täysin tunneta.

Synnynnäisen immuniteetin signalointireitit ovat säilyneet evoluutiossa hyönteisistä ihmisiin. Hyönteisillä ei nisäkkäistä poiketen ole lainkaan hankittua immuniteettia, vaan ne ovat täysin riippuvaisia synnynnäisestä immuniteetistaan. Näistä syistä banaanikärpänen (*Drosophila melanogaster*) sopii hyvin mallieläimeksi tutkittaessa synnynnäisen immuniteetin toimintaa. Banaanikärpäsellä immuunivaste Gram-negatiivisia bakteereita kohtaan välittyy Imd-signalointireitin kautta, joka solunsisäisiltä osiltaan vastaa nisäkkäiden TNFR-signalointireittiä. Tässä tutkimusprojektissa pyrimme RNAi (RNA-häirintä)-seulaa käyttäen tunnistamaan Imd (immune deficiency)-signalointireittiin kuuluvia ja sen säätelyyn osallistuvia proteiineja. Näistä kolmen uuden säätelijän, Tab2:n (Tak1-associated binding protein 2), Iap2:n (Inhibitor of apoptosis 2) ja Pirkin (Poor Imd response upon knock-in), toimintaa ja merkitystä banaanikärpäsen Imd-signaloinnille selvitettiin tarkemmin. Osoitimme solumallia apuna käyttäen, että Tab2 on välttämätön niin välittömälle kuin pitkäkestoiselle immuunivasteelle, kun taas Iap2 osallistuu vain pitkäkestoisen immuunivasteen säätelyyn. Lisäksi havaitsimme, että banaanikärpäset, joilta Iap2 on poistettu *in vivo* RNAi-menetelmää käyttäen, ovat herkkiä Gram-negatiivisten bakteerien aiheuttamille infektioille.

Pirk on aiemmin tuntematon proteiini, jonka osoitimme hillitsevän Imd signalointia sekä solumallissa että elävissä kärpäsissä. Pirkin vaikutus oli niin tehokas, että geenin yli-ilmentäminen kärpäsissä riitti herkistämään ne Gram-negatiivisten bakteerien aiheuttamille infektioille. Havaitsimme, että Pirk vuorovaikuttaa Imd-signalointireitin reseptorin, PGRP-LC:n (Peptidoglykaania

tunnistava proteiini LC), ja IMD:n kanssa. Sen toiminnan tarkkaa mekanismia ei kuitenkaan vielä tunneta.

Tehty tutkimus osoittaa, että Imd signalointi on tarkkaan säädeltyä ja aiempaa luultua monimutkaisempaa. Banaanikärpänen mallieläimenä tarjoaa mahdollisuuden tutkia tehokkaasti synnynnäisen immuniteetin säätelyä. Tutkimuksesta saadut tulokset tuovat uusia näkökulmia myös nisäkkäiden immuunisignaloinnin toiminnan selvittämiseen.

1. Introduction

The number of microorganisms in the human gut alone is approximately ten times the number of cells within the entire human body. Most of the microbes that accompany us throughout our lives are harmless commensals, or live in mutualistic relationship with us. But some species are harmful, and these we refer to as pathogens. To combat pathogens, organisms have developed efficient host defense systems that involve recognition of the microbe, a signaling machinery to activate the immune response, and a battery of arms to kill the infectious agent. Yet the immune system needs continuous balancing and regulation, because both over-responsiveness and non-responsiveness to microbes may lead to detrimental outcomes. In addition, maintaining immunity is costly. Therefore, the host must adjust and target its resources correctly to avoid unnecessary loss of precious energy that otherwise could be used to promote growth or reproduction.

From the microbe's perspective the host is a rather hostile environment, although rich in nutrients. To be able to survive, the microbe must find its own niche within the host and escape the host's immune system. Microbes achieve this by either avoiding detection or by suppressing the host's innate immune system. This requires evolution and adaptation from the microbe, but is usually followed by co-evolution of the host: microbial adaptation creates selective pressure for the host to re-establish and improve the immune system once again. Hence, host-pathogen interactions are dynamic, and often involve multiple aspects of immune defence strategies. Investigating these interactions requires a global view on the organism's responses. Due to the ethical problems related to carrying out the experiments in mammals, various non-mammalian model organisms are commonly used. Studies have revealed similarities in host defense mechanisms between species as well as in the strategies microbes use to avoid and survive these lines of defense.

This study used the well-characterized and genetically tractable model organism *Drosophila melanogaster* to study innate immune signaling and signal regulation from the host's perspective. The main focus was on the *Drosophila* Imd pathway. The Imd pathway is evolutionarily conserved and resembles the mammalian TNFR signaling pathway, which is implicated in inflammatory responses in mammals. By examining the Imd signaling in the *Drosophila* system, we hope to reveal new perspectives of host defense strategies also in mammals.

2. Review of the literature

2.1 Innate immunity

The human body provides a number of environmental niches for microbes to colonize in. The skin, respiratory tract, urogenital tract, and especially the intestine are crowded with various microorganisms that we are, under normal condition, happily unaware of. However, the same microbes contribute to the decay of our body after we have died. To prevent this from happening prematurely we need an efficient immune system to restrict the proliferation of commensal microbes, destroy the pathogenic ones, and deny their access to the parts of the body that need to be preserved sterile; in other words, to keep us alive. The epithelia and mucosa form a physical barrier between us and the environment. Proteolytic enzymes, low pH, reactive oxygen species (ROS) and the production of antimicrobial peptides (AMPs) limit microbial growth in our body. However, for more sophisticated defense mechanisms, the immune system needs to recognize putative pathogens by distinguishing self from non-self, and elicit an adjusted response against the detected target. In mammals, this involves both the innate and adaptive components of the immune system. This thesis focuses on innate immunity.

The innate immune response has ancient origins and is present in all eukaryotes from unicellular organisms to plants, invertebrates and vertebrates. It is based on the ability of genome-encoded proteins to recognize and bind microbial structures. These pattern recognition receptors (PRRs) were first described by Charles Janeway Jr. in 1989, when he presented his famous hypothesis in the Cold Spring Harbor Symposium on Immune Recognition (Janeway 1989). According to this theory, microbial structures are recognized by the PRRs of the antigen-precenting cells (APCs), which in turn signal the presence of non-self and stimulate lymphocyte activation. After 20 years of research and the identification of various PRRs and signaling molecules that transfer the message from APCs to the cells of adaptive immunity, we can now admire how accurate this hypothesis was (Medzhitov 2009).

PRRs are unique in their ability to bind a broad range of molecules that have a common structural motif referred to as pathogen associated molecular patterns (PAMPs) (Janeway 1989) or microbe associated molecular patterns (MAMPS). The detected molecules usually fulfil the following criteria: they are constantly present in certain types of microbes but not in host cells allowing the discrimination between self and non-self, and they play essential roles in

microbe physiology, which limits the possibility of the microbes to escape detection through the adaptive evolution of these molecules. MAMPs are therefore often structural components of the cell wall, such as peptidoglycan (PGN), lipopolysaccharide (LPS), lipoteichoic acid, and lipoproteins from bacteria, and β-glucan of the fungal cell wall. Viral particles are synthesized within the host cells and therefore can not be distinguished as non-self similarly to bacterial or fungal components. Viruses are therefore recognized on the basis of differences between host and viral DNA or RNA as well as the subcellular localization of these nucleic acids. A variety of PRRs have been identified so far, the Toll-like receptors (TLRs), C-type lectins and proteins of the NOD (nucleotide-binding oligomerization domain) family probably being the best characterized ones (Medzhitov 2007).

TLRs bind a large array of MAMPs and induce inflammatory and antimicrobial responses. They also activate macrophages, which in turn produce pro-inflammatory cytokines including tumour necrosis factor (TNF) and interleukins IL-1β and IL-6. These molecules trigger specific cell signaling pathways to activate the local and systemic inflammatory responses. The responses launch local coagulation cascades to prevent microbial dissemination via the blood circulation system and increase the permeability of blood vessels thus facilitating the recruitment of leukocytes and serum proteins to the site of infection. Furthermore, the acute phase proteins produced in hepatocytes activate complement and opsonize pathogens to be phagocytosed by neutrophils and macrophages. Dectin 1 is a transmembrane receptor present on dendritic cells and macrophages, and it belongs to the group of C-type lectins. Dectin 1 binds β-glucan and has therefore an important function in antifungal defense, being involved in the phagocytosis of fungal cells and the production of reactive oxygen species (ROS) and cytokines. NODs are responsible for PGN recognition in vertebrates, and initiate signaling pathways that regulate the production of pro-inflammatory cytokines and chemokines. In addition, NOD signaling recruits neutrophils to the site of infection and contributes to the initiation of adaptive immune responses (Hargreaves and Medzhitov 2005, Medzhitov 2007, Medzhitov 2009, Iwasaki and Medzhitov 2010).

Since PRRs themselves can not distinguish a pathogen from a symbiotic or commensal microbe, adjusting the innate immune response adequately requires delicate balancing via multiple cell signaling pathways. Many of these signaling pathways involve the activation of NF-κB transcription factors. The next chapters will provide a simplistic view on the regulatory mechanisms associated with these complex pathways.

2.2 NF-κB signaling cascades

NF-κB is a transcription factor that regulates the expression of multiple target genes involved in various cellular processes including the immune system

(Ghosh et al. 1998, Li and Verma 2002). In mammals, the NF-κB protein family consists of five related transcription factors, p50, p52, p65 or RelA, c-Rel and RelB (Moynagh 2005, Perkins 2007). Of these, p50 and p52 are derived from the larger precursor proteins p105 and p100, respectively. What is common to all these proteins is the N-terminal DNA-binding domain called the Rel homology domain (RHD), which mediates dimerization and binding of the proteins to their DNA target sequence at the promoter region of the genes whose expression they modulate. The transcriptional activity of NF-κB is regulated by the composition of the homo- or heterodimer, as well as interactions with inhibitory proteins called IκBs. Binding of IκB to NF-κB suppresses the transcription activity by preventing the nuclear translocation of NF-κB (Li and Verma 2002, Perkins 2007).

NF-κB signaling pathways are classified in two categories: the canonical and non-canonical pathways. In both pathways the fundamental steps in signal transduction are approximately the same. In the canonical pathway, ligand binding to a cell surface receptor leads to the recruitment of adaptor proteins to the cytoplasmic domain of the receptor. These adaptor proteins initiate signaling steps that activate the IκB kinase (IKK) complex. The IKK complex consists of the catalytic kinase subunits IKKα and IKKβ, and a regulatory subunit IKKγ, also called NEMO. The IKK complex phosphorylates the NF-κB inhibitor IκB, which leads to degradation of IκB and the release of NF-κB, which in turn can translocate into the nucleus and initiate the transcription of target genes (Gilmore 2006, Perkins 2007). IκB is one of these genes, providing a negative feed-back loop for the regulation of the pathway's activity.

While the canonical pathway activates NF-κB dimers consisting of RelA, RelB, c-Rel, and p50, the non-canonical pathway activates p100/RelB complexes. Signaling via the p100/RelB complex is implicated in the generation of B and T lymphocytes during the development of the lymphatic system, and triggered by a relatively small group of cell-differentiating stimuli, such as lymphotoxins and B cell activating factor (BAFF). The signal is propagated via NF-κB-inducing kinase (NIK) to the IKK complex, which in this case comprises of two IKKα subunits and lacks the regulatory subunit IKKγ. The IKKα complex then activates p100. p100 is cleaved into its active form p52, which dimerizes with RelB and translocates into the nucleus (Hayden and Ghosh 2004, Moynagh 2005). A simplified model of NF-κB signaling is depicted in Figure 1.

NF-κB signaling regulates the expression of pro-inflammatory cytokines, chemokines and other inflammatory molecules. It has been implicated in various autoimmune diseases as well as in cancer. Multiple upstream cell signaling pathways activate NF-κB signaling, one of them being the TNFR signaling pathway, which will be described next.

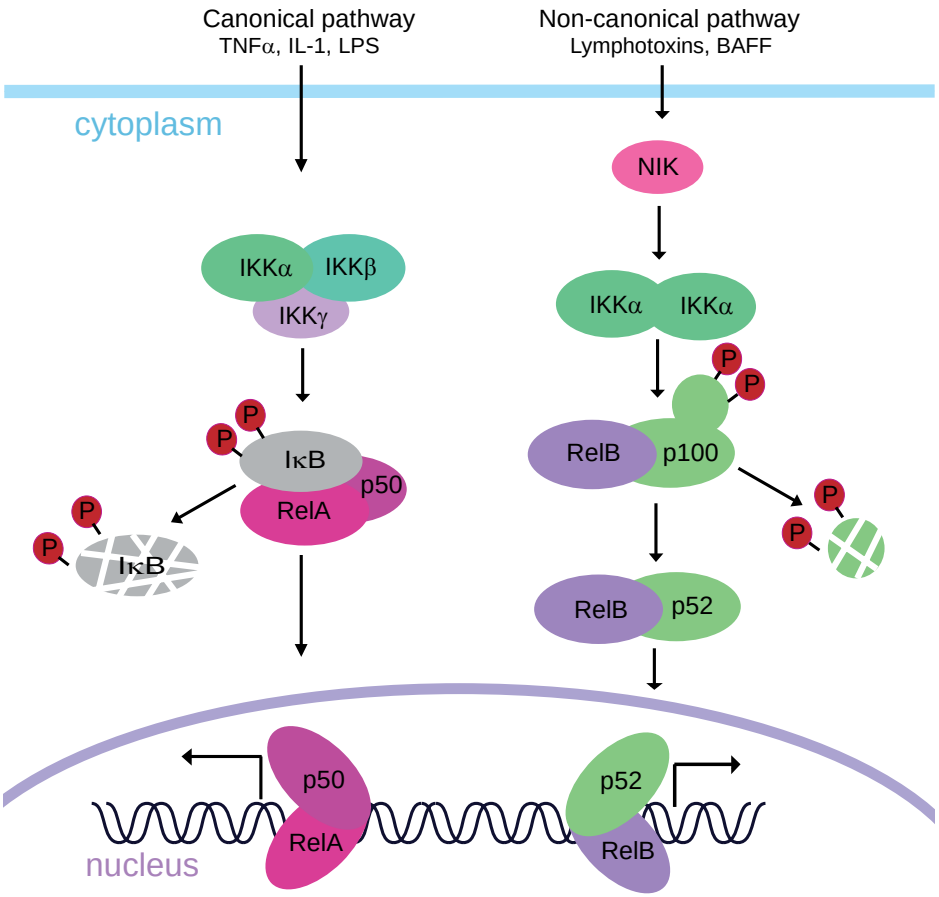

Figure 1. NF-κB signaling.

2.2.1 TNFR signaling

For more than a century it had been known that malignant tumors are often reduced in size due to bacterial infections. However, the molecule responsible for the tumor suppressing activity, tumor necrosis factor (TNF), was not discovered until 1984 (Pennica et al. 1984). Besides inducing apoptosis in certain tumor cells, TNF is involved in immune signaling, and necessary for a normal immune response. Disturbances in TNF production or signal regulation may have severe consequences, and TNFR signaling has been implicated in a large variety of human diseases, including sepsis, diabetes, cancer, and autoimmune diseases. Due to their importance to human health, TNF and TNFR signaling have been intensively studied, and many aspects of the signal regulation have already been unveiled. The signaling has turned out to be very complex, not only because there are multiple TNF receptors and partially redundant signaling pathway components that are activated in a tissue and cell-specific manner, but furthermore because TNFR signaling branches into other signaling pathways, and the sequential activation of these is not thoroughly understood. The TNFR1

signaling cascade described here is therefore a very simplified overview of the complex signaling regulatory networks that are triggered in response to TNF.

TNF is a cytokine that trimerizes and binds its transmembrane receptor TNFR on the plasma membrane. The binding releases a protein called SODD (silencer of death domain) from the intracellular domain of TNFR, which in turn leads to the recruitment of TRADD (TNF receptor associated death domain), RIP (receptor interacting protein), TRAF2, and FADD to the signaling complex (Hehlgans and Pfeffer 2005). From here, the pathway branches into three cascades: apoptosis signaling, mitogen activated protein kinase kinase kinase (MAPKKK) signaling, and NF-κB signaling. Apoptosis signaling is initiated by the FADD-mediated recruitment of caspase-8 to the TNFR signaling complex. Caspase-8 is thereafter activated, possibly by self-cleavage, which triggers a protease cascade leading to apoptosis. Inhibitor of apoptosis proteins c-Iap1 and c-Iap2, which also have E3 ubiquitin ligase activity, are recruited by TRAF2, and regulate the caspase-mediated apoptosis signaling. TRAF2 also signals to MAPKKKs, such as MEKK1 or ASK1. These kinases initiate kinase signaling cascades, which in turn result in JNK pathway activation. The third arm of TNFR signaling leads to the activation of NF-κB, and this requires RIP. The signal is propagated to the IKK complex, which consists of two catalytic subunits (IKKα, IKKβ) and the regulatory subunit IKKγ. In addition, two chaperone proteins Cdc37 and Hsp90 are involved in the regulation of the subcellular localization of the IKK complex. The IKK complex phosphorylates IκB, which releases NF-κB that sequentially translocates to the nucleus and initiates transcription of its target genes, such as inflammatory cytokines (Chen and Goeddel 2002). The TNFR1 signaling pathway is illustrated in Figure 2.

In mammals, research on TNFR signaling is often conducted in continuous cell lines or mouse-derived cells *ex vivo*, since many of the TNFR pathway mutants induce lethality. To gain an organismal view on the signaling and to avoid the compensatory effects of adaptive immunity, non-mammalian model organisms have been used. Innate immune signaling pathways are conserved throughout evolution from insects to mammals. Therefore it is not surprising that also the mammalian TNFR signaling cascade has its counterpart in *Drosophila*. Despite the receptor itself being different in flies, the intracellular components of the *Drosophila* Imd (immune deficiency) signaling pathway share striking similarities with TNFR pathway components. Although research performed in flies cannot be directly extrapolated to humans, understanding the signaling in *Drosophila* may still broaden our minds and fuel new ideas for how the signaling regulation works in the mammalian system.

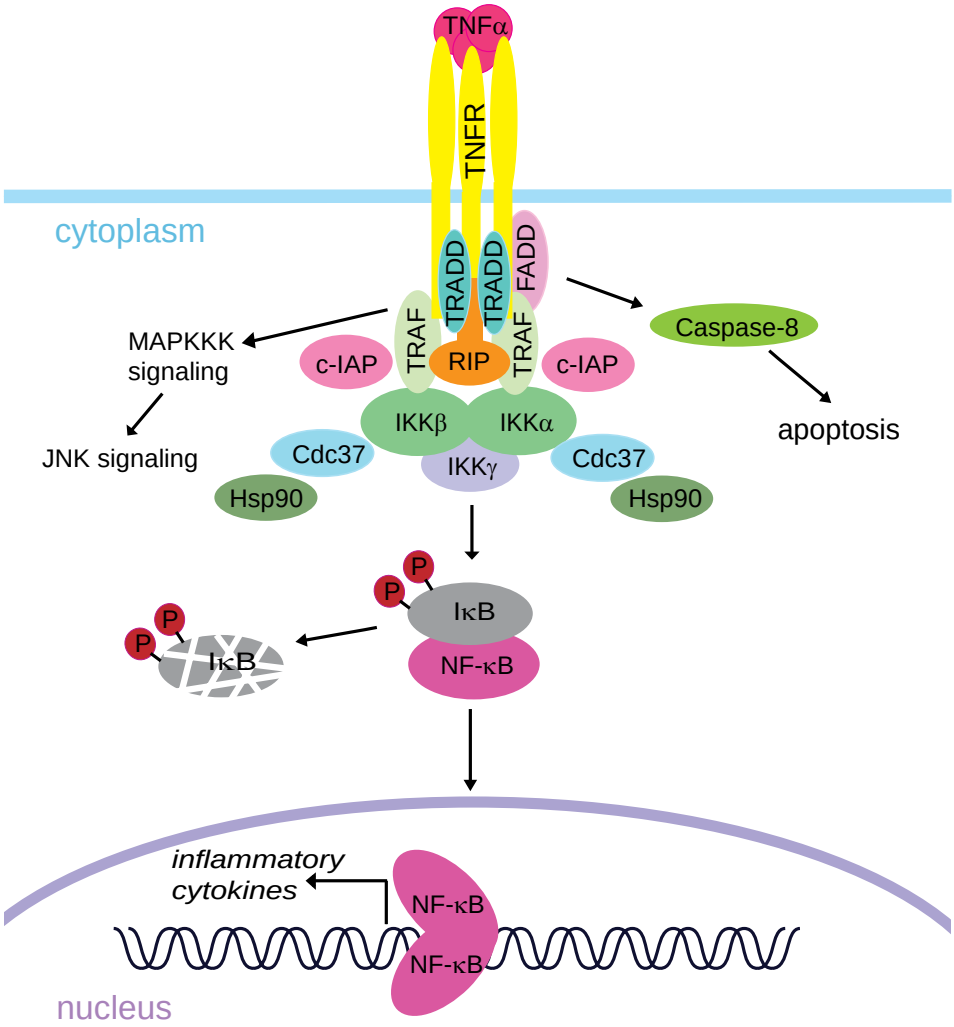

Figure 2. TNFR1 signaling.

2.3 *Drosophila* as a model organism

The human urge to conquer diseases, promote longevity and improve the quality of life drives us continuously to search for new drugs and treatments. Maintaining the balance that we call health requires complex cell signaling networks and careful regulation that we do not yet thoroughly understand. But due to obvious ethical and practical problems we cannot study humans on the organismal level to answer questions on how the human body functions in response to environmental stresses. There are also limitations to the use of other mammalian models. The life span of the mouse or rat, although short compared to humans, seems an eternity to a scientist who needs to complete his crossing scheme and carry out his experiments on the offspring. Ethical concerns reduce the amount of animals one can sacrifice for science, and the requirements of a

controlled environment and constant professional care make experiments in mammals expensive. The question therefore arises if we can overcome these problems by using non-vertebrate models, such as nematodes or flies, for studying human diseases.

