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Systems Biological Study of *Drosophila* Immune Signaling

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ACADEMIC DISSERTATION
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Life isn't about waiting for the storm to pass,
it's about learning to dance in the rain

To my family

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1 ABSTRACT

The development of systems biological high-throughput methods such as microarray analysis and genome-wide screens based on ribonucleic acid interference (RNAi) opened many doors for scientific research. Vast amounts of data can be obtained from a biological system and processed, while the system functions. . The ambitious goal of systems biological studies is to characterize the entire biological system: to identify all of its components and to determine how the system is regulated. However, a large scale systems biological study requires careful planning prior to experiments and taking into account any problems that might be related to the assay.

In this study, we used two systems biological methods, an oligonucleotide-microarray assay and a genome-wide RNAi screen, to characterize the *Drosophila melanogaster* immune response. *Drosophila* provides an excellent model for studying the principles of innate immunity. The fruit fly's evolutionary conserved immune system lacks adaptive immunity, so it relies solely on its innate immunity for defending against pathogens. In addition, many genetic and molecular techniques, including *in vivo* RNAi, are available for *Drosophila* enabling effective exploitation of the data obtained from *in vitro* studies. Also, *Drosophila* are cheap and easy to maintain, and RNAi in *Drosophila* is effective and straightforward to carry out.

In our systems biological study of the *Drosophila* innate immunity, we first used a microarray analysis to identify new gene products involved in innate immune signaling and phagocytosis. A microarray analysis was carried out for the genes induced in response to *E. coli* in *Drosophila* S2 cells. This was followed by an RNAi-based functional analysis of the up-regulated genes. Second, we carried out two separate genome-wide RNAi *in vitro* screens to identify gene products necessary for *Drosophila* immune signaling: the *Drosophila* nuclear factor kappa B (NF-κB) signaling and the *Drosophila* Janus tyrosine kinase/signal transducer and

activator of transcription (JAK/STAT) signaling pathway. We identified ten novel regulators of the *Drosophila* NF-κB signaling and five novel regulators of the *Drosophila* JAK/STAT pathway.

One gene from both RNAi screens was subjected to further functional studies. We carried out the *in vitro* characterization of G protein-coupled receptor kinase 2 (Gprk2) identified in the NF-κB screen. Similarly, we also characterized Eye transformer (ET), which was identified in the JAK/STAT screen. We were able to confirm both original phenotypes with targeted RNAi treatments. Furthermore, we validated these results by quantitative reverse transcription polymerase chain reaction (RT-PCR). Thereafter, we characterized both genes *in vivo.*

In our *Drosophila in vivo* RNAi assays, transgenic upstream activating sequence *(UAS)*-RNAi flies were crossed with selected *GAL4* driver flies, generating RNAi silencing of the target gene in the offspring. These flies were infected with *E. cloacae* to induce immune signaling and the RNAi phenotypes were validated by qRT-PCR from RNAs extracted from the flies. We concluded that Gprk2 has an essential role in the *Drosophila* Toll pathway mediated immunity *in vivo,* and that ET is a negative regulator of *Drosophila Tot* gene expression *in vivo.*

2 TIIVISTELMÄ

Systeemibiologiset menetelmät, kuten microarray analyysi ja genomin laajuinen kaksijuosteiseen ribonukleiinihappo(RNA)-häirintään perustuva seulonta-analyysi ovat kehittyneet huimasti viime vuosikymmenien aikana. Näiden menetelmien avulla tutkittavasta biologisesta systeemistä saadaan valtava määrä tietoa reaaliajassa systeemin yhä toimiessa. Systeemibiologisen tutkimuksen tavoitteet ovatkin aina melko kunnianhimoisia. Tutkimuksen avulla pyritään karakterisoimaan koko biologinen systeemi, eli selvittämään, mitkä proteiinit systeemiin osallistuvat ja miten sitä säädellään. Suuren luokan systeemibiologinen tutkimus edellyttää tarkkaa suunnittelua ja ongelmien kartoitusta ennen kokeiden toteutusta.

Tässä työssä pyrittiin karakterisoimaan banaanikärpäsen immuunivastetta kahden systeemibiologisen menetelmän, microarray-analyysin ja genominlaajuiseen RNA -häirintään perustuvan seulonta analyysin avulla. Banaanikärpänen on erinomainen mallieläin tutkittaessa synnynnäistä immuniteettia. Sen immuniteetti on säilynyt evoluutiossa melko muuttumattomana. Lisäksi siltä puuttuu hankittu immuniteetti, jolloin sen immuunipuolustus perustuu ainoastaan synnynnäiseen immuniteettiin. Banaanikärpäselle on saatavilla monia käteviä genetiikan ja molekyylibiologian työkaluja, kuten *in viv*o RNA-häirintä. Lisäksi kärpäsia on helppo ja halpa ylläpitää, ja RNA-häirintä on mentelmänä tehokas ja helppo toteuttaa.

Banaanikärpäsen immuniteetin systeemibiologinen tutkimus aloitettiin microarray analyysillä. Sen avulla pyrittiin selvittämään, mitkä geenituotteet osallistuvat banaanikärpäsellä synnynnäisen immuniteetin signalointiin ja fagosytoosiin. Analyysi toteutettiin banaanikärpäsen S2-soluissa, jotka oli altistettu kolibakteerille. Microarray-analyysin avulla selvitettiin, mitkä geenit aktivoituvat banaanikärpäsen S2-soluissa vasteena kolibakteeri-infektiolle. Näiden geenien toimintaa ja merkitystä immuunivasteelle pyrittiin kartoittamaan RNA-häirintään perustuvan analyysin avulla. Tässä työssä esitellään lisäksi kaksi genomin laajuista RNA-

häirintään perustuvaa analyysiä, joiden avulla pyrittiin selvittämään, mitkä geenituotteet osallistuvat banaanikärpäsen immuunisignalointiin. Genomin laajuiset analyysit tehtiin banaanikärpäsen NF-κB- sekä JAK/STAT -signalointireiteille. Näiden seulonta-analyysien avulla pystyimme tunnistamaan kymmenen ennestään tuntematonta säätelytekijää, jotka osallistuvat banaanikärpäsen NF-κB -signalointiin, sekä viisi uutta JAK/STAT - signalointireitin säätelijää.

Näistä geenituotteista valitsimme kaksi, Gprk2-proteiinin NF-κB -signalointireitiltä ja ET-proteiinin JAK/STAT -signalointireitiltä, joiden toimintaa halusimme tutkia tarkemmin. Alkuperäiset tulokset pystyttiin toistamaan molempien geenituotteiden osalta kohdennetun RNA-häirinnän avulla. Tulokset validoitiin lisäksi kvantitatiivisella käänteistranskriptiopolymeraasiketjureaktion (qRT-PCR) avulla. Tämän jälkeen molempien geenien toiminta pyrittiin myös karakterisoimaan banaanikärpäsellä *in vivo*.

In vivo analyysissä hyödynnettiin kaupallisia kärpäsiä, joiden perimään on istutettu RNA-häirintäominaisuus. RNA-häirintä näillä kärpäsillä toimii siten, että perimässään *UAS*-RNA - häirintägeeniä kantava kärpänen risteytetään *GAL*-geeniä kantavien kärpästen kanssa. Tuloksena syntyy jälkeläiskanta, jolla *GAL4* aktivoi *UAS*-RNA -häirinnän ja kohdegeenin ilmentäminen estyy. Nämä kärpäset infektoitiin *E. cloacae* -bakteerilla immuunivasteen käynnistämiseksi. RNA eristettiin ja analysoitiin qRT-PCR menetelmän avulla. Näiden systeemibiologisten tutkimusten perusteella tulimme siihen johtopäätökseen, että Gprk2 on välttämätön banaanikärpäsen Toll-signaloinnille. Lisäksi pystyimme osoittamaan, että ET - proteiini on banaanikärpäsen *Turandot* - geenin negatiivinen säätelijä.

3 LIST OF ORIGINAL COMMUNICATIONS

I. **Kallio J**, Kleino A, Ulvila J, Valanne S, Ezekowitz R.A., Rämet M
Functional analysis of immune response genes in *Drosophila* identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells
Microbes Infect. 2005 May;7(5-6):811-9

II. Valanne S, Myllymäki M[*], **Kallio J**[*], Schmid MR, Kleino A, Murumägi A, Airaksinen L, Kotipelto T, Kaustio M, Ulvila J, Esfahani SS, Engström Y, Silvennoinen O, Hultmark D, Parikka M, Rämet M
Genome-Wide RNA Interference in Drosophila Cells Identifies G Protein-Coupled Receptor Kinase 2 as a Conserved Regulator of NF-κB Signaling
J Immunol. 2010 Jun 1;184(11):6188-98.

III. **Kallio J**[*], Myllymäki H[*], Grönholm J, Armstrong M, Vanha-aho L-M, Mäkinen L, Silvennoinen O, Valanne S, Rämet M.
Eye Transformer is a negative regulator of Drosophila JAK/STAT signaling
FASEB J. 2010 Jul 12.

* equal contribution

The thesis is based on these three publications, which are referred to accordingly by their roman numerals.

4 ABBREVIATIONS

Act = Actin

AMP = antimicrobial peptide

aop = anterior open

Att = Attacin

β-gal = beta-galactosidase

bp = base pair

bsk = basket

CBM = cytokine binding module

cDNA = complementary DNA

CNTF = ciliary neurotrophic factor

CT-1 = cardiotrophin-1

C-terminal = carboxy-terminal

DAP = diaminopimelic acid

DD = death domain

Dif = Dorsal-related immunity factor

DNA = deoxyribonucleic acid

Dome = Domeless

dPIAS = *Drosophila* Protein Inhibitor of Activated Stat

Dredd = Death-related ced-3/Nedd2-like protein

Drs = Drosomycin

ds = double stranded

enok = enoki mushroom

ET = Eye Transformer

EtOH = ethanol

FADD = Fas associated death domain

G-CSF = granulocyte colony-stimulating factor

GFP = Green Fluorescence Protein

GNBP-1 = gram-negative bacteria binding protein

Hep = hemipterous

Hop = hopscotch

Hsp = heat-shock protein

Iap = inhibitor of apoptosis

IκB = inhibitor of κB

IKK = IκB kinase

IL = interleukin

Imd = Immune defiency

IRC = immune responsive catalase

Ird5 = immune responsive deficient 5

JAK = Janus tyrosine kinase

JNK = Jun kinase

Jra = Jun-related antigen

kay = kayak, *Drosophila* Fos

kb = kilobase

key = Kenny, Drosophila, IKKɣ

LB = Luria Bertani broth

LIF = leukemia inhibitory factor

LPS = lipopolysaccharide

LRR = leucine rich repeat

luc = luciferase

MAMP = microbe-associated molecular pattern

MAPKKK = Mitogen-Activated Protein Kinase Kinase Kinase

MBL = mannose binding lectin

Med27 = Mediator complex subunit 27

mRNA = messenger ribonucleic acid

MyD88 = myeloid differentiation factor 88

NFκB = nuclear factor kappa B

n.s. = non-significant

N-terminal = amino-terminal

OSM = oncostatin M

PAMP = pathogen associated molecular patterns

PBS = phosphate-buffered saline

PCR = polymerase chain reaction

PGN = peptidoglycan

PGRP = peptidoglygan recognition protein

Pirk = poor Imd response upon knock-in

PLB = passive lysis buffer

PRR = pattern recognition receptor

PTP61F = protein tyrosine phosphatase 61F

puc = puckered, *Drosophila* Jun kinase phospatase

qRT-PCR = quantitative reverse transcription PCR

RHD = REl homology domain

RHIM = RIP homotypic interaction

RING = really interesting new gene

RISC = RNA-induced silencing complex

RNA = ribonucleic acid

RNAi = ribonucleic acid interference

ROS = reactive oxygen species

S2 = Schneider-2

SH3 = Src homology 3

SCID = Severe combined immunodeficiency

siRNA = small interfering RNA

SOCS = suppressor of cytokine signaling proteins

SODD = silencer of deth domain

SP-A = surfactant apoprotein A

STAM = Signal Transducin Adaptor Molecule

STAT = signal transducer and activator of transcription

Su(var)2-10 = Suppressor of variegation 2-10

Tab2 = Tak1-associated binding protein 2

Taf1 = TBP-associated factor 1

Tak1 = TGF-β-activated kinase 1

TEP = thioester-containing protein

TGF = transforming growth factor

TIR = Toll/interleukin-1 receptor

TLR = Toll-like receptor

TNF = tumor necrosis factor

TNFR = tumor necrosis factor receptor

Tot = Turandot

TRADD = TNF receptor accociated death domain

TRAF = TNF associated factor

UAS = upstream activating sequence

Upd = Unpaired

UTR = untranslated region

Wg/Fz = Wingless-Frizzled

5 INTRODUCTION

Systems biology is a concept that can be seen as a way of scientific thinking. It began in the 20th century as a result of the discovery of many basic methods, such as PCR and DNA sequencing. The holistic perspective of systems biology quickly replaced the old practices of studying the characteristics of isolated parts of a cell or organism. Instead, increasing automation and computerization combined with a growing availability of genomic sequences allow processes of interest to be monitored in a functioning biological system.

Today, many high-throughput systems biological method are readily accessible, of these DNA microarray analysis is an eminent example. It comprises a series of thousands of microscopic spots of DNA oligonucleotides, which allows the examination of the entire genome in a single reaction. Microarrays can be used to detect temporal changes in expression levels in different cells or tissue samples.

Another example of a systems biological methods is a genome-wide RNAi based screen. It uses RNAi, an ancient gene silencing mechanism for host defense against parasitic nucleic acids such as viral genes and transposons. In the RNAi machinery, dsRNA, which is detected as foreign, activates a complex RNAi pathway. The RNAi pathway signals the degradation of the corresponding mRNA causing the expressional silencing of the target gene. RNAi was first observed by plant scientists attempting to alter flower colors in petunias, but was better understood when Andrew Fire and Craig C. Mello published their work on RNAi in *C. elegans* in Nature in 1998. A genome-wide RNAi based screen is an extremely powerful method for identifying gene products that are necessary for physiological events of interest. In a genome-wide RNAi screen, the systematical suppression of each gene in the cell by targeted dsRNA treatments can be used to identify the components necessary for different cellular processes.

The *Drosophila* immune system is well conserved through out evolution. This makes *Drosophila* an excellent model for studying the basic mechanisms of host defense, since the results obtained may be comparable to the human immune system. Although *Drosophila* lacks the adaptive immune system, it is highly resistant to microbial infections due to innate immune reactions. These include the encapsulation and phagocytosis of invading pathogens (cellular response), blood clotting, melanin formation and opsonization (local response) and the synthesis of potent antimicrobial peptides (AMPs) (humoral response). In addition, *Drosophila* resists viruses through the degradation of viral RNA by its RNAi machinery and via cytokine-mediated induction of many stress genes, which counter the viral infection.

There are at least four signaling pathways that regulate the immune responses in *Drosophila*. *Drosophila* immunity distinguishes between Gram-negative and Gram-positive bacteria through the Imd and the Toll pathways, respectively. In addition to NFκ-B signaling pathways, the JNK- and JAK/STAT pathways seem to have a role in regulating gene expression after septic injury and during viral infections.

6 REVIEW OF THE LITERATURE

6.1 Systems biology

For many decades the traditional approach of scientific study was to investigate the characteristics of isolated parts of a cell or organism. The discovery of many basic methods, such as PCR and DNA sequencing during the 80's paved the way to a whole new way of addressing scientific questions called systems biology. The beginning of the Human Genome Project in 1990 was one of the starting points of this revolution, which permanently changed the way of scientific thinking. !ncreasing automation and computerized systems combined with the availability of the genomic sequences of many species, including humans, allow processes of interest to be monitored in a functioning biological system (Kohl et al., 2010; Pitteri and Hanash, 2010; Schadt et al., 2010; Sharon et al., 2010).

6.1.1 Systems biology: history and practice

Systems biology is a scientific approach that applies a holistic perspective to the investigation of complex interactions in biological systems. This approach has been widely used in biosciences particularly from the year 2000 onwards. The basic goals of systems biology are to gain new insights into the functional interactions of macromolecules in a biological system of interest, and to better understand the entirety of the processes that take place in biological systems (Kohl et al., 2010; Pitteri and Hanash, 2010; Sharon et al., 2010).

The roots of the holistic thinking of systems biology can be seen as early as in 1950 when theoretician Ludwig von Bertalanffy published his book entitled "General Systems Theory in Physics and Biology". Two years later, neurophysiologists and Nobel prize winners Alan Lloyd Hodgkin and Andrew Fielding Huxley published a

mathematical model that explained the interaction between a potassium and sodium channel, giving rise to computational systems biology. The discovery of two major techniques, DNA sequencing in 1975 and PCR in 1985, further contributed to the beginning of this new scientific approach to biology. The scientific community started to increasingly acknowledge the digital nature of DNA and biology was redefined as an informational science (Bertalanffy, 1949; Bertalanffy, 1950; Hodgkin and Huxley, 1952)

The formal study of systems biology was launched in the 1990s when functional genomics made large quantities of data available, and computerized systems enabled the automation of their analysis. The scientific revolution started in 1990 in the form of The Human Genome Project. The first research institutes focusing entirely on systems biology were founded in 2000 in Seattle and in Tokyo (Sauer et al., 2007) and many more have been established since..

A holistic perspective of biological systems provides information about a system's structure, dynamics and control, all at the same time. The information controlling all biological systems can be divided into two main types. The most important aspect is the digital nature of DNA, i.e. of the genes which encode the proteins (Sauer et al., 2007). The expression of these genes in time and space is tightly controlled by a second level of biological information, the complex regulatory networks. Since vast amounts of data are obtained by high-throughput methods, such as large-scale DNA sequencing, microarrays and mass spectrometry, the aims of systems biological studies are always quite ambitious (Kohl et al., 2010; Nita-Lazar). The underlying goal of any systems biological study is to determine how the biological system of interest works and how it is regulated (Kohl et al., 2010; Nita-Lazar). This requires careful planning prior to experiments. The basic framework of a systems biological study is to first determine all of the components of the system and to then monitor the components by selected high-throughput biological tools and finally reconcile the original model with the results (Sauer et al., 2007; Schadt et al., 2010).

Systems biological approaches start with the definition of all of the components of the system under study. This includes determining the structure of the overall network of genes and proteins, their interactions and regulation. Another aspect is to predict the system dynamics in order to determine its functional properties; how the

system behaves over time under various conditions. The initial model is tested by disturbing the system by genetic or environmental perturbations and monitoring the system's response to these perturbation using large-scale discovery tools. Based on these experimental observations, the original hypothesis is then evaluated and modified to fit the results (Csete and Doyle, 2002; Davidson et al., 2002; Gardner et al., 2003; Ge et al., 2003; Hood, 2003a; Hood, 2003b; Ideker et al., 2001; Kitano, 2002)

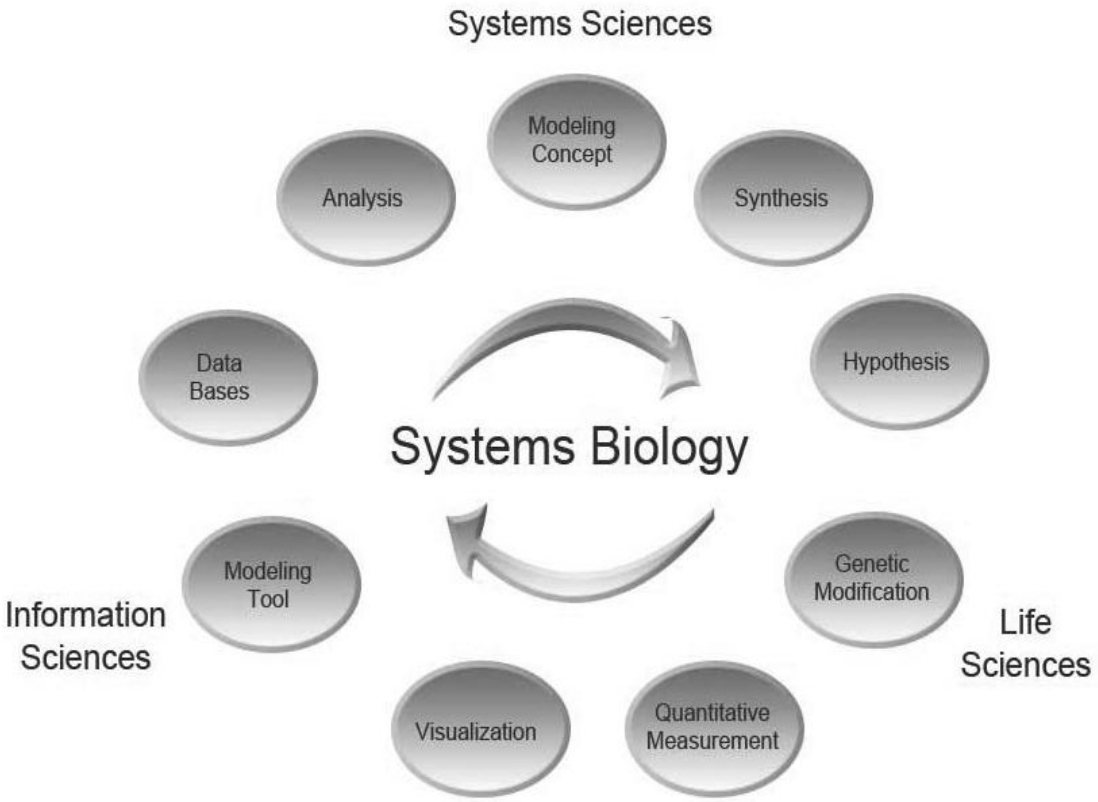


Figure 1. Systems biology diagram (modified from the picture on the Institute for Systems theory and Automatic control website: http://www.ist.uni-stuttgart.de/education/courses/sysbiointro/)

6.1.2 Microarray analysis

Microarrays are microscope slides that contain an ordered series of gene-specific probes. Since the probes are arranged on the slides in an ordered fashion, data obtained from an analysis can be traced back to any of the genes. Microarray assays can contain several thousands of addressable genes. Vast amounts of data are produced in a single assay.

A DNA microarray analysis is a great example of the multiplex technology used in systems biology. Microarrays comprise series of thousands of microscopic spots of DNA oligonucleotides each containing a specific DNA sequence as small as 20 base pairs. Microarrays can be used to detect temporal changes in the expression levels of different cells and tissue samples. Microarray assays are a genuine high-throughput method allowing the examination of the entire genome in a single reaction. A single microarray experiment can contain tens of thousands of probes and can therefore accomplish many genetic or expression analysis in parallel. Because of this, microarray technology has dramatically accelerated many types of biological and medical investigations (Schena et al., 1995).

The first expression analysis using microarray technology was described in 1987 when arrayed cDNAs were used to identify interferon regulated genes. In these early arrays the cDNA was spotted onto filter paper for analysis with a so called pin-spotting device (Kulesh et al., 1987). The first genome wide expression analysis became possible in 1997 when a complete eukaryotic genome (*Saccharomyces cerevisiae*) was published on a microarray (Kulesh et al., 1987) (Lashkari et al., 1997).

A standard microarray analysis typically follows the five experimental steps of selecting the genes of interest, sample preparation and microarray synthesis, biochemical reactions and array hydridization, detection, data analysis and modeling with controls. The oligonucleotides attached to a solid surface of glass or silicon can be synthesized either *in situ* or they can be pre-synthesized and then deposited onto the chip by a covalent bond. Microarrays can be used in a comparative genomic hybridization analysis to detect DNA or in an expression analysis to detect RNA or most commonly cDNA after reverse transcription.

Oligonucleotides on a chip can be short probes comprising a section of a gene or other DNA element such as a cDNA or cRNA sample target hybridized under high-stringency conditions. Targets are usually labeled with fluorophore-, silver-, or chemiluminescence tags to detect the probe by hybridization. The quantification of label localization and intensity provide information about temporal gene expression and relative abundance of nucleic acid sequences in the target (Pollack et al., 1999; Schena, 1996; Watson et al., 1998).

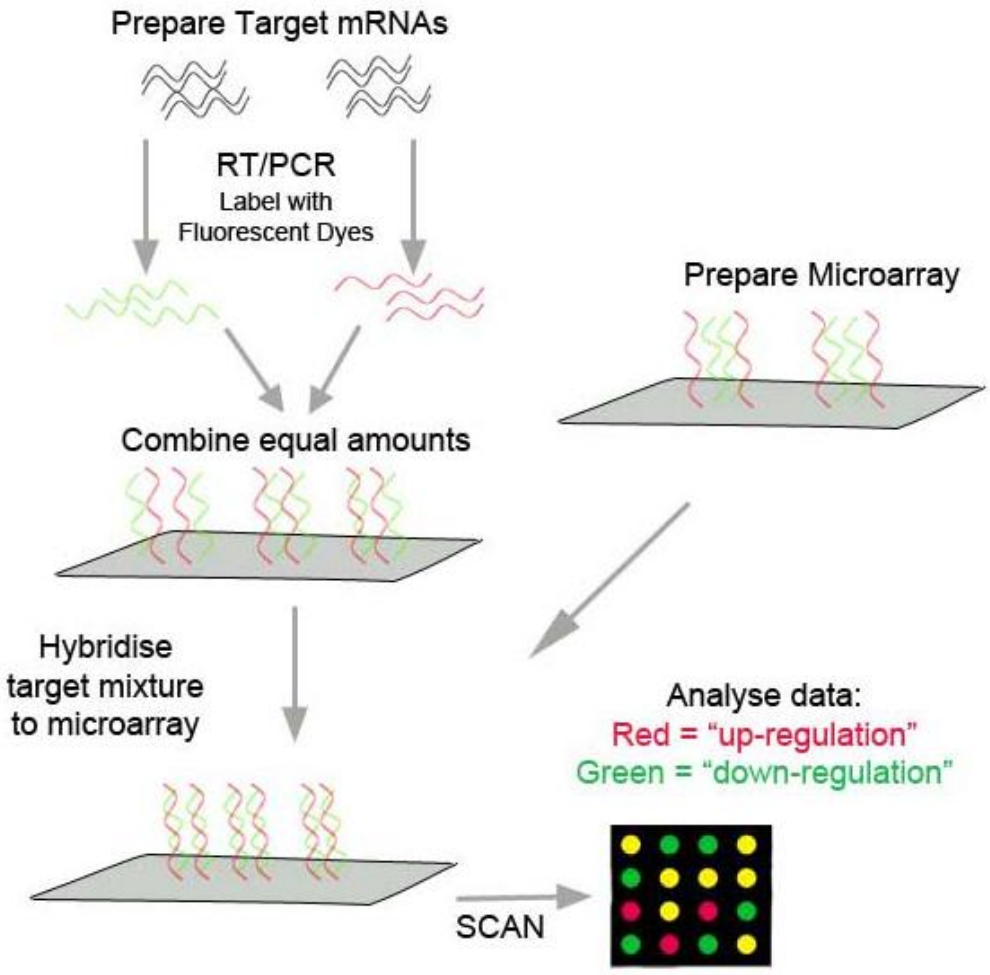


Figure 2. Example of a microarray analysis (Modified from the webpage of Microarray Center: http://www.microarray.lu/en/MICROARRAY_Overview.shtml)

6.1.3 RNA interference: Basic mechanisms of RNAi

RNA interference is an ancient gene silencing mechanism for host defense at the cellular level. It has a crucial role in the immune defense against the parasitic genes of viruses or transposons. RNAi is triggered by a double-stranded RNA normally introduced into the cells by viruses. dsRNA is recognized as foreign by cells and it therefore activates a complex RNA interference pathway leading to degradation of the corresponding mRNA, which causes the expressional silencing of the target gene (Hammond et al., 2000) (Bernstein et al., 2001a; Zamore et al., 2000) (Bernstein et al., 2001b; Macrae et al., 2006; Siomi and Siomi, 2009a) (Siomi and Siomi, 2009b).

RNAi machinery

The detection of foreign dsRNA in cells activates a complex RNAi machinery initiated by a nuclease of the RNase III family called Dicer. After detection of the dsRNA, the activated Dicer binds to dsRNA and cleaves it into small, approximately 20 nucleotides long fragments called small interfering RNAs (siRNAs). Each of these small fragments consists of two strands called the guide strand and the anti-guide strand corresponding to the original gene sequence. After cleavage the siRNAs are inserted into a large protein and RNA containing complex called the RNA-induced silencing complex (RISC). In the RISC complex one of the protein components, endonuclease of Argonaute AGO, cleaves off the anti-guide strands of the siRNAs. The remaining guide strand then attaches to the complementary endogenous mRNA marking it for degradation by cleavage also carried out by the AGO enzyme. A large number of siRNAs, each binding to the mRNA of the target gene, are made from a single strand of dsRNA. This generates the amplification effect of the pathway and only a small amount of original dsRNA is enough to activate the silencing effect of the target gene. Furthermore, the silencing is inherited by the next generation of cells (Hammond et al., 2000) (Bernstein et al., 2001a; Zamore et al., 2000) (Bernstein et al., 2001b; Macrae et al., 2006; Siomi and Siomi, 2009a) (Siomi and Siomi, 2009b) .

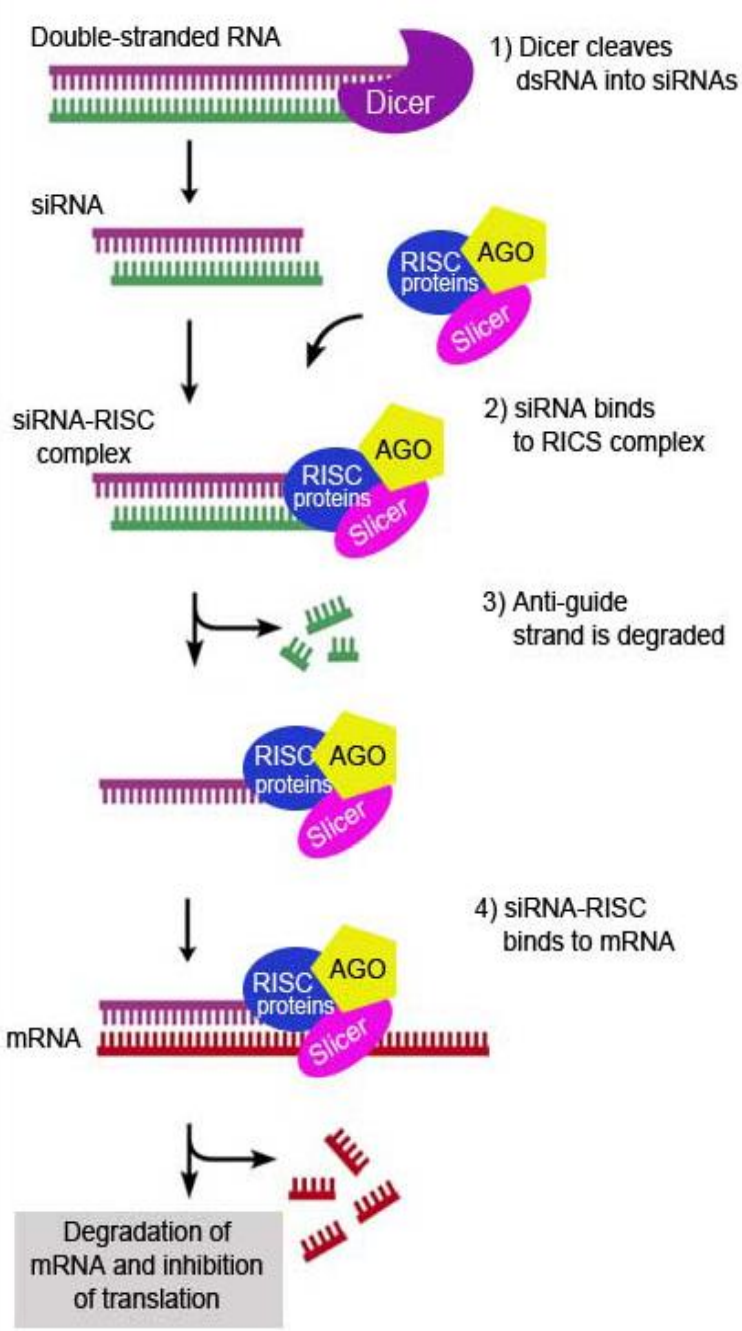


Figure 3. Schematic representation of the RNAi mechanism

History and discovery

RNAi was first observed by plant scientists attempting to alter flower colors in petunias. In order to produce darker flowers, they introduced additional copies of a gene encoding an enzyme responsible for flower pigmentation. This overexpression of chalcone synthase resulted unexpectedly in less pigmented white flowers, in which both endogenous and transgenes were completely downregulated. This was later discovered to be an outcome of post-transcriptional inhibition of gene expression via an increased rate of mRNA degradation, but the molecular mechanism remained unknown.

RNAi became better understood when Andrew Fire and Craig C. Mello published their work on RNA interference in *C. elegans* in Nature in 1998 (Fire et al., 1998). By that time, RNAi was known by other names such as co-suppression of gene expression and post-transcriptional gene silencing. Fire and his co-workers discovered that these are all part of the same phenomenon named RNAi. In 2006 Fire and Mello shared the Nobel Prize in Physiology or Medicine for their discovery (Fire et al., 1998).

RNAi is a valuable and easy-to-use research tool that can be used for large-scale screens (Ramet et al., 2002b).The effect of RNAi on gene expression is highly selective and robust and synthetic dsRNAs can be introduced into cultured cells or living organisms to induce the specific knock-down of a gene of interest. Systematic suppression of each gene in the cell by targeted dsRNA treatments can be used in systems biology to identify the components necessary for different cellular processes. The possible exploitation of RNAi in medicine is also under investigation (Castanotto and Rossi, 2009).

6.1.4 Large-scale genome-wide RNAi based screens

In current experimental biology, RNAi based methods are widely used to study the function of genes both in cell cultures and in appropriate model animals such as *Drosophila melanogaster*. With specific primers, a complementary dsRNA targeting the gene of interest can be synthesized and introduced into cells. The dsRNA is considered as exogenous genetic material and therefore it activates the RNAi pathway resulting in a drastic decrease in the expression of the target gene. The physiological effects of this decrease can provide information about the functional role of this gene product (Boutros et al., 2004; Cullen and Arndt, 2005).

One the most common model animals used in the functional genomics applications of RNAi is *Drosophila*. It is relatively easy to maintain, the RNAi is very effective and most importantly, the delivery of dsRNA into cultured *Drosophila* cells is extremely simple. *Drosophila* S2 cells spontaneously take up the dsRNA fragments so the RNAi can be accomplished simply by soaking the cells in dsRNA containing cell culture medium. dsRNA is subsequently taken up through scavenger receptor mediated endocytosis (Ulvila et al., 2006).

Genome-wide RNAi based screens are an extremely powerful method for identifying gene products necessary for physiological events of interest with many advantages and few downsides (Ramet et al., 2002b). One of the most disconcerting problems of RNAi is off-target effects. Off-target effects occur frequently with dsRNAs containing repetitive sequences and it has been estimated that such sequences occur in about 10% of possible RNAs(Ge et al., 2003; Qiu et al., 2005). Off-target effects appear when a dsRNA delivered into cells has a sequence that can align with multiple mRNAs and therefore potentially knock-down many genes at the same time. Computational biologists have directed extensive efforts into developping software tools for designing dsRNAs that are automatically checked for repetitive sequences and for possible cross-reactivity that might cause off-target effects (Ge et al., 2003; Qiu et al., 2005).

Another challenge of RNAi is the delivery of siRNAs into cells. Although *Drosophila* cells readily take up dsRNAs (Ulvila et al., 2006) delivery is more difficult in most other organisms. In general, large-scale genomic screening is seen as a promising method also when screening mammalian cells (Janitz et al., 2006). Designing genome-wide RNAi libraries also requires applications that are much more sophisticated than just the engineering of a defined set of experimental conditions for a single siRNA treatment. (Lu et al., 2005; Zhao et al., 2005) (Herranz and Cohen, 2010; Janitz et al., 2006; Wilkins et al., 2005).

6.2 *Drosophila* as a model for studying the immune system

The immune response of *Drosophila* has proven to be a useful model for studying the principles of the immune system (reviewed by Lemaitre and Hoffmann, Annu Rev Immunol (Lemaitre and Hoffmann, 2007)). *Drosophila* relies solely on innate immune reactions when defending against pathogens. This is an advantage over vertebrates, in which a highly sophisticated and complex immune system may complicate analyses. For instance, adaptive mechanisms may compensate for some knock-outs of innate immune mechanisms (Hoffmann et al., 1999). For this reason, the use of the *Drosophila* model facilitates the discovery of essential molecules and pathways involved in the innate immune response that is well-conserved from insects to human (Hoffmann et al., 1999; Hultmark and Ekengren, 2003), (Lemaitre and Hoffmann, 2007). On this premise, it is not surprising that the genetic and molecular techniques available for *Drosophila* have greatly facilitated the revealing of the basic mechanisms of the innate host response (Hoffmann et al., 1999; Hultmark and Ekengren, 2003).

6.2.1 The human immune system

The human immune system consists of the adaptive and innate immunity. Adaptive immunity is based on the selection of somatically recombined B and T cell receptors whereas innate immunity relies on germ-line encoded PRRs that selectively bind to pathogens. The innate immune system has an essential role in protecting the host during the first hours after exposure to an infectious agent before the clonal expansion of T- and B-cells (Hoffmann et al., 1999). Ligation of PRRs to microbes enhances phagocytosis (opsonisation), activates the complement system and/or activates inflammatory signal cascades that facilitate the destruction of an infectious agent. A highly specific immune response that is based on the selection and expansion of somatically recombined B and T cells is found only in vertebrates. Other animals and plants rely on innate immune defenses (Hoffmann et al., 1999; Hultmark and Ekengren, 2003; Janeway, 2005).

A key question is how an innate immunity system recognizes and discriminates between a pathogen's and the animal's own tissue. The answer relies on the absence of microbial markers on the surface of the host cells. Innate immunity is based on germ-line encoded PRRs that selectively bind to pathogens (PAMPs, pathogen associated molecular patterns). These receptors recognize different pathogen surface structures such as lipopolysaccharide (gram-negative bacteria), lipoteichoic acid (gram-positive bacteria), peptidoglycan and mannans (yeast). In additon, PRRs bind and recognize the most important PAMSs, microbe originated DNA and RNA (Hallman et al., 2001) (Hultmark and Ekengren, 2003) (Janeway, 2005). Microbe-specific receptors can be divided into three groups: Secreted molecules, phagocytic receptors and signaling molecules. One of the best-characterized **secreted receptors** is a mannose-binding lectin (MBL), which binds to carbohydrates on bacteria, yeast and viral surfaces. This group also includes, among many others, the surfactant apoproteins SP-A and SP-D and complement. **Phagocytic receptors** are expressed on the surface of phagocytes. These include, for example, a mannose receptor and a scavenger receptor. When these receptors recognize a pathogen, they phagocytose it into intracellular lysosomes, where it is destroyed. Components of the pathogen are moved to the surface of the antigen presenting cells for antigen presentation by

major histocompatibility complex molecules. This antigen presentation occurs only in vertebrates. **Signaling receptors,** including Toll receptors, originally found in *Drosophila* (Lemaitre et al., 1996), activate inflammatory cascades that induce the expression of the inflammatory cytokines that are important for the acute host defense (Hallman et al., 2001) (Janeway, 2005) (Takeda et al., 2003).

6.2.2 The *Drosophila* immune system

The fruitfly lacks the adaptive immune system and relies only on innate immune reactions for its defense. Nevertheless, *Drosophila* is resistant to microbial infections. This resistance is achieved through at least three mechanisms, all of which take place within a short period of time after exposure to a pathogen. (1) **phagocytosis** of micro-organisms by circulating blood cells called plasmatocytes, (2) activation of **proteolytic cascades** that lead to blood clotting, melanin formation and opsonization and (3) transient synthesis of potent **antimicrobial peptides (AMPs)** (Brennan and Anderson, 2004) (Ferrandon et al., 2004; Lemaitre and Hoffmann, 2007).

6.2.2.1 Local response

The *Drosophila* local response includes AMP production via surface epithelial cells followed by clotting, opsonization and melanization inside the organism, all mediated by sophisticated signaling cascades. When *Drosophila* epithelial cells get into contact with microbes, the activation of complex signaling pathways leads to the induction of potent AMP expression in these cells. This kind of local production of AMP in direct response to microbes is also seen in other animals, including mammals, and is considered an essential ancestral antimicrobial defense mechanism

(Brennan and Anderson, 2004; Ferrandon et al., 1998). Another local immune response event is wound healing including clotting of the hemolymph and melanization (Ramet et al., 2002a). Injury or invading large parasites activate a serine protease cascade which eventually leads to the synthesis of a large amount of melanin, clotting and encapsulation. Melanin formation can be detected under a microscope as a dark black band around the wound area (Brennan and Anderson, 2004) (Ferrandon et al., 2004). Melanization is coupled to the simultaneous production of cytotoxic oxygen species against micro-organisms possibly present in the wound (Hoffmann and Reichhart, 2002) (Ramet et al., 2002b) (Brennan and Anderson, 2004) (Ferrandon et al., 2004).

6.2.2.2 Cellular response

The key players in the *Drosophila* cellular response are the cells of the hemolymph, *Drosophila's* blood. These include lamellocytes which hunt and capture invading micro-organisms and encapsulate them. These large flattened cells comprise only a few percent of all the cells present in the *Drosophila* hemolymph (Meister and Lagueux, 2003). The most pre-dominant cell-type is plasmatocytes, which take care of phagocytosis. Plasmatocytes comprise over 95% of the hemolymph cells (Williams et al., 2007) (Meister and Lagueux, 2003). In addition, there are circulating hemocytes comprising approximately 5% of the hemolymph cells called crystal cells. These cells secrete components necessary for the melanization of invading organisms and wound repair (Williams et al., 2007) (Meister and Lagueux, 2003).

The basic mechanism of phagocytosis is well conserved throughout the evolution from invertebrates to vertebrates. Macrophages in mammals and plasmatocytes in *Drosophila* share similarities both in appearance and in phagocytic receptors such as

the scavenger receptors on their cell surface (Pearson et al., 1995) (Ramet et al., 2001). *Drosophila* phagocytic receptors have different ligand-binding properties and are able to phagocytose a wide spectrum of materials of different nature from apoptotic cells to large particles and micro-organisms. (Pearson et al., 1995) (Ramet et al., 2001) (Meister and Lagueux, 2003) Many recent studies have shown that phagocytosis is an important part of the *Drosophila* immune defense. Although able to orchestrate a normal humoral response, flies that do not express the phagocytic receptor *eater* are more sensitive to the gastro-intestinal tract infecting pathogen *S. marcescens* than are normal flies (Kocks et al., 2005). The crucial role of phagocytosis in the *Drosophila* host defense can be demonstrated by blocking phagocytosis with mutations or injecting polystyrene beads into the *Drosophila* body cavity (hemocoel). It has been shown by these studies that flies that are unable to phagocytose, and carry a mutation affecting the humoral response, are highly susceptible to infections (Braun et al., 1998) (Elrod-Erickson et al., 2000).

6.2.2.3 The humoral response

The *Drosophila* humoral response is based on the synthesis of potent AMPs and the signaling pathways that regulate their production.. AMPs act for example by permeabilizing the cell membranes of micro-organisms. They are produced mostly in the fat body and secreted rapidly into the hemolymph. There are at least 34 AMPs encoded in the *Drosophila* genome. These include AMPs with a broad-spectrum such as cecropins, and more specialized peptides like attacins. Defensins have a wide spectra of activities directed against bacteria, fungi and some enveloped viruses (Hoffmann et al., 1999) (Tzou et al., 2002) (Hultmark and Ekengren, 2003) (Hetru et al., 2003).

