The inducible antiviral immune response of *Drosophila melanogaster*.

vorgelegt von
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von der Fakultät III
Institut für Biotechnologie
der Technischen Universität Berlin
zur Erlangung des akademischen Grades

Doktorin der Ingenieurwissenschaften
- Dr.-Ing. -

genehmigte Dissertation

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Tag der wissenschaftlichen Aussprache: 30. Juli 2010

Berlin 2010
B83

MEINEN ELTERN.

DANKE.

This thesis is dedicated to my parents.
Thank you for all your love and support.

The research for this thesis was performed during
december 2006 to december 2009
in the *Institut de biologie moleculaire et cellulaire*, Strasbourg, France.

Acknowledgments

I would like to thank the many people who have been involved with this thesis.

First of all, I would like to thank Prof. Jules Hoffmann and Prof. Jean-Marc Reichhart for accepting me in the laboratory and allowing me to work in such a scientifically stimulating environment.

My sincere thank you to my supervisor Prof. Jean-Luc Imler for all of your support and guidance whilst working in the lab and during the process of writing my thesis. I have learned so much during the time I spent in your group.

I would also like to thank as well my second supervisor Prof. Roland Lauster who I started my scientific career with and who always had an open ear for me.

Thanks to everyone who participated to this work. I want to address a special thank you to all the members of the group and the institute that have shared this time of my life with me and became much missed friends. I would like to especially thank Safia, Steffi M., Ioannis, Stan, Yann, Steffi L. and Basti. There is not enough room to thank you for everything you did, let's simply say, this thesis would not have been finished without you. I wish you all the best for the future.

A big thank you to Estelle and Miriam for all your technical support. I equally would like to say thank you to Laurent Troxler and Charles Hetru for their bioinformatic support. I would like to thank as well the best student I ever had the pleasure to supervise, Francois and the recent team members Karim and Jessica. I wish you lots of luck for your projects.

A last but not least very special thank you to my wonderful husband and family that supported me all the way through. Thank you!

Abbreviations

aa	amino acids
AaSTAT	Aedes albopictus STAT
ACTH	adrenocorticotrophic hormone
AGO	Argonaute
AMP	antimicrobial peptide
ANV	American Nodavirus
bp	base pair
CARD	caspase recruitment domain
CARDIF	CARD adapter inducing IFN-β
cDNA	complementary DNA
cds	coding sequence
CGM	complete growth medium
CFM	Chifoumi
CrPV	Cricket paralysis virus
DAP	meso-diaminopimelic acid
DBV	Drosophila Birnavirus
DC	dendritic cell
DCL	Dicer-like
DCV	Drosophila C virus
DNA	desoxy ribonucleid acid
DREDD	Death related ced-3/Nedd2-like
dsRNA	double stranded RNA
DTV	Drosophila Totivirus
DXV	Drosophila X virus
eIF2	elongation initiation factor 2
ER	endoplasmatic reticulum
EST	expressed sequence tag
FADD	Fas-associated Death Domain
FHV	Flockhouse virus
GBP-1	guanylate binding protein-1
GNBP	Gram-negative binding protein
GPCR	G-protein-coupled receptors
GTPase	guanosine triphosphatase
h	hours
HBD	human β-defensin
HSPG	heparan sulphate proteoglycan
IFN	interferon
IIV 6	Invertebrate iridescent virus 6
imd	immune deficiency

IPS-1	IFN-β promotor stimulator-1
IRC	immune responsive catalase
IRES	internal ribosomal entry site
IRF	interferon regulatory factor
ISG	IFN-stimulated gene
ISRE	IFN-stimulated response element
Jak	Janus kinase
JEV	Japanese encephalitis virus
LDLR	low-density-lipoprotein receptor
MAP3K	Mitogen-Activated Protein 3 kinase
MAVS	mitochondrial antiviral signaling
MDA-5	melanoma differentiation-associated gene 5
μg	microgram
min	minute
ml	milliliter
Mx	myxovirus resistance
NCBI	National Center for Biotechnology Information
ng	nanogram
nt	nucleotide
NLR	NOD-like receptor
OAS	2'-5' oligo adenylate synthetase
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
PKC	protein kinase C
PRR	pattern recognition receptor
Q-PCR	quantitative real time PCR
RC2	retrocyclin 2
RdRP	RNA dependent RNA polymerase
RIG-I	retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RIP	receptor interacting protein
rpm	rounds per minute
SIGMAV	sigma virus
SH2	Src homology 2
SINV	Sindbis virus
siRNA	small interfering RNA
STAT	signal transducers and ativators of transcription

TBK1	TANK binding kinase 1
TCT	tracheal cytotoxin
TIR	Toll/IL-1 receptor
TLR	Toll-like recptor
TNF	tumor necrosis factor
TotA	Turandot A
TYK2	Tyrosine kinase 2
upd	unpaired
vRDRP	viral RNA dependent RNA polymerase
VAMP	virus associated molecular pattern
vir-1	virus-induced RNA1
VISA	virus-induced signaling adapter
VSR	viral suppressors of RNAi
VSV	vesicular stomatis virus

Zusammenfassung

In der vorliegenden Arbeit wurde *Drosophila melanogaster* als Modell genutzt, um die angeborene Immunantwort gegen virale Infektionen zu studieren.
Wir untersuchten mit Hilfe von genomweiten *microarrays* das Transkriptom von Fliegen, welche entweder mit dem Drosophila C Virus (DCV), dem Flockhouse Virus (FHV) oder dem Sindbis Virus (SINV) infiziert waren. Infektion mit diesen drei positiv orientierten Einzelstrang RNS Viren führte zu einer starken transkriptionellen Antwort, welche deutlich virusspezifische Induktionsmuster zeigte. Um die biologische Signifikanz dieser beobachteten Muster zu studieren, testeten wir Mutanten verschiedener Signalwege, nämlich den Imd, den Toll und den Jak-STAT Signalweg, für Suszebtibiltät nach viraler Infektion. Wir führten eine Vergleichsstudie durch, in welcher wir ein Set von 5 verschiedenen RNS Viren benutzten. Das Set beinhaltete die positiv orientierten RNS Viren DCV, Cricket Paralysis Virus (CrPV), FHV und SINV und den negativ orientierten RNS Virus Vesicular Stomatis Virus (VSV). Die Dicistroviren DCV und CrPV formten eine spezielle Gruppe unter den getesteten Viren, da Mutanten für den Jak-STAT und den Toll Signalweg anfällig fuer DCV und CrPV Infektion waren. Mutanten starben früher als Wildtyp Fliegen und zeigten erhöhte virale Titer. Im Gegensatz dazu, sahen wir keinen Phänotyp, weder im Überleben noch in den viralen Titern nach Infektion mit FHV, SINV oder VSV. Der Imd Signalweg wurde nicht als eine bedeutende Komponente der antiviralen Immunantwort identifiziert. Trotzdem zeigten Mutanten dieses Signalweges eine Tendenz zu reduzierten viralen Titern nach Dicistrovirus Infektion. Wir zeigten weiterhin, dass relativ gesehen, der Jak-STAT Signalweg genauso bedeutend für die Abwehr gegen Dicistriviren ist wie RNS Interferenz. Diese Daten unterstreichen die Wichtigkeit der induzierbaren Antwort in Drosophila.
Wir charakterisierten *Chifoumi* (*cfm*) alias *CG11501* als ein Beispiel fuer ein virusinduziertes Gen. Wir zeigten, dass *cfm* ein zirkulierendes Peptid kodiert, welches nach viraler Infektion im Fettkörper exprimiert und in den Hemolymph sekretiert wird. Mutanten für dieses Gen, zeigten reduzierte virale Titer und verlängertes Überleben nach Infektion mit DCV. Diese Ergebnisse legen Nahe, dass CFM möglicherweise ein wirtskodierter Faktor ist, welcher die virale Replikation in Drosophila fördert.

Abstract

The work presented in this thesis used the fruit fly *Drosophila melanogaster* as a model system to study the inducible innate immune response against virus infection.

We studied the transcriptome of flies infected with either Drosophila C virus (DCV), Flockhouse virus (FHV) or Sindbis virus (SINV) using genome-wide microarray analysis. Infection with these postive-sense single-stranded RNA viruses triggered a strong inducible response that showed evidence of virus-specific induction patterns. In order to study the biological significance of these gene inductions, we investigated susceptibility to viral infection of mutant flies for signaling pathways, such as the Imd, the Toll and the Jak-STAT pathway. We conducted a comparative analysis using a set of five distinct RNA viruses, that included the positive-sense RNA viruses DCV, Cricket paralysis virus (CrPV), FHV and SINV, and the negative-sense RNA virus Vesicular stomatis virus (VSV). We found that the Dicistroviruses DCV and CrPV formed a unique group among the viruses tested, as mutants of both, the Jak-STAT and the Toll pathway were susceptible to infection with DCV and CrPV. The observed susceptibility correlated with an increase in viral titers. No phenotype, neither in viral load nor in survival susceptibility was observed upon infection with FHV, SINV or VSV. We found no major role for the Imd pathway in the antiviral defense, however, mutant flies of this pathway showed a trend towards a reduction in Dicistrovirus load. Furthermore, we showed that the Jak-STAT pathway is as important for the defense against Dicistrovirus infection as RNA interference, highlighting the relative importance of the inducible response against this virus family.

As an example for a virus-induced gene, we characterized *Chifoumi* (*cfm*) alias *CG11501*. We showed that *cfm* codes for a circulating peptide that is induced in the fat body upon viral infection and secreted in the hemolymph. *cfm* mutant flies showed decreased viral titers and prolonged survival upon infection with DCV, suggesting that CFM may function as a host-encoded factor that propagates viral replication in drosophila.

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INTRODUCTION

Chapter 1

Introduction: Antibacterial and antifungal innate immunity in drosophila

The fruit fly *Drosphila melanogaster* solely relies on an innate immune system to combat infection. This defense is composed of multiple innate defense reactions, which include the protective nature of epithelial barriers as well as a local and systemic immune response. The uncovery of underlying regulatory mechanisms revealed the existence of two major immune signaling pathways, the Toll and Imd pathway. Relatives of the Toll receptor were found to also regulate innate immune responses in mammals. This discovery indicates that components of the innate immunity are evolutionary conserved between drosophila and mammals, and makes the fruit fly a potent model for studying general innate immune mechanisms in animals.

1.1 Epithelial and cellular defense in Drosophila

Barrier epithelia are in constant contact with a large variety of microorganisms. Besides acting as a physical barrier, these surfaces possess efficient systems for microbial recognition and control. Local expression of antimi-

crobial peptides (AMPs) has been discovered in several drosophila surface epithelia which are in contact with the external environment and include the epidermis, the reproductive system, the respiratory tract, and the digestive tract [1, 2]. This synthesis of antimicrobial effectors is referred to as the local immune response.

AMP expression in epithelia can be either constitutive or induced upon natural infection with Gram-negative bacteria. The induced local expression of AMP genes is mediated by the Imd pathway. For example, Drosomycin and Diptericin are induced in both trachea and gut via the Imd pathway in response to local infection by bacteria such as *Erwinia carotovora* [3].

In addition to the production of AMPs, natural infection with bacteria also induces rapid synthesis of reactive oxygen species (ROS) in the gut. ROS are generated by the Duox protein and are detoxified by the immune responsive catalase (IRC) [4, 5]. The combined action of Duox and IRC action creates a fine redox balance which is critical for microorganism control in the gut lumen.

The body cavity of drosophila is filled with a circulating hemolymph that contains free-floating and sessile blood cells (hemocytes), which mediate the cellular response. Drosophila larvae contain several thousand hemocytes which are classified into three cell types based on their structural and functional features, namely: plasmatocytes, crystal cells, and lamellocytes [6]. Plasmatocytes represent 90-95 percent of all mature larval hemocytes and are professional phagocytes similar to the mammalian monocyte/macrophage lineage. They are responsible for the disposal of both microorganisms and apoptotic cells, and can internalize within minutes a large variety of particles such as bacteria, yeast, double stranded RNA (dsRNA), Sephadex beads or ink [7]. The generation of plasmatocyte depleted drosophila revealed the importance of phagocytosis in adult flies. Plasmatocyte depleted adults showed susceptibility to systemic infection by various bacteria and fungi, although systemic antimicrobial peptide gene expression via the Toll and Imd pathway was not altered [8, 9]. Crystal cells constitute 5 percent of larval cells and are involved in melanization. Crystal cells express and store prophenoloxidase, which after disruption of the crystal cell is released into hemolymph.

Following activation by a cascade of serine proteases phenoloxidase catalyses the production of quinones, which will polymerize to form melanin. This process generates several cytotoxic intermediates, that are believed to participate in pathogen elimination [7]. Lamellocytes are not found in embryos and adults, and differentiate from hemocyte precursors upon infection of larvae with parasitoid wasp eggs (see section 1.3.3). Lamellocytes are flat adherent cells that will form a multilayered capsule around the invader, a process that is accompanied by melanization and results in parasite killing.
Autophagy, which is an evolutionally conserved homeostatic process for catabolizing cytoplasmic components, has been linked to the elimination of intracellular pathogens during mammalian innate immune responses. Recent evidence suggest, that this mechanism functions as well within the drosophila immune system. In drosophila, recognition of diaminopimelic acid-type peptidoglycan by the pattern-recognition receptor PGRP-LE was found to be crucial for the induction of autophagy, which occured independently of the Toll and IMD signaling cascade. Furthermore, autophagy prevented the intracellular growth of *Listeria monocytogenes* and promoted host survival after infection [10]. In addition to intracellular bacteria, autophagy was as well reported to participate in the antiviral defense of drosophila (see section 3.3.4).

1.2 The humoral response

1.2.1 Antimicrobial effectors

Injection of bacteria into the body cavity of flies induces antimicrobial activity in the hemolymph. This protection can persist for several days and can confer resistance against a second bacterial challenge [11]. The observed antimicrobial activity is mainly due to the production of secreted AMPs in the fat body, the major immune-responsive tissue in the adult fly. In drosophila AMPs group into seven classes and they are characterized by their small size (smaller than 10 kDa, with the exception of Attacin) and their cationic surface charge. They are predominantly membrane active and exhibit a broad

range of antibacterial and/or antifungal activity [12]. Some AMPs act against Gram-positive bacteria (Defensin [13]), others against Gram-negative bacteria (Diptericin, Drosocin, and Attacin [14, 15, 16]), whereas Drosomycin and Metchnikowin have been identified as antifungal agents [17, 18].

1.2.2 Microbial recognition

Flies which are challenged with different classes of microorganisms exhibit distinct AMP expression pattern [19]. This observation is in good agreement with the specialized activity of single AMPs, and suggests the existance of distinct host pattern recognition receptors (PRRs), which recognize molecular patterns shared by different classes of microorganisms [20]. Two families of PRRs, peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) have so far been described in drosophila. The name of the latter family reflects the properties of the first member of the family identified in insects, which bound cell wall from Gram-negative bacteria.

The PGRP family consists of 13 members. They share a common PGRP domain which is related to the bacteriophage type II amidases. PGRPs that actually lost amidase catalytic activity but kept the capacity to bind PGN were found to serve as microbial sensors [21]. Drosophila PGRPs are also categorized by their size. The short isoforms, PGRP-SA, -SB, -SC and SD are secreted proteins, while the long forms, PGRP-LA, -LB, -LC, -LD, -LE and -LF are transmembrane receptors, secreted or cytosolic.

The bacterial pattern sensed by PGRPs is peptidoglycan (PGN). PGN consists of long glycan chains of alternating N-acetylglucosamine and N-acetylmuramic acid residues, which are cross-linked via short peptide bridges [22]. PGN is found in the cell wall of Gram-negative and Gram-positive bacteria, but differs in the composition of the peptide chains. In Gram-negative bacteria, meso-diaminopimelic acid (DAP) is found at the third position of the peptide chain, which is replaced in most Gram-positive bacteria by a lysine residue. The use of highly purified bacterial compounds demonstrated that the Toll pathway is activated by Lys-type PGN, whereas DAP-type

PGN is activating the Imd pathway [23, 24] (Fig. 1.1 and Fig. 1.2). The Imd pathway can be activated by both, polymeric and monomeric DAP-type PGN. The minimum structure needed for activation is tracheal cytotoxin (TCT, GlcNAc-MurNAc(anhydro)-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala monomer) [24, 25]. TCT is an ideal pattern as this muropeptide is located at the end of the PGN strand and is released during cell growth and division. In contrast, the minimum structure needed for activation of the Toll pathway is a muropeptide dimer of lysine-type PGN [26].
GNBPs, which constitute the second family of PRRs in drosophila, display a significant overall homology to bacterial glucanases [27]. They contain an N-terminal β-1,3 glucan binding domain and a C-terminal most likely non-functional β-glucanase domain. The drosophila genome contains three GNBPs plus three related family members, known as GNBP-like proteins, which contain only an N-terminal domain similar to that of GNBP3.
In spite of their name, to date, drosophila GNBPs were described to participate in the activation of the Toll pathway by Gram-positive bacteria and fungi.

Recognition of Gram-negative bacteria by PGRP-LC/LE

PGRP-LC is the major receptor of the Imd pathway [28] and exists in three isofroms (LCa, LCx, LCy), which share the same intracellular (signaling) domain but differ in their extracellular (sensing) domains [24, 29]. PGRP-LCx is required for recognition of polymeric PGN, whereas PGRP-LCa and PGRP-LCx are mandatory for detection of monomeric PGN [24]. An extracellular fragment of PGRP-LE corresponding to the PGRP domain enhances PGRP-LC mediated PGN recognition [30]. Full length PGRP-LE is present in the cytoplasm where it acts as an intracellular receptor for monomeric PGN [30].

Recognition of Gram-positive bacteria

A mutation in PGRP-SA has been shown to block activation of the Toll pathway by Gram-positive bacteria [31]. PGRP-SA encodes a secreted PGRP,

which acts upstream of Toll by sensing Lys-type PGN. Two additional proteins, PGRP-SD and GNBP1, have been implicated in the recognition of Gram-positive bacteria. Experimental data indicate that GNBP1 and PGRP-SA form a complex in the hemolymph [32, 33] . GNBP1 has been proposed to hydrolyze Gram-positive PGN into small fragments, which may then be detected by PGRPSA [26, 34].

Fungal recognition

Flies mutant for GNBP3, another gene encoding a GNBP family member, fail to activate the Toll pathway in response to killed fungi and succumb rapidly to fungal but not bacterial infections [35]. GNBP3 is believed to activate a proteolytic cascade, which partially overlaps that triggered by the GNBP1/ PGRP-SA complex [36]. Hence, although belonging to the same family and activating the same pathway, GNBP1 and GNBP3 are required for sensing distinct classes of microorganisms. It was shown recently, that a recombinant polypeptide encoding the N-terminal domain of GNBP3 is able to bind to fungi and to long β-1,3-glucan chains but not to short laminari-oligosaccharides, identifying β-1,3-glucan as the sensed pattern [37].

1.3 Signaling pathways implicated in innate immunity of drosophila

1.3.1 The Toll pathway

The Toll pathway is evolutionary conserved and was first found to participate in the formation of the dorso-ventral axis in drosophila embryo and other processes during development [38]. Canonic members of this pathway are the extracellular cytokine Spaetzle, the transmembrane receptor Toll, the adaptor molecules Tube and MyD88, the kinase Pelle, the inhibitor molecule Cactus and the transcription factors Dorsal and Dif [38, 39, 40]. With the exception of Cactus and Dorsal, deletion of any of this pathway components results in reduced expression of AMPs including Drosomycin and a marked

susceptibility to fungal and Gram-positive bacterial infection [41, 42]. During the larval stage Dif and Dorsal seem to be redundant in the control of Drosomycin expression. Dif seems to be sufficient in adults to mediate Toll activation [43, 44, 45], however, subtle roles for Dorsal have been proposed [46]. Several components of the pathway are in fact regulated in a Toll-dependent manner [41, 47]. In particular, induction of Cactus, which is an inhibitor molecule, enables the establishment of a negative feedback loop on this cascade [48]. In contrast to vertebrate Toll-like receptors (TLRs), Toll itself does not function as a pattern recognition receptor and is activated by the cleaved form of the cytokine Spaetzle (reviewed in [7]). This immune induced cleavage is initiated in part by secreted PRRs, which activate a proteolytic cascade. This process is conceptually similar to vertebrate blood coagulation or complement activation cascades [49]. The functional core of this protelytic cascades consists of several serine proteases (SPs) that undergo zymogen activation, followed by cleavage of a substrate by the terminal downstream protease. Inappropriate activation is prevented by the existance of SP inhibitors (serpins) [50].
To date, the number of cascades that lead to Spaetzle cleavage is still a matter of debate. Genetic evidence points to the existance of at least two extracellular pathways: A PRR-dependent pathway, which is activated by the presence of pathogen associated molecular patterns (PAMPs), as well as a Persephone-dependent pathway, which senses danger signals such as abnormal proteolytic activity in the hemolymph caused by fungal and bacterial proteases [35, 36, 51] (Fig. 1.1).

1.3.2 The Imd pahway

A mutation named *immune deficiency* (*imd*) led to the discovery of the Imd pathway. Imd mutants showed impaired expression of several antimicrobial peptide genes, but only marginally affected *Drosomycin* induction [52, 53, 54]. *imd* mutant flies are sensitive to Gram-negative bacteria but are more resistant to fungi and Gram-positive bacteria than Toll mutant flies. *imd* encodes a Death domain-containing protein related to the mam-

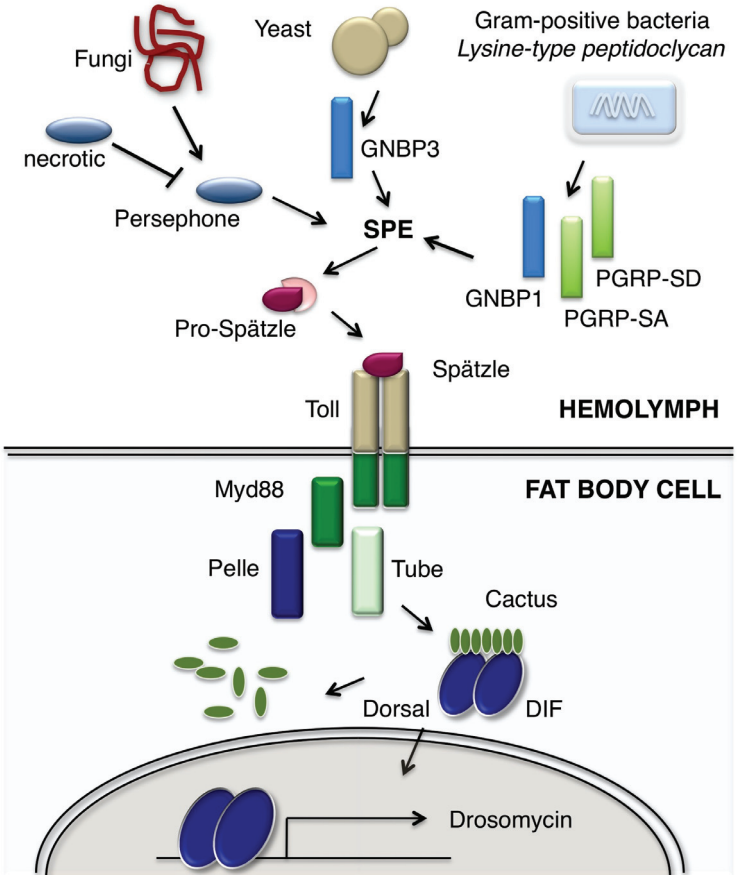


Figure 1.1: The Toll pathway. The Toll receptor is activated upon binding of the cleaved form of Spaetzle that is processed by proteolytic cascades. SPE is the Spaetzle processing enzyme and integrates signals from secreted PRRs, as well as the protease Persephone. Necrotic is a Serpin acting on Persephone. PGRP-SA, PGRP-SD and GNBP1 mediate the recognition of Gram-positive bacteria. GNBP3 binds to fungi upon identifying long chains of beta-1,3-glucans in the fungal cell wall. Binding of mature Spaetzle leads to dimerization of Toll and further signaling via Death domain-containing proteins, MyD88, Tube, and Pelle, the latter also being a kinase. Cactus is phosphorylated by an uncharacterized mechanism and degraded by the proteosome leading to the nuclear translocation of Dif and Dorsal.

malian factor RIP-1, and its overexpression triggers the expression of antibacterial peptide genes in the absence of any infection [55]. Reverse genetic approaches and genetic screens identified eight additional canonical members of the Imd pathway (reviewed in [7] and Fig. 1.2): the PGRP-LC receptor; the Mitogen-Activated Protein 3 kinase (MAP3K) TAK1 and its associated cofactor TAB2; DIAP2, a member of Inhibitor of Apoptosis proteins; IKKβ (encoded by the gene *ird5* and IKKγ (encoded by *Kenny*, which form a drosophila equivalent of the mammalian IKK signalosome; the dFADD adaptor; the Dredd caspase; and the transcription factor Relish (Fig. 1.2). Mutations affecting any of these components result in an immune deficiency phenotype similar to that of *imd*. No developmental role has been atributed to this pathway, since mutant flies are perfectly viable.
Sensing of DAP-type PGN by PGRP-LC results in the recruitment of the adaptor Imd. Imd interacts with dFADD, which itself interacts with Dredd. Dredd is suggested to associate with Relish, which is activated by Dredd mediated cleavage. The IKK complex controls Relish by direct phosphorylation on two distinct serine residues. Surprisingly, these phosphorylation sites are not required for Relish cleavage, nuclear translocation, or DNA binding. Instead they are critical for recruitment of RNA polymerase II and antimicrobial peptide gene induction, whereas IKK functions noncatalytically to support Dredd-mediated cleavage of Relish [56].