Drosophila melanogaster has been used as an experimental model organism for more than a hundred years. It is big enough to be easy to handle under the microscope but small enough not to require too much laboratory space. *Drosophila* are inexpensive to maintain, fast in reproduction and relatively short-lived. Before anyone even knew how genetic information was encoded in the chromosomes the first studies on linkage groups were performed by Calvin Bridges in *Drosophila*. The large polytene chromosomes in *Drosophila* larval salivary glands served as the first rough map of the fly genome, greatly facilitating the scientific work before releasing the first annotated sequence of the entire fly genome in 2000 (Adams et al. 2000). The intensive and systematic genetic research carried out in *Drosophila* for so many decades has also yielded wonderful tools for scientists. The balancer chromosomes, EMS mutagenesis screening, and stock collections of mutant fly lines and deficiencies have long been part of the fly scientist's tool box. More recently, P element techniques developed by Rubin and Spradling (Rubin and Spradling 1982, Spradling and Rubin 1982), tissue-specific expression of genes or RNAi constructs using the UAS-GAL4 expression system by Brand and Perrimon (Brand and Perrimon 1993), and the genome-wide collection of RNAi fly lines (Dietzl et al. 2007) have further broadened the possibilities of studying gene function in *Drosophila in vivo*. Moreover, the availability of the genome sequences of twelve related *Drosophila* species provides a unique opportunity for evolutionary studies (The 12 Genomes Consortium 2007). And if these factors are not attractive enough, *Drosophila* offers a compact genome with less redundancy compared to humans. This means that the effects of gene knock-outs, which in mammals might be masked by related proteins sharing the same functions, are more likely to be detected in *Drosophila*. In addition, the fundamental mechanisms of cell biology are evolutionarily conserved, and it has been estimated that 77% of the genes associated with human diseases have corresponding ones in the fly genome (Reiter et al. 2001, Chien et al. 2002). This also applies to the innate immune signaling pathways, of which the most famous example is the discovery of the Toll-like receptors (Medzhitov et al. 1997) soon after the importance of Toll for the fly immune system had first been described (Rosetto et al. 1995, Lemaitre et al. 1996).

Insects do not have adaptive immunity, which makes them totally dependent on their innate immune response. Compared to mammalian models, in which the adaptive immune response can compensate and hide the phenotype of interest, experiments carried out in flies are easier to interpret. Together with the less-redundant, well annotated genome, the ease of performing genetic screens, and the possibility of generating inducible knock-down and overexpression constructs have made *Drosophila* a popular model organism in studying conserved cellular mechanisms and signaling pathways.

2.4 Overview of the *Drosophila* immune system

Drosophila lives in a natural environment that consists of decomposing fruits and is rich in bacteria and fungi. The fly therefore needs an effective and well-adjusted immune response to be protected from pathogens, simultaneously tolerating the commensal bacteria in the gut. In contrast to mammals, *Drosophila* has no adaptive immunity. However, *Drosophila* has developed a sophisticated innate immune defense (reviewed by Lemaitre and Hoffmann 2007) and is highly resistant to microbial infections, which makes it a good model for studying innate immunity.

Drosophila immunity relies on multiple mechanisms that can be roughly divided into cellular and humoral responses. *Drosophila's* circulating blood cells, hemocytes, are capable of phagocytosing bacteria. Bigger objects that are too large to be phagocytosed are encapsulated by specialized cells called lamellocytes. The activation of proteolytic cascades leads to clotting of hemolymph and melanin formation. Finally, the recognition of microbial patterns triggers the activation of multiple cell signaling cascades and the synthesis of effector molecules, such as the antimicrobial peptides (AMPs), especially in the fat body. Figure 3 represents an overview of the *Drosophila* immune system.

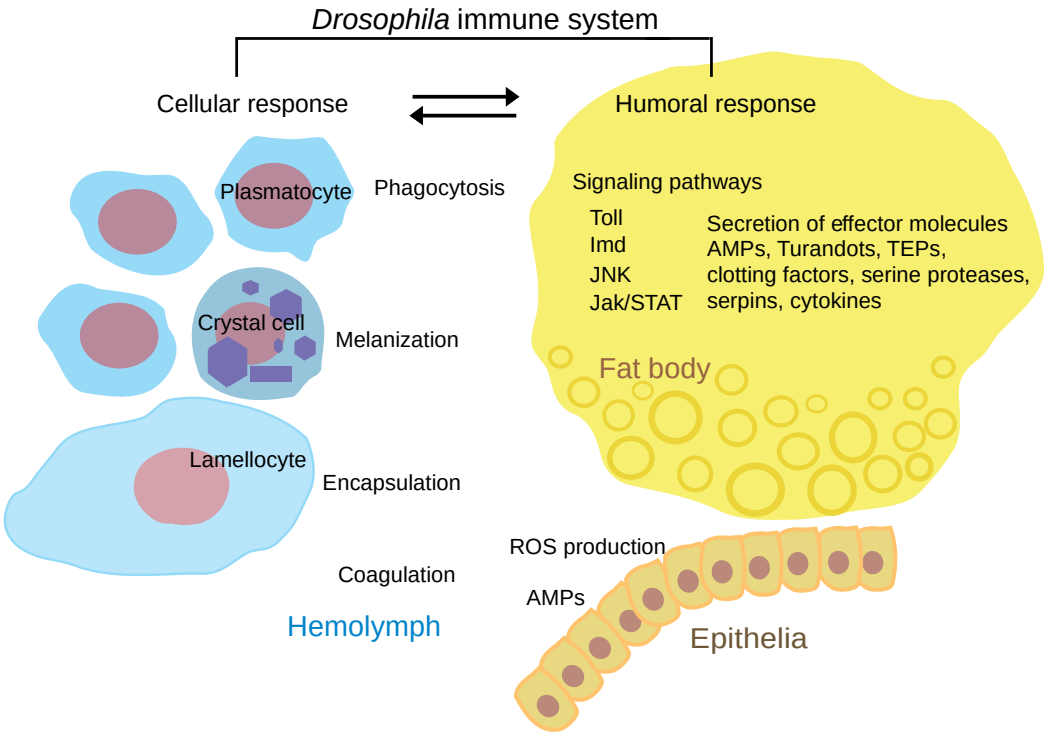

Figure 3. Schematic presentation of the *Drosophila* immune system.

2.5 Cellular response

Unlike mammals, insects do not have a closed blood-circulation system. Instead, the body cavity of both larvae and adult flies is filled with free-floating and sessile blood cells that are called hemocytes. *Drosophila* blood cells can be divided into three classes based on their appearance and functional properties: plasmatocytes, lamellocytes, and crystal cells (for a review, see Williams 2007). Due to the relative easiness of studying blood cells in larvae compared to adult flies, the following aspects are based on larval hemocytes. Plasmatocytes form the largest group by representing more than 90% of the hemocytes in non-infected animals. Plasmatocytes are responsible for phagocytosing both apoptotic cells that need to be removed, as well as invading microbes. Lamellocytes appear large and flat, and they can only be found in larvae, although they are rare in healthy animals. In contrast, parasitoid wasp infestation readily induces the formation of lamellocytes. Lamellocytes primarily function by encapsulating foreign objects, such as parasitoid wasp eggs that are too large to be phagocytosed. The third group of cells, crystal cells, comprises approximately 5% of the larval hemocytes. They are nonphagocytic and involved in the melanization process. The name crystal cell derives from the prophenoloxidase (pro-PO) crystals that the cells carry and release in response to activation.

Phagocytosis is an evolutionarily conserved defense mechanism originally discovered in invertebrates, and recent research has even emphasized the similarities between invertebrate and vertebrate phagocytic machineries. Phagocytosis is a step-by-step process that involves the recognition and binding of the target followed by cytoskeletal reorganization, engulfment, internalization and destruction of the target within phagosomes. These mechanisms have been intensively studied in *Drosophila*, since the *Drosophila* embryonic hemocyte-derived cell lines are capable of phagocytosis, and are easily manipulated by RNAi (Clemens et al. 2000). Based on multiple studies utilizing RNAi screening (Rämet et al. 2001, Rämet et al. 2002b, Pearson et al. 2003, Kocks et al. 2005) and mass spectrometry (Stuart et al. 2007), hundreds of proteins have been implicated in phagocytosis. Stuart et al. (2007) alone reported 617 proteins to be associated with phagosome formation, and their interaction network was mapped using a systems biology approach. Approximately 70% of these proteins have mammalian orthologs (Stuart et al. 2007). How the protein interaction network functions remains elusive.

Several types of receptor proteins have been implicated in triggering phagocytosis. The two best-characterized ones are the multiple EGF-repeats containing protein called Eater, and Scavenger Receptor CI (SR-CI) (Kocks et al. 2005, Pearson et al. 1995, Rämet et al. 2001). Other proteins belonging to the family of scavenger receptors, Croquemorte and Peste, have also been implicated in the phagocytosis of micro-organisms (Philips et al. 2005, Stuart et al. 2005). In addition, Croquemorte is required for the removal of apoptotic cells (Franc et al. 1996, Franc et al. 1999). PGRP-LC has been associated with phagocytosis

(Rämet et al. 2002b), although it is principally thought to mediate microbial recognition leading to the activation of the humoral arm of the *Drosophila* immune response. Last but not least, a very peculiar protein with more than 18,000 putative isoforms, Dscam, has been reported to be required for efficient phagocytosis of bacteria (Watson et al. 2005).

The engulfment and internalization of the target require the synergistic action of multiple pathways, especially those involved in cytoskeleton remodeling, vesicle trafficking, and other cellular functions, such as energy metabolism (Pearson et al. 2003, Stuart et al. 2007). Finally, the target is destroyed inside the phagosomes by a cocktail of lysosomal enzymes, reactive oxygen species, nitric oxide, and intracellular antimicrobial peptides. The exact mechanisms of this killing are not yet thoroughly understood.

Opsonins are molecules that bind microbial surfaces and thereby promote microbial killing and phagocytosis. In *Drosophila*, thioester-containing proteins (TEPs) are a family of six secreted proteins, three of which are known to be induced upon infection (Lagueux et al. 2000). They are structurally related to the α2-macroglobulins and components of the mammalian complement system (Blandin and Levashina 2004). TEPs have been suggested to function as opsonins in *Drosophila*, but this has not yet been verified. Instead, in cultured cells of *Anopheles gambiae* TEP-1 has been associated with the opsonization of both Gram-negative and Gram-positive bacteria (Levashina et al. 2001).

Foreign objects that are too big to be phagocytosed are insulated from the host tissues by encapsulation. This defense mechanism has developed to protect the host from parasitoid wasps that lay their eggs into *Drosophila* larvae. The process involves the detection and recognition of the wasp egg by plasmatocytes, which triggers a still poorly characterized signaling network resulting in the proliferation and activation of lamellocytes. Lamellocytes surround the foreign body and form a capsule around it. The intruder is eventually killed, although not always, inside the capsule probably due to ROS production or toxic intermediates of the melanization cascade. The molecular mechanisms involved in this process are largely unknown.

Unlike mammals, insects have an open circulatory system. This means that to prevent the loss of body fluids after injury in the fly cuticle, efficient hemolymph clotting mechanisms are essential for the insect's survival. Coagulation of the hemolymph also protects the host from micro-organisms that would otherwise enter through the wound. Instead of freely spreading into the host system, microbes are trapped into the clot. Clotting often involves melanization as well, but the latter is not required for the formation of the clot, since the coagulation system is effective also in the absence of PO activity (Scherfer et al. 2004). Instead, melanization of the site of injury is thought to contribute towards killing the microbes through the toxic intermediates released during the process (Scherfer et al. 2004, Theopold et al. 2004). Hemolymph clotting in *Drosophila* is relatively poorly understood compared to the other aspects of the immune defense. However, some proteins have been associated with coagulation, the best-studied ones being Hemolectin (Goto et al. 2001, Goto et al. 2003) and

Fondue (Scherfer et al. 2006). Hemolectin is a large hemocyte-specific protein that is the major structural component of the fibers forming the clot. In contrast, Fondue is not required for the formation of these fibers, but instead needed to cross-link them together (Scherfer et al. 2006). In addition, pull-down and proteomic analyses have identified other factors associated with clotting, such as mucin-like proteins and proteins involved in phenoloxidase cascades (Karlsson et al. 2004, Scherfer et al. 2004).

Melanization is a rapid reaction that occurs at the site of cuticular injury or on the surface of an encapsulated parasitoid egg, and can be detected easily due to the black-coloured melanin that is synthesized in the process. Melanization is thought to be activated primarily by crystal cells that rupture in response to JNK signaling (Bidla et al. 2007) and release pro-PO. However, one of the three pro-PO encoding genes in *Drosophila* is exclusively expressed in lamellocytes, suggesting that it might be involved in melanization during encapsulation (Irving et al. 2005). After pro-PO is released it is cleaved by a serine protease, and the cleaved PO sequentially activates the cascade leading to the production of melanin and toxic intermediates that may contribute to the killing of the microbes. The PO cascade must be strictly regulated to prevent excess melanization, and this inhibitory action is mediated by Serpin27A, a serine protease inhibitor that prevents pro-PO cleavage (De Gregorio et al. 2002).

Overall, *Drosophila* has a sophisticated cellular response to pathogens consisting of both features that have been conserved in evolution and features that are unique to invertebrates. In addition, *Drosophila* has an efficient humoral immune response involving the production of potent antimicrobial peptides (AMPs). AMPs and the signaling events leading to the production of AMPs are described in the following chapters.

2.6 Humoral response

Besides the cellular responses, microbial challenge in *Drosophila* triggers the secretion of effector molecules from the fat body into the hemolymph. The fat body is an immune-responsive tissue, which is also involved in other functions, such as energy metabolism. It is therefore often referred to as the *Drosophila* equivalent of the mammalian liver. Being large in size, the fat body of a third instar larva extends almost throughout the entire larval body. Due to the open circulatory system hemolymph can freely surround the fat body, which in turn can, at least in theory, recognize the microbial components in the hemolymph and initiate AMP production. The role of hemocytes in the microbial recognition and initiation of AMP production is not completely understood, yet it is reasonable to assume that the circulating hemocytes that float around in the hemolymph might be able to recognize microbes and signal to the fat body that AMP production is needed. To support this, there is increasing evidence that signaling between hemocytes and the fat body is required for the full activation

of the humoral immune response (Agaisse et al. 2003, Foley and O'Farrell 2003, Dijkers and O'Farrell 2007, Shia et al. 2009).

In a normal homeostatic state no *AMP*s are transcribed and therefore they are not detected in the hemolymph. However, AMPs are readily synthesized and secreted during systemic infection, and the synthesis is paused once the intruding microbes have been destroyed. This process requires signaling, and multiple pathways are involved in the regulation of humoral responses. The most important ones for the production of AMPs are the Imd and Toll pathways. The *Drosophila* Imd pathway bifurcates to the JNK pathway, which besides controlling cell survival and apoptosis also plays a role in the regulation of AMP production. These three pathways will be discussed later in more detail. In addition, the Jak-STAT signaling pathway is known to regulate certain immune-responsive genes, such as the genes encoding the complement-like protein Tep2 implicated in opsonization, and Turandots, which have an unknown function but are known to be induced as a response to stress and septic injury (Ekengren and Hultmark 2001, Agaisse et al. 2003). However, Jak-STAT signaling is not essential to AMP release, since Jak-STAT deficient flies are shown to be as resistant to bacteria and fungi as wild-type flies. Instead, the mutants are more sensitive to the *Drosophila* C virus (Dostert et al. 2005). Understanding the function and importance of Jak-STAT signaling for the fly immune response requires further studies.

2.6.1 Antimicrobial peptides

Of all the various effector molecules produced upon infection, antimicrobial peptides (AMPs) are the best characterized. They are a heterogeneous group of peptides that share some common features: a relatively small size, positive charge, and antimicrobial properties against a variety of bacteria and even fungi. The *Drosophila* genome encodes 4 Attacins, 4 Cecropins, 7 Drosomycins, 2 Diptericins, and one Drosocin, Defensin, and Metchnikowin (Imler and Bulet 2005). These peptides have traditionally been classified into three families according to their microbe specificities, but since most of them are produced in response to both Gram-negative and Gram-positive bacteria, this kind of division can be confusing. In addition, *Drosophila* has several lysozymes that also play a role as digestive enzymes, and a male-specific AMP called Andropin, which is likely to locally protect the seminal fluid and reproductive tract from microbial infections (Samakovlis et al. 1991). Furthermore, Attacin C has been shown to be further processed into another antimicrobial peptide called maturated pro-domain of Attacin C (MPAC). Cecropin and Drosomycin were the first AMPs to be associated with Toll pathway activation (Rosetto et al. 1995, Lemaitre et al. 1996), but later it has been shown that their production can also be triggered by Imd signaling. Diptericin seems to be the only Imd pathway-specific antimicrobial peptide, and has therefore been widely used as a read-out in various assays monitoring Imd pathway activation.

The molecular mechanisms of how AMPs contribute to microbial killing are not yet understood. It has been proposed that cationic AMPs bind to the negatively charged microbial surfaces and break down the bacterial integrity by creating holes through which the bacterial contents leak out (Yang et al. 2000), by depolarizing the bacterial membrane (Westerhoff et al. 1989), or by inducing hydrolases that degrade the bacterial cell wall (Bierbaum et al. 1985). Since AMPs are structurally diverse, it seems plausible to think that more than one mechanism for bacterial killing exists. A model that could explain the function of most AMPs is referred to as the Shai-Matzuaki-Huang model (Matzuzaki et al. 1999, Shai et al. 1999, Yang et al. 2000). According to this model the AMPs interact with the bacterial membrane, which leads to the displacement of lipids in the membrane, alteration of the membrane structure and in some cases penetration of the AMPs into the bacterial cell. Cholesterol in the host cell membranes is thought to stabilize the lipid bilayer thereby reducing the AMP activity that would otherwise harm the host as well. Whatever the mechanism behind the activity of AMPs, they are highly efficient and kill microbes at micromolar concentrations (Zasloff 2002, Imler and Bulet 2005).

2.6.2 The Toll pathway

Since the early 1980s the developmental function of the Toll pathway has been known crucial for the establishment of dorso-ventral patterning (Nüsslein-Volhard at al. 1980). However, it wasn't until the mid-1990s when the first indications of the importance of Toll signaling to the fly immune system were obtained. The first pieces of evidence came from Rosetto et al. (1995) who found that *CecA1* could be induced by overexpressing the constitutively active form of Toll, $Toll^{10B}$ in *Drosophila* hemocyte-like mbn-2 cells. In addition, they showed that this induction was dependent on a κB binding site in the *CecA1* promoter region. These findings were soon accompanied by a report from Lemaitre et al. (1996), where they demonstrated that the Toll pathway mutants were susceptible to certain fungi but had normal resistance to bacteria, such as the Gram-negative *Escherichia coli.* Shortly after, Medzhitov et al. (1997) identified the human homolog of Toll, and reported that the constitutively active form of human Toll (i.e. TLR) could induce NF-κB activation and expression of NF-κB target genes, such as the inflammatory cytokines. After these pioneering experiments, the Toll pathway has been even more extensively studied, and this has yielded a better but still incomplete understanding of the signal regulation.

The intracellular components involved in development and immune signaling are nearly the same. Toll, which in *Drosophila* is not a PRR but a transmembrane receptor binding an extracellular protein Spätzle (Weber et al. 2003), becomes dimerized after ligand binding, and this leads to the interaction of the adaptor protein MyD88 and Toll via their TIR domains. MyD88 also contains a death domain (DD), which sequentially recruits other DD-containing proteins, such as Tube and the kinase Pelle (Shelton and Wasserman 1993), to

the signaling complex (Sun et al. 2002). Tube apparently has separate binding sites for both MyD88 and Pelle, and can form heterodimeric or heterotrimeric complexes with these two proteins. In contrast, no interaction between the DDs of MyD88 and Pelle was detected, which implies that their interaction is mediated by Tube (Moncrieffe et al. 2008). An additional adaptor protein Weckle is required for embryonic development (Chen et al. 2006). It has been suggested that Pelle undergoes autophosphorylation (Shen and Manley 1998) and phosphorylates Toll and Tube before being released from the signaling complex (Sun et al. 2004). Then either activated Pelle or a yet unidentified additional kinase phosphorylates Cactus, which is the *Drosophila* IκB protein suppressing the Toll pathway activity. Phosphorylation of Cactus releases the NF-κB proteins Dorsal and Dif, which in turn translocate into the nucleus and initiate the transcription of their target genes. There are indications that additional phosphorylation steps are needed to further activate Dorsal (Edwards et al. 1997, Drier et al. 1999, Isoda and Nüsslein-Volhard 1994). Dorsal is thought to be more important for the transcription of genes involved in the formation of the dorsal-ventral axis, while Dif is thought to be activated in response to an immune challenge and to be involved in the transcription of immune-responsive genes, such as the AMPs *Drosomycin* and *Metchnikowin* (Meng et al. 1999, Rutschmann et al. 2000a).

What then separates the dorso-ventral patterning from immune signaling, and contributes to the detection of both fungi (Lemaitre et al. 1996) and Gram-positive bacteria (Michel et al. 2001)? The answer is the PRRs that in *Drosophila* Toll signaling are not membrane-bound but secreted proteins. A Lys-type peptidoglycan, which is typical for Gram-positive bacteria, is recognized by PGRP-SA, PGRP-SD, and GNBP1 (This abbreviation stands for Gram-negative binding protein 1 due to a historical misnomery. Nowadays it is sometimes referred to as Glucan-binding protein 1). Two of these proteins, PGRP-SA and GNBP1, have been implied to associate with each other in the hemolymph (Gobert et al. 2003, Pili-Floury et al. 2004). It is not yet clear if these proteins have redundant functions or different specificities for various Gram-positive bacteria. The present evidence supports the hypothesis of partial redundancy (Bischoff et al. 2004).

The response to fungi is based on the recognition of β-glucan that is a structural component of the fungal cell wall. A member of the GNBP family, GNBP3, structurally resembles the lepidopteran β-glucan recognition proteins. GNBP3 mutant flies are sensitive to fungal infections and fail to induce Toll signaling, which implies that GNBP3 is indeed required for the pathway's activity (Gottar et al. 2006). In addition, the fungal virulence factors, such as proteases and chitinases that the entomopathogenic fungi secrete to cross the chitinous epitelia, trigger the Toll pathway via a serine protease called Persephone (Psh) (Ligoxygakis et al. 2002). Persephone is known to be negatively regulated by the serpin Necrotic (Levashina et al. 1999).