In past decades, scientist paid a great deal of attention to *Drosophila* immune signaling and revealed four different signaling pathways regulating innate immune responses after bacterial challenge or septic injury. The Toll pathway is activated primarily in response to gram-positive bacteria and fungi (Lemaitre et al., 1996), whereas the Imd (immune deficiency) pathway is activated by gram-negative bacteria (Lemaitre et al., 1995). Both the Toll and the Imd pathways are involved in

regulating gene production after septic injury (Hetru and Hoffmann, 2009). In addition, there is growing evidence for the involvement of the Janus tyrosine kinase/signal transducer and activation of transcription (JAK/STAT) and JNK signaling pathways in controlling immune response genes (Ramet et al., 2002a) (Baeg et al., 2005) (Dostert et al., 2005) (Brun et al., 2006; Galiana-Arnoux et al., 2006; Muller et al., 2005).

6.2.2.4 The *Drosophila* antiviral response

Drosophila resists viruses through two types of responses: degradation of viral RNA by the RNA interference machinery and cytokine-mediated induction of many stress genes, which counter the viral infection. RNAi function is to limit the viral infection in the infected cells by preventing viral replication. There is some recent evidence, that *Drosophila* may also have a systemic RNAi response similar to vertebrate protein-based immunity, where the virus-specific immunity signal is systematically spread throughout the animal (Costa et al., 2009; Saleh et al., 2009). This defense mechanism is then coupled to other immune reactions induced by JAK/STAT signaling in uninfected cells (Galiana-Arnoux et al., 2006, {Costa, 2009 #177) (Jing-Huan Wang, 2010).

Many scientists have demonstrated that RNAi has an essential role in the immune response against viruses and other foreign genetic material. In plants, RNAi also has a vital role in preventing self-propagation by transposons (Stram and Kuzntzova, 2006). In some animals, including *Drosophila*, RNAi has also shown to have a role in initiating the antiviral response and is active against pathogens such as the *Drosophila* X virus (Zambon et al., 2006) (Wang et al., 2006). A similar antiviral response mechanism may be present in *C. elegans*, since RNAi related argonaute proteins are upregulated in *C. elegan* in response to viruses. In addition, worms that overexpress RNAi pathway components are more resistant to viral infections (Lu et al., 2005) (Wilkins et al., 2005) (Fritz et al., 2006) (Zambon et al., 2006) (Wang et al., 2006).

There are relatively little data available concerning the role of RNAi in mammalian innate immunity in response to viral infections and it is therefore poorly understood.

Some evidence of an RNAi-dependent viral response in mammals comes from the existence of viruses encoding genes able to suppress the RNAi response in mammalian cells. However, this hypothesis is poorly substantiated due to the lack of adequate data on the mammalian RNAi-dependent viral response (Berkhout and Haasnoot, 2006) (Schutz and Sarnow, 2006) (Cullen, 2006).

6.3 Signaling pathways regulating the *Drosophila* immune response

Innate immunity is strikingly well conserved throughout evolution (Hoffmann and Reichhart, 2002) (Hoffmann and Reichhart, 2002). For example, mammalian Toll-like receptors (TLRs) were discovered based on their similarity to the insect Toll protein (Beutler, 2009; Lemaitre and Hoffmann, 2007). The *Drosophila* host defense relies on the rapid detection of microbes by PRRs. The initial recognition by PRRs triggers phagocytosis, proteolytic cascades leading to coagulation and melanization in order to limit the infection and production of AMPs (Brennan and Anderson, 2004) (Ferrandon et al., 1998) (Hoffmann and Reichhart, 2002). Similar responses are also seen in mammalian immunity. For example, proteolytic cascades are used in blood clotting and the complement system. Signaling cascades leading to the release of antimicrobial peptides in *Drosophila* immunity distinguish between Gram-negative and Gram-positive bacteria through the Imd and the Toll pathways, respectively (Lemaitre et al., 1997). These two distinct pathways that are very similar to mammalian Toll/IL and TNF-receptor pathways are the major regulators of the immune response in *Drosophila in vivo* (Hetru and Hoffmann, 2009; Mishima et al., 2009).

Current data suggest the involvement of two additional pathways in the *Drosophila* immune response. In addition to the Toll and Imd pathways, the JNK- and JAK/STAT pathways seem to have a role in regulating gene expression/protein production after septic injury and in viral infections. There are also indications that the mitogen-activated protein kinase kinase kinase (MAPKKK) Mekk1 regulates the

transcription of some genes induced by septic injury, such as the Turandot stress genes (Baeg et al., 2005) (Dostert et al., 2005) (Muller et al., 2005) (Brun et al., 2006) (Galiana-Arnoux et al., 2006)

6.3.1 The Imd pathway

The *Drosophila* immune response against Gram-negative bacteria is carried out via peptidoglycan recognition proteins (PGRPs), a conserved family of microbial recognition proteins. There is a total of 13 PGRPs in *Drosophila* of which several have been implicated in immune response reactions. A transmembrane protein PGRP-LC has been shown to be a receptor of the Imd pathway. In addition, three secreted proteins, PGRP-LE, PGRP-SA and PGRP-SD, cooperate with PGRP-LC by binding Gram-negative peptidoglygan (Werner et al., 2000) (Choe et al., 2002) (Ramet et al., 2002b) (Gottar et al., 2002).

The Imd pathway is a reasonably complex cascade comprising several different components. The pathway is initially activated by Gram-negative bacteria binding to the extracellular peptidoglycan recognition domain of the receptor PGRP-LC (Choe et al., 2002) (Ramet et al., 2002b) (Gottar et al., 2002). There are several indications that this binding is assisted by secreted PGRP-LE. Activated PGRP-LC then interacts via its intracellular domain with the C terminal death domain (DD) of Imd. Activated Imd delivers the signal to various other components of the pathway (**fig 4**), leading finally to phosphorylation of the transcription factor Relish. As Relish becomes phosphorylated, its C terminal inhibitory domain is cleaved and activated, and it translocates into the nucleus and activates the expression of several antimicrobial peptides, including *Attacin, Diptericin* and *Drosocin* (Elrod-Erickson et al., 2000) (Rutschmann et al., 2000) (Georgel et al., 2001) (Vidal et al., 2001) (Wang et al., 2001) (Naitza et al., 2002) (Giot et al., 2003) (Silverman et al., 2003) (Kleino et al., 2005) (Zhou et al., 2005) (Aymeric et al., 2010; Beutler and Moresco, 2008; Leulier et al., 2006; Lhocine et al., 2008; Matova and Anderson, 2010; Wiklund et al., 2009).

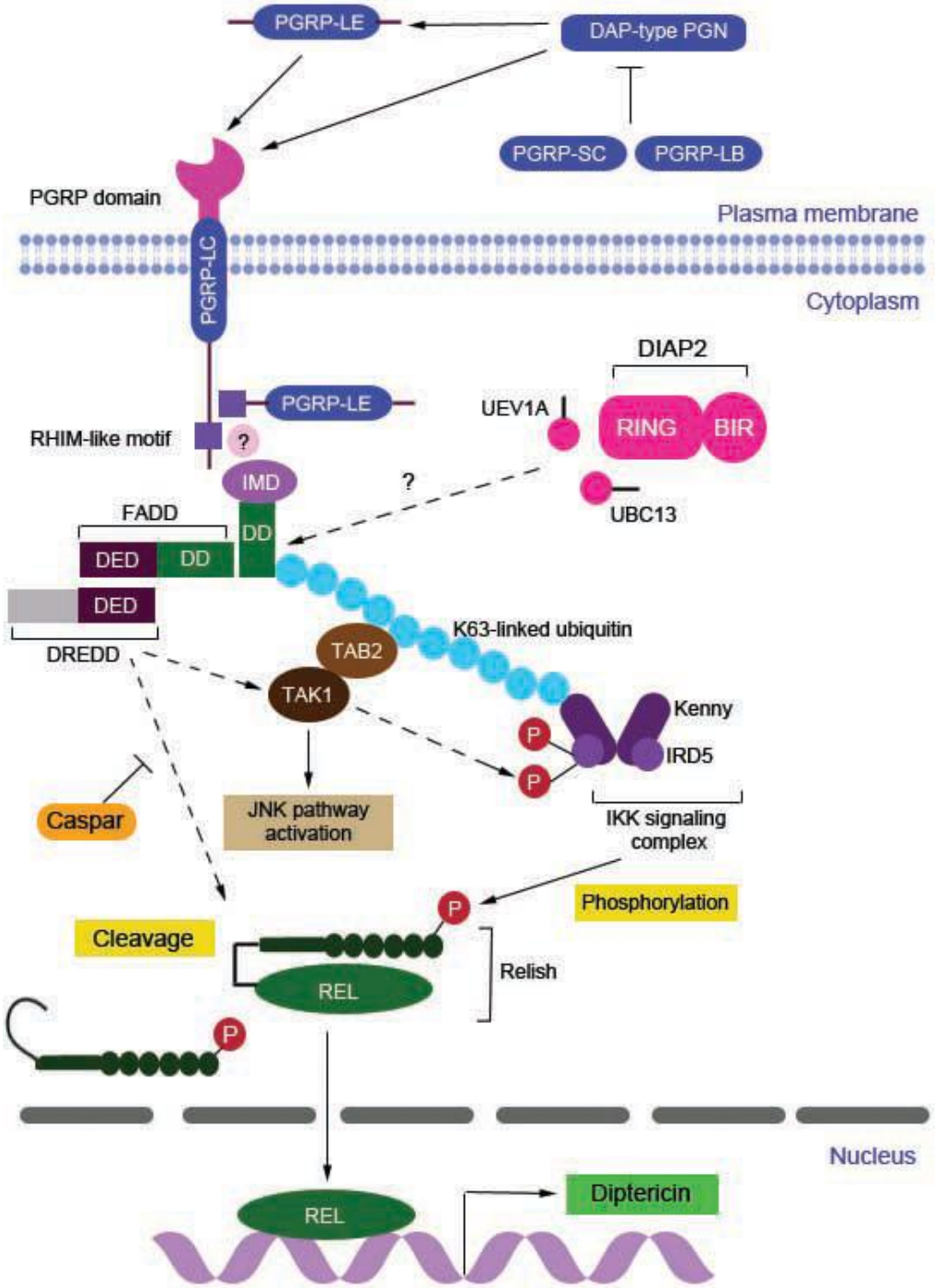


Figure 4. The Imd pathway. PGRP-LC and PGRP-LE recognize Peptidoglycan (PGN) on Gram-negative bacteria and activate Imd via an unidentified protein. PGRP-LE is both an intracellular and extracellular receptor for monomeric peptidoglycan and contains both the RIP homotypic interaction motif (RHIM) and the PGRP domain. Activated Imd recruits FADD and DREDD, each containing a death domain (DD) and/or death effector domain (DED). DREDD passes the signal by activating TAK1. TAB2 is thought to participate in assembling the IKK signaling complex, which is composed of IRD5 and Kenny, the *Drosophila* homologs of IKKβ and IKKγ, respectively (Erturk-Hasdemir et al., 2009). IRD5 phosphorylates Relish, triggering its cleavage. The ankyrin repeats cleaved of Relish remain in the cytoplasm, and the REL moiety translocates to the nucleus and activates the ranscription of target genes (Ferrandon et al., 2007)

6.3.2 The JNK pathway

The *Drosophila* Imd and the mammalian TLR signaling pathways both branche into another conserved signaling cascade, the Jun N-terminal kinase (JNK) signaling pathway at the level of Tak1 (Wang et al., 2001) (Silverman et al., 2003) (Park et al., 2004) (Bond and Foley, 2009; Stronach, 2005). The JNK pathway controls several biological processes from cytoskeletal remodeling, dorsal closure, tumor growth and invasion, stem cell regeneration and wound healing to transcriptional regulation of a wide variety of gene products in response to pathogens and tissue damage (Lesch et al., 2010). Like most of the signaling pathways involved in immune responses, the JNK pathway is highly conserved throughout evolution and has been implicated in stress responses, cell migration and proliferation, differentiation and morphogenesis, apoptosis as well as immune responses in both insects and mammals (Boutros et al., 2002) (Bond and Foley, 2009; Igaki, 2009). Despite of its well-known importance in immune signaling, the actual contribution of JNK signaling to the *Drosophila* immune response is more or less unclear. It has been shown that the JNK pathway is essential for sufficient Imd signaling and it is most likely involved in Dif-controlled *Drosomycin* expression via the Toll pathway. The most recent data suggest that this rather complex cascade can be activated by several kinases leading to various biological outcomes (Boutros et al., 2002) (Ramet et al., 2002a) (Park et al., 2004) (Delaney et al., 2006) (Bond and Foley, 2009; Igaki, 2009).

The JNK pathway can be activated by one of three cell surface ligand-receptor systems, TNF-TNFR (Eiger/Wengen), PVF/PVR (PDGF/PDGFR) or Wingless/ Frizzled (Wg/Fz) depending on the biological process it mediates (Igaki, 2009). The signal is then passed on to intracellular adaptor proteins such as small GTPases (Rac), Dishevelled (Dsh), the tumor necrosis factor (TNF) receptor-associated factors (TRAFs) and the Ste20-related kinase Misshapen (msn) (Boutros et al., 2002) (Igaki, 2009; Ramet et al., 2002a). These adapter proteins function as waypoints and mediate the activation of the core JNK signaling proteins, JNKKKs dTAK1, DASK1, Slpr, dMekk1, JNKK hemipterous (Hep) and JNK basket (bsk). Activation of the Hep-Bsk cascade finally leads to the phosphorylation of the transcription factors DJun homologue of Jun-related antigen (Jra) and kayak (kay),

the *Drosophila* homologue of the human Fos, the transcriptional repressor anterior open (aop) and the dual-specificity phosphatase puckered (puc) (Igaki, 2009). Being a transcriptional target of the AP-1 (Jun/Fos) transcription factor, Puc forms a negative feedback loop and acts as a negative regulator of JNK signaling (Boutros et al., 2002) (Ramet et al., 2002a) (Park et al., 2004) (Delaney et al., 2006) (Igaki, 2009).

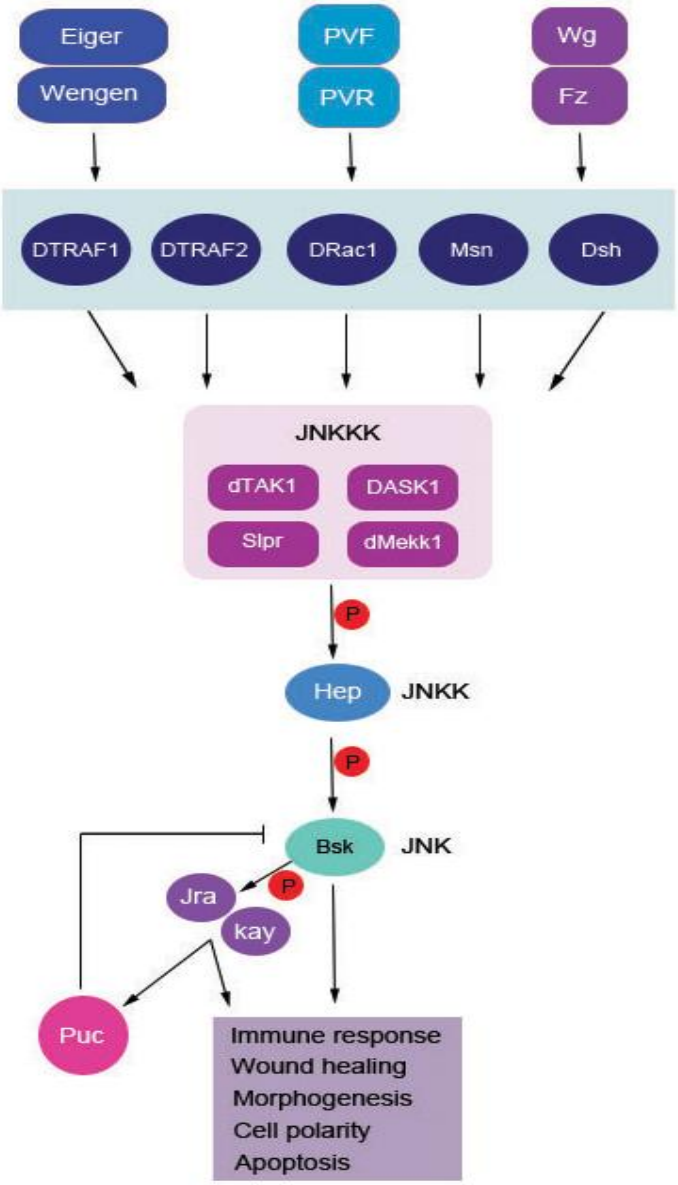


Figure 5. The JNK pathway is activated by one of tree cell surface ligand-receptor systems, TNF-TNFR (Eiger/Wengen), PVF/PVR (PDGF/PDGFR) or Wingless/Frizzled. These pass the signal to the adaptor proteins TRAFs, Dishevelled (*Dsh*), small GTPases or the Ste20-related kinase Misshapen (*Msn*). Adaptor proteins activate the core JNK signaling complex consisting of JNKKK (dTAK1, DASK1, Slpr, and dMekk1), JNKK (Hep), and JNK (Bsk). The activated JNK cascade triggers the phosphorylation of the transcription factors Jra and Kay (Jun and Fos homologs) (Igaki, 2009)

6.3.3 The Toll pathway

The *Drosophila* Toll pathway functions in response to Gram-positive bacteria and fungi. It also has a role in establishing the dorsal-ventral patterns of the early embryo (Anderson et al., 1985; Royet et al., 2005{Jin, 2008 #201)}(Lund et al., 2010). The *Drosophila* Toll receptor is a cytokine receptor, thus it does not directly bind pathogens like do the human Toll-like receptors (Medzhitov et al., 1997) (Huang et al., 2010; Tabuchi et al., 2010; Tauszig et al., 2000). Recognition of Gram-positive bacteria is mediated via the circulating receptors PGRP-SA and PGRP-SD. There are some indications that PGRP-SA actually functions in a complex together with a lysine-type peptidoglygan processing enzyme, Gram-negative binding protein 1 (GNBP-1). Nevertheless, bacterial and fungal recognition leads to serine protease activation and the cleavage of a cytokine called Spätzle. Once cleaved, Spätzle binds to the Toll receptor inducing Toll dimerization (Lemaitre et al., 1996) (Michel et al., 2001) (Wang et al., 2002) (Gobert et al., 2003) (Bischoff et al., 2004; Weber et al., 2003) (Pili-Floury et al., 2004) (Kambris et al., 2006) (El Chamy et al., 2008)

Activated Toll delivers the signal into the intracellular components of the Toll pathway. These include the DD proteins dMyd88, Tube and Pelle. After dimerization, Toll binds to a pre-existing Myd88/Tube complex that eventually recruits the Pelle kinase, homologous to the mammalian IRAK (Medzhitov et al., 1998; Shen and Manley, 2002). This then leads to the phosphorylation and degradation of the NFκ-B inhibitory protein Cactus. The kinase, which directly phosphorylates Cactus, remains to be discovered, but the degradation of Cactus releases DIF and /or Dorsal to translocate into the nucleus and activate the transcription of many immune response genes such as *Defensin*, *Drosomycin*, *Cecropin* and *Metchnikowin* (Medzhitov and Janeway, 1998) (Rosetto et al., 1995) (Horng and Medzhitov, 2001) (Bilak et al., 2003; Tauszig-Delamasure et al., 2002) (Tanji and Ip, 2005) (Kuttenkeuler et al., 2010; Zhang and Zhu, 2009).

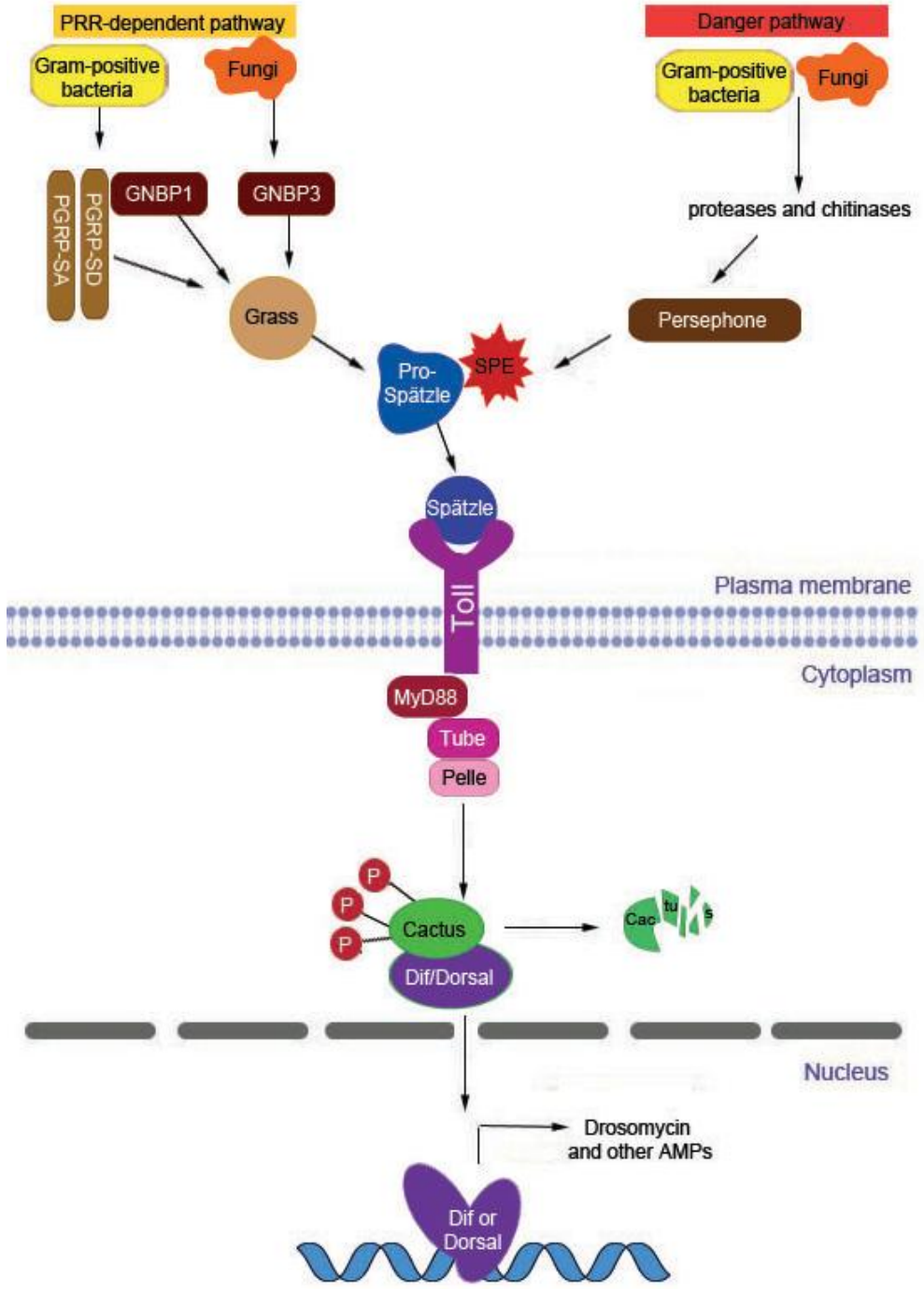


Figure 6. The Toll pathway Gram-positive bacteria are recognized by PGRP-SA cooperatively with GNBP1 and PGRP-SD. Similarly, fungi are recognized by GNBP3. Mediated by Grass, this recognition results in the activation of a proteolytic cascade leading to the cleavage of Spätzle by the serine protease Spätzle Processing Enzyme (SPE). Cleaved Spätzle binds to the Toll receptor, causing Toll dimerization and the activation of the intracellular signaling cascade comprising MyD88, Tube and Pelle, and culminating in the phosphorylation and degradation of the NFκB inhibitory protein Cactus. This allows Dif/Dorsal to translocate into the nucleus and induce the transcription of immune response genes. The Toll pathway can also be activated by entomopathogenic fungi through virulence factors such as secreted proteases and chitinases, which activate the circulating serine protease Persephone.

6.3.4 The JAK/STAT pathway

The *Drosophila* JAK/STAT pathway is involved in multiple processes, including embryonic segmentation, larval hematopoiesis and the development of various organs, the regulation of stem cell maintenance and cellular proliferation (Beebe et al., 2010; Bina et al., 2010; Conder and Knoblich, 2009; Flaherty et al., 2010; Wang and Huang, 2010). In addition, *Drosophila* JAK/STAT signaling has an important role in controlling immune and stress responses after septic injury. After septic injury, the activation of the JAK/STAT pathway leads to the expression of a number of genes including the *Turandot (Tot)* stress genes in the fat body. (Ekengren and Hultmark, 2001; Ekengren et al., 2001) (Hombria and Brown, 2002) (Muller et al., 2005) (Baeg et al., 2005) (Dostert et al., 2005) (Singh et al., 2005) (Brun et al., 2006).

The main components of the JAK/STAT signaling cascade are well characterized and conserved in evolution from flies to mammals, with the exception that *Drosophila* only has one Janus tyrosine kinase (JAK), *hopscotch (hop),* and a single STAT transcription factor, Stat92E. Binding of an extracellular ligand, the cytokine-like molecule *Unpaired (Upd)* to the transmembrane receptor *Domeless (Dome),* which shares homology with the *interleukin 6 (IL-6)* receptor family, induces a conformational change in the receptor (Brown et al., 2001). This leads to the activation of a nearby Hopscotch by auto- and/or trans-phosphorylation. Once phosphorylated, Hop phosphorylates tyrosine residues on the receptors thereby introducing docking sites for STATs, which concurrently are phosphorylated by JAKs as well. Phosphorylation activates STAT causing it to dimerise and translocate in the nucleus where STAT dimers act as transcriptional activators of target genes such as *TotM*. (Perrimon and Mahowald, 1986) (Binari and Perrimon, 1994) (Harrison et al., 1998) (Hou et al., 2002) (Agaisse et al., 2003) (Gilbert et al., 2005) (Hombria et al., 2005).

The JAK/STAT pathway is tightly regulated by a number of cytokines and growth factors. There are few known regulators of the JAK/STAT pathway that are also found functionally unchanged in the mammalian system. These include a family of SOCS-like genes (*Drosophila* homolog of suppressor of cytokine signaling gene

family), *dPIAS/Su(var)2-10* (*Drosophila* Protein Inhibitor of Activated Stat) and *STAM* (Signal Transducin Adaptor Molecule) (Hou et al., 2002) (Baeg et al., 2005) (Muller et al., 2005) (Arbouzova and Zeidler, 2006; Gronholm et al., 2010).

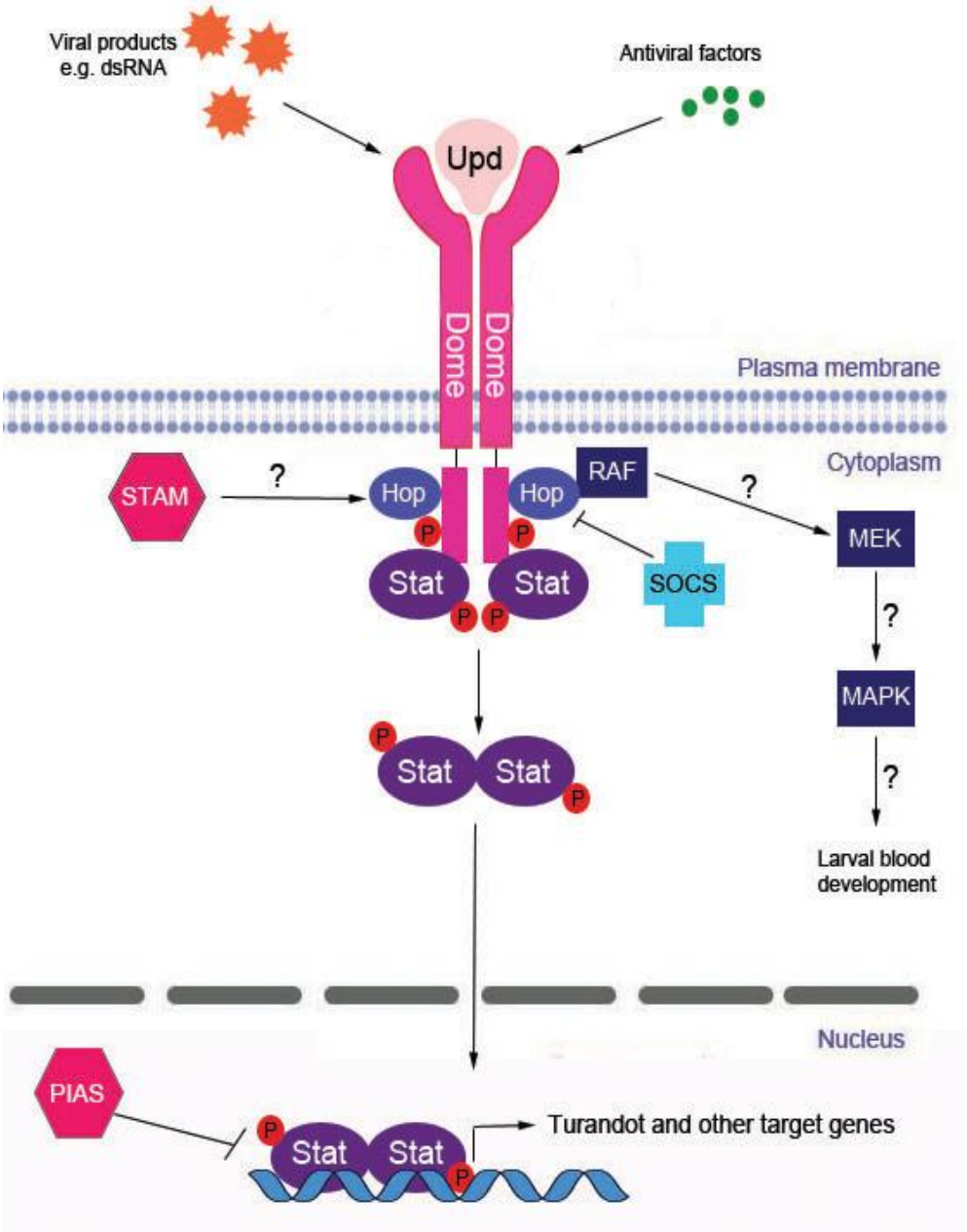


Figure 7. The JAK/STAT signaling pathway in *Drosophila* Binding of the extracellular ligand Upd to the receptor Dome causes a conformational change and allows Hop (attached to the intracellular domain of Dome) to trans-phosphorylate. Activated Hop phosphorylates Dome and activates cytoplasmic STAT. Activated STAT dimers translocate into the nucleus and activate the transcription of Turandot genes. PIAS inhibits activated STAT by targeting it for degradation. STAM is predicted to act as a positive and SOCS as a negative regulator of Hop.

7 AIMS OF THE STUDY

The overall aim of this study was to gain a detailed view of the signaling pathways regulating *Drosophila* host defense. This included determining all of the gene products involved in *Drosophila* NFκ-B- and the JAK/STAT signaling. Thus, we set out to to validate previously characterized genes as well as to identify new gene products involved in signaling via these pathways. We approached this goal using two powerful systems biological methods: microarrays and genome-wide RNAi screening.

More specifically, the aims were:

1. To find new gene products involved in the *Drosophila* host response using microarray analysis to determine genes induced in response to microbes.

2. Determine the role of unknown immune-response genes for microbial binding, phagocytosis and AMP release controlled by NFκ-B signaling.

3. To identify all of the gene products involved in both NFκ-B and JAK/STAT signaling using genome-wide RNAi screens in *Drosophila* cells. And to determine the *in vivo* significance of selected genes using a *UAS-GAL4*-based *in vivo* RNAi system.

8 MATERIALS AND METHODS

8.1 Cell lines and culture

8.1.1 S2 cells

Drosophila Schneider-2 (S2) cells were grown in Schneider medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% Fetal bovine serum (FBS, Gibco/Life Technologies, Carlsbad, CA), 100units/ml of penicillin and 100 µg/ml of streptomycin at +25°C.

8.1.2 Hela cells

Human HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus GlutaMAx cell culture medium (Gibco) supplemented with 10% FBS, 100U/ml of penicillin, 100 µg/ml of streptomycin and 1% nonessentials amino acids (Sigma) at +37°C.

8.2 Oligonucleotide microarray analysis

A 3.0 x 10^6 culture of S2 cells was incubated with 3.0 x 10^7 heat-killed *Escherichia coli* for 6 hours. Total RNA was extracted from S2 cells using the RNeasy Mini kit (Qiagen) and gene expression was analyzed using the Affymetrix (Santa Clara) *Drosophila* Genechips according to standard protocols provided by the manufactures. The extracted RNA was reverse transcribed into cDNA, which was used as template for the synthesis of labeled RNA. The labeled RNA was fragmented and analyzed using gene chips. Each gene is represented as a probe set of 10-25 oligonucleotide pairs in a single chip. The probe pair comprises a nucleotide sequence, which perfectly matches the gene (PM) and one that has a

mismatch (MM). In the experiment, biotin-labeled RNA is hybridized to the array which is stained with phycoerytrin-conjugated streptavidin after washing, and scanned using the Gene Array Scanner. A grid is automatically laid over the array image and the intensities of each probe pair are used to make expression measurements with the Affymetrix Microarray Suite. The gene expression levels of four uninduced and control dsRNA treated S2 cells were compared pair-wisely to four *E. coli* induced samples and to three *E. coli* induced and PGRP-LC RNAi treated samples. For more details, see *Drosophila* GeneChip Expression Analysis Technical Manual by Affymetrix (2001).

8.3 Flow cytometry

8.3.1 Synthesis of targeted dsRNAs

Total RNA was extracted from cultured S2 cells using TRIZOL® Reagent (Gibgo). Thereafter, first-strand cDNA was synthesized from 1.0 µg of total RNA. Gene specific primers for targeted dsRNA treatments were designed based on the sequences found at www.fruitfly.org. The templates for dsRNA synthesis were generated from cDNA by a two-step PCR reaction. The first set of primers contained 15 base gene specific sequences and the second PCR reaction was performed using nested primers containing a T7 promoter sequence on its 5'-end (GAATTAATACGACTCACTATAGGGAGA) attached to gene-specific sequences. Both sense and antisense RNAs are synthesized simultaneously from a single PCR product using the T7 MegaScript RNA polymerase (Ambion, Austin, TX) (Ramet et al., 2001). dsRNA was precipitated with LiCl. The quality of DNAase-treated dsRNA was analyzed by agarose gel electrophoresis and the concentration of the RNA was measured with a spectrophotometer.

8.3.2 dsRNA treatments and FACS analysis

The ability of targeted dsRNA treated S2 cells to bind and phagocytose heat-killed, FITC-labeled *E. coli* and *Staphylococcus aureus* (Molecular Probes, Leiden, Netherlands) compared to GFP dsRNA treated control cells was quantified using flow cytometry (Ramet et al., 2002b). For dsRNA treatment, 10 µg dsRNA per 10^6 S2 cells were added directly into the cell culture medium 72 h prior to measurements. A total of 5.0 x 10^5 dsRNA treated S2 cells were transferred onto 48-well plates and cells were allowed to attach onto plates for 1 h at room temperature. Thereafter, cells were kept at +4°C for 30min. Once cooled, cells were exposed to FITC-labelled *E. coli* or *S. aureus*, plates were centrifuged for 1 min at 100 x g at +4°C and incubated for 1 h at +4°C to allow binding to occur. An additional incubation at +26°C was carried out to allow phagocytosis, 16 min for *E. coli* and 30 min for *S. aureus*. Binding and phagocytosis were stopped by placing cell plates on ice and plates kept on ice at all times during the analysis. For measurements, the cell culture medium was replaced with PBS and cells were transferred into flow cytometry tubes. Flow cytometric analyses were done by FACS using the EXPO32 program (Beckman Coulter, Ordior). For the phagocytosis analysis, 0.2% Trypan blue in 1 x PBS (pH 4.85) was added before the measurement to quench the fluorescence of non-internalized particles on cells surface. 5,000-10,000 cells were counted for each sample and the rate of phagocytosis was quantified as the percentage of fluorescence positive cells multiplied by the mean fluorescence.

8.4 RNAi screens

8.4.1 *Drosophila* dsRNA libraries

For our genome-wide RNAi screen we produced a total of 16,025 dsRNAs. For synthesis, we used a commercial *Drosophila* genome RNAi library consisting of a set of 13,625 PCR products with dual T7 promoter sequences (Medical Research Council (MRC) Geneservice Ltd (Cambridge, UK)) as template for the synthesis of dsRNAs. In addition, we used our own S2 cell-derived cDNA library comprising of

2,400 plasmid templates. dsRNAs were synthesized with the T7 MegaScript RNA polymerase kit (Ambion, Austin, TX) according to the manufacturer's instructions. The concentration of each dsRNA was measured using the PicoGreen® dsDNA Quantitation Kit (Molecular probes) or NanoDrop (Thermo Scientific). Targeted dsRNAs were produced in a two-step PCR with nested primers containing a T7 promoter sequence on its 5'-end as described above. The dsRNA targeting *GFP* used as a negative control was produced from the pMT/BiP/V5-His/GFP plasmid (Invitrogen).

8.4.2 *Luciferase* reporter assay

The effects of dsRNA treatments on signaling cascades were quantified by luciferase reporter assays. The basic idea is to transfect the cells with reporter plasmids containing the target gene attached to *luciferase* promoter sequences. The intensity of the light reaction generated by luciferase can be measured and it corresponds to the expression level of the target gene. An *Attacin-luciferase* expression vector was used for the Imd pathway, a *Drosomycin-luciferase* reporter was used for the Toll pathway and a *TurandotM-luciferase* reporter was used to monitor the activity of the JAK/STAT signaling pathway. An *Actin 5C-β-galactosidase* reporter was co-transfected to control cell viability and transfection efficiency as well as to normalize results. 72 h after transfection and RNAi treatment, S2 cells were lysed in reporter lysis buffer (Promega) and luciferase and β-galactosidase activities were measured using standard procedures (Ramet et al., 2001).

8.4.3 Reporter plasmids, transfections and dsRNA treatments

Transfections, dsRNA-treatments and reporter assays were all performed according to same protocol. For the NF-κB signaling and for the Toll pathway alone, 1.0 x 10^6 S2 cells were transfected with 0.1 µg (if not stated otherwise) of the *Drosomycin-luciferase* (*Drs-luc*; (Tauszig et al., 2000)) and 0.1 µg of the *Actin 5C-β-galactosidase* (*Act 5C-β-gal*) reporter plasmids using Fugene reagent (Roche). 0.5 µg of dsRNAs was added into transfection mixture. 0.1 µg of the *Toll*^{10b}-construct

was co-transfected with the reporter constructs to activate the Toll pathway. For the genome-wide screen, in order to screen both the Toll and the Imd pathway simultaneously, the Imd pathway was later activated by adding heat-killed *E. coli* 48 h after transfection, 24 h prior to measurements. The transfections, dsRNA treatments and reporter assays were performed accordingly when assessing the Imd pathway, except that S2 cells were transfected with 0.1 μg of the *Att-luc* reporter construct, Ecdysone was added into the cell culture media to a 1μmol concentration 48 h after transfection, and the pathway was activated by adding heat-killed *E. coli* as described above (Zhang and Palli, 2009). When analyzing JAK/STAT signaling, 0.1 μg of the *TotM-luc* reporter was used and the pathway was activated by co-transfecting cells with 0.1 μg of the hop^{Tum-l} reporter construct.

Human HeLa cells were cultured as described above. For transfections, HeLa cells were seeded onto a 24-well plate, 6 x 10^4 cells per well and incubated for 24 h. Thereafter, cells were transfected with 0.1 μg of the *NF-κB-luciferase* and 0.05 μg of the *CMV*-β-*galactosidase* reporter plasmid and 50pmol of siRNAs (Amersham) using Lipofectamine transfection reagent (Invitrogen) and OPTI-MEM medium (Gibco). siRNAs used were as follows: *GFP* siRNA (eGFP; cat# AM4626, neg. control), *GRK5* siRNA (cat# AM16704; ID: 110898) and *RelA* (cat# AM16704; ID: 216912, positive control). NF-κB signaling was induced for 48 h post transfection with 10 ng/ml of TNF-α (Sigma). 6 h later the cells were lysed and luciferase and β-galactosidase activities were measured from the lysates.

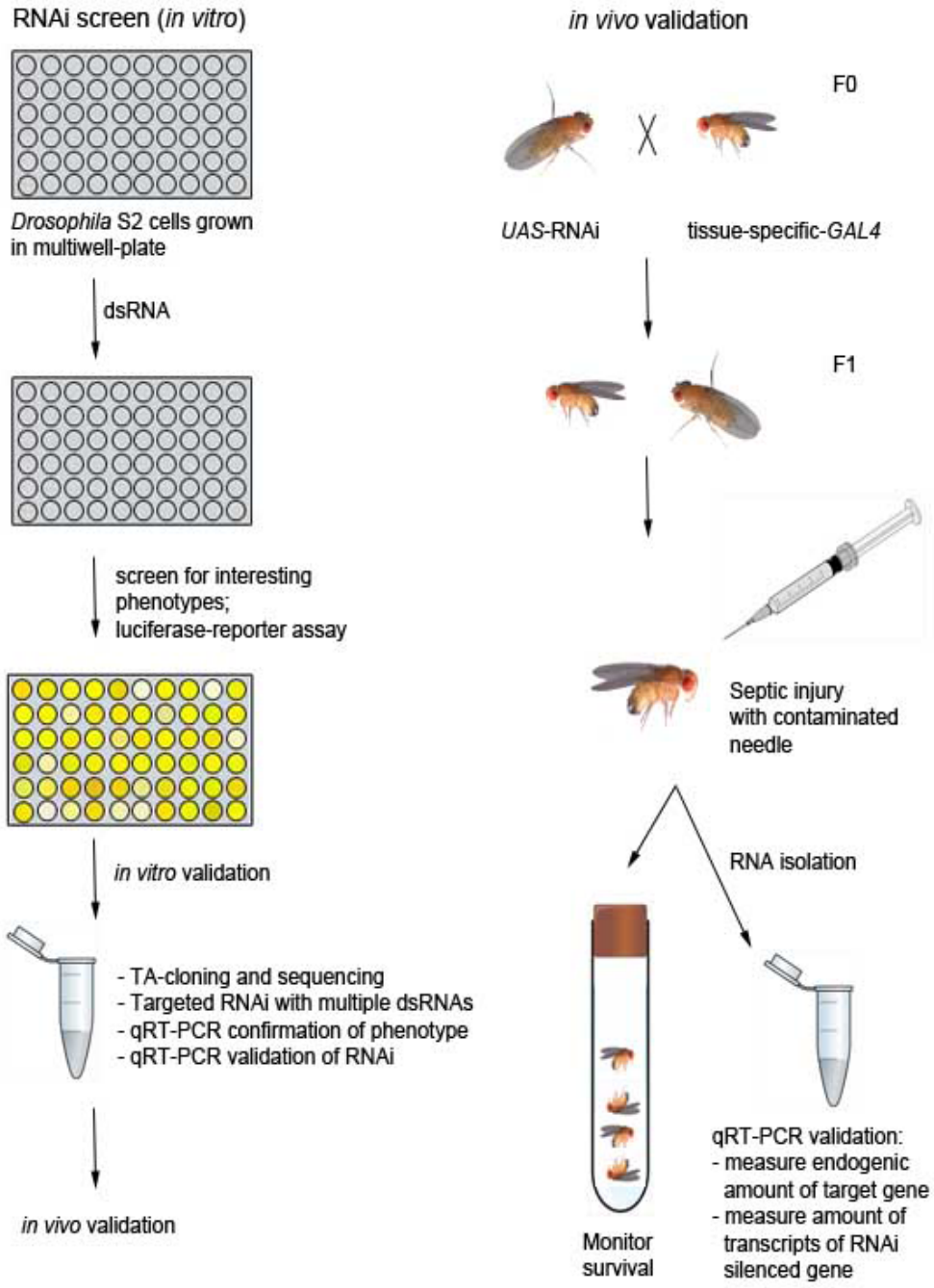


Figure 8. Schematic representation of the methods used in the systems biological study of *Drosophila* immune signaling.