1.3.3 The Jak-STAT pathway

In flies, the Jak-STAT pathway was first identified for its role in embryonic segmentation [57]. The canonical pathway consists of four main components, a ligand that belongs to the unpaired (upd) family, the transmembrane receptor domeless (dome), the non-receptor tyrosine kinase JAK (hopscotch), and the transcription factor STAT (STAT92E/Marelle). Vertebrate homologues can be identified for all of the pathway components, except that no obvious interferon or interleukin-like molecules are found in the fly genome. However, members of the upd family were shown to act as pathway ligands. The upd family consists of three different members. They are expressed dur-

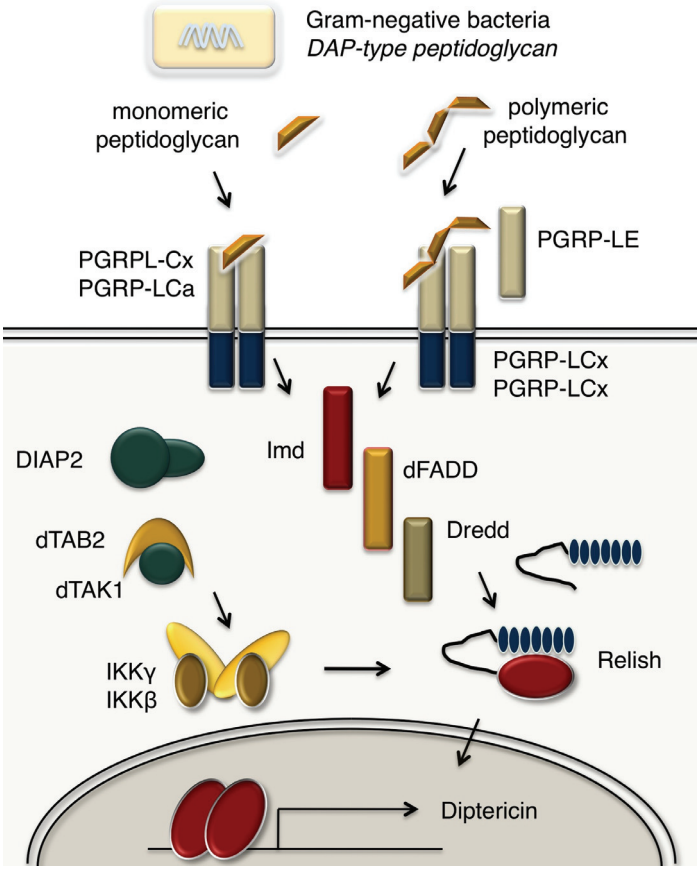


Figure 1.2: The IMD pathway. Direct binding of bacterial elicitors to PGRP-LC recruits the adaptor Imd. Imd interacts with dFADD, which itself binds the apical caspase Dredd. This caspase has been suggested to associate with Relish. The cleaved Rel domain translocates to the nucleus, while the inhibitory domain remains stable in the cytoplasm. Relish is phosophorylated by the IKK signaling complex, which is believed to be activated by TAK1 and its adaptor TAB2 in an Imd- and possibly dFADD-dependent manner. DIAP2 may activate TAK1. To date, the mechanisms that link Imd to the IKK complex and the precise role of TAK1 and DIAP2 are not known.

ing embryogenesis, and upd3 has been additionally reported to be expressed in hemocytes after septic injury [58]. Tissue culture assays showed that both upd and upd2 are secreted molecules able to efficiently activate the Jak-STAT pathway. Interestingly, upd was found to be associated primarly with the extracellular matrix, whereas upd2 was secreted into the extracellular medium [59]. Of note, upd carries a signal peptide, whereas no conventional signal peptide is predicted for upd2 or upd3. There is indirect evidence, however, that upd3 is as well a secreted molecule, as upd3 expression in hemocytes affects *Turandot A* (*TotA*) expression in the fat body [58].
Binding of the ligand to the gp130-related receptor Dome results in receptor dimerization and recruitment of two JAK molecules. JAKs in juxtaposition will transphosphorylate each other and phosphorylate tyrosine residues in their associated receptor. This allows cytosolic STAT molecules to dock onto the receptor complex via their Src homology 2 (SH2) domains. The recruited STAT molecules are then activated by JAK mediated phosphorylation. Activated STAT molecules dimerize and translocate into the nucleus, where they will bind to their consensus DNA target sites and induce gene expression.
Studies performed in the mosquito *Anopheles* provided the first evidence for a role of the Jak-STAT pathway in insect immunity [60]. Subsequent work in *Drosophila* revealed an involvement of this pathway in the humoral and cellular response to septic injury. More recently, the Jak-STAT has been reported to participate in the host defense against natural infection with Gram-negative bacteria, as well as infection with the Drosophila C virus (DCV, see chapter 3).

Jak-STAT and the humoral immune response

The drosophila genome contains a family of 6 genes (Tep1 - Tep6) that share significant similarities with the thiolester-containing proteins of the complement C3/α2-macroglobulin superfamily [61, 62]. Tep proteins were proposed to either function as opsonins that promote phagocytosis (like complement C3) or as protease inhibitor (like α2-macroglobulin). The drosophila Teps are transcribed at a low level during all stages of development, and their expres-

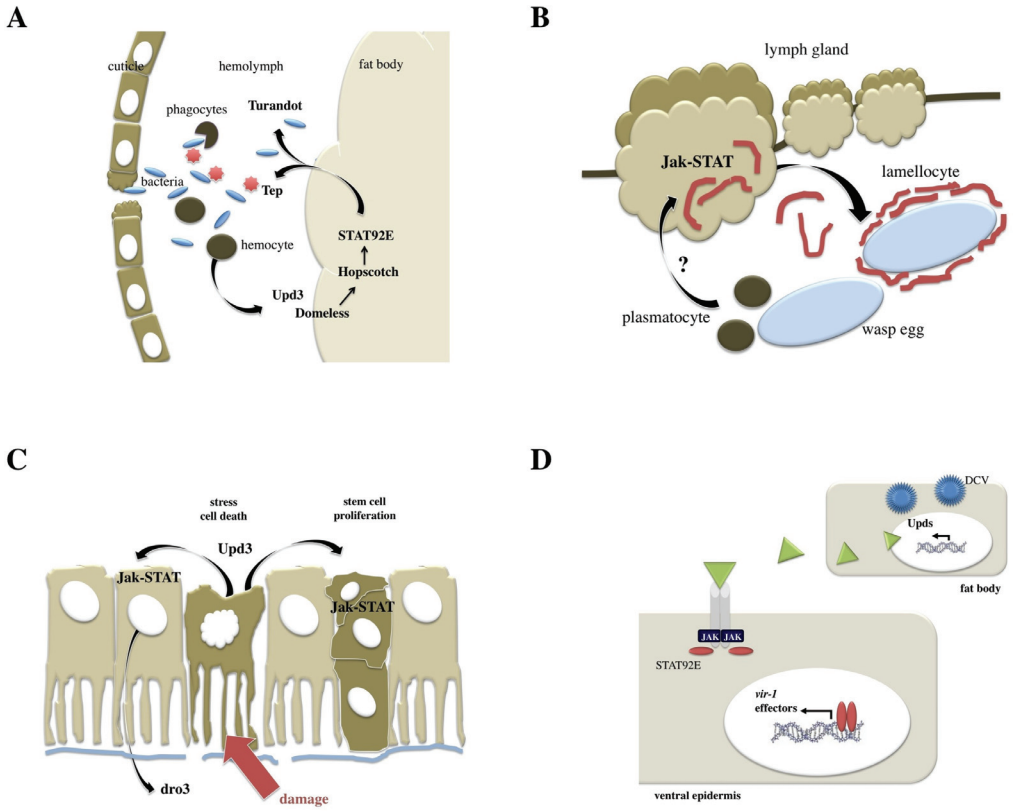


Figure 1.3: Roles of the Jak-STAT pathway in insect immunity. The Jak-STAT pathway has been shown to participate in the humorol response (A), cellular response (B), gut immunity (C) and antiviral response (D). For explanation see text.

sion is markedly up-regulated after immune challenge. Tep1 was found to be predominantly expressed in the larval fat body. Gain-of-function *hopscotch* mutant flies showed constitutive expression of Tep1, whereas a reduced inducibility was observed in loss-of-function *hopscotch* mutants, suggesting that the Jak-STAT pathway participates in the regulation of Tep1 upon immune challenge [61].

TotA was the first member of the Turandot family that was reported to be induced upon immune challenge in a Jak-STAT dependent manner in the fat body [58]. In addition to TotA, TotC and TotM were also found to be controlled by the Jak-STAT pathway [63]. Turandot are drosophila-specific genes of unknown function that are induced by various stress conditions, especially by septic injury [64, 65]. The transcriptional regulation of these genes seem complex, with additional inputs from both the Imd and MAPK (mitogen-activated protein kinase) pathways [66]. Computational analysis of gene expression profiles revealed that TotM displayed a temporal profile after septic injury that was similar to the gene CG11501 [67]. This gene has been named Chifoumi (CFM) and was reported to be regulated like the Tot family members in a Jak-STAT dependent manner. CFM was also identified in a screen for Jak-STAT pathway components as a putative negative regulator of the pathway [68].

Curiously, Jak-STAT deficient flies resist to bacterial and fungal infections like wild-type flies and no impaired AMP expression is observed. It has been proposed, that this pathway could respond to tissue damage and that its activation is achieved by a hemocyte released cytokine (upd3), that will bind to the receptor Domeless in the fat body of infected flies [58] (Fig. 1.3 A).

Jak-STAT and the cellular response

A common defense reaction of drosophila against foreign objects and parasites in the hemolymph is encapsulation (see below). Lamellocytes differentiate in the lymph gland [69] and are released in the hemolymph. *hopscotch* gain-of-function mutant flies contain numerous lamellocytes in the absence of any immune challenge that will contribute to black masses/ melanotic tumors

[70, 71, 72]. This data strongly suggests that the Jak-STAT pathway may participate to lamellocyte differentiation upon wasp infestation. Indeed, larvae carrying a loss-of-function mutation in *hopscotch* were no longer able to mount an encapsulation response [73]. Hence, the Jak-STAT pathway seems to be not only involved in the humoral response, but probably contributes to the cellular response of the lymph glands (Fig. 1.3 B).

Jak-STAT and the natural infection model

In the classic infection model, flies are pricked with a thin needle that has been previously dipped into a concentrated solution of bacteria. Epithelial barriers are mechanically broken, and the bacteria are directly introduced into the circulating hemolymph. Recently, natural infection models by feeding insect pathogens have been established, in order to study the fly's intestine-specific responses to bacterial infection. Two different approaches were made that included a genome-wide *in vivo* drosophila RNA interference screen using the Gram-negative bacterium *Serratia marcescens* as well as global gene expression analysis of intestinal tissue after infection with the Gram-negative bacterium *Erwinia carotovora* [74, 75]. Both groups found that immune responses in the gut are mediated by the Imd pathway, but not the Toll pathway. Furthermore, they both showed that the Jak-STAT signaling pathway participates to the host defense by regulating stem cell proliferation and therefore epithelial renewal and homeostasis. In the case of Erwinia infection the Jak-STAT pathway was additionally shown to contribute to the AMP transcription in the gut, as the expression of *dro3* was dependent on hopscotch loss-of-function flies and constitutive in hop $Tum - l$ gain of-function flies. Thus, these recent data point to a novel role of the Jak-STAT pathway in fly immunity (Fig. 1.3 C).

1.4 Conserved aspects of drosophila innate immunity

Drosophila is a very good model to study innate immunity. Not only is it devoid of an adaptive immune system, but more importantly, existing parallels with mammalian signaling pathways point to a common ancestry of host-defense mechanism.

1.4.1 Toll and TLRs: lessons from drosophila

The discovery that the Toll pathway participates to the immune response in drosophila led to the search for homologs in the mammalian system. Analysis of expressed sequence tags (ESTs) libraries led to the identification of the Toll receptor orthologue in mice and human. One of these orthologues, named h-Toll or TLR4, was shown to mediate nuclear translocation of NF-κB and expression of cytokines and costimulatory molecules in transfected tissue-culture cells [76]. Later on, *in vivo* work in mice proved that TLR4 as well as TLR2, where indeed PRRs [77, 78]. A family of Toll like receptors (TLRs) comprising 10 and 13 members in humans and mice respectively, was subsequently described. TLRs are transmembrane receptors which contain leucine-rich repeats (LRRs), a transmembrane domain, and a cytoplasmic domain referred to as the Toll/Il-1 receptor (IL-1R) homology (TIR) domain. Mammalian TLRs sense a large spectrum of microbial patterns, such as flagellin (TLR5), LPS (TLR4), single-stranded U-rich RNA (TLR7,8), dsRNA (TLR3) and DNA enriched in non-methylated CpG motifs (TLR9). In comparison, only one (Toll) of the 9 distinct Toll genes is strictly required for the defense against fungi and Gram-positive bacteria. Another difference between *Drosophila* and mammals lies in the sensing of microbial patterns. In drosophila, PAMPs are recognized by circulating PRRs that will trigger a proteolytic cascade which will lead to the cleavage of Spaetzle (Fig. 1.4). Thus, Toll functions as a cytokine receptor that will activate NF-κB signaling, a function reminiscent of that of the IL-1 receptor (IL-1R) in mammals. In contrast, mammalian TLRs directly interact with microbial

patterns, as examplified for the TLR1-TLR2-lipopeptide complex, the TLR3-dsRNA complex and the TLR4-MD-2-LPS complex [79]. TLRs can function in association with co-receptors or associated proteins. For example, LPS-sensing by the TLR4 complex involves the LPS binding protein (LBP) and the CD14 and MD2 co-receptors. In summary, these findings suggest that insects and mammals independently developed a cytokine-activated family of receptors, namely Toll in drosophila and IL-1R mammals, which originated from an ancestral TLR system activating NF-κB signaling upon sensing microbial agents.

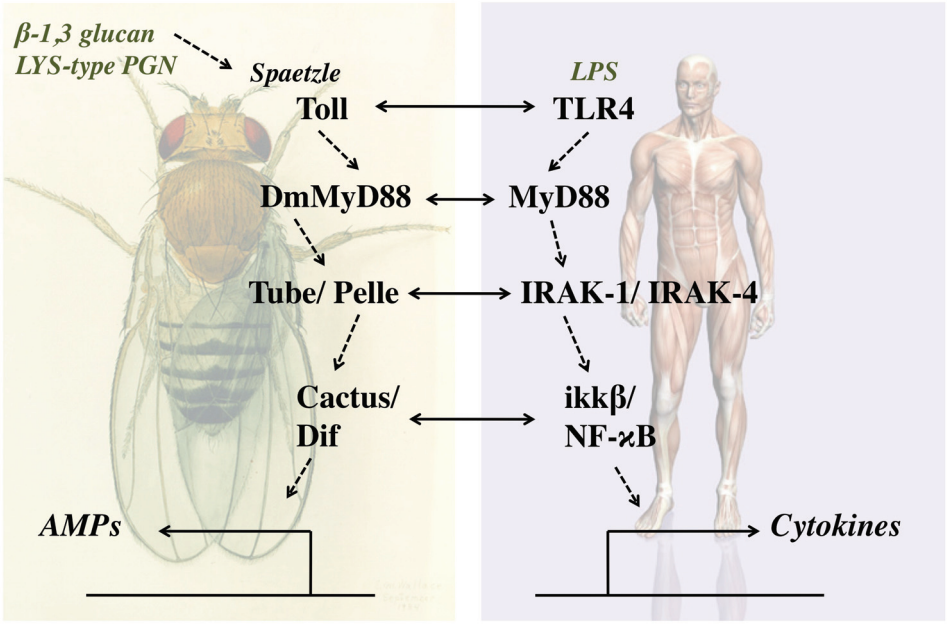


Figure 1.4: Conserved aspects of the drosophila Toll and the mammalian TLR4 pathway. The Toll pathway is indirectly activated by the cytokine Spaetzle, whereas TLR4 directly senses LPS. Various Toll pathway components are conserved in the mammalian TLR4 signaling pathway.

1.4.2 NF-κB and apoptosis: The TNF-Imd connection

Counterparts of components that function in the Tumor Necrosis Factor (TNF) pathway are found in the Imd pathway. This is illustrated by the presence of signaling molecules with striking similarities. For example Receptor Interacting Protein (RIP) and Imd, Fas-associated Death Domain (FADD) and drosophila FADD, caspase-8 and Death related ced-3/Nedd2-like (DREDD) (Fig. 1.5). TNF-α signaling can lead to NF-κB activation or apoptosis in mammals. Imd overexpression promotes apoptosis in flies and it induces expression of the pro-apoptotic reaper gene [80]. Despite these existing parallels, much more work is needed to understand the precise significance of the connection between the Imd pathway and apoptosis. To date, there is no link between apoptosis and the host-defense of drosophila against Gram-negative bacteria. Again, the described parallels between TNF-α and Imd signaling point to a common ancestry of host-defense mechanism, which predated the separation of the lineages giving rise to insects and vertebrates. All together, drosophila has proven to be a suitable model organisms to study innate immunity. Initial work has focused on innate defense mechanisms against bacteria and fungi, and the investigation of innate antiviral immunity has not been adressed until recently. The next chapters will briefly describe known innate antiviral defense mechanisms in plants and mammals, as well as our current understanding of drosophila antiviral immunity.

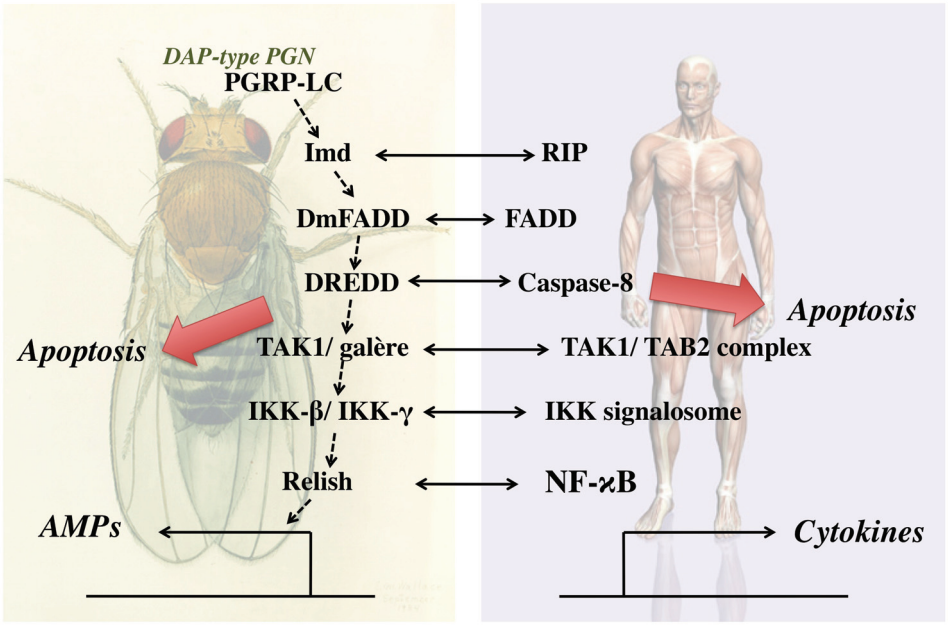


Figure 1.5: Conserved aspects of the drosophila Imd and the mammalian TNF-α pathway. The Imd pathway regulates the immune response towards infection with Gram-negative bacteria, but can also induce apoptosis. The mammalian TNF-α receptor signaling pathway shares compelling similarities with the Imd pathway.

Chapter 2

Introduction: Innate antiviral defense strategies in plants and mammals

Plants and mammals have developed different innate defense strategies to combat viral invaders. Mammals are known to mainly rely on the potent interferon (IFN) system to fight infection, whereas plants use the RNA interference (RNAi) machinery to control viral replication.

2.1 Plant antiviral defense

Plants rely on the RNAi machinery to counteract viral infection. The possibility of an antiviral function of RNAi was first raised, when experimentally induced "gene silencing" was found to provide resistance to viruses carrying an identical sequence (reviewed in [81]).
The Dicer family found in *Arabidopsis thaliana* consists of four different members, Dicer-like (DCL) 1-4. Individual mutations in the DCL genes does not significantly affect antiviral defense. However, inactivation of both DCL2 and DCL4 was found to strongly interfere with the control of several RNA viruses. The observed high susceptibilty correlated with decreased levels of virus-derived siRNAs [82]. Three out of the four DCLs have been found to be

implicated in the antiviral defense. DCL1 mainly functions in the production of miRNAs and is required for plant development. DCL2, 3 and 4, however, are involved in the control of RNA viruses (DCL2, DCL4) or DNA viruses (DCL3) (reviewed in [81]). Once generated, virus derived siRNAs will be incorporated in an AGO1 containing RISC complex, which will target viral degradation. The plant RNAi machinery includes an amplification process by a host-encoded RNA dependent RNA polymerase (RdRP) enzyme and non-cell autonomous spread of siRNAs through the plant phloem (reviewed in [81]).