Irrespective of whether the initial stimulus originates from bacteria or fungi, the recognition triggers proteolytic cascades that were previously thought to

converge at Spätzle processing enzyme, SPE (Jang et al. 2006, Kambris et al. 2006). SPE cleaves Spätzle, whereafter the activated Spätzle can bind Toll. SPE shares remarkable similarity with Easter, which is the Spätzle processing enzyme, which is active in the embryo (Chasan and Anderson 1989). Recently, El Chamy et al. (2008) demonstrated that a serine protease upstream of SPE, Grass, is involved in the recognition of both fungi and Gram-positive bacteria.

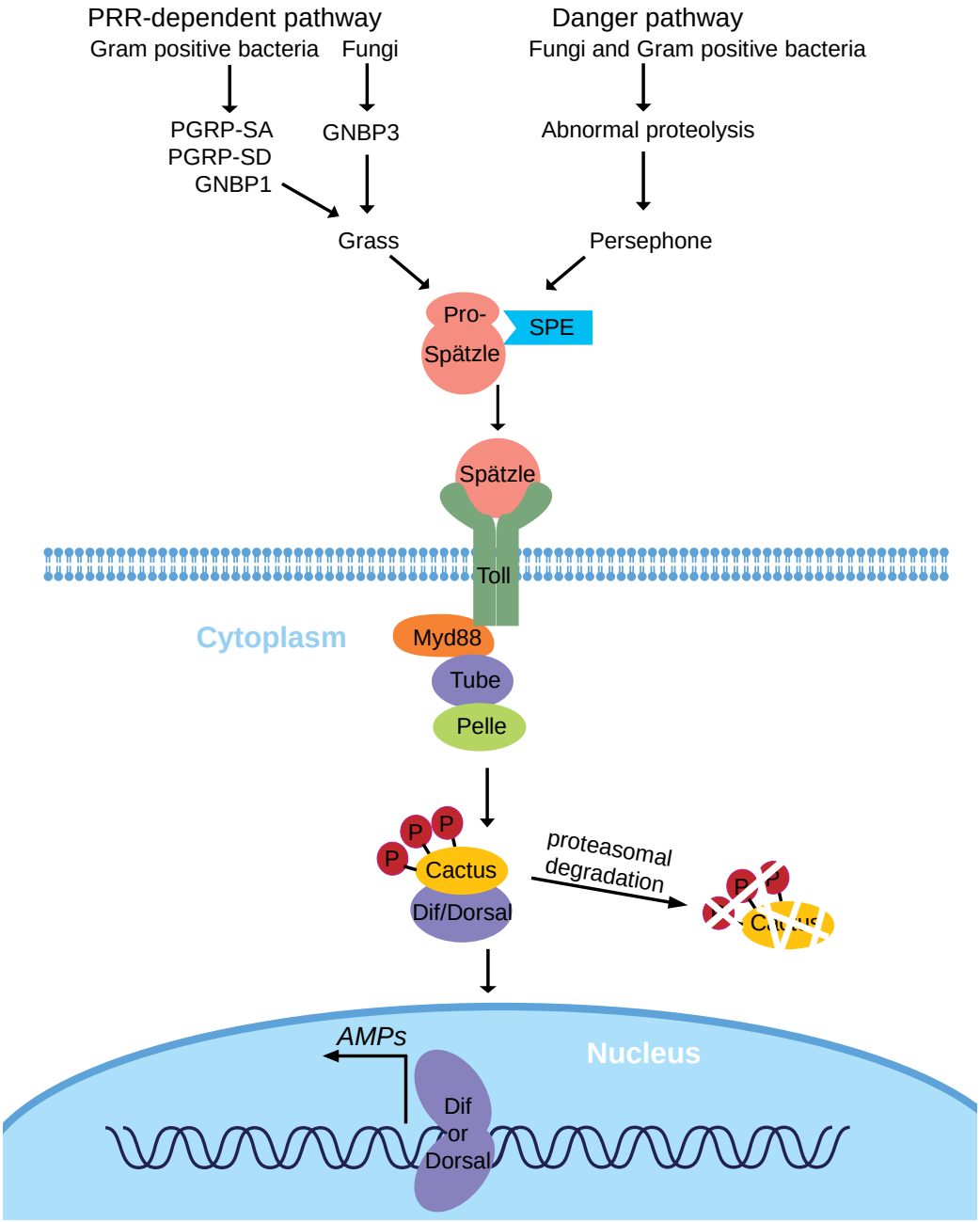

Figure 4. The Toll pathway.

Grass then activates another serine protease Spirit, which in sequence activates SPE. Based on this and the earlier data, the serine protease cascades leading to the activation of the Toll pathway have been proposed to divide into a PRR-dependent pathway responding to bacterial or fungal cell wall components, and a so-called danger pathway responding to proteases and an abnormal proteolysis activating Psh (El Chamy et al. 2008, Ashok 2009). The current model for Toll pathway signaling is illustrated in Figure 4.

Curiously, there are eight other Toll-resembling proteins in *Drosophila*. It has been speculated whether these would also play a role in immune signaling, but so far there is no evidence supporting this hypothesis. Toll signaling in general has been vigorously studied during the last couple of years, but many aspects of the signal regulation remain elusive. For example, the kinase that phosphorylates Cactus has not yet been identified. Furthermore, the negative regulation of the Toll pathway is poorly documented. The only negative regulator reported besides the IκB protein Cactus is WntD, which represses Toll signaling through a negative feedback loop. WntD is associated with embryonic development, but also reported to possess an immunity-related function (Ganguly et al. 2005, Gordon et al. 2005). Gordon et al. (2005) reported that *WntD* mutant flies showed elevated transcript levels of certain *AMP*s. Surprisingly, in *wntD* mutant flies the transcription of *Dpt* was most severely affected, while the transcript levels of *Drs* did not differ from controls. Although the transcription of *AMP*s can be activated by multiple pathways *Dpt* is considered Imd pathway-specific. In addition, *wntD* mutant flies were apparently more susceptible to *Listeria monocytogenes* infections than to wild-type flies (Gordon et al. 2005). The PGN of *L. monocytogenes* is a DAP-type PGN that is known to activate the Imd pathway instead of Toll signaling. Recently, Gordon et al. (2008) reported that both the *Drosophila* TNF homolog *eiger*, which encodes a known component of the JNK signaling pathway (Igaki et al. 2002), and a novel immunity gene *edin* are upregulated in *Listeria*-infected *wntD* flies.

Together, these data suggest that Toll pathway signaling is more complex than was previously thought and involves interactions with other immune signaling pathways via mechanisms that are largely unknown. Innate immunity signaling cascades are meant to act synergistically and to a certain extent redundantly to fully protect the host from a variety of microbes. In addition, the Toll and Imd pathways have different temporal profiles. The Toll pathway response to microbes is observed later but is sustained longer, while the Imd and JNK pathways respond within minutes (Lemaitre and Hoffmann 2007). This may also reflect the different microbe specificities of these pathways, since the fungi recognized by PRRs signaling via Toll grow slowly compared to bacteria detected by the Imd pathway receptor (Steiner 2004). The regulation of Imd pathway signaling will be described next in more detail.

2.6.3 The Imd pathway

The first indications of the existence of the Imd pathway were initially obtained by studying a fly line with a homozygous viable mutation in the second chromosome and with a very peculiar phenotype. The larvae of *Bc* (*Black cells*) flies have melanized black crystal cells in their hemolymph, and they develop melanotic tumors at temperatures higher than +21ºC. In addition, the transcription of *AMP*s was severely impaired in adult flies, excluding *Drs* (Lemaitre et al. 1995). This data implied that *AMP* induction was regulated by two separate pathways, one regulating the production of antibacterial and the other antifungal AMPs. The defective *AMP* response was shown to be independent of the *Bc* phenotype but dependent on a nearby mutation. According to this previously uncharacterized mutation, the corresponding gene was thereafter called *imd* (immune deficiency) (Lemaitre et al. 1995) and gave its name to the pathway.

The activation of the Imd pathway is triggered by DAP-type PGN, and not LPS like was initially believed. The reason why LPS is still commonly used for activating the Imd response in experiments is because PGN is a common contaminant in commercial LPS preparations (Werner et al. 2003, Kaneko et al. 2004). PGN, also called murein, is an essential component of the bacterial cell wall, whose molecular structure varies greatly from one bacterial species to another. The backbone of this large polymer is formed by chains of alternating *N*-acetylmuramic acid and *N*-acetylglucosamine residues that are cross-linked together by short amino acid chains of 4 to 5 residues. If the third residue in this chain is L-lysine, the PGN is defined as Lys-type PGN, which is more common in Gram-positive bacteria. Instead, if the third residue is *meso*-diaminopimelic acid, the PGN is called DAP-type PGN. DAP-type PGN can be found in Gram-negative bacteria but also in some species of Gram-positive bacteria, such as *Bacillus* and *Listeria*.

DAP-type PGN is recognized by a member of the peptidoglycan recognition protein family, PGRP-LC (Choe et al. 2002, Gottar et al. 2002, Rämet et al. 2002b). *PGRP-LC* encodes three alternative splice variants, *PGRP-LCa*, *PGRP-LCx*, and *PGRP-LCy*. The biological function of PGRP-LCy is not known. *PGRP-LCa* and *PGRP-LCx* encode transmembrane proteins that have identical transmembrane and cytoplasmic parts but different PGRP domains (Werner et al. 2000), corresponding to the different specificities of these two isoforms. Unlike PGRP-LCx, PGRP-LCa is not capable of binding polymeric PGN. In contrast, it participates in the recognition of monomeric PGN, also called the tracheal cytotoxin (TCT). Structural and biochemical studies suggest that PGRP-LCa and PGRP-LCx bind TCT as heterodimers (Stenbak et al. 2004, Chang et al. 2005, Kaneko et al. 2005, Mellroth et al. 2005, Chang et al. 2006, Lim et al. 2006). Furthermore, another PGRP family member, PGRP-LE has been associated with PGN recognition (Takehana et al. 2004, Kaneko et al. 2006, Lim et al. 2006). *PGRP-LE* encodes a protein without an obvious transmembrane domain or signal sequence, and curiously has been suggested to be both intracellular and

secreted. The detection of the PGRP-LE ectodomain in hemolymph supports this theory. PGRP-LE has been suggested to act synergistically with PGRP-LC in TCT recognition, and to participate in the recognition of intracellular pathogens, such as *Listeria* (Yano et al. 2008). Upon TCT binding, PGRP-LE is known to homodimerize (Lim et al. 2006). How this results in Imd pathway activation is still elusive, although both PGRP-LC and PGRP-LE are known to co-immunoprecipitate with Imd (Aggarwal and Silverman 2008).

After the initial recognition of the bacterial components, the signal is propagated from the PGRP-LC cytoplasmic domain to IMD. IMD is a death domain-containing protein and homologous to the mammalian RIP1 (Georgel et al. 2001). The DD of IMD mediates the recruitment of other DD containing proteins, such as the *Drosophila* homolog of the Fas-associated death domain (FADD) often referred to as dFADD or BG4. dFADD in turn recruits a caspase called Death-related ced-3/Nedd2-like protein (Dredd) via its caspase recruitment domain to the signaling complex. Dredd is homologous to the mammalian caspase-8, and has been proposed to play a dual role in Imd pathway activation. First, Dredd has been shown to cleave IMD (Paquette et al. 2010), which apparently contributes to its activation. Second, Dredd is required for cleavage of the transcription factor Relish (Leulier et al. 2000), although it is not certain if Relish is actually cleaved by Dredd. A physical interaction of Dredd and Relish has been demonstrated *in vitro* (Leulier et al. 2000, Stöven et al. 2003). In addition, the caspase cleavage sites of IMD and Relish are identical, which further supports the hypothesis that Dredd is the caspase contributing to the activation of Relish.

In addition to the components described above, Imd pathway signaling requires the activation of kinases. The *Drosophila* homolog of the TGF-β activated kinase 1 (Tak1) functions downstream of Imd and is required for the activation of the *Drosophila* IKK complex. (Rutschmann et al. 2000b, Lu et al. 2001, Vidal et al. 2001, Silverman et al. 2003). The *Drosophila* IKK complex consists of two subunits, the kinase called Immune response deficient 5 (Ird5) and the regulatory subunit Kenny, which are homologous to mammalian IKKβ and IKKγ, respectively (Rutschmann et al. 2000b, Silverman et al. 2000). The IKK complex is also required for Relish cleavage (Silverman et al. 2000), although the molecular mechanism for this is not perfectly clear yet.

Relish is the third *Drosophila* NF-κB protein homologous with the mammalian proteins p100 and p105, and the transcription factor essential for the expression of the Imd pathway target genes. Unlike the Toll pathway NF-κB proteins Dif and Dorsal, Relish consists of both the N-terminal Rel homology domain (RHD) and a C-terminal inhibitory IκB part. The IκB part must be removed by endoproteolytic cleavage to release the NF- κB part, which thereafter translocates to the nucleus and initiates the transcription of its target genes, such as the *AMP*s, while the C-terminal part remains in the cytoplasm (Stöven et al. 2000, Stöven et al. 2003). Besides cleavage, Relish activation is further promoted by phosphorylation by the IKK complex (Silverman et al. 2000). Recently, Ertürk-Hasdemir et al. (2009) reported that Ird5, the *Drosophila*

IKKβ, phosphorylates two serine residues in the N-terminal part of Relish, serines 528 and 529. They discovered that these phosphorylation sites were not required for Relish cleavage, translocation to the nucleus, or binding to the DNA target sequence. Instead, phosphorylation of these residues appears essential for the Relish transcription factor activity, possibly by promoting the recruitment of RNA polymerase II into the promoter region (Ertürk-Hasdemir et al. 2009).

Besides Relish, the induction of *AMP*s requires the presence and function of the transcription machinery proteins. Recently, Goto et al. (2008) performed an RNAi screen in *Drosophila* S2 cells, and identified a previously unknown Imd pathway regulator Akirin. The *Drosophila* genome contains only one *Akirin*, which encodes a protein with no other recognizable motifs than a nuclear localization signal. The nuclear localization was confirmed by immunocytochemistry and epistasis analysis, which implied that Akirin would act downstream of Relish. Furthermore, RNAi knock-down of Akirin *in vivo* sensitized the flies to Gram-negative bacterial infections. Interestingly, the Akirin null mutation is lethal, suggesting that Akirin might play other roles than merely assisting the NF-κB function. Mammals have two Akirins, Akirin1 and Akirin2. The role of Akirin1 is not known, but Akirin2 seems to be essential for development, since homozygous knock-out mice die before embryonic day 9.5. The embryonic fibroblasts derived from these animals show defects in their ability to express genes dependent on NF-κB signaling (Goto et al. 2008). The discovery of Akirin demonstrates the power and value of novel reverse genetic approaches, as well as the relevance of invertebrate model organisms in deciphering the mechanisms of evolutionarily conserved signaling pathways.

2.6.4 The JNK pathway

JNK signaling is usually triggered by environmental stress and often leads to apoptosis. It has, however, also been implicated in hemocyte activation (Boutros et al. 2002, Silverman et al. 2003, Bidla et al. 2007) and wound healing (Rämet et al. 2001). The JNK pathway components upstream of Tak1 are Mishapen and the mammalian TNFR-resembling Wengen, which binds the *Drosophila* cytokine Eiger at the plasmamembrane (Igaki et al. 2002, Moreno et al. 2002). These proteins have not been associated with the humoral immune response. However, overexpressing Eiger and Wengen is known to induce apoptosis in a JNK dependent manner (Igaki et al. 2002, Geuking et al. 2005).

In addition to stress, the JNK pathway can also be activated via the Imd pathway. The Imd pathway branches into the JNK signaling pathway at the level of Tak1, which is an essential component of both pathways. Tak1 activates the *Drosophila* MKK7/JNKK homolog Hemipterous (Hep) (Glise et al. 1995), which in sequence phosphorylates the *Drosophila* JNK called Basket. Basket then activates the *Drosophila* Jun and Fos homologs Jun-related antigen (Jra) and Kayak (Kay), respectively, which initiate transcription and induce target genes (Riesgo-Escovar et al. 1996, Sluss et al. 1996) such as the negative

regulator *puckered*, and genes involved in cytoskeleton remodeling and hemocyte activation. In addition, the JNK pathway has been implied to be involved in the up-regulation of *AMP*s during the early response to bacteria (Kallio et al. 2005, Delaney et al. 2006), although this is somewhat controversial. Others have reported that the JNK pathway would in fact down-regulate *AMP*s via the inhibitory action of AP-1 (the heterodimer of Jun and Fos), and STAT (Kim et al. 2005, Kim et al. 2007). The interplay of the Imd and JNK pathways in the regulation of the humoral response therefore requires clarification.

Besides Puckered, two negative regulators of the JNK pathway have been identified. A putative E3 ligase Plenty of SH3 (Posh) has been reported to regulate the signaling of both the Imd and the JNK pathways (Tsuda et al. 2005), possibly by regulating Tak1 stability by targeting it for proteasomal degradation. In addition, the Imd pathway negative regulator dUSP36 also suppresses JNK signaling (Thevenon et al. 2009) in an Imd-dependent manner.

2.6.5 Negative regulation of the Imd pathway

A hyperactivated immune response can have detrimental effects on the fitness and survival of the organism in both invertebrates and vertebrates. Immune signaling must therefore be carefully regulated. The Imd pathway activation is indeed modulated to ensure a properly timed and adjusted immune response. This modulation takes place at multiple levels and involves a variety of regulatory proteins emphasizing the significance of control over the expression of the target genes.

The Imd pathway is activated by binding of a DAP-type peptidoglycan to the receptor PGRP-LC (Leulier et al. 2003, Choe et al. 2005), which leads to receptor dimerization (Mellroth et al. 2005) and signal transduction via the PGRP-LC intracellular domain to IMD (Choe et al. 2005). The first level of regulation is therefore the dampening of the initial stimulus. This can be achieved by the enzyme-catalyzed degradation of PGN into smaller subunits, which attenuates the pathway activation (Werner et al. 2003, Kaneko et al. 2004). Members of the PGRP family, namely PGRP-LB, PGRP-SB1, and PGRP-SC1 encode active amidases and others, such as PGRP-SB2, and PGRP-SC2 have predicted amidase activity (Werner et al. 2000, Mellroth et al. 2003). These proteins have been suggested to modulate Imd pathway signaling by reducing the immunostimulatory capacity of PGN (Mellroth et al. 2003, Bishoff et al. 2006, Zaidman-Rémy et al. 2006). Yet another family member, PRGP-LF, has been reported to suppress both Imd and JNK signaling (Persson et al. 2007, Maillet et al. 2008), but the mechanism of this action is not known. PGRP proteins suppressing the Imd pathway response have different specificities for PGN. For example, PGRP-LB is capable of binding the DAP-type PGN only, whereas PGRP-SC can digest both DAP-type and Lys-type PGNs (Mellroth et al. 2005, Zaidman-Rémy et al. 2006).

PGRP-LB is expressed in the fat body, from which the protein is secreted into the hemolymph (Zaidman-Rémy et al. 2006). In addition, both *PGRP-LB* and *PGRP-SC* are expressed in the gut epithelium (Bischoff et al. 2006, Zaidman-Rémy et al. 2006). Since the fly must tolerate the presence of a certain amount of commensal bacteria and the PGN fragments that they release, this kind of regulation appears to be necessary in order to avoid excess and redundant Imd pathway signaling that might trigger the production of AMPs and even provoke apoptosis in non-infective conditions. Indeed, the effects of RNAi silencing both PGRP-LB and PGRP-SC *in vivo* were quite dramatic. Depletion of these proteins led to prominent expression of AMPs under normal rearing conditions (Zaidman-Rémy et al. 2006) and during infection (Bischoff et al. 2006, Zaidman-Rémy et al. 2006,). In addition, most of the PGRP-SC-deficient larvae succumbed to *Erwinia carotovora* natural infection, and the minority that survived until adulthood showed developmental defects, such as wing notching. These defects were shown to be due to increased apoptosis in the wing disc in response to bacterial infection (Bischoff et al. 2006), which highlights the importance of properly adjusted immune signaling for the host's survival.

In mammals, NF-κB signaling is regulated by post-translational modifications of the pathway proteins as well as the IκB proteins, and this regulation is tissue-specific and delicately controlled. The *Drosophila* transcription factor Relish differs from the other NF-κB proteins. It contains both the NF-κB and IκB parts, and is activated when the IκB part is enzymatically cleaved. This reaction is not reversible, and therefore it seems reasonable to assume that fine-tuning of the Imd pathway activity requires the presence of other negative regulators besides the IκB.

The caspase Dredd is required for the cleavage and activation of both IMD and Relish (Ertürk-Hasdemir et al. 2009, Paquette et al. 2010), which makes it a good target for negative regulation. Two proteins have so far been proposed to modulate Dredd activity: the Defense repressor 1 (Dnr1), and Caspar (Foley and O'Farrell 2004, Kim et al. 2006). Dnr1 is a conserved protein, which consists of an ezrin/radixin/moesin domain and a C-terminal RING finger domain, suggesting that Dnr1 might function as an E3 ligase. It has been shown that RNAi silencing of Dnr1 in S2 cells is sufficient to induce the expression of a *Dpt*-lacZ reporter construct in the absence of infection (Foley and O'Farrell 2004). However, the molecular mechanism of the inhibitory action of Dnr1 is not thoroughly understood, neither has it been verified *in vivo*. The function of the other Dredd regulator, Caspar, has been documented better. Caspar is homologous to the human Fas associated factor 1 (FAF1), which has been reported to associate with signaling components of the NF-κB pathway, such as FAS, FADD, caspase-8 and NF- κB (Chu et al. 1995, Ryu et al. 2003, Park et al. 2004b). Caspar was identified in a screen of *Drosophila* mutants for hyperactivated immune responses, in which *caspar* mutant flies were found to express *AMP*s constitutively even in the absence of infection. In addition, the mutants were more resistant to Gram-negative bacterial infections than the wild-type flies. Overexpression of Caspar was sufficient to suppress the Imd pathway

activity but did not affect Toll pathway activation. Furthermore, Caspar was shown to inhibit the Dredd-dependent cleavage of Relish *in vivo* (Kim et al. 2006). The inhibitory mechanism of Caspar has not been revealed yet. However, the association of the human homolog FAF1 in ubiquitin-proteasome signaling suggests that Caspar might have similar functions in *Drosophila* (Song et al. 2005).