8.5 Immunodetection

8.5.1 Confocal microscopy with Gprk2/GRK5 constructs

For immunodetection, the *Drosophila* Gprk2 was cloned into a *Drosophila* expression vector. The full length cDNA for the Gprk2 gene was first amplified by PCR and cloned into the KpnI site of the modified *Drosophila* expression vector pMT/GFP/V5/His, (gift from Dr. Iivari Kleino) to create a Gprk2-GFP fusion protein. S2 cells were seeded onto 24-well plates and transfected with 0.1 µg of the Gprk2-GFP fusion construct using Fugene reagent (Roche). Overexpression of the Gprk2-GFP fusion proteins was induced 24 h post-transfection by adding CuSO4 to a final concentration of 350µM. Cells were harvested 36 h later and passed 1:3 in Schneider's cell culture medium onto 6-well plates. The cells were incubated for 30 min to allow the cells to attach to coverslips on the bottom on each well. Thereafter, the culture medium was removed and the cells were fixed with 3.7% formaldehyde and 5% sucrose for 20 min. The coverslips were washed three times with PBS and mounted to objective slides with Vectashield mounting medium for fluorescence with 4_,6-diamidino-2-phenylindole (Vector Laboratories). For HeLa cell transfections, cells were grown on coverslips on the bottom 6-well plates for 24 h after which the cells were transfected with 0.1 µg of the *GRK5-GFP* construct. Transfected HeLa cells were grown for 36 h and the coverslips were collected and washed. Coverslips were mounted to objective slides as described above. Both S2 and HeLa cells were imaged with a Olympus IX70 confocal microscope and analyzed with the Andor iQ software.

8.5.2 Coimmunoprecipitation and Western blotting

For coimmunoprecipitations and Western blotting analyss, S2 cells were transferred into 6-well plates and transfected with 0.5 µg of Gprk2-V5 full length or deletion constructs and Cactus-myc constructs in the pMT/V5/HisA vector (Invitrogen Life Technologies). 24 h post-transfection, the expression of the tagged proteins was induced by adding $CuSO_4$ directly to cell culture medium to a final concentration of

250µM.. After a 48 h incubation, cells were lysed in 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.5% sodium deoxycholate, 20 mM NaF, 1% Nonidet-P40, 10% glycerol, 100 µg/ml PMSF, and a Complete Mini protease inhibitor cocktail (Roche Applied Science)). Protein concentrations of lysates were measured with the BCA protein assay kit (Pierce) and a lysate volume corresponding to 1mg of total protein for each sample was treated with 25 µl of a 1:1 suspension of protein GSepharose (GE Healthcare) in lysis buffer and incubated rotating for 50 min at 4°C. Cleared supernatants were transferred to fresh tubes in which 25 µl of protein G-beads were added to 1µg of anti-c-Myc rabbit IgG Ab. The samples were incubated overnight rotating at 4°C to allow co-immunoprecipitation and immunoprecipitates were washed for 4 x 10 min with PBS containing PMSF and protease inhibitors. Immunoprecipitates were released from the beads by adding 25 µl of 2 x SDS loading buffer, vortex, and incubated at 95°C for 5 min and proteins were separated by electrophoresis in a NuPAGE 10% Bis-Tris gel (Invitrogen Life Technologies). Protein samples were blotted on to a nitrocellulose membrane and detected with a 1/5000 dilution of a mouse anti-V5 or rabbit anti-c-Myc primary Ab and by goat anti-mouse or anti-rabbit Ab HRP conjugates (Invitrogen Life Technologies), respectively, and visualized with the enhanced chemiluminescence method (ECL) Plus Western blotting detection system (GE Healthcare).

8.5.3 Stable S2/epidermal growth factor receptor-Toll cells

The response of the Toll signaling pathway to EGF was analysed in stable S2/EGFR-Toll cells using the *Drs-luc* construct. For the analysis, S2 cells with a stable integration of a chimeric EGFR-Toll construct were established according to (Sun H, 2004). Stable S2/EGFR-Toll cells were seeded onto 6-well cell culture plates in total volume of 3 ml and treated with 5 µg/ml of dsRNA. After 4 days, the Toll pathway was induced by adding 0.5 µg/ml of EGF (Molecular Probes) for 30 min, cells were lysed and cytoplasmic extracts were separated by electrophoresis. The separated proteins were transferred to a Hybond-P membrane (Amersham Biosciences) and blocked. A polyclonal rabbit anti-Cactus antibody and a HRP-

linked donkey anti-rabbit IgG (GE Healthcare) were used to detect the cactus protein on the membrane. As a loading control, we used a rabbit polyclonal anti-GM130 antibody (Abcam) that recognises the *Drosophila* Golgi protein GM130. Signals intensities were measured on three separate Western blots and band quantifications were done with the Adobe Photoshop 7 software (Adobe systems, San Jose, CA). All experiments with the stable S2/EGFR-Toll cells were carried out by Ylva Engström and her co-workers at the Department of Molecular Biology and Functional Genomics, Stockholm University, Sweden.

8.6 Quantitative RT-PCR

8.6.1 Total RNA extraction from S2 cells and *in vitro* qRT-PCR

Approximately 1.0 x 10^6 S2 cells were seeded onto 24-well cell culture dishes and transfected with 0.2 μg of $hop^{Tum\text{-}l}$ and 0.5 μg of control or experimental dsRNA. After a 72 h incubation, cells were harvested and lysed in TRIsure® reagent (Bioline) by pipetting up and down for 10 times. Total RNAs were extracted essentially as stated in the manufacturer's protocol. Quantitative RT-PCR for *TotM, TotA, and CG14225* and *Act5C* levels was carried out from the dilutions of extracted RNAs using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) and the ABI7000 instrument (Applied Biosystems) according to the manufacturer's instructions. qRT-PCR to determine the expression levels of chosen genes related to NF-κB signaling was carried out accordingly, except that the Toll signaling cascade was activated by transfection of 0.1 μg $Toll^{10b}$.

8.6.2 Fly RNA extraction and *in vivo* qRT-PCR

For the *in vivo* qRT-PCR, the JAK/STAT pathway was activated by pricking flies with a tungsten needle dipped into a concentrated culture of *E. cloacae*. 16h after infection, flies were collected and snap-frozen in dry ice. Groups of five flies were homogenized manually directly into Eppendorf tubes, and total RNAs were

extracted from the homogenates with TriSURE reagent (Bioline) according to the manufacturer's instructions. Quantitative RT-PCR for *TotM*, *TotA* and *Act5C* levels, as well as expression levels of chosen genes related to NF-κB signaling, was carried out as described above.

8.7 Cloning

8.7.1 TA-cloning and sequencing

The genes identified in the genome-wide screens were sequenced in order to design targeted PCR primers for dsRNA synthesis to confirm the original findings. PCR products corresponding to the genes of interest were used as templates for *Taq* polymerase-amplified (TA) cloning using the TOPO TA Cloning® system (Invitrogen) according to the manufacturer's instructions. For the sequence analysis, the sequence of interest was blasted with NCBI blast at the NCBI web site (http://www.ncbi.nlm.nih.gov/). In order to identify similar regions between ET, Dome and gp130, ClustalW alignments were done at the EBI web site.

8.8 *Drosophila in vivo* experiments

8.8.1 Fly stocks and maintenance

Drosophila stocks were kept on a standard mashed potato diet at RT or at 25ºC. *C564-GAL4* driver flies, a kind gift from Professor Bruno Lemaitre (CNRS, France), express *GAL4* in the adult fatbody; the *P{UAS-Tl^{10b}:11}* stock carries a *Toll^{10b}* insert on the X chromosome and the *hml$^{\Delta}$-GAL4, UAS-eGFP* stock constitutively expresses GFP in the majority of its blood cells (Sinenko and Mathey-Prevot, 2004). The *UAS*-RNAi fly stocks were purchased from the Vienna *Drosophila* RNAi Center [VDRC, Vienna, Austria; (Dietzl et al., 2007)] or the Kyoto NIG-Fly stock center (*Drosophila* Genetic Resource Center, Kyoto Institute

of Technology, Japan). *UAS-RNAi* flies were crossed with fat body and hemocyte specific *C564-GAL4* flies, ubiquitous *GeneSwitch-GAL4* driver flies or, w^{1118} flies for controls. Week-old offspring carrying one copy of the *UAS-RNAi* construct and one copy of the *GAL4* driver were used for infections.

8.8.2 Fly infections and survival experiments

In order to study the Toll pathway-mediated immunity, experimental fly crosses were first pricked with a thin tungsten needle dipped into a concentrated culture of *M. luteus* to active the Toll pathway. 24h later the flies were additionally infected with *E. faecalis* by pricking them as above and their survival was monitored for 24 h at RT. For Imd pathway -mediated immunity, flies were infected with the gram-negative bacterium *Enterobacter cloacae* (*E. cloacae*) as described above, kept at RT and their survival was monitored for 48 h.

8.8.3 Fly larvae experiments

To assess the distribution of blood cells in *Drosophila* larvae *in vivo*, parental crosses of experimental flies were kept on a stained mashed potato diet, which permits the staging of larval progeny (Zettervall et al., 2004) at 29°C for two days. Fly vials were assigned with arbitrary numbers in order to blind the experiment. After 48 h, larvae were collected, washed gently and embedded in 50% chilled glycerol with their dorsal side up between an objective glass and a cover slip. Slides were kept at -20°C for 18 min for immobilization and examined under UV-light on an Axioplan microscope (Carl Zeiss Jena). Digital pictures were taken with a Hamamatsu C4742-95 video unit, controlled by the Openlab program (Improvision, Coventry, UK).

Twenty F1 progeny larvae from each cross were divided into four grades depending on the percentage of segments showing a band formed by islets of sessile hemocytes under the epidermis. In the grading system, larvae were graded as follows: grade 1 larvae showed sessile hemocyte bands in 100% of the segments. Grade 2 or 3 larvae showed bands in less than 75 or 50% of their segments and larvae showing no bands, or bands only in the most posterior 25% of their segments, received grade 4. All crosses were repeated three times and the average grades were calculated as triplicates. These experiments with fly larvae were carried out in Dan Hultmark's laboratory at the Department of Molecular Biology, Umeå University, Umeå, Sweden.

8.9 Data analysis

The statistical analyses of reporter assays, qRT-PCR and Western Blot band quantifications were carried out using one-way ANOVA. The statistical analysis of fly larvae *in vivo* experiments was performed using one-way ANOVA and Bonferroni as a post hoc method. The statistical analysis of fly survival experiments was carried out using the logrank (Mantel-Cox) test. $P < 0.05$ was considered to be statistically significant.

Band quantifications with stable S2/EGFR-Toll cells Western blotting were done with the Adobe Photoshop 7 software: To obtain the absolute intensity, the mean value of each band was multiplied by the pixel value. The relative intensity was calculated by normalizing absolute intensities with the absolute intensity of the negative control, which was set to1.

In a microarray analyses, genes whose RNA levels were affected in response to *E. coli* were analyzed using three criteria: the majority of 16 comparisons had to be affected and the remaining ones had to be not affected, the relative expression levels had to be changed at least three fold and the increase or decrease of the relative expression level had to be significant in the *t*-test ($P < 0.05$).

9 RESULTS

9.1 Functional analysis of *Drosophila* immune response genes

Microarray analysis is a convenient and widely used method for analyzing a large number of genes simultaneously. We used oligonucleotide microarrays to identify genes involved in the *Drosophila* immune response to heat-killed *E. coli*. The significance and biological roles of these genes were further analyzed in more detail by targeted RNAi-based functional studies.

9.1.1 Microarray analysis of the genes induced in response to *E. coli* in *Drosophila* S2 cells

In our microarray analysis, total RNA was extracted from S2 cells after challenging them with heat-killed *E. coli*. The expression levels of more than 13, 500 *Drosophila* genes were measured using Affymetrix *Drosophila* genechips. We identified 53 genes that were up-reguated/whose expression was induced in response to *E. coli* by at least three-fold compared to untreated controls. The induction was peptidoglycan recognition protein LC (PGRP-LC) dependent for each of the 53 genes. Twenty-two had already been identified as differentially expressed proteins involved in the *Drosophila* host defense (De Gregorio et al., 2002). In our follow up studies, we aimed to determine the role of 31 genes with unknown function by targeted dsRNA treatments. We carried out an RNAi-based functional analysis of the genes with unknown function and evaluated their role in microbial binding, phagocytosis and antimicrobial peptide (AMP) release as regulators of both the Imd- and the Toll pathway.

The 53 genes up-regulated in *E. coli* treated S2 cells are listed in **Figure 9**. Ten of these genes code for known antimicrobial peptides: *Attacin B1* and *D, Cecropin A1*,

A2 and *B, Diptericin B, Drosocin, Metchnikowin, Cecropin C* and *Drosomycin.* The expression levels of three PGRP genes, *PGRP-SA, PGRP-SD* and *PGRP-like gene (CG4437)* were also induced in our assay. However, *PGRP-SB1, PGRP-SC2* and *PGRP-LB,* which all are shown to be up-regulated in septic injury *in vivo*, were not induced (De Gregorio et al., 2002) (Irving et al., 2005).

Among the induced genes were five putative serine protease-coding genes *CG16731, CG8215, CG9370, CG3505* and *CG10232, which may* possibly contribute to melanization. In addition, we found two genes coding for the complement-like proteins *Tep4* and *Tep-like CG18589* as well as one homologue of the vertebrate α-2-macroglobulin receptor gene (*CG4823*) and the transcription factor gene *Relish* significantly up-regulated in response to the Gram-negative bacterium, *E. coli*.

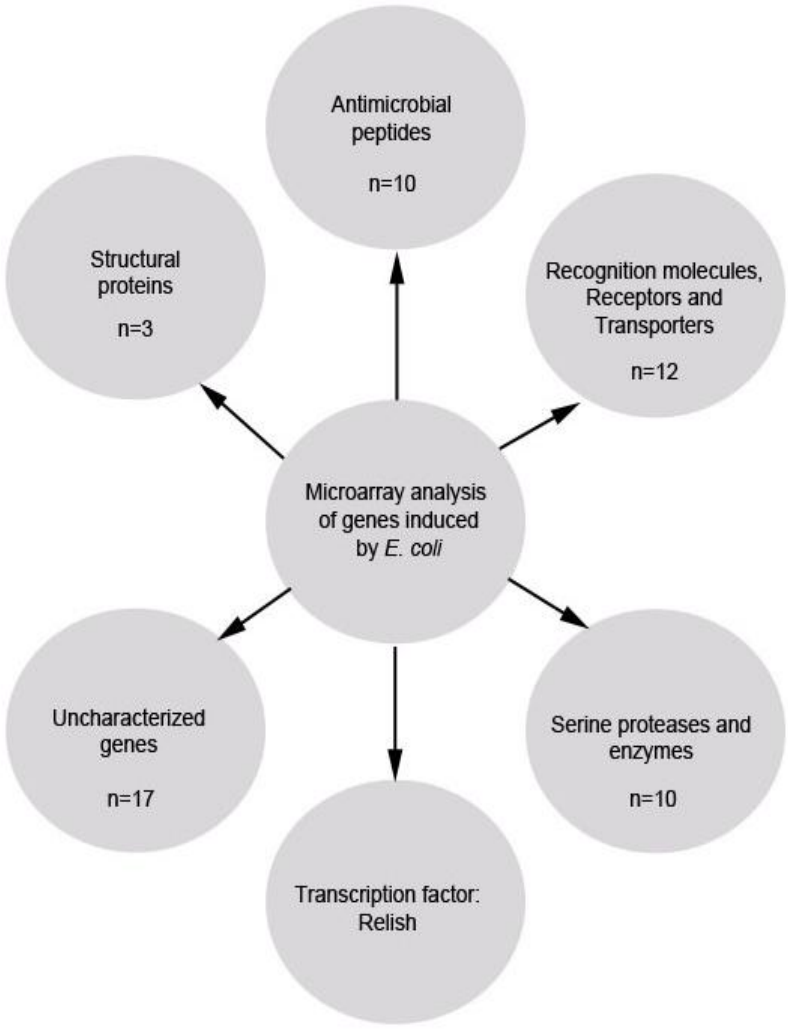


Figure 9: Genes induced in S2 cells by *E.coli*

9.1.2 The importance of immune response genes for *Drosophila* phagocytosis and immune signaling

In our functional analysis, we used targeted RNAi treatments to determine the biological role of the genes with unknown function. At first, we designed primers for dsRNA treatments against the genes of interest according to the sequences found at the www.fruitfly.org website. The templates for dsRNA synthesis were generated from cDNA by a two-step PCR reaction resulting in T7 promoter sequence containing templates. Both sense and antisense RNAs were synthesized simultaneously from a single PCR product using the T7 MegaScript RNA polymerase.

The binding and phagocytosis of FITC-labeled heat-killed *E. coli* and *S. aureus* were quantified using flow cytometry. Labeled heat-killed bacteria were incubated with RNAi treated S2 cells and the amount of bound or phagocytosed microbes was determined using flow cytometry (Ramet et al., 2002b). Our hypothesis was that upon microbial challenge, some of the genes expressed in the challenged S2 cells would encode opsonin-like proteins with roles in either phagocytosis or binding of the invading microbes. However, none of the studied genes proved to be important for the recognition or internalization of bacteria, since RNAi against any of the genes had no significant affect on these processes in our experimental setting.

The possible effect of targeted dsRNA treatment on AMP release was determined using a Luciferase reporter assays. An *Attacin-luciferase* (*Att-luc*) reporter was used for the Imd–pathway and a *Drosomycin-luciferase* (*Drs-luc*) reporter was used for the Toll-pathway. An *Actin 5C β-galactosidase* (*Act5C-β-gal*) reporter was used to normalize results. S2 cells were transfected with either *Attacin*- or *Drosomycin* –reporter plasmids together with a *β-galactosidase* expression vector. After RNAi treatment, cells were lysed and luciferase and β-galactosidase activities were measured.

In our luciferase reporter assay, we found only one dsRNA treatment that affected the *Drs-luc* reporter activity like it did the the activity of the Toll pathway. RNAi against *CG15507* caused more than a 50% reduction in luciferase activity (**Fig. 10**).

This effect was also observed in our phagocytosis assay as the dsRNA treatment targeting *CG15507* resulted in a 42±18% reduction in phagocytosis. However, we came to the conclusion that this effect is not specific to either phagocytosis or Toll signaling, since the RNAi targeting/ *CG15507* also decreased cell viability as measured by the *Act5C-β-gal* reporter. A microscopic examination of the *CG15507* dsRNA treated cells also reveiled them to be unhealthy.

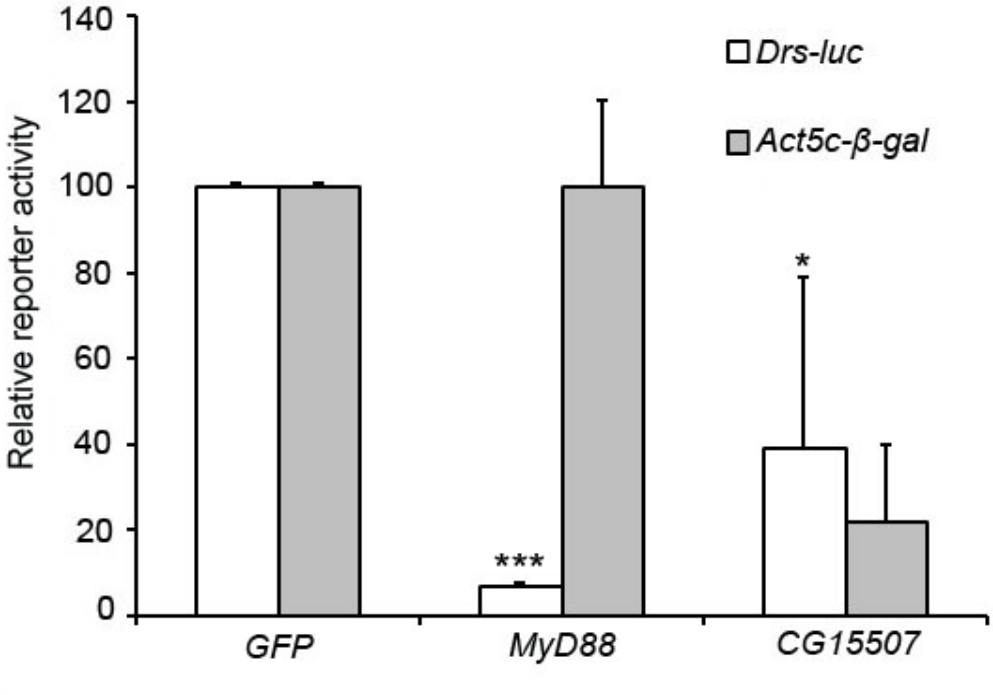


Figure 10: RNAi against the gene *CG15507* caused a marked decrease in the *Drs-luc* reporter activity but it also affected cell viability (*Act5C-β-gal* activity)

Only one dsRNA treatment strongly decreased the *Att-luc* reporter activity mediated via the Imd pathway in S2 cells without affecting cell viability. RNAi against *CG7097*, a Ste20 family kinase named *happyhour* (Corl et al 2009), caused a 52±30% reduction in *Att-luc* reporter activity in response to *E. coli* (**Fig. 11**). *CG7097* presumably encodes a protein highly homologous to the mammalian mitogen activated protein kinase kinase kinase kinase (MAP4 K) isoform 3 (e^{-132}) and to a germinal center kinase related protein kinase (e^{-132}), since it has an N-terminal Serine/Threonine kinase domain and a C-terminal CNH domain. To test whether *CG7097* interacts with JNK, we carried out targeted dsRNA treatments against several known components of the JNK pathway. As shown in figure 10, dsRNA treatments targeting either *kayak, msn, hep* or *aop* caused a significant decrease in Imd pathway activity.

In addition, we identified two potential negative regulators of the Imd pathway. RNAi silencing the expression of *CG15678* and *β-Tubulin 60D* resulted in the hyperactivation of the *Att-luc* reporter in response to Gram-negative *E. coli*. dsRNA treatments targeting *CG15678* caused on average a 284±102% induction of the Imd pathway compared to GFP dsRNA treated controls, and *CG15678* expression was also highly increased (10.3 fold) in response to *E. coli* in our microarray assay (**Fig. 12**). *CG15678* encodes a protein with unknown molecular function and it has no close mammalian homologues. It has been shown by previous studies to be induced by septic injury (De Gregorio et al., 2002). In addition, dsRNA treatment against *β-Tubulin 60D* caused a 370±170% increase in the activity of the Imd pathway as measured by the *Att-luc* reporter assay. We studied the role of Tubulin-associated proteins for the Imd pathway signaling further by targeted dsRNA treatments against *α-Tubulin 84D (CG1913),* and detected a strong, 738±107% increase in Imd pathway activity. Of note, the expression of hemocytes was further demonstrated by Irwing et al. in their studies on whole animals. (Irving P et al. 2005).

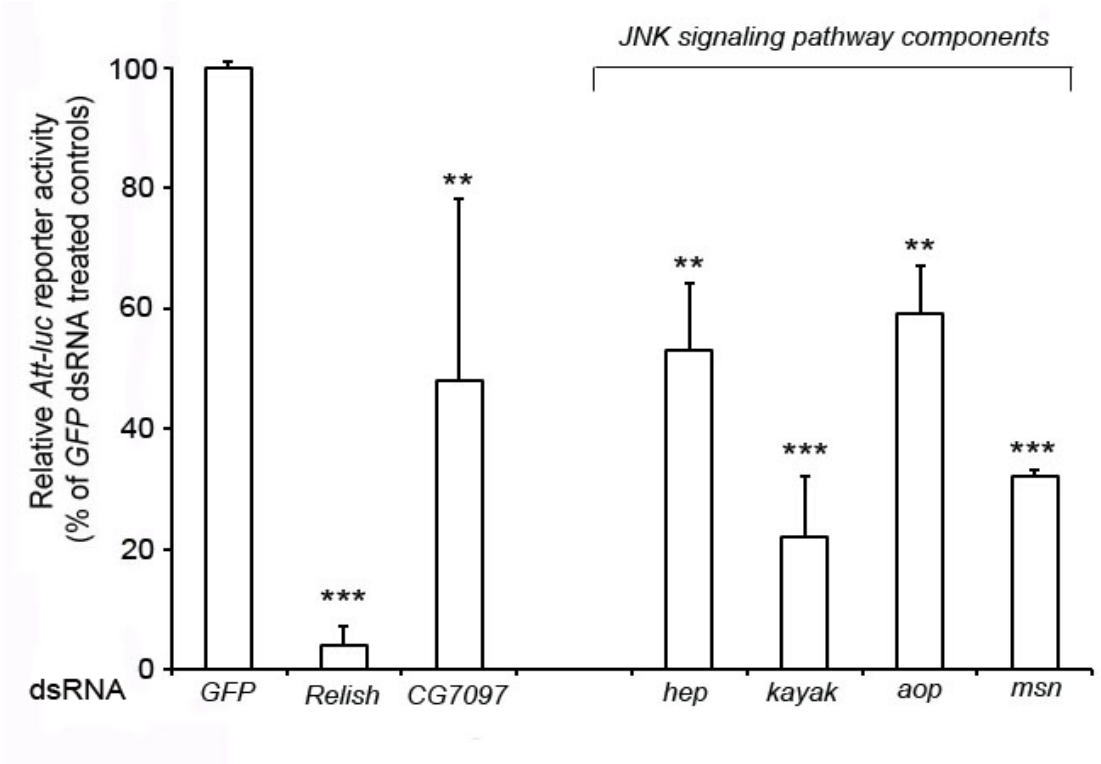


Figure 11: *Att-luc* reporter assay (targeted RNAi against CG7097 and JNK pathway components, [* p≤0.001, ** p≤0.01])**

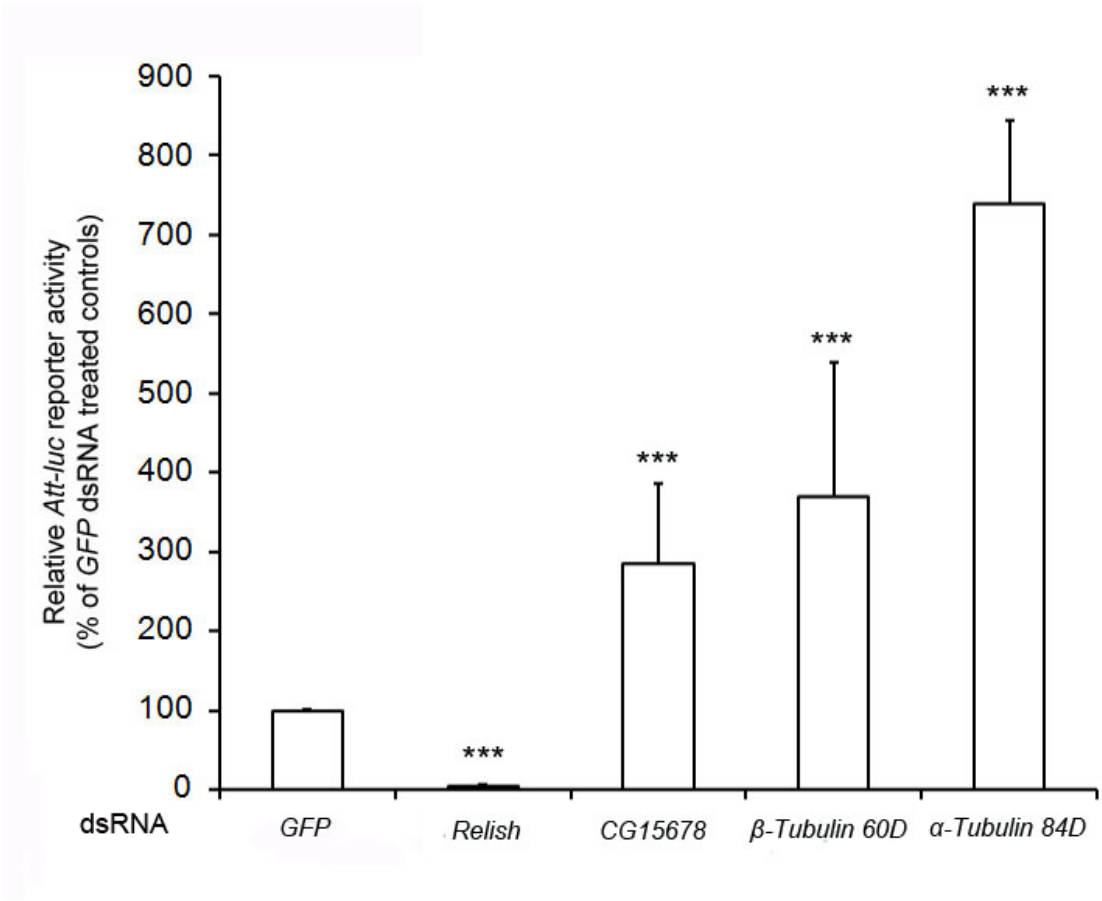


Figure 12: Negative regulators: *Att-luc* reporter assay (dsRNA treatments targeting *CG15678*, *β-Tubulin 60D* and *α-Tubulin 84D* [* p≤0.001])**

9.2 Genome-wide RNAi *in vitro* screens to identify gene products necessary for *Drosophila* immune signaling

Based on our previous work with *Drosophila melanogaster we* reasoned that genome-wide RNAi performed *in vitro* would be an extremely powerful method for identifying gene products necessary for antimicrobial peptide release in *Drosophila* (Kleino et al., 2005; Ramet et al., 2002b). First, *Drosophila* makes an excellent model for studying innate immune signaling, since it lacks adaptive immunity and relies only on innate immunity for defending against pathogen. This is an advantage compared to vertebrates, in which a highly sophisticated and complex immune system can compensate for the effects of RNAi against genes of interest. Second, innate immunity is well-conserved throughout evolution from insects to human. Therefore the fruit fly can be used as a model organism to study these responses and the results may be comparable to humans. Third, *Drosophila* is cheap and easy to maintain, and the RNAi is relatively simple to carry out. For example, *Drosophila* S2 cells take up dsRNA from the growth medium by scavenger receptor-mediated endocytosis (Ulvila et al., 2006), so the delivery of dsRNA into the cells can be accomplished simply by soaking cells in culture media containing dsRNAs. In addition, there is a plethora of genetic and molecular techniques available for *Drosophila melanogaster* enabling effective exploitation of the data obtained from these assays.

We carried out two separate genome-wide RNAi-based *in vitro* screens to identify novel genes involved in immune signaling: a screen for genes involved in NF-κB signaling and another screen for JAK/STAT signaling. Our aim was to identify all the gene products involved in the Toll, Imd and the JAK/STAT pathways in a *Drosophila in vitro* model. The most interesting genes were later subjected to further functional studies.

In our genome-wide RNAi-based *in vitro* screens, we used commercial PCR products representing the entire *Drosophila* genome (13,607 genes, MRC Geneservice, Babraham Bioincubator, Cambridge) and an additional 2,418 genes

from an S2 cell-derived cDNA library as template for dsRNA synthesis. The second PCR reaction and dsRNA synthesis were performed as stated above except that both reactions were carried out on 96-well plates. The concentration of each dsRNA was measured using the PicoGreen® dsRNA Quantitation Kit (Molecular probes).

9.2.1 A genome-wide RNAi *in vitro* screen to identify novel regulators of *Drosophila* NF-κB signaling

Drosophila NFκ-B signaling comprises two signaling pathways, the Toll and the Imd pathway (Ganesan et al., 2010; Lemaitre et al., 1995; Tanji et al., 2010). In our genome-wide RNAi *in vitro* screen, we used our earlier finding, indicating that in *Drosophila* S2 cells RNAi mediated knock-down of the Imd pathway components decreases the $Toll^{10B}$ induced *Drosomycin* promoter-driven luciferase activity (Kleino et al., 2005). In order to define the specific roles of the Toll and the Imd pathways in regulating *Drosomycin* expression, we carried out an assay to analyze *Drs-luc* expression induced by both $Toll^{10B}$ and *E. coli.* The S2 cells, transfected with appropriate reporter constructs, were co-transfected with $Toll^{10B}$ to induce Toll signaling. Later, the cells were also exposed to heat killed *E. coli* to activate Imd signaling. *Drs-luc* reporter activity was measured to analyze *Drs* expression levels in response to both $Toll^{10B}$ and *E. coli.* Our results demonstrate that *Drs* expression is controlled by both NFκ-B signaling pathways, the Toll and the Imd pathway,, in S2 cells. Overexpression of $Toll^{10B}$ caused the induction of *Drs* expression as expected, and *Drs* expression was further induced when the Imd pathway was also activated with *E. coli* (**Fig. 13**). This Toll and Imd pathway stimulated induction of *Drs* expression was significantly decreased by RNAi against the Toll pathway components, *Toll*, *Myd88* or *dorsal*. Furthermore, our results indicate, that the Imd pathway components, PGRP-LC, Imd, Tab2 and Relish are also essential for the normal induction of *Drs* expression in this assay. *Drs* induction is completely abolished if both NFκ-B pathways are silenced by RNAi against both *Relish* and *Myd88*. These results are in line with previous reports and confirm that *Drs-luc* activity can be used to discover and identify novel components of both the Toll and the Imd signaling pathways.

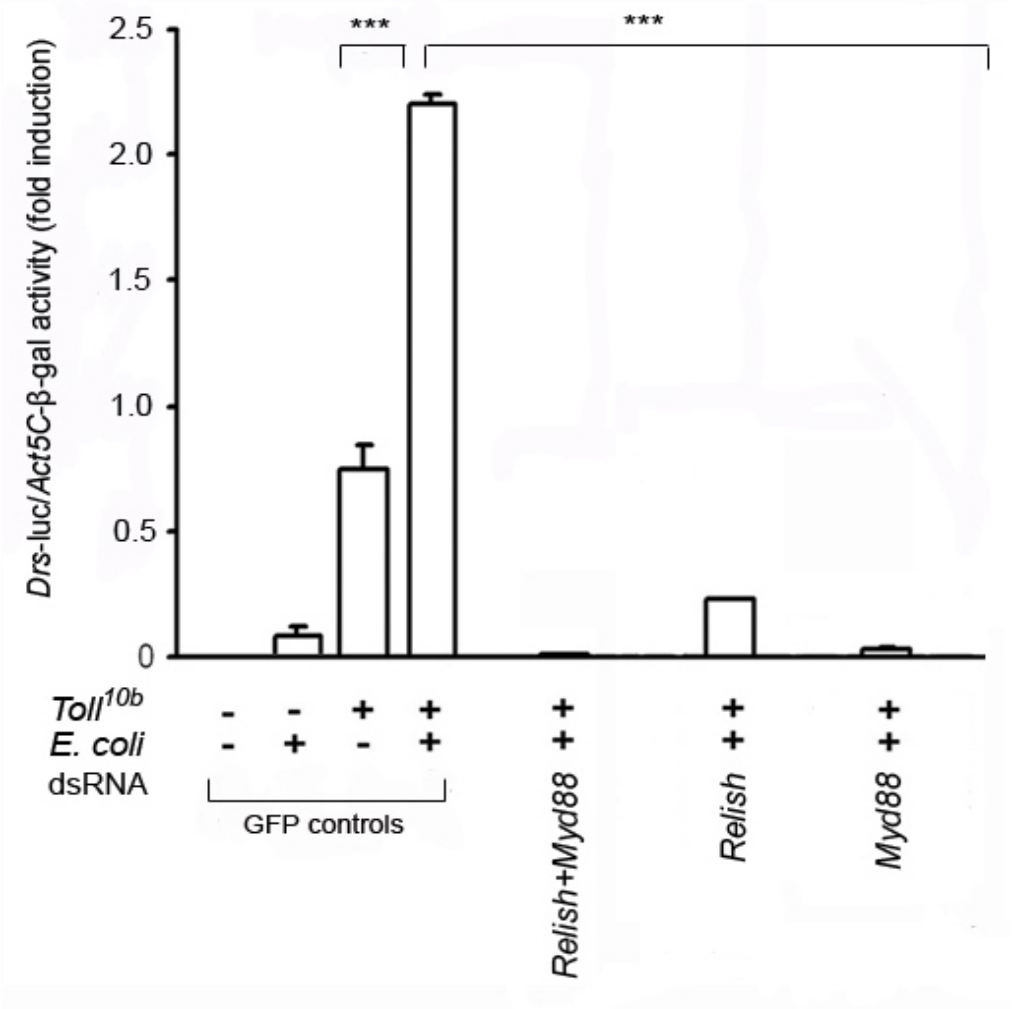


Figure 13. *Drs-luc* reporter activity in both *Toll*^{10B} and *E. coli* induced S2 cells (Data is shown as the mean ± SD, n≥3, [* p≤0.001])**

On this premise, we analyzed the effects of 16,025 dsRNA treatments on the *Drs-luc* reporter activity in *Toll*^{10B} and *E. coli* induced *Drosophila* S2 cells. Our aim was to identify all of the gene products required for signaling via the Toll and Imd pathways. With a cut-off of 50%, we found twenty-three out of 16, 025 dsRNA treatments that repeatedly decreased the *Drs-luc* reporter activity without affecting cell viability (**Fig 14**). To validate these findings and to confirm that the RNAi effect really was due to dsRNA targeting the identified gene and to rule out off-target effects, the 23 genes were TA-cloned, sequenced and targeted PCR primers were designed based on the sequencing results. These PCR products were used as templates to synthesize targeted dsRNAs against the genes. With these targeted dsRNA treatments, we were able to verify, that our RNAi screen effectively found

components of both of the *Drosophila* NFκ-B signaling pathways. Five of the identified genes represented known components of the Toll pathway (*Toll*, *Myd88*, *tube*, *pelle* and *dorsal*) and eight were known components of the Imd pathway (*Relish*, *Kenny*, *Fadd*, *Tak1*, *imd*, *Tab2*, *Ird5* and *Iap2*) (**III fig. 1C**). Most importantly, we identified ten novel regulators of the *Drosophila* NFκ-B signaling pathways. These genes were subjected to further studies.

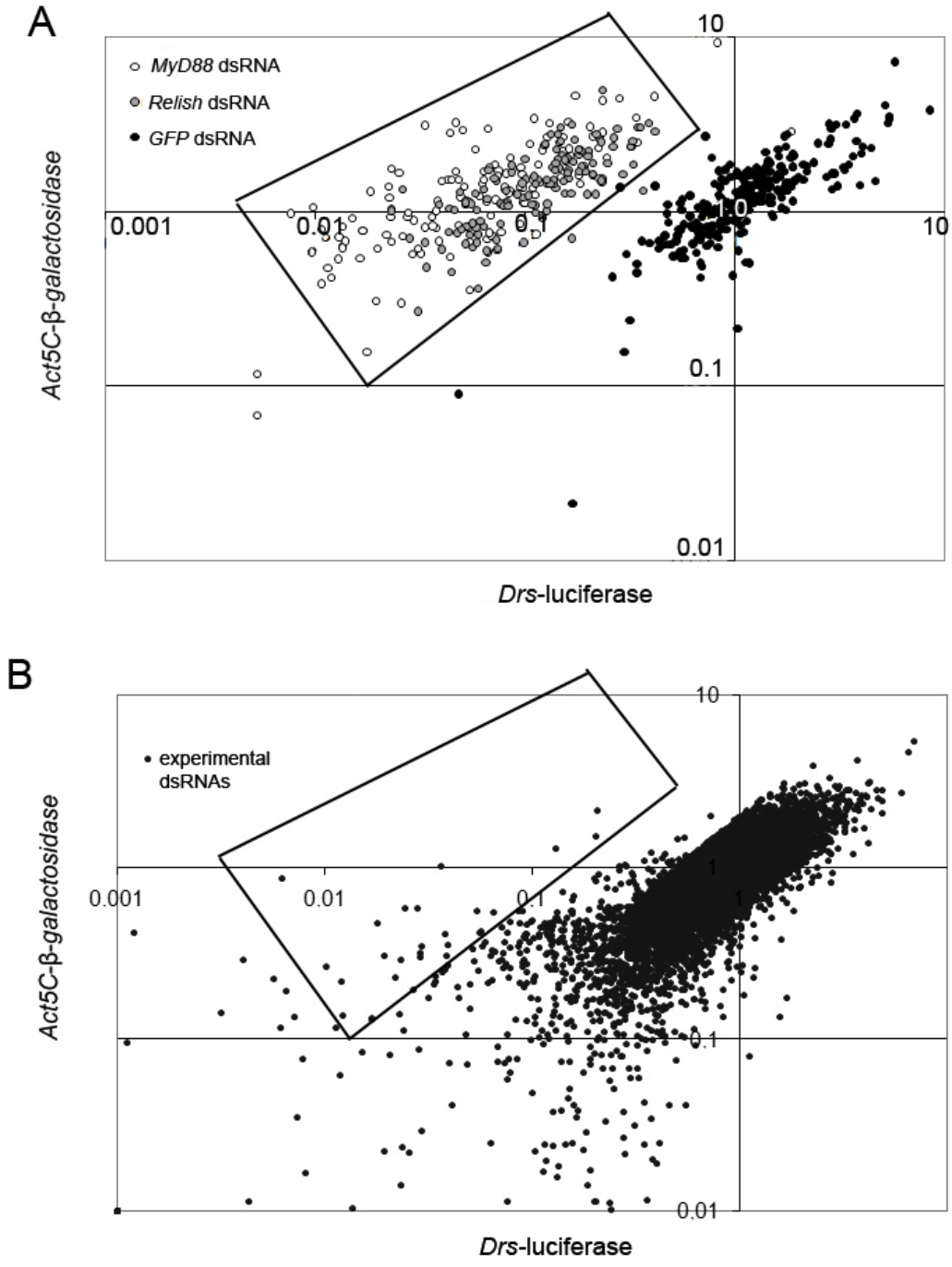


Figure 14. Results of the genome-wide RNAi screen of *Drosophila* NFκ-B signaling

To find out which pathway is affected by these ten dsRNA treatments and which pathway they potentially regulate, we assayed the pathways separately. We carried out luciferase reporter assays in dsRNA treated and induced S2 cells. We used an *AttA-luc* construct to measure the activity of the Imd pathway in dsRNA treated and heat-killed *E. coli* induced S2 cells. Accordingly, we used a *Drs-luc* construct in $Toll^{10b}$ induced and dsRNA treated S2 cells to measure the activity of the Toll pathway.

Out of these ten candidate genes, only one RNAi treatment, *Mediator complex subunit 25* (*MED25*), affected Imd signaling approximately at the level of RNAi against known components of the Imd pathway (data not shown). In addition, RNAi against *u-shaped* caused a significant decrease (52%) in Imd pathway activity, and RNAi targeting *achaete, Gprk2, pannier, CG4325* and *CG32133* caused hyperactivation of the Imd pathway, whereas RNAi against *Spt6, CG31660* and *CG15737* had only a minor effect on pathway activity.

Nine of the original candidate genes turned out to be regulators of the Toll signaling pathway, since RNAi against these genes decreased the *Drs-luc* reporter activity in $Toll^{10b}$ induced S2 cells by more than 60% (**II fig. 2B**). These findings were further confirmed with an qRT-PCR analysis of endogenous *Drosomycin* expression in dsRNA treated S2 cells. With regard to these findings, we concluded that *u-shaped, pannier, CG4325, Gprk2, CG15737, CG32133, CG31660, achaete and Spt6* are novel regulators of and are required for accurate signaling via the *Drosophila* Toll pathway *in vitro* in S2 cells. Of note, *MED25* RNAi had no significant effect on the Toll pathway alone.