In good agreement with the proposed antiviral function of RNAi, several plant viruses were found to possess viral suppressors of RNAi (VSRs) (reviewed in [81]). VSRs are extremely diverse, both structurally and in terms of mode of action. Some plant VSRs encode dsRNA binding proteins, which will prevent Dicer action (e.g. Turnip Crinkle Virus p38). Others bind directly to small RNAs and prevent loading in the RISC complex (e.g. Tomato bushy stunt virus P19 or Peanut clumb virus P15). Finally, some VSRs do not interact with RNA but are able to prevent assembly of the RISC complex (e.g. Gemini virus AC4), interfere with slicing (e.g. Cauliflower Mosaic Virus 2b), or promote ubiquitin-dependent proteolysis of key molecules such as AGO1 (e.g. Polerovirus P0).

2.2 Mammalian innate antiviral immunity

In contrast to plants, the mammalian antiviral innate immunity does not rely on RNAi mechanisms. Although the microRNA pathway is preserved in mammals, viral recognition by PRRs results in the secretion of multiple cytokines, among which type I and III interferons play a central role in the orchestration of the antiviral response (Fig. 2.2).

2.2.1 Viral recognition

Two classes of PRRs, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and Toll-like receptors (TLRs) have been described to induce inter-

feron (IFN) mediated innate antiviral immunity in mammals. While TLRs essentially sense viral infection in plasmacytoid dendritic cells, RLRs play an essential role in virus recognition in various cell types. TLR and RLR downstream signaling does not only lead to the secretion of type I IFNs, but mediates as well the induction of proinflammatory cytokines and chemokines, as well as increased expression of costimulatory factors. A third class of receptors, NOD-like receptors (NLRs) do not trigger the induction of IFN but participate in virus-induced inflammasome activation.

RLR family

The RLR family is composed of three different members, RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology-2 (LGP-2) (reviewed in [89]). RIG-I and MDA5 consist of two N-terminal caspase-recruitment domains (CARDs), a DExD/H box RNA helicase domain, and a C-terminal regulatory domain (RD). The RD and helicase domains are important for recognition, whereas signaling is mediated by the CARD domains. LGP2 lacks a CARD domain and seems to function as a negative regulator [90, 91]. RIG-I and MDA5 differentially recognize RNA viruses. RIG-I is involved in the recognition of Sendai and Newcastle disease virus (*Paramyxoviridae*), Influenza virus (*Orthomyxoviridae*), VSV (*Rhabdoviridae*), and Japanese encephalitis and Hepatitis C virus (*Flaviviridae*). In contrast, MDA-5 recognizes EMCV, Theiler's virus and Mengo virus (*Picornaviridae*). Viruses such as West Nile or Dengue virus (*Flaviviridae*) and Reovirus (*Reoviridae*) are recognized by both, RIG-I and MDA5 (reviewed in [89]). These differential roles in RNA virus recognition are reflected in the molecular pattern sensed by RIG-I and MDA-5. Infection with either dsRNA or positive-sense ssRNA virus results in the presence of dsRNA, which is normally not found in cells. Work with RIG-I-/- and MDA5-/- mice revealed that only MDA5 mediates the IFN response to stimulation with the synthetic dsRNA analog polyI:C [92]. Partial digestion of polyI:C with a dsRNA-specific endonuclease, RNaseIII, converted a MDA5 ligand into a RIG-I ligand in a dsRNA length dependent manner [93]. In summary, RIG-

I seems to recognize dsRNA of up to 1 kb, whereas dsRNA bigger than 2 kb can be recognized by MDA-5. RIG-I was as well reported to recognize ssRNA bearing a 5'-triphosphate [94]. A recent report, however, suggested, that the RNA requires some double-strandedness (panhandle structures) and that previous results were because of unintended hairpins produced by T7 *in vitro* transcription [95].

The RLR signaling pathway

Upon detection of viral RNAs, RIG-I and MDA-5 associate with the adaptor protein IFN-β promotor stimulator-1 (IPS-1), also known as mitochondrial antiviral signaling (MAVS), virus-induced signaling adapter (VISA) or CARD adapter inducing IFN-β (CARDIF) (reviewed in [89]). IFN induction was enhanced by IPS-1 over-expression and conversely impaired in IPS-1 *knockout* cells in response to either RIG-1 or MDA-5 specific stimulation [96, 97]. IPS-1 contains a CARD domain in its N-terminus, with which it interacts with the receptors, a proline-rich region in the middle, and a transmembrane domain at its C-terminus. It is localized on the outer membrane of mitochondria and predicted to interact with RIG-I or MDA-5 by homotypic CARD-CARD interaction [98]. Interaction results in the recruitment of downstream signaling molecules including TBK-1/IKK-i/IKKϵ and FADD/RIP/IKKα/IKKβ, which are involved in the activation of the transcription factors IRF-3/7 and NF-κB, respectively. Activation of IRF-3/7 results in the expression of type I and type III IFN, whereas activation of NF-κB induces pro-inflammatory cytokines.

TLR family

Besides cytoplasmic RLRs, TLRs play a central role as PRRs and recognize diverse virus associated molecular patterns (VAMPs) such as viral nucleic acids (either RNA or DNA) and viral protein motifs. TLRs are capable of detecting extracellular viral patterns as well as viral components in cytoplasmic vacuoles after phagocytosis or endocytosis. TLR2, TLR3, TLR4, TLR7 and TLR9 are involved in the recognition of viral components. TLR 2 and 4,

which are present on the plasma membrane have been reported to recognize viral envelope proteins on the cell surface [89]. By contrast TLR3, TLR7 and TLR9 are localized on cytoplasmic vesicles, such as endosomes and the endoplasmatic reticulum (ER) and reported to recognize microbial nucleotides. TLR 3 and TLR7 recognize dsRNA and ssRNA, respectively, whereas TLR9 recognises DNA with CpG motifs [89]. TLR3 functions in conventional DCs (cDCs) and possibly epithelial cells. In contrast, TLR7 and TLR9 are highly expressed in pDCs, which are known to produce extremely high levels of type I IFNs upon viral infection.

TLR signaling upon viral detection

The activation of TLR signaling is mediated by cytoplasmic Toll/IL-1 receptor (TIR) domains. Each TLR associates with a different combination of four TIR domain containing adaptors (MyD88, TIRAP/MAL, TRIF and TRAM) through homophylic interaction [99]. All TLRs except TLR3 associate with MyD88, either directly or indirectly through TIRAP (Fig. 2.1).
Ligand binding to TLR3 and TLR4 recruits TRIF. In the case of TLR4, TRIF is recruited through TRAM. This TRIF dependent pathway then leads to the expression of either inflammatory cytokines or type I IFN. IFN induction is achieved through IRF3. IRF3 is phosphorylated and activated by TANK binding kinase 1 (TBK1) and IKKi. TRAF3 acts as a linker between TRIF and TBK1(reviewed in [100]) .
Stimulation of pDCs with TLR7 and TLR9 ligands induces robust secretion of type I IFN (mainly IFNα) through activation of IRF7 or induction of inflammatory cytokines through activation of NF-κB and AP-1 in a MyD88 dependent manner. IFN induction is mediated by direct interaction of MyD88 with IRF7 along with IRAK1, IRAK4, IKKα, TRAF3 and TRAF6. IRF7 is phosphorylated by IRAK1 and/or IKKα and translocated to the nucleus where it induces IFN expression (reviewed in [100]).

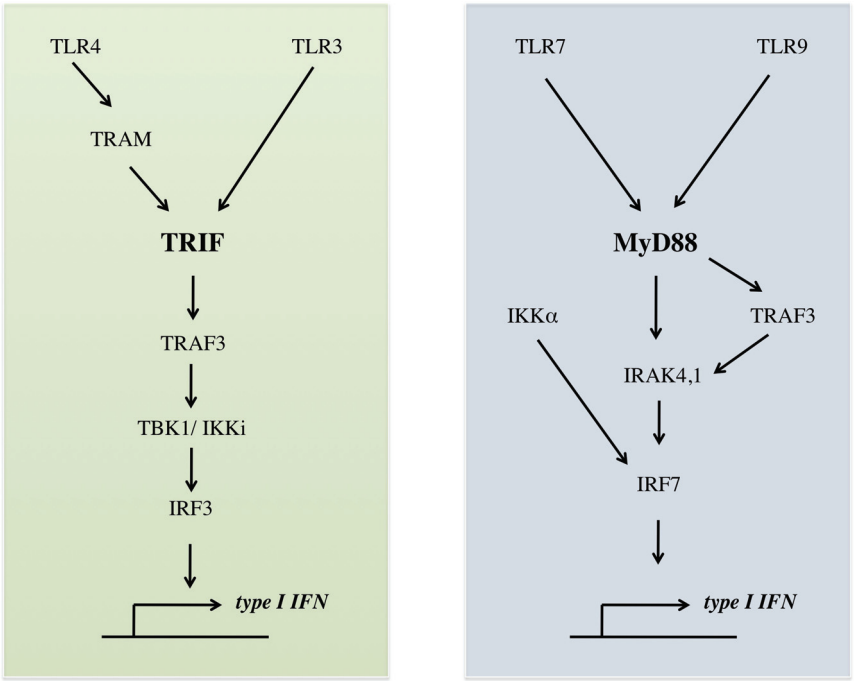


Figure 2.1: TLR mediated IFN induction in response to virus infection. Signaling is either TRIF or MyD88 dependent. For explanation see text.

NOD-like receptors

In addition to the secretion of IFN, viruses are also able to induce activation of proinflammatory cytokines including IL-1β and IL-18 in a variety of cell types. Activation of IL-1β and IL-18 involves the inflammasome, a complex composed of specific NOD-like receptors (NLRs) that oligomerize upon stimulation and recruit caspase 1 through CARD-CARD interactions. This interaction will lead to the self-cleavage and maturation of caspase 1, which then cleaves pro-IL-1β and pro-IL-18 to release the mature forms of these proinflammatory cytokines for secretion, thus driving the inflammatory response that recruits immune cells to the site of infection (reviewed in [101]). The NLRP3 inflammasome, made up of NLRP3 with its adaptor molecule apoptotic speck-like protein containing a CARD (ASC) and caspase 1, was found to be stimulated during acute infection by the DNA viruses adenovirus and vaccinia virus, leading to IL-1β secretion [101]. In addition, influenza A, an RNA virus, was as well reported to trigger inflammasome signaling through NLRP3 [101]. Taken together these studies indicate a role of NLRP3 inflammasomes in cytosolic detection of DNA and RNA viruses. However, it is not clear if NLRP3 directly detects viral nucleic acids, or if these inflammasomes are activated by a less specific stimulus such as cellular stress or alterations of host metabolites downstream of pathogen infection.

2.2.2 The IFN system

Type I IFNs include multiple IFN-α isoforms, a single IFN-β, and other members, such as IFN-ϵ, -κ or-ω. Upon secretion, they regulate a range of immune responses through the type I IFN receptor. This transmembrane receptor is composed of two subunits, IFN-receptor 1 (IFNR1) and IFNR2 [83, 84]. The cytoplasmic tails are associated with two members of the Janus kinase (Jak) family, Jak1 and Tyrosine kinase 2 (TYK2). Binding of type I IFN results in rapid autophosphorylation of the receptor and activation of Jak1 and TYK2 by phosphorylation [85, 86]. Jak1 and TYK2 will in turn phosphorylate and activate Signal Transducers and Activators of Transcription (STAT) factors [87, 88]. STATs activated in this manner will dimerize

and translocate to the nucleus where they will initiate transcription by binding specific sites in the promoters of IFN-Stimulated Genes (ISGs). Activated STATs can form homo- or heterodimers and can additionaly associate with other factors. One important transcriptional complex is ISG factor 3 (ISGF3) complex. Mature ISGF3 is composed of activated froms of STAT1 and STAT2, as well as Interferon Regulatory Factor 9 (IRF9), which itself does not undergo tyrosine phosphorylation. This complex is known to bind to IFN-Stimulated Response Elements (ISREs) that are present in the promoters of some IFN-stimulated genes (ISGs). Another known IFN induced STAT complex is a homodimer formed by two activated STAT1 molecules. Both of these complexes control the transcription of more than 300 ISGs, whose combined action will lead to the generation of an "antiviral state". Despite the considerable high number of IFN regulated molecules and more than 20 years of research, only a few are well characterized for their antiviral function (reviewed in [102]). In addition to these known antiviral effectors, AMPs were as well found to interfere with viral growth.

IFN induced antiviral effectors

The best-characterized ISGs with antiviral activity are the myxovirus resistance gene (Mx), the protein kinase (PK) stimulated by dsRNA PKR, and the 2'-5' Oligo Adenylate Synthetase (OAS). Mx is a guanosine triphosphatase (GTPase), which belongs to the dynamin family and was found to sequester viral ribonucleoproteins to specific subcellular compartments. PKR is a serine-threonine kinase that phosphorylates downstream substrates upon recognition of dsRNA. One of its target molecules is elongation Initiation Factor eIF2. Phosphorylation of eIF2 results in translation inhibition of viral as well as cellular proteins. It should be noted that virus containing an internal ribosomal entry site (IRES) in their genome are not dependend on eIF2 action and therefore not directly affected by PKR. Like PKR, the OAS proteins are activated by dsRNA. Activation leads to the generation of 2'-5' oligoadenylates, which activate ribonuclease L (RNase L) that will degrade

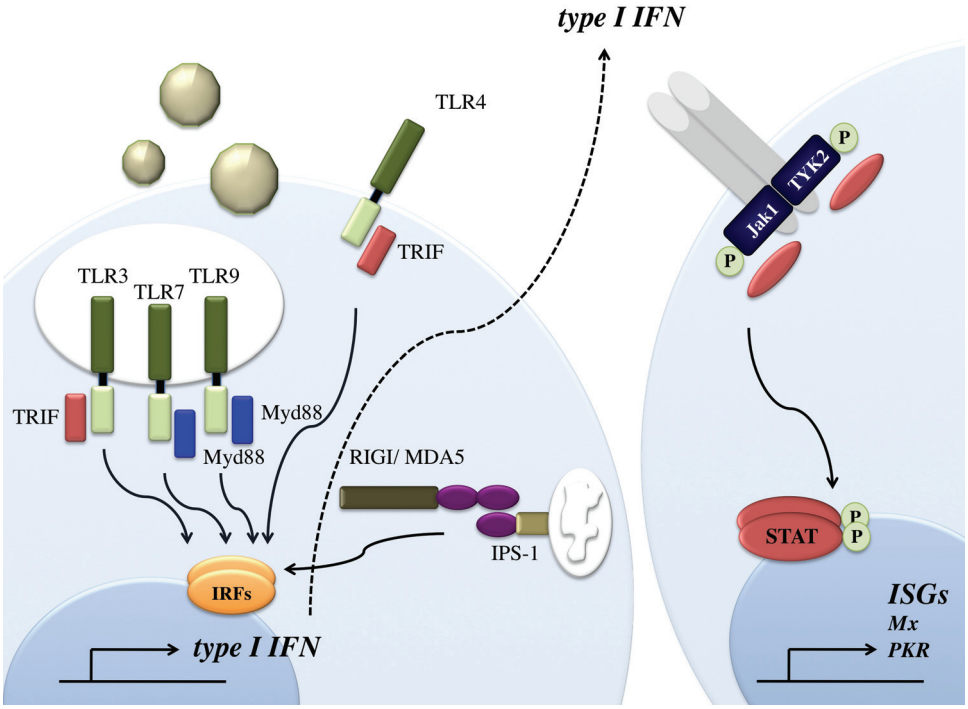


Figure 2.2: The IFN system. Virus recognition by either TLRs or RLRs will lead to the induction of type I IFN, which will in turn induce the expression of more than 300 genes, including several known antiviral effector molecules.

cellular and viral RNAs. Both PKR and the OAS/RNaseL system have profound inhibitory effects on basal cellular processes that will lead to the elimination of virus-infected cells by programmed cell death. Additional proteins with potentially important antiviral activities were described, namely ISG15, ISG20, P56 and guanylate-binding protein-1 (GBP-1). ISG15 is a 15 kD Ubiquitine-like protein and is present in cells either free or conjugated to various cellular substrates. It was found to act against influenza, herpes, Sindbis, and human immunodeficiency virus type 1 [103, 104]. ISG20 is a 3'-5' exonuclease that specifically degrades ssRNA *in vitro* [105] and reduces VSV replication in cell culure. P56 interferes with the translation of viral and cellular RNA by binding to the eukaryotic Initiation Factor 3e (eIF3e) subunit of the translation initiation factor eIF3 [106]. GBP-1 belongs to the same Dynamin superfamily like Mx and has been shown to confer antiviral activity against VSV [107]. Recently, it was discovered that the interferon-inducible transmembrane proteins IFITM1, 2, and 3 restrict an early step in influenza A viral replication. The IFITM proteins do not only confer basal resistance to influenza A virus but are also inducible by interferons type I and II and are critical for interferon's virustatic actions. Further characterization revealed that the IFITM proteins inhibit the early replication of flaviviruses, including dengue virus and West Nile virus [108].
In summary, it is clear that the collective action of ISGs establishes a strong antiviral state in cells. However, only a few ISGs are well characterized and the physiological importance of the majority of ISGs remains to be characterized.

Antiviral effects of AMPs

AMPs such as defensins and cathelicidins are small molecules that are mainly produced by leukocytes and epithelial cells. They possess a broad range of actions against bacteria and fungi, as well as viruses (reviewed in [109]).
Defensins are cationic cysteine-rich peptides with β-pleated sheet structures. They are stabilized by three intramolecular disulphide bonds. Mammalian defensins are mainly produced by leukocytes and epithelial cells and are

classified dependent on their arrangement of the disulphide bonds into three subfamilies, the α-, β- and θ defensins. Some α- and β-defensins have been shown to have chemotactic activity for T cells, monocytes and immature DCs, and can induce cytokine production by monocytes and epithelial cells. Hence, one of their roles might be to control viral replication by modulating the immune system. Furthermore, defensins have a dual role in antiviral activity that involves on one hand direct interaction with viral envelopes, possibly in a similar way to the antibacterial activity of defensins, and on the other hand indirect antiviral activity through interactions with potential target cells. In the absence of serum (e.g. mucosal surfaces), defensins were shown to actively inactivate enveloped virus particles by disrupting viral envelopes or by interacting with viral glycoproteins, such as HIV gp120 [109]. Moreover, Defensins are able to prevent viral entry by directly acting on cellular surface proteins. The θ-defensin retrocyclin 2 (RC2) was shown to block membrane fusion between influenza virus encoded hemaglutinin and target cells. RC2 crosslinks sugars on the surface of cells, thereby hindering the fusion step that precedes viral entry. In addition to RC2, human β-defensin 3 and mannan-binding lectin were also shown to block viral fusion by immobilizing cell membrane proteins [110].

Serum and high salt concentrations were found to alter the ability of defensins for direct interaction. Thus, in the presence of serum, defensins function indirectly by acting on target cells. Defensins possibly interact with G-protein-coupled receptors (GPCRs) and/or other cell surface receptors, such as adrenocorticotrophic hormone (ACTH) receptor, heparan sulphate proteoglycan (HSPG) and low-density-lipoprotein receptor (LDLR). This interaction will result in alterated downstream signaling, as it was shown for protein kinase C (PKC). Antiretroviral activity is achieved by blocking either nuclear import of the pre-integration complex or transcription of viral RNA [109].

Furthermore, some α- and β defensins were shown to have chemotactic activity for T cells, monocytes and immature dendritic cells (DCs), and to induce cytokine production by monocytes and epithelial cells. In the case of human β-defensin (HBD) 1, HBD2 and HBD3, the chemotactic activity

for memory T cells and immature DCs was shown to be receptor mediated through binding to CCR6, which is the receptor for CC-chemokine ligand 20 (CCL20; also known as MIP3α) [109].
Finally, cathelicidins were found as another important group of mammalian AMPs with antiviral properties. Human cathelicidin LL37 was shown to have chemotactic activity and to alter receptor-mediated signaling in a similar manner to that observed for defensins [109].

Chapter 3

Introduction: Antiviral immunity of D. melanogaster

Insects are susceptible to a wide range of RNA and DNA viruses. More than 25 viruses have been documented to infect *Drosophila*, and the effects of few of them have been recently characterized (reviewed in [111]). The immune system of the fruit fly seems to possess two axes to counteract viral invaders. Like plants, *D.melanogaster* relies largely on the RNA interference (RNAi) machinery to suppress viral replication. In addition to RNAi, virus infection triggers the induction of several hundred genes and signaling pathways, such as the Jak-STAT, Toll and Imd pathway, which have been reported to participate to the anitiviral defense. (Fig. 3.1).

3.1 Models of *Drosophila* viral infection

Viruses used to study the antiviral immune defense of drosophila included natural pathogens as well as other insect viruses that were shown to efficiently infect the fruit fly.

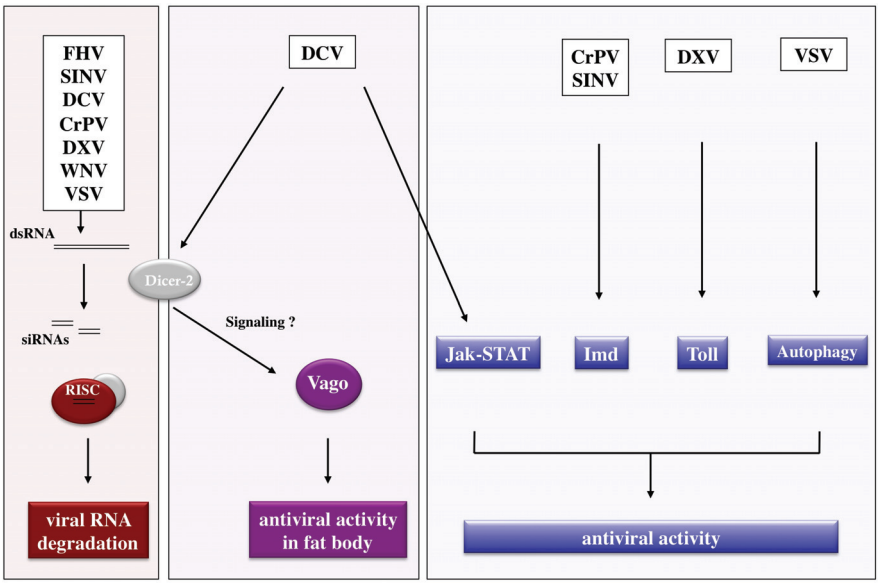


Figure 3.1: Antiviral host-defense of drosophila. Drosophila relies on RNAi as well as on an inducible response to combat virus infection.

3.1.1 Drosophila viruses

Drososphila C virus (DCV)

DCV has been identified in 1972 in a drosophila laboratory stock that exhibited an unexplained high mortility rate. It is the best studied drosophila virus. DCV is a non-enveloped positive-sense single-stranded RNA virus that shares many aspects with the *Picornaviridae* in terms of structural properties and replication life cycle [112]. The DCV replication cycle has been extensively studied. Viral particles are internalized by chlathrin-mediated endocytosis [113] and replication takes place on Golgi apparatus derived cellular vesicles [114]. The outcome of the infection depends on the infection route. DCV is extremely pathogenic when injected intrathorically into adult flies. It kills flies in a dose-dependent manner within a few days (Fig. 5.3) and replicates to high levels in multiple tissues, including the fat-body and ovaries. By contrast, natural infection by the oral or respiratory tract gen-

erally does not lead to major symptoms.