The ubiquitin-proteasome pathway has been implicated in Imd pathway regulation also in *Drosophila*. Khush et al. (2002) performed an EMS mutagenesis screen in order to identify negative regulators of the Imd pathway, and found two independent mutations of *skpA* that resulted in the constitutive expression of *Dpt* in larvae and adults. SkpA is homologous to the mammalian and yeast Skp1 proteins, which are known components of Skp1/Cullin/F-box protein (SCF)-E3 ubiquitin ligases (reviewed in Cardozo and Paganino 2004). The SCF complex is involved in the K48-linked ubiquitination of proteins targeting them for proteasomal degradation. Khush et al. further demonstrated that a deficiency in other components of the SCF complex, such as dCullin or Slimb, resulted in the expression of *Dpt* in a non-infective state. In addition, RNAi silencing of *skpA* or *slimb* was shown to increase the levels of both full-length and cleaved Relish suggesting that the SCF complex might regulate the stability of Relish and thereby modulate the Imd pathway activity (Khush et al. 2002).

Another target for ubiquitin-mediated regulation was recently found to be IMD itself. Similar to the mammalian RIP1, IMD is activated by the conjugation of K63-linked polyubiquitin chains. In mammalian cells, these ubiquitin chains are recognized by TAB2, which thereafter recruits TAK1 into the complex (Legler et al. 2003, Ea et al. 2006, Newton et al. 2008). The constitutive and non-specific activation of IMD was reported to be suppressed by Scrawny (Scny), also called dUSP36, which is homologous to mammalian ubiquitin-specific proteases (Thevenon et al. 2009). The authors suggest that the removal of K63-linked ubiquitin chains would sensitize IMD to K48-linked ubiquitination and promote its proteasomal degradation. Since dUSP36 acts at the level of IMD, it is not surprising that it was found to suppress JNK signaling as well (Thevenon et al. 2009).

In addition to the previously described suppressors, Imd pathway activity is dampened by regulating the transcriptional activity of Relish. The transcription factors of the JNK and Jak/STAT signaling pathways, AP-1 and STAT, have been implicated in this type of regulation together with other proteins. Kim et al. (2007) suggested that as a response to continuous immune signaling causing increased levels of dAP1 and Stat92E (*Drosophila* homolog of STAT), these proteins would form a repressosome complex with a *Drosophila* High mobility group (HMG) protein called Dorsal switch protein 1 (Dsp1). The complex would replace Relish at the promoter and recruit a histone deacetylase to the complex to inhibit transcription of the target genes. Mutations in dAP-1, Stat92E or Dsp1 were shown to induce hyperactivation of Relish target genes. Furthermore, the mutant flies were more susceptible to *E. coli* infections compared to wild-type

flies. This phenotype could be rescued by reducing the copy number of *relish*, suggesting that the reduced survival of the repressosome complex mutants was in fact due to the defective regulation of Relish, and not the inhibited expression of dAP-1 or Stat92E target genes (Kim et al. 2007). The positive and negative regulators of the Imd pathway are summarized in Figure 5.

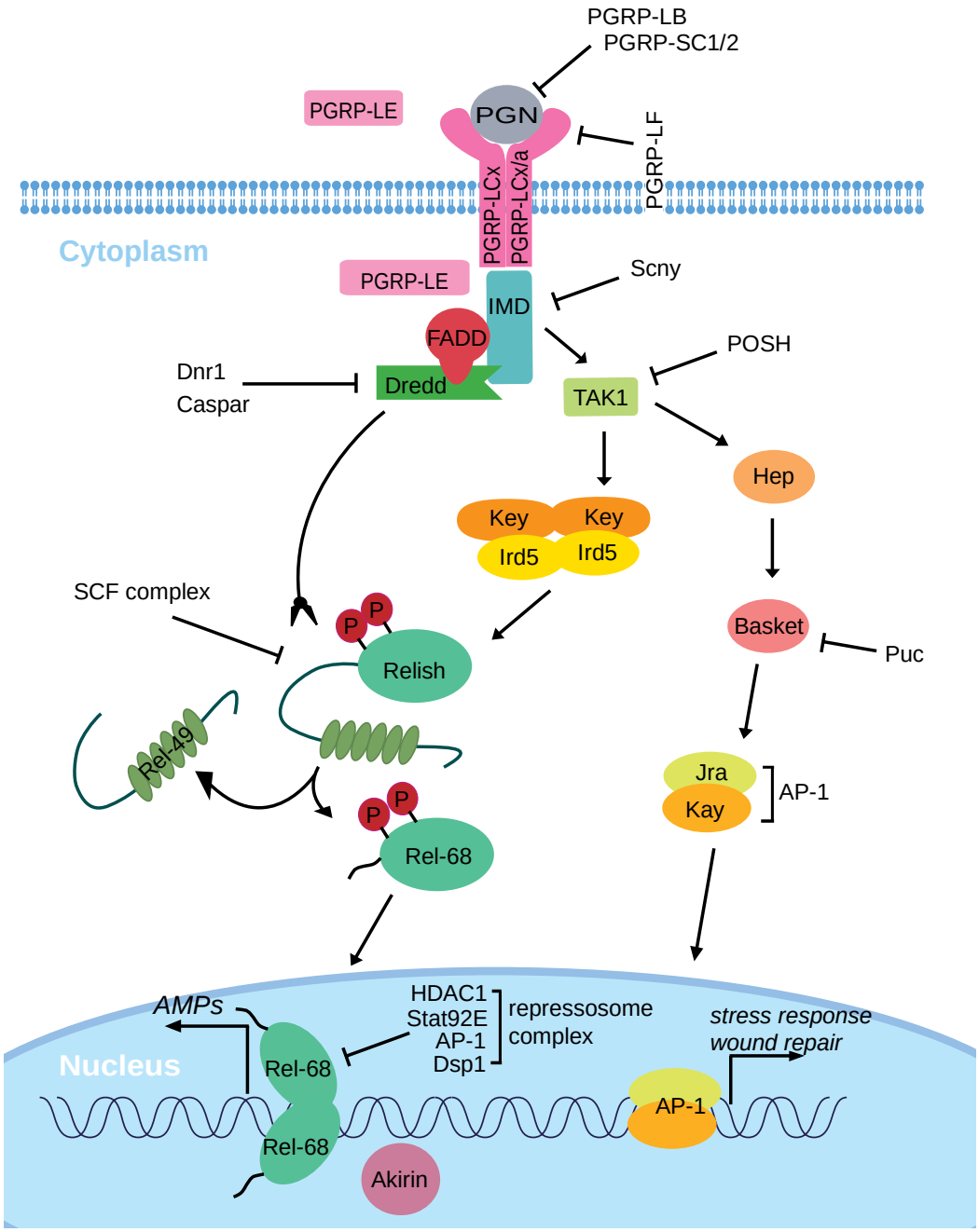

Figure 5. Regulation of the Imd/JNK pathway.

2.6.6. Tissue-specific immune response

The fat body is the main tissue responsible for the synthesis of AMPs and other immunomodulatory molecules during a systemic immune response in *Drosophila*. However, to prevent microbes from entering the body, other tissues, especially the barrier epithelia, are capable of producing AMPs. The living environment and diet of *Drosophila* mainly consist of fermenting fruits that are rich in bacteria, yeast and mold. The local immune response in epithelia and especially in the gut is therefore vital for the fly's survival.

The first evidence of AMPs produced in epithelia was reported by Ferrandon et al. (1998). They used a *Drs*-GFP reporter fly line to monitor the expression of *Drosomycin*, which at the time was thought to be solely a Toll pathway target gene. Expression of *Drs* was detected in a variety of epithelial tissues that are in direct contact with the environment, such as the epithelia of the respiratory, digestive and reproductive tracts. Furthermore, this expression was independent of the Toll pathway, suggesting another yet unidentified regulatory mechanism for the local immune response (Ferrandon et al. 1998). These findings were soon confirmed and expanded by Tzou et al. (2000), who studied the tissue-specific expression of all *Drosophila* AMP types. They detected localized AMP expression in both unchallenged larvae and adults, but also noticed tissue-dependent differences in the expression patterns of different AMPs. In larvae, *Dro* and *Drs* were often observed in tracheal epithelia, while *Def* and *Mtk* were detected in the oral region and sometimes in the pharynx. The expression of *Dpt*, *Def* and *Att* was prominent in the digestive tract. In adults, most of the AMPs were expressed in the reproductive tract, suggesting that it too could serve as an entry siten for pathogens. In addition, *Def* and *Mtk* were detected in the labelar glands, *Dpt* in the gut, and *Cec* and *Mtk* in the malpighian tubules. Importantly, the tissue-specific expression of AMPs was found to be absent in *imd* mutant flies, suggesting that the local immune response is mediated by the activation of the Imd pathway (Tzou et al. 2000).

AMP production in epithelial tissues appears to be an evolutionarily conserved feature that is seen in both invertebrates and vertebrates. Instead, secretion of AMPs into the hemolymph during systemic infection seems to be a defense response that is typical only to insects. It has therefore been proposed that the epithelial immune response would actually be of a more ancient origin, and the recruitment of the fat body and specialized cell types in response to AMP release has occured later in evolution (Tzou et al. 2000).

The *Drosophila* gut harbors a considerable amount of different kinds of microbes (Cox et al. 2007, Ryu et al. 2008). Like other microorganisms, the ones in the gut continuously generate MAMPs that can potentially evoke a massive defense response in the gut epithelial cells. The constitutive expression of *AMP*s in the gut can be detected, but at a surprisingly low level. Maintenance of the flies in axenic conditions completely abolishes this expression, which suggests that it is after all conditional and triggered by the commensal microbiota (Ryu et al. 2008). Imd pathway activation must therefore be dampened in the gut, and

this is achieved by the synergistic action of various proteins. PGN degrading PGRPs, such as PGRP-LB and PGRP-SC, have been shown to be expressed especially in the gut (Bischoff et al. 2006, Zaidman-Rémy et al. 2006). In addition, the intestinal homeobox protein Caudal specifically suppresses AMP production in the gut, simultaneously allowing the expression of NF-κB-independent target genes. Uncontrolled AMP production in the gut appears to have severe consequences for the host. *Caudal* knock-down has been reported to induce AMP expression, which in turn reduces the levels of *Acetobacteriae* that normally represent the majority of the bacteria colonizing the gut. The freed niche is consequently invaded by *Gluconoacetobacter*, which by an unknown mechanism provokes apoptosis of the gut epithelia resulting in the death of the host (Ryu et al. 2008). This implies that it is not AMP production itself but its deleterious effects on the homeostasis of commensal microbes, which kills the fly. It has also been suggested that the battery of AMPs only serves as a back-up system to protect the gut from pathogenic bacteria, and that the levels of commensals would be mainly regulated by the production of reactive oxygen species (ROS). ROS production in flies requires the protein Duox (Ha et al. 2005a), which contains an NADPH domain and an extracellular peroxidase domain (PHD). PHD can convert hydrogen peroxidase into hypochlorous acid that is a potent microbicide. Excessive production of ROS is regulated by the immune responsive catalase (IRC) (Ha et al. 2005b). Imd pathway mutant flies are susceptible to ROS-resistant microbes but can normally tolerate the presence of ROS-sensitive ones (Ha et al. 2005, Ryu et al. 2006) underscoring the efficiency and importance of ROS for the local immune response *in vivo*.

3. Aims of the study

Overall, the aim of this study was to gain a genome-wide view of the defense strategies and the regulation of the immune system of the fruit fly *Drosophila melanogaster*. Our studies focused on the positive and negative regulation of the Imd pathway, which in *Drosophila* mainly responds to Gram-negative bacterial infections. Although the Imd pathway, and insect immunity in general, have been under intense investigation during the last decades, all of the components had not been thoroughly studied, and especially the negative regulation was poorly understood at the time this project was launched. Moreover, the intracellular parts of the Imd pathway resemble the mammalian TNFR signaling pathway, and hence we were also hoping to acquire some useful data regarding the regulation of mammalian immune signaling.

The specific aims of the study were:

1. To identify changes in gene expression during Gram-negative bacterial infection of *Drosophila melanogaster in vitro* by oligonucleotide microarrays.

2. To identify previously unknown components of the Imd pathway by RNAi screening.

3. To molecularly characterize the selected target genes *in vitro* and *in vivo* in order to understand their role in the regulation of the Imd pathway signaling.

4. Materials and methods

4.1 Cells and cell culture (I-III)

Schneider-2 (S2) cells are *Drosophila* hemocyte-like cells, which are derived from embryonic cells and are able to phagocytose. The S2 cells used in our laboratory were originally brought from R. A. Ezekowitz's laboratory (Harvard Medical School, Boston, MA, USA) in 2002, and are known to phagocytose better than the ATCC line of S2 cells. S2 cells were used in most of the *in vitro* experiments, except in the western blot analysis of Relish cleavage in original publication III. In that experiment we used another *Drosophila* hemocyte-like cell line, malignant blood neoplasm-2 (mbn-2) cells (Gateff et al, 1978), because these cells contain less cleaved Relish under normal conditions, and therefore the results were easier to interpret. Mbn-2 cells are derived from circulating larval hemocytes and like S2 cells, they have retained the ability to phagocytose and express antimicrobial peptide genes.

S2 cells were cultured in Schneider medium (Sigma) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µl/ml streptomycin. For mbn-2 cells, the medium was subsequently supplemented with L-glutamine (Sigma) to a 10 mM concentration. Both S2 and mbn-2 cells were cultured at +25ºC.

4.2 dsRNA synthesis (I-III)

4.2.1 Generation of the dsRNA library

The dsRNAs used in the RNAi screen (III) were synthesized using an S2 cell-derived cDNA library cloned into pcDNAI plasmids (Invitrogen) as template (Pearson et al. 1995). The library was transformed into competent MC1061/P3 *E. coli*, and the bacteria were plated onto ampicillin (20 µg/ml) and tetracycline (8 µg/ml) containing LB plates. Subsequently, individual bacterial colonies were collected and the plasmids from these randomly picked colonies were isolated. The inserted sequence was amplified by PCR using the following primers: 5'-CAA GCT TGG TAC CGA GCT C-3' and 5'-CTG CTC CCA TTC ATC AGT TC-3'. Then, binding sites for T7 RNA polymerase were introduced by performing the second round of PCR with the following nested primers: 5'-TAA TAC GAC TCA CTA TAG GGC GGA TCC ACT AGT AAC GG-3' and 5'-

TAA TAC GAC TCA CTA TAG GGA GGT GAC ACT ATA GAA TAG G-3'. The resulting PCR product was used as a template in an *in vitro* transcription reaction performed using the T7 MegaScript RNA polymerase kit (Ambion) according to the manufacturer's instructions.

4.2.2 Generation of targeted dsRNAs

Targeted dsRNAs were generated using gene specific primers (see original communication III, Supplementary Table IV). S2 cell-derived cDNA was used as template. dsRNA against green fluorescent protein (GFP) was used as a negative control, and it was generated using pMT/BiP/V5-His/GFP plasmid (Invitrogen) as template. PCR and the synthesis of dsRNAs were performed as in the generation of the dsRNAs library.

4.3 Oligonucleotide microarrays (I, II)

S2 cells were seeded onto 24-well plates (500,000 cells per well) and 4 µg of experimental or control dsRNA was applied to the cell culture medium. After 48 h heat-killed BL21 *E. coli* were added and the cells were incubated for 0.5, 1, 4, 24 or 32 h. Uninduced cells were used as the 0 h time point. At given time points the cells were harvested and total RNAs extracted (from ~$2x10^6$ cells per sample) using the RNeasy Mini Kit (Qiagen CA, USA). The RNA samples were sent to the University of Oulu, where the expression analysis was performed using the Affymetrix (Santa Clara) *Drosophila* Genechips according to the Affymetrix GeneChip Expression Analysis Technical Manual.

4.4. Sequencing (III)

Sequencing of constructs and genomic DNA samples was performed as follows: 0.3 µg of DNA was applied to a PCR reaction mix containing 1 µl of either upstream or downstream primer (final conc. 0.2 µM), 1µl of BigDye v. 3.1 Reaction mix, 2 µl of 5xDilution buffer and nuclease free H_2O up to 10 µl. The PCR was performed according to the manufacturer's instructions. The synthesized DNA fragments were precipitated with 90 µl of 70% EtOH (Final EtOH conc. ~63%) for 15-20 min in RT. The precipitate was harvested by centrifugation (3,000 x g, 60 min. at 20ºC), and the supernatant was discarded. The precipitate was washed with excess 70% EtOH, centrifuged at 2000 x g for 15 min, and the EtOH was discarded. The left-over EtOH was removed by centrifuging the tubes up-side-down on a pile of filter papers for 1 min at 700 x g. The precipitate was then dissolved into 15 µl of HiDi Formamide (Applied

Biosystems), denatured at 95ºC for 2 min. and cooled on ice before inserting the tubes into the sequencer (ABI Prism 3130x Genetic Analyzer). The sequencing was performed using POP7 polymer with a 36 cm capillary according to the instrument user manual.

4.5 Western blotting (II, III)

Protein samples were electrophoresed using NuPAGE 10% Bis-Tris gels and running buffer supplemented with NuPAGE antioxidant (Invitrogen Life Technologies) and blotted onto a nitrocellulose membrane. The membrane was thereafter incubated for 2h in blocking buffer (5% milk powder in PBST) before adding the 1:5000 diluted primary antibody to the blocking buffer. The primary antibodies used were mouse anti-V5 (Invitrogen Life Technologies) (II) and mouse anti-C-Relish antibody (a gift from Dr. Svenja Stöven from the Umeå University) (III). The primary antibody was incubated on a rocking platform for 1.5 h, then rinsed twice and washed 3 x in PBST for 5 min. The secondary antibody, also diluted 1:5000 in blocking buffer, was added to the membrane and incubated for 1 h before rinsing twice and washing 5 x 5 min in PBST. The secondary antibodies used were the goat anti-mouse Ab HRP (Molecular Probes) (II) and anti-rabbit Ab HRP conjugates (Bio-Rad). The bound antibodies were detected with the ECL Plus Western blotting detection system (GE Healthcare).

4.6 Epistasis analysis

The epistasis analyses were carried out *in vitro*. S2 cells were transfected with experimental or control (*GFP*) dsRNA together with constructs encoding known components of the Imd pathway, such as full-length IMD, Relish or a truncated constitutively active form of Relish, Rel ΔS29-S45. The effect of the dsRNA treatment to the Imd pathway activity was assessed by luciferase assay, and therefore the S2 cells were also transfected with the *Attacin*-luciferase reporter together with the *Actin 5C*-β-galactosidase reporter, which was used for monitoring cell viability.

4.7 Assays for measuring Imd pathway activation

4.7.1 Reporter assays (I-III)

Luciferase reporter-based assays were used for measuring the Imd, Toll, and Jak-STAT pathway activities *in vitro*. *Actin 5C*-β-galactosidase (*Act5C*- β-gal) was used in all experiments to control cell viability. The reporter constructs were kindly provided by Prof J-L. Imler.

To study the Imd pathway, *Attacin A*-luciferase (*AttA*-luc) (Tauszig et al, 2000) or *Cecropin A1*-luciferase reporters were used. The S2 cells were transfected with luciferase and β-galactosidase reporters together with control or experimental dsRNA using the Fugene transfection reagent (Roche). *GFP* dsRNA was used as the negative and *Relish* dsRNA as the positive control. After 48 h, 20-hydroxyecdysone (Sigma) was added to a 1 μM concentration to promote immune competence (Dimarcq et al. 1997), and 60 h after the transfection the Imd pathway was activated by adding heat-killed BL21 *E. coli* to the cells. 90 h post-transfection the S2 cells were harvested by centrifugation and lysed in Passive Lysis Buffer (PLB) (Promega).

For the analysis of Toll pathway activity S2 cells were transfected with *Drosomycin*-luciferase (*Drs*-luc) reporter and, to activate the pathway, with the constitutively active form of Toll, Toll10B (Rosetto et al, 1995). *GFP* and *Myd88* dsRNAs were used as negative and positive control dsRNAs, respectively. The S2 cells were harvested by centrifugation 72 h after transfection and lysed in PLB.

Jak-STAT pathway activation was assessed by transfecting the S2 cells with the *TurandotM*-luciferase (*TotM*-luc) reporter together with constitutively active hopscotch, which activates the pathway. *GFP* and *STAT* dsRNAs were used as negative and positive control dsRNAs, respectively. The S2 cells were harvested 72 h post-transfection and lysed in PLB.

The cell lysates were centrifuged to remove the cell debris, and luciferase and β-galactosidase activities were measured from the cleared lysates.

4.7.2 Semi-quantitative RT-PCR (I) *and quantitative RT-PCR (qRT-PCR)* (II)

Semi-quantitative PCR was performed using SuperScript™ II One-Step RT-PCR with Platinum Taq kit (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen) with a LightCycler (Roche) or an ABI7000 (Applied Biosystems) instrument. The results were analyzed with the LightCycler version 3.5 software or the ABI 7000 System SDS software version 1.2.3., respectively. Total RNA extracted either from S2 cells or flies with TRIzol® Reagent (Invitrogen) was used as a template in both semi-quantitative and qRT-PCR.

4.7.3 Immunocytochemistry (III)

S2 cells were cultured on a 24-well plate with glass slides on the bottom of the wells. 4 μg of control or experimental dsRNA was introduced to the cells by soaking, and the cleavage of Relish was induced after 72 h by adding heat-activated LPS (10 μg/μl) to the cells. 10 min. after the induction the cells were spun down onto the glass slides, fixed and labeled with an anti-RHD antibody (Stöven et al. 2000).

4.8 *In vivo* experiments

4.8.1 Drosophila *stocks* (I-III)

Canton S, *Relish*^{E20}, and *w*^{1118} flies were obtained from Prof. Dan Hultmark from the Umeå University. *Iap2* null mutant flies were a gift from Dr. Francois Leulier (Centre de Génétique Moléculaire, Centre National de la Recherche Scientifique, Gif-Sur-Yvette, France).