Epistasis analysis of the identified regulators of the Toll pathway

To further investigate the role of the novel regulators identified in the screen, we carried out a *cactus* RNAi based epistasis analysis of these genes. Cactus is a *Drosophila* homolog for human Iκ-B, and silencing *cactus* releases the Dif/Dorsal complex to translocate into the nucleus resulting in over a 40-fold induction of the *Drs-luc* reporter activity (**II fig. 2E**). In the epistasis experiment, *Drs-luc* expression was induced by *cactus* RNAi instead of $Toll^{10b}$. This induction was blocked by ten

dsRNA treatments, *dorsal, pannier, CG15737, Relish, Spt6, Gprk2,* and *CG4325, u-shaped, CG32133* and *achaete,* indicating that these gene products act downstream or independently of Cactus. In contrast, dsRNA treatments targeting *Toll, tube, pelle, CG31660 and MyD88* had very little or no effect on this induction, therefore these gene products appear to act upstream of Cactus.

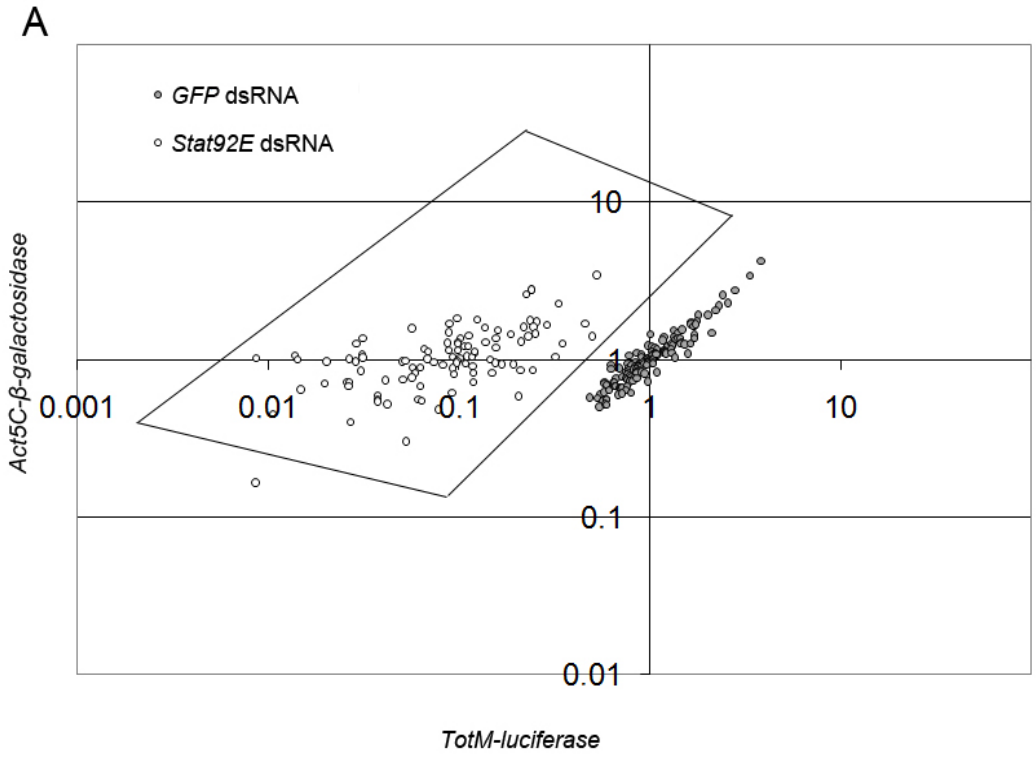
9.2.2. Genome-wide RNAi analysis of the *Drosophila* JAK/STAT pathway

In addition to the genome-wide analysis of the *Drosophila* NFκ-B signaling pathways, we carried out a genome-wide RNAi-based *in vitro* screen to analyze *Drosophila* JAK/STAT signaling. It has been shown that under stressful conditions, such as after septic injury, several *Turandot* (*Tot*) stress genes are expressed in the *Drosophila* fat body, and that this expression is regulated via the JAK/STAT pathway (Ekengren and Hultmark, 2001; Ekengren et al., 2001) (Agaisse et al., 2003) (Brun et al., 2006). Based on this information, we used a Stat92E responsive *TurandotM-luciferase* (*TotM-luc*) reporter based assay to monitor JAK/STAT pathway activity in *Drosophila* S2 cells. Since the intracellular part of the JAK/STAT pathway downstream of the JAK kinase is especially well conserved, we decided to activate signaling using the constitutively active form of the *Drosophila* JAK kinase *hopscotch (hop^{Tum-l})* (Harrison et al., 1995) (Luo et al., 1995).

As shown in **III fig. 1A**, hop^{Tum-l}-induced *TotM-luc* reporter activity is a valid means of studying the regulation of the JAK/STAT pathway in *Drosophila* S2 cells. First, *TotM-luc* reporter activity is induced by *hop^{Tum-l}* in *Drosophila* S2 cells in a STAT dependent manner, as RNAi targeting the transcription factor *Stat92E* almost completely blocks *TotM* expression. Second, our assay is specific for JAK/STAT

signaling because RNAi targeting the Imd pathway transcription factor *Relish* has no effect on hop^{Tum-l}-induced *TotM* expression (data not shown).

After validating our assay, we carried out a genome-wide RNAi analysis of the *Drosophila* JAK/STAT pathway in *Drosophila* S2 cells in order to identify all of the regulators of the JAK/STAT pathway downstream of hop. Altogether, we monitored the effects of 16,025 different dsRNA treatments on hop^{Tum-l}-induced *TotM-luc* reporter activity in *Drosophila* S2 cells using the same pool of dsRNAs as described earlier. Again, *Act5C-β-gal* activity was used to monitor cell viability and *GFP* RNAi served as a negative control. The luciferase and β-galactosidase values for *GFP* dsRNA treated cells were set to one and used as reference values for experimental dsRNAs. RNAi against *STAT92E* was used as a positive control in each assay.



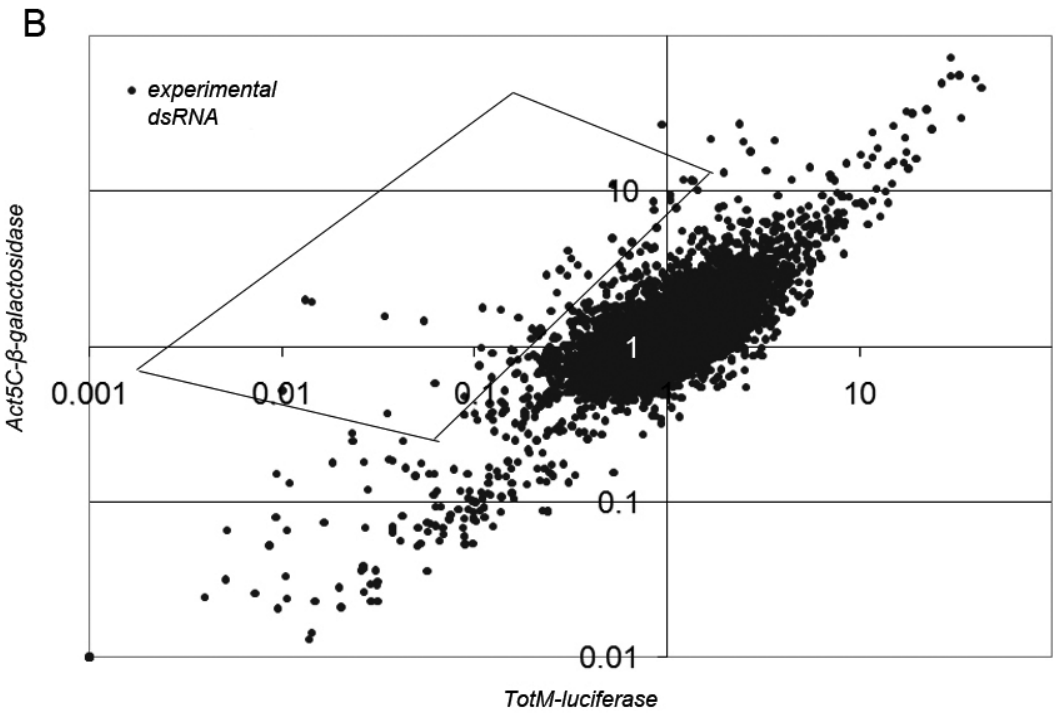


Figure 15. Results of the genome-wide screen for JAK/STAT pathway signaling

Figure 15 shows the results of 16,025 different dsRNA treatments analyzed for their effect on hop^{Tum-l} –induced *TotM-luc* activity. As expected, most dsRNA treatments had little or no effect. Importantly however, we found seven dsRNA treatments that repeatedly decreased *TotM-luc* activity by more than 50% without significantly affecting cell viability as represented by *Act5C-β-gal* activity. Out of these seven dsRNA treatments, two targeted known components of the JAK/STAT pathway (*Stat92E* and *hop*) and one targeted a previously characterized regulator of the JAK/STAT pathway (*enok*) (Baeg et al., 2005) (Muller et al., 2005), demonstrating the efficiency of our screen to find components of the JAK/STAT signaling pathway. In addition, we found four putative novel regulators (*Taf1*, *CG31716*, *CG14225* and *Med27*) essential for JAK/STAT signaling. Again, the corresponding templates from the original library were TA-cloned and sequenced and gene specific primers were designed. Targeted independent dsRNAs were synthesized against these novel regulators and the reporter assays were repeated with these dsRNAs. As shown in **III fig. 1C**, all five targeted dsRNA treatments decreased *TotM-luc* reporter activity comparably to dsRNAs from the library.

To further confirm our findings and to ensure that the results were not due to any artifact related to the reporter assay, we used qRT-PCR to study endogenous *TotM* and *Act5C* expression levels in $hop^{Tum\text{-}l}$ -transfected and dsRNA treated S2 cells. The results of this qRT-PCR assay further confirmed our findings that *enok, Taf1, CG31716, CG14225* and *Med27* are potential regulators of the *Drosophila* JAK/STAT pathway and that these genes are required for a normal $hop^{Tum\text{-}l}$ -induced *TotM* response in S2 cells. RNAi targeting any of the identified genes resulted in at least a 50% reduction in relative *TotM* expression levels (**III fig 1D**). We decided to subject these five genes to further studies.

9.3 Functional characterization of the selected genes identified in the screens

To gain more insight into the role of the genes identified in the RNAi screens, we carried out a set of functional *in vitro* studies of the selected genes. In addition, we carried out a set of functional studies for the five potential regulators of the JAK/STAT signaling pathway.

9.3.1 Functional analysis of the G protein-coupled receptor kinase in *Drosophila* cells

Of the genes identified in the RNAi screens, we chose to study the function of Gprk2 in more detail. Gprk2 has a strong *in vitro* phenotype and is evolutionarily conserved. In fact, *Drosophila* Gprk2 shares high sequence similarity with the human, mouse and zebrafish GRK5s. *Drosophila* Gprk2 belongs to a family of multifunctional GTPase-accelerating proteins (De Vries L, 2000). It is a 714-aa protein with three known domain structures: a regulator of G protein signaling (RGS) domain, a serine/threonine protein kinase catalytic domain and a kinase domain extension. To investigate the cellular localization of Gprk2, we

overexpressed a *Gprk2-GFP* construct in S2 cells. As shown in **Figure 16A**, Gprk2 is localized on the cell membrane or in the cytoplasm. A similar localization pattern is observed with a *GRK5-GFP* construct when overexpressed in HeLa cells (**Fig 16B**). On this premise, we decided to investigate whether GRK5 has the same evolutionary conserved role in NFκ-B signaling. A similar RNAi-based luciferase reporter assay was carried out with GRK5 (**II fig. 4B**). HeLa cells were transfected with *NFκ-B luciferase* and *CMV-β-galactosidase* reporters together with *GRK5* or control siRNAs, and NFκ-B signaling was induced with TNFα six hours prior to measurements. The relative *NFκ-B-luc* activity was reduced more than 60% in *GRK5* siRNA treated HeLa cells indicating that GRK5 is an important regulator of human NFκ-B signaling *in vitro*. These findings were further confirmed in our *in vivo* studies with zebrafish, indicating that GRK5 is necessary for proper NFκ-B signaling in the vertebrate immune system *in vivo* (**II fig. 4D**).

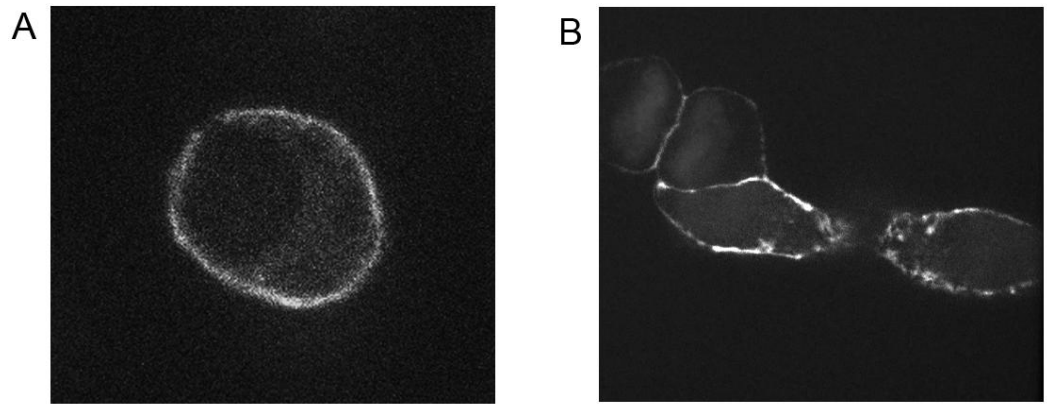


Figure 16. Gprk2/GRK5 is localized on the cell membrane or in the cytoplasm in both *Drosophila* S2 cells (A) and in HeLa cells (B)

Because we observed both sequence and functional similarities between *Drosophila* Gprk2 and vertebrate GRK5, and because of reports on mammalian GRK5 interacting with members of the Iκ-B family (Parameswaran et al., 2006) (Sorriento et al., 2008) (Patial et al.), we decided to study the potential interaction of Gprk2 with Cactus and Dorsal. We co-immunoprecipitated myc-tagged Cactus and Dorsal with V5-tagged full-length Gprk2 or with three deletion constructs: a calmodulin (CaM) binding site deletion (ΔCaM1), an RGS-domain deletion (ΔRGS) and a

kinase deletion (Δkinase) in S2 cells. The full-length Grpk2 interacted with Cactus as did the ΔCaM1 and ΔRGS deletion constructs, indicating that these domains are not essential for the Grpk2 and Cactus interaction (**Fig. 17**). Conversely, an interaction between Cactus and the Δkinase construct could not be detected. This suggests that either the kinase domain is important for the interaction or such a large deletion itself causes the loss of function of the whole protein due to improper folding. Interactions between the V5-tagged Gprk2 constructs and Dorsal-myc were not detected.

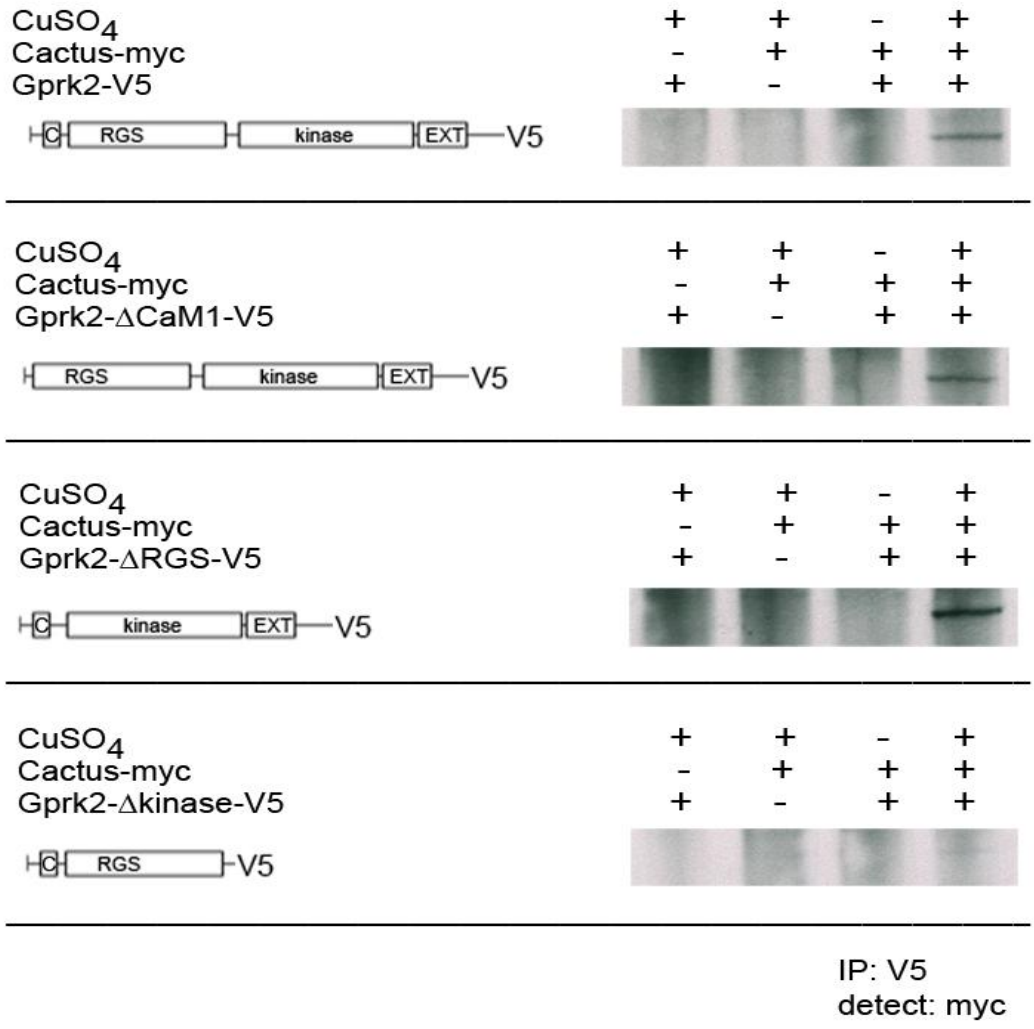


Figure 17. The full length Gprk2, the Calmodulin binding-site deletion (ΔCaM1) and the RGS-domain deletion (ΔRGS) constructs interact with the Cactus protein

In order to indentify the functional significance of the interaction between Cactus and Gprk2, we decided to investigate the potential role of Gprk2 in Cactus degradation. We set up an EGFR-Toll pathway induction system (Sun H, 2004) with S2 cells expressing a chimeric EGFR-construct. These cells were treated with *GFP*, *Gprk2* and *MyD88* dsRNAs and Cactus degradation was monitored by Western-blot (**II fig. 5C**). Additionally, we carried out kinase experiments with coimmunoprecipitated Gprk2 and Cactus. *Grpk2* RNAi had no effect on Cactus degradation nor did the kinase experiments show any Gprk2-mediated Cactus phosphorylation, indicating that Gprk2 is not required for Cactus degradation upon signaling although it interacts directly or indirectly with the Cactus protein.

9.3.2 *In vitro* characterization of Eye transformer

We identified five putative regulators of the *Drosophila* JAK/STAT pathway in our genome-wide RNAi *in vitro* screen. Of these, CG14225, which we call Eye Transformer (ET), was subjected to further functional studies.

ET is a negative regulator of the JAK/STAT pathway in Drosophila *S2 cells*

To investigate the role of *ET* in a more physiological context, we overexpressed the natural ligand of the JAK/STAT pathway, *unpaired* (*upd*) to activate the pathway. *TotM-luc* and *10xStat92E-luc* reporters were used to measure JAK/STAT pathway activity in *ET* dsRNA treated S2 cells. *10xStat92E-luc* is a reporter consisting of a sequence containing a double Stat92E binding site from the *SOCS36E* enhancer region multiplied 10 times. This construct allowed us to study if ET is specific for regulating *TotM* expression or if it affects JAK/STAT target genes in a more general manner. As is shown in **III fig. 2**, the RNAi targeting *CG14225* caused strong hyperactivation of both reporters compared to *GFP* dsRNA treated controls. These results are in total contrast to our results with the constitutively active hop^{Tum-l} induction, and thus prompted us to investigate *CG14225* gene more thoroughly.

According to the literature, *CG14225*/*ET* is a 3.3 kb gene located next to the *Drosophila* JAK/STAT pathway receptor *Domeless* (*Dome*) and codes for a 713-aa type-I transmembrane protein sharing structural similarities with *Dome* (Hombria

and Brown, 2002; Makki et al., 2010). Intriguingly, ET's closest human homologue is the signal transducing protein gp130, a protein associating with interleukin-6 (IL-6) receptors, the mammalian receptors, which Dome resembles (Taga and Kishimoto, 1997). Thus, ET shares structural similarities with Dome and with mammalian gp130, both of which play key roles in JAK/STAT pathway regulation.

In order to rule out the possibility that our results are due to unintended off-target effects or other artifacts of the assay, we designed an additional dsRNA targeting the third exon of *ET* (**III fig. 3A**). *10xStat92E-luc* reporter assays were then carried out with both $hop^{Tum\text{-}l}$ and *upd* induced S2 cells (**III fig. 3B and 3C**). The *ET* RNAi phenotype was confirmed with these results, since both *ET*-specific RNAi treatments caused a strong inhibition of the $hop^{Tum\text{-}l}$ induced *10xStat92E-luc* reporter activity and conversely, hyperactivation of *upd* induced the *10xStat92E-luc* reporter activity. The efficiency of the RNAi was further validated by analyzing *ET* mRNA levels in targeted dsRNA treated S2 cells by qRT-PCR (**III fig. 3D**). Both dsRNA treatments sufficiently suppressed *ET* expression compared to *GFP* dsRNA treated control cells.

These RNAi phenotype results were further confirmed with our overexpression experiments. As shown in **III fig. 3E**, overexpression of *ET* decreased the upd-induced *10xStat92E-luc* activity, but increased hop-induced *TotM-luc* activity. Of note, since *ET* mRNA lacks UTR regions, we were not able to carry out any rescue experiments, where *ET* would be first silenced by dsRNAs targeting the UTR regions and this phenotype then rescued by overexpressing *ET*.

Epistasis analysis of ET

To gain more insight into the function of ET and to explain the opposite effects of *ET* RNAi in the reporter assays, depending on whether JAK/STAT signaling was activated using $hop^{Tum\text{-}l}$ or upd, we performed an epistasis analysis of ET in S2 cells. We carried out dual RNAi treatments targeting, in addition to *ET*, the known components of the *Drosophila* JAK/STAT signaling pathway, *hop*, *Stat92E* or *Dome*, after which the activity of the JAK/STAT pathway was measured by the *TotM-luc* reporter. Our results indicate that ET is a negative regulator of the JAK/STAT pathway and functions at the level or upstream of the known

components, *hop*, *Stat92E* or *Dome* in S2 cells (**III fig. 4A**). *ET* RNAi caused a slight enhancement of the *TotM-luc* reporter activity and this activation was prevented by RNAi targeting any of the known *Drosophila* JAK/STAT pathway components (*hop*, *Stat92E* or *Dome)*. These results explain the conflicting results gained from *ET* RNAi reporter assays depending on the level of JAK/STAT pathway activation.

9.4 *In vivo* characterization of selected genes regulating *Drosophila* immune signaling

To investigate if the nine candidate genes identified in the Toll screen are important for Toll signaling *in vivo*, the RNAi flies, carrying *UAS*-RNAi constructs targeting these nine genes were crossed with the *C564-GAL4* driver line, which drives the expression of the dsRNA in the fat body. Flies that were crossed with w^{1118} i.e. flies without the driver, which therefore did not express any dsRNA were used as a negative control, and *MyD88* RNAi flies crossed with the driver line were used as a positive control. Flies were pricked with a *M. luteus* contaminated needle to generate septic injury to activate the Toll pathway. After infection, total RNAs were isolated and relative *Drosomycin* expression levels were measured by qRT-PCR. As a result, we detected four *in vivo* RNAi fly lines with significantly decreased *Drosomycin* expression (**II fig. 3**). First, two *Gprk2* fly lines, *Gprk2 R-1* and *Gprk2 R-3*, crossed with *C564-GAL4* driver lines showed a significant degrease in *Drosomycin* expression. In addition, fly lines expressing the *CG15737* and *u-shaped* RNAi constructs showed a statistically relevant reduction in *Drosomycin* expression compared to control flies. We decided to name the *CG15737* gene *Toll pathway activation mediating protein* (*TAMP*). Flies carrying RNAi constructs against other genes identified in the screen showed no significant difference in *Drosomycin* expression compared to controls.

9.4.1 Gprk2 has an essential role in *Drosophila* Toll pathway mediated immunity *in vivo*

To investigate the role of Gprk2 on Toll pathway mediated immunity *in vivo*, we carried out a series of experiments with *Gprk2* RNAi flies. First, we assayed the expression levels of Toll pathway target genes after a natural fungal infection. In addition, we examined the survival of *Gprk2* RNAi flies upon *E. faecalis* infection. We also carried out *in vivo* experiments in *Drosophila* larvae in order to determine if *Gprk2* RNAi can inhibit blood cell activation triggered by a constitutively activated Toll pathway.

In order to assess the role of Gprk2 on signaling via the Toll pathway, we studied the expression levels of Toll pathway target genes, namely *Drosomycin, IM1* and *IM2*, under a natural fungal infection. *Gprk2* RNAi fly lines, *Gprk2 R-1* and *Gprk2 R-3*, were crossed with *C564-GAL4* driver lines and these flies, as well as control flies, were subjected to the insect pathogen *B. bassiana*. After a 48 hour infection, total RNA was isolated and the expression of Toll pathway target genes was measured by qRT-PCR. w^{1118} flies crossed with *C564-GAL4* flies were used as negative and *MyD88* RNAi flies over *C564-GAL4* flies as a positive control. RNAs isolated from the noninfected flies were used as a control for the infection. As a result, we discovered that Gprk2 is essential for Toll pathway-mediated immunity and that the expression of Toll pathway target genes is poorly activated in the *Gprk2*-silenced flies. The expression of Toll pathway induced genes was reduced in both *Gprk2 R-1* and *Gprk2 R-3* RNAi flies crossed with the driver *C564-GAL4* (**II fig. 6**).

Gprk2 is also needed for a normal defense against the Gram-positive bacteria *E. faecalis*. In our survival experiment, we used septic injury with *E. faecalis* to examine if the *Gprk2* silencing affects the Toll pathway enough to impair the fly's survival. Both Gprk2 RNAi flies were crossed with *C564-GAL4* driver flies. These flies were first immunized by pricking them with a needle dipped into a culture of *M. luteus* in order to activate the Toll pathway response, including *Drosomycin* expression. After a 24 hour immunization, flies were infected with *E. faecalis* by septic injury generated by pricking as above and the survival rate of the flies was monitored. Both Gprk2 RNAi flies crossed with the *C564-GAL4* driver were more

susceptible to the *E. faecalis* infection and showed a statistically significant reduction in survival compared with control flies lacking the driver (**II 7A and 7B**).

To further examine the role of Gprk2 in Toll signaling *in vivo*, we studied whether RNAi targeting *Gprk2* can inhibit hemocyte activation generated by *UAS-Toll*^{10b}. To do this, we combined *Gprk2 R-3* RNAi fly lines and *MyD88* as a control, with the blood-cell specific *hml*$^{\Delta}$*-GAL4*, *UAS-GFP* driver. Males originating from these stocks were crossed with females carrying *UAS-Toll*^{10b}. As a negative control, males from the original driver line were crossed with females of the *UAS-Toll*^{10b} and with the *w*^{1118} line to gain treatment control. Progeny third-instar larvae were graded into four groups for the percentage of their segments. Segments showing bands were formed by islets of sessile hemocytes gathering under the epidermis. The constitutive activation of the Toll pathway in blood cells caused a largely disturbed sessile hemocyte banding pattern in the larvae of the negative control (**II fig 7C and 7D**). This loss of the sessile hemocyte banding pattern could be rescued by reversing the constitutive Toll pathway activation i.e. by introducing *Gprk2* or *MyD88* RNAi constructs. In conclusion, RNAi targeting *Gprk2* can rescue *UAS-Toll*^{10b} blood cell activation in *Drosophila* larvae *in vivo*.

9.4.2 ET is a negative regulator of *Tot* gene expression *in vivo*

Our functional studies clearly showed, that ET negatively regulates the *Drosophila* JAK/STAT pathway signaling *in vitro*. In order to investigate whether ET is important for JAK/STAT signaling *in vivo,* we carried out in vivo RNAi experiments with fly lines carrying *UAS*-RNAi constructs targeting *ET* (*ET IR*^{1} and *ET IR*^{2}) (Dietzl et al., 2007). RNAi flies and w^{1118} control flies were crossed with the *C564-GAL4* driver line and flies were pricked with a *E. cloacae* contaminated needle. Thereafter, total RNA was isolated and the relative expression levels of both *TotM* and *TotA* were measured by qRT-PCR. As expected, the *C564-GAL4* driven expression of *UAS-Stat92E* dsRNA significantly degreased both the *TotM* and *TotA* response compared to w^{1118} control flies. Moreover, *ET* RNAi flies crossed with *C564-GAL4* driver flies showed a significant increase in both *TotM* and *TotA* expression in response to septic injury with *E. cloacae* (**III fig. 6A and 6B**). These results are in line with the earlier results from the *in vitro* studies and further confirm ET as a negative regulator of *Tot* gene expression in *Drosophila in vivo.* The relative expression level of *ET* was also measured in *E. cloacae* infected flies, but the infection showed no effect on *ET* expression compared to uninfected controls, indicating that *ET* expression is not regulated by the JAK/STAT or the Imd pathway activated in response to *E. cloacae* infection (data not shown).

According to the literature, *Tot* gene expression is co-operatively regulated by the JAK/STAT and the Imd pathways upon septic injury in *Drosophila* (Agaisse et al., 2003) (Brun et al., 2006). To this end, we tested if the effect of *ET* RNAi on *Tot* gene expression is mediated by the Imd pathway. *ET* RNAi flies were again crossed with *C564-GAL4* driver line, pricked with a needle dipped into a *E. cloacae* culture, after which total RNAs were isolated. The relative expression level of Imd pathway mediated *Attacin B* (*AttB*) in response to the *E. cloacae* infection was measured (**Fig 18**). Our results indicate, that ET does not have role in host defence regulation via the Imd pathway *in vivo*, since *ET* RNAi had no effect on *AttB* expression upon septic injury with the Gram-negative bacterium *E. cloacae*.

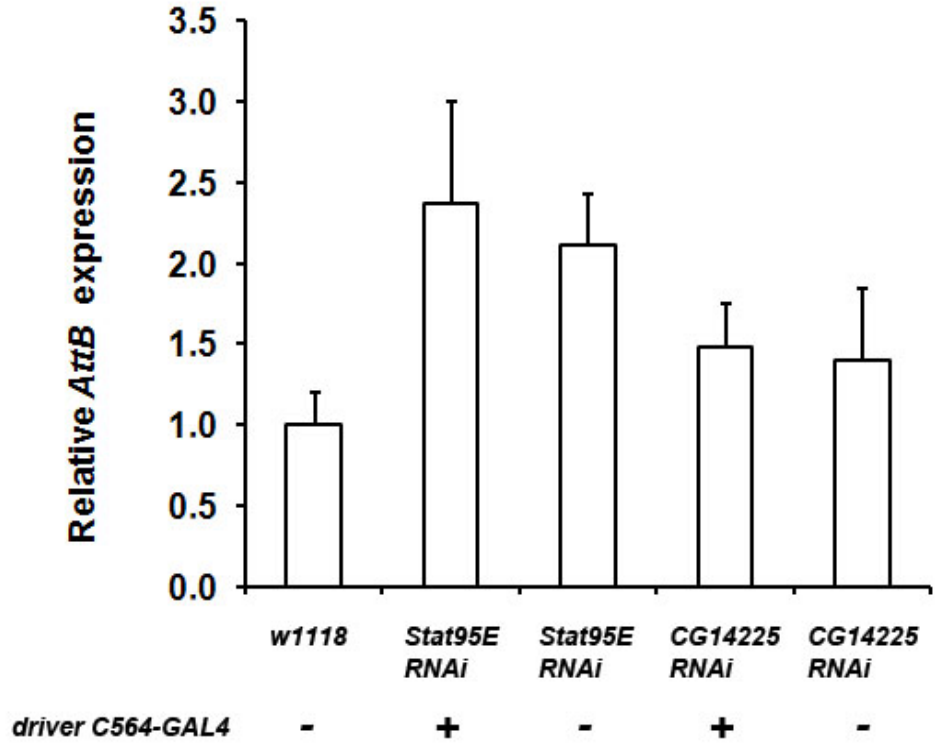


Figure 18. *ET* RNAi had no effect on *AttB* expression upon septic injury with the gram-negative bacterium *E. cloacae*, indicating that ET does not participate in host defence regulation via the Imd pathway *in vivo*.

Because genetic background can have an effect on gene expression levels under experimental conditions, we decided to further analyze the *ET* RNAi *in vivo* phenotype using the *GeneSwitch-GAL4* driver. *GeneSwitch-GAL4* is a drug-inducible driver, which activates the expression of a *UAS*-RNAi construct only when drug called Mifepristone is added to the fly food vials. These conditions provide a genetically relevant control, since the offspring from each cross can be monitored with and without the expression of dsRNA. In response to septic injury with *E. cloacae*, *ET* RNAi flies crossed with *GeneSwitch-GAL4* driver flies and induced by Mifepristone showed a significant increase in both *TotM* and *TotA* expression levels measured as above. In contrast, *Stat92E* RNAi strongly inhibited the expression of both genes, demonstrating that the *GeneSwitch-GAL4* driver functioned as expected (**III fig. 6C and 6D**). Taken together, our results indicate that ET negatively regulates *Tot* gene expression in *Drosophila in vivo*, and functions independently of the Imd pathway mediated regulation. The importance of ET/latran was later demonstrated by another research group (Makki et al., 2010).

10 DISCUSSION

Automation and computerized systems together with the availability of the genomic sequences of many species enabled the systems biological revolution in the twentieth century. With high-throughput methods, such as microarrays and genome-wide screens, vast amounts of data can be obtained and processed. Therefore, the aims of systems biological studies are ambitious; the goal is to determine the basic mechanism of the biological system, identify all its components and define how it is regulated. This requires careful planning prior to experiments. It includes determining the hypothetical model of the system under study and defining potential problems related to the assay.

Large-scale RNAi screening

In the past decade, large-scale RNAi based *in vitro* screening has become a commonly used systems biological method for identifying gene products involved in a variety of biological processes (Ramet et al., 2002b) (Boutros et al., 2004). Nevertheless, one could argue whether RNAi based genome-wide *in vitro* screening is a genuine systems biological method. After all, only one gene is studied at a time. However, the screens do provide information on the entire system, and allow us to determine all of the components involved in the biological system under study, such as *Drosophila* immune signaling, as well as the regulation of the system. There are many benefits to *Drosophila* RNAi screening. The delivery of dsRNAs is easy; they can be introduced into S2 cells simply by soaking and S2 cells spontaneously take up dsRNA fragments as long as 1,000 bp from the cell culture medium (Ulvila et al., 2006). As a technique RNAi itself is fast, effective and easy to carry out, and off-target effects rarely present any problems.

However, in general, off-target effects are a genuine problem related to RNAi based screening (Ge et al., 2003; Qiu et al., 2005). Off-target effects may occur, when a dsRNA delivered into cells contains a sequence that can bind and therefore silence multiple genes at the same time. In our assay set-up however, off-target effects presents rarely any problems. At least in one particular case, RNAi was shown, by microarray, to be completely specific (Kleino et al., 2005). In our experimental setting, we typically deliver 700-1000bp of sequence specific dsRNA into *Drosophila* S2 cells. This gives rise to approximately 35-50 siRNAs. If one or two of these siRNAs are able to bind multiple genes, the remaining 33-48 siRNAs are likely more specific or at least do not target the same off-target gene. It is likely that the off-target effects are a greater problem in mammalian systems, where the siRNAs are delivered into cells one or only a few at a time. Nevertheless, one should always verify the observed phenotypes with at least two independent dsRNAs and if possible, carry out rescue experiments with dsRNAs that target the UTR of the endogenous gene (**Fig 19**). Of note, since *ET* mRNA lacks UTR regions, we were not able to carry out any rescue experiments, in which *ET* would be first silenced by dsRNA targeting the UTR regions and this phenotype would then be rescued by overexpressing *ET* from constructs lacking UTR regions. In addition, it is also important to control and monitor cell viability, especially when the rate of expression (in our assay luciferase expression) equals the activity of the signaling pathway. In our RNAi screen, the general well being of the cells was assessed using the *Act5C-β-gal* reporter.

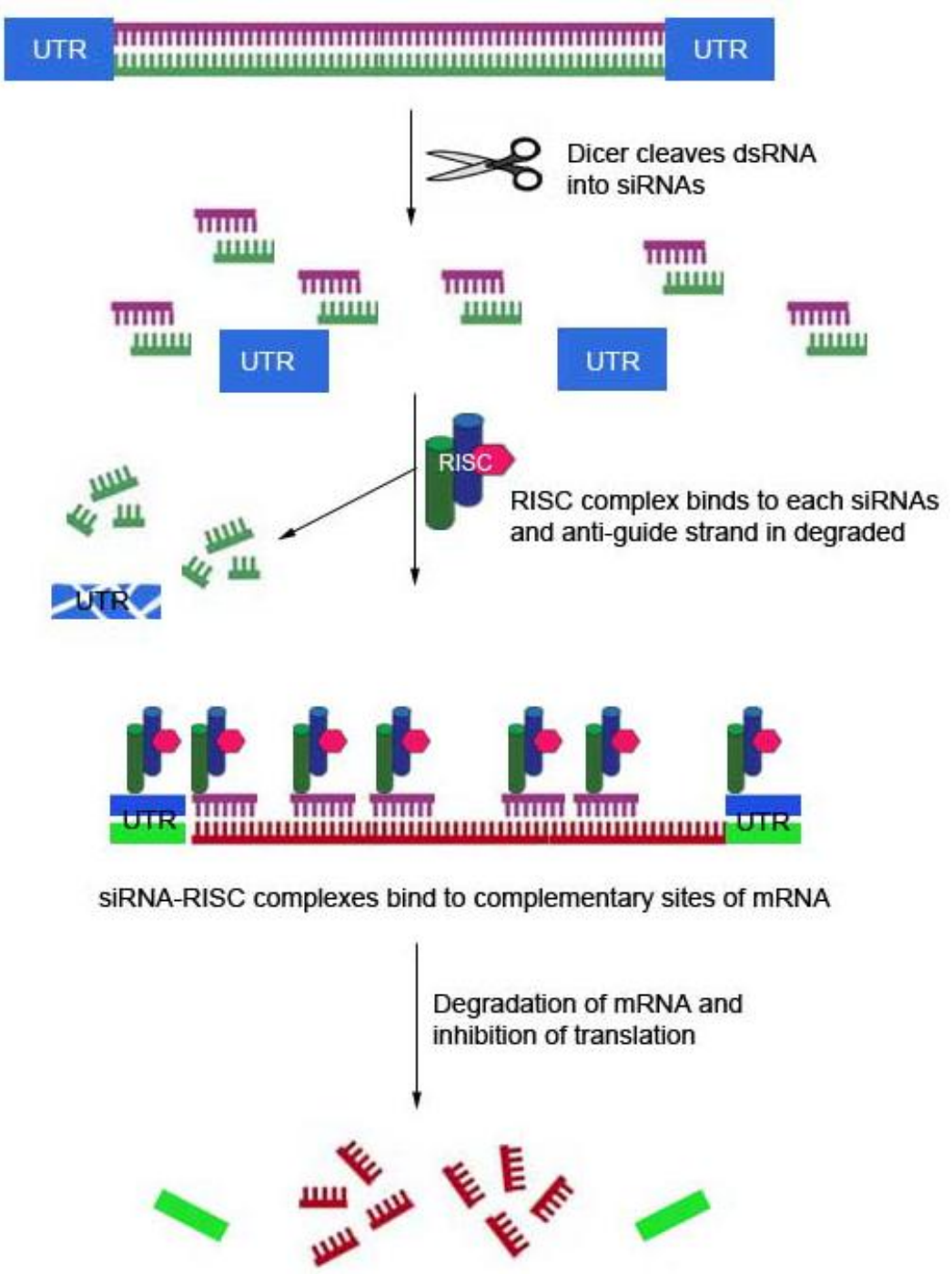


Figure 19. Schematic representation of RNAi. The untranslated regions (UTRs) can be used to validate the efficiency of RNAi. The gene of interest is first silenced by dsRNA targeting the UTR regions and then rescued by overexpressing the same gene from constructs without UTR regions

In addition to our work, two large-scale RNAi based screens have been carried out earlier to identify regulators of the *Drosophila* JAK/STAT signaling (Baeg et al., 2005; Muller et al., 2005). Interestingly, the findings of these three screens show only minor overlap (**Fig 20**). In fact, only one gene, *enok,* was identified in all three screens. A similar divergence of the results can be seen when comparing our RNAi screen for regulators of the *Drosophila* NF-κB signaling to three corresponding RNAi screens (Foley and O'Farrell, 2003) (Kambris et al., 2006) (Kuttenkeuler et al.).

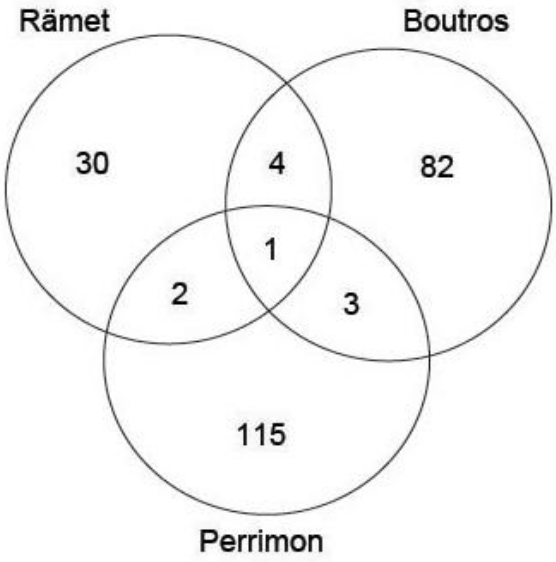


Figure 20. Results of the three screens identifying regulators of *Drosophila* JAK/STAT signaling showed very little overlap. Only one gene, enok, was identified in all three screens.

This can be due to variety of issues, such as biological differences between the assay set-ups, cell lines chosen, reporters used and means of pathway activation, as well as different normalization approaches. All these factors create variation in the RNAi phenotypes rising from the screens. The smaller number of RNAi phenotypes in our screen corresponds to biologically meaningful cut-offs in our assay set up. In addition, we were able to repeat our original findings with targeted dsRNA treatments and importantly, validate our results at least with the one gene we chose

to study *in vivo*, with secondary and tertiary assays. This variation between independent screens underlines the importance of careful planning of the screen and the validation of the results obtained. To confirm our screening results, we found it essential to TA-clone and sequence PCR templates corresponding to interesting RNAi phenotypes and design gene-specific primers to carry out independent RNAi with targeted dsRNAs. Thereafter, RNAi phenotypes were further validated with qRT-PCR quantification, where expression levels of endogenous target genes (*Drs* or *TotM*) were measured from S2 cell lysates treated with targeted dsRNAs. Similarly, the efficiency of the RNAi itself was demonstrated by qRT-PCR by measuring the absence of endogenous transcripts in the RNA extracted from S2 cells treated with a targeted dsRNA against the gene in question.