Sigma virus (SIGMAV)

SIGMAV is a negative-sense single-stranded RNA virus, which is widespread in natural drosophila population. This virus is a member of the *Rhabdoviridae*, a family that is known to infect animals as well as plants [115]. SIGMAV is atypical as it has no other hosts than flies where it is vertically transmitted via germ cells. SIGMAV infection leads to reduced viability of eggs and lower survival over winter [116]. Another symptom is high sensitivity to CO_2 exposure.

Drosophila X virus (DXV)

DXV is a dsRNA virus and member of the *Birnaviridae*. It was initially identified as a contaminant of SIGMAV and later found in various drosophila cell lines. The name DXV reflects the enigmatic origin of the virus. DXV infected flies die slowly from infection after 10 to 20 days depending on the viral dose injected [117]. Similar to SIGMAV infection, infected flies become sensitive to CO_2 exposure.

Other Drosophila RNA viruses

Drosophila F virus, which belongs to the *Reoviridae*, or non-classified viruses such as Drosophila P virus and Nora virus have been reported in drosophila cell lines or fly population [111]. A recent approach led to the discovery of four previously unknown viruses present in drosophila cell lines. Small RNA libraries were used for viral genome assembly and revealed the presence of two new positive-sense ssRNA virus, the *Alphanodavirus* American Nodavirus (ANV) and the *Tetravirus* Drosophila Tetravirus (DTrV). Furthermore, two dsRNA virus were discovered, namely the *Birnavirus* Drosophila Birnavirus (DBV) and the *Totivirus* Drosophila Totivirus (DTV) [118]. However, the pathogenicity and replication cycle of these viruses is poorly characterized.

3.1.2 Non-drosophila viruses

Flock House virus (FHV)

FHV is a small positive stranded RNA virus and belongs to the *Nodaviridae*. It was originally isolated from the grass grub *Colestelytra zealandic* near the Flock House Agricultural research station in New Zealand in 1983 and is not a natural pathogen of drosophila. FHV is one of the simplest known viruses and well studied (reviewed in [119]). FHV possesses a bipartite genome consisting of RNA1, which encodes the replicase, and RNA 2, which encodes the precursor of the capsid protein. During infection, a subgenomic RNA3 is produced from RNA1, that encodes the viral supressor of RNAi (VSR) B2. FHV efficiently infects flies and drosophila cell lines, where it replicates in the outer mitochondrial membrane. Upon intrathoric injection, FHV infects multiple tissues, including muscle, fat body and trachea [120].

Sindbis virus (SINV)

SINV was originally identified in 1952 in the mosquito *Culex univittatus* near the town Sindbis in Egypt. It is an enveloped positive stranded RNA alphavirus. Alphavirus comprise a widespread group of significant human and animal pathogens that belong to the *Togaviridae*. One particularly important example is the O'nyong-nyong virus. Alphaviruses are "arboviruses" (arthropod-borne) that are maintained in nature by transmission between vertebrate hosts and invertebrate vectors. In nature SINV is maintained between birds and mosquitos and it was shown to efficiently replicate in drosophila where it causes persistant infection [120].

3.1.3 Insect DNA viruses

Although members of the *Iridoviridae*, *Ascoviridae* and *Baculoviridae* are found in many insect species, no DNA virus has yet been isolated from drosophila. DNA viruses are known to have a distinct and relatively narrow host-range. Among those with a broader host-range, the baculovirus AcMNPV was shown to efficiently enter drosophila cells. However, the cells

are not permissive for infection as virus entry triggered apoptosis [121]. Another DNA virus which is able to infect drosophila is Invertebrate iridescent virus 6 (IIV 6) (Imler unpublished data and [122]).

3.2 RNA interference: A sequence-specific antiviral defense

The term RNA interference (RNAi) descripes the concept of RNA mediated gene silencing, which was first observed in the nematode roundworm *Caenorhabditis elegans*. Small RNAs are found in many multicellular organisms, such as plants, insects and mammals, where they regulate a broad spectrum of different cellular processes. Three classes of small RNAs have been described in drosophila. First, micro RNAs, which are of endogenous origin and regulate gene expression during development. Second, small interfering RNAs, which are of either endogenous (endo-siRNAs) or exogenous (exo-siRNAs) origin. Endo-siRNAs participate in transposon or gene silencing, whereas exo-siRNAs are implicated in the degradation of viral nucleic acids and are often referred to as virus derived small interfering RNAs (viRNAs). Finally, Piwi-associated RNAs (piRNAs), which are involved in the control of transposable elements (TEs) in the germline (Fig. 3.2).

3.2.1 Biogenesis and functions of small RNAs

The microRNA pathway

miRNAs originate from cellular genes, which are transcribed into pri-miRNAs in the nucleus. In drosophila, this stem-loop containing precursors are then processed into pre-miRNAs by the nuclear RNase Drosha [123]. Pre-miRNAs translocate from the nucleus to the cytosol via the exportin-5 system, where they are further processed into 22 nt long double-stranded miRNAs by the RNaseIII enzyme Dicer-1 [124]. Dicer-1 in complex with the dsRNA binding protein R3D1 [125], catalyzes miRNA maturation in faciliating dissociation and loading of the resulting single-stranded miRNA into the miRNA-

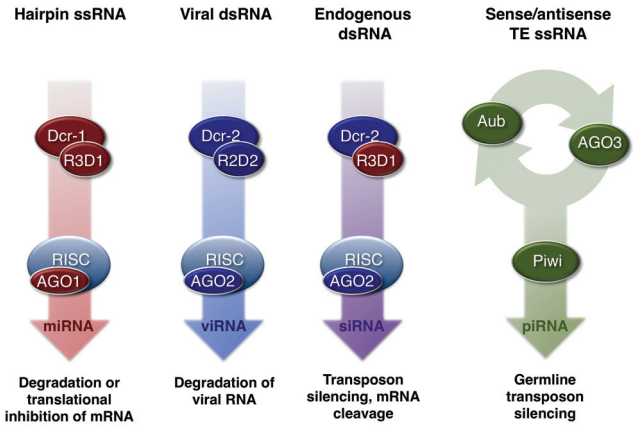


Figure 3.2: Small RNA pathways in *Drosophila melanogaster*. Schematic presentation of distinct drosophila small RNA pathways and functions.

dependent RNAi silencing complex (miRISC). Part of this complex is the RNaseH-like enzyme Argonaute-1 (AGO1), which guides the loaded miRNA to complementary sequences [126] (Fig. 3.2). MiRNAs are found in insects, vertebrates and plants, where they carry out important regulatory functions during development. Consistant with this developmental function, mutant flies for Dicer-1 or AGO1 are embryonic lethal. Interestingly, miRNAs are able to regulate gene expression in various ways. Target RNAs will be cleaved in the case of perfect sequence complementary between miRNA and target. Imperfect sequence complementary, however, will lead to translational inhibition [127]. In addition, miRNAs have been reported to regulate gene transcription by modulating chromatin structure.

The small interfering RNA pathway

siRNAs are either of endogenous (endo-siRNA) or exogenous (exo-siRNA) origine. They have a size of 22 bp and are processed from dsRNA by the second Dicer enzyme encoded by the the drosophila genome, Dicer-2 [124]. Biogenesis of exo-siRNAs involves Dicer-2, R2D2 and AGO2. Virus derived exo-siRNAs are known for their dominant antiviral function (see section 3.2.2). Endo-siRNAs originate from annealed RNAs generated by bidirec-

tional transcription of overlapping genes, from palindromic sequence transcripts generating hairpin structures, or from inverted repeat pseudogenes. They associate with AGO2, which leads to repression of TEs or cellular mRNAs. Interestingly, R3D1 is required for endo-siRNA biogenesis, which is normally functioning within the miRNA pathway (see 3.2).

The Piwi-associated RNA pathway

piRNAs are longer than miRNAs or siRNAs and have a size of 24-30 nt. Interestingly, piRNAs do not seem to originate from dsRNA precursors, as biogenesis of piRNAs is not dependent on Dicer [128]. piRNAs are maternally deposited and/ or are generated by a yet unknown mechanism. They are associated with Piwi proteins, who form a distinct subfamily within the five members of the AGO superfamily. The AGO superfamily is made up of two different subfamilies, the Argonaute and the Piwi subfamily. Piwi, Aubergine and AGO3, which are involved in the production and function of piRNAs belong to the Piwi subfamily, whereas AGO1 and AGO2 join the Argonaute subfamily. All three members of the Piwi subfamily are expressed in the male and female germlines. However, Piwi was found to be additionally expressed in somatic cells surrounding germline cells. In agreement with this restricted expression pattern, drosophila Piwi is required for the maintenance of germline stem cells in both testis and ovaries. In addition, genetic studies pointed for a role of the Piwi pathway in the control of mobile genetic elements, such as endogenous retroviruses and transposons.

3.2.2 Small interfering RNAs: Tiny helpers against viral infection

RNAi has been shown to be a very efficient antiviral defense mechanism. Three independent lines of evidence support this finding. First, there is strong genetic evidence that RNAi controls very efficiently virus replication in the fruit fly. Second, virus-derived small RNAs are found in infected flies,

and third, at least some insect viruses express viral suppressors of RNAi (VSRs).

RNAi and virus control in drosophila

Genetic studies in the fruit fly have been greatly faciliated by the fact, that mutant flies for Dicer-2, AGO2 and R2D2 are homozygous viable. Dicer-2 mutant flies showed increased susceptibility to infection with the ssRNA viruses DCV, CrPV, FHV and SINV [129, 120, 130]. Mutant flies died faster than wildtype controls and the higher lethality correlated perfectly with increased viral titers. Same phenotypes were observed in AGO2 and R2D2 mutant flies for DCV, CrPV and the dsRNA virus DXV [131]. In addition to this strong genetic evidence, virus derived siRNAs are found in infected flies [132]. In summary, all these observations point to the following model. dsRNA is detected by Dicer-2 and cleaved into viRNAs (Fig. 3.3). Dicing by Dicer-2 is strucure dependent and requires the presence of dsRNA. Given an infection with an ssRNA virus, dsRNA intermediates are formed during the replication process by the viral RNA dependent RNA polymerase (vRDRP). The guide strand of the viRNAs is then incorporated into the AGO2 containing RISC complex, which will degrade viral RNA in the cytosol in a sequence specific manner (reviewed in [133]). Consistant with this model, transgenic flies expressing FHV dsRNA, are protected against FHV but not DCV infection [120]. Furthermore, the introduction of naked dsRNA into flies was shown to protect against SINV infection in a sequence-specific manner [136]. This data demonstrates that RNAi provides a highly specific antiviral defense system that is based on complementary base pairing.

There was strong evidence that flies were unable to mount a systemic RNAi response and therefore RNAi mediated antiviral defense was always described as cell-autonomous [134]. However, it has been reported that drosophila S2 cells can internalize dsRNA via the endocytic pathway [135] and that introduction of naked dsRNA into flies elicited a sequence-specific antiviral immunity against SINV that was dependent on an intact dsRNA uptake pathway [136]. Hence, drosophila seems to be able to release and uptake

dsRNA from lysed cells, which will trigger RNAi. However, this mechanism largely differs from systemic RNAi seen in plants and nematodes, where host-encoded RDRPs generate dsRNA that will enhance RNAi.

Interestingly, no phenotype was observed in Dicer-2 mutants in the case of

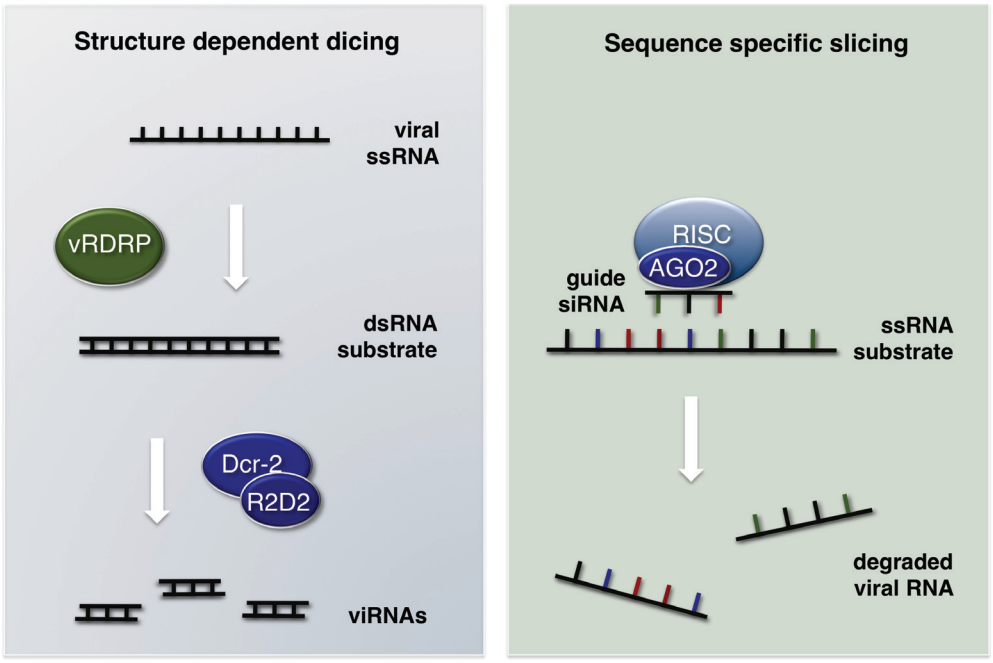


Figure 3.3: Degradation of viral nucleic acids by RNAi. Dicer-2 senses dsRNA produced by the viral RNA dependent RNA polymerase (vRDRP) during replication of the virus. Following recognition, Dicer-2, which is found in a complex with the dsRNA binding protein R2D2, processes dsRNA into viRNAs. These siRNAs are then incorporated into the RISC complex, mediating a sequence specific slicing of viral ssRNA by its core component AGO2.

DXV infection [131]. Similar results were found for West-Nile virus (WNV). WNV is an arbovirus belonging to the Flavoviridae, that can readily infect drosophila and replicates to higher levels in AGO2 mutants than in wild-type flies [137]. However, as for DXV, no increase in viral titers was observed in Dicer-2 mutants.

Viral suppressors of RNAi

Consistant with the proposed critical function of RNAi in the antiviral defense of insects, various insect viruses have been shown to express VSRs. The best characterized VSR is the B2 protein from FHV. B2 is a small 106 aa molecule, whose dimer forms a four-helix bundle. It is synthesized at high levels in infected cells, and binds dsRNA with nanomolar affinity. B2 is not able to bind to DNA, ssRNA or DNA-RNA hybrids. The interaction with dsRNA is sequence-independent, and B2 can bind with high affinity to dsRNA as short as 17 bp. Hence, B2 efficiently interacts with both, long dsRNA as well siRNA, and can therefore interfere with the RNAi machinery upstream and downstream of Dicer [138]. In good agreement with this proposed function, B2 deficient virus are not virulent and are barely detectable in wild-type flies, but can replicate in Dicer-2 or AGO2 mutant flies, proving that B2 acts *in vivo* as a suppressor of RNAi [120, 130].
Among the viruses that can infect drosophila, DCV and CrPV were also shown to encode VSRs. The two VSRs map to the N-terminus of Open reading frame 1 (ORF1). Despite the fact that CrpV and DCV are highly related, the VSRs show no sequence similarities and appear to function differently. Indeed the N-terminus of ORF1 of DCV encodes a canonical dsRNA binding domain, whereas no known structural motifs are found in the N-terminus of CrPV. *In vivo* experiments with recombinant protein confirmed that the DCV VSR DCV-1A prevents Dicer-2 processing by binding long dsRNA with high affinity in a sequence-independent manner [129]. The mode of action of CrPV remains to be identified [130]. Examination of the N-terminus of ORF1 from other dicistroviruses reveals that they are not conserved. This suggests that each virus has evolved individual strategies to combat host-defense. The fact that some viral proteins target components of the RNAi machinery is indirectly supported by the fact that Dicer-2, AGO2 and R2D2 are among the fastest evolving genes in the drosophila genome [139].

3.3 The inducible response

3.3.1 The Toll and Imd pathway

The humoral response, which describes the secretion of antimicrobial effector molecules into the hemolymph upon immune challenge, plays a vital role in the defense against various pathogens. In order to characterize the drosophila immune response towards infection with DCV, proteomic analysis was performed on hemolymph of DCV infected flies [140]. Interestingly, no antimicrobial peptides regulated by the Toll or IMD pathway were detected. An independent proteomic analysis of FHV infected drosophila tissue-culture cells identified some 150 up-regulated proteins. Again, the known drosophila antimicrobial peptides were not found [141].
Genome-wide microarray analysis of the transcriptome of DCV infected flies suggested the induction of some Toll or Imd controlled AMP coding genes. However, these findings were not confirmed using quantitative analysis by RNA blot hybridization or Q-PCR, as these genes were only weakly up-regulated compared to bacterial or fungal challenges [142]. Thus, neither DCV nor FHV seem to induce the expression of AMPs by activation of the Toll or Imd pathway.
Infection with DXV, however, was reported to induce several AMP encoding genes to the same levels as infection with the Gram-negative bacterium *Escherichia coli* [117]. However, overexpression of single AMPs did not influence survival or viral load. Genetic analysis revealed that flies mutant for Dif, the NF-κB-like transcription factor regulated by the Toll pathway, were more susceptible to DXV infection than wild-type flies. Surprisingly, loss-of-function mutants for other Toll pathway components, such as *Toll*, *spaetzle*, *tube* and *pelle*, died similar to wild-type flies [117]. These findings suggest, that Dif could mediate resistance to DXV infection independently of the canonical Toll pathway.
It should be mentioned, that the gene $ref(2)P$, which mediates refractoriness to SIGMAV infection, has been proposed to encode a component of the Toll pathway [143]. However, flies carrying a permissive allele of $ref(2)P$

are more susceptible to SIGMAV infection than flies deficient for the gene. This data indicates that the control of SIGMAV by $ref(2)P$ may not result from a host defense mechanism [144]. SIGMAV has been reported to induce the AMPs diptericin and drosocin, which are regulated by Imd, but not drosomycin [145], which is regulated by Toll. these findings would suggest an involvement of the IMD pathway rather than the Toll pathway in the defense against SIGMAV. However, it was not shown that induction of AMPs can actually confer protection against SIGMAV infection. Recently, another study analyzed the transcriptome of SIGMAV infected flies using micro arrays and found no evidence for the activation of the Toll, Imd or Jak-STAT pathway [146].

Two recent reports using different viruses linked the Imd pathway to antiviral immunity. The Imd pathway has been shown to interfere with the replication of the alphavirus SINV [147]. Various mutants of the pathway showed increased expression of SINV replicon RNA and induction of the Imd controlled AMP Diptericin and the Imd and Toll controlled Metchnikowin. In good agreement with this results, injection of SINV into the thorax of Relish mutant flies resulted in increased viral titers. Similar results were observed after infection with CrPV [148]. Intrathorical injection of CrPV resulted in increased susceptibility of several Imd pathway mutants (e.g. PGRP-LC, rel^{E20}). Again, the observed susceptibility correlated with higher viral loads. In contrast to the reported AMP induction after infection with SINV, no induction of Diptericin, Defensin or Drosomycin was observed after CrPV infection. In summary, there have been various reports, linking either the Toll or IMD pathway to antiviral immunity. However, these studies focused on single viruses and it is not clear if these pathways have a broad antiviral function, or specifically counteract some viruses or virus families.

3.3.2 The Jak-STAT pathway

Genome-wide microarray analysis of the transcriptome of DCV infected flies identified some 150 genes, which were induced by a factor of at least two [142]. Pathways different from the Toll and Imd are activated in response to DCV,

as only one-third of the induced genes were also up-regulated upon bacterial or fungal challenge. In order to identify participating pathways, the regulation of the gene *virus-induced RNA 1* (*vir-1*) was studied, a gene which is not expressed in adult flies, and is strongly induced after infection with DCV and FHV, but not bacteria and fungi [142] Hedges and Johnson 2008). *vir-1* is a previously unrecognized transcript of the gene *CG31764*. Promoter truncation experiments in transgenic flies mapped the virus-responsive element to a 190 bp fragment, that contains a consensus binding site for the transcription factor STAT92E. Point mutations in this binding site strongly reduced the promoter activity and putative STAT binding sites were found in the proximal upstream regions of several other virus-regulated genes. Genetic experiments showed that the Jak kinase Hopscotch was required but not sufficient for the induction of some virus-induced genes, including *vir-1*. Interestingly, mutants of the Jak-STAT pathway were more susceptible to infection with DCV and showed higher viral loads ([142]). This data strongly suggests that genes regulated by the Jak-STAT pathway participate in the control of viral replication. However, *vir-1* itself has not been shown to possess antiviral properties, and the identity of the antiviral mediators induced by the Jak-STAT pathway remain unknown. *vir-1* was found to be expressed in the ventral epidermis of DCV infected flies, a tissue which is normally not virus infected. This observation let to propose that virus detection in infected tissues, such as the fat-body, leads to the production of a cytokine, most likely a member of the upd family, that will activate the pathway in non-infected tissues (Fig 1.3 D).

To date, there has been no other report that links Jak-STAT with antiviral immunity in drosophila. However, an involvement of the Jak-STAT pathway in the antiviral defense was shown in mosquitos [149]. Dengue virus infection in *Aedes aegypti* was found to activate Jak-STAT, and pathway suppression by RNAi depletion of the receptor Domeless or the Janus kinase Hop resulted in increased susceptibility of the mosquito. Conversely, overactivation of the pathway confered increased resistance. Moreover, the authors could identify two Jak-STAT regulated and infection-responsive viral restriction factors. Another study found that infection of C6/36 cells with Japanese en-

cephalitis virus (JEV) diminished DNA binding activity of *Aedes albopictus* STAT (AaSTAT) in nuclear extracts. Furthermore, these authors observed decreased tyrosine phosphorylation of AaSTAT, suggesting that JEV infection may interfere with the tyrosine phosphorylation of AaSTAT, probably through the induction of cellular phosphatase(s) or the inactivation of JAK or other tyrosine kinase(s) by viral products [150].
Overall, these studies provide evidence that the Jak-STAT pathway is involved in the defense against DCV in drosophila and other viruses in dipteran insects. This raises the question, whether this pathway exhibits a broad antiviral function in flies or if its activity is specific against some virus or virus families.

3.3.3 Dicer-2 mediated inducible antiviral activity

Besides the genes like the marker $vir-1$ that are regulated by the Jak-STAT pathway, some genes remain up-regulated after DCV infection in *hopscotch* mutant flies [142]. One of these genes is *Vago* (*CG2081*). It encodes a 160 aa protein with a signal peptide and eight cysteine residues forming a conserved $CX_{20}CX_4CX_{1011}CX_{79}CX_{1314}CCX_4C$ (where X is any aa) [151, 152]. Interestingly, *Vago* loss-of-function mutant flies showed an increase in viral load in the fat body of DCV infected flies. This data suggests that *Vago* controls viral replication in a tissue-specific manner. Indeed, analysis of transgenic *Vago* :: *lacZ* reporter lines confirmed that the *Vago* promoter is specifically induced in fat-body cells upon DCV infection. This tissue-specific induction was as well virus specific. Indeed, bacteria and fungi failed to induce *Vago*. Interestingly, although *Vago* was induced after infection with DCV and SINV, a third positive sense RNA virus, FHV did not trigger induction. In addition, pre-infection with FHV prior to infection with DCV abolished up-regulation of the *Vago* transcript, suggesting that FHV actively supresses *Vago* induction. FHV is a very simple virus that encodes, in addition to the polymerase and capsid protein, VSR B2. Expression of B2 protein in transgenic flies negatively interfered with *Vago* up-regulation, indicating that dsRNA mediates the induction and suggesting an involvement of the RNAi

machinery. Indeed, *Vago* expression was significantly reduced in *Dcr-2* mutant flies. Phylogenetic analysis further revealed that Dicer enzymes share a conserved DExD/H box helicase domain with the RLRs, which sense viral infections and trigger IFN production in mammals [153]. In summary, *Dcr-2* seems to have a dual function in drosophila after sensing viral RNA in infected cells. In addition to its involvement in RNAi, this RLR-like helicase also regulates the induction of molecules like *Vago*, which helps to control the viral load in some tissues.