RNAi fly lines *15678-R1* and *15678-R2* for *pirk*, as well as *Iap2-IR* and *dFADD-IR* were obtained from Prof. Ryu Ueda (National Institute of Genetics, Mishima, Shizuoka, Japan). The genetic background of these flies is *w*^{1118}.

C564-GAL4 driver flies were obtained from Prof. B. Lemaitre (Centre de Génétique Moléculaire, Centre National de la Recherche Scientifique, Gif-Sur-Yvette, France, and Global Health Institute, EPFL, Lausanne, Switzerland).

Pirk overexpression flies were generated by microinjecting a *UAS-pirk-V5* construct into *w*^{1118} embryos in the Umeå Fly and Worm Transgene Facility, Umeå, Sweden. The independent Pirk-overexpressing fly lines UAS-pirk^{221}, UAS-pirk^{351}, UAS-pirk^{432}, and UAS-pirk^{721} harbor the constructs in their 3rd, X, 3rd and 2nd chromosomes, respectively.

P-element lines *4440*, *14838*, and *15039* were ordered from the Bloomington Drosophila Stock Center at Indiana University. Δ2-3 transposase carrying stocks were received from Prof. Dan Hultmark. *Rescue*^{82} and *rescue*^{461} flies were generated by mobilizing the *EY00723* P element in *15039* flies and screening for white-eyed offspring after standard crossings.

4.8.2 RNA extraction from flies (II)

Equal amounts of male and female flies were anesthetized with CO_2, collected into microtubes and snap-frozen by immersing the tubes in dry ice. The frozen

flies were then homogenized in TRIzol® Reagent (Invitrogen), and the extraction of total RNA was carried out according to the manufacturer's instructions.

4.8.3 DNA extraction from flies (II)

Genomic DNA was extracted from 25 snap-frozen flies. At first, the flies were homogenized on ice in a buffer containing 0.1M Tris-HCl (pH 9.0), 0.1M EDTA, and 1% SDS. The homogenate was then incubated at 70ºC for 30 min, KAc was added, and again the homogenate was incubated on ice for 30 min prior to centrifugation at 16,000 x g for 15 min. The supernatant was then transferred to another tube and the DNA extracted by adding 1 volume of 25:24:1 phenol-chlorophorm-isoamyl alcohol, and centrifuged at 16,000 x g for 5 min. The supernatant containing the DNA was transferred to a clean tube, and the DNA was precipitated by adding 0.6 x volume of isopropanol. The DNA pellet was harvested by centrifugation (5 min at 16,000 x g), and washed with an excess (1 ml) of 70% EtOH prior to drying and resuspending in TE buffer or water.

4.8.4 Protein extraction from flies (II)

Total protein was extracted from flies by homogenizing 10 snap-frozen flies in 80 µl of lysis buffer containing 10 mM Tris (pH 8.0), 140 mM NaCl, 1% Triton X-100, and the Complete Mini protease inhibitor cocktail (Roche Applied Science). The homogenate was incubated on ice for 45 min and centrifuged at 16,000 x g for 15 min to remove solid particles. The supernatant was pipetted into a new tube, and the protein concentration of the supernatant was measured by using the BCA protein assay kit (Pierce).

4.8.5 Infection assays (I-III)

One-week-old healthy flies were infected by pricking them with a thin tungsten needle dipped into a fresh, concentrated bacterial culture. For survival assays, the flies were kept at 25ºC and monitored regularly for 72 h. *E. coli* and *Enterobacter cloacae* were used as representatives for Gram-negative bacteria, and *Micrococcus luteus* as Gram-positive bacteria. Equal amounts of males and females were used in all experiments

5. Results

5.1 Gram-negative bacteria-induced changes in the gene expression profiles in S2 cells (I)

Organisms, single and multicellular, have developed ways of responding to environmental changes and combatting intruders. The response involves multiple changes in gene expression, and varies naturally depending on the stimulus. In addition, transcriptional changes are often specific to a certain tissue and cell type. To get an overview of the signaling events and genes involved in the defense mechanisms of *Drosophila* against Gram-negative bacteria, we chose S2 cells as our model system. S2 cells are derived from embryonic cells and share many features with hemocytes, like for instance the ability to phagocytose. Microbial attack was mimicked by adding heat-killed Gram-negative bacteria, namely *E. coli*, to the cell culture medium. The cells were collected at different time points, total RNA was extracted, and gene expression levels were analyzed by oligonucleotide microarrays.

In total, the expression levels of 47 transcripts were greater than two-fold at the 4h time point compared to naïve cells (I Table 1). The Imd pathway transcription factor *relish* was among these genes, which suggests a positive feedback loop in Imd signaling. In S2 cells, where Relish was removed by RNAi, only 14 of these transcripts were induced more than two-fold. Based on this, the 46 upregulated transcripts were further divided into 32 Relish-dependent and 14 Relish-independent genes. The group of Relish-dependent genes includes many well known immune responsive genes, such as the antimicrobial peptides *Attacin B, Diptericin B, Attacin D, Metchnikowin, Cecropin B, Drosomycin,* and *Drosocin*, and the PGRP-proteins *PGRP-LB, PGRP-SB1, PGRP-SD*, and *PGRP-LF*. The group of Relish-independent genes includes the matrix metalloproteinase *Mmp1*, *wunen 2*, *sulphated,* and *Annexin IX,* which are predicted to be involved in multiple cellular functions. The rest of the identified genes have unknown functions.

The general trend among the Relish-independent genes was that their transcript levels reached their maximum before the 24 h time point. In contrast, the transcript levels of the Relish-dependent genes were either still increasing or sustained at high levels after 24 h. Therefore the Relish-independent and Relish-dependent genes are also referred to as the early response genes, and the sustained response genes, respectively (I Table 1). The only exception to the rule was the gene *CG15678*, which we call *pirk* (poor Imd response upon knock-in).

Expression of *pirk* peaked already at 1 h and decreased before 24 h. Previously, we have demonstrated that removing Pirk from cells by RNAi leads to increased Imd, but not Toll signaling (Kallio et al. 2005). This led us to speculate if *pirk* played a role in the negative regulation of the Imd signaling pathway.

5.2 Identification of CG15678/Pirk as a negative regulator of the Imd pathway (I, II)

Drosophila has a highly sophisticated and efficient immune response. Immunity, however, can not be achieved without costs. For example, Ye et al. (2009) reported that flies, which had been selected for an improved defense against the bacterium *Pseudomonas aeruginosa*, showed reduced longevity and larval viability compared to control flies. The improved immunity was also rapidly lost in the absence of selection. In addition, Valtonen at al. (2010) demonstrated that $Relish^{E20}$ flies, which fail to produce Imd pathway-mediated antimicrobial peptides, lived longer under starvation compared to the wild-type flies. Thus, maintaining an adequate level of disease resistance and concurrently avoiding unwanted and prolonged responses that would harm the host requires delicate regulation of the immune signaling.

Mammalian TNFR signaling, which shares many similarities with *Drosophila* Imd signaling, is regulated temporarily by a negative feedback loop. The TNFR signaling pathway transcription factor induces the transcription of the inhibitory protein IκB, which sequentially binds to NF-κB and displaces it from the DNA binding sites. In *Drosophila*, the Imd pathway transcription factor Relish contains both the NF-κB and inhibitory domains, and is activated by cleavage of the inhibitory part of the protein. We therefore hypothesized that the putative negative feedback loop in the Imd pathway might act at a different level than in the mammalian system and involve still unknown regulators.

Previously, we showed that *pirk* expression was highly induced in *Drosophila* S2 cells in response to Gram-negative bacteria (Kallio et al. 2005), and that the induction was Relish-dependent (II Fig. 1). To confirm this *in vivo*, we infected wild-type (*Canton S*) and mutant flies lacking the functional transcription factor Relish ($Relish^{E20}$) with Gram-negative bacteria, and compared the levels of the *pirk* transcript at different time points (II Fig. 1C) by qRT-PCR. In *Canton S* flies, the expression of *pirk* was greatly induced, the relative induction being 23-fold at the 4 h time point. In contrast, *pirk* expression in $Relish^{E20}$ mutant flies was below the level of detection.

To study if Pirk was important to the Imd signal regulation, we first analyzed the effects of knocking down *pirk* in S2 cells in luciferase-based reporter assays (Fig. 6, II Fig. 2A). In the luciferase reporter constructs the *luciferase* coding sequence is under the control of a promoter region of an Imd pathway dependent antimicrobial peptide gene, such as *Attacin A* or *Cecropin A1*. The promoter activity can thus be quantitated by measuring luciferase activity from the lyzed

cells by a luminometer. We transfected S2 cells with either control (*GFP* as negative and *relish* as positive control), or *pirk* dsRNAs together with the reporter construct. Imd pathway activation was triggered by adding heat-killed *E. coli* to the cells. As Figure 6 shows, in cells treated with *pirk* dsRNA, *CecA1* promoter activity was significantly higher than in *GFP* dsRNA-treated cells. The results were similar when we used the *AttA* promoter (II Fig. 2A), which suggests that the higher activity is not limited to *CecA1*.

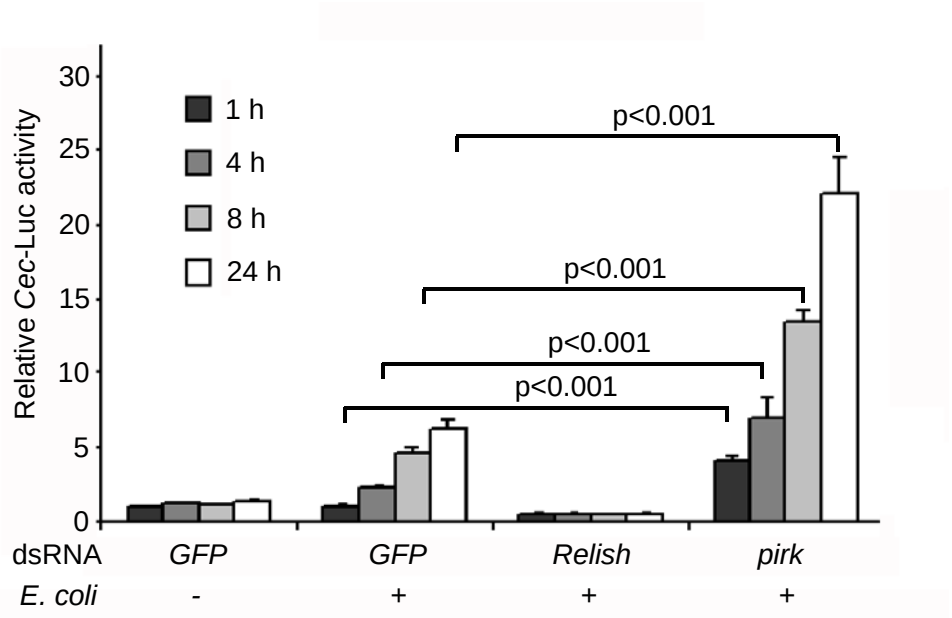

Figure 6. Pirk is a negative regulator of the *Drosophila* Imd pathway in vitro. *pirk* RNAi increases Imd pathway-mediated *CecA1* response at 1, 4, 8 and 24 h compared to *GFP* controls. S2 cells were transfected with a *CecA1*-luc reporter plasmid and treated with dsRNAs. Activation of the Imd pathway was induced 48 h later by adding heat-killed *E. coli* in the cell culture medium. *GFP* and *Relish* dsRNAs were used as negative and positive controls, respectively.

Next, we studied whether the effect of *pirk* RNAi was specific to the Imd pathway. We transfected S2 cells with reporter constructs specific to the Toll and Jak/STAT pathways together with control and *pirk* dsRNAs. *pirk* RNAi had no effect on the Toll pathway. In contrast, Jak/STAT signaling was significantly reduced by more than 30%. This implies that Pirk is not a general inhibitor of signaling in S2 cells (II Fig. 2F-G).

To eliminate the possibility that these results were merely a reporter construct-related artifact, we monitored the levels of AMP gene expression also by qRT-PCR. S2 cells were transfected with either *pirk* or control (*GFP*) dsRNA, and the Imd pathway was activated by adding heat-killed *E. coli* to the cell culture medium. The cells were collected at 0, 1 h, 4 h, and 24 h after induction, and total RNA was extracted and used as template in one-step qRT-PCR. In *GFP* dsRNA-treated cells, the expression profile of *pirk* was as expected: strongly induced already at 1 h, reaching its maximum at 4 h, and then decreasing. Some *pirk* expression was detected also in *pirk* dsRNA-treated cells,

yet it was significantly suppressed at all time points. The expression levels of the Imd pathway-dependent AMP genes *Attacin B* (*AttB*) and *Diptericin B* (*DptB*), were significantly higher in *CG15678* knock-down cells compared to the control at the 4 h and 24 h time points (II Fig. 2B-D). Furthermore, this effect was also seen with *Metchnikowin* (*Mtk*) expression at 24 h (data not shown). This indicates that the RNAi-induced reduction in the *pirk* transcription is sufficient to decrease the protein levels, which leads to increased Imd pathway signaling.

To study whether overexpression of *pirk* suppressed the Imd pathway signaling, we transfected S2 cells with a metal-inducible *pMT-pirk* construct together with the *AttA*-luciferase reporter construct. An empty *pMT* vector was used as control, and *Actin5C*-β-galactosidase reporter construct was used to assess cell viability. *pirk* expression was induced by adding $CuSO_4$ to a 300 μM concentration and the Imd pathway was activated by heat-killed *E. coli*. Overexpression of *pirk* reduced the Imd pathway activity more than 70% (II Fig. 2E). Altogether these results indicate that Pirk suppresses Imd pathway signaling *in vitro*.

5.2.1 Pirk suppresses Imd pathway activation in vivo *(II)*

To examine if Pirk suppressed the Imd pathway signaling also *in vivo*, we analyzed the Imd pathway response of Pirk mutant flies. Fly line *15039* from the Bloomington Stock Center carries a P element at the 5' untranslated region (UTR) of the *pirk* gene. We hypothesized that this might affect the transcription of *pirk* and sequentially alter the fly's defense responses to Gram-negative bacteria. Therefore we infected *15039* and wild-type (*Canton S*) flies by pricking them with a needle dipped into a concentrated culture of *E. cloacae,* extracted the total RNAs at 0, 1h, 4 h, and 8 h post-infection, and measured the transcript levels of *pirk* and *AMP*s by qRT-PCR. In *Canton S* flies, the level of the *pirk* transcript increased upon infection, reaching its maximum at 4 h. In contrast, no induction was detected in *15039* flies (Fig. 7A). Furthermore, in *15039* flies the transcript levels of *AttA*, *Dpt*, *Mtk*, and *Dro* did not differ from *Canton S*, but the levels of *AttB*, *AttC*, *AttD*, *CecB,* and *DptB* were elevated (Fig. 7).

P elements are transposable elements, which naturally occur around the fly genome and can jump and integrate into different loci. The structure and integration mechanisms of naturally occurring P elements have been used in creating transgenic flies but also in designing artificial P elements that then have been introduced into the fly genome in order to disrupt the function of a gene. Fly line *15039* had been generated for the latter purpose. To ensure that the increase in the AMP expression that we detected was in fact due to low expression of *pirk* and not the genetic background of the flies or an unknown environmental factor, we aimed at mobilizing this P element. In this case, P element mobilization could result in precise excisions that would rescue *pirk* transcription. However, sometimes the mobilized P element removes part of the neighbouring sequence and creates a deletion mutant. We therefore hoped that

we would simultaneously rescue the P element phenotype and obtain a *pirk* knock-out fly. Curiously, we obtained five precise excisions, but no knock-out line. Two of the precise excision lines, *rescue*^{82} and *rescue*^{461} were studied further. Removal of the P element from the 5' UTR restored *pirk* expression levels in both rescue lines (Fig. 8A). Surprisingly, we did not detect significant differences in the transcript levels of *AttB* or *DptB* when comparing *15039* flies to the rescue flies with restored *pirk* activity (Fig. 8B and C).

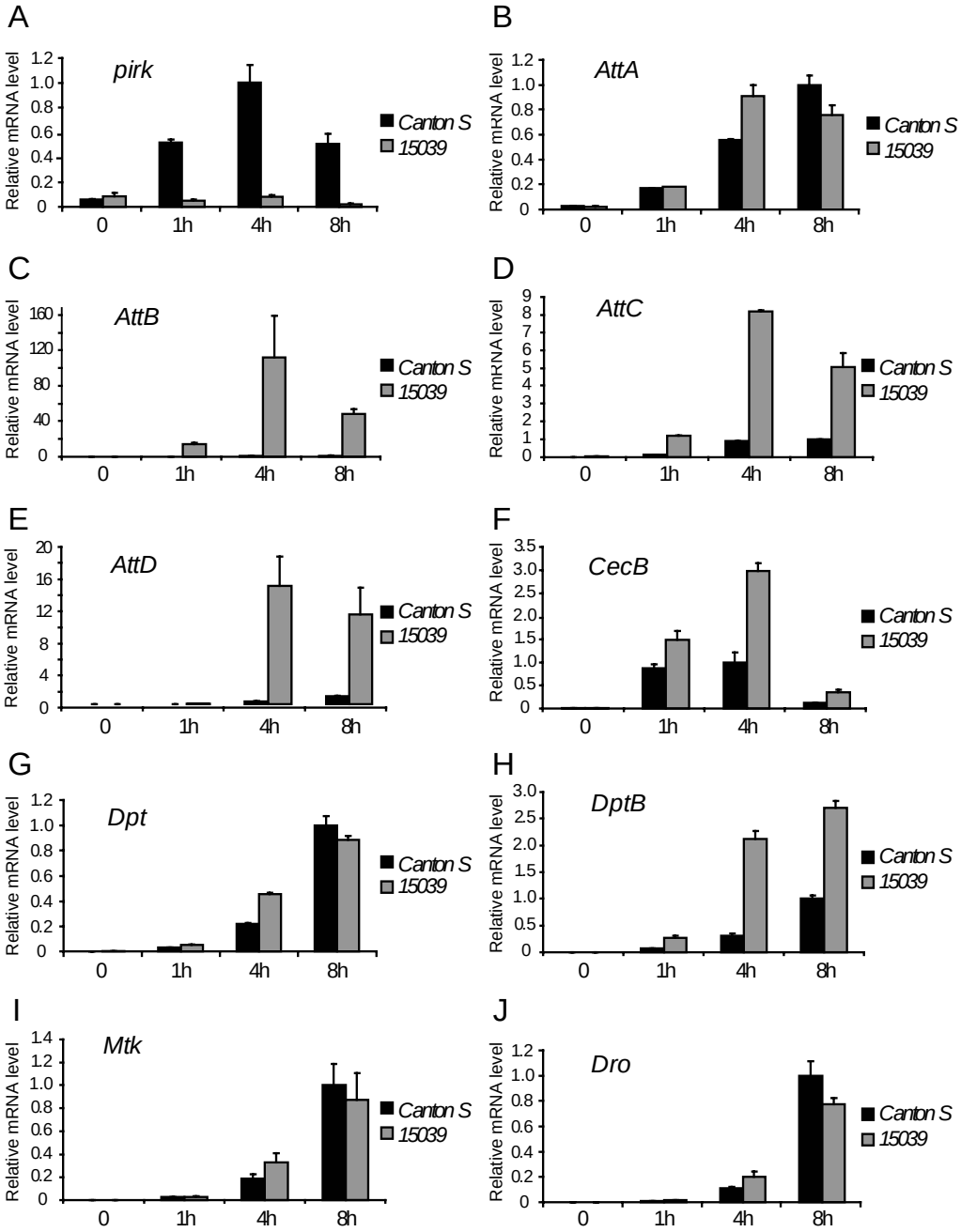

Figure 7. Relative expression levels of *pirk* and *AMP*s in *pirk* hypomorphic *15039* flies and wild-type flies (*Canton S*). The flies were infected with *E. cloacae* by pricking and the total RNAs were extracted at 0, 1, 4, and 8 h post-infection. mRNA levels of *pirk* (A), *AttA* (B), *AttB* (C), *AttC* (D), *AttD* (E), *CecB* (F), *Dpt* (G), *DptB* (H), *Dro* (I), and *Mtk* (J) were measured by qRT-PCR and normalized to the levels of *Act5C* mRNA.

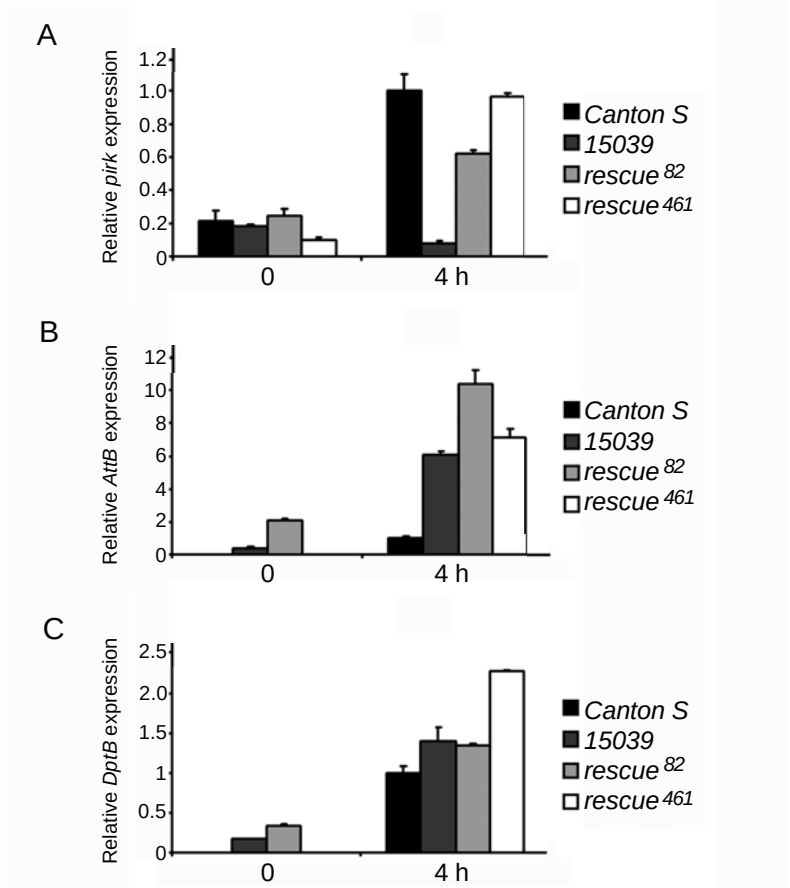

Figure 8. Transcript levels of *pirk* (A) in *15039* flies do not correlate with transcript levels of Imd pathway-regulated *AMP*s *AttB* (B) and *DptB* (C). *15039* flies harbor the P element *EY00273* at the 5' UTR of *pirk*, which disrupts the gene function. *Rescue*^{82} and *rescue*^{461} are individual fly lines from which the P element *EY00723* was removed. The flies were infected by pricking them with a needle dipped in a concentrated culture of *E. cloacae*, and the expression levels of *pirk, AttB,* and *DptB* were analyzed by qRT-PCR 0 and 4 h post-infection.