In both genome-wide screens (Toll and JAK/STAT), we chose to activate the pathways with constitutively active forms of pathway components, the Toll pathway with $Toll^{10b}$ and the JAK/STAT pathway with Hop^{Tuml} , instead of cytokine induction. The approach was taken to study the evolutionarily conserved intracellular part of these pathways and to exclude the extracellular part of the signaling cascade. Due to this arrangement, some of the upstream regulators of both pathways remain unknown, and may to some extent explain the smaller number of genes identified in our studies.

In vivo validation of the results

The fruit fly's evolutionarily conserved immune system makes *Drosophila melanogaster* an excellent model for studying the principles of innate immunity. For our purposes, studying the innate immune reactions using RNAi, *Drosophila* is the best possible model to use. The basic mechanisms of *Drosophila* immunity are highly conserved throughout evolution and the data obtained may have relevance to mammalian models. *Drosophila* lacks adaptive immunity, so the RNAi mediated knock-down of the gene studied is not compensated by adaptive immune reactions. Finally, *Drosophila* is cheap and easy to maintain and there is a large amount of

genetic and molecular techniques available for *Drosophila*, enabling effective exploitation of the data obtained from the studies.

In vivo RNAi in *Drosophila* provides a readily accessible tool for observing the functional importance of a selected gene product on the scale of the whole organism., Transgenic *Drosophila* RNAi fly collections, namely the VDRC (Vienna, Austria) and NIG-fly (Kyoto, Japan) are commercially available for this purpose. In these flies the target gene is silenced through the production of hairpin dsRNAs when the flies are crossed with an appropriate GAL4 driver fly line. We used this approach to validate the selected RNAi phenotypes of both screens *in vivo.* For the *ET in vivo* validation, flies carrying *UAS*-RNAi constructs for *ET* (*ET IR*^{1}, *ET IR*^{2}) were crossed with the *C564-GAL4* driver and the offspring was infected with *E. cloacae* to induce JAK/STAT signaling. Total RNA was extracted and the expression levels of *TotM* and *TotA* were measured by qRT-PCR. With these validations, we were able to demonstrate, that *ET* RNAi strongly increases both of the JAK/STAT signaling pathway target genes (*TotM* and *TotA*) in response to septic injury in *Drosophila in vivo*. Thus, we confirmed our original *in vitro* findings also *in vivo*.

Curiously, however, we were able to confirm only three *in vivo* phenotypes (*Gprk2, TAMP, u-shaped*) in our Toll screen out of ten candidates originating from the screen. This can be due to certain limitations of the approach. It is acknowledged that, in some cases, silencing of the targeted gene is far from sufficient. In fact, as much as 35%-40% of fly-lines may give a false-negative result (Dietzl et al., 2007). These problems may be due to several causes related to the RNAi, the driver GAL4 strain and/or the assay chosen (Dietzl et al., 2007). Along these lines, since we were able to confirm three out of ten candidates, the results of our Toll pathway *in vivo* infection assays are actually in accordance with the estimates presented by Dietzl and co-workers.

Microarray analysis

Our genome-wide microarray analysis was carried out in macrophage-like S2 cells in contrast to earlier genome-wide microarray analyses, that were carried out with adult flies (De Gregorio et al., 2001; Irving et al., 2001). However, our results are in good agreement with these earlier studies, but also include several interesting differences, perhaps due to better sensitivity of our *in vitro* model. We analyzed genes that were induced in S2 cells by exposure to heat-killed *E. coli* and further evaluated the role of PGRP-LC in this response. Due to these experimental arrangements, the accuracy of detecting hemocyte-specific changes at the transcriptional level may be better compared to previous *in vivo* studies (De Gregorio et al., 2001; Irving et al., 2001). On the other hand, we were not able to detect events that are regulated at levels other than transcription. Furthermore, we carried out a targeted RNAi analysis of the up-regulated genes in order to determine the role of these gene products in microbial recognition, phagocytosis and signaling via the Imd and the Toll pathway.

In this context, it is quite surprising that we failed to identify any opsonin-like proteins or any other gene products necessary for binding or phagocytosis in our functional studies of the genes induced by *E. coli*. We anticipated that these proteins would be expressed (induced?) in response to microbial challenge. However, none of the genes we identified appeared to be necessary for the binding or phagocytosis of bacteria. This led us to conclude that the most important genes for the recognition and engulfment of invading microbes are not transcriptionally regulated. This makes sense as phagocytosis is a rapid process occurring instantly after initial recognition. Instead of transcriptional regulation, it seems that these genes appear to be expressed at relatively high levels constantly, and S2 cells are always surveying pathogens ready to initiate immune responses. In general, the data obtained from our microarray study are in good agreement with earlier studies. We identified twenty-two genes previously shown to be up-regulated upon septic injury *in vivo*. These included ten well characterized and highly conservative antimicrobial peptides, PGRP proteins and other recognition and receptor proteins as well as one transcription factor, *Relish*. The expression of structural proteins coding for genes like *β-Tubulin 60D* as well as *Tep4* and *Tep-like CG18589*, putative candidates for

complement cascade, were up-regulated in our assay. In addition, the expression of thirty-one previously uncharacterized genes was induced in response to *E. coli*. Further functional studies identified three new gene products regulating antimicrobial peptide release *in vitro*. Silencing *CG7097*, a Ste20 family kinase named *happyhour* (Corl et al., 2009), caused a significant decrease in *Attacin* expression in response to *E. coli*. Silencing the components of the JNK pathway had a similar effect indicating that induction of the JNK pathway is important for a optimal response via the Imd pathway. In contrast, RNAi targeting *CG15678* or the structural protein coding genes *β-Tubulin 60D* and *α-Tubulin 84D* caused a clear increase in the Imd pathway response. Of note, *CG15678* was later confirmed to be a negative regulator of the *Drosophila* NFκ-B signaling and was named Pirk (Kleino et al., 2008). None of the genes studied seem to be import for the Toll pathway response. This is unsurprising since *Drosophila* humoral immunity distinguishes between different classes of pathogens and the Toll response is not activated in response to the Gram-negative *E. coli*.

Future perspectives

The past few years have brought answers to many key questions concerning the *Drosophila* innate immunity. With powerful systems biological methods such as microarrays and large-scale RNAi screening, vast amounts of data can be obtained from biological processes, including *Drosophila* immunity. Genes involved in these processes can all be monitored simultaneously, which is a huge advantage compared to the traditional mutation-based genetic assays. Using these systems biological methods, scientists have been able to determine many of the key players in the field of *Drosophila* immunity. For example, the main phagocytic receptor, Eater, was eventually identified by Kocks et al in 2005 (Kocks et al., 2005). Similarly, the comprehesive models for the *Drosophila* NFκ-B signaling pathways are coming together. The complexity of the Imd signaling pathway is currently well understood and the main components of the Toll pathway are known.

Our work has, for its part, provided a lot of new insight into *Drosophila* immunity. We have found new regulators of the Imd pathway in our microarray assay and further confirmed the involvement of the JNK pathway in the Imd response. Our microarray data also addresses many new questions to be answered by future experiments. The potential role of Tep-like proteins in the complement cascade remains to be shown although recent studies on *Drosophila* intestine-specific responses have pointed in this direction (Buchon et al., 2009). Similarly, the molecular means of how Tubulin-associated proteins affect Imd signaling remain speculative. We identified five putative new regulators of the *Drosophila* JAK/STAT pathway and nine potential novel regulators of the Toll pathway. Our *in vitro* and *in vivo* studies confirmed ET as a negative regulator of the JAK/STAT pathway involved in Stat92E phosphorylation and showed that ET function is Dome and Hop dependent. However, the exact role of ET in Stat92E phosphorylation remains to be discovered. We have also proved that Gprk2 is an evolutionary conserved regulator of immune signaling and is required for normal microbial resistance *in vivo*. Interestingly, although it interacts with Cactus, Gprk2 is not necessary for Cactus degradation. The exact molecular mechanism of the Gprk2-Cactus interaction and its purpose will certainly be a target for great scientific interest in the near future.

To address these questions one could use a more recent systems biological method, *Drosophila in vivo* RNAi screening (Kambris et al., 2006) (Lesch et al., 2010). This techique allows genes involved in designated biological processes to be inactivated in a tissue specific manner and at a defined time point. The method is particularly convenient for analysing secreted gene products (Kambris et al., 2006) (Lesch et al., 2010). In the future, it would be interesting to carry out a *Drosophila in vivo* RNAi screen of the Toll and the JAK/STAT pathways in order to determine the upstream regulators that weren't identified in this work.

11 SUMMARY AND CONCLUSIONS

With systems biological high-throughput methods, such as microarrays and genome-wide screens, genes involved in several different biological processes can be monitored all at the same time. This is an epoch-making advantage compared to traditional mutation-based genetic assays. With these methods, scientists have been able to determine many of the key players in the field of *Drosophila* immunity. Our systems biological studies have provided new insights into *Drosophila* immunity. In our microarray analysis, we identified new regulators for the Imd pathway and confirmed the involvement of the JNK pathway in the Imd response. In addition, we carried out two genome-wide RNAi screens and were able to identify five putative new regulators of the *Drosophila* JAK/STAT pathway and nine potential novel regulators of the Toll pathway.

Our systems biological studies also indicate many new issues for future experiments. The potential role of Tep-like proteins in the complement cascade remains to be discovered. In addition, the exact molecular role of Tubulin-associated proteins in Imd signaling is yet to be determined. Furthermore, our *in vitro* and *in vivo* studies characterized ET as a negative regulator of the JAK/STAT pathway involved in Stat92E phosphorylation. We were also able to show that ET function is Dome and Hop dependent, but the exact role of ET in Stat92E phosphorylation remains to be discovered. We have also confirmed that Gprk2 is an evolutionary conserved regulator of immune signaling and is required for normal microbial resistance *in vivo*. We have shown that Gprk2 interacts with Cactus. Nevertheless, Gprk2 is not necessary for Cactus degradation. The exact molecular mechanism of the Gprk2-Cactus interactions and the precise function of this interaction are still to be discovered. Moreover, since we chose to activate the pathways with constitutively active forms of pathway components, instead of cytokine induction, some of the upstream regulators of both pathways remain uncharacterized.

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Original article

Functional analysis of immune response genes in *Drosophila* identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells

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Abstract

The templates of innate immunity have ancient origins. Thus, such model animals as the fruit fly, *Drosophila melanogaster*, can be used to identify gene products that also play a key role in the innate immunity in mammals. We have used oligonucleotide microarrays to identify genes that are responsive to Gram-negative bacteria in *Drosophila* macrophage-like S2 cells. In total, 53 genes were induced by greater than threefold in response to *Escherichia coli*. The induction of all these genes was peptidoglycan recognition protein LC (PGRP-LC) dependent. Twenty-two genes including 10 of the most strongly induced genes are also known to be up-regulated by septic injury in vivo. Importantly, we identified 31 genes that are not known to respond to bacterial challenge. We carried out targeted dsRNA treatments to assess the functional importance of these gene products for microbial recognition, phagocytosis and antimicrobial peptide release in *Drosophila* S2 cells in vitro. RNAi targeting three of these genes, *CG7097*, *CG15678* and *β-Tubulin 60D*, caused altered antimicrobial peptide release in vitro. Our results indicate that the JNK pathway is essential for normal antimicrobial peptide release in *Drosophila* in vitro.

Keywords: *Drosophila melanogaster*; Innate immunity; JNK signaling

1. Introduction

Recent progress in understanding the basic templates of innate immunity has revealed striking conservation in the first-line host defense from insects to human [1,2]. This similarity is clearly illustrated by the role of Toll-like receptors and related intracellular pathways in acute humoral response to microbial infection [3,4]. Consequently, genetically tractable model organisms like *Drosophila*, in particular, have been widely used to dissect genes and pathways that are of importance in innate immune response of the host.

Upon microbial challenge, both cellular and humoral arms of the *Drosophila* innate host defense are required for optimal immune competence in vivo [5]. Invading microbes are phagocytosed by circulating macrophages and the antimicrobial peptides are synthesized by both hemocytes and the fat body, a functional equivalent of the mammalian liver. In addition, proteolytic cascade that leads to activation of phenoloxidase, which catalyses the conversion of dopamine to microbicidal melanine, is activated. *Drosophila* humoral immunity distinguishes between different classes of pathogens through the immune defiency (Imd) and the Toll pathways. These two pathways, which are very similar to mammalian Toll/IL and

Abbreviations: aop, *anterior open*; dSR-CI, *Drosophila* scavenger receptor CI; GNBP, Gram-negative bacteria-binding protein; *hep*, *hemipterous*; Imd, immune defiency; *kay*, *kayak*; MAPK, mitogen-activated protein kinase; *msn*, *misshapen*; PAMP, pathogen associated molecular pattern; PGRP, peptidoglycan recognition protein; PRR, pattern recognition receptor; TNF, tumor necrosis factor.

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tumor necrosis factor (TNF)-receptor pathways [2], are the major regulators of the immune response in *Drosophila* in vivo [6]. Remarkable conservation between human and *Drosophila* pathways points to an evolutionary link from insects to man.

Earlier we have shown that *Drosophila* S2 cells are macrophage-like [7,8], which makes them a valid in vitro system to study hemocyte-mediated response to microbial challenge. In these cells, exposure to Gram-negative bacteria leads to rapid transcriptional induction of several well-characterized antimicrobial peptide genes including *Attacin*, *Cecropin* and *Metchnikowin* via the Imd pathway [8]. In addition, S2 cells recognize and phagocytose efficiently both Gram-negative and Gram-positive bacteria. In contrast, the activation of the Toll pathway, which is initiated by Gram-positive bacteria through a circulating peptidoglycan recognition protein (PGRP) SA in vivo [9], requires introduction of the active form of Spätzle by transfection in these cells [10]. Therefore, S2 cells provide an opportunity to study the Imd pathway mediated response to Gram-negative bacteria.

2. Materials and methods

2.1. Cell cultures

S2 cells were maintained as described earlier [11].

2.2. Microbial challenge, RNA isolation and oligonucleotide microarray analysis

3.0×10^6 S2 cells were incubated for 6 h with 3.0×10^7 heat-killed *Escherichia coli* and thereafter total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Gene expression analysis was performed using the Affymetrix (Santa Clara) *Drosophila* Genechips according to the standard Affymetrix Genechip protocol as outlined in the GeneChip Expression Analysis Technical Manual by Affymetrix (2001). Gene expression levels of four unchallenged, control dsRNA treated S2 cells were compared pair-wisely to four *E. coli* exposed samples (altogether 16 comparisons) and to three *E. coli* exposed, PGRP-LC dsRNA treated samples (12 comparisons).

2.3. Data analysis

We identified the genes whose RNA levels are affected by an exposure to *E. coli* using the following criteria. First, the majority of the 16 comparisons had to be called increased (or decreased) and none of the remaining ones was called decreased (or increased). Second, there must be at least a threefold increase (or decrease) in the relative expression level. Finally, the *t*-test was used to measure statistical significance. A *P*-value of < 0.05 was considered to be significant. Those genes that fulfilled all of these criteria are shown in Table 1.

2.4. dsRNA treatments

Total RNA was isolated with TRIZOL® Reagent (GIBCO BRL, Gaithersburg, MD) and first-strand cDNA was synthesized from 1.0 μg of total RNA. The templates for dsRNA synthesis were generated from cDNA by a two-step PCR reaction. The first primers consisted of 15 base gene-specific sequences designed for each gene of interest. A second PCR reaction was performed using primers containing a T7 promoter sequence (GAATTAATACGACTCACTATAGGGAGA) attached to the 5′-end of the gene-specific sequences. Both sense and antisense RNAs were synthesized simultaneously from a single PCR product using the T7 MegaScript RNA polymerase (Ambion, Austin, TX) as recommended by the manufacturer. dsRNA was precipitated with LiCl and treated with DNAase. Quality of dsRNA was analyzed by agarose gel electrophoresis and the concentration was measured by spectrophotometer. The concentration of dsRNA was 10 μg per 10^6 S2 cells in each experiment (if not stated otherwise). Cells were incubated with dsRNA for 72 h.

2.5. FACS analysis to quantify phagocytosis and microbial binding

Flow cytometry was used to analyze the ability of the dsRNA treated cells to bind and phagocytose heat-killed, FITC-labeled *E. coli* and *Staphylococcus aureus* (Molecular probes, Leiden, the Netherlands) compared to GFP dsRNA treated control cells. A total of 5.0×10^5 dsRNA treated S2 cells were plated onto 48-well plates and incubated for 1 h at room temperature. Thereafter the cells were cooled down to 4 °C for 30 min, and heat-killed, FITC-labeled *E. coli* or *S. aureus* was added. Plates were centrifuged for 1 min at $100 \times g$ (at 4 °C) and incubated for 1 h at 4 °C to let S2 cells bind bacteria. To allow phagocytosis, the plates were incubated at 26 °C (16 min for *E. coli* and 30 min for *S. aureus*). Thereafter plates were placed on ice, medium was replaced with ice-cold PBS and cells transformed into flow cytometry tubes. Samples were analyzed with FACS using EXPO32 program (Beckman Coulter, Ordior). The fluorescence of extracellular particles was quenched by adding 0.2% Trypan blue in 1× PBS (pH 4.85) shortly before the actual measurement. The amount of phagocytosis was quantified as the percentage of fluorescence-positive cells multiplied by the mean fluorescence of these cells. 5000–10,000 cells were counted from each sample. The ability of dsRNA treated cells to bind bacteria was measured accordingly except plates were kept at +4 °C at all times and no Trypan blue was added prior to measurements.

2.6. Luciferase reporter assay

A luciferase reporter assay was used to analyze the effect of dsRNA treatments on antimicrobial peptide release by both the Imd and the Toll pathway. The S2 cells were transfected either with 0.5 μg of *Attacin*-reporter plasmid (the Imd path-

Table 1
Microarray analysis of genes induced by *E.coli*

Gene	Name or function	Naïve expression	*E. coli* stimulated	Fold change	*P*-value	Decrease by PGRP-LC RNAi
Antimicrobial peptides						
CG7629 [a]	*Attacin*-D	162 ± 113	43788 ± 11750	281.6	<0.001	92 ± 1%
CG1367 [a]	*Cecropin* A2	229 ± 538	42282 ± 16122	184.5	0.002	77 ± 9%
CG8175 [a]	*Metchnikowin*	436 ± 224	49119 ± 15181	112.7	<0.001	82 ± 8%
CG10816 [a]	*Drosocin*	162 ± 138	14728 ± 2913	90.7	<0.001	84 ± 5%
CG10794 [a]	*Diptericin* B	1206 ± 577	67329 ± 23964	55.8	0.001	85 ± 7%
CG1878 [a]	*Cecropin* B	143 ± 128	6266 ± 2333	43.7	0.002	87 ± 3%
CG1365 [a]	*Cecropin* A1	2210 ± 1044	90405 ± 26110	40.9	<0.001	78 ± 6%
CG18372 [a]	*Attacin*-B	624 ± 102	23692 ± 7418	38.0	<0.001	78 ± 5%
CG10810 [a]	*Drosomycin*	540 ± 72	6266 ± 87	5.0	<0.001	79 ± 14%
CG1373 [a]	*Cecropin* C	451 ± 29	2707 ± 391	3.80	<0.001	85 ± 17%
Recognition molecules, Receptors and Transporters						
CG7496 [a]	PGRP-SD	736 ± 118	12466 ± 2376	16.90	<0.001	69 ± 3%
CG11709 [a]	PGRP-SA	1252 ± 206	16496 ± 5352	13.20	0.001	86 ± 5%
CG11897	Transporter	1183 ± 812	11297 ± 2096	9.50	<0.001	80 ± 11%
CG6231	Transporter	4653 ± 821	25420 ± 4120	5.50	<0.001	83 ± 3%
CG7052 [a]	Tep2	376 ± 141	2003 ± 881	5.30	0.01	55 ± 28%
CG4823 [a]	LDL-receptor	88 ± 152	412 ± 136	4.70	0.02	73 ± 31%
CG8805	Receptor	406 ± 199	1580 ± 234	3.90	<0.001	97 ± 18%
CG16700	Transporter	280 ± 118	967 ± 163	3.50	<0.001	106 ± 11%
CG9753	Receptor	352 ± 96	1202 ± 176	3.40	<0.001	93 ± 15%
CG4437	PGRP-like	484 ± 161	1566 ± 636	3.20	0.02	58 ± 16%
CG18589	Tep-like	1192 ± 253	3726 ± 795	3.10	<0.001	39 ± 8%
CG5372	Integrin	–5 ± 118	301 ± 101	~	0.008	66 ± 15%
Transcription factor						
CG11992 [a]	Relish	5278 ± 528	15429 ± 2478	3.00	<0.001	83 ± 22%
Serine proteases and enzymes						
CG16731	Serine protease	158 ± 50	1681 ± 213	10.60	<0.001	67 ± 6%
CG8215 [a]	Serine protease	261 ± 67	1685 ± 167	6.50	<0.001	82 ± 8%
CG9370	Serine protease	344 ± 42	1966 ± 131	5.70	<0.001	88 ± 8%
CG3505 [a]	ProPO-AE	363 ± 362	1266 ± 179	3.50	0.004	74 ± 35%
CG10232	ProPO-AE	–75 ± 31	190 ± 102	~	0.003	57 ± 29%
CG4837	5′-Nucleotidase	44 ± 93	267 ± 64	6.10	0.007	98 ± 68%
CG5992	Adenosine deaminase	2472 ± 491	11182 ± 1273	4.50	<0.001	89 ± 2%
CG7097	Protein kinase	130 ± 78	537 ± 116	4.10	0.001	27 ± 26%
CG10564	Adenylyl cyclase 78C	139 ± 94	555 ± 261	4.00	0.02	53 ± 43%
CG2171	Triosephosphate isomerase	633 ± 544	2088 ± 553	3.30	0.01	38 ± 21%
Structural proteins						
CG3401	β-Tubulin 60D	3580 ± 727	13427 ± 1043	3.80	<0.001	88 ± 5%
CG8772	Nemy	526 ± 265	1609 ± 328	3.10	0.002	77 ± 21%
CG11312	Inscuteable	–42 ± 142	382 ± 172	~	0.009	75 ± 61%
Uncharacterized genes induced by E. coli						
CG15678 [a]		753 ± 136	7744 ± 473	10.30	<0.001	79 ± 8%
CG18356		153 ± 226	1173 ± 75	7.60	<0.001	73 ± 27%
CG17218		75 ± 149	514 ± 126	6.90	0.004	62 ± 25%
CG10006		125 ± 88	833 ± 189	6.70	0.02	89 ± 3%
CG6804		56 ± 128	348 ± 57	6.20	0.006	53 ± 33%
CG3831		153 ± 134	729 ± 123	4.80	<0.001	71 ± 37%
CG3304		104 ± 171	491 ± 95	4.70	0.008	84 ± 40%
CG1572		1922 ± 356	8629 ± 1009	4.50	<0.001	77 ± 4%
CG5847		335 ± 233	1507 ± 114	4.50	<0.001	89 ± 11%
CG9815		836 ± 446	3602 ± 163	4.30	<0.001	90 ± 15%
CG15507		221 ± 76	862 ± 296	3.90	0.006	78 ± 30%
CG14253		4074 ± 768	14142 ± 679	3.50	<0.001	69 ± 10%
CG7778 [a]		–594 ± 440	1469 ± 207	~	<0.001	57 ± 4%
CG4250 [a]		–693 ± 626	562 ± 436	~	0.02	66 ± 73%
CG13654		–205 ± 232	695 ± 187	~	<0.001	59 ± 14%
CG17124		6 ± 93	566 ± 213	~	0.003	78 ± 16%
CG6051		–93 ± 40	436 ± 186	~	0.001	80 ± 62%

[a] Induced also in vivo [12].

way) or with 0.4 μg of *Drosomycin*-reporter plasmid together with 0.4 μg of constitutively active form of Toll (Toll10B) (the Toll pathway) using Fugene reagent (Roche). A β-galactosidase expression vector (0.6 μg) was included in the reaction mix to control transfection efficiency and the viability. In addition, 2 μg of dsRNA was added directly to the transfection medium. The *Drosomycin*-reporter driven luciferase activity was measured 72 h afterwards to measure the activity of the Toll pathway. When assessing the Imd pathway, Ecdysone was added into the cell media to 1 μmol concentration 48 h after the initial transfection followed by addition of heat-killed *E. coli* 24 h later. After another 24 h, cells were lyzed in reporter lysis buffer (Promega) and luciferase and β-galactosidase activities were measured using standard procedures.

3. Results

3.1. Genes induced in S2 cells in response to E. coli

To identify all the genes that are induced in macrophage-like S2 cells by a 6-h-exposure to Gram-negative bacteria, we first extracted the total RNA from both *E. coli* exposed cells and control cells, and then used Affymetrix *Drosophila* genechips to detect the expression level of more than 13,500 *Drosophila* genes. Four independent RNA preparations from control S2 cells and *E. coli* induced cells were hybridized. The data from altogether 16 pair-wise comparisons between *E. coli* treated and untreated cells were analyzed as stated in Section 2. Altogether 53 genes were induced by greater than threefold in response to *E. coli*. Twenty-two genes including 10 of the most strongly induced genes were previously shown to be up-regulated by septic injury in vivo (Table 1, [12]). All of the 10 most strongly induced genes code for well-characterized, immunity-related peptides. In addition, we identified 31 genes that have not been described to be induced in vivo (Table 1).

Eight of the most strongly induced genes are known antimicrobial peptides. These include two *Attacin* (*B1* and *D*), three *Cecropin* (*A1*, *A2* and *B*), *Diptericin B*, *Drosocin* and *Metchnikowin* genes (Table 1). In addition, *Cecropin C* was moderately induced as well as antifungal peptide *Drosomycin*. The remaining five antimicrobial peptides, *Attacin A1* and *A2*, *Defensin*, *Drosomycin B* and *Diptericin* were not significantly up-regulated. Interestingly, our Northern blot data indicate that *Diptericin* gene expression is induced by bacteria in S2 cells only if the cells are pretreated with Ecdysone (data not shown). Therefore, it is likely that in S2 cells a specific co-factor is required for efficient *Diptericin* response to *E. coli*.

As shown in Table 1, we found three *PGRP* genes to be induced by *E. coli* in our assay: *PGRP-SA*, *PGRP-SD* and *PGRP-like* gene (*CG4437*). Interestingly, *PGRP-SB1*, *PGRP-SC2* and *PGRP-LB* which all are up-regulated in response to septic injury in vivo [12,13] were not significantly induced. None of the *Gram-negative bacteria-binding protein (GNBP)* genes were up-regulated by *E. coli* in the present study. This is in line with the notion that both *GNBPs* (*CG12780* and *CG13422*) are regulated in vivo selectively by the Toll pathway [6] and as stated above, our experimental setting was to identify genes induced by the Imd pathway in response to Gram-negative bacteria.

In our experimental setting using macrophage-like S2 cells, five genes coding for putative serine proteases *CG16731*, *CG8215*, *CG9370*, *CG3505* and *CG10232*, possibly involved in melanization, were induced. The fewer number of genes induced in our assay compared to previous in vivo studies may indicate that at least a portion of the in vivo induced genes that participate in melanization are induced by the wound itself. In addition, the expression of two genes coding for complement-like proteins, Tep4 and Tep-like CG18589, was up-regulated. Furthermore, a homologue of vertebrate α-2-macroglobulin receptor gene (*CG4823*) was among genes up-regulated significantly by an exposure to *E. coli*.

PGRP-LC is important for antimicrobial peptide response against Gram-negative bacteria both in vitro and in vivo [8,14,15]. Furthermore, PGRP-LC deficient flies are susceptible to Gram-negative, but not Gram-positive, bacterial infection [8,15]. Interestingly, however, PGRP-LC null mutant flies are less prone to Gram-negative bacterial infection compared to flies harboring mutation in other key gene, such as Relish, in the Imd pathway. Therefore, it is plausible that only a subset of genes that are under transcriptional control of Relish is regulated by PGRP-LC. Our data indicate that in S2 cells, induction of all the genes that respond to *E. coli* is PGRP-LC dependent (Table 1). These results combined with the notion that PGRP-LC mutant flies have impaired—not totally abolished—*Diptericin* response to Gram-negative bacteria in vivo argue that PGRP-LC is not absolutely required for the activation of the Imd pathway.

3.2. Functional analysis of immune response genes

3.2.1. Microbial binding and phagocytosis

Many of the genes induced by *E. coli* in S2 cells fit well into the current concept of *Drosophila* immunity. However, we found transcription of several yet uncharacterized genes to be up-regulated upon bacterial challenge in S2 cells (Table 1). To assess functional significance of these genes, we carried out targeted dsRNA treatments and evaluated the role of these gene products for microbial recognition, phagocytosis and antimicrobial peptide release in *Drosophila* S2 cells in vitro. To measure if any of the genes induced by *E. coli* encode protein(s) necessary for binding or engulfment of bacteria, we performed targeted dsRNA treatments and measured the rate of microbial binding and phagocytosis of both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria by S2 cells using flow cytometry (data not shown). We anticipated that upon microbial challenge, S2 cells might express opsonin-like protein(s) that would have a role in binding or phagocytosis. However, none of the targeted genes stud-

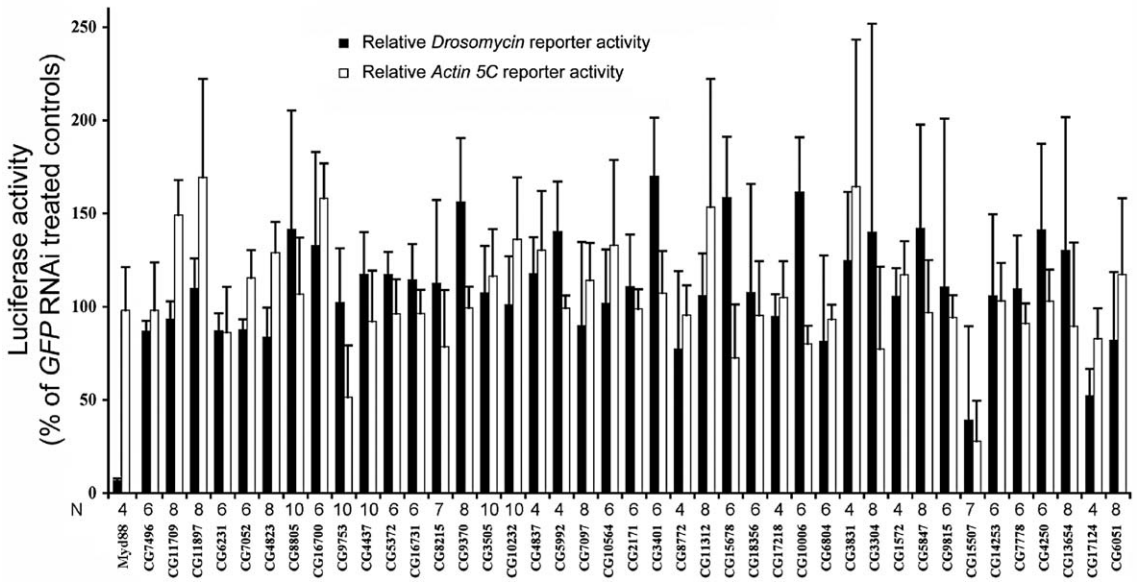

Fig. 1. The effect of dsRNA treatments targeting immune response genes on antimicrobial peptide release via the Toll pathway in S2 cells measured by a luciferase reporter assay. The dsRNA treated S2 cells were transfected with *Drosomycin*–luciferase reporter plasmid to measure antimicrobial peptide release (black columns), and *Actin 5C*-β-galactosidase reporter plasmid was used as a control for transfection efficiency and viability (white columns). The *Drosomycin*-reporter driven luciferase activity was measured 72 h after activating the Toll pathway using a constitutively active form of Toll (Toll10B). Results are presented as an average of independent dsRNA treatments (% of *GFP* dsRNA treated controls) ± S.D. Myd88 RNAi, resulting in 93 ± 1% reduction of the Toll pathway activity, was used as a positive control. *N* is the number of independent dsRNA treatments.

ied appeared very important for recognition or engulfment of bacteria. RNAi targeting *SCAR*, known to be involved in phagocytosis [16], was used as positive control and resulted in 42 ± 18% reduction in phagocytosis. Of note, dsRNA treatment targeting *CG15507* strongly decreased the phagocytosis of *S. aureus*. As *CG15507* has been shown to be the first exon of the *kayak* gene [17] we further analyzed the role of the JNK pathway for normal phagocytosis. We carried out targeted dsRNA treatments against several well-characterized JNK pathway components including *p21-activated kinase* (*PAK*), *misshapen* (*msn*), *hemipterous* (*hep*) and *anterior open* (*aop*). None of these treatments drastically affected phagocytosis (data not shown). Furthermore, RNAi targeting *CG15507* decreased cell viability as measured by *Actin 5C* promoter-driven β-galactosidase activity in S2 cells (Figs. 1 and 2). This let us to conclude that the effect of RNAi targeting *CG15507* is not specific for phagocytosis.

3.2.2. Luciferase reporter assay for measuring antimicrobial peptide release in S2 cells

In addition to binding and phagocytosis, we analyzed whether any of the induced genes are involved in the release of antimicrobial peptides. Again, we performed targeted dsRNA treatments and analyzed the transcription rate of *Drosomycin* (the Toll pathway) and *Attacin* (the Imd pathway) genes using luciferase reporter assay (Fig. 1). There was only one dsRNA treatment that decreased *Drosomycin* reporter-driven luciferase activity by more than 50% (Fig. 1). This dsRNA treatment targeted *CG15507* and caused an average of 61% reduction in the luciferase activity. However, it also very drastically decreased the *Actin 5C* promoter-driven β-galactosidase activity indicating unhealthiness of cells. Decreased viability was also clear by microscopic examination of *CG15507* dsRNA treated cells.

There was one dsRNA treatment that decreased the Imd pathway mediated *Attacin*-reporter driven luciferase activity in S2 cells by more than 50% without affecting viability. dsRNA targeting a putative protein kinase encoding gene *CG7097* decreased the Imd pathway activity in response to *E. coli* by 52 ± 30% (Fig. 2). *CG7097* is predicted to code for a protein of 1218 amino acids, which has an N-terminal Serine/Threonine kinase domain and a C-terminal CNH domain. It has high homology to a mammalian mitogen-activated protein kinase kinase kinase kinase (MAP4 K) isoform 3 (e^{-132}) and to a germinal center kinase related protein kinase (e^{-132}). Therefore, it may have a role in activating the JNK pathway also in *Drosophila*. To this end we carried out targeted dsRNA treatments to silence several known components of the JNK pathway. RNAi silencing either *kayak*, *msn*, *hep* or *aop* resulted in marked decrease in the Imd pathway activity (Table 2).

In addition, two of the genes induced by *E. coli* appear to be negative regulators of the Imd pathway: *CG15678* and *β-Tubulin 60D*. First, silencing the expression of *CG15678* caused an average of 284 ± 102% enhancement of the activity of the Imd pathway in response to Gram-negative bacteria. *CG15678* expression was strongly increased (10.3-fold induction) in response to *E. coli* in our experimental setting. It is also induced by septic injury in vivo [12]. *CG15678* is predicted to encode a protein of 197 amino acids with no conserved domains and it has no close mammalian homologues. Molecular function of the *CG15678* gene product is

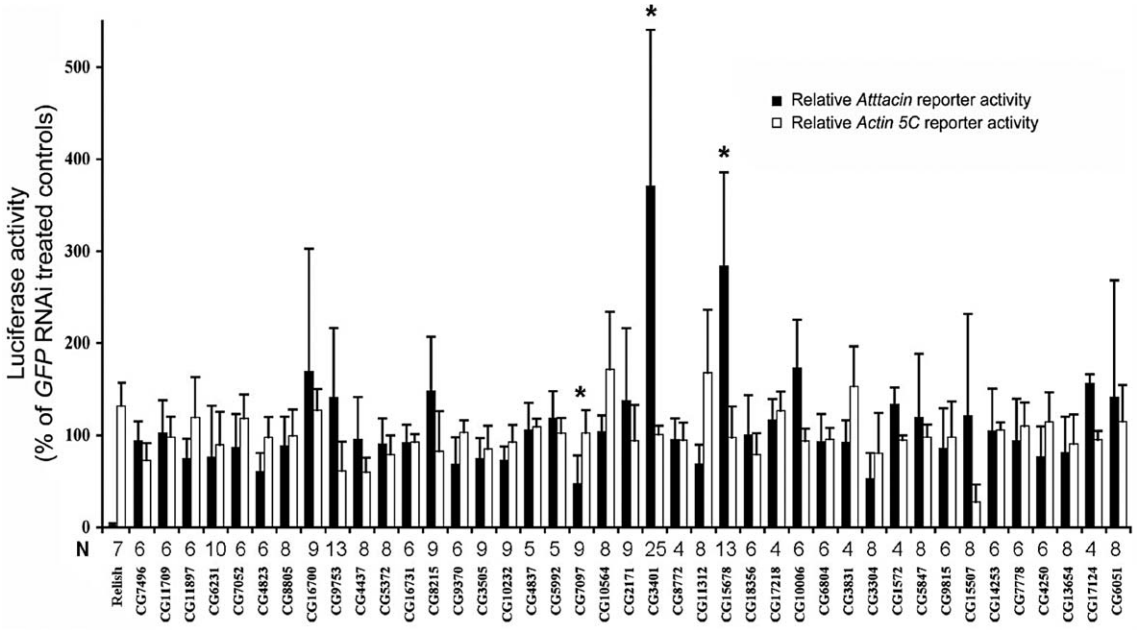

Fig. 2. The effect of dsRNA treatments targeting immune response genes on antimicrobial peptide release via the Imd pathway in S2 cells measured by a luciferase reporter assay. The dsRNA treated S2 cells were transfected with *Attacin*–luciferase reporter plasmid to measure antimicrobial peptide release (black columns), and *Actin 5C*-β-galactosidase reporter plasmid was used as a control for transfection efficiency and viability (white columns). The Imd pathway was activated by adding 1 μmol of Ecdysone to the culture medium followed by addition of heat-killed *E. coli* 24 h later. After another 24 h cells were lyzed in reporter lysis buffer (Promega) and luciferase and β-galactosidase activities were measured using standard procedure. Results are presented as an average of independent dsRNA treatments (% of *GFP* dsRNA treated controls) ± S.D. Relish RNAi, resulting in 96 ± 3% reduction of the Imd pathway, was used as a positive control. Statistically significant results ($P < 0.01$) are marked with an asterisk (*) and *N* is the number of independent dsRNA treatments.

Table 2
Attacin and *Drosomycin*-reporter activities after dsRNA treatments of the JNK signaling pathway components

Gene	Name	Function	*Attacin* reporter activity [a]		*Drs* reporter activity [a]		*Act 5C* reporter activity [b]	
CG4353	*hep*	MAPKK	53 ± 11	(5)	79 ± 14	(5)	90 ± 15	(10)
CG15509	*kayak*	*fos* homologue	22 ± 10	(5)	71 ± 12	(5)	105 ± 67	(10)
CG3166	*aop*	Transcript. repressor	59 ± 8	(5)	72 ± 19	(5)	123 ± 17	(10)
CG16973	*msn*	MAPKKK	31 ± 1	(2)	29 ± 4	(3)	69 ± 1	(5)

[a] Relative luciferase activity (% of *GFP* dsRNA treated controls ± standard deviation (S.D.) [number of experiments]).
[b] Relative β-galactosidase activity (% of *GFP* dsRNA treated controls ± S.D. [number of experiments]).

unknown. Second, RNAi targeting *β-Tubulin 60D* increased *Attacin*-reporter activity by 370 ± 170%. To further evaluate the role of Tubulin-associated proteins for the Imd pathway signaling, we targeted *α-Tubulin 84D* (*CG1913*) and observed a striking 738 ± 107% increase in *Attacin*-reporter activity in response to *E. coli*.

4. Discussion

In this study, we have used a genome-wide analysis of genes that are induced by a 6-h-exposure to *E. coli*. Furthermore, we evaluated the role of PGRP-LC in this response. In general, our data are in good agreement with the earlier genome-wide analysis of immune responses in *Drosophila* [12,13]. However, several interesting differences were observed that perhaps reflect better sensitivity of our experimental setting to detect hemocyte specific changes in transcription levels compared to previous in vivo studies. In addition, we assessed functional importance of the up-regulated genes in vitro by carrying out targeted dsRNA treatments and evaluating the role of these gene products for microbial recognition, phagocytosis and antimicrobial peptide release.

Importantly, we found three new gene products that appear to regulate significantly the antimicrobial peptide release in S2 cells in vitro. RNAi targeting *CG7097* caused a 52 ± 30% decrease in *Attacin* expression in response to *E. coli*. In contrast, silencing *CG15678*, *β-Tubulin 60D* or *α-Tubulin 84D* (*CG1913*) by RNAi caused a marked increase in the Imd pathway response. In a recent paper by Foley and O'Farrell [18], RNAi targeting *α-Tubulin 84D* increased the *Diptericin* response via the Imd pathway in response to lipopolysaccharide. The molecular mechanisms of how the Tubulin-associated proteins affect the Imd pathway remain speculative.

CG7097 codes for a protein which is very similar to a mammalian MAP4 K isoform 3 and to a germinal center kinase related protein kinase. These proteins are known to be involved in regulating the JNK pathway in mammals and it is possible that the *CG7097* gene product has a similar role in

Drosophila. This is highly interesting as regulatory cross-talk between the JNK and NF-κB pathways was recently identified in *Drosophila* [19]. In their study, rapid and transient activation of the JNK pathway was terminated by sustained induction of Relish-dependent genes [19]. Others have also shown the importance of the JNK pathway in the *Drosophila* immune response [20,21]. In our study, RNAis silencing the components of the JNK signaling cascade caused decreased *Attacin* promoter-driven luciferase response to *E. coli*. Our results indicate that appropriate induction of the JNK pathway is important for optimal response via the Imd pathway.

Microbial recognition by immune cells is of paramount importance for immune response. The initial recognition is mediated by germline encoded pattern recognition receptors (PRRs) that are capable of selectively recognizing pathogen associated molecular patterns (PAMPs) [22]. Currently, PGRPs and GNBPs are regarded as the main PRRs in *Drosophila*. PGRPs were first identified as PRRs from the moth *Trichoplusia ni* and the silkworm *Bombyx mori* [23,24]. Similar proteins are also found in flies, mice and humans [25–27]. There are altogether 13 predicted PGRP and five Gram-negative bacteria-binding protein (GNBP) genes in the *Drosophila* genome. Today, several *Drosophila* PRRs are molecularly characterized, including *Drosophila* scavenger receptor CI (dSR-CI), PGRP-SA, PGRP-LC, PGRP-LE, PGRP-SC1b and PGRP-LB and GNBP1 [7–9,14,15,28–30]. Seven of the *PGRP* and two of the *GNBP* genes, including *PGRP-SB1*, *PGRP-SC2* and *PGRP-LB*, are induced upon septic injury in vivo [12,13]. PGRP-LC was shown to be important for antimicrobial peptide response against Gram-negative bacteria both in vitro and in vivo [8,14,15]. dSR-CI is able to recognize both Gram-positive and Gram-negative bacteria and has a role in phagocytosis of both *E. coli* and *S. aureus* in vitro but it is not required for antimicrobial peptide response [7]. PGRP-SA, PGRP-LC and PGRP-LE are required in vivo for antimicrobial peptide response [8,9,14,15,29]. In addition, PGRP-SD was recently shown to be involved in activation of the Toll pathway [31]. As shown in Table 1, we found *PGRP-SA*, *PGRP-SD* and *PGRP-like* gene (*CG4437*) to be induced by *E. coli* in our assay, but interestingly, *PGRP-SB1*, *PGRP-SC2* and *PGRP-LB* were not significantly induced. This argues for a difference in regulation of these *PGRP* genes in S2 cells. This is likely the case also in vivo since *PGRP-SA* is induced by natural fungal infection whereas *PGRP-LB*, for instance, is not [12,13]. It is interesting to note that both *E. coli* and natural fungal infection induce the expression of the gene that codes for the molecule (PGRP-SA) required for antimicrobial peptide response to Gram-positive bacteria.