3.3.4 Autophagy

Autophagy is an intrinsic mechanism that can degrade cytoplasmic components. Recently, autophagy was reported to participate to the host-defense against VSV in drosophila. This report identified the surface glycoprotein, VSV-G, as the likely pathogen-associated molecular pattern (PAMP) that would initiate this cell-autonomous response. Once activated, autophagy decreased viral replication, and repression of autophagy led to increased viral replication and pathogenesis in cells and animals. This antiviral response was dependent on the phosphatidylinositol 3-kinase (PI3K)-Akt-signaling pathway, which normally regulates autophagy in response to nutrient availability [154].

3.4 Importance of studying virus-insect interaction

As abundant infectious agents, viruses are able to infect a broad range of species. Infection of arthropods and insects in particular can cause serious economic consequences. Moreover, hematophagous insects constitute vectors for viruses that have a severe impact on human health.

3.4.1 Economical aspects

Infection of beneficial arthropods and insects by viruses can cause serious economical consequences. Therefore, studies historically focused on the interaction between viruses and silkworms or honeybees. Especially honeybees, which are important pollinators of crops and fruits, are indispensable for a sustainable and profitable agriculture. Honeybees are threatened by numerous pathogens including viruses, bacteria, fungi and parasites. For most, if not all of these diseases, the molecular pathogenesis is poorly understood, hampering the development of prevention or therapy strategies. A few years ago, a sudden bee mortality syndrome called Colony Collapse Disorder (CCD) appeared. CCD was originally observed in Western honeybee colonies in North America in 2006, and similar phenomena were also found in Europe. Although this disease is causing severe ecomonic loss to beekeepers, the cause or causes of this syndrome are not well understood. Recently, a newly identified virus, Israeli acute paralysis virus (IAPV) [155], has been suggested as a possible cause [156].
Viral infections are also a great concern for other arthropods such as shrimps or crayfish. The White spot syndrome virus is one of the most virulent pathogens of cultured shrimps, and causes massive financial loss [157]. In order to develop counteracting strategies, it is important to understand the pathology and the immune response of the affected animals.

3.4.2 Insect disease vectors

Insects, such as mosquitos, ticks or sandflies are common vectors for diseases that have a serious impact on human health. For example, yellow fever virus, West Nile virus (WNV) and Dengue virus (DENV) are mosquito borne and can cause severe illness such as encephalitis in humans [158]. Until the early 20th century, vector borne diseases led to more deaths in humans than all other causes combined. Some studies suggest that climate change and increased climate variability are fostering the spread of infectious diseases beyond their traditional geographic domain [159]. WNV, for example, was confined to Africa, Asia and Europe, but has recently spread to North Amer-

ica [160]. Consequently, a genetic model for studying host-virus interactions in insects would be beneficial to society in many ways.
It was assumed that insects acted as passive vectors, that could transmit pathogens by three non-specific interactions: carriage on the body, regurgitation and defecation. Today most researchers think that dissemination of pathogens by insect-vectors involves specific interactions between the virus and the host. Studying the mechanisms that ensure virus persistence and transmission might therefore allow the development of strategies to prevent the spread of infectious diseases. Recently, model systems for host-pathogen interactions, such as the fruit fly *Drosophila melanogaster* have been used to dissect the crosstalk between the virus and the insect defense mechanisms.

3.4.3 Aims of this thesis

The general aim of this PhD thesis was to use the common fruit fly *Drosophila melanogaster* as a model to study the insect host immune response against viral infection. My work mostly focused on the characterization of the inducible response towards virus infection, and the main findings are presented in this manuscript.
At the start of my thesis, several groups had independently shown that RNAi is a major antiviral defense mechanism that protects drosophila against a broad range of viral invaders (see section 3.2.2). Furthermore, there was strong evidence that in addition to RNAi, drosophila mounts an inducible antiviral response, that is partially mediated by the Jak-STAT pathway [142]. However, the data available at that time were restricted to the study of a single virus infection, namely DCV. Hence, nothing was known about the participation of this pathway in the defense against viruses other than DCV. This lack of knowledge prompted us to conduct a comparative study, that included members of four distinct RNA virus families. Our aim was (i) to decipher whether the Jak-STAT pathway constitutes a broad antiviral defense mechanism and (ii) to investigate if the relative contribution of this pathway to the fly's antiviral immunity is as important as RNAi.
In the meantime, several publications pointed to a role of the Toll and Imd

pathway in the antiviral defense of the fruit fly. Again, these studies were performed using individual viruses, and we decided to extend our comparative analysis to these additional pathways. Like for the Jak-STAT pathway we were interested to know if these pathways are active against a certain type of virus or virus family, or if they exhibit a broad antiviral function. The results of this comparative study are summarized in Chapter 5.

Another focus of my work was the characterization of virus induced effector molecules. Infection with DCV leads to the induction of some 150 genes [142]. Several of these induced genes including the marker *vir-1* were known to be regulated by the Jak-STAT pathway. We hypothesized that genes regulated by this pathway may function within the antiviral defense, and decided to focus our attention on the gene *CG11501* (*Chifoumi*). *Chifoumi* was of particular interest for us, as it was reported to (i) be regulated by the Jak-STAT pathway in response to septic injury [67], and (ii) function as a putative negative regulator of the Jak-STAT pathway [68]. The results of this characterization are summarized in Chapter 6.

In addition to the research presented in this thesis, I contributed to the study of *Vago*, where I co-authored the manuscript published in Nature Immunology in 2008.

MATERIAL AND METHODS

Chapter 4

Material and Methods

4.1 Fly strains

OregonR (OR), *yw*, *DD1 cn bw*, w^{A5001} and w^{1118} flies were used as wild-type control flies. The *hop* alleles used (*M38* and *msv1*) have been described [58] as well as the $ago2^{414}$ and $dcr2^{R416X}$ mutant lines [126, 164]. $MyD88^{c03881}$, Dif^{1} and *key* mutant flies were described [161, 45, 162]. CFMex flies were a gift from M. Boutros (Heidelberg, Germany) and the Df(3R)ED6316 (Def8925) and Df(3R)Ptp99A[R3] (Def5091) lines were obtained from Bloomington stock center.

4.2 Infections and survival

Adult flies 3-10 days in age were used for survival experiments. Infections were performed by injecting 4.6 nl of a viral suspension into the thoraces of adult flies (Nanoject II apparatus, Drummond Scientific). Viral stocks were prepared in 10 mM Tris-HCL, pH 7.5 with the exception of VSV which was used directly from Vero cell culture supernatant (DMEM). Injection of the same volume of 10 mM Tris-HCl, pH 7.5, was used as a control. Infected flies were then incubated at 22°C or at 29°C if the experiment involved thermosensative mutants. For survival experiments, two tubes of 10 flies were injected per virus and flies were kept on standard cornmeal-agar. For

RNA isolation, 20-25 flies were injected. In the case of bacteria, flies were pricked with a thin needle previously dipped in a concentrated overnight culture of *Escherichia coli* and *Micrococcus luteus* in LB medium.

4.3 Cell culture

Full Schneider medium (Biowest) for drosophila cell lines contained 10% fetal calf serum (PerBio), 1x Glutamax (100x, 200 mM, Invitrogen), 1x Penicillin/ Streptomycin (100x mix, 10 mg/ml/ 10000 U, Invitrogen).
DMEM for Vero R cells contained DMEM high glucose medium (Invitrogen), 10 % fetal calf serum (PerBio), 1x Penicillin/ Streptomycin (100x mix, 10 mg/ml/ 10000 U, Invitrogen), 1x non essential amino acid mix (100x, Invitrogen), 10 mM Pyruvat (Gibco) and 200 mM L-Glutamin (Invitrogen).

4.4 DNA extraction

DNA from whole flies was extracted by smashing single flies in 50 μl squishing buffer (10 mM Tris-Cl pH=8.2, 1mM EDTA, 25 mM NaCL and 200 μg/ml Proteinase K). The resulting suspension was incubated for 30 min at 37°C followed by heat inactivation of the proteinase at 95°C for 15 min.

4.5 Plaque assay

Vero R cells were seeded in 24 well plates (Falcon, 1Mio cells in 12 ml DMEM per plate) and incubated overnight at 37°C and 5% CO_2. Infected flies were smashed in 300 μl DMEM and centrifuged at full speed (13000 rpm). The supernatant was collected and applied to a 0.22 μm filter unit (Millipore) using a 1 ml syringe. The filtrate was then serially diluted with DMEM (1:10 dilutions). The media was removed from the cells and 100 μl of the virus-inoculum added (each dilution in duplicates). After one hour of infection at 37°C and 5% CO_2, free virus was removed by washing with PBS (Biowest). After adding the overlay (2/3 DMEM, 1/3 5% methylcellulose for SINV and

2.5% methylcellulose for SINV (methylcellulose 400 cp/ Sigman-Altrich in water autoclaved), cells were incubated at 37°C and 5% CO_2 for 2 days. Cells were then fixed using 4% formaldehyde (Electron Microscopy Sciences)/ 1X PBS (Biowest) for 20 min at RT and cells were stained for 10 min at RT using crystal-violet (10x crystal-violet solution 100 ml, formaldehyd 37% 100 ml and water 800 ml). 10x crystal-violet solution contained crystal-violet (Sigma-Altrich) 10 g, formaldehyde 37% 50 ml, EtOH (96%) 100 ml and water 350 ml. Plaques were counted and results expressed in PFU/ ml or PFU/ fly.

4.6 TCID50

1,2*10^5 KC 167 cells were seeded per well in a volume of 100 μl full Schneider media in a 96 well plate (Falcon). Cells were led to attach for a minimum of 20 min at RT. 5 flies were smashed in 300 μl of full Schneider media and spun for 10 sec at full speed (13000 rpm). The supernatant was filtrated through 0.22 μm filter units (Millipore) using a 1 ml syringe. The filtrate was then serially diluted with full Schneider media (1:10 dilututions). The media was removed from the seeded cells by turning the plate upside down and 50 μl of the serially diluted virus inoculum was added. Four wells per sample and dilution were infected and kept in the insect cell incubator for 2 days at 23°C. To fix the cells, 50 μl of 8% formaldehyde (Electron Microscopy Sciences) in 1x PBS (Biowest) was added and incubated for 20 min at RT. Afterwards, cells were washed twice with 100 μl of 1x PBT (1x PBS (Biowest), 0.1% Triton-X-100) solution. Then 100 μl of blocking solution (1x PBT, 10% fetal calf serum (PerBio)) was added and incubated for 30 min at RT. The primary antibody was added for 2 h at RT 1:500 diluted in 50 μl blocking solution. Rabbit anti-DCV F3 was used for the detection of DCV. Polyclonal antibodies directed against FHV were a gift from A. Schneemann. Cells were then washed twice as described above, before the secondary antibody (goat-anti-rabbit FITC labelled, Jackson Immuno Research) was added 1:500 diluted in 50 μl blocking solution for 1 h at RT. Cells were again washed twice with 1x PBT and dried. Positive and negative stained wells were identified

using a fluorescent microscope.
The TCID50 per ml was calculated by identifying the inoculum dilution at which 50% of the wells were positively stained. The proportionate distance (PD) between the two dilutions in between 50% infected wells was calculated as followed: (% of positive wells in the dilution above 50% dilution) - 50%) divided by ((% of positive wells in the dilution above 50% dilution) - (% of positive wells in the dilution below 50% dilution)). Then the 50% endpoint was calculaed by taken the log of the dilution next above the 50% dilution. PD and 50% endpoint are then used to calculate the TCID50 per ml.

4.7 RNA analysis

4.7.1 RNA extraction

For RNA extraction 10 to 15 flies were smashed in 350 μl of a TRI Reagent RT bromoanisole solution (50 μl of bromoanisole per 1 ml of TRI Reagenz RT (MRC)). Tubes were vortexed for 15 sec and incubated at RT for 5 min. Following incubation, tubes were centrifuged for 15 min at 12500 rpm at 4°C. After centrifugation the aqueous phase was transferred into a fresh tube and 300 μl of isopropanol was added. Samples were mixed and incubated for 10 min at RT before being centrifuged for 10 min at 12500 rpm at 4°C. The supernatant was removed and the pellet washed with 500 μl 70% ethanol. After centrifugation for 5 min at 7500 rpm, the supernatant was removed and samples were dried for 5-10 min at 65°C and resolved in 30 μl of pre-heated (65°C) water. RNA samples were quantified using Nanodrop.

4.7.2 cDNA synthesis

cDNA was synthesized using the iScriptTM cDNA synthesis Kit (Biorad). Reactions took place in a final volume of 20 μl, containing 4 μl 5x reaction mix, 1 μl reverse transcriptase and 1 μg of RNA plus nuclease free water. For reverse transcription, the T3000 Thermocycler (Biometra) was used with following PCR program. Step 1: 65 °C 5 min, step 2: 4°C 5 min, step 3:

25°C 10 min, step 4: 42°C 60 min, step 5: 70°C 15 min, step 6: 16°C pause. For QPCR analysis, cDNA was diluted 20 times.

4.7.3 Semiquantitative reverse transcriptase PCR

We performed semiquantitative reverse transcriptase (RT) PCR using Brilliant II QRT-PCR Core Reagent Kit 1-step (Stratagene). 200 ng of RNA was used per reaction, in a total volume of 25 μl (2.5 μl core buffer, 0.75 μl MgCL2, 1 μl dNTPs, 5 μl forward primer (100 μM), 5 μl reverse primer (100 μM), 1 μl reverse transcriptase and 0.25 μl Taq polymerase. Primers used were as follows.
CrPV forward (OJL1369) 5'-GAGACGCAAACACTCTTCAGG-3'
CrPV reverse (OJL1372) 5'-ATGACCCTTGAGATAATCCTC-3'
RpL32 forward (IMU 85) 5'-GTGTATTCCGACCACGTTACA-3'
RpL32 reverse (IMU 86) 5'-ATACAGGCCCAAGATCGTGA-3'
Following PCR program was used: step 1: 45°C 30 min, step 2: 95°C 10 min, step 3: 95°C 30 sec, step 4: 60°C 1min 30 sec go to step 3 and repeat 34 times, step 5: 16°C pause. The PCR product was applied to a 1% agarose gel and band intensenties were quantified using Image J (Open source).

4.7.4 Quantitative real time PCR

1-step QPCR

We used the Brilliant II QRT-PCR Core Reagent Kit, 1-step (Stratagene). The reaction took place in a total volume of 20 μl containing 2 μl core buffer, 0.8 μl dNTPs, 0.8 μl MgCl2, Taq man gene expression assay (Applied Biosystems) 1μl, reverse transcriptase 1 μl, Taq polymerse 0.2 μl, water 9.2 μl and 5 μl of RNA (40 ng/ μl). epMotion 5070 (Eppendorf) was used to pipette the plates.
We used the 7500 Fast Real-Time PCR System (Applied Biosystems) with following PCR program. Step 1 45°C 30 min, step 2: 95°C 10 min, step 3: 95°C 15 sec, step 4: 60°C 1 min. Go to step3 and repeat 39 times.

Following Taq man gene expression assays (Applied Biosystems) were used. CG11501 (Dm02146136 s1), TotM (Dm02362087 s1), upd (Dm01843792 g1), upd2 (Dm01844134 g1), upd3 (custom designed upd3exon2-ANY), Attacin (Dm02362218 s1), RpL32 (Dm02151827 g1). Gene expression was normalized to the expression of RNA encoding the ribisomal protein (RpL32).

2-step QPCR

We used the iQTM Custom SYBR Green Supermix Kit (Biorad). The reaction took place in a total volume of 10 μl, containung 2.4 μl Supermix, 0.3 μl forward oligo, 0.3 μl reverse oligo, water 5 μl and 2 μl 20 times diluted cDNA. epMotion 5070 (Eppendorf) was used to pipette the plates. The PCR was performed using the CFX384TM Real-Time System (Biorad) with the following program. Step1: 95°C 3 min, step 2: 95°C 10 sec, step3: 55°C 30 sec. Go to step 2 and repeat 39 times.
Primers used for QPCR were as follows:
RpL32 forward 5'-GACGCTTCAAGGGACAGTATCTG-3'
Rpl32 reverse 5'-AAACGCGGTTCTGCATGAG-3'
Vago forward 5'-TGCAACTCTGGGAGGATAGC-3'
Vago reverse 5'-AATTGCCCTGCGTCAGTTT-3'
vir-1 forward 5'-GATCCCAATTTTCCCATCAA-3'
vir-1 reverse 5'-GATTACAGCTGGGTGCACAA-3'
DCV forward 5'-TCATCGGTATGCACATTGCT-3'
DCV reverse 5'-CGCATAA CCATGCTCTTCTG-3'
FHV RNA 2 forward 5'-CAACGTCGAACTTGATGCAG-3'
FHV RNA 2 reverse 5'-GCTTTACAGGCATTTCCAA-3'
Drosomycin forward 5'-CGTGAGAACCTTTTCCAATATGATG-3'
Drosomycin reverse 5'-TCCCAGGACCACCAGCAT-3'
Diptericin forward 5'-GCTGCGCAATCGCTTCTACT-3'
Diptericin reverse 5'-TGGTGGAGTGGGCTTCATG-3'
Gene expression was normalized to the expression of RNA encoding the ribisomal protein (RpL32).

4.7.5 Microarray analysis

For each experimental challenge, three biologically independent samples samples comprising 45 male OR flies were used. Flies were infected with either 500 particles of DCV, $1*10^5$ particles of FHV or 200 particles of SINV and mRNA was extracted 24 h and 48 h or 48 h and 72 h or 4 d and 8 d after infection, respectively. RNA extraction, biotinylation and hybridization to Affymetrix drosophila GeneChip microarrays (Affymetrix) were done as described [163]. The Affymetrix Microarray Suite 5.0 (Affymetrix) or Excel (Microsoft) with a combination of built-in functions and custom formulae were used for data analysis. Raw data were sorted with the "absent-marginal-present flags" generated by the Microarray Suite functions. Although an "absent flag" might indicate that there was no mRNA of particular type present in a sampe, "marginal flags" and "absent flags" may indicate problems with the hybridization. Therefore, only data points marked as "present" were retained. The remaining data mass for each microarray was then normalized to itself, making the median of all the meassurements one. Subsequent analysis of the results was done on the genes present in all three replicates of a sample type.

4.7.6 In situ hybridisation

Cloning

Primers were chosen to amplify the whole coding region of CG11501 as well as the 5 and 3 UTR. DNA from OR wild-type flies was used as template and oligos used were in situ forward 5-GTTCTCCCAGTAAATCCAATCA-3 (CK-C) and in situ reverse 5-GCTTCTCTTTATTGTTTATGTGATGC-3 and (CK-D). The reaction took place in a totale volume of 50 μl and contained 5 μl 10x buffer, MgCl2 (50mM) 1.5 μl, dNTPs (mM) 5 μl forward ologi (100 μM) 1 μl, reverse oligo (100 μM) 1 μl, Taq 0.5 μl, water 34 μl. Following PCR program was used: 94°C for 2 min, 94°C for 30 s, 60°C for 30 s, 72°C for 1min (24 cycles), 72°C for 10 min. The PCR product was cloned using the TOPO TA Dual Promotor cloning kit (Invitrogen).

Resulting clones were verified by sequnecing and linearized using KpnI and PvuI (NEB). 4μg of plasmid was used and the digestion mix was incubated for 1h at 37°C. Linearization of the plasmids were verified by applying 3 μl of the restriction mix to the gel. 100 μl of linearized plasmids were mixed with 100 μl phenol/chloroform (vol/vol). The aqueous phase was taken, filled up with water to 200 μl, and DNA was precipitated by adding 800 μl EtOH 100% and 20 μl sodium acetate (3M). The mixture was kept at -80°C for 30 min. The precipitation mixture was centriged for 30 min at 12500 rpm, and the pellet washed with EtOH 70%. After centrifugation at 12500 rpm for 10 min, the pellet was dried and resuspended in water (15-20 μl) to reach a final concentration of 1 μg/ μl.

In vitro transcription and DIG labelling

We used the DIG-RNA labelling Kit from Roche. Plasmids linearized with PvuI were used as template for transcription from the SP6 promotor, creating the antisense probe, whereas KpnI digested plasmids were used for T7 transcrption, creating the sense probe. The reaction took place in a total volume of DNA (1 μg/μl) 2 μl, 10x reaction buffer 2 μl, T7 or SP6 enzyme 2 μl, RNAse OUT 1 μl, labelled nucleotide mix (dGTP, dCTP, dATP, dUTP) 2μl and water 11 μl. The reaction mix was incubated for 2 h at 37°C. RNA was precipitated at -80°C for 30 min using EDTA (0.2M) 2 μl, LiCl (4M, RNAse free, Sigma) 2.5 μl, EtOH 100% 75 μl. Following centrifugation at 12500 rpm (4°C) for 30 min, the pellet was washed with EtOH 70% and centrifuged for further 10 min. The pellet was dried and resuspended in 10 μl water, before addition of 90 μl hybridisation (HS) buffer (deionised formamide 50% final volume, 20x SSC 5% final volume, salmon sperm 100 μg/ ml, heparin 50 μg/ ml, TritonX 0.1% final volume, fill up to final volume with water).

Preparation of anti-DIG-alcaline phosphatase antibody and test of probes

In order to minimize the background, anti-DIG-alcaline phosphatase antibodies were pre-absorbed using embryos. For this purpose embryos were

conserved overnight in EtOH -20°C. Approximately 100 μl of these dehydrated embryos were then washed in 1ml of a 1:1 (v:v) mixture of EtOH and PBT (PBS, 0.1% Tween-20) whilst rotating for 5 min. Afterwards, embryos were washed twice with 1ml PBT whilst rotating for 10 min. The antibody solution was then diluted 1:100 in PBT . 1 ml of this dilution was then incubated with the rehydrated embryos overnight at 4°C. After removal of the embryos, the pre-absorbed antibody solution was then stored at 4°C. In order to test the DIG-labelled probes, 2 μl of different probe concentrations were immobilized (ultraviolet cross-linking) on a nitrocellulose membrane. The membranes were then saturated with BSA (3% BSA in PBT) for 30 min at RT. Membranes were then washed twice with PBT, followed by a 30 min PBT wash whilst shaking. The antibody was then diluted in PBT, 0.5% BSA (1:2000) and incubated with the membrane for 30 min at RT. Afterwards, the membrane was washed twice with PBT followed by two washes with AP buffer (100mM TrisHCl pH 9.5, 100mM NaCl, 50mM MgCl2, 0.1% Tween-20, fill up with water to final volume). Coloration was achieved by adding NBT and BCIP in AP buffer (Roche) according to the manufacturers instructions. The coloration reaction was then stopped by adding PBT.