The strength of the immune response is known to vary from one fly line to another due to the influence of the genetic background on the phenotype. To overcome the possible background effect, we analyzed the impact of *pirk* RNAi on the AMP gene expression in *Drosophila in vivo*. We received two parallel RNAi mutant fly lines from Prof. Ryu Ueda in Kyoto. These transgenic flies have in their genome an incorporated *UAS* construct that encodes a *pirk* hairpin structure. The expression of this hairpin can be targeted to a given tissue using the UAS-GAL4 approach (Brand and Perrimon 1993), where it acts in a way comparable to that of dsRNA in the cell-based system. In *Drosophila* the main immunological organs or tissues involved in AMP release are the fat body and hemocytes. To target the expression of the *pirk* RNAi construct to these biologically relevant sites, we crossed the flies with a constitutively active *C564-GAL4* driver (Harrison et al. 1995). Control flies were crossed with w^{1118}. The week-old healthy offspring were subjected to septic injury by pricking them with

a needle dipped into a concentrated culture of *E. cloacae*, and the flies were collected at different time points for total RNA extraction and qRT-PCR. In agreement with the *in vitro* results, *pirk* RNAi significantly increased the relative transcript levels of *AttB*, *Cecropin B* (*CecB*), and *DptB* at the 4 h and 8 h time points compared to the control (II Fig. 3).

To analyze whether Pirk could suppress Imd pathway activity *in vivo*, we generated Pirk overexpressing flies using the UAS-GAL4 expression system. *pirk* was cloned into the pUAST vector, which was subsequently microinjected into w^{1118} embryos at the Umeå Fly and Worm Transgene Facility. To confirm that the expression of this construct led to protein production, and to facilitate its detection, *pirk* was tagged with a V5 epitope. We obtained several parallel *UAS-pirk-V5* fly lines, of which we used four (referred to with the superscript numbers 221, 351, 432, and 721) in the following experiments. The induction of the transgene was driven by crossing the flies with *C564-GAL4* flies. Flies crossed with w^{1118} were used as uninduced controls. The offspring of both crosses seemed healthy. The flies were snap-frozen and homogenized in lysis buffer, and the production of Pirk was detected by Western blotting using the anti-V5 antibody. In uninduced flies, a faint band of Pirk could be seen. This was not surprising, since the UAS-GAL4 system is known to be somewhat leaky at +25ºC. *C564-GAL4*–induced flies, however, produced much more Pirk compared to the uninduced controls (II Fig. 4A).

Next, we studied the expression of AMP genes in *C564-GAL4*-induced and uninduced flies by qRT-PCR. The Imd pathway was activated by pricking the flies with a needle contaminated with *E. cloacae*, and the flies were collected and total RNA extracted 4 h after infection. Transcript levels of the tested AMP genes, *AttB*, *CecB*, and *DptB*, were all significantly decreased compared to uninduced controls (II Fig. 4B-D); hence the gene name *pirk* (poor Imd response upon knock-in).

To examine if these changes in the AMP gene expression were sufficient to impair the *Drosophila* host defense against Gram-negative bacteria, we infected *pirk*-overexpressing flies with *E. cloacae* and monitored their survival. Three parallel *UAS-pirk* fly lines were crossed with *C564-GAL4* driver (induced) or w^{1118} (uninduced) flies. *C564-GAL4* flies crossed with w^{1118} were considered wild-type in this experiment. Relish null-mutant flies ($Relish^{E20}$) do not express Imd pathway-dependent AMPs, and are thus very sensitive to Gram-negative bacterial infections. They were therefore used as a control to assess the efficiency of the septic injury. As expected, only few uninduced or wild-type flies died during the 48 h period of surveillance monitoring, while $Relish^{E20}$ flies all succumbed at 20 h post-infection. Moreover, the survival of the C564-GAL4-induced flies was decreased (II Fig. 8). Although the decrease in survival rate was less dramatic than that of the $Relish^{E20}$ mutant, it was statistically significant. This implies that overexpression of Pirk is sufficient to weaken the fly's immune response to Gram-negative bacteria.

Taken together, these results indicate that Pirk specifically suppresses the *Drosophila* Imd pathway-mediated immune response *in vivo*.

5.2.2 Characterization of Pirk's mechanism of action (II)

We found Pirk to be both a structurally and functionally poorly characterized protein. According to predictions, it consists of 197 amino acids with no recognizable domain structure that would suggest a mechanism of action. Nor does it have an N-terminal signal sequence, which implies that Pirk is a cytoplasmic protein. We were able to verify this by expressing a GFP-Pirk fusion protein in S2 cells, and detecting its cellular localization by confocal microscopy.

To gain mechanistic insight into Pirk's role in the Imd signaling cascade, we performed a luciferase assay-based epistasis analysis *in vitro*. S2 cells were transfected with either the empty *pMT* vector or full-length Pirk together with a construct encoding the active form of Relish to activate the Imd pathway. Overexpression of Pirk did not affect Imd pathway signaling, which implies that Pirk acts upstream of Relish. Since the *Drosophila* Imd pathway branches at the level of Tak1 to the JNK pathway, we next examined whether *pirk* RNAi could also affect JNK signaling. Therefore we assessed the transcript levels of a JNK pathway target gene *puckered* (*puc*) upon induction with heat-killed *E. coli* in S2 cells by qRT-PCR. In *pirk* dsRNA-treated cells, transcript levels of *puc* were significantly higher at 1 h and 2 h after adding the *E. coli* (II Fig. 6C). This suggests that Pirk also suppresses the JNK pathway mediated early response of host defense. Hence, Pirk is likely to act at the level of or upstream of Tak1.

To examine the mechanism of Pirk-mediated Imd pathway down-regulation, we concentrated on the Imd pathway components that, according to current knowledge, act at the level of or upstream of Tak1. We performed protein interaction studies by coimmunoprecipitating full-length V5-tagged Pirk with Myc-tagged Tak1, IMD, and the cytoplasmic part of PGRP-LC in S2 cells. Pirk coimmunoprecipitated with IMD and the cytoplasmic part of PGRP-LC but not with Tak1 (II Fig. 6C). To further characterize the interactions between Pirk, PGRP-LC and IMD, we aimed to take a closer look at the structure of Pirk.

No Pirk homologs had been previously described. Fortunately, the *Drosophila* 12 genomes consortium provided us with the genome sequences of the close relatives of *Drosophila melanogaster* (*Drosophila* 12 genomes consortium, 2007). Combining these with the other assembled genomic sequences on Flybase (http://flybase.org), we identified putative orthologous proteins from altogether 18 insect species by a tblastn search. However, no mammalian or *Anopheles* orthologs were found. The 18 identified orthologs had different N- and C-terminal regions, but the central part of the protein was conserved (II Fig. 5). Although this conserved region or domain was previously unknown, we used the information as a starting point and designed three Pirk deletion constructs coding for N-terminal, central, and C-terminal parts of Pirk. Coimmunoprecipitation studies with these constructs revealed that Pirk interacted with IMD via its central part, and with the cytoplasmic part of PGRP-LC via its C-terminal part. In addition, overexpression of either one of these domains in S2 cells was sufficient to suppress the Imd pathway (II Fig. 7).

5.3 Identification of novel Imd pathway components by RNAi screening in S2 cells (III)

During the last decade, a number of completed genome sequencing projects have yielded a vast amount of available DNA sequence data. However, to understand the bigger picture, this sequence data needs to be annotated and the genes within the sequence to be identified and characterized to elucidate the functions and interactions of the proteins that they encode. By RNAi, individual genes can be easily knocked down in cell culture systems or in whole organisms, which enables searching the genomes to identify the genes related to the loss-of-function phenotypes of interest. We therefore aimed at identifying the Imd pathway components by performing a large-scale RNAi screen in *Drosophila* S2 cells.

5.3.1 RNAi-based screening and validation of the screen in S2 cells (III)

Before initiating the RNAi screen, we assessed the specificity and efficiency of gene silencing in our system. *CG5210*, which encodes a chitinase-like protein, was silenced by adding *CG5210* dsRNA to the cell culture medium of S2 cells. Total RNAs were extracted from both dsRNA-treated and untreated S2 cells, and the expression levels of more than 13,500 transcripts were measured by oligonucleotide microarrays. The expression level of *CG5210* was greatly decreased, while the expression levels of the other transcripts remained unaffected (III Fig. 1A). From this we concluded that the RNAi in S2 cells was both efficient and specific.

Next, we assayed the reliability and reproducibility of our method of screening. We performed targeted RNAi against the Imd pathway transcription factor Relish by transfecting S2 cells with increasing amounts of *Relish* dsRNA together with luciferase assay reporter constructs. The Imd pathway was activated by adding heat-killed *E. coli* to the culture medium 24 h before harvesting the cells. We found that Relish RNAi suppressed the Imd pathway activity in a dose-dependent manner, and that small concentrations (0.1 ng/ul) of dsRNA were enough to almost completely block signaling (III Fig. 1C).

To generate the dsRNAs for the screening, we used a non-commercial S2 cell-derived cDNA library as a source of templates (Pearson et al. 1995). From this library, we synthesized and analyzed altogether 6,713 dsRNAs for their effect on Imd signaling by a *AttA*-luciferase reporter-based assay. Cell viability was assessed by a *Act5C*-β-galactosidase reporter assay to exclude the gene products that affected S2 cell homeostasis, or the experiments where transfection efficiency was not high enough. As expected, most of the tested dsRNAs had little or no effect on *AttA*-luc or *Act5C*-β-gal activities. However, RNAi against seven genes decreased *AttA*-luc activity by more than 80% without

clearly affecting the *Act5C*-β-gal activity indicating that none of these dsRNA treatments significantly affected cell viability or proliferation (III Table I). Three of these genes encoded the known components of the Imd pathway: *PGRP-LC*, *imd*, and *relish*. *Relish* was identified altogether three times, which reflects the reproducibility of our screen. The other four genes were *kayak*, *longitudinals lacking* (*lola*), *inhibitor of apoptosis 2* (*Iap2*), and *CG7417*. *kayak* encodes a homolog of the mammalian Fos, and is thus a known component of the JNK pathway. Moreover, *lola* was reported to be required for *Drosophila* neural development (Madden et al. 1999) and normal phagocytosis of bacteria in S2 cells (Rämet et al. 2002b). We therefore focused on *Iap2* and *CG7417*, whose RNAi knock-down decreased the *AttA*-luc activity by 98%, almost as dramatically as the RNAi for *relish*.

5.4 CG7417/Tab2 as a component of the Imd pathway (III, I)

According to predictions, *CG7417* encodes a protein of 831 aa with two recognizable domains: a CUE domain (CUE) at the N-terminus, and a zinc finger domain (ZnF) at the C-terminus. These domains are characteristic for mammalian TAK1-binding proteins (TABs). The CUE domain has been implicated in binding to ubiquitin-conjugated enzymes. ZnF includes a coiled-coil region, which may mediate protein-protein interactions. Although these domains are homologous with the CUE and ZnF domains of mammalian TABs, there is very little sequence similarity outside these domains. Since *CG7417* is the only *Drosophila* protein containing both CUE and ZnF domains, and the protein product CG7417 has been reported to bind TAK1 in a yeast two-hybrid interaction study (Giot et al. 2003), *CG7417* is referred to as *Tab2*.

To confirm our screening results, we first performed semi-quantitative RT-PCR to rule out that this result was merely a reporter artifact. In agreement with the luciferase-assay results, the induction of the Imd pathway-regulated AMP genes, *CecA1*, *Dpt,* and *AttD*, was decreased in *Tab2* dsRNA-treated S2 cells compared to GFP dsRNA-treated controls (III Fig. 2B). An epistasis analysis was then performed by overexpressing either *imd*, full-length *relish*, or the constitutively active *RelΔS29-S45* together with the luciferase assay reporters in S2 cells. *Tab2* RNAi in *Rel* or *RelΔS29-S45* overexpressing cells had no effect on *AttA*-luc activity. However, in *imd* overexpressing cells, *Tab2* RNAi completely abolished the *AttA*-luc activity, indicating that Tab2 was acting upstream of Relish but downstream of IMD (III Fig. 3A). This was in line with the interaction study by Giot et al. (2003), where Tab2 was reported to interact with TAK1.

To study if Tab2 was essential for the Imd pathway mediated immune response *in vivo*, we aimed at generating a Tab2 null mutant fly line by transposase-mediated imprecise excision. For this, we ordered two P element

carrying fly lines, *4440* and *14838*, from the Bloomington stock center, and mobilized the P elements by crossing the flies with a Δ2-3 transposase element carrier line. Homozygous offspring of the flies that had lost their P element were screened for the absence of Tab2 by RT-PCR. Curiously, we were not able to obtain a homozygous viable Tab2 null mutant.

Unlike other NF-κB proteins, the Imd pathway transcription factor Relish contains both the transcription factor part and the inhibitory IκB part, the latter of which must be cleaved before the activated transcription factor can translocate to the nucleus. This cleavage could be induced by a bacterial peptidoglycan, and was thought to be mediated by the caspase Dredd. To better understand the role of Tab2 in the Imd pathway signaling, we next investigated if Tab2 was required for Relish cleavage. *Drosophila* hemocyte-like mbn-2 and S2 cells were treated with dsRNAs, and the Imd pathway was activated by adding commercial *E. coli* LPS, known to contain contaminating peptidoglycan, to the cell culture medium. Relish cleavage was thereafter detected by Western blotting with an α-C Relish antibody (Stöven et al. 2000). In *GFP* dsRNA-treated cells, Relish was cleaved rapidly after the LPS stimulus. However, RNAi of *Dredd* and *kenny* (*key*), which encodes a component of the Relish-activating IKK complex, blocked the cleavage of Relish. RNAi of *Tab2* did not affect Relish cleavage (III Fig. 4A-B).

We next examined if Tab2 was involved in the nuclear translocation of Relish. We treated S2 cells with control and *Tab2* dsRNAs, stimulated the cells with LPS, and assessed the nuclear localization by immunostaining Relish with α-RHD antibody (Stöven et al. 2000). Unlike in *GFP* dsRNA-treated cells, no Relish signal could be detected in the nuclei of *Dredd* or *key* dsRNA-treated cells after LPS stimulation. *Tab2* RNAi also affected Relish translocation to the nucleus (III Fig. 4C). This was surprising, since Relish was detected in the nuclei of *Tak1* dsRNA-treated cells. The requirement of Tab2 but not Tak1 in the nuclear localization of Relish implies that the regulation of Relish activation is more complex and involves more components than was previously thought.

Besides being a component of the Imd pathway, Tak1 also plays a role in the sequential activation of the c-Jun N-terminal kinase (JNK). In addition to embryonic development, JNK signaling is required for various processes, such as apoptosis, wound healing, and the immune response in *Drosophila*. In S2 cells, the JNK pathway is activated prior to the Relish-dependent Imd pathway in response to a Gram-negative bacterial infection (Boutros et al. 2002, Park et al. 2004a). JNK signaling is terminated approximately 4 h later (Park et al. 2004a), after which the Imd pathway is fully activated. Since Tak1 and Tab2 appeared necessary for the activation of both pathways (Vidal et al. 2001, Boutros et al. 2002, Park et al. 2004a, Zhuang et al. 2006), we aimed to investigate, whether the roles of Tak1 and Tab2 in Imd pathway activation were mediated via the JNK pathway.

To elucidate this, we used a *AttA*-luciferase reporter assay and *AttD* semi-quantitative RT-PCR to study the roles of the JNK pathway downstream components Hemipterous (hep), Basket (bsk), and Kayak (kay) in the expression of AMP genes in comparison to Tak1 and Tab2 by (I Fig. 1). In the reporter

assay, *hep*, *bsk*, and *kay* RNAi slightly decreased the luciferase activity compared to the *GFP* control RNAi, especially after the 4 h time point. A similar trend could be seen with *AttD* semi-qRT-PCR. In contrast, Tab2 and Tak1 dsRNA treatments completely abolished the *AttA*-luc activity and *AttD* expression. This indicates that the JNK pathway is involved in the regulation of AMP gene expression as reported previously (Kallio et al. 2005), but Tab2 and Tak1 are more important than the downstream components. Moreover, the functions of Tak1 and Tab2 are not restricted to the JNK pathway regulation. This was further supported by oligonucleotide microarray analysis of *Tab2* and *Tak1* dsRNA-treated S2 cells, which revealed that *Tab2* and *Tak1* RNAi eliminate the induction of Relish-dependent sustained response genes similarly to *Relish* and *imd* RNAi 4 h post-infection. Tab2 and Tak1 are thus required for the induction of all of the Imd pathway target genes (I Fig. 2). The exact mechanism for promoting this induction, however, remains elusive.

5.5 Iap2 as a regulator of the Imd pathway activation (III, I, II)

Another novel component identified in our RNAi screen was Iap2, an evolutionarily conserved protein consisting of 498 amino acids. It had previously been implicated as an inhibitor of Reaper and FADD-induced apoptosis in insect cells (Vucic et al. 1997). Characteristically for other IAPs, Iap2 contains three N-terminal baculovirus IAP repeat (BIR) domains and a C-terminal really interesting new gene (RING) domain. However, the caspase recruitment (CARD) domain, which is present in the mammalian homolog, was absent in the *Drosophila* Iap2. Various RING domains have been associated with E3 ubiquitin-protein ligase activity, and binding activity towards E2 ubiquitin-conjugating enzymes. The N-terminal BIR domains in human c-Iap2 have been suggested to be involved in protein interactions especially with TRAF proteins (Rothe et al. 1995). In our screen, however, RNAi targeting of *TRAF1* or *TRAF2* (nowadays referred to as *Traf4* and *Traf6*, respectively) did not alter the activity of the Imd pathway. We therefore aimed to further characterize the role of Iap2 in the signal propagation in the Imd pathway.

We started by performing an *in vitro* epistasis analysis by overexpressing either *imd*, full-length *Relish*, or the constitutively active *RelΔS29-S45* together with the luciferase assay reporters in S2 cells. In *Rel* or *RelΔS29-S45* overexpressing cells, *Iap2* RNAi did not affect the *AttA*-luc activity. Yet, *Iap2* RNAi in *imd* overexpressing cells blocked the *AttA*-luc activity (III Fig. 3). This implied that Iap2 was acting upstream of Relish but downstream of IMD. Similarly to Tab2, we also asked if Iap2 was required for Relish cleavage or nuclear translocation. RNAi of *Iap2* did not affect Relish cleavage, but like in *Tab2* dsRNA-treated cells, the nuclear translocation of Relish was altered. This suggested a new approach for Imd pathway regulation.

Curiously, when we confirmed our reporter assay results by semi-quantitative RT-PCR, we noticed that unlike in the *Tab2* dsRNA-treated cells, *CecA1* expression was not completely abolished 6 h post-infection in *Iap2* dsRNA-treated S2 cells (III Fig. 2B). We first speculated if this was due to an incomplete inhibitory action of the RNAi, but this was not the case. Our luciferase assays with *AttA*-luc and *CecA1*-luc reporters had been performed 24 h after inducing the Imd signaling by *E. coli*, and *Iap2* RNAi had repeatedly blocked the Imd pathway signaling entirely. Hence we hypothesized that the difference might be due to the different time points used in these experiments. To study this, we assessed the expression kinetics of *AttD* upon *E. coli*-induction in *Iap2* dsRNA-treated S2 cells compared to *Tab2* and *Tak1* dsRNA-treated cells, and *GFP* dsRNA-treated controls by semi-quantitative RT-PCR. While *Tak1* and *Tab2* RNAi totally abolished *AttD* expression, the effect of *Iap2* RNAi was once again a more moderate. In fact, *AttD* expression in *Iap2* dsRNA-treated cells was as strong as in the *GFP* control at the 4 h time point, and decreased gradually over time (I Fig. 1). This was further confirmed by oligonucleotide microarray analysis of *Iap2* dsRNA-treated S2 cells, which demonstrated that *Iap2* RNAi had no effect on the induction of Relish-dependent sustained response genes 4 h post-infection. After 24 h, Iap2 RNAi moderately decreased the relative expression levels of Relish-dependent immune response genes (I Fig. 2). *Iap2* RNAi had merely a minor effect on the expression levels of the Relish-independent immune response genes,. This implied that Iap2 was necessary for the sustained AMP response rather than for the early response in S2 cells.

To examine if this phenomenon was restricted to S2 cells only, we analyzed the expression levels of *AttA*, *AttD*, and *CecA1* in *Iap2* null mutant flies (Leulier et al. 2006a) at 0, 1h, 4h, and 8h after *E. cloacae* infection by semi-quantitative RT-PCR (I Fig. 3). Compared to *Canton S* flies, *AttD* and *CecA1* expression was almost abolished in $Relish^{E20}$ flies, while some *AttA* expression could still be detected. In *Iap2* null mutants *AttA* expression was completely abolished, while *AttD* and *CecA1* were clearly expressed (I Fig. 3). Due to the semi-quantitative nature of this assay, we can not provide exact measurements for the expression levels of *AttD* or *CecA1*. However, they are clearly detectable both in *Canton S* and in *Iap2* null flies, suggesting that Iap2 is required for a sustained response rather than an early response also *in vivo*. Transcription of *AttA* seems to be independent of these regulatory mechanisms.