It is a longstanding observation that an injection of bacteria into *Drosophila* larvae or adults results in synthesis of immune proteins. More recently, *Drosophila* humoral immunity has been shown to distinguish between different classes of pathogens via the Imd and the Toll pathways [2,3]. Flies harboring a mutation in a key molecule in the Toll pathway are susceptible to certain Gram-positive bacteria and fungal infections due to impaired antimicrobial peptide response. Similarly, mutants having insufficient response via the Imd pathway are susceptible to certain Gram-negative bacteria. In this study, we performed microarray analysis of the *Drosophila* genes induced by *E. coli*. Eight of the most strongly induced genes are known antimicrobial peptides. Our results concerning antimicrobial peptides are in good agreement with the earlier in vivo studies [6,12,13] and confirm that S2 cells are a valid model to investigate the antimicrobial response mediated via the Imd pathway. Furthermore, the lack of induction of several antimicrobial peptides that are induced in vivo by the Imd pathway points to an additional level of regulation beyond linear PGRP-LC/Imd/Tak1/IKK/Relish signaling cascades.

Drosophila has circulating macrophages that phagocytose microbes comparably to mammalian macrophages [7]. Phagocytosis appears to have an important part in *Drosophila* host defense [5]. Although the exact mechanisms by which the initial ligation of a microbe to the phagocytic receptors leads to an engulfment of the bound particle are insufficiently understood, several gene products are necessary for optimal phagocytosis in *Drosophila*, including, for instance, SCAR, Abi and RN-tre [8,16]. The regulation of these genes is poorly understood. In our experimental setting, none of these or other gene products necessary for optimal phagocytosis in *Drosophila* was induced upon exposure to Gram-negative bacteria. Our results indicate that the most important genes for phagocytosis are not transcriptionally regulated. This is not unexpected as phagocytosis is a very rapid phenomenon occurring within minutes after the initial recognition. Similarly, PRRs responsible for binding of bacteria are not under transcriptional regulation. There are two hemocyte PRRs that have a role in phagocytosis of bacteria in S2 cells: dSR-CI and PGRP-LC [7,8], out of which *E. coli* induced neither. Therefore, it seems that phagocytic receptors are not transcriptionally regulated upon *E. coli* challenge in S2 cells and these genes appear to be expressed in relatively high levels in unchallenged cells also. Therefore, it is likely that both dSR-CI and PGRP-LC proteins are constantly surveying pathogens and as such, always ready to initiate both cellular and humoral immune responses.

Phagocytosis-capable plasmatocytes account for approximately 90% of all the hemocytes. The remaining 10% consists of cells characterized by crystalline inclusions that contain both substrates and enzymes for melanization. In *Drosophila*-like in many other arthropods—injury triggers proteolytic cascades that lead to blood clotting and melanization at the site of the injury. Similar processes are also initiated also by foreign particles such as microbes that contaminate the body cavity. Previous studies [12] have shown that especially septic injury induces several genes that are likely involved in these cascades. It is less clear whether these genes are induced merely due to injury or whether septicemia is required. In this study, five genes coding for putative serine proteases, possibly involved in melanization, were induced. This is much less compared to previous in vivo studies, which may indicate that at least some of these genes are induced by

the wound itself. In addition, it is likely that some of the previously reported genes that are up-regulated upon a septic injury react only to Gram-positive bacteria. For example, Irving et al. [13] showed that *masquerade*-like gene was minimally induced by Gram-negative bacteria but responded dramatically to both Gram-positive bacteria and fungal infection. In addition, we have shown earlier that S2 cells are macrophage-like and express plasmatocyte-determining factor *glial cell missing* but do not express crystal-cell-determining factor *lozenge* [8]. Therefore, the cells used in this study are not professional melanizers, which may well explain the lack of induction of the genes participating in the melanization cascade. In conclusion, our data indicate that the transcription of certain genes coding for proteins involved in melanization is induced by *E. coli* without injury. The exact role of these proteases in host defense remains to be studied.

In mammals, complement proteins C3 and α-macroglobulin bind covalently to conserved surface structures of microorganisms through a thiolester bond initiating a cascade that leads to lysis of the target microbe and enhanced phagocytosis. Although no similar cascade has been detected in insects, there are four thiolester-containing, complement-like proteins in *Drosophila* [32]. Of these, *Tep1*, *Tep2* and *Tep4* genes are strongly up-regulated after bacterial challenge in vivo [12,13,32]. In our analysis, the expression of *Tep4* and *Tep-like* CG18589 was up-regulated. In addition, a homologue of vertebrate α-2-macroglobulin receptor gene (*CG4823*) was among genes significantly up-regulated by an exposure to *E. coli*. This gene is induced by a septic injury also in vivo [12]. Future experiments will tell whether *Tep4* gene product—or any other Tep-like molecule—has a similar role in *Drosophila* immunity as it appears to have in the mosquito, *Anopheles gambiae* [33].

Two hundred and nine of the genes up-regulated by septic injury in vivo were not induced in our experimental setting. These included, for example, *puckered* (*puc*), which codes for a well-characterized down-stream target of the JNK pathway [34]. As discussed earlier, the JNK pathway has been shown to be activated upon microbial challenge in *Drosophila* both in vitro and in vivo [19,20]. This discrepancy is likely explained by the earlier time point used by Park and colleagues [19]. In their analysis, *puc* was induced very rapidly (maximum at 2 h after induction) and transiently (induction was over by 4 h after induction). It is noteworthy that induction of *puc* in vivo may also be caused by pricking—not by the host's response to microbial challenge itself—as the JNK pathway was recently shown to be involved in wound healing in *Drosophila* [34]. This speculation is in line with the notion that transcription of the *puc* gene is not induced by natural fungal infection in vivo [12]. Altogether, our more moderate number of genes induced by *E. coli* argues that our assay is more specific in identifying genes induced by microbial challenge itself than previous in vivo studies. Alternatively, fat body is the principal immune tissue in *Drosophila* and thus blood cells may produce only a subset of immune response genes in response to bacteria.

The last few years have brought a lot of new insight into *Drosophila* immunity. There are, however, several key questions that remain to be solved. For example, the main phagocytic receptor that recognizes both Gram-negative and Gram-positive bacteria is currently unidentified. Similarly, it is currently unknown if there are any circulating molecules that opsonize microbes for more efficient phagocytosis. Tep4 expression is induced by an exposure to microbes both in vivo and in vitro. It is interesting to see whether Tep4 protein acts as an opsonin and whether it has any role in immune response in vivo. The fungal PRR as well as receptors that trigger melanization and coagulation cascades are still unknown. Progress in understanding *Drosophila* immunity will likely continue to fuel experiments in mammalian systems.

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

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Because NF-κB signaling pathways are highly conserved in evolution, the fruit fly *Drosophila melanogaster* provides a good model to study these cascades. We carried out an RNA interference (RNAi)-based genome-wide in vitro reporter assay screen in *Drosophila* for components of NF-κB pathways. We analyzed 16,025 dsRNA-treatments and identified 10 novel NF-κB regulators. Of these, nine dsRNA-treatments affect primarily the Toll pathway. G protein-coupled receptor kinase (Gprk)2, CG15737/Toll pathway activation mediating protein, and u-shaped were required for normal *Drosomycin* response in vivo. Interaction studies revealed that Gprk2 interacts with the *Drosophila* IκB homolog Cactus, but is not required in Cactus degradation, indicating a novel mechanism for NF-κB regulation. Morpholino silencing of the zebrafish ortholog of Gprk2 in fish embryos caused impaired cytokine expression after *Escherichia coli* infection, indicating a conserved role in NF-κB signaling. Moreover, small interfering RNA silencing of the human ortholog *GRK5* in HeLa cells impaired NF-κB reporter activity. *Gprk2* RNAi flies are susceptible to infection with *Enterococcus faecalis* and *Gprk2* RNAi rescues *Toll^{10b}*-induced blood cell activation in *Drosophila* larvae in vivo. We conclude that Gprk2/GRK5 has an evolutionarily conserved role in regulating NF-κB signaling. *The Journal of Immunology*, 2010, 184: 6188–6198.

Nuclear factor-κB signaling is involved in a variety of cellular processes, including control of both the innate and adaptive immune systems. The NF-κB/Rel family of transcription factors consists of five members in humans. These proteins control the expression of hundreds of target genes, including various cytokines and chemokines, in a tightly regulated manner (1). In mammals, immune-related NF-κB activation mainly occurs via two signaling pathways, the TNFR pathway and the TLR pathway.

NF-κB signaling pathways are highly conserved in evolution, and therefore, similar signaling cascades are found in lower eukaryotes, such as the fruit fly *Drosophila melanogaster. Drosophila* systemic immune response is largely mediated by two NF-κB signaling cascades, the Toll and the immune deficiency (Imd) pathway, which closely resemble mammalian TLR and TNFR signaling cascades, respectively (2). Both signaling cascades lead to activation and nuclear localization of *Drosophila* NF-κB family protein and expression of a distinct but overlapping set of antimicrobial peptide genes (3–5). Thus, lacking adaptive immunity, the fruit fly makes a useful and simpler model to study the signaling cascades and their involvement in innate immune responses.

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

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Abbreviations used in this paper: *Act5C–β-gal*, *Actin 5C–β-galactosidase*; C, CaM binding site; CaM, Calmodulin; Dif, Dorsal-related immunity factor; EGF, epidermal growth factor; EGFR, EGF receptor; EXT, extension to kinase domain; FADD, Fas-associated death domain protein; Gprk, G protein-coupled receptor kinase; GRK, G protein-coupled receptor kinase; *hml$^{\Delta}$*, *hemolectin$^{\Delta}$*; IM, immune-induced molecule; Imd, immune deficiency; kinase, kinase domain; MED25, mediator complex subunit 25; MRC, Medical Research Council; NIG-FLY, Fly Stocks of the National Institute of Genetics; PGRP, peptidoglycan recognition protein; qRT-PCR, quantitative RT-PCR; RGS, regulator of G protein signaling; RNAi, RNA interference; siRNA, small interfering RNA; Tab2, TGF-β–activated kinase 1-associated binding protein 2; Tak1, TGF-β–activated kinase 1; UAS, upstream activating sequence; VDRC, Vienna *Drosophila* RNAi Center.

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

To identify novel gene products involved in *Drosophila* NF-κB signaling, we carried out a genome-wide screen for 16,025 dsRNAs using a *Drosomycin* luciferase reporter-based assay that enables us to monitor both the Toll and Imd pathways. We identified 10 novel NF-κB regulators, of which 9 act primarily on the Toll pathway. Furthermore, we identified G protein-coupled receptor kinase (Gprk)2/GRK5 as an evolutionarily conserved regulator of NF-κB signaling.

Materials and Methods

Drosophila *dsRNA libraries and synthesis of targeted dsRNAs*

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

S2 cell treatments and reporter assays

S2 cell culture, transfections, dsRNA treatments, and reporter assays for the Toll and Imd pathways were performed essentially as previously described (17, 28). For the genome-wide screen, S2 cells were transfected with 0.1 μg *Drosomycin luciferase* (29) and 0.1 μg *Actin 5C–β-galactosidase* (*Act5C–β-gal*) reporter plasmids. In addition, the cells were treated with 0.5 μg dsRNAs. To screen both Toll and Imd pathways simultaneously, the Toll pathway was first activated by transfecting S2 cells with 0.1 μg $Toll^{10b}$ construct. Forty-eight hours posttransfection and 24 h prior to measurements, the Imd pathway was activated by adding heat-killed *Escherichia coli*.

S2 cell transfections for quantitative RT-PCR

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

HeLa cell culture and transfections

HeLa cells were grown in DMEM plus GlutaMAX (Gibco/Life Technologies, Carlsbad, CA) with 10% FBS, 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin. For transfection, 6×10^4 cells per well were seeded onto 24-well plate. Twenty-four hours later, the cells were transfected with 0.1 μg *NF-κB luciferase*, 0.05 μg *CMV–β-galactosidase* reporter plasmid, and 50 pmol small interfering RNAs (siRNAs) (Ambion, Austin, TX) using Lipofectamine transfection reagent (Invitrogen/Life Technologies) and Opti-MEM medium (Life Technologies). siRNAs used were as follows: *GFP* siRNA (Silencer GFP [eGFP]; catalog number AM4626, negative control), *GRK5* siRNA (catalog number AM16704; ID 110898), and *RelA* (catalog number AM16704; ID 216912, positive control). Forty-eight hours post-transfection, NF-κB signaling was induced with 10 ng/ml TNF-α (Sigma-Aldrich), and 6 h later, luciferase and β-galactosidase activities were measured from the cell lysates.

Immunohistochemistry with Gprk2/GRK5 constructs

To create a Gprk2-GFP fusion protein, the full-length cDNA for *Drosophila Gprk2* gene was amplified by PCR and cloned into the KpnI site of the modified *Drosophila* expression vector pMT/GFP/V5/His, a kind gift from Dr. I. Kleino (University of Helsinki, Helsinki, Finland). S2 cells were transfected with the *Gprk2-GFP* fusion construct essentially as described previously (30). Overexpression of Gprk2-GFP fusion protein in S2 cells was induced with 350 μM $CuSO_4$ for 36 h. For HeLa cell transfection, cells were seeded onto coverslips on six-well plates, and 24 h later, the cells were transfected with 0.1 μg *GRK5-GFP* construct. Thirty-six hours later, the coverslips were mounted to slides with Vectashield mounting medium for fluorescence with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Cells were imaged with an Olympus IX70 confocal microscope (Olympus, Tokyo, Japan) and analyzed with Andor iQ software (Andor Technology, Belfast, U.K.).

Zebrafish maintenance and morpholino gene silencing

AB wild-type zebrafish strain was maintained according to standard protocols (31). Translation-blocking morpholinos targeting zebrafish GRK5 (ZDB-GENE-060929-1198) (5′-GGCCACGATATTCTCAATCTCCATT-3′) and MyD88 (ZDB-GENE-040219-3) (5′-GGTCTATACTTAACTTTGATGCCAT-3′), as well as a mismatch-GRK5 control morpholino (5′-GCCCACCATATTGTCAATGTCGATT-3′), were obtained from GeneTools (Philomath, OR). A total of 1 nl 250 μM morpholinos in 0.2 M KCl was injected into the yolk sacs of AB wild-type embryos at the 1-2 cell stage.

Zebrafish infections

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

Coimmunoprecipitation

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

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

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

Fly stocks and maintenance

Drosophila stocks were kept on a standard mashed potato diet at room temperature or at 25°C. *C564-GAL4* flies express *GAL4* in the adult fatbody; the $P\{UAS\text{-}Tl^{10b}\text{:}11\}$ stock carries a $Toll^{10b}$ insert on the X

chromosome and the *hemolectin*$^{\Delta}$ (*hml*$^{\Delta}$)*-GAL4, UAS-GFP* stock constitutively expresses GFP in the majority of blood cells (33). The upstream activating sequence (UAS)-RNAi fly stocks listed in Supplemental Table I were obtained from the Vienna *Drosophila* RNAi Center [VDRC; Vienna, Austria (34)] or the Kyoto Fly Stocks of the National Institute of Genetics (NIG-FLY) (*Drosophila* Genetic Resource Center, Kyoto Institute of Technology, Kyoto, Japan). The *C564-GAL4* flies were crossed with *UAS-RNAi* flies, and the adult flies carrying one copy of the *UAS-RNAi* construct and one copy of the *GAL4* driver were used in infections. *UAS-RNAi* flies crossed to w^{1118} were used as controls in infection experiments.

Fly infection and RNA extraction

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

qRT-PCR

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

Fly survival experiments

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

Fly larvae in vivo experiments

To assess the distribution of blood cells in *Drosophila* larvae in vivo, parental crosses were kept for 2 d at 29˚C in stained mashed potato food, which permits the staging of larval progeny as described previously (27). The experiment was blinded by assigning arbitrary numbers to the fly bottles. Collected larvae were gently washed and embedded with the dorsal side up in 50% chilled glycerol between a glass slide and a coverslip. For immobilization, slides were kept at −20˚C for 18 min before examining under UV light on an Axioplan microscope (Carl Zeiss, Jena, Germany). Digital pictures were taken with a Hamamatsu C4742-95 video unit (Hamamatsu Photonics, K.K., Hamamatsu City, Japan), controlled by the Openlap program (Improvision, Coventry, U.K.).

For each cross, 20 F1 progeny larvae were graded for the percentage of their segments showing a band formed by islets of sessile hemocytes under the epidermis. In the grading system, grade 1 larvae showed sessile hemocyte bands in 100% of the segments. Larvae receiving grade 2 or 3 showed bands in <75 or 50% of their segments, respectively. Larvae showing no bands or bands only in the most posterior 25% of their segments received grade 4. All crosses were repeated three times and the average grades of three independent experiments calculated.

Data analysis

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

Results

Drosomycin *expression is controlled by both the Toll and Imd pathways in* Drosophila *S2 cells*

Drosomycin promoter-driven luciferase activity has been used to monitor the *Drosophila* Toll pathway activity (29). However, we have observed earlier (17) that in S2 cells, RNAi targeting components of the Imd pathway decreased the *Drosomycin luciferase* expression induced by the constitutively active form of Toll (*Toll*^{10b}). To investigate the respective roles of the Toll and Imd pathways to the regulation of *Drosomycin*, we analyzed the *Drosomycin luciferase* activity in S2 cells activated with both *Toll*^{10b} overexpression and heat-killed *E. coli*. As shown in Fig. 1*A*, *Drosomycin* expression was induced by *Toll*^{10b}, and this induction was further enhanced if the Imd pathway was also activated by *E. coli*. This induction, stimulated by both pathways, can be drastically decreased by silencing known components of the Toll pathway, namely *Toll*, *MyD88*, or *dorsal*. Imd pathway components, namely *PGRP-LC*, *Imd*, *Tab2*, and *Relish*, are also required for normal induction in this assay. If both pathways are silenced by targeting both *Relish* and *MyD88*, *Drosomycin* activation is totally blocked (Fig. 1*A*). These results are in line with previous studies indicating that *Drosomycin* expression can be induced by both the Toll and Imd pathways in vivo and in vitro (5, 36). These results also show that *Drosomycin luciferase* activity can be used in screening for components of both the Toll and Imd signaling cascades in *Drosophila* S2 cells.

Genome-wide analysis of the Toll and Imd pathways in Drosophila *S2 cells*

To identify gene products required for signaling via the Toll and Imd pathways, we examined the effect of 16, 025 dsRNA treatments for *Drosomycin luciferase* reporter activity in response to induction with *Toll*^{10b} and *E. coli* in *Drosophila* S2 cells. The dsRNA collection was obtained by transcribing PCR products from the commercial *Drosophila* genome-wide library (MRC Geneservice) to dsRNAs (13,607) and transcribing dsRNAs (2,418) from S2 cell-derived cDNA library (37).

Drosophila S2 cells were transfected with *Drosomycin luciferase* and *Act5C–β-gal* reporter constructs, *Toll*^{10b}, and dsRNAs. dsRNAs targeting *MyD88* and *Relish* were used as positive controls and *GFP* as a negative control in each experiment. *E. coli* was added 24 h prior to luciferase and β-galactosidase measurements. Out of 16,025 dsRNA treatments, 23 repeatedly decreased the *Drosomycin luciferase* reporter activity >50% without considerably affecting the cell viability as measured by *Act5C–β-gal* reporter activity (Fig. 1*B*). Corresponding templates were sequenced and targeted PCR primers for dsRNA synthesis designed to confirm that the effect had been due to dsRNA according to the library data and not due to contaminating dsRNAs. As shown in Fig. 1*C*, five dsRNA treatments representing known components of the Toll pathway (*Toll*, *MyD88*, *tube*, *pelle*, and *dorsal*), and eight dsRNA treatments representing known Imd pathway components (*Relish*, *kenny*, *FADD*, *Tak1*, *imd*, *Tab2*, *Ird5*, and *inhibitor of apoptosis 2*) were identified, indicating that our screen effectively found components of both of the *Drosophila* NF-κB signaling pathways. Importantly, 10 novel regulators of NF-κB signaling were identified (Fig. 1*C*). Corresponding genes were subjected to further studies.

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

Our RNAi screen effectively identified components of both the Toll and Imd signaling cascades. To find out which pathway is affected by these regulators, we carried out separate assays for the Toll and Imd pathways in S2 cells with targeted dsRNAs. Imd pathway activity was measured with a reporter assay in which *Attacin A*-driven *luciferase* (*AttA-luc*) construct, *Act5C–β-gal*, and dsRNAs were transfected into S2 cells, cells treated with *E. coli*, and reporter activities measured (Fig. 2*A*). Out of the 10 novel candidate genes, mediator complex subunit 25 (*MED25*) RNAi was shown to affect Imd pathway at the same level as RNAi to known Imd pathway

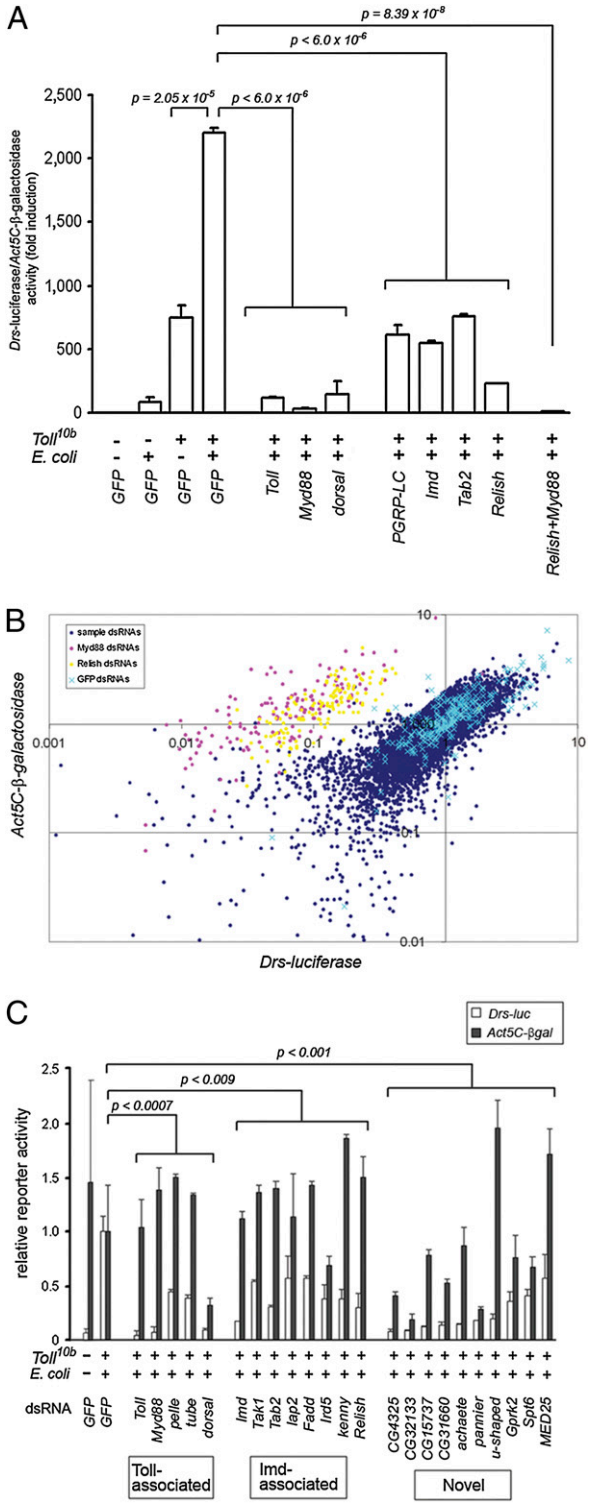

FIGURE 1. Genome-wide RNAi screen to identify genes required for NF-κB signaling in *Drosophila* S2 cells. *A*, *Drosomycin luciferase* reporter activity is regulated by both the Toll and Imd pathways in S2 cells. S2 cells were transfected with *Drosomycin luciferase* and *Act5C–β-gal* reporters. Toll pathway was induced by overexpression of $Toll^{10b}$ and the Imd pathway by heat-killed *E. coli* treatment for 24 h. RNAi targeting known components of the Toll pathway (*Toll, MyD88*, or *dorsal*) or components of the Imd pathway (*Tab2*, *PGRP-LC*, *imd*, or *Relish*) caused a strong reduction in the *Drosomycin luciferase*/*Act5C–β-gal* activity. *Relish* + *MyD88* RNAi completely blocked the relative *Drosomycin* activation. Data are shown as mean ± SD; $n \geq 3$. *B*, Results of the genome-wide RNAi screen of the *Drosophila* NF-κB signaling. The 16,025 independent dsRNA treatments were analyzed for effect on *Drosomycin luciferase* reporter activity induced via both the Toll and Imd signaling pathways. S2 cells were transfected with *Drosomycin luciferase* and *Act5C–β-gal* reporters, $Toll^{10b}$, and dsRNAs and treated with heat-killed *E. coli*. Luciferase and β-galactosidase values were plotted on a log-scale. Negative control samples (*GFP* RNAi) are illustrated with light blue crosses. Toll pathway positive controls (*MyD88* RNAi) are shown in purple, and Imd pathway positive controls (*Relish* RNAi) are shown in yellow. Samples with values in the top left corner of the plot include the most potential regulators of NF-κB signaling in *Drosophila* S2 cells. *C*, Targeted dsRNA treatments of potential regulators of the *Drosophila* NF-κB signaling confirm 23 dsRNA treatments that decrease *Drosomycin luciferase* reporter activity by >40%. *Drosomycin luciferase* and *Act5C–β-gal* values of induced *GFP* dsRNA-treated cells were set to 1. Data are shown as mean ± SD; $n \geq 3$. Statistics refer to *Drosomycin luciferase* values.

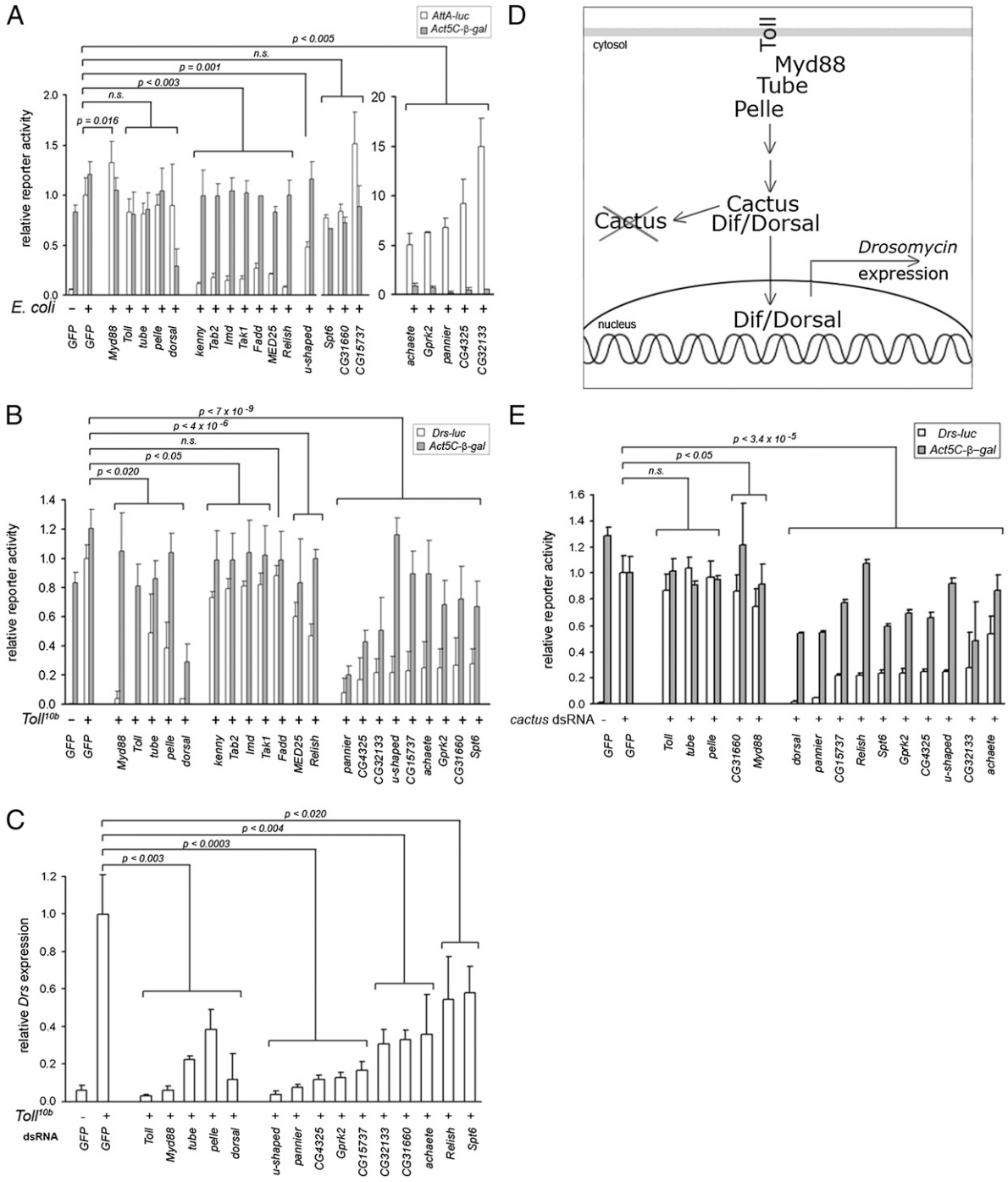

FIGURE 2. MED25 affects mainly the Imd signaling cascade, U-shaped affects both Toll and Imd signaling, and the other eight identified novel NF-κB regulators affect mainly Toll signaling. Data are shown as mean ± SD; $n \geq 3$. In each panel, statistics refer to white bars. *A*, For Imd pathway, S2 cells were transfected with *AttA-luc* reporter, *Act5C–β-gal* reporter, and indicated dsRNAs and induced with heat-killed *E. coli*. In addition to known Imd pathway components (*kenny*, *Tab2*, *Imd*, *Tak1*, *FADD*, and *Relish*), *MED25* RNAi decreased the *AttA-luc* reporter activity comparably to the known components. *u-shaped* RNAi also significantly affected the *AttA-luc* reporter activity. *B*, For Toll pathway activity, S2 cells were transfected with *Toll*^{10b}, *Drosomycin luciferase* reporter, *Act5C–β-gal* reporter, and indicated dsRNAs. Of the novel regulators, *pannier*, *CG4325, CG32133*, *u-shaped*, *CG15737*, *achaete*, *Gprk2, CG31660*, and *Spt6* dsRNAs considerably decreased the *Drosomycin luciferase* reporter activity. Also, most of the Imd pathway components/regulators (*kenny*, *Tab2*, *imd*, *Tak1*, *MED25*, and *Relish*) affected the *Drosomycin luciferase* activity. *C*, *Drosomycin* expression is inhibited by dsRNA treatments targeting all of the known tested components of Toll pathway and 10 dsRNAs identified from our collections. Endogenous *Drosomycin* expression in *Toll*^{10b}-transfected dsRNA-treated S2 cells was analyzed using qRT-PCR and normalized to *Act5C* expression values. *D*, Schematic representation of the *Drosophila* Toll pathway. *E*, Epistasis analysis of the identified regulators of Toll signaling. *Drosomycin luciferase* expression was induced by *cactus* RNAi. Ten dsRNA treatments (*dorsal*, *pannier*, *CG15737*, *Relish*, *Spt6*, *Gprk2*, *CG4325*, *u-shaped*, *CG32133*, and *achaete*) blocked this induction and therefore appear to act downstream or independently of Cactus. Five dsRNA treatments (*Toll*, *tube*, *pelle*, *CG31660*, and *MyD88*) did not considerably affect this induction, indicating that these gene products act upstream of Cactus.

components (21% of pathway activity left; Fig. 2*A*). Also, *u-shaped* RNAi decreased significantly the Imd pathway activity (48% of activity left). *Spt6*, *CG31660*, and *CG15737* RNAi had little effect on Imd pathway activity, whereas RNAi targeting *achaete*, *Gprk2*, *pannier*, *CG4325*, and *CG32133* resulted in hyperactivation of the pathway (Fig. 2*A*, on the right with a separate scale).

Out of the 10 novel candidate genes, 9 dsRNA treatments decreased the *Drosomycin luciferase* activity of $Toll^{10b}$-induced S2 cells >60% (Fig. 2*B*). The *Drosomycin luciferase* activity of S2 cells treated with these dsRNAs was also reduced by at least 50% when the Toll pathway was activated by overexpression of the cleaved, active Spätzle ligand (Spz^{C106}; data not shown). MED25 dsRNA treatment did not have as strong an effect on Toll pathway alone (Fig. 2*B*), so it was omitted from further analyses.

To ensure that our results were not due to an artifact related to the use of a reporter construct, we analyzed the relative endogenous *Drosomycin* expression levels of $Toll^{10b}$-induced S2 cells by qRT-PCR. In this assay, $Toll^{10b}$ transfection to S2 cells induced the relative endogenous *Drosomycin* expression (*Drosomycin*/*Act5C*) in S2 cells ~25-fold (Fig. 2*C*). Treating cells with dsRNAs targeting the known components of the Toll pathway, *Toll*, *MyD88*, *tube*, *pelle*, or *dorsal*, decreased the pathway activity by >55%. Similarly, RNAi targeting all of the nine novel Toll pathway regulators identified in the reporter assay, namely *u-shaped*, *pannier*, *CG4325, Gprk2, CG15737*, *CG32133*, *CG31660*, *achaete*, and *Spt6* caused a statistically significant reduction in endogenous *Drosomycin* expression. Of note, RNAi targeting *Relish*, the NF-κB factor in the Imd pathway, also caused a statistically significant reduction in $Toll^{10b}$-induced *Drosomycin* expression.

To gain more insight into the mechanism of how the novel regulators are functioning on the Toll pathway, we silenced *cactus*, the *Drosophila* homolog of human IκB, by RNAi (Fig. 2*D*, 2*E*). Silencing *cactus* results in Dif/Dorsal translocation into the nucleus (Fig. 2*D*) and >40-fold induction of the Toll pathway in a *Drosomycin luciferase* reporter assay (Fig. 2*E*). RNAi targeting known components of the pathway upstream of Cactus, namely *Toll*, *tube*, *pelle*, and *MyD88* have no or very little effect on *Cactus* RNAi-induced *Drosomycin luciferase* activity. Conversely, RNAi targeting *dorsal*, the *Drosophila* NF-κB homolog in the Toll pathway (downstream of Cactus), blocks this induction completely (Fig. 2*E*). Results indicate that CG31660 appears to act upstream of Cactus, whereas pannier, CG15737, Spt6, Gprk2, CG4325, u-shaped, CG32133, and achaete appear to act downstream or independently of Cactus. Relish also acts downstream of Cactus.

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

To investigate whether the identified nine genes are important for the Toll pathway signaling in vivo, we carried out in vivo RNAi experiments with fly lines carrying *UAS*-RNAi constructs targeting these genes. RNAi flies (Supplemental Table I) were crossed with the *C564-GAL4* driver line, which drives expression of the dsRNA in the fatbody. Fly strains without the driver (i.e., RNAi strains over w^{1118} flies) were used as controls. The Toll pathway was activated by *M. luteus* septic injury for 24 h, after which total RNAs were isolated. Relative *Drosomycin* expression in RNA samples was measured by qRT-PCR (Fig. 3). *MyD88* RNAi crossed with *C564-GAL4* flies were used as positive controls (Fig. 3*A*). Two *Gprk2* RNAi fly lines, namely *Gprk2 R-1* and *Gprk2 R-3*, crossed with *C564-GAL4* showed a significant decrease in *Drosomycin* expression (Fig. 3*B*, 3*C*). Moreover, the fly line expressing the *CG15737* RNAi construct showed a statistically significant reduction in *Drosomycin* expression compared with control flies, so we decided to name the *CG15737* gene *TAMP* (Fig. 3*D*). Also, flies expressing the *u-shaped* RNAi construct showed a statistically significant reduction in *Drosomycin* expression compared with control flies (Fig. 3*E*). In vivo RNAi targeting other identified Toll pathway candidate genes did not significantly decrease *Drosomycin* expression (Supplemental Fig. 1).

Drosophila *Gprk2 is homologous to GRK5 from other organisms*

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

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

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

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

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

Because Gprk2 acts at the level or downstream Cactus in the cactus dsRNA epistasis experiment (Fig. 2*E*), and because of reports of mammalian GRK5 interaction with members of the IκB family (39–41), we decided to investigate the interaction of Gprk2 with Cactus and Dorsal. V5-tagged full-length Gprk2 and deletion constructs were coimmunoprecipitated with myc-tagged Cactus and Dorsal in S2 cells. The full-length Gprk2, Calmodulin (CaM) binding-site deletion (ΔCaM1), and RGS-domain deletion (ΔRGS) constructs interact with Cactus protein, indicating that RGS and CaM1 domains are not needed in Gprk2 and Cactus interaction. In the kinase deletion (Δkinase) construct, this interaction is virtually not detectable anymore, which suggests that the kinase domain is important for the interaction, or that the protein, lacking a large domain, is not correctly folded anymore, resulting in loss of the interaction (Fig. 5*A*,

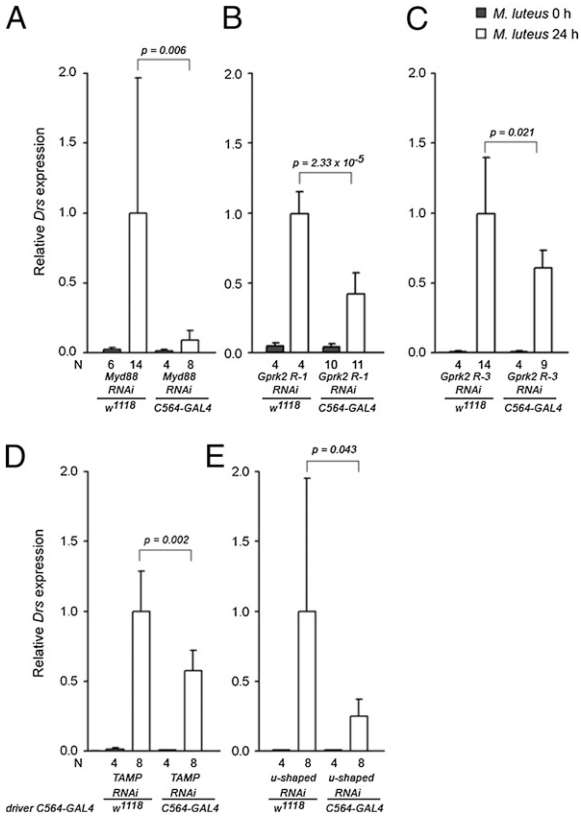

FIGURE 3. RNAi targeting *Gprk2*, *CG15737/TAMP*, and *u-shaped* reduces *Drosomycin* expression in *Drosophila* in vivo. Fly lines containing indicated *UAS*-RNAi constructs crossed over the *C564-GAL4*-driver flies, and controls were infected with *M. luteus* by pricking and collected 24 h later. Total RNAs were extracted and *Drosomycin* expression levels measured by qRT-PCR. Results were normalized to *Act5C* expression values. In each experiment, the relative *Drosomycin* expression value of the control flies was set to 1. *A*, *MyD88* RNAi flies were used as a positive control. *Gprk2* (*B*, *C*), *TAMP* (*D*), and *u-shaped* (*E*) RNAi flies show impaired *Drosomycin* expression ($p < 0.05$) compared with controls without the driver. Data are shown as mean ± SD.

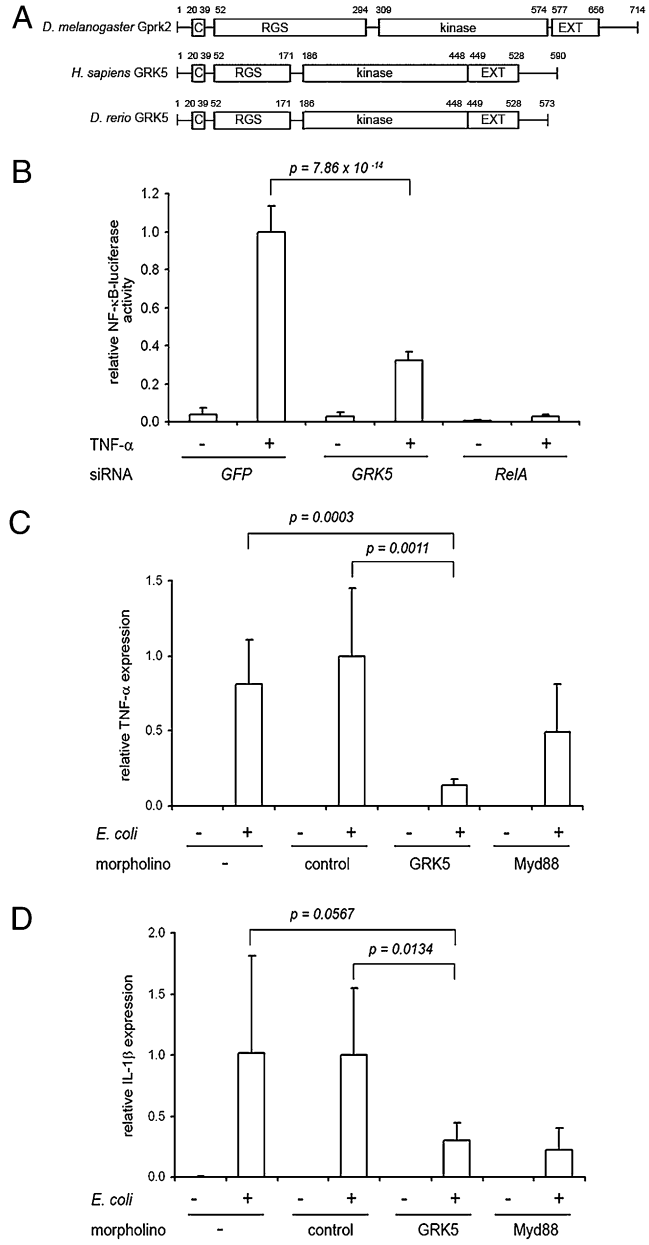

FIGURE 4. Gprk2/GRK5 has an evolutionarily conserved role in NF-κB–mediated immune signaling in human HeLa cells in vitro and in zebrafish embryos in vivo. *A*, Schematic diagram of *Drosophila* Gprk2, *Homo sapiens* GRK5, and *Danio rerio* GRK5 proteins. *B*, GRK5 is required for TNF-α–triggered NF-κB signaling in HeLa cells. HeLa cells were transfected with *NF-κB–luciferase* and *CMV–β-galactosidase* reporters and with *GRK5* or control siRNAs. Cells were induced with TNF-α 6 h prior to luciferase and β-galactosidase measurements. *C* and *D*, GRK5 is required for *E. coli*-induced activation of NF-κB signaling in zebrafish embryos. Zebrafish embryos were injected with GRK5 and control morpholinos, and 24 h later, embryos were infected by *E. coli* injection. After 18 h incubation, embryos were collected and total RNAs extracted. Relative expression of TNF-α (*C*) or IL-1β (*D*) in infected and control embryos was analyzed by qRT-PCR. Data are shown as mean ± SD; $n \geq 5$. C, CaM binding site; EXT, extension to kinase domain; kinase, kinase domain.