Fixation

Flies were dissected and kept in PBT (PBS, 0.1% Tween-20) on ice (less than 30 min to avoid RNA degradation). The PBT was replaced by first adding 280 μl PBT, then 120 μl 10% formaldehyde and finally 500 μl heptan. Shake gently by turning the tube upside down (30-45 sec). Replace the first fixation solution by adding 520 μl PBT, 240 μl 10% formaldehyde and 40 μl DMSO and incubate for 20 min at RT. Afterwards, the fixed tissues are washed twice for 5 min with 1 ml MeOH. Samples can be stored in MeOH at -20°C for weeks.

Post-fixation and permeabilisation

Replace the MeOH with 980 μl MeOH and 20 μl water and incubate for 30 min. Wash three times with 1 ml PBT for 5 min. Afterwards, incubate for 30

min with 1 ml PP (PBS, 4% formaldehyde, 0.1% Triton-X) and wash twice with 1ml PTX (PBS, 0.3% Triton-X). Incubate for 10 min in 500 μl PTX and 500 μl HS, followed by a 5 min wash with 1ml HS.

Prehybridization

The samples were placed for 2-3h in HS buffer (50% deionized formamide, 5x SSC, 50 μg/ ml denatured salmon sperm DNA) at 65°C in a waterbath. Remove HS buffer and leave a volume smaller than 100 μl. Then add 5 μl of pre-absorbed and denatured probe and hybridize overnight at 65°C without mixing.

Hybridization

The DIG-labelled probe was prepared as described by Roche. 1 μl of the probe was then mixed with 4 μl of HS, denatured at 65°C for 4 min and placed on ice. The probe was then added to the sample and hybrdized overnight at 65°C. The next day samples were washed. All washing steps took place at 65°C. First, samples were washed twice in HS for 20 min. Then samples were washed in 1ml HS/PBT (3 volume/ 1 volume) followed by HS/PBT (1 volume/ 1 volume) and finally HS/PBT (1 volume/ 3 volume). Samples were then placed back at RT and washed four times for 10 min with 1 ml PBT. Finally, samples were incubated with pre-absorbed anti-DIG antibody diluted 1:2000 in PBT for 1h at RT, followed by three 10 min washes in 1ml PBT.

Coloration

Coloration was achieved according to the manufacturers specification (Roche). Samples were washed twice for 5 min in AP buffer and transferred in 24 well plates (Corning). The coloration substrate for the alcaline phosphatase was prepared during the secondwash by mixing 1 ml AP buffer with 4.5 μl NBT (50 mg/ml) and 3.5 μl BCIP (50 mg/ml). The substrate mixture was then applied to the samples and stored in dark until the coloration became apparent. The reaction was then stopped by adding and rinsing several times

with PBT. Samples were then mounted in glycerol and analyzed.

4.8 Protein analysis

4.8.1 Sample preparation and protein Quantification

Protein was quantified using Bradford reagent, Biorad Protein Assay (Biorad). Fly samples were prepared by smashing 2 to 3 flies in 150 μl lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Trition X100 and 1 dose of protease inhibitor cocktail complete mini (Roche)). 200 μl of Protein Assay was mixed with 800 μl water plus 1 μl of sample in 1 ml cuvettes (Sarstedt). BSA was used to create a standard curve ranging from 0 μg/ml to 21 μg/ml. Optical density (OD) was meassured at 595 nm, and 30 μg was loaded on a SDS-PAGE gel. Hemolymph was extracted from 20 flies using Nanoject II apparatus (Drummond Scientific) and mixed with 10 μl lysis buffer. The whole hemolymph/ lysis buffer mixture was loaded on a SDS-PAGE gel and equal protein amounts were verified by staining later the membrane with Ponceau red (Ponceau S solution, Sigma). Purified recombinant protein was quantified by meassuring the OD at 280 nm (Nanodrop). Samples were mixed with 10μl of 5x Laemmli loading buffer (400 mM Tris Base, 8% LiDS, 30% Glycerol, 408 mM MAC, 0.1% bromophenol blue, final pH 6.8), incubated at 95°C for 5 min and applied to the gel.

4.8.2 SDS-PAGE

Samples were loaded on a 12% SDS-PAGE gel. The migration gel contained: Water 4.1 ml, Acrylamide/ Bisacrylamide ratio 29/1 (Euromedex) 5 ml, Tris 1.5 M pH=8.8 3.1 ml, SDS 20% (Euromedex) 62.5 μl , APS 10% (Euromedex) 125 μl, Temed 5 μl (Roth). The stacking gel contained: Water 4.1 ml, Acrylamide/ Bisacrylamide ratio 29/1 (Euromedex) 1 ml, Tris 1 M pH=6.8 750 μl, SDS 20% (Euromedex) 30 μl , APS 10% (Euromedex) 30 μl, Temed 6 μl (Roth). Migration was carried out in 1x TCG buffer (Tris/ glycine/ SDS Buffer, Biorad) for 90 min at 90 V.

4.8.3 Western Blot

Proteins were transferred under standard conditions to nitrocellulose membranes for 90 min at 100 V under agitation using a commercially available transfer buffer (Biorad). The membrane was saturated in TBST 2x 3% milk (Biorad) for 1 h at RT (10x TBST pH 7.6 contained Tris HCl pH 7.2 100 mM, NaCl 90g/ l and 0.5 % Tween 20). Afterwards, the membrane was washed twice with TBST 2x and the primary antibody was incubated overnight at 4 °C in TBST 2x 0.3% milk . After incubation the membrane was washed twice with TBST 2x and the secondary antibody was added in TBST 2x for 1 h at RT. The membrane was washed times with TBST 2x and proteins on blots were visualized with ECL Chemiluminescent Detection reagents (Amersham) according to the manufacurer's instructions. Polyclonal mouse antiserum directed against CFM was used in a dilution of 1:2500. The actin-specific monoclonal antibody MAB1501R was from Chemicon and used in a dilution of 1:1000. Secondary antibodies to mouse (W4021) or rabbit (W4011) conjugated to horseradish peroxidase were from Promega and used in a dilution of 1:10000.

4.9 Generation of CFM specific antiserum

Recombinant CFM carrying a V5-Tag (2 mg/ml) was mixed 1:1 with Freund's adjuvant (CFA) and around 200 μl of the resulting emulsion was injected per mouse. 15 days later, a 2nd immunization was performed and after a further 15 days mice were bleeded. The blood was kept for 1 h at RT and at 4°C overnight. The serum was recovered the next morning. Prior to the first injection, preimmune serum was taken.

4.10 Statistical analysis

An unpaired two-tailed Student's t-test was used to determine statistically significant differences. P values of less than 0.05 were considered statistically significant. GraphPad Prism version 4 for Macintosh (GraphPad Software)

was used for statistical analysis of data.

4.11 Cell based Jak-STAT reporter assay

4.11.1 Preparation of dsRNA

dsRNAs directed against the genes of interest were prepared as follows. PCR was performed using oligos carrying the T7 promoter sequence and PCR products were puriefied using the QIAquick Rnase free Kit (Quiagen). The DNA was then quantified using Nanodrop and should have a minimum concentration of 125 ng/μl.
For RNA transcription, the MEGAscript T7 Kit (Ambion) was used according to the manufacturer's instructions with a total of 1 μg DNA. For precipitation, the RNA was then mixed with sodium acetate/ ethanol and incubated for 15 min at -20°C. After centrifugation for 15 min at 12000 rpm and 4°C, the RNA was washed with ethanol -70°C. The centrifugation step was repeated, the pellet dried and the RNA was reconstituted in 30-50 μl nuclease free water.

4.11.2 Transfection

3*10^6 S2 or Kc167 cells were seeded with in a cell petri dish (Corning, diameter 6cm) in 3ml of Schneider medium. The reporter plasmids contained fragments of the TotM promotor or Draf promotor upstream of the firefly luciferase gene. For the transfection, 0.1 μg of the reporter plasmid, 4 μg of dsRNA (GFP, hop, cfm or Ptp61f), 20 ng of internal transfection control (renilla lucifrase) and 1 μg of either empty pAc5V5HisA or pAC containing the upd gene under the control of an actin promotor were mixed in a total of 195 μl of water. 28 μl of 2M CaCl2 were added to this mix while vortexing. While continuing vortexing, the mix was then transferred dropwise in 225 μl of 2X HeBs (280mM Nacl, 50mM Hepes, 1,5mM Na2HPO4, pH 7,12). The precipitate is then kept at RT for 30 min, before it is transferred dropwise to the cells. 24 h post transfection, cells were washed with PBS (Biowest) and

Schneider medium added. 4 days after transfection, cells were collected and centrifuged for 10 min at 1500 rpm at RT. The cell pellet was lysed using 70 μl of lysis buffer (Dual Luciferase reporter Assay System, Promega) whilst shaking for 30 min at RT. The lysate was then centrifuged for 10 min at 10000 rpm at RT and 10 μl of supernatant transferred in a Lux 96 black well plate (Thermoelectron). Luciferase activity was read using the Dual Luciferase reporter Assay System kit (Promega).

4.12 In vitro assays using recombinant Chifoumi

The recombinant CFM used in the following assays was dissolved in PBS (Biowest) and devoid of any tag. The protein was expressed in S2 cells and purified by the group of Alain Roussel, Orleans, France.

4.12.1 Antibacterial assays

The antibacterial function of CFM against either gram(-) or gram(+) bacteria was tested using liquid-growth-inhibition assays. Overnight suspension cultures of bacteria were 1000-fold diluted and distributed in 96 well plates (100 μl per well). Recombinant CFM and Ampicillin were 2-fold serially diluted with PBS (Biowest). The serial dilutions were added to the bacteria (Escherichia coli strain D31 or Micrococcus luteus). After overnight incubation at 37°C, bacterial growth was determined by meassuring the optical density (OD) at 570 nm. The experiment was performed in triplicates. The highest final concentration of CFM tested was 50 μM.

4.12.2 Pretreatment of viruses with CFM

It was tested if incubation of CFM with either SFV, EMCV or RFV inhibits infection of Vero cells. $5 \cdot 10^5$ Vero cells per well were seeded in 6 well plates (Falcon) the day prior to infection. As a positive control, Interferon β (1000 U/ml) was added. The following day, 18 μl CFM (2.53 mM) or PBS

(Biowest) were incubated with 2 μl of virus stock (SFV $2.5 \cdot 10^7$ PFU/ml, EMCV $3 \cdot 10^7$ PFU/ml, RVF $2 \cdot 10^8$ PFU/ml) at RT for 90 min. Preincubated virus was diluted and Vero cells were infected with an MOI of 0.001. Infection took place in 250 μl media for 1 h at 37°C. After infection, the inoculum was removed and cells were washed with 1 ml media. The following days, cell culture supernatant was harvested and viral titers were determined using plaque assay.
The direct effect of CFM on SINV was investigated using a plaque reduction assay. $4 \cdot 10^5$ PFU were incubated with 100 μM CFM or BSA in a total volume of 40 μl sera free DMEM for 1h at RT. Samples were serially diluted and directly titrated on Vero cells.

4.12.3 Cytokine assay

To analyse if CFM functions as a cytokine, 10^6 S2 cells per well were seeded in 24 well plates (Falcon) in 300 μl full Schneider, containing 5% serum. 6 μl of CFM (2.53 mM) was added, leading to a final concentration of 50 μM. After 15 h of incubation, 200 μl of supernatant was removed from each sample and cells were infected with either DCV or VSV (MOI=1) in the remaining volume of 100 μl. Thus, CFM was still present during infection (15 μM). After 1h of infection at 22°C, 1,5 ml full Schneider containing 10% serum was added to non-infected and DCV infected samples, whereas 1ml full Schneider containing 10% serum was added to VSV infected wells. Samples were taken the following days after infection, and viral loads were determined using Q-PCR for DCV and plaque assay for VSV, respectively. In the case of DCV, viral RNA was isolated using QIAamp Viral RNA Mini Kit (Quiagen). 7 μl of each RNA sample was used for Q-OCR.
In the case of RFV, 10^6 S2 cells per well were seeded in 6 well plates (Falcon) in 1 ml full Schneider, containing 5% serum. CFM was added in a final concentration of 50 μl.

RESULTS

Chapter 5

Results: A comparative study of the drosophila immune response towards infection with RNA viruses

RNA interference (RNAi) is widely accepted to be a major defense mechanisms of drosophila against virus infection. However, infection with DCV triggers the induction of some 150 genes [142], suggesting that in addition to RNAi, drosophila relies as well on an inducible response to combat infection. Some of these induced genes were shown to be regulated by the Jak-STAT pathway, and loss-of-function mutants of this pathway were more susceptible to infection with DCV. Other pathways, such as the Toll and Imd pathway, have been reported to participate to the host-defense against other viruses. However, it is not clear if these inducible pathways participate to an universal antiviral defense mechanism in drosophila, like it has been proposed for RNAi, or if they are involved in virus-specific mechanisms of host-defense.

5.1 RNA virus infection triggers a complex transcriptional response

5.1.1 Microarray analyisis of DCV, FHV and SINV infected flies

Our group has previously studied the transcriptome of flies infected with three positive RNA viruses, DCV, FHV and SINV using Affymetrix DNA chip technology. Male OR flies were infected with either 500 particles of DCV, $1*10^5$ particles of FHV or 200 particles of SINV and mRNA was extracted 24 h and 48 h or 48 h and 72 h or 4 d and 8 d after infection, respectively. We then compared global transcriptional profiles of buffer- or virus-injected flies for every single virus.

This genome-wide, unbiased approach showed that drosophila mounts an inducible reponse to these three viruses, as we identified a large number of genes, that were induced by a factor of at least 2 following infection (Fig. 5.1).

Among these up-regulated genes, we found a group of 45 genes that were induced by all three RNA viruses (Fig. 5.1). According to Flybase, 22 of these commonly induced genes have no known molecular function (Table 5.1). Among the genes with known function, we found 8 genes that are associated with drosophila immunity. PGRP-SD and PGRP-SA, belong to the PGRP family of PRRs and are implicated in the recognition of Gram-positive bacteria upstream of Toll. GNBP-L1 (*CG12780*) belongs to the second family of PRRs in drosophila, and contains an N-terminal β-1,3 glucan binding domain similar to that of GNBP3, the PRR involved in fungal detection. Interestingly, GNBP-L1 has no predicted signal peptide, suggesting that it could function as a PRR against intracellular pathogens such as viruses. Immune induced molecule 2 (IM2, *CG18106*) and DIM23a (*CG15066*) are genes that have been shown to be induced in response to microbial infection in a Toll pathway dependent manner [67, 47]. Thus, PGRP-SD, PGRP-SA, IM2 as well as DIM23a are associated with the Toll pathway and therefore linked to microbial infection. Furthermore, the Toll pathway was shown to be in-

volved in the defense against DXV [117], suggesting that this pathway might be involved in the control of certain viruses.
Sp7 is an endopeptidase that has been found to be involved in the positive regulation of the melanization response [165]. SOCS36E (*CG15006*) has been shown to function as a negative regulator of Jak-STAT signaling [166], a pathway that was reported to participate to the host-defense of drosophila against DCV [142]. Finally, Vago (*CG2081*) which has been shown to regulate viral replication in a tissue specific manner [153].
Hence, the fact that one fifth of shared induced genes have an immune-related function indicates, that the inducible response observed, can indeed be directly linked to viral infection and not solely attribuated to other stresses caused by the infection.
Interestingly, we found evidence for virus-specific gene induction (Fig. 5.1). Although DCV and SINV induced 149 and 250 genes, respectively, 63 and 39 genes were specific to either DCV or SINV infection. FHV was found to be the strongest inducer of gene expression and triggered a 4-fold stronger induction than DCV with a total of 590 up-regulated genes. Although around 60% of these genes were specific for FHV, we could identify a large group of 163 genes that were shared between FHV and SINV. Taken together, these data suggest that virus infection leads to an inducible response in drosophila, that is specific for individual viruses. However, we can identify distinct virus groups as we observed a larger overlap of FHV and SINV induced genes than we did for FHV and DCV.
Among the five strongest induced genes by FHV were *CG11501* and *TotM*, which ranked as the two strongest induced genes upon SINV infection (Table 5.2). DCV infection, however, failed to trigger expression of these genes. *TotM* is a member of the Turandot family. Turandot genes are induced in certain stress conditions, including septic injury. Their function is unknown and their regulation complex and includes input from the Jak-STAT, the Imd and the MAPK pathway (reviewed in [7]). *CG11501*, termed *Chifoumi* (*cfm*) was shown to be regulated by the Jak-STAT pathway upon septic injury and proposed to function as a negative regulator of Jak-STAT signaling [67, 68]. A characterization of this gene is presented in chapter 6.

Table 5.2 lists as well expression values for the markers of virus infection *vir-1* and *Vago*. Both, *vir-1* and *Vago* induction was observed after infection with all three viruses. However, subsequent more quantitative analysis of *vir-1* and *Vago* gene expression revealed, that *vir-1* was induced by DCV and FHV but only weak by SINV ([142] and Fig. 5.5 A), whereas *Vago* was induced by DCV and as well SINV but only marginally by FHV [153]. These discrepancies might be explained by the fact that microarrays are not very quantitative, as hybridization occurs between the nucleic acid samples and short oligo sequences.

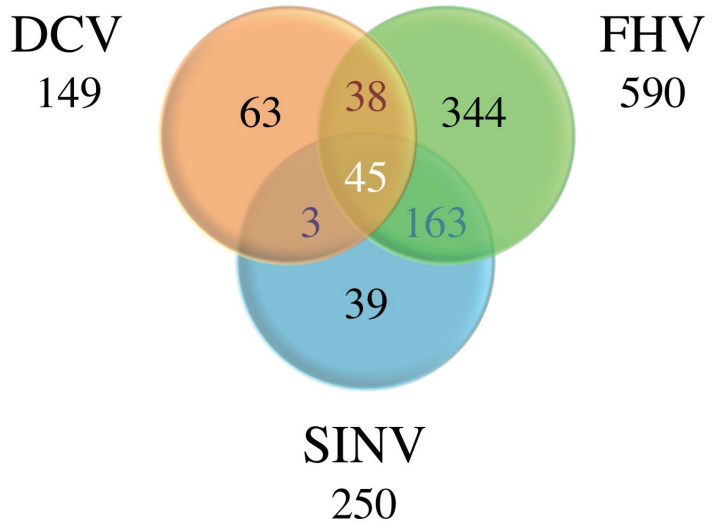


Figure 5.1: Virus specific transcriptional profiles. Venn diagram of genes induced by a factor of ≥ 2 after infection with DCV, FHV or SINV.

These observations reveal, that viral infection does trigger an inducible response in drosophila. This response, however, is largely dependent on the type of virus, and we can distinguish groups of viruses that show bigger overlap in their expression profiles than others. Several of the induced genes, including *TotM*, *CG11501* and *vir-1* were reported to be regulated by the Jak-STAT pathway. However, genes like *Vago* are known to be regulated independently of Jak-STAT, implying that several pathways will be involved. Hence, the inducible response to RNA virus infection appears to be rather

complex, and might reflect virus-specific differences, such as replication cycle, tissue tropism or pathophysiology. This high level complexity prompted us to not only investigate the inducible response towards a single virus but to compare a set of five distinct viruses. We decided to include the three positive-sense ssRNA virus, DCV, FHV and SINV, that were previously used for the microarrays. In addition, we chose two other viruses, Cricket paralysis virus (CrPV) and Vesicular stomatis virus (VSV).

Table 5.1: List of genes commonly induced by DCV, FHV and SINV by a factor of $\geq$ 2.

Gene	DCV	FHV	SINV	Function
Sp7 (CG3066)	2.2	4.1	3.0	Serine-type endopeptidase involved in positive regulation of melanization defense response
IM2	2.4	2.6	2.7	Experimental evidence that it is involved in the defense response
CG2081	5.3	6.0	4.7	Experiemtal evidence that it controls DCV replication in a tissue-specific manner
PGRP-SA	3.2	3.9	2.9	Peptidoglycon-binding; defense response to Gram(+) bacteria
Socs36E	6.3	10.0	6.6	Negative regulator of Jak-STAT signaling
CG12780	6.5	5.4	3.1	Sequence similarity with Gram-negative binding proteins (GNBPs)
DIM23a	3.1	2.2	3.2	Defense/ immunity protein
PGRP-SD	2.0	3.9	2.7	Peptidoglycon-binding; defense response to Gram(+) bacteria
CG6687	4.5	10.3	6.5	Serpin
Nmda1	2.0	4.9	2.9	N-methyl-D-aspartate selective glutamate receptor
EG:22E5.5	2.4	5.8	4.5	Enzyme
Uro	2.2	2.2	2.1	Urate oxidase (allantoin symthesis)
Tig	2.6	3.9	3.4	Motor
Cyp4e3	4.6	9.9	4.3	Cytochrome P450
CG14934	3.1	7.7	3.7	Alpha-glucosidase
Cyp4p3	2.5	2.1	2.0	Cytochrome P450
CG13941	4.3	22.3	8.8	Signal transduction
Hsp70Bc	3.1	5.5	5.4	Heat shock protein (chaperone)
Obp99b	3.8	10.8	4.6	Odorant-binding protein 99b (ligand binding or carrier)
Nmdmc	2.5	5.4	5.1	Methenyltetrahydrofolate cyclohydrolase
CG32412	3.1	3.5	2.0	Receptor guanylate cyclase
CG9505	2.6	5.3	3.0	Endothelin-converting enzyme (endopeptidase)
Idgf1	3.4	3.2	2.8	Imaginal Disc Growth Factor 1
Eip75B	2.5	3.3	2.1	Ligand-dependent nuclear receptor
CG8791	7.5	3.7	2.5	High affinity inorganic phosphate:sodium symporter
CG7635	4.8	2.9	2.2	No known function
CG14906	2.3	4.4	2.8	No known function
CG15745	3.4	10.9	8.5	No known function
CG15043	6.9	7.3	2.1	No known function
CG31764	7.1	9.5	4.6	No known function
Tsp42El	2.1	4.0	2.2	No known function
Tsp42Ed	2.4	4.0	2.5	No known function
CG13324	3.5	9.2	5.2	No known function
CG10911	2.3	2.9	2.3	No known function
CG18348	2.5	7.3	2.2	No known function
CG13075	2.1	3.1	2.2	No known function
CG11671	3.1	5.0	2.0	No known function
CG14907	2.4	5.4	2.6	No known function
CG10912	3.6	7.7	2.3	No known function
CG4680	2.9	13.7	8.6	No known function
CG1572	2.4	2.1	2.0	No known function
Frost	19.7	10.9	2.9	No known function
CG4713	2.1	3.3	2.3	No known function
CG4618	2.5	2.5	2.3	No known function
CG10916	2.0	6.7	4.3	No known function

Table 5.2: List of the 10 strongest FHV induced genes and corresponding expression values for SINV and DCV infection. / indicates absent value.

Gene	FHV	SINV	DCV	Function
CG14957	141.2	/	57	Chitin binding (based on InterPro)
CG11501	113.6	65.9	/	Putative negative regulator of Jak-STAT pathway
CG6639	97.6	/	/	Endopeptidase
CG3117	61.1	/	/	Endopeptidase
TotM	47.4	42.4	1.9	Stress response
Ets21C	33.8	/	18.5	DNA binding
CG9989	32.3	5.9	10.8	No known function
GstD2	26.1	3.2	1.5	Glutathion S transferase D2
CG1304	23.5	/	/	Endopeptidase
Arc2 (CG13941)	22.3	8.8	4.3	No known function
vir-1 (CG31764)	9.5	4.6	7.1	Induced upon DCV infection in a Jak-STST depedent manner
Vago (CG2081)	6	4.7	5.3	Restricts DCV replication in a tissue-specific manner

5.1.2 The RNA viruses CrPV and VSV can also be used to study antiviral immunity in drosophila

CrPV was originally isolated from Australian field crickets (*Teleogryllus oceanicus* and *T.commudus*). CrPV was of interest for us, as it is a member of the *Dicistroviridae* and closely related to DCV (Fig. 5.2). DCV and CrPV significantly differ in the VSRs they encode (see section 3.2). CrPV infects a large host range within insects (Diptera, Lepitodtera, Orthoptera and Heteroptera). It replicates efficiently in drosophila cells and is pathogenic when injected into flies [130].