5.5.1 Iap2 is required for resistance towards Gram-negative bacteria in vivo *(I, III)*

To examine the importance of Iap2 for the *Drosophila* immune response *in vivo*, we studied Imd pathway activation in *Iap2* mutant flies. In collaboration with Prof. Lemaitre's and Prof. Ueda's laboratories, we acquired flies carrying a *UAS-Iap2-IR* construct, which encoded two inverted repeats of the *Iap2* gene separated by a spacer sequence to form a hairpin-shaped RNA, which *in vivo*

functions as a dsRNA in S2 cells. These UAS-RNAi flies were then crossed with flies carrying various GAL4 drivers to activate the expression of the hairpin construct. Interestingly, overexpression of *UAS-Iap2-IR* by the ubiquitous *daughterless*-GAL4 driver resulted in lethality at the pupal stage. The expression of *UAS-Iap2-IR* was therefore driven by the *C564-GAL4* driver, which targets the expression of the construct into the adult fat body and hemocytes. These flies appeared healthy despite the *Iap2* RNAi, and were therefore used when assessing Imd pathway activity. *Iap2* knock-down flies were infected with *E. carotovora*, and the relative *Dpt* mRNA levels of the flies were monitored 6 h post-infection by qRT-PCR. *Dpt* expression in Iap2 knock-down flies was significantly reduced compared to wild-type flies, and almost as low as in the *dFADD* RNAi flies that were used as a positive control (III Fig. 2C). This indicates that Iap2 is required for Imd pathway-mediated AMP gene expression also *in vivo*.

Next, we aimed to investigate if *Iap2* RNAi-induced reduction in AMP gene expression was sufficient to affect the fly's resistance to bacterial pathogens. We infected healthy week-old Iap2 RNAi flies by pricking them with a needle contaminated with *E. cloacae*, and compared their survival to similarly treated wild-type (*Canton S*), $Relish^{E20}$, and *dFADD* RNAi flies. $Relish^{E20}$ flies succumbed to the Gram-negative bacterial infection within 20 h, while the wild-type flies were totally resistant to *E. cloacae*. The survival rates of both *dFADD* RNAi flies and *Iap2* RNAi flies were greatly reduced, only 35% of the *Iap2* RNAi flies still being alive at 44 h post-infection (I Fig. 4). This indicates that Iap2 is required for normal resistance towards Gram-negative bacteria *in vivo*.

6. Discussion

6.1 Methodological aspects

6.1.1 Drosophila *as a model in studying innate immunity*

Drosophila has a long history as a model organism in genetic and developmental studies, and from the 1980s it has been used increasingly also in immunology. In addition to generally having many advantages as model organisms, flies have qualities that make them good models also for immunological research. First, the pathways of innate immunity are evolutionarily well conserved. Although the signaling mechanisms and signal regulation between species may be somewhat different, the identification of important signaling components in *Drosophila* has fueled and accelerated the discovery of the components of mammalian signal transduction pathways. The most famous example is probably the discovery of the mammalian TLRs (Medzhitov et al. 1997) soon after Toll was reported to be involved in fly immunity (Rosetto et al. 1995) and to be essential to the fly's resistance to fungal pathogens (Lemaitre et al. 1996). Second, flies have no adaptive immunity that would compensate or hide the effects of knocking out an important immunity-related gene. Their total dependence on innate immunity and reduced genetic redundancy facilitates the interpretation of results. Third, a vast variety of tools is available for studying the different aspects of *Drosophila* immunity from the genomic level to complex regulatory networks of proteins both *in vitro* and *in vivo*. However, one should remember that no model is perfect for describing everything. The methods and tools we use should therefore be chosen carefully based on their validity for answering the simple questions that we ask, bearing in mind the possible drawbacks that the use of the particular model involves. Although we are often looking for similarities between the human and *Drosophila* systems, humans can not be seen as scaled-up flies, or flies as scaled-down humans. Flies have the right to be considered important and interesting simply for what they are: not merely lower organisms or simple and ethically trouble-free laboratory animals.

Both cell-based and *in vivo*-assays were performed in this thesis study,. Of the available tools we used RNAi screening and oligonucleotide microarrays, as well as the UAS-GAL4 expression system for the generation of both RNAi knock-down and overexpression flies. The possible advantages and disadvantages are discussed below.

6.1.2 Drosophila *cell lines*

The cell-based assays described in this thesis were performed using two continuous cell lines, S2 and mbn-2, which are both derived from embryonic cells (Schneider 1972, Gateff 1978). They resemble hemocytes in their ability to phagocytose, produce various AMPs and express receptors typical for immune responsive cells (Samakovlis et al. 1990, Dimarcq et al. 1997, Rämet et al. 2001, Rämet et al. 2002b). Although both lines have their limitations, they provide an unlimited and more standardized source of cells for different assays compared to hemocytes bled from *Drosophila* larvae. Moreover, efficient gene silencing can be induced in these cells simply by adding dsRNAs into the culture medium (Clemens et al. 2000). S2 cells are known to be capable of binding and internalizing dsRNA via their cell surface receptors (Ulvila et al. 2006), and have an efficient RNAi machinery to dice the dsRNA into shorter functional siRNAs. Although the use of cell-lines that originate from embryonic blood cells limits the possible phenotypes to be mapped, the ease of triggering RNAi and culturing these cells explains their wide-spread use in RNAi screening (Rämet et al. 2002b, Lum et al. 2003, Boutros et al. 2004, Foley and O'Farrell 2004, DasGupta et al. 2005, Müller et al. 2005).

6.1.3 Microarrays

Genome-wide transcription analysis became a reality in 1997 when the first eukaryotic genome-wide microarray was published (Lashkari et al. 1997). Microarrays covering a few thousand *Drosophila* genes had been custom-made even before the genome project was completed (White et al. 1999). But as soon as the sequence of the *Drosophila* genome was published in 2000 (Adams et al. 2000), and commercial oligonucleotide microarrays became available, microarrays made their final break-through as a tool that is at hand in every laboratory. However, microarray technology is still relatively novel and microarray chips have evolved and improved over time. The genomic sequence data has been updated and the gene annotation edited, which adds more complexity to the interpretation of results when reviewing old and more recent microarray data. Still, the most important variables are the source and quality of the mRNA to be analyzed. Hence the initial experimental settings before and during the RNA extraction should be carefully controlled.

We used oligonucleotide microarrays to assess, which genes are expressed in S2 cells in response to an *E. coli*-infection. This was performed at different time points and after the RNAi silencing of *relish*. Similar experiment could have been performed *in vivo* comparing *E. coli*-infected *relish* mutant and wild-type flies. Then, however, the model would have already included many more variables, such as the genetic background of the flies, whether we infected the flies by septic injury or feeding, whether we extracted the RNA from entire flies or just certain tissues, whether we chose male or female flies, and so on. To

acquire a rough overview of transcriptional modifications occurring upon Gram-negative bacterial infection, we therefore simplified our system and studied a genetically homogeneous population of cells that had been subjected to excess amounts of heat-killed *E. coli* in the cell culture medium. It is likely that we would have detected a slightly different set of induced genes had we used mbn-2 cells instead of S2 cells, other bacterial species, live *E. coli*, different amounts of bacteria, or only fragments of the components of the bacterial cell wall.

Mainly for reasons described above, microarray studies deciphering the genes involved in the regulation of the immune system produced lists with some but not complete overlap. In accordance with our microarray data, the most induced genes (*AMP*s, *PGRP*s, and *puc*) were also detected by others (De Gregorio et al. 2001, Irving et al. 2001, Rämet et al. 2002b, Agaisse et al. 2003, Johansson et al. 2005, Vodovar et al. 2005). In addition, *Mmp1* was reported to be upregulated in two of the studies (Agaisse et al. 2003, Johansson et al. 2005). Curiously, Johansson and colleagues reported that challenging mbn-2 cells with *E. coli* resulted in the down-regulation of certain *AMP*s, such as *AttA*, *AttC*, *Dpt*, *Drs*, and *Dro*, while they showed increased expression after treatment with crude LPS. Similar profiles could be detected also with some of the short *PGRP*s. Why the expression patterns of these genes differ depending on the initial stimulus remains unfortunately unclear. In conclusion, one can, from the published microarray data, deduce some guide lines for which pathways are involved in the signaling, but the expression levels of individual genes vary greatly from one experiment to the other.

6.1.4 In vitro RNAi screening

Before the discovery of RNAi, *Drosophila* was favored as a model organism due to its potential for carrying out forward genetics, such as mutagenesis screening. RNAi has simply broadened the use of *Drosophila* by enabling the study of genes that could not be knocked out by traditional methods due to lethality. RNAi in *Drosophila* works exceptionally well in cells as well as in the whole organism from larvae to adults. This is probably due to many reasons. First of all, it has been shown that fly S2 cells can bind and internalize dsRNA by scavenger-receptor mediated endocytosis (Ulvila et al. 2006). This means that dsRNAs can either simply be added to the cell culture medium, or transfected into the cells. Second, *Drosophila* cells have an intact RNAi machinery, which makes them capable of dicing 200-700 bp long dsRNAs into 21-23 bp fragments. This way, the concentration of each small fragment is relatively small compared to the situation where two or three siRNAs would be transfected into the cells. The lower concentration might limit the amount of off-target effects. It is also plausible to assume that off-target effects are less likely in *Drosophila* due to a lower redundancy of the genome. The use of long dsRNAs has another advantage over using siRNAs: dsRNAs are easy to synthesize, which enabled the creation of dsRNA libraries very soon after RNAi was discovered. Because

RNAi is both efficient and relatively straight-forward, it has been used ever since to perform large-scale screens aiming to identify components involved in various biological processes (Perrimon and Mathey-Prevot, 2007). However, like all experimental settings, RNAi screening requires careful planning and optimization steps before reasonably reliable data can be acquired (for a review, see Boutros and Ahringer 2008).

In this thesis study, we carried out a large-scale RNAi screen in S2 cells using dsRNAs synthesized from an S2 cell-derived cDNA library (Pearson et al. 1995). The work-flow and optimization steps for designing an RNAi screen are illustrated in Figure 9. At the time when we started planning the screen, there were no commercial dsRNA libraries available. We therefore chose to proceed by synthesizing a collection of our own. The colonies containing the plasmids used as template were picked up randomly. The advantage of this approach was that we did not have any presumptions about what the results should be like, since the genes we were targeting were not known until the plasmids from the library were sequenced.

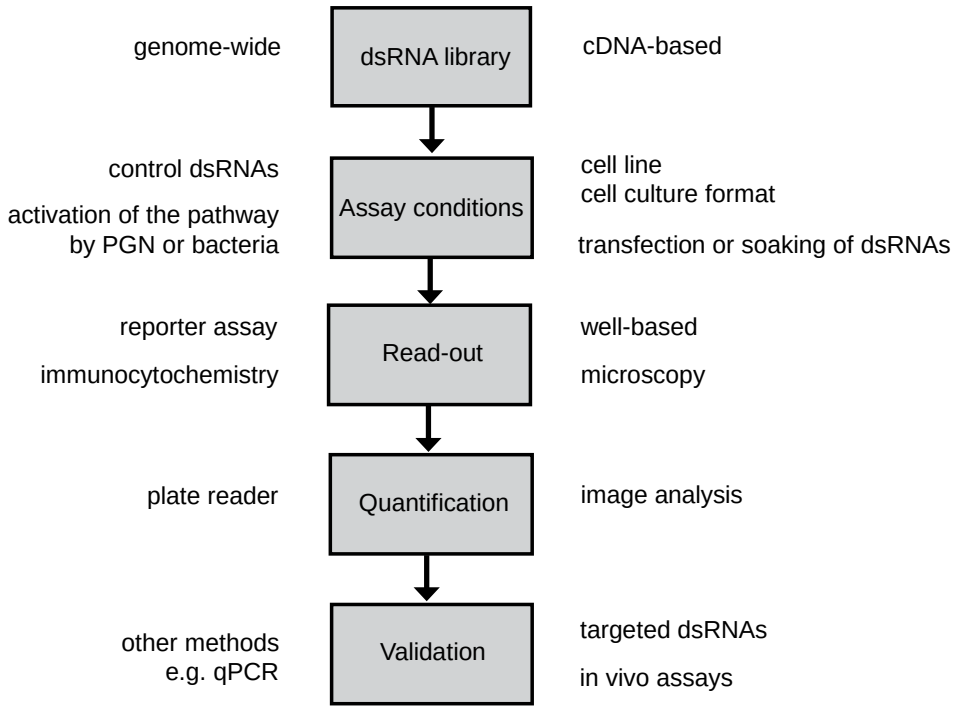

Figure 9. Work-flow of designing an RNAi screen.

Using cDNA from a continuous cell line as the template for dsRNA synthesis has both disadvantages and advantages. On the one hand no cDNA represents the whole fly genome. On the other hand, cDNA derived from immune-competent hemocytes will only represent the genes transcribed in those cells. This means that we were likely to pick up the genes that had relevance for the immune response in hemocytes. However, we might have lost some genes that are

important for the immune response in the fat body, or those that are needed for signal transduction between different immune responsive cell types or tissues. In addition, the most abundant transcripts are likely to be tested repeatedly, and less frequent ones maybe not be picked up at all. Since our cDNA was derived from naïve S2 cells instead of ones challenged with PGN or bacteria, our collection of dsRNAs lacked genes that are transcribed only in response to microbes, such as *pirk*.

To examine the activity of the Imd pathway, we decided to use an *AttA*-luciferase reporter-based assay. Other *AMP*-luc reporters, such as *Dpt*-luc that is generally thought to be more specific for the Imd pathway, were tested. In our hands the *AttA*-luc reporter gave the strongest induction, and was therefore chosen as our read-out. To exclude dsRNA treatments that would affect cell viability, we assayed the level of actin biosynthesis by an *Act5C*-β-galactosidase reporter. S2 cells were cultured on 24-well plates to maximize the amount of cells and minimize the standard deviation between samples, and reporter constructs were transfected into the cells together with dsRNAs to exclude the possibility that the dsRNA treatments might affect the internalization of dsRNAs. Imd pathway activation was triggered by adding heat-killed *E. coli* to the cell culture medium to gain a robust and intense response. Before harvesting the cells for lysis and measuring the luciferase activity, cells were viewed under a microscope to further confirm that the dsRNA treatments did not affect cell viability. The luciferase and β-galactosidase activities were measured from cleared cell lysates in a 96-well format. The dsRNAs that gave a positive result in our screen were further validated by using other reporter constructs and targeted RNAi. We were diligent in excluding experimental artifacts, and therefore ended up with such a short list of hit genes.

Others too have screened Imd pathway regulators by RNAi, but chosen different experimental settings. Foley and O'Farrell published their genome-wide screen as early as in 2004 (Foley and O'Farrell 2004). They created a stable cell line expressing a *Dipt*-LacZ reporter, induced Imd pathway activation by PGN-contaminated LPS, and detected the β-galactosidase activity by microscopy in cells seeded on 96-well plates. Their dsRNA library consisted of 7,216 dsRNAs that represented most of the phylogenetically conserved genes of *Drosophila*. However, they did not assay for cytotoxicity of their dsRNA treatments in their screen. Foley and O'Farrell identified 121 of the genes involved in the Imd pathway, and they further divided them into three categories: positive regulators (49 genes), and two types of negative regulators. The first group of negative regulators included 26 genes that constitutively activated the reporter in the absence of LPS, and the second group of 46 genes that increased the responsiveness to the PGN induction. They identified some of the known components in their screen, namely PGRP-LC, Dredd, and Relish. Interestingly, they did not identify Tab2 or Iap2. Since both of these proteins have a conserved domain structure and mammalian orthologs, it would be surprising if dsRNAs targeting these genes had been absent from their dsRNA library. It is therefore

reasonable to assume that due to different experimental settings our screening results have very little overlap.

Another screen was published by Gesellchen and colleagues in 2005 (Gesellchen et al. 2005). They used a *Mtk*-luc reporter as a read out in their preliminary screen, and confirmed their candidate gene list using a *AttA*-luc reporter. In addition, they normalized their results with a constitutive *Renilla* reporter. Their list of candidate Imd pathway regulators is relatively short, but has still only partial overlap with the results of our screen. However, Gesellchen and colleagues identified both Iap2 and Tab2 as positive regulators of the Imd pathway. Once again, the differences in the results may be due to the different experimental settings. These examples of published studies emphasize the need of careful planning, optimization and validation steps in RNAi screening.

6.1.5 Drosophila *in vivo* assays

Many decades of extensive research carried out in *Drosophila* have yielded a variety of available research tools. We have greatly benefited from the publicly available stock collections, but also from the generosity of the *Drosophila* research community, that is famous for kindly sharing fly lines and assay methodology when requested. In this thesis study, we used the available P-element lines, GAL4-driver lines, RNAi lines, and generated some overexpression lines of our own. Acquiring fly lines from different sources always has one problem: the genetic background of the flies. Immune response is one of those features that varies greatly from one stock to another and even between individuals. The reasons for this are probably both the genetic background and environmental factors, such as the presence of the endosymbiotic bacterium *Wolbachia pipientis*. It is therefore essential to try to reduce the effect of the genetic background by back-crossing, which unfortunately takes several generations. In the case of *Wolbachia*, it has been reported that around 30% of the stocks at the Bloomington Stock Center are *Wolbachia*-infected (Clark et al. 2005). *Wolbachia* can infect a wide range of different arthropods and nematodes, and both manipulate the host's reproduction and alter its phenotype, such as longevity and viral response (Toivonen et al. 2009, Hedges et al. 2008), in multiple ways that are still largely unknown. So far the stock collections do not provide information about the presence of *Wolbachia* in individual stocks. If one can not feed all the flies with antibiotics to get rid of *Wolbachia*, one should at least bear in mind the possibility of the alterations that it might induce. We do not know whether the stocks that we used in our experiments contained *Wolbachia*. Since we did not rely solely on *in vivo* phenotypical analysis when sorting out the regulators of the Imd pathway, it seems reasonable to assume that the possible *Wolbachia*-induced phenotypic changes did not significantly affect our results.

The infection assays that we used to assess the strength of the Imd pathway response were conducted by pricking the flies with a needle contaminated with a

concentrated bacterial culture. This model of septic injury is commonly used when studying the innate immune response in *Drosophila*, but one can argue if it has little or any relevance outside the laboratory. The bacteria that we and others widely use in the laboratory are usually of human origin, and are less likely to be found in the natural environment of *Drosophila*. The advantages of using *E. cloacae* and *E. coli* are that they are easy to access and culture, and more standardized and tractable as bacterial species than the ones found in fermenting cactus fruits in Latin America. In addition, the survival assays are convenient to perform using *E. cloacae*, since the results can be obtained within a few days. The Imd pathway mutants succumb relatively quickly to the infection. Using this method, however, may leave the milder phenotypes undetected. As an alternative to the septic injury model, the Imd response can be studied by orally infecting *Drosophila* with *Erwinia carotovora carotovora* (*Ecc15*), which is an entomopathogenic Gram-negative bacterium. *Ecc15* has been used for pricking flies as well, and it also probably serves as a more relevant model for that purpose. Ideally, it would be good if the infection assays were performed using different bacteria as well as both oral infection and septic injury, depending on the type of response under study.

In vivo RNAi in *Drosophila* was first performed by Kennerdell and Carthew in 1998. They induced gene silencing by injecting dsRNAs straight into the embryos (Kennerdell and Carthew 1998). This technique could be applied to smaller screens and for the silencing of individual genes, but was labor-intensive and technically rather difficult. RNAi became more feasible when the dsRNA was expressed *in vivo* in a UAS-driven hairpin construct containing a long inverted repeat of the target sequence (Kennerdell and Carthew 2000). The expression of this kind of a construct could be targeted to any tissue or cell type for which a GAL4 driver line was available (for details of UAS-GAL4 expression system, see Brand and Perrimon 1993). In addition, the expression of the RNAi construct could be triggered at the developmental stage of interest. This enabled the creation of knock-down animals also for those genes whose knock-down in the embryo would otherwise result in lethality.

Gene silencing by *in vivo* RNAi has the same limitations as *in vitro* RNAi when it comes to off-target effects, efficiency and specificity. In addition, the developmental stage of the fly, the type of tissue targeted, and the gene to be silenced may all affect the efficiency of RNAi. For example, genes that are regulated through efficient feedback mechanisms might react to the depletion of mRNA levels with an increased rate of transcription. Furthermore, the integration site of the transgene may influence the knock-down efficiency. Techniques for site-specific integration might at least partially solve this problem, and are currently being developed (Bischof et al. 2007, Ni et al. 2008).

Currently, two publicly available collections of transgenic RNAi fly lines exist: the National Institutes of Genetics Fly Stocks (NIG-FLY), and the Vienna Drosophila RNAi Center (VDRC) (Dietzl et al. 2007). The first genome-wide *in vivo* RNAi screen was performed using the latter collection (Cronin et al. 2009). The flies that were used in our experiments were from Prof. Ryu Ueda and

essentially the same as the NIG-FLY stocks, since the VDRC collection had not yet been opened for common use at that time. The transgene insertion sites of these flies are less well documented. In addition, some stocks suffer from balancing problems probably caused by multiple insertions of the transgene. Nevertheless, we have been able to rule out that these problems would have biased our results. Our *in vivo* experiments regarding Iap2 were confirmed by a study performed using a null mutant (Leulier et al. 2006a, Huh et al. 2007), and the Pirk results corresponded well with the phenotype of the P element-induced hypomorph studied by others (Aggarwal et al. 2008, Lhocine et al. 2008).

In original article III we reported that crossing *Iap2* RNAi with a ubiquitous *da-GAL4* driver resulted in pupal lethality. The reasons for this are unknown but probably due to the ubiquitous driver. *Iap2* null mutant flies are viable (Leulier et al. 2006a, Huh et al. 2007), unlike Iap1 null mutants. Although these proteins share homology at the protein level, their DNA sequence contains only 8 identical nucleotides. Since dsRNA or the hairpin construct in *Drosophila* cells is cleaved into 21-23 nt siRNA-duplexes that target the complementary mRNAs, it is unlikely that 8 nucleotides would be enough to efficiently silence the gene encoding Iap1, *thread*, and cause lethality. Curiously, Iap1 has recently been associated with the cellular immune response. Matova and Anderson reported *thread* to be the primary transcriptional target gene of the NF-κB transcription factors Dorsal and Dif, and Iap1 to be essential for blood-cell survival. Dorsal and Dif double-mutant flies have fewer hemocytes, and the existing hemocytes have abnormal morphology and reduced phagocytic ability (Matova and Anderson 2006). The constitutive expression of *thread* in hemocytes was sufficient to rescue all these phenotypes (Matova and Anderson 2010). However, it remains unclear if the defects in cellular response and phagocytosis result in the lethality of *thread* mutant.