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

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

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

To investigate the role of Gprk2 on Toll pathway-mediated immunity in vivo, we carried out an experiment in which *Grpk2* RNAi flies and controls were subjected to natural fungal infection with an insect pathogen *B. bassiana* at +29°C for 48 h, after which total RNAs were isolated. RNAs from noninfected flies were isolated as a control for the infection. Expression of Toll pathway target genes, namely *Drosomycin*, *IM1*, and *IM2* was measured by qRT-PCR (Fig. 6*A–C*, respectively). Results were normalized to *Act5C*

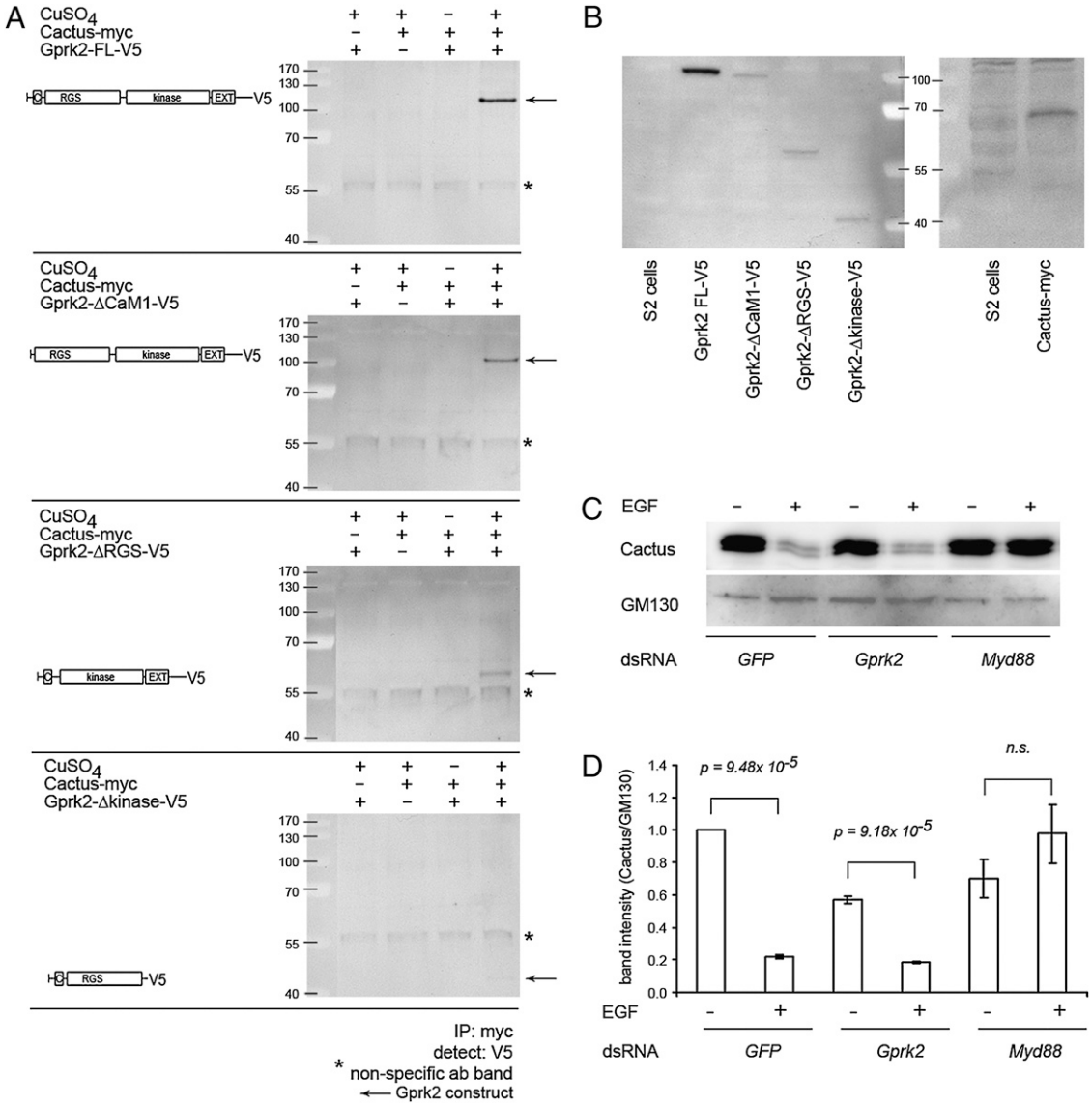

FIGURE 5. Gprk2 interacts with Cactus, but is not required for Cactus degradation upon signaling. *A*, The full-length Gprk2, CaM binding site deletion (ΔCaM1) and RGS-domain deletion (ΔRGS) constructs interact with Cactus protein. In the kinase deletion (Δkinase) construct, this interaction is virtually nondetectable. Protein expression was induced with $CuSO_4$ (250 μM). Proteins were coimmunoprecipitated with anti-myc Ab and detected with anti-V5 Ab. *B*, Expression of pMT-Gprk2-V5 and pMT-Cactus-myc constructs in S2 cells induced with $CuSO_4$ (250 μM) detected with anti-V5 and anti-myc Abs, respectively. *C*, *Gprk2* RNAi does not affect Cactus degradation upon signaling. S2 cells expressing a chimeric EGFR-Toll construct were treated with *GFP*, *Gprk2*, and *MyD88* dsRNAs. Toll pathway was activated with EGF, and Cactus protein in cytoplasmic extracts was detected by SDS-PAGE and Western blotting with anti-Cactus Ab. Gel loading was controlled using anti-GM130 Ab targeting a *Drosophila* Golgi protein. *D*, Cactus band intensities were quantified from three separate Western blots and normalized to the loading control GM130 band intensities. Data are shown as mean ± SD; $n = 3$.

expression values. Both *Gprk2 R-1* and *Gprk2 R-3* RNAi lines crossed with the driver *C564-GAL4* showed a reduced expression of Toll pathway target genes. w^{1118} flies over the *C564-GAL4* driver and *MyD88* RNAi flies over the *C564-GAL4* driver were used as negative and positive controls, respectively. These results indicate that in *Gprk2*-silenced flies, Toll pathway-induced genes are poorly activated after fungal infection.

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

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

Gprk2 *RNAi construct can rescue* UAS-Toll^{10b} *blood cell activation in* Drosophila *larvae in vivo*

To examine if *Gprk2* RNAi can inhibit blood cell activation caused by a constitutively activated Toll pathway in vivo, transgenic RNAi fly lines of *Gprk2 R-3*, and *MyD88* as a control, were combined with blood cell-specific hml^{Δ}*-GAL4*, *UAS-GFP* driver. Males originating from these stocks were crossed to females of the *UAS-Toll*^{10b}

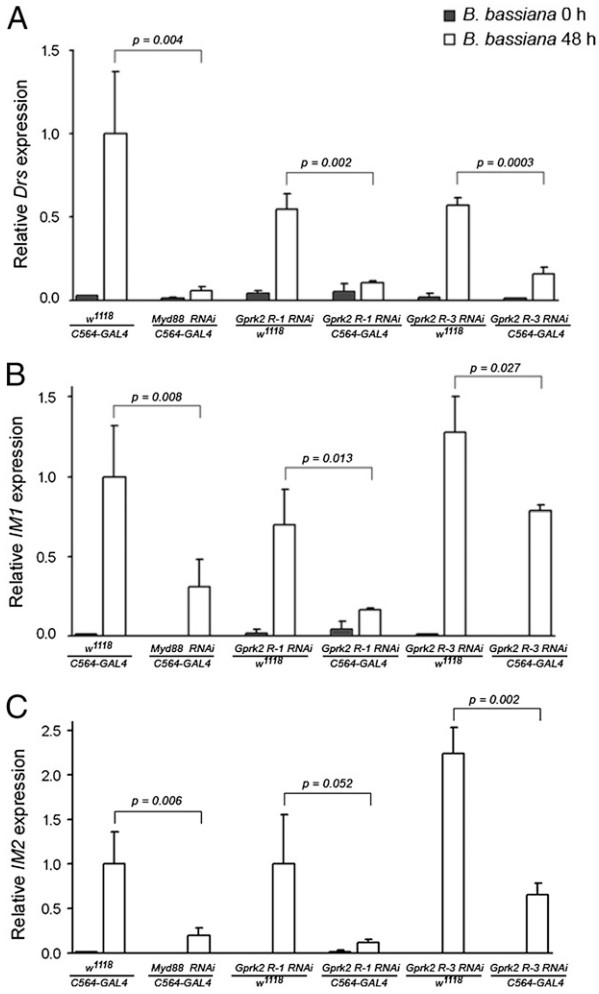

FIGURE 6. *Gprk2* RNAi flies have impaired expression of Toll pathway target genes when subjected to *B. bassiana* natural fungal infection. *Gprk2* RNAi flies crossed with *C564-GAL4* driver and controls were infected with *B. bassiana*, incubated for 48 h at 29°C, and collected. Total RNAs extracted from flies were subjected to qRT-PCR analysis. The relative *Drosomycin*, *IM1*, or *IM2* value of the control flies (w^{1118}/*C564*) was set to 1. *A*, Relative *Drosomycin* expression. Relative *IM1* expression (*B*) and relative *IM2* expression (*C*) in infected flies. Data are shown as mean ± SD, $n = 3$.

line. Males from the original driver line were crossed to females of either the *UAS-Toll*^{10b} line (negative control) or w^{1118} (treatment control). Progeny third-instar larvae were graded for the percentage of their segments showing bands formed by islets of sessile hemocytes under the epidermis. Offspring of the treatment control showed bands of sessile cells in all segments, indicating that larval handling had little effect on the blood cell distribution (Fig. 7*Ci*). In contrast, larvae of the negative control showed a largely disturbed sessile hemocyte banding pattern caused by the constitutive activation of the Toll signaling pathway in blood cells (Fig. 7*Cii*) (27). This difference is reproducible and reflected in the average grades calculated, with significantly higher average grades for crosses of the negative control compared with the treatment control (Fig. 7*D*). The loss of sessile hemocyte banding pattern could be rescued by introducing the RNAi constructs targeting *Gprk2* or *MyD88* (Fig. 7*Ciii*, 7*Civ*, 7*D*). These results indicate that RNAi targeting *Gprk2* can inhibit hemocyte activation caused by *UAS-Toll*^{10b}.

Discussion

Large-scale in vitro RNAi screening has become a commonly used method to identify gene products involved in numerous cellular processes. In this study, we used a luciferase-based reporter assay, with which we were able to monitor both the Toll and the Imd pathway activities simultaneously. Based on careful setup of the assay, biologically meaningful cutoffs and assessment of the general well-being of the cells using *Act5C–β-gal* reporter, we obtained a sensible hit list of 23 genes. These included 5 known components of the Toll signaling pathway, 8 known components of the Imd pathway, and 10 previously uncharacterized novel regulators of *Drosophila* NF-κB signaling. Noteworthy, only one new regulator (MED25) strongly affected the Imd pathway, whereas there were nine dsRNA treatments that primarily decreased the Toll pathway activity. This is in accordance with the notion that several RNAi screens have already been carried out for the Imd pathway, whereas the Toll pathway is less thoroughly studied using RNAi. The results related to Toll pathway activity were further confirmed by secondary (*Spz*^{C106}-based) and tertiary (qRT-PCR for endogenous *Drosomycin*) assays. After these confirmation and validation steps, we ended up with a solid hit list of nine novel modifiers of the Toll signaling pathway, namely *u-shaped*, *pannier*, *achaete*, *TAMP*, *CG4325*, *CG32133*, *CG31660*, *Spt6*, and *Gprk2*.

Our screen failed to identify one known positive regulator of the Toll pathway downstream of Toll receptor (Dif) and two components of the Imd pathway (PGRP-LC and Dredd). Targeted RNAi for Dif did not decrease the *Drosomycin luciferase* reporter activity either when induced by *Toll*^{10b} or *Toll*^{10b} together with *E. coli*. This suggests that in S2 cells, dorsal has a more important role in Toll pathway-mediated signaling than Dif. As for PGRP-LC and Dredd, we conclude that these two were not successfully targeted by our dsRNA libraries. In fact, there were several instances in which there was more than one PCR product, occasionally none of them corresponding to the indicated gene, in a single well of the MRC Geneservice PCR product library. Therefore, we found it imperative to TA-clone and sequence every PCR template corresponding to interesting RNAi phenotypes, to design gene-specific primers, and to carry out independent RNAi with targeted dsRNAs. If there were multiple PCR products, all corresponding targeted dsRNAs were tested to identify the one that caused the observed phenotype.

U-shaped is the *Drosophila* Friend of GATA homolog with a known important role in hemocytes. U-shaped has been shown to interact with and negatively regulate pannier, a *Drosophila* GATA transcription factor (42). U-shaped and pannier together with achaete (and scute) also regulate the bristle formation in *Drosophila* (43). This suggests that u-shaped, pannier, and achaete may act together in the process of Toll pathway regulation. *TAMP* (*CG15737*) encodes a protein with an N-terminal domain homologous to poly (A) polymerase proteins. CG4325 is a small protein with a RING finger domain, which is likely to bear E3 ubiquitin-protein ligase activity and is often involved in mediating protein-protein interactions. CG32133 is a large protein with postulated molecular functions in transcription factor binding, but the biological processes it mediates are unknown. *Drosophila* Spt6 has homology to *Saccharomyces cerevisiae* Spt6p, which has been implicated in transcription initiation and maintaining normal chromatin structure during transcription elongation (44). CG31660 bears homology to human G protein-coupled receptor 158, and it contains a domain typical for a metabotropic glutamate family. Metabotropic glutamate receptors are coupled to G proteins and stimulate the inositol phosphate/Ca^{2+} intracellular signaling pathway (45). It is likely that understanding the exact molecular functions of these genes will reveal novel levels and means to delicately control NF-κB pathway-mediated immune response.

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

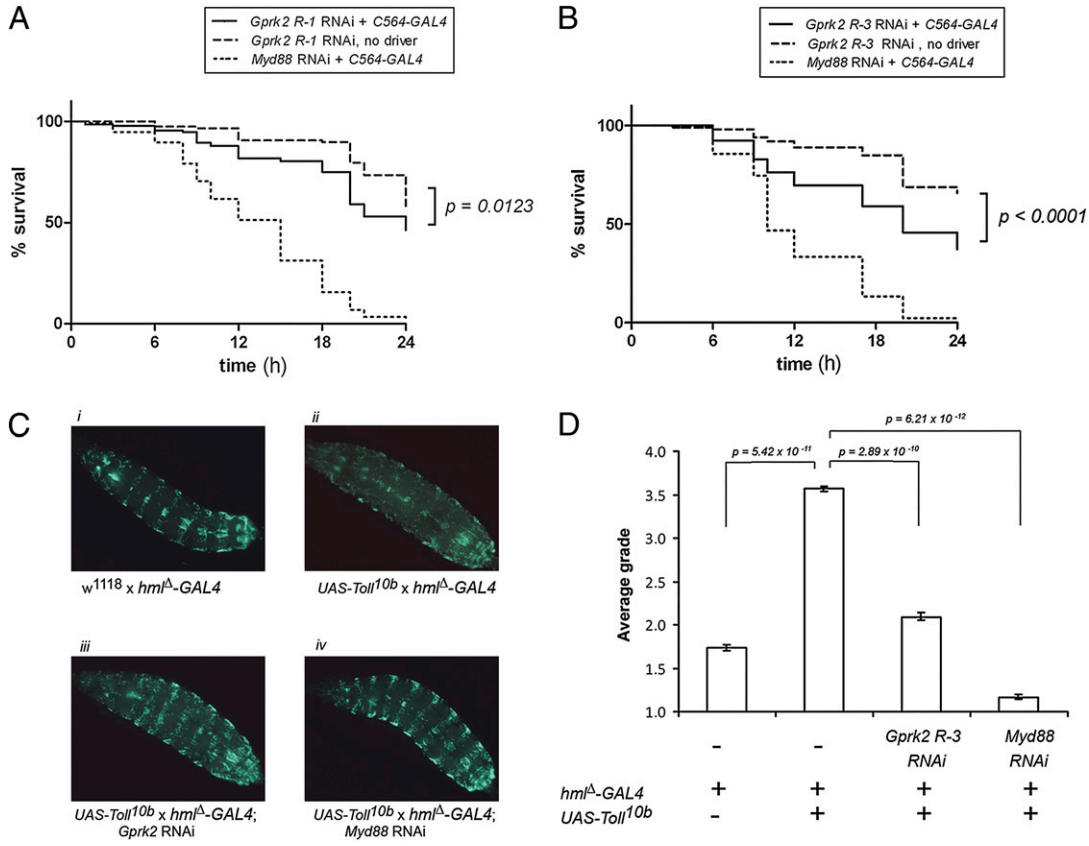

FIGURE 7. *Gprk2* RNAi flies are susceptible to infection with *E. faecalis*, and *Gprk2* RNAi rescues *Toll*^{10b}-induced blood cell activation in *Drosophila* larvae in vivo. *A* and *B*, The Toll pathway was activated by pricking flies with *M. luteus*, and 24 h later, the flies were pricked with *E. faecalis*. The survival of the flies was monitored for 24 h. *MyD88* RNAi flies were used as a positive control. Both *Gprk2* RNAi lines (*Gprk2 R-1* and *Gprk2 R-3*) crossed over the *C564-GAL4* driver show a statistically significant reduction in survival compared with controls. *Gprk2 R-1* × *C564-GAL4* (n = 132); *Gprk2 R-1* control without the driver (n = 117); *Gprk2 R-3* × *C564-GAL4* (n = 105); *Gprk2 R-3* control without the driver (n = 99). *C*, Sessile hemocyte banding pattern (*i*), lost upon constitutive activation of the Toll signaling pathway in blood cells (*ii*), could be rescued by *Gprk2* (*iii*) or *MyD88* RNAi (*iv*). *D*, The average grades of three independent crosses. n = 20 larvae per cross, ± SEM. Grade 1, sessile hemocyte bands in 100% of segments; grade 2, bands in <75% of segments; grade 3, bands in <50% of segments; and grade 4, no bands or bands only in the most posterior 25% of segments.

GAL4 driver fly line results in silencing of the targeted gene in the chosen tissue in the progeny. However, it is recognized that as much as 35–40% of fly lines may give a false-negative result, which may be due to multiple reasons related to RNAi, driver GAL4 strain, and/or the assay chosen (34). In our in vivo infection assays, we found a phenotype with a statistical difference to controls in 5 out of 11 strains (Fig. 3, Supplemental Fig. 1). In addition, two other strains (*Spt6* and *pannier* RNAi strains; Supplemental Fig. 1) showed a similar but nonsignificant trend. Therefore, our results are in line with the estimates presented by Dietzl and coworkers (34). Of note, the false-positive rate is estimated to be <2%, which means that it is very likely the reduction in *Drosomycin* expression in our driver-induced strains is due to silencing of the gene in question (34).

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

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

NF-κB signaling is of paramount importance for regulating immune response both in flies and vertebrates. The power of the *Drosophila* model includes the possibility of combining large-scale RNAi screening with sophisticated in vivo tools. In this study, we carried out a genome-wide RNAi screen in cultured *Drosophila* cells and identified 10 novel regulators of *Drosophila* NF-κB signaling. The evolutionarily conserved role for Gprk2/GRK5 in NF-κB pathway activation was shown using human HeLa cells in vitro and zebrafish embryos in vivo. Finally, the importance of Gprk2 for *Drosophila* NF-κB signaling was demonstrated both in vitro and in vivo.

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Disclosures

The authors have no financial conflicts of interest.

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Eye transformer is a negative regulator of *Drosophila* JAK/STAT signaling

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ABSTRACT **JAK/STAT signaling pathway is evolutionarily conserved and tightly regulated. We carried out a reporter-based genome-wide RNAi *in vitro* screen to identify genes that regulate *Drosophila* JAK/STAT pathway and found 5 novel regulators. Of these, CG14225 is a negative regulator structurally related to the *Drosophila* JAK/STAT pathway receptor Domeless, especially in the extracellular domain, and to the mammalian IL-6 receptor and the signal transducer gp130. CG14225 coimmunoprecipitates with Domeless and its associated kinase hopscotch in S2 cells. *CG14225* RNAi caused hyperphosphorylation of the transcription factor Stat92E in S2 cells on stimulation with the *Drosophila* JAK/STAT pathway ligand unpaired. *CG14225* RNAi *in vivo* hyperactivated JAK/STAT target genes on septic injury and enhanced unpaired-induced eye overgrowth, and was thus named the *eye transformer (ET)*. In the gastrointestinal infection model, where JAK/STAT signaling is important for stem cell renewal, *CG14225/ET* RNAi was protective *in vivo*. In conclusion, we have identified ET as a novel negative regulator of the *Drosophila* JAK/STAT pathway both *in vitro* and *in vivo*, and it functions in regulating Stat92E phosphorylation.—Kallio, J., Myllymäki, H., Grönholm, J., Armstrong, M., Vanha-aho, L.-M., Mäkinen, L., Silvennoinen, O., Valanne, S., Rämet, M. *Eye transformer* is a negative regulator of *Drosophila* JAK/STAT signaling. *FASEB J.* 24, 000–000 (2010). www.fasebj.org**

Key Words: RNAi screen · cytokine signaling · stress response · Domeless · unpaired

The evolutionarily conserved janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway controls responses to hematopoietic cytokines that orchestrate inflammatory and immune responses in mammals (1). In humans, selective utilization of 4 different Janus kinases (JAKs) and 7 STAT transcription factors leads to specific changes in the activity of a set of target genes providing complexity for JAK/STAT-mediated responses. Disturbances in JAK/STAT signaling may cause serious human diseases, including cancer, polycytemia vera, severe immune deficiencies such as SCID, autoimmunity, allergies, and neurological defects (1–5).

Drosophila has been widely used as a model for JAK/STAT signaling. The core signaling pathway is conserved in evolution from flies to humans, but *Drosophila* has only 1 JAK, hopscotch (6, 7), and a single STAT transcription factor, Stat92E (8, 9), making the pathway simpler and less redundant. Like in mammals, the *Drosophila* JAK/STAT pathway is also involved in multiple processes, which include embryonic segmentation, larval hematopoiesis and development of various organs, regulation of stem cell maintenance, and cellular proliferation. In addition, *Drosophila* JAK/STAT signaling is required to control immune and stress responses (7, 10; reviewed in refs. 11–14). After septic injury, the activation of JAK/STAT pathway leads to the expression of a number of genes, including *Turandot* (*Tot*) stress genes in the fatbody (15–17).

JAK/STAT pathway activation is mediated by secreted cytokine-like molecules unpaired (upd), upd2, and upd3 (18–20), which bind to the transmembrane receptor Domeless (Dome) (21, 22). Dome shares homology with members of the interleukin 6 (IL-6) receptor family and, like the mammalian cytokine receptors, forms dimers (22, 23). In the canonical model of JAK/STAT signaling cascade, ligand binding induces a conformational change in the receptor leading to activation of associated JAKs by auto- and/or trans-phosphorylation. Activated JAKs phosphorylate tyrosine residues in the receptors, thereby creating docking sites for STATs, which subsequently become phosphorylated by JAKs too. Activated STATs dimerize and translocate in the nucleus, where they bind their target sites in DNA and act as transcriptional activators (8, 24). In *Drosophila,* the *Socs36E* (suppressor of cytokine signaling 36E) gene is known to be a target gene of the JAK/STAT pathway, forming a negative feedback loop by inhibiting hop activity (25, 26). *Drosophila* protein inhibitor of activated stat (dPIAS) is shown to

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be a negative regulator of the *Drosophila* JAK/STAT pathway too, but the mechanism remains elusive (27) compared to humans, where, for example, hPias1 is shown to promote sumoylation of hStat1 (28).

JAK/STAT signaling has been studied in the *Drosophila* system because of its importance in human diseases. S2 cell-based large-scale RNAi screening provides a powerful tool to identify theoretically all genes required for given cellular function (29, 30). Two *Drosophila in vitro* RNAi screens have been carried out to find genes involved in JAK/STAT signaling (31, 32), and several new modifiers of this pathway have been identified. Curiously, however, there were differences between the sets of identified genes. To study regulation of the *Drosophila* JAK/STAT signaling and to elucidate the events involved in the signaling, we carried out a genome-wide RNAi based *in vitro* screen in *Drosophila* S2 cells. As the intracellular part of the JAK/STAT pathway downstream of the JAK kinase is particularly well conserved, we chose to activate the signaling using the constitutively active form of the *Drosophila* JAK kinase *hopscotch* ($hop^{Tum\text{-}l}$) (33, 34). Activity of the pathway was monitored using a Stat92E responsive *TotM-luciferase* (*TotM-luc*) reporter-based assay. We screened 16,025 dsRNAs for their effect on *TotM* reporter activity. In addition to the known JAK/STAT signaling pathway components, we identified 5 genes that regulated *TotM* response in S2 cells. Of these, the *CG14225* gene we call *eye transformer (ET)* was identified as a negative regulator of JAK/STAT signaling both *in vitro* and *in vivo*.

MATERIALS AND METHODS

dsRNA synthesis

The dsRNAs used in the RNAi screen were produced from a commercial *Drosophila* genome RNAi library consisting of a set of 13,625 PCR products with dual T7 promoter sequences [Medical Research Council (MRC) Geneservice Ltd., Cambridge, UK]. An additional 2400 dsRNAs were transcribed from the S2 cell-derived cDNA library (35). Targeted dsRNAs were synthesized from S2 cDNA essentially as described in ref. 36. pMT/BiP/V5-His/GFP plasmid (Invitrogen, Carlsbad, CA, USA) was used as a template for the production of the negative control *GFP* dsRNA. Primers used for targeted dsRNAs were *GFP* 5′-T7+GCTCGGGAGATCTCC-3′ and 5′-T7+CTAGACTCGAGCGGC-3′; *Stat92E* 5′-T7+CCGATTAGCCAACGC-3′ and 5′-T7+GGACCCCAGTGATCT-3′; *hop* 5′-T7+GGAGCAGCAGATAGC-3′ and 5′-T7+GGCGGTAGAGGAACT-3′; *Dome* 5′-T7+TAACGGCAAGAGCGC-3′ and 5′-T7+AGGTTCTGGCCAGGT-3′; *ET dsRNA*[1] 5′-T7+TGCGAAGGCAGGGCACAATAGAATC-3′ and 5′-T7+CAAGTCTGGTTGGGCGTTTGTATCA-3′; *ET dsRNA*[2] 5′-T7+CGGAGAATGCGTTGC-3′ and 5′-T7+AGTTGGGCAGCTTGG-3′; *ET dsRNA*[3] 5′-T7+GACATCCGGGATCGACG-3′ and 5′-T7+CGTGGGCTCCTCTTCCG-3′. Additional information related to primers is presented in Supplemental Table S1.

Cell culture, transfections, dsRNA treatments, and overexpression constructs

Drosophila S2 cells were cultured in Schneider medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 25°C. Transfections and dsRNA treatments were performed essentially as described previously (37). We transfected 1.0×10^6 S2 cells with 0.2 μg of a constitutively active form of the Janus kinase $hop^{Tum\text{-}l}$ together with *TotM-luc* reporter plasmid for activating the JAK/STAT pathway and for quantifying *TotM* expression, respectively. Cells were also transfected with 0.2 μg *Act5C-β-gal* reporter plasmid for monitoring cell viability and transfection effiency. We used 0.5 μg of control and experimental dsRNAs for RNAi. Reporter activities were measured 72 h after transfection. Transfections with other reporters were carried out similarly. *CG14225* was cloned from S2 cell cDNA to *Eco*RI and *Not*I sites of *Drosophila* expression vector pMT-HisA. Protein production was induced by addition of $CuSO_4$ to a final concentration of 500 μM 24 h prior to cell lysis.

Quantitative RT-PCR

Quantitative RT-PCR for *TotM, TotA,* and *CG14225* and *Act5C* levels was carried out using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, USA) and the ABI7000 instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. Primers used for qRT-PCR were *Act5C* 5′-CGAAGAAGTTGCTGCTCTGG-3′ and AGAACGATACCGGTGGTACG; *TotM* 5′-ACCGGAACATCGACAGCC-3′ and 5′-CCAGAATCCGCCTTGTGC-3′; *TotA* 5′-CCCAGTTTGACCCCTGAG-3′ and 5′-GCCCTTCACACCTGGAGA-3′; *ET* 5′-CGGAGAAAGGAGCACCCA-3′ and 5′-GGGACTGCATCTCGCAGT-3′.

Sequence analysis

Sequences were analyzed with the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/). ClustalW alignments (http://www.ebi.ac.uk/Tools/clustalw2/index.html) were carried out in order to identify similar regions between ET, Dome, and gp130.

Coimmunoprecipitation

S2 cells were transfected with constructs of cDNAs cloned in the pMT/V5/HisA vector (Invitrogen). Protein production was induced with $CuSO_4$. The following tagged full-length constructs were used: hopscotch-V5, Dome-myc, ET-myc, ET-V5, and Dome-V5. Constructs were cotransfected in the combinations shown and immunoprecipitated with Protein G Sepharose beads (GE Healthcare, Little Chalfont, UK) or Protein G Dynabeads (Invitrogen), separated, transferred to nitrocellulose membrane, and detected essentially as described previously (38).

Immunodetection

We transfected 5.0×10^6 S2 cells with a total amount of 3.0 μg of dsRNA and 1.0 μg of pMT-upd plasmid or 1.0 μg of empty pMT-V5-HisA using Fugene6 reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Then 48 h after transfection, cells were treated with 500 μM $CuSO_4$ for 24 h. Cells were lysed in Triton-X lysis buffer. The protein amounts were determined by a Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany). Membranes were blocked with 5% milk in TBS-0.1% Tween 20, incubated with anti-Stat92E-N-terminal antibody (dN-17;

Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with anti-phospho-Tyrosine antibody (PY99; Santa Cruz Biotechnology) and with a biotinylated anti-goat or anti-mouse secondary antibody (DakoCytomation, Copenhagen, Denmark). Immunodetection was performed with the enhanced chemiluminescence method (GE Healthcare). TBS buffer containing 1% β-mercapthoethanol and 0.2% SDS was used for stripping. Phospho-Stat92E bands were quantified by ImageQuant TL image analysis software (GE Healthcare) and analyzed after background subtraction.

Fly stocks and maintenance

Drosophila stocks were kept on a standard mashed potato diet at RT or at 25°C. The RNAi transgenic fly stocks were obtained from the Vienna *Drosophila* RNAi Center [VDRC; Vienna, Austria; VDRC transformants 19756 (*ET IR*^{1}), 100881 (*ET IR*^{2}), and 43866 (*Stat92E IR*)]. *UAS-RNAi* flies were crossed over a fatbody-specific *C564-GAL4* or ubiquitous *GeneSwitch-GAL4* driver flies or to w^{1118} flies for controls. In flies crossed over the *GeneSwitch-GAL4* driver, the *GAL4* construct was induced with Mifepristone (200 μM). Week-old offspring were used for experiments.

To study the eye phenotype, flies carrying *GMR-upd*Δ*3′* were first crossed over *ET* IR^{1}/*CyO* flies. The F1 flies with 1 copy of *GMR-upd*Δ*3′* and 1 copy of *ET* IR^{1} were then crossed over eye-specific driver *ey-GAL4* to induce *ET* RNAi. The offspring from the first cross without the *ET* RNAi construct were used as controls.

Fly infections

For *Enterobacter cloacae* infection, week-old flies were pricked with a thin tungsten needle dipped in a concentrated culture of bacteria. *Serratia marcescens* feeding infection experiment was performed as described previously (39, 40). Survival of the flies was recorded daily.

Data analysis

Statistical analyses of reporter assays and qRT-PCR results were carried out using 1-way ANOVA. Statistical analysis of fly survival experiments was carried out using the log-rank (Mantel-Cox) test. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

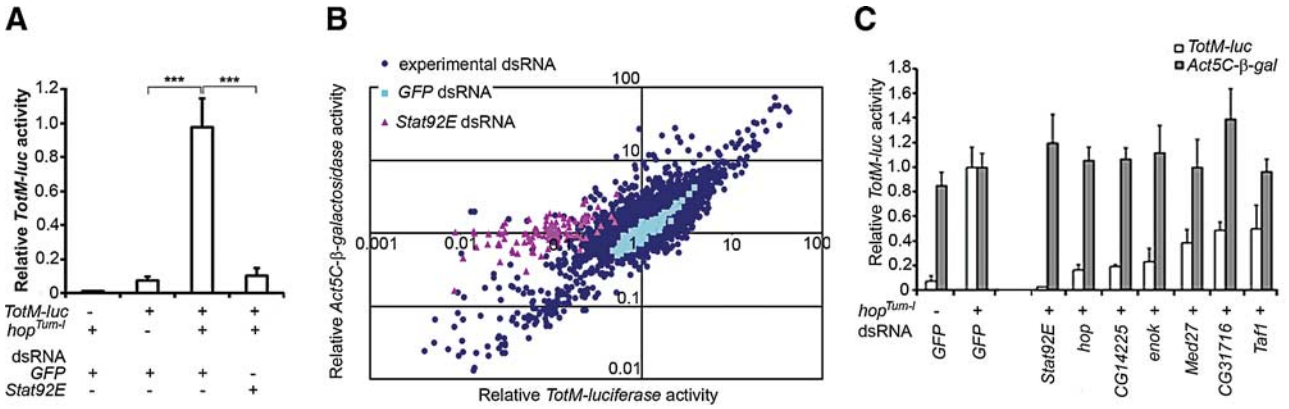
Luciferase-based reporter assay to monitor JAK/STAT signaling pathway activity in *Drosophila* S2 cells

Under stressful conditions, including septic injury, several *Tot* genes are expressed in the *Drosophila* fatbody, the functional equivalent of mammalian liver (15, 16). It has been shown that the activation of *TotA* is JAK/STAT pathway dependent but is also partly regulated by the Imd pathway and requires MAPKKK Mekk1 (17, 41). As shown in **Fig. 1*A***, *TotM-luc* reporter activity is induced by a constitutively active form of Janus kinase *hopscotch* (*hop*$^{Tum\text{-}l}$) in *Drosophila* S2 cells. This induction is STAT dependent, as RNAi targeting the transcription factor *Stat92E* blocks the *TotM* expression. On the other hand, RNAi targeting the Imd pathway transcription factor *Relish* has no effect on *hop*$^{Tum\text{-}l}$-induced *TotM* expression, demonstrating the specificity of our assay to JAK/STAT signaling (data not shown). These results indicate that *hop*$^{Tum\text{-}l}$-induced *TotM-luc* reporter activity can be used to study the regulation of the JAK/STAT pathway in *Drosophila* S2 cells.

Genome-wide RNAi analysis of the *Drosophila* JAK/STAT pathway in *Drosophila* S2 cells

To identify all regulators of the JAK/STAT pathway downstream of hop, we carried out a genome-wide RNAi screen and monitored the effects of 16,025 dsRNA treatments on *hop*$^{Tum\text{-}l}$-induced *TotM-luc* reporter activity in S2 cells. The dsRNAs were produced by *in vitro* transcription from a commercial *Drosophila* genome-wide library (MRC Geneservice; 13,625 PCR products), and an additional 2400 dsRNAs were transcribed from S2 cell-derived cDNA library (35). S2 cells were transfected with a *hop*$^{Tum\text{-}l}$ expression vector and *TotM-luc*-reporter together with experimental or control dsRNAs. *Act5C-β-gal* reporter was used to control cell viability. The luciferase and β-galactosidase activities were measured 72 h after transfection. dsRNA targeting *Stat92E* and dsRNA targeting a gene encoding *Green Fluorescence Protein* (*GFP*), which is not expressed in S2 cells, were used as positive and negative controls in each experiment, respectively. The luciferase and β-galactosidase values for *GFP* dsRNA-treated cells were used as reference values for experimental dsRNAs. As shown in Fig. 1*B,* most dsRNA treatments had little or no effect on *TotM-luc* or *Act5C-β-gal* activity. Notably, there were 7 dsRNA treatments that repeatedly decreased *TotM-luc* activity by more than 50% without significantly affecting *Act5C-β-gal* activity. These targeted 2 known JAK/STAT pathway components (*Stat92E* and *hop*), one gene previously shown to be involved in JAK/STAT pathway regulation (*enok*) (31, 32), and 4 novel regulators (*Taf1, CG31716, CG14225,* and *Med27*). Corresponding templates from the original library were TA-cloned and sequenced. Based on the sequencing results, we designed gene-specific primers and synthesized targeted independent dsRNAs against these novel regulators to confirm that the RNAi phenotype had been due to presumed dsRNA and not due to contaminating dsRNAs or any off-target effect. As shown in Fig. 1*C,* all 5 targeted dsRNA treatments decreased *TotM-luc* reporter activity comparably to the library dsRNAs.

To ensure that the obtained results were not caused by an artifact related to the reporter assay, we studied the endogenous *TotM* and *Act5C* expression levels of *hop*$^{Tum\text{-}l}$-transfected and dsRNA-treated S2 cells using qRT-PCR (Fig. 1*D*). RNAi targeting any of the identified genes resulted in at least a 50% reduction in relative *TotM* expression level, indicating that these genes are required for normal hop$^{Tum\text{-}l}$ -induced *TotM* response in S2 cells. Based on these results, *enok, Taf1,*



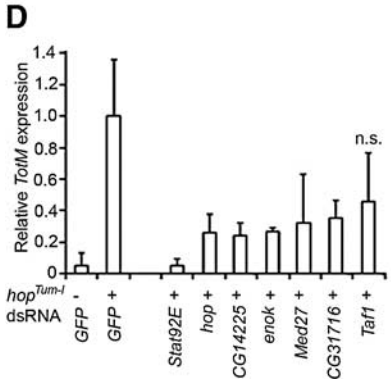


Figure 1. Genome-wide RNAi screen to identify gene products required for *Drosophila* JAK/STAT signaling. *A*) *TotM-luc* reporter activity is induced by hop^{Tum-l} in a Stat92E-dependent manner in S2 cells. Expression of hop^{Tum-l} caused more than 5-fold induction in the *TotM-luc* activity, which was blocked by RNAi targeting *Stat92E*. S2 cells were transfected with *TotM-luc* reporter plasmid together with hop^{Tum-l} and *Act5C-β-gal* reporter. $^{***}P \leq 0.001$. *B*) Results of the genome-wide RNAi screen of the *Drosophila* JAK/STAT signaling. Luciferase and β-galactosidase values are plotted on a log-scale. Light blue dots represent *GFP* dsRNA-treated negative control samples, and *Stat92E* dsRNA-treated positive controls are shown in purple. Samples located near the purple dots in the top left corner of the plot represent most potential positive regulators of JAK/STAT signaling. S2 cells were transfected with hop^{Tum-l}, *TotM-luc,* and *Act5C-β-gal* plasmids together with experimental or control dsRNAs. In total, 16,025 independent dsRNAs for their effect on *TotM-luc* reporter activity induced by hop^{Tum-l} were analyzed. *C*) Targeted dsRNA-treatments against potential regulators of the *Drosophila* JAK/STAT pathway. To confirm the initial findings of the screen, independent dsRNAs targeting indicated genes were designed and tested for their effect on hop^{Tum-l}-induced *TotM*-reporter activity as in panel *A*. All targeted dsRNAs decrease hop^{Tum-l}-induced *TotM-luc* reporter activity by more than 50% in S2 cells as compared to *GFP* dsRNA-treated cells. *D*) Endogenous *TotM* expression is reduced by dsRNA treatments targeting any of the 5 novel regulators of the *Drosophila* JAK/STAT pathway. dsRNAs targeting indicated genes were transfected into S2 cells together with hop^{Tum-l}. Endogenous *TotM* expression levels were measured by qRT-PCR and results normalized to *Act5C* expression values. All data are shown as means ± SD, $n \geq 4$. Values of $P \leq 0.05$ unless indicated otherwise.

CG31716, CG14225, and *Med27* are potential regulators of *Drosophila* JAK/STAT signaling, and these 5 genes were subjected to further studies.

CG31716 and CG14225 are general modifiers of JAK/STAT signaling, whereas enok, Med27, and Taf1 are more context sensitive

To assess the role of identified genes in JAK/STAT signaling in more physiological context, we activated the JAK/STAT signaling in S2 cells by overexpressing the ligand *upd,* and we used *TotM-luc* reporter to measure the pathway activity. As shown in **Fig. 2*A***, dsRNA treatments targeting *enok, Taf1,* and *CG31716* reduced upd-induced *TotM* expression in a similar manner compared to hop^{Tum-l}-induced *TotM* response, whereas RNAi against *Med27* showed no effect. Intriguingly, RNAi targeting *CG14225* caused strong hyperactivation of the *TotM* reporter activity in this setting.

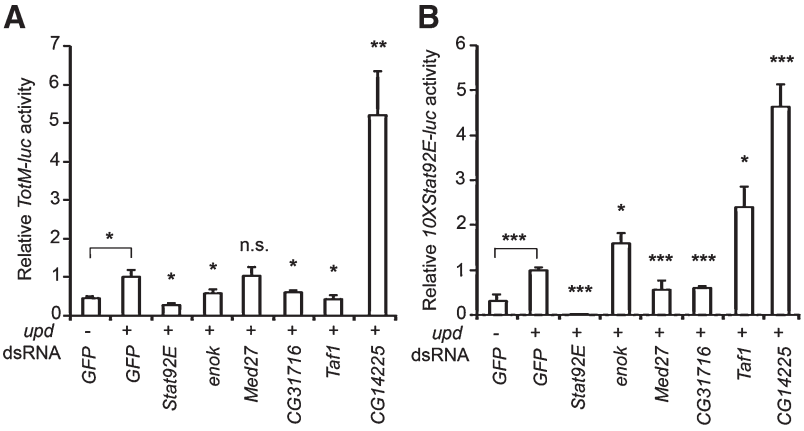


Figure 2. CG31716 and CG14225 are general modifiers of JAK/STAT signaling, whereas enok, Med27, and Taf1 are more context sensitive. *A*) RNAi targeting *enok, CG31716,* and *Taf1* decreases upd-induced *TotM-luc* activity in S2 cells, whereas *CG14225* dsRNA strongly enhances *TotM-luc* response. *B*) RNAi targeting *Med27* and *CG31716* decreases upd-induced *10xStat92E-luc activity* in S2 cells, whereas *CG14225* and *Taf1* RNAi enhances the response. S2 cells were transfected with *TotM-luc* (*A*) or *10xStat92E-luc* (*B*) and *Act5C-β-gal* reporter constructs together with indicated dsRNAs and *upd* to induce the pathway. Data are shown as means ± SD, $n \geq 4$, $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

Next, we tested whether these novel regulators are specific for regulating *TotM* expression, or whether they affect JAK/STAT target genes in a more general manner. To this end, we induced JAK/STAT signaling by expressing *upd,* and we used *10xStat92E-luc* reporter consisting of a sequence containing a double Stat92E binding site from a *SOCS36E* enhancer region multiplied 10 times, to measure the JAK/STAT pathway activity (31). As shown in Fig. 2*B, upd* expression caused 3.1-fold induction in *10xStat92E-luc* reporter activity. This induction is strongly inhibited by dsRNA treatments targeting *CG31716* and *Med27,* suggesting that these genes are required for Stat92E-dependent activation of target genes in *Drosophila* S2 cells. Interestingly, RNAi targeting *enok* or *Taf1* increased upd-induced *10xStat92E* reporter activity, indicating that these gene products have a more specific effect on regulating *TotM* expression. Of note, as enok also had been identified earlier as a negative regulator of *Drosophila* JAK/STAT signaling (31, 32), our results with *10xStat92E-luc* reporter are in line with the earlier reports. *CG14225* RNAi caused clear hyperactivation of *10xStat92E-luc* reporter also in this setting. Furthermore, *CG14225* dsRNA treatment caused a strong increase in another Stat92E-responsive reporter, *3 × 2xDraf-luciferase* activity (32) in response to *upd* expression in S2 cells (more than 10-fold induction compared to GFP dsRNA-treated controls, data not shown). These results related to the role of CG14425 in upd-induced JAK/STAT signaling are in striking contrast compared to our results with $hop^{Tum\text{-}l}$ induction and prompted us to study this gene in more detail.