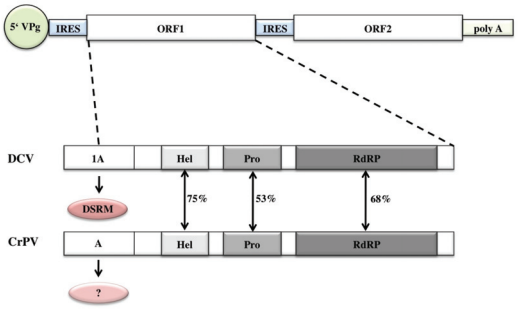


Figure 5.2: Schematic representation of the genome structure of *Dicistroviridae* members. The positive-sense ssRNA genome is covalently linked to a viral protein (VPg) at the 5' end and carries a polyA tail at the 3' end. It contains 2 ORFs encoding non-structural (ORF1) and capsid (ORF2) proteins. Viral translation is mediated by two internal ribosome entry sites (IRES). The percentage of identity between the sequence of non-structural proteins of DCV and CrPV is indicated. Hel: helicase, Pro: protease, RDRP: RNA dependent RNA polymerase, DSRM: double-stranded RNA binding motif.

Infection of drosophila with 500 particles of CrPV or DCV resulted in similar pathogenicity, leading to death in 9-12 days in a dose dependent manner at the standard temperature of 22°C (Fig 5.3 A). However, we found that the pathogenicity of DCV was temperature dependent, as infection at a higher temperature of 29°C led to a 1 to 2 day shift in survival (Fig. 5.3 B). Surprisingly we observed a much stronger influence of the temperature on the virulence of CrPV. Flies infected with 500 particles of CrPV died

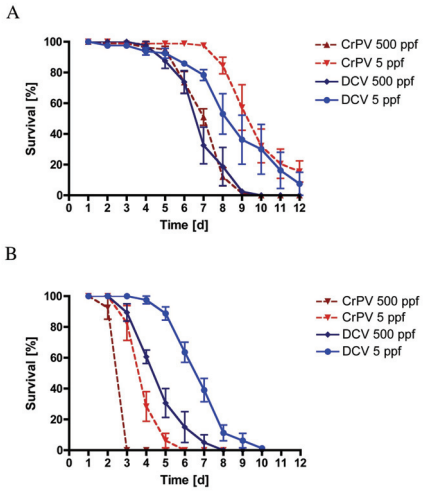


Figure 5.3: Relative pathogenicity of DCV and CrPV. A and B: OR wild-type flies were infected with the indicated dose of either DCV or CrPV and survival was monitored daily. Survivals were carried out at 22°C (A) and 29°C (B).

very rapidly within 3 days of infection, reflecting a shift in survival by 6 days when compared to 22°C. Flies infected with 5 particles of CrPV died within 6 days, thus, even faster than flies infected with a 100-fold higher dose of DCV. Hence, a change in temperature by 7 degrees resulted in a profound difference of the virulence of CrPV.

The last virus, VSV, was chosen as a prototype for negative-sense ssRNA virus. VSV is a member of the *Rhabdovirdae* and able to infect insects and mammals. Due to its ability to infect cattle, VSV has particular importance to farmers in certain regions. It is also a common laboratory virus that is used to study the properties of viruses in the *Rhabdovirdae* family. VSV is an arbovirus whose natural infections encompass two steps: cytolytic infections of mammalian hosts and transmission by insects. In cultured drosophila cells, VSV establishes a persistant, noncytopathic infection [167]. VSV was introduced to our laboratory by Stefanie Mueller. At standard temperature, VSV replicates to high numbers in wild-type flies without killing the host. Infection at 29°C did not effect the ability of flies to tolerate the infection(Fig. 5.4).

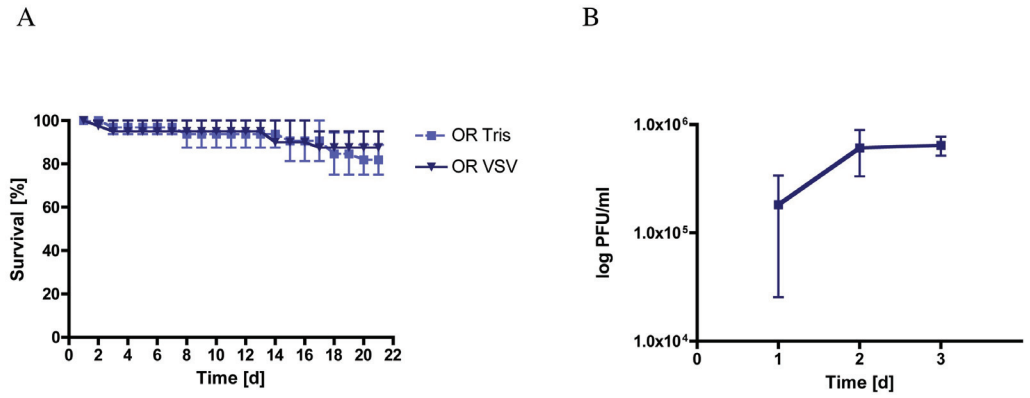


Figure 5.4: VSV causes a persistant infection in wild-type flies. A: OR wild-type flies were infected with 1.8*10^4 particles of VSV and survival was monitored daily. B: Or wild-type flies were injected with 1.8*10^4 particles of VSV and viral titers were determined at indicated timepoints. Experiments were carried out at 29°C.

5.1.3 Induction of marker genes by RNA viruses in drosophila: a comparative analysis

First, we aimed to characterize the transcriptional response triggered by CrPV and VSV by analysing their ability to induce known marker genes of viral infection. Like previously published work, we observed a strong induction of *vir-1* by DCV and FHV, whereas septic injury failed to induce transcription of *vir-1* ([142] and Fig. 5.5 A). The induction level was comparable for DCV and FHV and increased with time. Interestingly, we could observe induction of the *vir-1* transcript after infection with CrPV. However, *vir-1* expression by CrPV was less pronounced than after infection with DCV or FHV. SINV failed to induce *vir-1* within 4 days of infection and similar results were obtained for VSV.

Infection with DCV led to a robust induction of *Vago*, whereas no upregulation was observed upon FHV infection or septic injury confirming previous reports ([153] and Fig. 5.5 B). Surprisingly, although closely related to DCV, CrPV also failed to induce *Vago* expression. VSV infection resulted in a minor and timepoint restricted increase of *Vago* transcript numbers, that was only noticeable after 2 days of infection and much lower than *Vago* induction triggered by DCV. Finally, we could not detect upregulation of *Vago* after infection with SINV, which is at odds with previously published data ([153] and Fig. 5.5 B). This discrepancy may reflect differences in the dose of virus used to infect flies, and experiments are in progress to test this possibility.

Our microarray analysis identified *cfm* (*CG11501*) and *TotM* as the two strongest induced genes by SINV. SINV infection triggered a robust induction of the *cfm* transcript as early as 6 h post injection, which decreased with time (Fig. 5.6 A). Interestingly, we observed a similar induction pattern after infection with VSV. In agreement with the microarray data, FHV also induced *cfm* expression at 24 h post injection, albeit not as strong as SINV and VSV (Fig. 5.6 B). Consistant with the microarray results, DCV failed to induce *cfm* within the first 2 days of infection. However, *cfm* expression was slightly triggered by DCV 3 and 4 days after infection. No induction of *cfm* was observed upon expression of CrPV. Surprisingly, we observed a

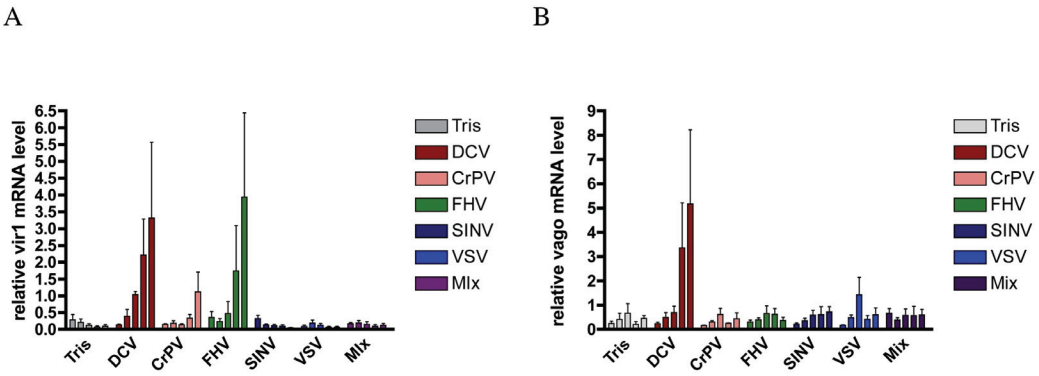


Figure 5.5: ***vir-1*** **and** ***Vago*** **expression profiles after virus infection. A and B: OR wildtype-flies were injected either with Tris, DCV (500ppf), CrPV (500 ppf), FHV (2*10^4 ppf), SINV (2.5*10^3 ppf), VSV (1.8*10^4 ppf) or pricked with a mix of Gram-positive (M. luteus) and Gram-negative (E. coli) bacteria. Flies were frozen at following timepoints: 6h, 24h, 48h, 72 h and 96 h post injection. RNA was extracted and** *vir-1* **(A) and** *Vago* **(B) expression determined using Q-PCR. Values shown represent the mean and standard deviation of 3 independent experiments.**

very minor induction of *cfm* by bacteria compared to viruses, which was only present in some experiments performed (see as well Fig. 6.3).

Interestingly, *TotM* expression patterns were almost mirror images of *cfm* profiles, confirming previous reports pointing to co-regulation of these genes (Fig. 5.6 C and D) [67]. In summary, *cfm* as well as *TotM* were very strongly induced by SINV and VSV very early after infection. FHV and DCV, as well as bacteria induced *cfm* and *TotM*, although to a much lower extent. Inducible expression of *cfm* and *TotM* following SINV, VSV, FHV or bacterial infection was transient and decreased after 2 days of infection. By contrast DCV induced expression of these genes increased with time.

We next examined the induction pattern of AMPs upon virus infection. We chose to monitor expression of *drosomycin* and *diptericin*, which are standard read-outs for the Toll- and Imd- pathways, respectively. Infection with a mix of Gram-positive and Gram-negative bacteria led to an expected strong induction of *drosomycin* expression, which peaked after 24 h. *drosomycin* mRNA levels were much weaker in virus injected flies. This expression most likely reflects the injury effect of the injection, as it is similar to Tris injected

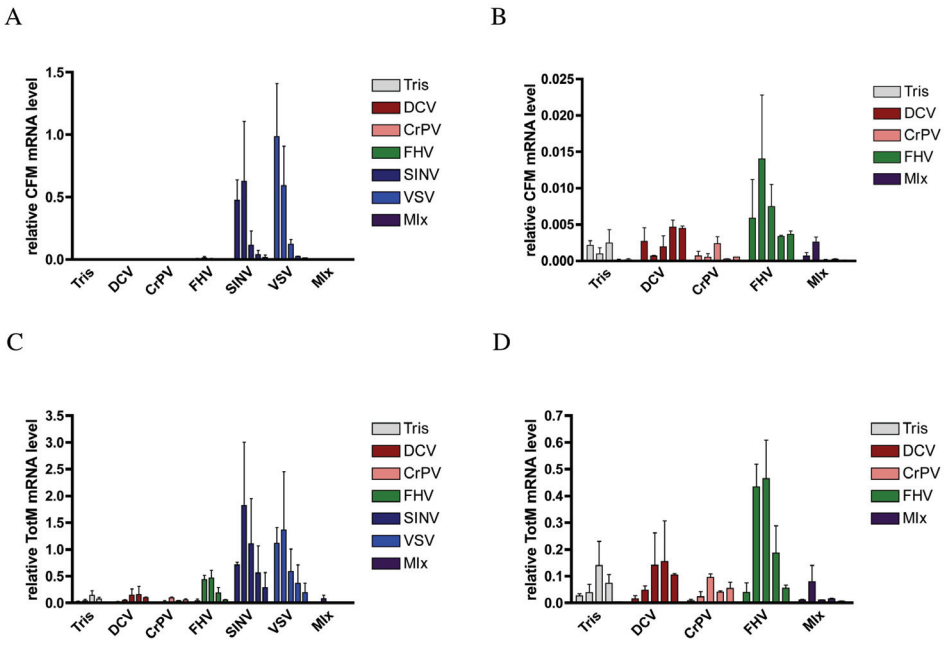


Figure 5.6: *cfm* **and** *TotM* **expression profiles after virus infection. A and C: OR wildtype-flies were injected either with Tris, DCV (500ppf), CrPV (500 ppf), FHV (2*10^4 ppf), SINV (2.5*10^3 ppf), VSV (1.8*10^4 ppf) or pricked with a mix of Gram-positive (M. luteus) and Gram-negative (E. coli) bacteria). Flies were frozen at following timepoints: 6h, 24h, 48h, 72 h and 96 h post injection. RNA was extracted and** *cfm* **(A) and** *TotM* **(C) expression determined using Q-PCR. Values shown represent the mean and standard deviation of 2 independent experiments. B and D: SINV and VSV were removed from the dataset.**

control flies (Fig. 5.7 A and B).

Bacterial infection led to a strong expression of *diptericin*, with a maximal induction 6 h post infection. As for *drosomycin*, virus infection did not lead to an induction of *diptericin* that was as strong as for bacteria. However, *diptericin* profiles of virus infected flies were not generally superposable with *diptericin* profiles of Tris injected control flies (Fig. 5.7 C and D). Weak *diptericin* expression was observed after infection with CrPV as well as SINV. This data makes clear that virus are, if at all, weak inducers of AMP expression compared to bacteria. However, this observation might simply reflect

the fact that viral infection might lead to a local immune response, whereas septic injury triggers a systemic response. Furthermore, other AMPs need to be tested.

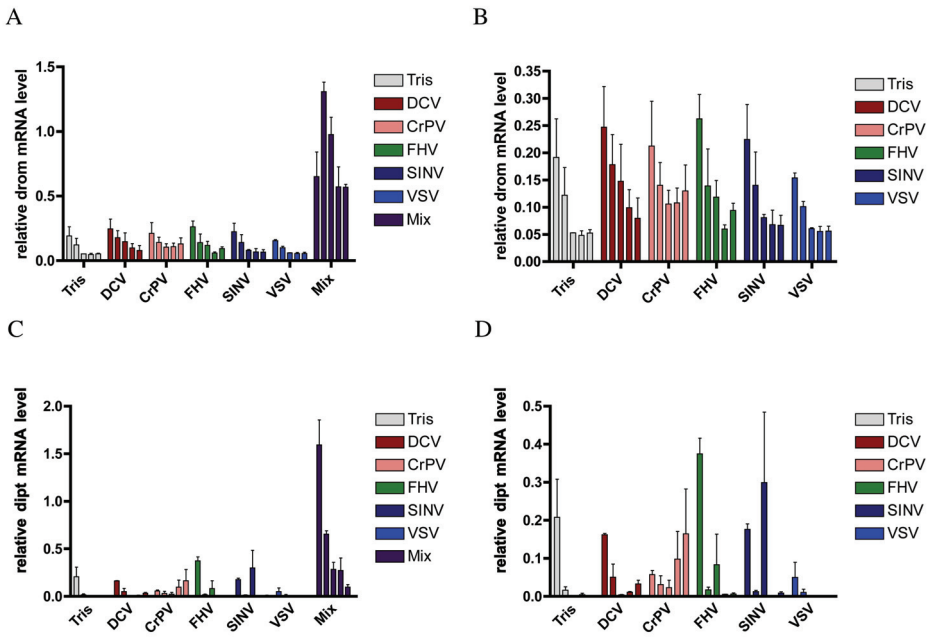


Figure 5.7: *drosomycin* **and** *diptericin* **expression profiles after virus infection. A and C: OR wildtype-flies were injected either with Tris, DCV (500ppf), CrPV (500 ppf), FHV (2*10^4 ppf), SINV (2.5*10^3 ppf), VSV (1.8*10^4 ppf) or pricked with a mix of Gram-positive (M. luteus) and Gram-negative (E. coli) bacteria). Flies were frozen at following timepoints: 6h, 24h, 48h, 72 h and 96 h post injection. RNA was extracted and** *drosomycin* **(A) and** *diptericin* **(C) expression determined using Q-PCR. Values shown represent the mean and standard deviation of 2 (A) and 3 (C) independent experiments. B and D: Bacteria were removed from the dataset.**

5.1.4 Summary

In summary we found evidence for an inducible response towards virus infection in drosophila. Our microarray data as well as the study of marker genes revealed profound differences in the expression profiles of distinct viruses al-

though we can distinguish groups of viruses that show bigger overlap in their expression profiles than others. Table 5.3 summarizes the induction patterns for known marker genes of viral infection. Genes like *vir-1*, *cfm* and *TotM* have been reported to be regulated by the Jak-STAT pathway, which was shown to participate to the host-defense against DCV infection. However, although we could observe induction of Jak-STAT regulated genes by CrPV, FHV, SINV and VSV nothing is known to date about the importance of this pathway in the defense against this viruses. Vago has been shown to control DCV replication in the fat body. At odds with published data, we could not observe any Vago induction by SINV within 4 days of infection, a situation that requires further investigation.The Toll and Imd pathway were as well described to participate to the antiviral defense of drosophila. We could not detect induction of the Toll pathway controlled *drosomycin* by any of our virus. However, expression of *diptericin* was slightly altered by CrPV and SINV in comparison to Tris injected control flies, but much weaker than the expression caused by septic injury.

Hence, our findings raise the question of the biological significance of these gene inductions and prompted us to investigate susceptibility to viral infection of mutant flies for signaling pathways, such as the Imd, the Toll and the Jak-STAT pathway.

Table 5.3: Virus induced genes. *At odds with published data.

Gene	DCV	CrPV	FHV	SINV	VSV	Bacteria
vir-1	↑	↗	↑	-	-	-
Vago	↑	-	-	-*	-	-
cfm	↗	-	↗	↑	↑	↗
TotM	↗	-	↗	↑	↑	↗
Drosomycin	-	-	-	-	-	↑
Diptericin	-	↗	-	↗	-	↑

↑ strong induction ↗ weak induction - no induction

5.2 The role of the Jak-STAT, Toll and Imd pathway in the antiviral defense

5.2.1 The Jak-STAT pathway participates to host-defense against Dicistrovirus

Previous work showed, that mutants of the Jak kinase *hopscotch* were susceptible to infection with DCV [142]. However, recent work revealed, that the endosymbiont *Wolbachia* protects flies from infection with several viruses, including DCV. As this bacteria is commonly found in drosophila, we decided to verify our stocks and found that *hopscotch* mutant flies were indeed infected with *Wolbachia*. This finding made it necessary to confirm the previously observed phenotype in *Wolbachia* cured flies.
Furthermore it was known, that the DCV-induced gene *vir-1* is regulated by the Jak-STAT pathway upon infection [142]. *vir-1* was found to be induced in the ventral epidermis, a tissue that is not targeted by DCV. This observation led to the following model in which virus infection leads to the secretion of a cytokine, which in turn would activate the Jak-STAT pathway in yet uninfected tissues. However, the cytokine remained to be identified.

Members of the upd family are inducded upon infection with DCV

Members of the upd family were shown to act as Jak-STAT pathway ligands (see section 1.3.3). We challenged OR wild-type flies with 500 particles of DCV and monitored expression of all three family members the following 4 days after infection (Fig. 5.8). No induction was observed for *upd* following DCV infection, as Tris injected control flies showed similar *upd* expression level. However, a clear induction of *upd2*, as well as *upd3* was observed starting from day 2 of the infection. These data strongly suggest, that the activation of the Jak-STAT pathway upon viral infection is mediated by members of the upd family, namely *upd2* and *upd3*.

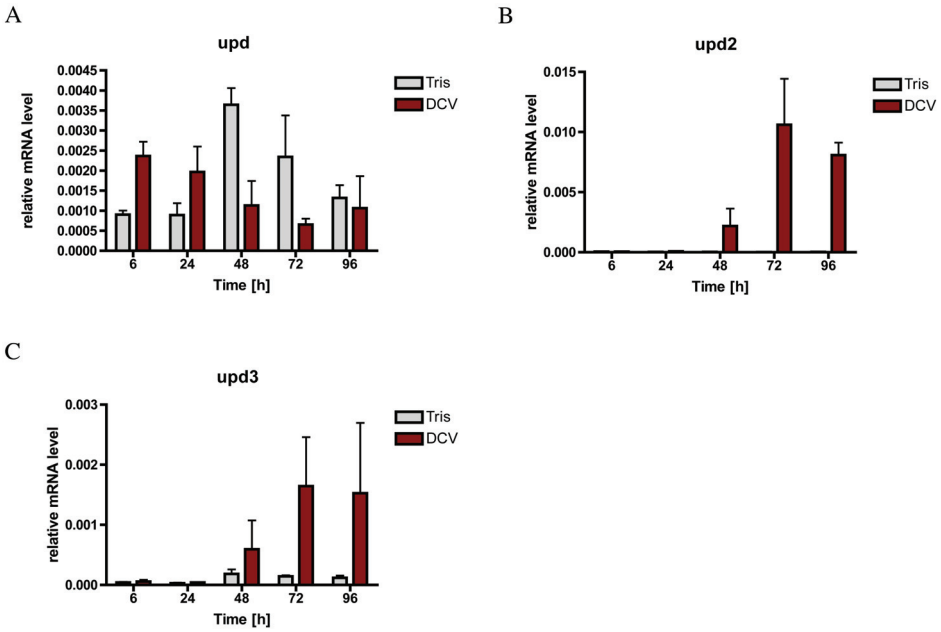


Figure 5.8: Members of the upd family are induced upon infection with DCV. A and C: OR wildtype-flies were injected either with Tris or DCV (500ppf) and RNA was extracted at indicated time-points. The expression level of *upd* (A), *upd2* (B) and *upd3* (C) was determined using Q-PCR. Values shown represent the mean and standard deviation of 2 independent experiments.

Antiviral resistance of *hopscotch* mutant flies

We monitored survival of *Wolbachia* cured loss-of-function $hop^{M38/msv1}$ flies after infection and observed, as previously reported, higher susceptibility of the mutant to DCV. Mutant flies died notably earlier (3-4 days) than wild-type control flies with less than 20% survival of mutant flies 5 days after infection, compared to 9 days for OR wild-type flies. (Fig. 5.9 A). In agreement with this observation, we detected significantly 12-fold higher viral titers in mutant flies (Fig. 5.9 B). This data confirms, that the Jak-STAT pathway is involved in the control of DCV infection in drosophila.