In conclusion, UAS-driven RNAi is a valid method for creating knock-down alleles, silencing multiple genes, and even individual isoforms of genes, but like in any other RNAi experiment, adequate controls must be used to exclude driver or insertion site-related artifacts. However, the phenotype of a knock-down allele is sometimes not dramatic enough, and a null allele, if available, is then the only option.

6.2 Pirk as an Imd pathway negative regulator

At the time this thesis work began, hardly anything was known about the negative regulation of Imd pathway signaling, but it has been intensively studied since. Recent evidence shows that the intensity, duration, and localization of the Imd pathway-mediated responses are under tight regulation, and that the down-modulation takes place on multiple levels by different types of regulatory proteins (for a review, see Aggarwal and Silverman 2008). In this thesis study we show that Pirk is a negative regulator of the Imd pathway and that it interacts

either directly or indirectly with PGRP-LC and Imd. These results were soon confirmed by two other groups (Aggarwal et al. 2008, Lhocine et al. 2008).

Lhocine and colleagues reported that Pirk, which they refer to as PGRP-LC-interacting inhibitor of Imd signaling (PIMS), is an essential modifier of the Imd pathway response to commensal bacteria particularly in the gut, but also during systemic infection. They initially identified *pirk* as a strongly induced, Imd-dependently expressed gene in a large-scale microarray analysis (De Gregorio et al. 2001). This is in line with our findings (Kallio et al. 2005, original communication I). Lhocine et al. (2008) showed that *pirk* expression is Relish-dependent, and that there are four putative NF-κB binding sites in the predicted regulatory region of *pirk*, one of which perfectly matches the DNA-binding motif of Relish. In addition to $Relish^{E20}$ mutant flies, *pirk* expression was significantly reduced in flies reared in axenic conditions, in *pirk* RNAi flies, and in $pims^{EY00723}$ flies, which have the corresponding P element insertion as the Bloomington stock *15039* that we also briefly used in our studies. Lhocine et al. reported clear differences between $pims^{EY00723}$ and wild type flies, and demonstrated that this P element insertion is haploinsufficient at certain time points during infection, since $pims^{EY00723}$/+ flies displayed elevated levels of *Dpt* 8 h after septic injury with *Ecc15* or *E. coli*. Furthermore, they showed that *Pirk* co-immunoprecipitated with PGRP-LC. However, they detected a much weaker signal from the immunoprecipitation experiments with IMD than we did. Hence they suggest that the Pirk interaction with IMD could be indirect, and possibly mediated by PGRP-LC. They also observed that coexpression of Pirk and PGRP-LC altered the subcellular localization of PGRP-LC. They therefore hypothesize that Pirk suppresses Imd pathway activity by promoting the internalization of PGRP-LC from the plasmamembrane, or by preventing PGRP-LC from reaching it. Pirk would primarily act as a negative regulator protecting the gut and to a lesser extent the fat body in order to maintain normal bacterial homeostasis (Lhocine et al. 2008).

The study by Aggarwal et al. (2008) also identified Pirk, which they refer to as Rudra, as a negative regulator of the Imd pathway. Instead of using microarray data as a starting point, they performed a yeast two-hybrid screen to identify proteins interacting with the cytoplasmic domain of PGRP-LC. They further confirmed the yeast two-hybrid screening results by performing co-immunoprecipitation experiments, which demonstrated that Pirk binds both PGRP-LC and PGRP-LE, and that this interaction is at least to some extent mediated by the RHIM domain, although the interaction with PGRP-LC requires also the IMD interaction domain. In addition, they found that Pirk also co-immunoprecipitated with IMD. Similar to Lhocine et al., Aggarwal and colleagues showed that $rudra^{EY00723}$ flies are strong hypomorphs with a hyperactivated immune response after infecting them with *E. coli*. When infected by *Ecc15*, $rudra^{EY00723}$ flies were more resistant to the infection than the wild-type flies. For the mechanism of Pirk action, Aggarwal et al. suggest that Pirk disrupts the signaling complex formed by PGRP-LC and IMD, which in turn leads to the downregulation of the signaling.

In their *in vivo* experiments both Aggarwal et al. and Lhocine et al. used flies that carry the P-element insertion *EY00723* at the *pirk* 5' UTR. The same insertion is in the Bloomington stock *15039* that we used in our experiments. Both groups report a significant decrease in Pirk expression, and a significant increase in AMP gene expression in infected *EY00723*-carrying flies. Lhocine et al. further suggest that *EY00723*-carrying flies are Pirk null mutants. In our *15039* flies, *pirk* expression was markedly lower than in wild-type flies, and removing the P element restored expression levels to normal (*rescue*^{82} and *rescue*^{461} flies). However, when we compared the AMP expression of P element harboring and rescue flies, we could not detect significant differences (Fig. 8). This was probably due to the different genetic backgrounds of the flies. The strength of the immune defense and the expression levels of immune responsive genes are known to vary more than many other traits between individual flies and fly lines, which makes controlling the genetic background absolutely necessary. By completing a more thorough back-crossing scheme we might have detected the differences between P-element harboring and rescue flies, but since we were worried about the initial results being a mere artifact related to the use of a cell line and reporter assays, we chose to proceed to use RNAi fly lines to verify our results *in vivo*. Otherwise, the results that we acquired using *pirk* RNAi flies are in line with the data obtained by Aggarwal et al. and Lhocine et al. using the *pirk* hypomorph. Pirk knock-out flies of which *pirk* had been removed by precise excision, have not been published yet.

Besides being an Imd pathway inhibitor, Pirk has also been implicated in JNK pathway signal regulation (II, Bond and Foley 2009). This is not surprising since the Imd pathway bifurcates at the level of Tak1 (i.e. down-stream of Pirk). However, according to data from our lab and Aggarwal et al., Pirk is not involved in Toll signaling. Lhocine et al. further demonstrated that Pirk does not bind Wengen, a *Drosophila* member of TNFR superfamily, and is therefore not likely to act as a general suppressor of immune regulatory pathways in *Drosophila*. Surprisingly, we detected a decrease in Jak/STAT signaling after *pirk* RNAi in S2 cells. Although this decrease was quite modest, it was still significant. This may be due to the interplay of the Imd and Jak/STAT pathways, or a mere reporter artifact.

Based on the data published so far, one can conclude that Pirk negatively regulates Imd pathway activity by binding PGRP-LC and IMD either by disrupting the signaling complex or triggering PGRP-LC internalization from the membrane (Fig. 10). However, how the activity of Pirk is regulated at the post-transcriptional level, is not known. Relish is required for *pirk* transcription, but the mechanisms that fine-tune the Imd pathway response are elusive. The question that still remains to be answered is how the fly maintains the homeostasis of normal flora, being simultaneously ready to trigger the full battery of AMPs as a response to systemic infection. Unveiling the detailed mechanism for this requires further studies.

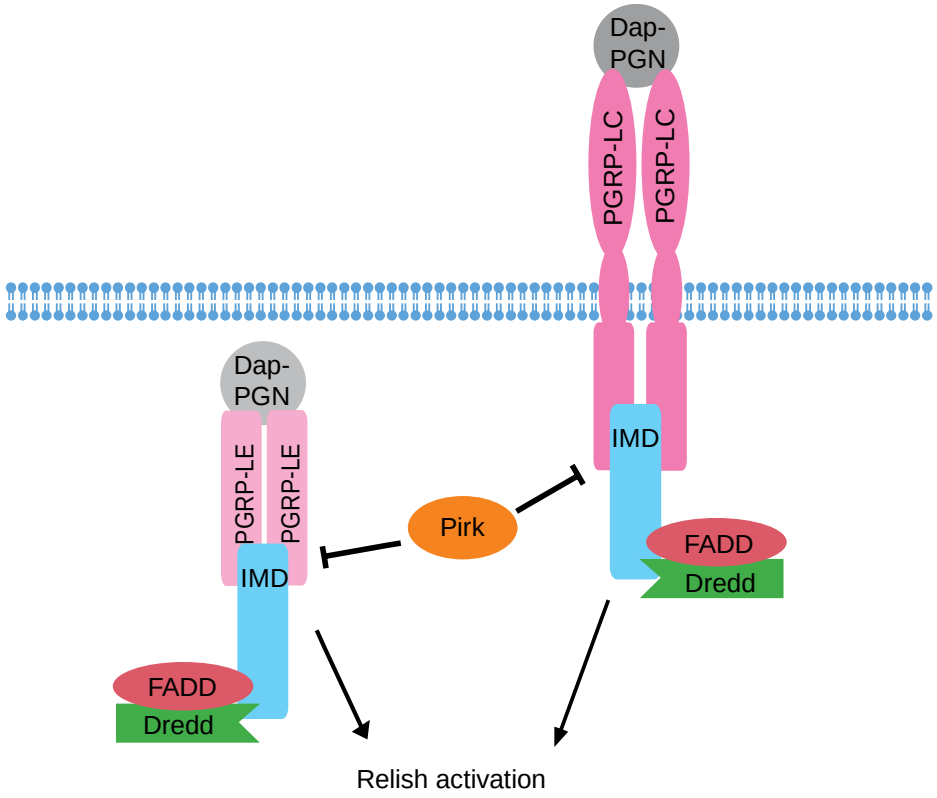

Figure 10. The current hypothesis for Pirk function. Pirk interacts with PGRP-LC, PGRP-LE, and IMD, and suppresses Imd signaling by disrupting the signaling complex or by eliciting internalization of PGRP-LC from the plasma membrane.

6.3 Iap2 and Tab2 as novel components of the Imd pathway

Before the publication of original communication III, *Drosophila* Iap2 was regarded as a caspase regulator and apoptosis antagonist rather than an essential component of the innate immune signaling machinery. Indeed, Iap2 plays a partially redundant role with Iap1 in controlling the cell death machinery (Leulier et al. 2006b), although Iap1 seems to be more important for caspase regulation. However, the association of Iap2 with the immune response was soon verified by other groups both *in vitro* (Gesellchen et al. 2005) and *in vivo* (Leulier et al. 2006a, Huh et al. 2007). The exact mechanism of Iap2 function is still not completely understood. Based on the predicted domain structure, Iap2 could act as an E3 ubiquitin ligase. This is supported by the observation that the

RING domain of Iap2 is indispensable for its function in immune response (Huh et al. 2007).

Ubiquitination has been reported to be involved in the activation of the Imd pathway (Zhou et al. 2005), and it has also been implicated in mammalian NF-κB signaling. Like Iap2, mammalian TRAF proteins contain the N-terminal RING domain, typical for ubiquitin E3 ligases. It has been shown *in vitro* that the Tak1/TAB2 complex is activated by TRAF6, which in turn is activated via K63-linked ubiquitination (Wang et al. 2001). This requires the E2 proteins Ubc13 and UEV1a (Deng et al. 2000), the orthologs of which have been reported to participate in the activation of Tak1 and the IKK complex in *Drosophila* (Zhou et al. 2005). In addition, the mammalian homolog of IMD, RIP1, has been shown to be K63 polyubiquitinated as well (Lee et al. 2004). However, the E3 ligase involving these ubiquitination steps still remains unknown.

Due to the complexity of mammalian TNFR signaling, the effector molecules involved in the ubiquitination-mediated regulation of the pathway activity in mammals have not been thoroughly mapped. Instead, Thevenon et al. (2009) recently reported a *Drosophila* deubiquitinating enzyme dUSP36 (or Scrawny) to be important for the negative regulation of the Imd pathway *in vivo*. They demonstrated that IMD was K63-polyubiquitinated in response to pathway activation, and that dUSP36 reduced the amount of K63-ubiquitinated IMD. This in turn led to increased proteasomal degradation of IMD (Thevenon et al. 2009). Based on this they proposed a model, where IMD is activated by K63-linked polyubiquitination, and these ubiquitins are then removed by dUSP36, which deactivates Imd, targets it to degradation and suppresses the activation of the Imd pathway. The E3 ligase activating IMD is missing from this model, but the authors proposed that Iap2 could be a potential candidate.

Recently, Paquette et al. (2010) demonstrated that Iap2 is an active E3 ligase interacting with IMD. They reported that upon PGN stimulation, IMD is enzymatically cleaved by the caspase Dredd. This cleavage reveals an interaction site for Iap2, which binds to IMD via its BIR2 domain, and to a lesser extent via its BIR3 domain. E2 ligases Bendless (Bend), Uev1a, and Effete (Eff) participate in the K63-linked polyubiquitination mediated by the Iap2 RING domain. The authors speculate that K63-linked polyubiquitin chains could recruit both Tab2/Tak1 and IKK complexes, which sequentially leads to kinase activation and signal transduction. Ubiquitination of cleaved IMD was shown to be rapid and to occur within minutes after a PGN or bacterial stimulus, reaching its maximum in 10 minutes. Thereafter the levels of polyubiquitinated IMD started decreasing until the ubiquitination was almost undetectable at 30 min (Paquette et al. 2010).

When studying the kinetics of the Imd pathway response by microarray and PCR-based methods, we found that knocking down *Iap2* in S2 cells could not block signaling within the first hours of infection (I Fig. 2, III Fig. 2B). This was also seen *in vivo* in *Iap2* null mutant flies (I Fig. 3), which confirms that this was not an RNAi-related artifact. In contrast, knocking down other Imd pathway components, such as *Tab2*, completely abolished the transcription of *AMP*s. This

implies that Iap2 is not required for the early response. It could therefore be hypothesized that Iap2-induced K63-polyubiquitination of IMD could be the trigger for switching from the JNK pathway-mediated early response to a sustained response. Ubiquitination of IMD would therefore be unnecessary for activation and signaling via the JNK branch, but instead essential for maintaining Imd pathway activity high after a few hours. Prolonged signaling may ensure host survival during a systemic infection, but as a response to commensal bacteria in the gut this could severely harm the host. Therefore suppressing the constitutive activation of IMD by dUSP36 as suggested by Thevenon et al. (2009) would be required for the temporal control of the immune response. To test this, one should first determine if IMD cleavage alone is sufficient to initiate Imd signaling at all, or if the K63-linked polyubiquitination of IMD is crucial for signal transduction. In addition, the signaling steps required for Dredd recruitment and activation in response to bacterial recognition, and how this activation is regulated, require further research. Dnr1 and Caspar have been implicated as negative regulators of the Imd pathway possibly targeting Dredd (Foley and O'Farrell 2004, Kim et al. 2006), but the mechanisms for this are not fully understood. Furthermore, the putative E3 ligase POSH has been reported to regulate Tak1 stability and to be required for the accurately timed termination of JNK activation (Tsuda et al. 2005). How all these factors work in concert to carefully adjust the *Drosophila* Imd and JNK-mediated responses, requires the study of the complex signaling network as a whole, as well as the understanding of the function of the individual proteins. The current model for signal regulation at the level of IMD is illustrated in Fig. 11.

In mammals, the activation of NF-κB and IKK requires a complex formed by Tak1 together with the adaptor proteins TABs. It has been reported that mammalian TAB2 and TAB3 proteins bind K63-polyubiquitin via the ZnF domain. Mutations in the ZnF domain can abolish this binding ability, resulting in the deactivation of Tak1 and IKK. Furthermore, TAB2 has been shown to bind RIP1 after TNFα stimulation (Kanayama et al. 2004). TABs also contain a CUE domain, which has been suggested to be involved in binding ubiquitin conjugating enzymes. It is therefore tempting to speculate if Tab2 in *Drosophila* acts as a mediator between IMD and the proteins involved in the ubiquitinylation machinery, such as the E2 ligases Bendless (*Drosophila* Ubc13) and Uev1A. Nevertheless, this is a further indication of the significance of ubiquitinylation in the regulation of immune signaling also in *Drosophila*.

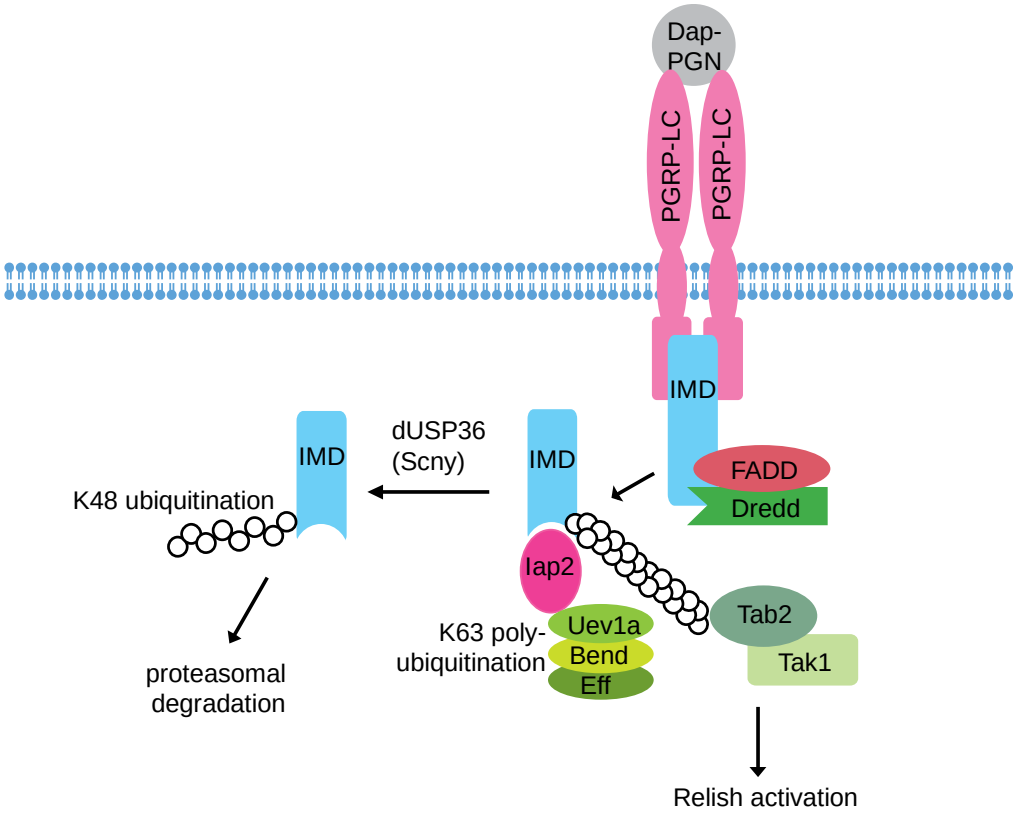

Figure 11. The current hypothesis for the ubiquitin-dependent regulation of IMD. Dredd-dependent cleavage of IMD reveals a binding site for the E3 ligase Iap2, which together with the E2 ligases Uev1a, Bendless (Bend) and Effete (Eff) link K63 polyubiquitin chains to IMD. Tab2 contains a domain that can bind K63 polyubiquitin chains, and this interaction is likely to mediate the recruitment of Tak1 to the signaling complex leading to the activation of both Imd and JNK signaling. IMD activation can be suppressed by the ubiquitin protease dUSP36, which removes the K63 polyubiquitin chains promoting K48-linked polyubiquitination and proteasomal degradation of IMD.

7. Conclusions and future perspectives

During the past decades studies conducted using *Drosophila melanogaster* as a model have been essential in expanding our knowledge and understanding of various biological processes. A compact genome combined with relatively low redundancy, evolutionarily conserved cell signaling pathways, the broad variety of tools available for genetic studies, and the fact that flies lack the adaptive immune system, have fueled the use of *Drosophila* in studying innate immunity. After the implication of Toll involvement in immune signaling led to the discovery of mammalian TLRs, the fly has become even more popular. Traditionally the use of *Drosophila* was more limited to forward genetic screening, but since RNAi was found to work exceptionally well in *Drosophila in vitro* and *in vivo*, both reverse and forward genetics have been used. This has enabled scientists to uncover new connections between cellular and environmental factors that otherwise would have been difficult to examine.

In this study, we used the reverse genetic approach by performing a large-scale RNAi screen in *Drosophila* S2 cells to identify novel Imd pathway components. Thereafter we used the more traditional tools of molecular biology and genetics to verify our findings *in vitro* and *in vivo*. We found that Tab2 and Iap2 are essential for Imd pathway signaling. Both of these proteins have mammalian homologs, and mammalian TABs have been implicated in the regulation of NF-κB signaling. Tab2 is required for both the early and sustained response to Gram-negative bacteria, while Iap2 is required for the sustained response only. Furthermore, flies that lack Iap2 succumb to Gram-negative bacterial infections. Recently, others have demonstrated that Iap2 is a functional E3 ligase activating IMD by K63-linked ubiquitination. The interaction between Iap2 and IMD requires the cleavage of IMD by the caspase Dredd (Paquette et al. 2010). Dredd has a dual role in Imd pathway activation, since it is required for the cleavage of both IMD and Relish. Recruitment of Dredd to the PGRP-LC-IMD signaling complex is possibly mediated by the death domain-containing adaptor protein dFADD, but the exact mechanism of Dredd activation remains elusive. Nor is it known which proteins are responsible for the temporal control of the early and sustained responses, and how this process is regulated.

In addition to the two positive regulators, we identified an Imd pathway negative regulator, Pirk. Pirk suppresses Imd pathway activity *in vitro* and *in vivo*, and the overexpression of Pirk sensitizes the flies to Gram-negative bacterial infections. Pirk is likely to interact with PGRP-LC and IMD directly, but there might be other proteins involved that further facilitate the silencing of the pathway. The exact mechanism for how Pirk suppresses the Imd pathway is

not thoroughly understood. Nor is it known how the activity of Pirk is regulated under normal conditions or during infection.

Overall, the present study further verifies that *Drosophila* is a relevant model for studying innate immune signaling pathways, and demonstrates that Imd signaling is strictly regulated and more complex than was previously thought. The identification of both positive and negative Imd pathway regulators hopefully opens up novel insights also into the regulation of mammalian NF-κB signaling.

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Anni Kleino

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10. Original communications