CG14225/ET is a *Dome*-related gene that negatively regulates the JAK/STAT signaling in *Drosophila* S2 cells

CG14225/ET is a 3.3 kb gene comprising 3 separate coding sequences (**Fig. 3*A***). It is located next to the

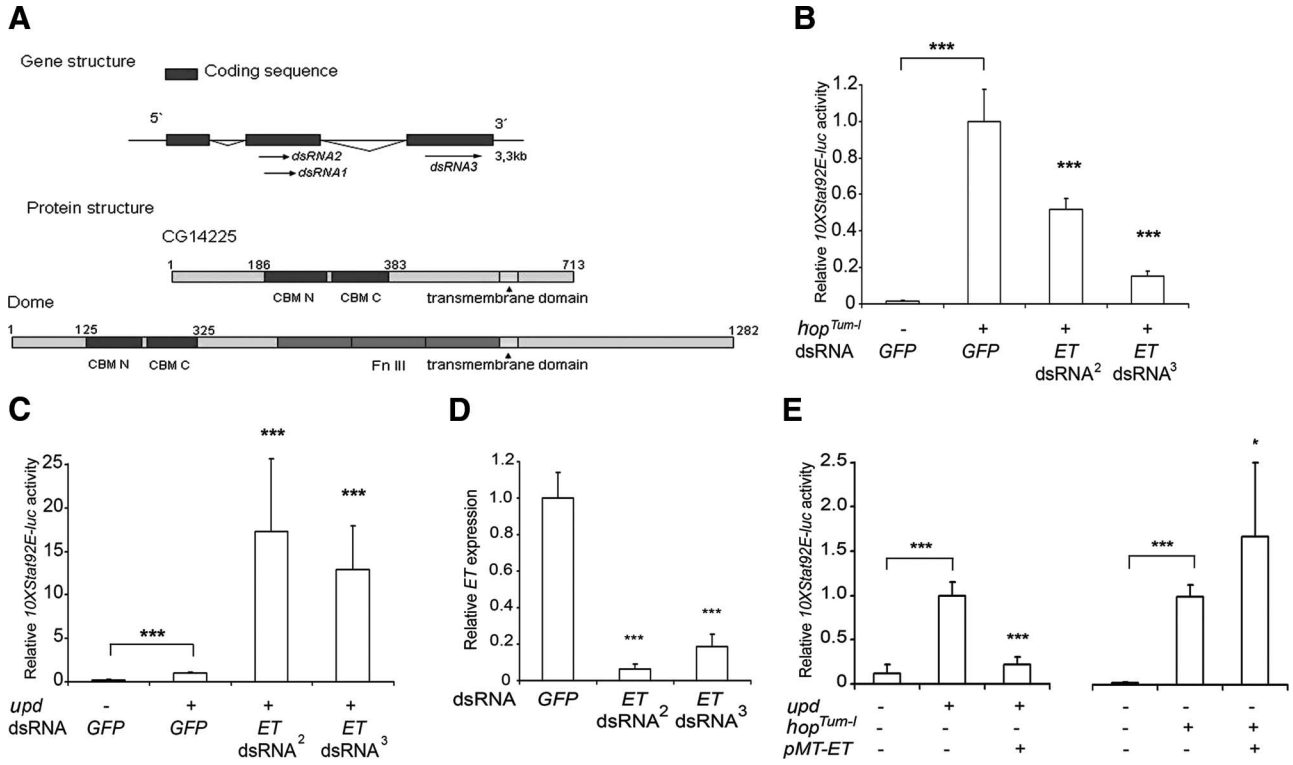


Figure 3. ET is a negative regulator of the *Drosophila* JAK/STAT pathway and is structurally related to Dome. *A*) Schematic representation of gene and protein structures of *CG14225/ET*. The *ET* gene consists of 3 exons and has no UTR regions. Sequence regions where *ET* dsRNAs ($dsRNA^{1-3}$) were designed are shown. Domain structure of ET protein is illustrated and compared to domain structure of Dome in the bottom panel. N-terminal fibronectin-type III domain of the cytokine-binding module (CBM N) and C-terminal fibronectin-type III domain of the cytokine-binding module (CBM C) are highly conserved in both proteins. The fibronectin-type III (FnIII) domain triplet near the transmembrane domain in Dome is absent in ET. *B, C*) RNAi targeting different regions of *ET* has a similar effect on both $hop^{Tum\text{-}l}$- and upd-induced *10xStat92E-luc* activity. S2 cells were transfected with *10xStat92E-luc* reporter plasmid together with $hop^{Tum\text{-}l}$ or upd constructs for induction of the pathway. Two dsRNAs targeting different parts of the *ET* gene decrease $hop^{Tum\text{-}l}$-induced *10xStat92E-luc* activity (*B*) and increase upd-induced *10xStat92E-luc* activity (*C*), compared to *GFP* dsRNA-treated controls in S2 cells. *D*) RNAi targeting *ET* strongly decreases the amount of *ET* transcripts in S2 cells compared to *GFP* dsRNA-treated controls. Endogenous *ET* mRNA levels after dsRNA treatments were measured using qRT-PCR. *E*) Overexpression of *ET* blocks upd-induced *10xStat92E-luc* activity in S2 cells, but enhances $hop^{Tum\text{-}l}$-induced *10xStat92E-luc* response. S2 cells were transfected with an *ET* overexpression construct or an empty vector together with $hop^{Tum\text{-}l}$ or upd to induce the pathway. All data are shown as means ± SD, $n \geq 4$. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

Drosophila JAK/STAT pathway receptor *Dome* in the genome. Furthermore, the *ET* gene encodes a 713 amino acid type-I transmembrane protein that shares structural similarities with Dome (11). Like Dome, *ET* has a cytokine binding module (CBM) in the N terminus but lacks 3 FnIII domains near the transmembrane domain. The CBM domain of vertebrate interleukin receptors is composed of 2 FnIII domains, containing 4 conserved cysteine residues in the N-terminal domain and a conserved WSXWS motif in the C-terminal domain (42). CBM in Dome and CG14225/ET share these features, but the WSXWS motif is incomplete in both (NTLWS/GSPWS). Intriguingly, although Dome shares similarities with the mammalian interleukin-6 (IL-6) receptor family members, ET's closest human homologue is the signal transducing protein gp130 with 12.7% identity, mostly found in the extracellular region. In mammalian IL-6 signaling, gp130 associates with IL-6 receptors that have bound their ligand and dimerizes, therefore allowing transduction of the signal to activate target molecules, including JAKs and STATs (43). Thus, ET shares structural similarities with Dome and mammalian gp130, which plays an important role in regulation of JAK/STAT signaling. The structure of the *ET* gene product explains in part why we observed opposing results with *ET* RNAi depending on what level the JAK/STAT pathway was activated at, because with the transmembrane domain, *ET* is likely to function at the level of Dome, thus epistatically between upd and hop.

To verify that the observed *ET* RNAi phenotype was not due to off-target effects, we generated another dsRNA targeting the third exon of *ET* (the 3 different dsRNAs targeting *ET* are shown schematically in Fig. 3*A*). As shown in Fig. 3*B,* both targeted dsRNAs ($dsRNA^2$ and $dsRNA^3$) caused strong inhibition of $hop^{Tum\text{-}l}$-induced *10xStat92E-luc* reporter activity. Furthermore, both targeted dsRNAs caused hyperactivation of the upd-induced *10xStat92E-luc* reporter activity (Fig. 3*C*), indicating that independent dsRNAs targeting *ET* cause similar phenotypes. Furthermore, hyperactivation of *10xStat92E-luc* reporter was also observed when upd-conditioned medium (32) was added on *ET* dsRNA-treated cells (data not shown). To confirm efficiency of RNAi on the *ET* mRNA level, we treated S2 cells with both targeted dsRNAs against *ET* and analyzed mRNA levels using qRT-PCR (Fig. 3*D*). Both dsRNAs resulted in strongly decreased *ET* mRNA levels compared to GFP dsRNA-treated control cells, indicating that these treatments effectively suppress *ET* expression in S2 cells. Taken together, these results indicate that *ET* RNAi causes hyperactivation of upd-induced JAK/STAT response in S2 cells.

To test whether overexpression of *ET* has an effect on JAK/STAT signaling in S2 cells, we cloned *ET* to a *Drosophila* expression vector. As shown in Fig. 3*E,* overexpression of *ET* caused a marked decrease in upd-induced *10xStat92E-luc* reporter activity in S2 cells and consistently with the RNAi phenotype results, 1.6-fold increase in $hop^{Tum\text{-}l}$-induced *TotM-luc* activity. Of note, *ET* mRNA contains no UTR regions, and thus it was not possible to carry out a rescue experiment where endogenous *ET* would be knocked down by dsRNA targeting the UTR regions and the resulting phenotype would then be rescued by overexpression of *ET* construct.

To analyze whether *ET* RNAi affects signaling cascades in S2 cells in a more general manner, we investigated the Toll and the Imd pathway signaling in *ET* dsRNA-treated S2 cells. *ET* RNAi did not significantly affect the heat-killed *E. coli*-induced *Attacin* (Imd pathway target gene) reporter activity, and had only a minor effect on $Toll^{10b}$-induced *Drosomycin* (Toll pathway target gene) reporter activity in S2 cells (data not shown). These results indicate that *ET* is not a general regulator of signaling pathways in S2 cells, but its function is more specific to the JAK/STAT pathway.

ET is an intrinsic component of the Dome receptor complex, and it functions as a regulator of Stat92E phosphorylation

Curiously, RNAi targeting *ET* caused different phenotypes in *TotM-luc* and *10xStat92E-luc* reporter assays depending on whether $hop^{Tum\text{-}l}$ or upd was used to trigger the JAK/STAT pathway signaling. To investigate the function of ET, we tested whether *ET* RNAi phenotype can be suppressed by dual RNAi treatments targeting known regulators of *Drosophila* JAK/STAT signaling in S2 cells. S2 cells were treated with *ET* dsRNA and with dsRNA targeting known components of the JAK/STAT pathway, *hop, Stat92E,* or *Dome.* The JAK/STAT pathway activity was measured using *TotM-luc* reporter. *ET* RNAi caused a subtle activation of the *TotM-luc* reporter activity (**Fig. 4*A***), suggesting that ET acts as a constitutive negative regulator of the JAK/STAT pathway in S2 cells. RNAi targeting any of the known *Drosophila* JAK/STAT pathway components (*Dome, hop,* or *Stat92E*) prevented this activation, which suggests that ET function is dependent on these components and that ET acts upstream or at their level in S2 cells.

To gain a more mechanistic insight about ET-mediated inhibition of JAK/STAT signaling, we coimmunoprecipitated overexpressed V5-tagged components of the *Drosophila* JAK/STAT signalosome with myc-tagged ET in S2 cells (Fig. 4*B*). At first, we confirmed the method by hop and Dome coimmunoprecipitation, and as expected, hop-V5 coimmunoprecipitated with Dome-myc (Fig. 4*B*). Notably, both hop-V5 and Dome-V5 coimmunoprecipitated with ET-myc, suggesting that overexpressed ET interacts directly with key regulators of the *Drosophila* JAK/STAT signalosome in S2 cells. Of note, we were unable to coimmunoprecipitate upd or Stat92E with ET (data not shown).

To gain further insight to the molecular function of ET, we analyzed whether ET suppresses JAK/STAT signaling by affecting the dimerization of Dome. As shown in **Fig. 5*A***, Dome-Dome interaction, as analyzed by immunoprecipitating V5-tagged Dome with myc-

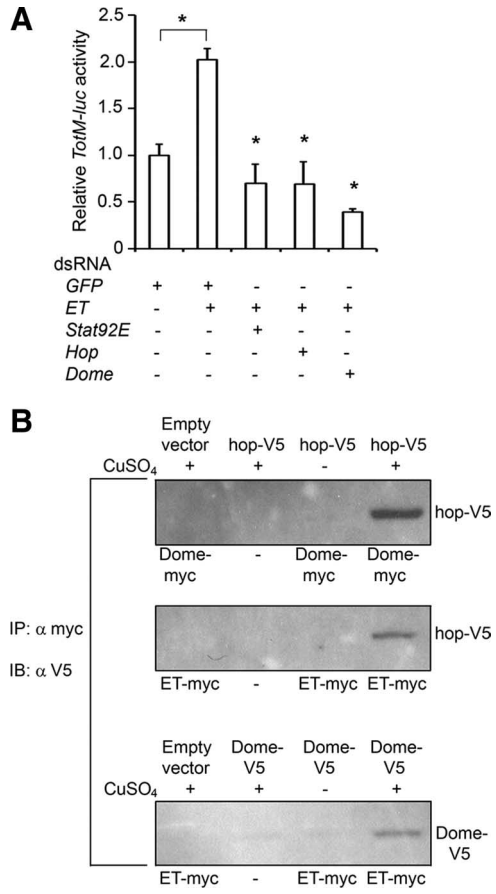


Figure 4. ET functions at the level or upstream of Dome and coimmunoprecipitates with hop and Dome. *A*) ET functions at the level or upstream of Dome. *TotM*-luc reporter was activated by RNAi targeting *ET*. RNAi targeting known components (*hop, Stat92E, Dome*) of the *Drosophila* JAK/STAT pathway abolishes *ET* RNAi -induced *TotM* activity, indicating that these components are located functionally downstream of ET. Data are shown as means ± SD, $n \geq 4$. $^*P \leq 0.05$. *B*) ET coimmunoprecipitates with both hop and Dome. S2 cells were transfected with either ET-myc or Dome-myc and hop-V5 or Dome-V5. Immunoprecipitation (IP) was done with anti-myc (α myc) antibody and immunoblotting (IB) with anti-V5 (α V5) antibody. $CuSO_4$ was used to induce the expression of the constructs.

tagged Dome, was not affected by overexpression of *ET* (or *ET* RNAi). Similarly, overexpression of *ET* (or *ET* RNAi) did not alter the interaction between hop-V5 and Dome-myc (Fig. 5*A*). These results indicate that ET is not likely to function by preventing dimerization of Dome, or by disrupting Dome-hop interaction in S2 cells.

Next, we studied whether ET affects the kinase activity of the *Drosophila* JAK/STAT signalosome by investigating Stat92E phosphorylation upon upd induction in S2 cells (Fig. 5*B*). S2 cells were transfected with indicated dsRNAs and *upd* to activate signaling leading to Stat92E phosphorylation. Then 72 h after transfection, S2 cell protein lysates were separated by SDS-PAGE and analyzed by Western blotting with anti-Stat92E N-terminal antibody. RNAi targeting *ET* caused Stat92E band shift upon activation with upd compared to *GFP* dsRNA-treated controls, suggesting hyperphosphorylation of Stat92E. To ensure that this upper band represents a phosphorylated form of the protein, Stat92E was immunoprecipitated from S2 cell lysates with anti-Stat92E N-terminal antibody and detected with phospho-tyrosine-specific antibody (Fig. 5*B*). Hyperphosphorylation of Stat92E was abolished when *Dome* or *hop* dsRNA was cotransfected with *ET* dsRNA (Fig. 5*B*), indicating that ET function is dependent on these factors. *Stat92E* dsRNA strongly decreases the signal demonstrating the specificity of the antibody.

Taking these data together, we see that ET is a negative regulator of the JAK/STAT pathway in *Drosophila* S2 cells. ET functions as a regulator of Stat92E phosphorylation and is located functionally at the level or upstream of the receptor Dome. Furthermore, ET appears as an intrinsic component of the Dome receptor complex as it coimmunoprecipitates with both hop and Dome when overexpressed in S2 cells, but it does not affect Dome dimerization or Dome-hop interaction. The mechanistic function of ET is shown schematically in Fig. 5*C*.

ET negatively regulates *Tot* gene expression *in vivo*

ET functions as a negative regulator of JAK/STAT signaling *in vitro*. JAK/STAT pathway is required for *Tot* gene expression under stressful conditions in *Drosophila in vivo* (17, 41). To investigate the role of *ET* in JAK/STAT signaling *in vivo,* we crossed fly lines carrying *UAS-RNAi* constructs targeting *ET* (*ET IR*^{1} and *ET IR*^{2}) (44) over *C564-GAL4* flies, which drive GAL4 expression in the adult fatbody. Thereafter the relative expression levels of both *TotM* and *TotA* in response to septic injury with *E. cloacae* were measured in experimental and control progeny flies by qRT-PCR (**Fig. 6*A, B***). As expected, *C564-GAL4*-driven expression of *UAS-Stat92E* RNAi strongly impaired both *TotM* and *TotA* response to *E. cloacae* compared to controls crossed over w^{1118} (Fig. 6*A, B*). On the contrary, *C564-GAL4*-driven *UAS-ET* RNAi markedly enhanced both *TotM* and *TotA* expression. These results are in agreement with the results obtained using S2 cells and indicate that *ET* negatively regulates *Tot* gene expression in adult *Drosophila*. Of note, infection with *E. cloacae* did not affect the level of *ET* expression, which suggests that *ET* expression is not regulated by the JAK/STAT (or the Imd) pathway (data not shown).

Genetic background may affect gene expression levels under experimental conditions. To avoid bias caused by genetic background, we analyzed the *in vivo ET* RNAi phenotype using a drug-inducible ubiquitous driver *GeneSwitch-GAL4,* which activates the expression of the RNAi construct when Mifepristone is added to the food vials. This enables monitoring the offspring from each cross with and without expression of the RNAi construct, therefore providing a genetically relevant control. Figure 6*C, D* shows that in Mifepristone-induced *ET* RNAi flies, both *TotM* and *TotA* expression in response to septic injury with *E. cloacae* are hyperac-

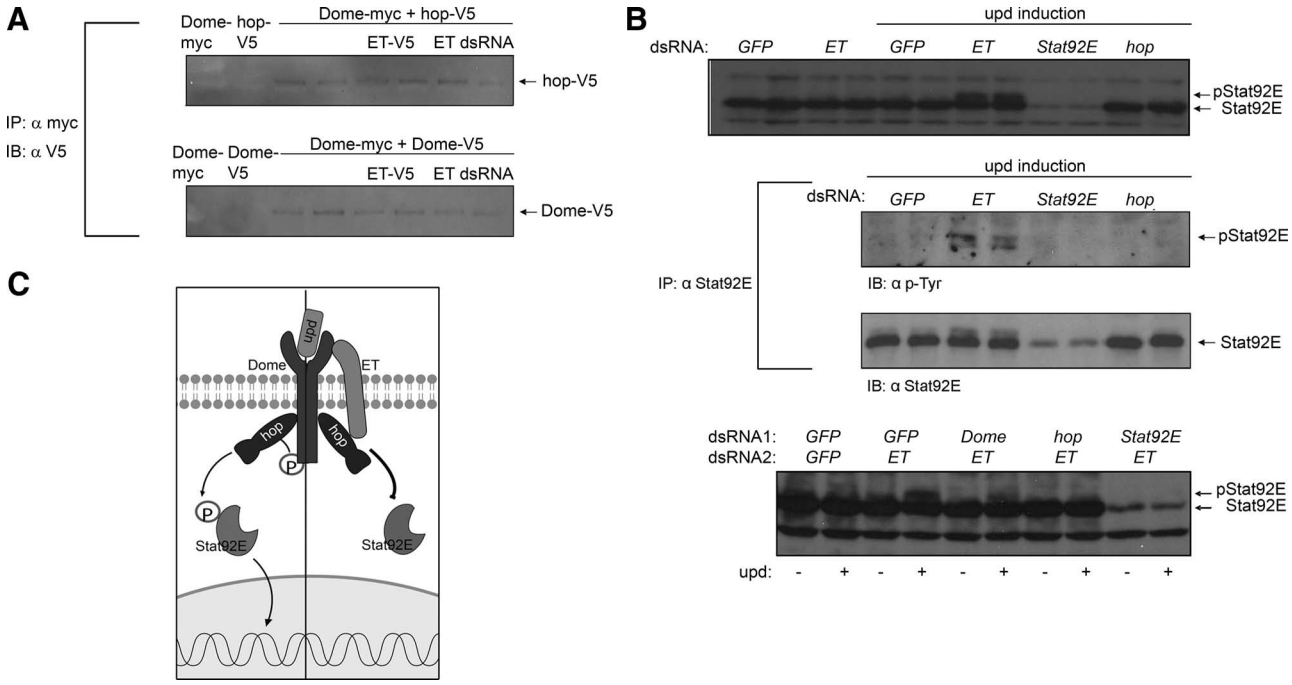


Figure 5. *ET* RNAi causes hyperphosphorylation of Stat92E in response to *upd* expression in S2 cells. *A*) ET does not affect the interaction between hop and Dome (top panel) or homodimerization of Dome (bottom panel). The effect of *ET* overexpression or RNAi was studied by transfecting S2 cells with Dome-myc and hop-V5 or Dome-myc and ET-V5 or treated with *ET* dsRNA. Immunoprecipitation (IP) was done with antimyc (α-myc) antibody and immunoblotting (IB) with anti-V5 (α V5) antibody. *B*) *ET* RNAi causes Stat92E hyperphosphorylation in response to activation of JAK/STAT signaling with upd (3 top panels). The intensity of the phosphorylation bands of Stat92E is significantly increased in *ET* dsRNA-treated samples compared to *GFP* dsRNA-treated controls. Hyperphosphorylation of Stat92E is abolished when *Dome* or *hop* dsRNA is cotransfected with *ET* dsRNA (bottom panel), indicating that function of ET is dependent on these factors. S2 cells were transfected with dsRNAs as indicated with or without *upd* to activate the JAK/STAT pathway. Cells were lyzed 72 h after transfection, and proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-Stat92E N-terminal antibody. IP was done with anti-Stat92E N-terminal antibody followed by SDS-PAGE and IB with antiphospho-Tyrosine antibody. After stripping, the same membrane was reprobed with anti-Stat92E N-terminal antibody showing equal amounts of protein in the immunoprecipitates (middle panels). *C*) Schematic representation of the canonical *Drosophila* JAK/STAT signaling (left) and the inhibitory function of ET (right).

tivated compared to control flies with a drug-free diet. As expected, *Stat92E* RNAi strongly inhibits both *TotM* and *TotA* induction under these conditions, demonstrating that the *GeneSwitch-GAL4* driver was operative.

Tot gene expression is controlled jointly by the JAK/STAT and by the Imd pathway on septic injury in *Drosophila* (17, 41). By crossing *UAS-ET* RNAi flies over *C564-GAL4* flies and measuring *Attacin B* expression on *E. cloacae* septic injury in the offspring, we confirmed that the effect of *ET* RNAi on *Tot* gene expression is not mediated by the Imd pathway (data not shown).

ET RNAi is protective in a gastrointestinal infection model with *Serratia marcescens*

Recently, the *Drosophila* JAK/STAT signaling has been shown to be important for survival of the flies after intestinal bacterial infection (45, 46). In response to infection or damage, enterocytes in the *Drosophila* midgut produce upd, upd2, and upd3, which activate JAK/STAT signaling in intestinal stem cells, leading to cell division and regeneration of the gut epithelium (45). *Serratia marcescens* is an entomopathogenic bacterium that can infect *Drosophila* through the digestive tract. It was shown that despite the local immune response induced, *S. marcescens* infection causes disruption of the gut morphology, which contributes to death of the flies that follows in a few days (40). Thus, we used the *S. marcescens* intestinal infection assay to study the role of ET in microbial resistance *in vivo* (39, 40, 46). *ET* RNAi lines (*ET IR*^{1} and *ET IR*^{2}) were crossed over the *GeneSwitch-GAL4* driver line, and w^{1118} and *Stat92E* over *GeneSwitch-GAL4* were used as controls. As shown in **Fig. 7**, *ET* RNAi flies survived better in food contaminated with *S. marcescens* than flies in which RNAi was not induced by Mifepristone. In contrast, flies with induced *Stat92E* RNAi were more susceptible than flies with no RNAi induction (Fig. 7*A, B*). These data support previous reports suggesting that JAK/STAT signaling is involved in survival of the flies from *S. marcescens* infection *in vivo,* and that *ET* RNAi enhances their resistance to *S. marcescens.* The enhanced resistance against gastrointestinal infection may be due to improved stem cell renewal caused by hyperactivated JAK/STAT pathway (45), although it is plausible that the protective effect of *ET* RNAi is caused by mechanisms independent of JAK/STAT signaling.

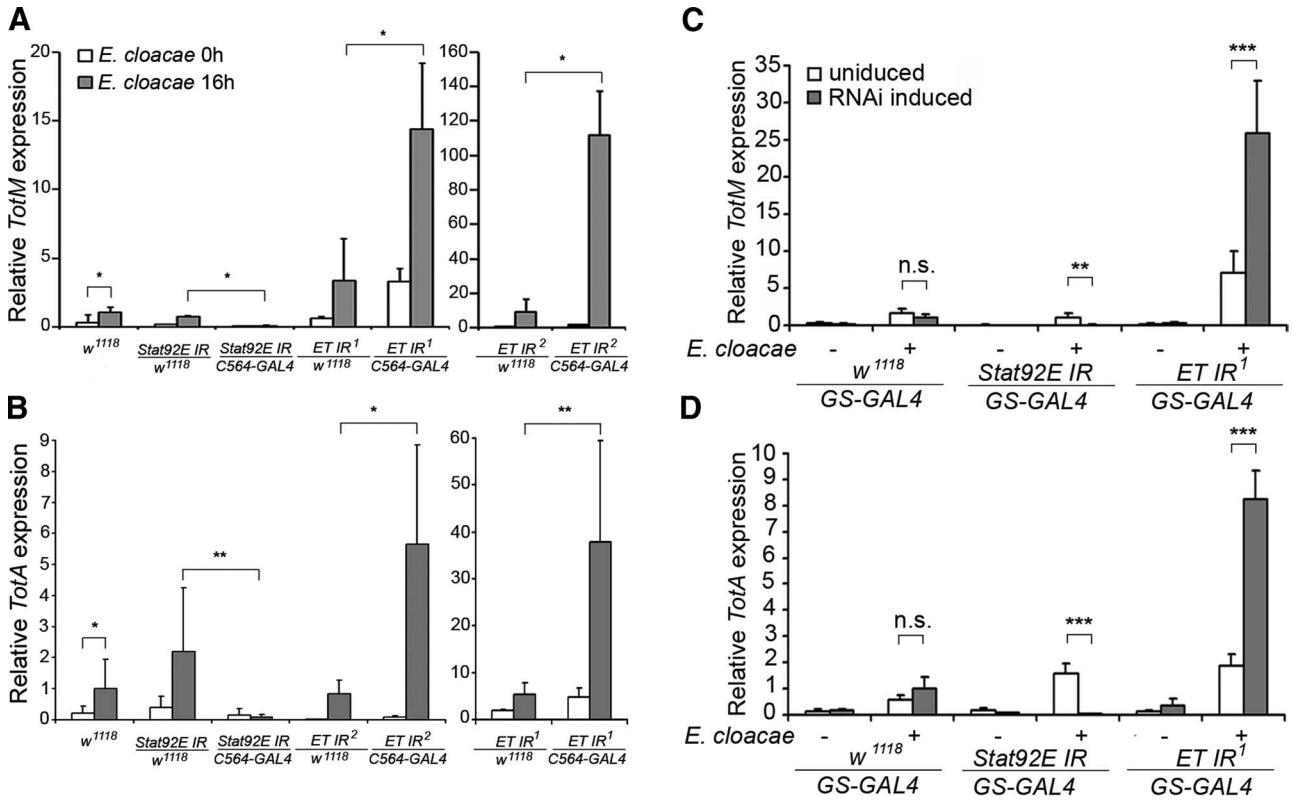


Figure 6. *ET in vivo* RNAi increases JAK/STAT pathway response. *A, B*) *ET* RNAi strongly increases *TotM* (*A*) and *TotA* (*B*) expression in response to septic injury in *Drosophila in vivo,* whereas *Stat92E* RNAi abolishes *Tot* gene expression. Flies carrying the *UAS*-RNAi constructs *ET IR* [1], *ET IR*[2], or *Stat92E IR* (positive control) were crossed over either *C564-GAL4* driver or w^{1118} flies (negative control). The offspring were infected with *E. cloacae* to induce JAK/STAT signaling. *TotM* and *TotA* expression levels were measured from extracted total RNAs by qRT-PCR. *C, D*) *ET* RNAi also dramatically increases *TotM* (*C*) and *TotA* (*D*) expression with an inducible ubiquitous *GeneSwicth-GAL4* driver as compared to flies with the same genetic background with no RNAi induction. In the positive control (*Stat92E* RNAi flies), induction of RNAi abolished *Tot* gene expression, whereas in the negative control (w^{1118}) *Tot* gene expression was not affected by induction. In flies crossed over *GeneSwitch-GAL4* driver, RNAi was induced by adding the drug Mifepristone to food vials, and flies with a drug-free diet (uninduced) were used as controls. All data are shown as means ± SD, $n \geq 4$. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

ET RNAi causes eye overgrowth in adult flies

To gain more direct evidence that ET negatively regulates JAK/STAT signaling *in vivo,* we investigated whether *ET* RNAi affects upd-induced eye overgrowth. JAK/STAT signaling has been shown to be important in regulation of *Drosophila* eye imaginal disc development (47, 48). This is demonstrated by the fact that hyperactivation of the pathway by ectopic overexpression of the ligand *upd* in the developing eye leads to distinct overgrowth (48; Fig. 7*C*). Furthermore, the overgrowth phenotype has been shown to be modulated by overexpression or removal of several JAK/STAT pathway components and regulators (*e.g.,* refs. 32, 48–50). To this end, we tested whether *ET* RNAi affects eye development. Expressing the *ET* RNAi construct under an eye-specific driver (*ey-GAL4*) alone did not affect eye development (Supplemental Fig. S1). However, when these flies were further crossed to *GMR-upd*Δ*3′* flies that overexpress *upd* in the eye, the resulting offspring had more severe eye overgrowth than flies from the same cross without *ET* RNAi (Fig. 7*C* and Supplemental Fig. S1). To evaluate the eye phenotypes objectively, we created a scoring system to quantify the observations. Pictures of each fly's eyes were independently evaluated by 5 experienced researchers from our group as a blind test, and the eye phenotypes were given scores 0–5, 0 representing wild type and 5 the most severe phenotype. The results in Fig. 7*D* show that the eye phenotype of flies carrying both *GMR-upd*Δ*3′* and *ET* RNAi construct together with the *ey-GAL4* driver is more severe than that of flies with only *GMR-upd*Δ*3′*. These results suggest that ET regulates JAK/STAT pathway-mediated eye overgrowth in *Drosophila.*

DISCUSSION

JAK/STAT signaling is involved in a variety of processes in both *Drosophila* and mammals. To identify gene products involved in regulation of *Drosophila* JAK/STAT signaling, we carried out a genome-wide reporter assay-based RNAi screen in S2 cells. A constantly active form of hop ($hop^{Tum\text{-}l}$) was chosen for pathway activation in order to focus on the more evolutionarily

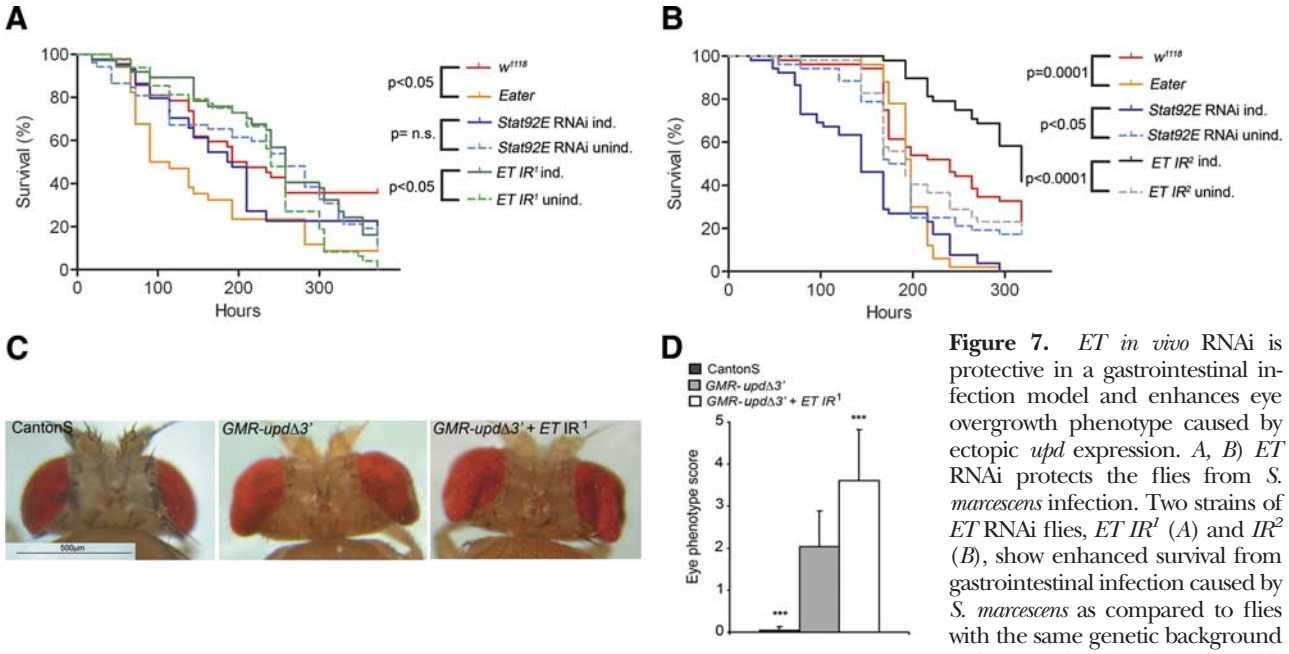


Figure 7. *ET in vivo* RNAi is protective in a gastrointestinal infection model and enhances eye overgrowth phenotype caused by ectopic *upd* expression. *A, B*) *ET* RNAi protects the flies from *S. marcescens* infection. Two strains of *ET* RNAi flies, *ET IR*^{1} (*A*) and *IR*^{2} (*B*), show enhanced survival from gastrointestinal infection caused by *S. marcescens* as compared to flies with the same genetic background with no RNAi induction. Flies with induced *Stat92E* RNAi show decreased survival, as well as *eater* mutant flies, which were used as controls. *ET* RNAi flies were crossed over the drug-inducible driver *GeneSwitch-GAL4*. Mifepristone was used to induce the expression of the RNAi construct. w^{1118} and *Stat92E* RNAi flies crossed over *GeneSwitch-GAL4* flies were used as an additional control. *C*) *ET* RNAi enhances upd-induced eye overgrowth. Eye overgrowth phenotype caused by overexpression of *upd* in the developing eye (*GMR-updΔ3′*) is more severe in flies that also express an *ET* RNAi construct with an eye-specific driver (*ey-GAL4*). *D*) The eye phenotype in flies with ectopic expression of both *upd* and *ET* RNAi in their eyes was significantly more severe than in flies with *upd* alone. Grades: 0 = wild-type, 5 = most severe eye phenotype. $n = 8$ for CantonS flies and $n \geq 16$ for *GMR-updΔ3′* and *GMR-updΔ3′ + ET* IR^{1} flies. Data are shown as means ± SD. $^{***}P < 0.001$ *vs. GMR-updΔ3′*.

conserved intracellular part of the signaling cascade. To identify the most important regulators, we subjected the genes, whose RNAi repeatedly caused >50% decrease on the luciferase activity, to further studies. The original findings of the screen were confirmed *in vitro* by several means using different dsRNAs, different reporters, and different ways to induce the JAK/STAT signaling. In this way, we identified 5 novel putative regulators of *Drosophila* JAK/STAT signaling. This is a reasonable number of gene products that can be directly involved in JAK/STAT pathway regulation and probably excludes factors that affect JAK/STAT pathway activity indirectly *via* crosstalk with other signaling pathways. In addition, our screen found 2 previously known intracellular components of the pathway, *hop* and *Stat92E,* confirming the validity of our screen. Notably, we identified the gp130/IL-6R related transmembrane protein ET as a novel negative regulator of *Drosophila* JAK/STAT pathway.

ET is a negative regulator of *Drosophila* JAK/STAT signaling

Our RNAi screen was originally set out to identify novel positive regulators of *Drosophila* JAK/STAT pathway. *ET* RNAi caused strong decrease in hop$^{Tum\text{-}l}$-induced *TotM-luc* activity in our primary screen. Curiously, when the JAK/STAT signaling was induced using upd, the ligand of the pathway, the phenotype of *ET* RNAi was the exact opposite. In this setting, *ET* RNAi increased the activity of all reporters tested (*TotM-luc, 10xStat92E-luc,* and *3×2xDraf-luc*) by at least 5-fold, indicating that the RNAi phenotype was not reporter sensitive, and therefore not likely to be highly context-dependent or due to an artifact. Furthermore, overexpression of *ET* in S2 cells also produced opposite luciferase assay phenotypes with different activating molecules. Even though these results are consistent with the RNAi phenotypes observed, the reason for different phenotypes of *ET* knockdown or overexpression observed depending on the activating molecule remains speculative. However, several pieces of evidence suggest partial explanations for this phenomenon.

In the *Drosophila* genome, the sequence coding for *ET* is located next to that of *Drosophila* JAK/STAT pathway receptor *Dome*. ET polypeptide carries a putative transmembrane domain and shares sequence similarity to Dome, especially in the extracellular domain (11); therefore, *ET* is likely a result of gene duplication.

Both the proposed protein structure of ET and our experimental data from double-RNAi and coimmunoprecipitation assays suggest that ET functions on the cell membrane, epistatically at the level upd and hop. hop$^{Tum\text{-}l}$ causes strong activation of *Drosophila* JAK/STAT signaling, but this induction appears to take a somehow pathological form. Interestingly, Dome was not among the known components identified in our screen for positive regulators. We therefore tested the

RNAi phenotype for *Dome* with both upd and hop^{Tum-l}-induction in S2 cells. As expected, when upd was used, *Dome* RNAi abolished reporter activity. In line with our ET results, when hop^{Tum-l} was used to activate JAK/STAT signaling, the phenotype for *Dome* RNAi was the opposite, the reporter activity being doubled (data not shown). These results implicate that the mutant hop is likely to behave abnormally in *Drosophila* cells acting independently of Dome, perhaps by using ET's short cytoplasmic tail as a docking site: This results in constitutive activation of JAK/STAT signaling and may explain the hop^{Tum-l}-mutant phenotype. Using ectopic expression of upd as an inducer of JAK/STAT pathway provides more physiological information about ET's function, and thus we conclude that ET is a negative regulator of the *Drosophila* JAK/STAT signaling.

ET's exact molecular function in regulation of *Drosophila* JAK/STAT signaling remains to be studied, but since it has putative cytokine binding motifs in the ectodomain, it could function as a decoy receptor that captures upd ligands from Dome. ET could also inhibit Dome activation by forming a nonsignaling heterodimer with Dome, or by inhibiting Dome homodimer-hop signalosome in some other manner. Since ET coimmunoprecipitated with Dome but did not affect Dome homodimerization, or interaction of hop with Dome, the latter statement appears more likely.

Regulation of JAK/STAT signaling in *Drosophila* and mammals: common mechanisms

The core JAK/STAT signaling pathway is evolutionarily conserved. Because of its role in diverse cellular processes, the JAK/STAT pathway needs to be strictly controlled at different levels of the cascade. Several regulatory mechanisms appear to be conserved from flies to humans, as many of the positive and negative regulators of *Drosophila* JAK/STAT signaling have been identified based on their homology with the mammalian counterparts. The *Drosophila* model has also provided important information about regulation of the JAK/STAT pathway. For example, the first evidence for the critical role of the JH2 pseudokinase domain in regulation of JAK activity and hematopoietic homeostasis was obtained in *Drosophila* (51). Of the known conserved negative regulators of the JAK/STAT pathway, *SOCS36E* is strongly induced by Stat92E, forming a negative feedback loop. dPIAS, on the other hand, interacts directly with Stat92E and affects its nuclear functions. According to our results, ET negatively regulates pathway at the level or upstream to Dome and is not induced *via* a negative feedback loop.

Both Dome and ET show homology with mammalian IL-6 receptor family members and the signal transducer gp130. In mammals, gp130 is able to form functional signaling complexes with several cytokine receptors, such as interleukin-6, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, cardiotrophin-1, the granulocyte colony-stimulating factor, IL-11, and IL-27 (43, 52–56). In these ligand-induced receptor complexes gp130 mediates the activation signal to multiple cytoplasmic signaling molecules *via* activation of JAKs that are constitutively associated with gp130 cytoplasmic domain. In *Drosophila,* the signaling cascade is simpler, and Dome is the only characterized JAK/STAT pathway receptor (22, 23). Dome homodimerization is needed for a proper upd-induced signal transduction and Stat92E activation (23), but it is still unclear if Dome participates in larger multisubunit receptor complexes resembling those seen in mammals.

The role of ET in *Drosophila in vivo*

Notably, we also assessed ET's role in *Drosophila* JAK/STAT signaling *in vivo*. Two *ET* RNAi strains crossed over a driver expressed in the fatbody and lymph glands (*C564-GAL4*) or a ubiquitous drug-inducible driver (*GeneSwitch-GAL4*) were tested by qRT-PCR for their effect on JAK/STAT pathway target gene expression in response to septic injury with *E. cloacae*. Both *ET* RNAi lines showed a significant increase in JAK/STAT pathway-dependent stress response compared to controls, indicating hyperactivation of JAK/STAT signaling. More direct *in vivo* model to study JAK/STAT activity in *Drosophila* is overexpression of *upd* in the developing eye, which leads to eye overgrowth due to hyperactivated JAK/STAT signaling (48). The eye overgrowth phenotype is shown to be modified by overexpression and removal of several JAK/STAT pathway components and regulators (32, 48–50). Notably, overexpression of *upd* together with *ET* RNAi construct under an eye-specific driver *ey-GAL4* lead to a significantly more severe eye overgrowth than overexpression of *upd* alone. In addition, *ET* RNAi appears to have a protective role in an *S. marcescens* gastrointestinal infection model. Enhanced JAK/STAT signaling activity may be advantageous in *S. marcescens*-infected flies for renewal of the injured gut wall (45). Accordingly, *Stat92E* RNAi flies were more susceptible to infection in this assay. It is possible, however, that the protective effect of *ET* RNAi is due to another mechanism unrelated to JAK/STAT signaling.

In summary, we identified 5 putative novel regulators of *Drosophila* JAK/STAT signaling in this study. Of these, ET is a negative regulator of JAK/STAT pathway signaling both *in vitro* and *in vivo*. ET is involved in Stat92E phosphorylation and coimmunoprecipitates with Dome and hop. The exact molecular mechanisms of how ET regulates Stat92E phosphorylation remains to be studied. FJ

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structs, Prof. Michael Boutros (German Cancer Research Center, Heidelberg, Germany) for *GMR-upd*Δ*3′* and *ey-GAL4* fly stocks and *pMT-upd* and *3* × *2-Draf-luc* constructs, and Erica Bach (New York University Langone Medical Center, New York, NY, USA) for *10xStat92E-luc* reporter. The authors also thank Prof. Jussi Taipale, Dr. Minna Taipale, and Dr. Mikael Björklund (University of Helsinki, Helsinki, Finland) for their help in generating our dsRNA library. This research was supported by grants from the Academy of Finland, the Foundation for Pediatric Research, the Sigrid Juselius Foundation, the Emil Aaltonen Foundation (to M.R.), the Competitive Research Funding of the Pirkanmaa Hospital District, and the Tampere Tuberculosis Foundation (to M.R. and S.V.). The authors declare they have no conflicts of interest. All authors approved the final version of the text.

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