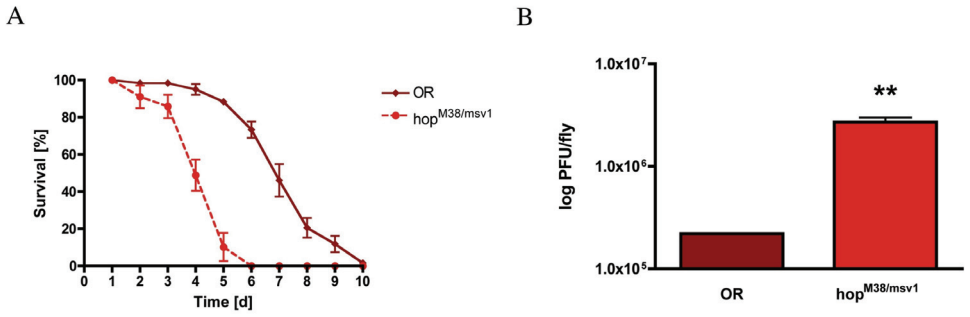


Figure 5.9: Jak kinase loss-of-function flies are more susceptible to DCV infection and show higher viral titers. A: OR wild-type and $hop^{M38/msv1}$ mutant flies were injected with 5 particles of DCV and survival was monitored daily. Values shown represent the mean and standard deviation of 3 independent experiments. B: Flies were injected with 5 particles of DCV and viral titers were determined 2 days after infection using TCID50. The higher susceptibilty in survival correlated with a 12-fold increase in viral load. Values shown are the mean and standard deviation of 3 independent experiments (P=0.001). Flies were kept at 29°C 3-4 days before injection and during experiments.

However, it remains unclear, if this pathway can be considered as a universal antiviral defense mechanism in drosophila, or if its importance is specific for the type of virus. We therefore infected loss-of-function $hop^{M38/msv1}$ flies with a subset of different viruses and monitored survival and viral load. Interestingly, infection with CrPV, which belongs like DCV to the Dicistroviridae, resulted in a similar phenotype. (Fig. 5.10). Jak kinase mutants died notably faster from infection than OR wild-type flies, with less than 20% survival after 4 and 5 days of infection, respectively. The observed shift in survival by 1 day correlated with an increase in viral RNA 1 day post injection, pointing to an involvement of the Jak-STAT pathway in the defense against CrPV.

We next tested for susceptibility of $hop^{M38/msv1}$ mutants to the Nodavirus FHV. Infection with a standard dose of $2*10^4$ viral particles showed no difference in survival. Mutant and wild-type flies died at the same rate, with less than 20% surviving flies after 5 days (Fig. 5.11 A). Similar results were obtained using a 10- and 100-fold lower dose of FHV (data not shown). A minor 2- to 3-fold increase in viral RNA 2 was observed in $hop^{M38/msv1}$ mutants (Fig. 5.11 B). However, viral RNA 2 levels did not reflect the amount of infectious particles, as no difference in viral load was observed using TCID50 (Fig. 5.11 C). This data suggests that the Jak-STAT pathway is not involved in the defense against FHV.

Similar results were obtained after infection with the Rhabdovirus VSV (Fig. 5.12). Neither wild-type nor mutant flies died from infection, as survival curves for Tris and VSV injected flies were superimposable. No significant difference in viral load was observed 1 and 2 days after injection. We saw a slight but statistically significant increase in viral load in loss-of-function $hop^{M38/msv1}$ flies 3 days post infection (2.7 fold compared to wild-type), which did not seem high enough to affect survival of the fly.

We last tested the Alphavirus SINV. Infection with SINV showed no phenotype in survival. Both, wild-type and mutant flies slowly started to die 18 days post infection. (Fig. 5.13 A). Likewise, no difference in viral load was observed 1, 2 and 3 days post infection (Fig. 5.13 B).

Overall, we did not see an involvement of the Jak-STAT pathway for all

viruses tested. Infection with either FHV, VSV or SINV was not affected in $hop^{M38/msv1}$ mutant flies. However, we could observe striking similarities between two members of the same virus family. Both DCV and CrPV showed increased susceptibility in survival and a significant increase in viral load (for a summary see Table 5.4) .

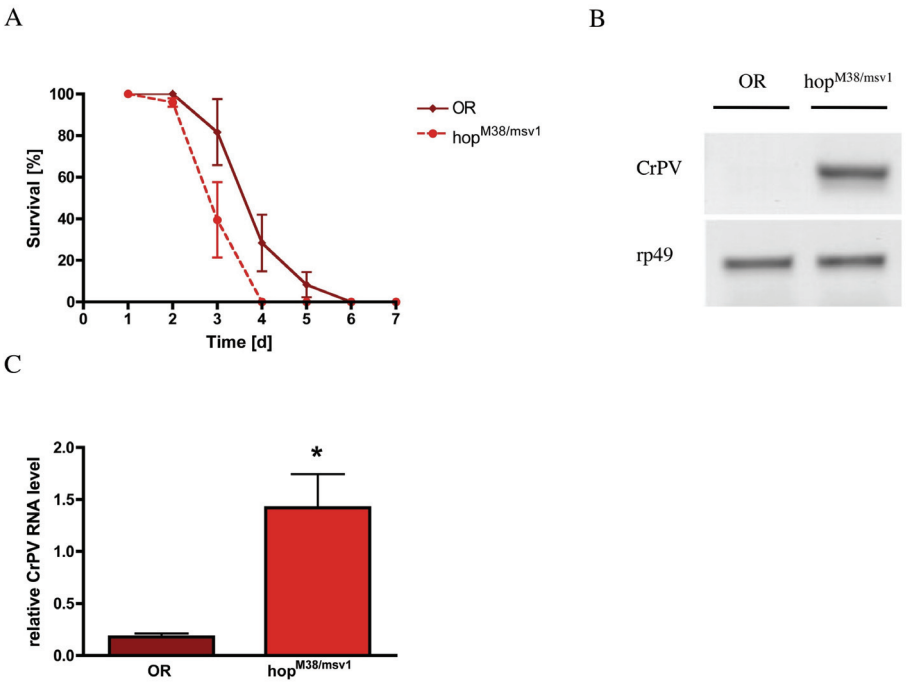


Figure 5.10: Jak-STAT mutant flies are more susceptible to CrPV infection and show higher viral titers. A: OR wild-type and $hop^{M38/msv1}$ mutant flies were injected with 5 particles of CrPV and survival was monitored daily. Values shown represent the mean and standard deviation of 3 independent experiments. B: Flies were injected with 5 particles of CrPV and viral load was determined 1 day after infection using semiquantative PCR. In OR wild-type flies, CrPV RNA was not detectable under the chosen conditions. One representative out of 3 independent experiments is shown. C: The band intensity of 3 independent gels was quantified and normalized to rp49, showing a significant higher virus load after 1d of infection for $hop^{M38/msv1}$ mutants (P=0.0185). Flies were kept at 29°C 3-4 days before injection and during the experiment.

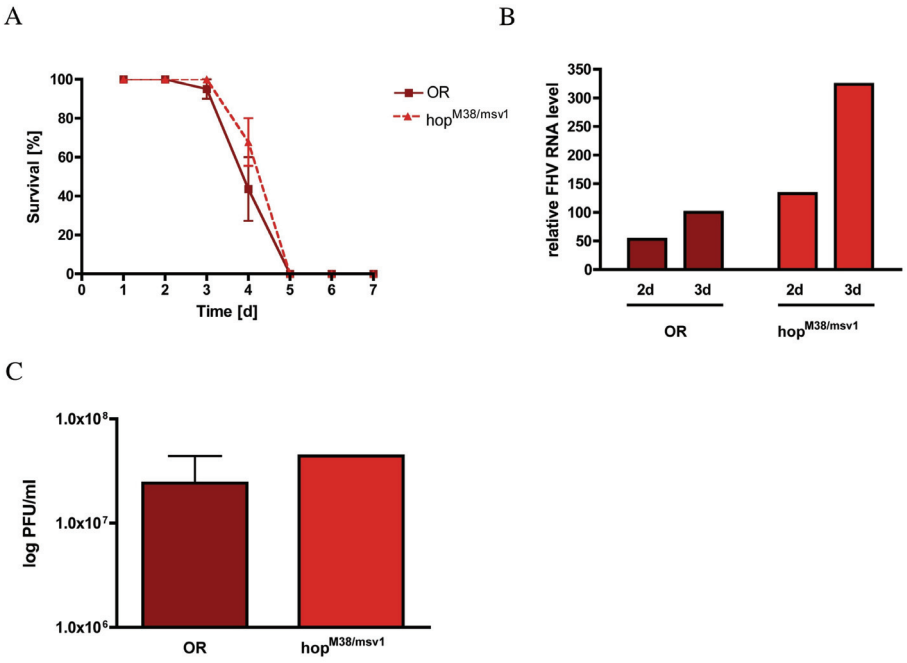


Figure 5.11: Jak kinase mutants show no phenotype upon FHV infection. A: OR wildtype flies and $hop^{M38/msv1}$ mutant were injected with 2*104 particles of FHV and survival was monitored daily. Values shown represent the mean of 2 groups of ten flies. B: $hop^{M38/msv1}$ mutants were injected with 2*10^4 particles of FHV and viral load was determined 2 and 3 days post infection. One representative out of 3 independent experiments is shown. C: No difference in viral load was detected 3 days post infection using TCID50. Values shown represent the mean and standard deviation of 2 independent experiments (P=0.4207). Flies were kept at 29°C 3-4 days before injection and during the experiment.

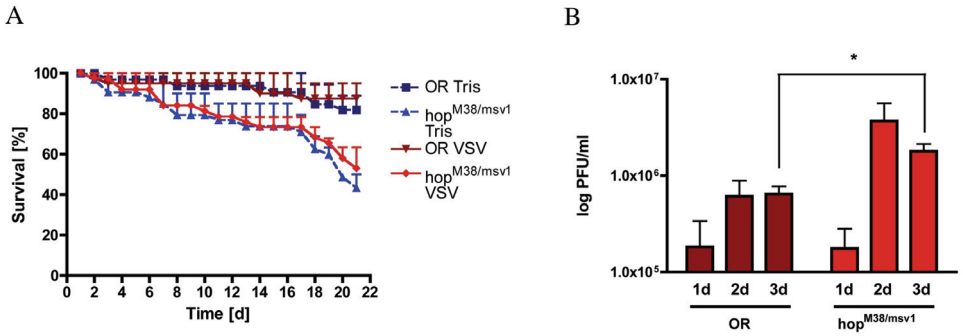


Figure 5.12: JAK kinase mutant flies show a slight increase in VSV titer but no survival phenotype. A: OR wild-type flies and $hop^{M38/msv1}$ mutant flies were injected with 1.8*10^4 particles of VSV and survival was monitored daily. Values shown are the mean and standard deviation of 2 independent experiments. B: Flies were injected with 1.8*10^4 particles of VSV and viral titers were monitored at indicated timepoints. After 3 days of infection a slight but significant 2.7-fold increase was observed in $hop^{M38/msv1}$ mutant flies (1d P=0.9754, 2d P= 0.1896, 3d P =0.0359). Values shown are the mean and standard deviation of 3 independent experiments. Flies were kept at 29°C 3-4 days before injection and during the experiment.

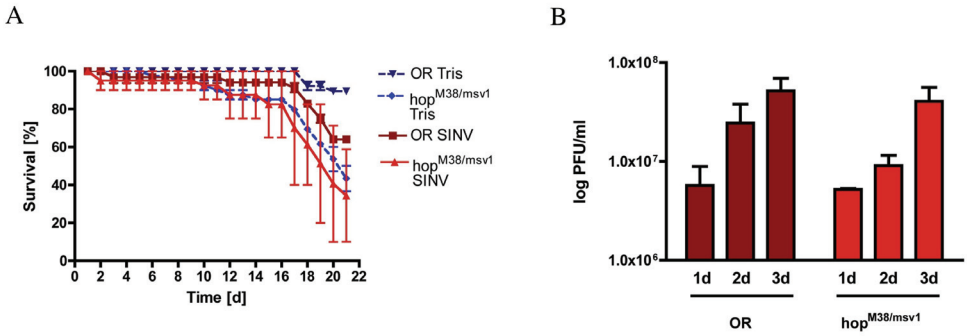


Figure 5.13: Jak kinase loss-of-function flies show no phenotype for SINV. A: OR wild-type and $hop^{M38/msv1}$ mutant flies were injected with 2.5*10^3 particles of SINV and survival was monitored daily. Values shown represent the mean and standard deviation of 2 independent experiments. B: Flies were injected with SINV and viral titers were determined for the following 3 days. No significant differences were detected (1d P= 0.8915, 2d P= 0.3778, 3d P=0.6844). Values shown are the mean and standard deviation of 2 independent experiments. Flies were kept at 29°C 3-4 days before injection and during experiment.

The Jak-STAT phenotype for Dicistrovirus is as strong as for RNAi mutants

We next addressed the question of the importance of the Jak-STAT pathway compared to RNAi in the context of *Dicistrovirus* infection. We decided to compare the Jak-STAT phenotypes observed for DCV and CrPV with the phenotypes for two null mutants of the RNAi machinery $ago2^{414}$ and $dcr2^{R416X}$ (Fig. 5.14 B). Both, $ago2^{414}$ and $dcr2^{R416X}$mutant flies died 3 days earlier from infection compared to yw wild-type control flies, with less than 20% surviving flies after 7 days of infection for yw and 4 days of infection for $dcr2^{R416X}$ and $ago2^{414}$ flies. In comparison, $hop^{M38/msv1}$ mutant flies died 3-4 days faster from infection compared to OR wild-type control flies, with less than 20 % surviving flies 5 and 9 days after infection, respectively (Fig. 5.14 A) We next monitored viral load in $dcr2^{R416X}$ and $ago2^{414}$ mutant flies 2 days post infection and compared the x-fold titer to the one optained in $hop^{M38/msv1}$ mutant flies (Fig. 5.14 C). The higher susceptibility of $dcr2^{R416X}$ and $ago2^{414}$ mutant flies correlates with a 10-fold increase in viral titer, hence, an increase similar to that observed in $hop^{M38/msv1}$ mutant flies. These data indicate that the Jak-STAT pathway is as important for control of DCV as RNAi.

Similar results were obtained in the case of the second *Dicistrovirus* CrPV. $ago2^{414}$ mutant flies died from infection 1 day earlier than yw wild-type control flies, with less than 20% surviving flies 5 and 4 days after injection, respectively. This result confirms that RNAi is involved in the host-defense against CrPV infection [129]. A similar difference was observed for $hop^{M38/msv1}$ mutant flies with less than 20% survival after 5 days of infection for OR wild-type flies and 4 days for $hop^{M38/msv1}$ mutant flies. In agreement with the survival data, both $ago2^{414}$ and $hop^{M38/msv1}$ mutant flies showed an increase in CrPV load 1 day post infection (Fig 5.15 C). However, we noted that yw flies supported replication of CrPV to higher levels than OR flies and, as a result, the x-fold increase in viral titer is more important for $hop^{M38/msv1}$ mutants than $ago2^{414}$ mutants (Fig 5.15 D). Surprisingly, $dcr2^{R416X}$ mutant flies did not show any phenotype for CrPV, neither in survival nor viral load

(Fig 5.15 B, C and D). This result is at odds with previous work from others [129], and probably reflects the fact that our experiments were carried out at 29°C.

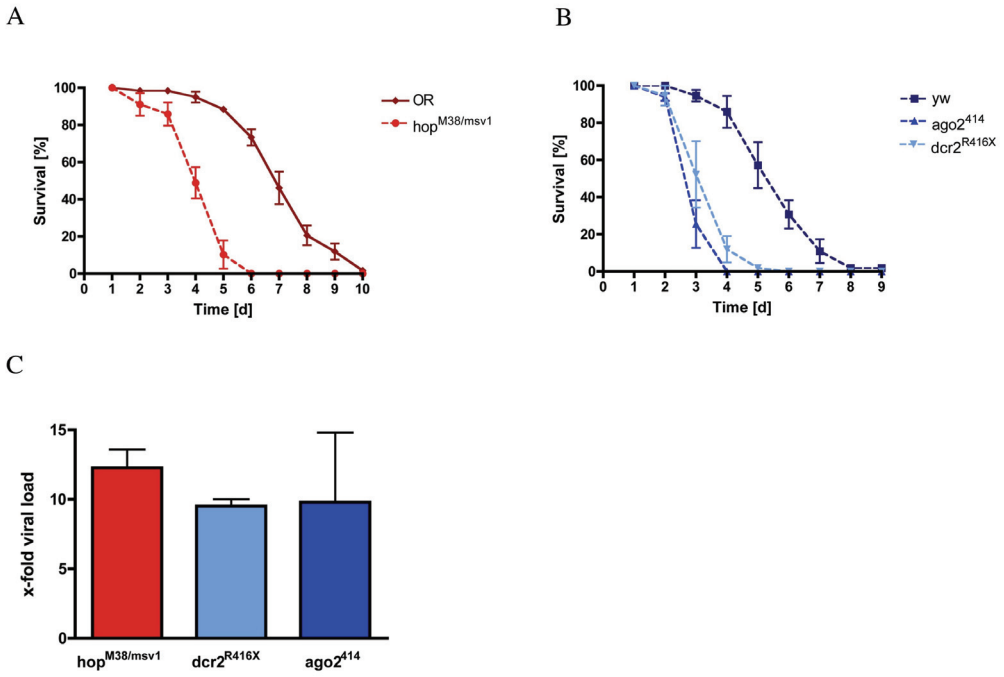


Figure 5.14: Equal contribution of the Jak-STAT pathway and RNAi for the control of DCV infection. A and B: Flies of the indicated genotypes were infected with 5 particles of DCV and survival was monitored daily. Values shown are the mean and standard deviation of 3 independent experiments. C: Flies of the indicated genotypes were injected with 5 particles of DCV and viral titers were determined 2 days post infection using TCID50 and expressed as x-fold, normalized to the titer obtained in corresponding wild-type flies. Values shown are the mean and standard deviation of 3 independent experiments. No significant differences were found between Jak-STAT and RNAi mutant flies ($hop^{M38/msv1}$ vs $dcr2^{R416X}$ P=0.12; $hop^{M38/msv1}$ vs $ago2^{414}$ P=0.6573; $ago2^{414}$ vs $dcr2^{R416X}$ P=0.9594). Flies were kept at 29°C 3-4 days before injection and during the experiment.

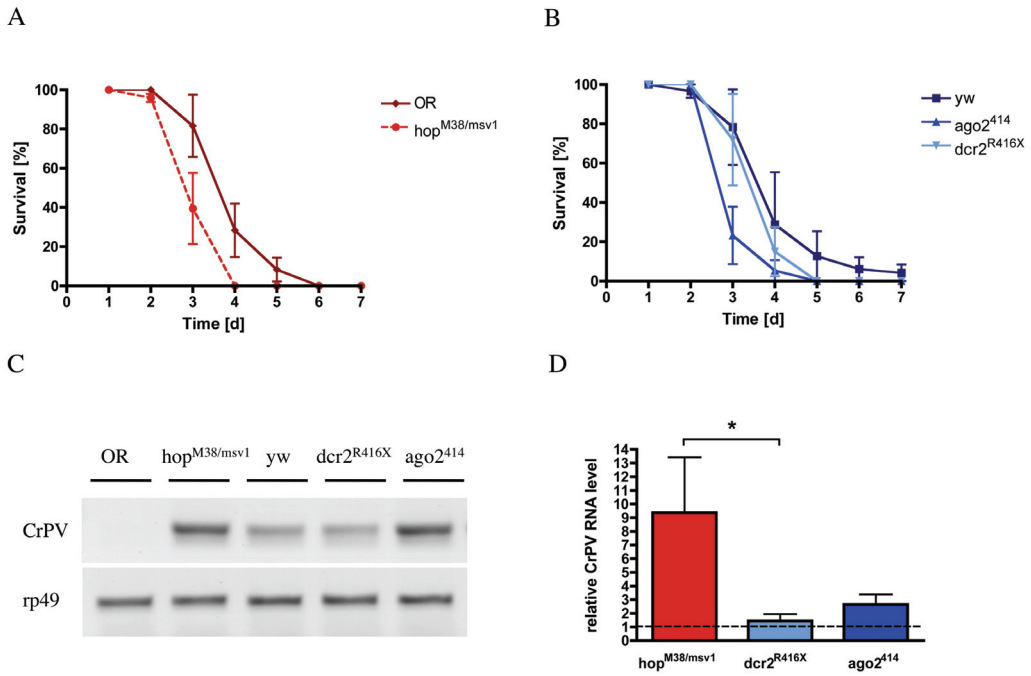


Figure 5.15: Comparison of Jak-STAT and RNAi phenotypes for CrPV. A and B: Flies of the indicated genotype were injected with 5 particles CrPV and survival was monitored daily. Values shown represent the mean and standard deviation of 3 independent experiments. C: Flies of the indicated genotypes were injected with 5 particles of CrPV and viral load was determined 1 day after infection using semiquantative PCR. D: The band intensity was quantified and normalized to rp49. Presented are the x-fold viral titers compared to wild-type flies. Values shown represent the mean and standard deviation of 3 independent experiments for $hop^{M38/msv1}$ and 5 independent experiments for $ago2^{414}$ and $dcr2^{R416X}$ ($hop^{M38/msv1}$ vs $dcr2^{R416X}$ P=0.043; $ago2^{414}$ vs $dcr2^{R416X}$ P=0.221; $hop^{M38/msv1}$ vs $ago2^{414}$ P=0.075). Flies were kept at 29°C 3-4 days before injection and during the experiment.

5.2.2 Toll pathway mutants are susceptible to DCV infection

We first examined survival of flies mutant for the Toll pathway transcription factor Dif and observed increased susceptibility of the mutant to infection with DCV (Fig. 5.16 B). Dif^1 loss-of-function flies died 4 days earlier compared to DD1 *cn bw* wild-type flies, with less than 20% survival after 6 and 10 days post infection, when infected with a standard dose of 500 ppf. The susceptibility in survival correlated with a significant 7.9-fold increase in viral RNA 2 days post infection (see Fig. 5.16 C). We decided to test another mutant of the Toll pathway affecting the adaptor molecule MyD88, that functions upstream of Dif. The survival phenotype was weaker in $MyD88^{c03881}$ mutant flies infected with 500 particles of DCV than the previously observed phenotype using Dif^1 mutants. $MyD88^{c03881}$ mutants died 1 day earlier from infection compared to w^{A5001} wild-type flies, with less than 20% survival 5 and 6 d after after infection, respectively (see Fig. 5.17 B). Although the shift in survival by 1 day was less pronounced than the one observed in Dif^1 mutant flies we could detect a considerable 16.8-fold increase in viral RNA 2 days post infection (Fig. 5.17 C). Taken together, these findings point to an involvement of the Toll pathway in the defense against DCV.

As we observed similar phenotypes for Jak-STAT pathway mutants after infection with DCV and CrPV, we decided to infect Dif^1 and $MyD88^{c03881}$ mutant flies with CrPV. Dif^1 mutant flies showed a 1 day shift in survival with less than 20% survival 6 days post infection for Dif^1 flies and 7 days post infection for DD1 *cn bw* wild-type flies after infection with 500 particles of CrPV. The mild susceptibility in survival correlated with a slight 2 to 4-fold increase in viral RNA 2 days post infection (see Fig. 5.18 C). However, we observed no phenotype for $MyD88^{c03881}$ mutants, neither in survival nor in viral load (Fig. 5.19).

Infection with the Nodavirus FHV did not show any phenotype, neither in survival, nor viral load (Fig. 5.20). Both, Dif^1 as well as $MyD88^{c03881}$ mutant flies died like their wild-type controls within 8 to 9 days of infection with FHV. Likewise, no difference in viral RNA was observed the following 3 days

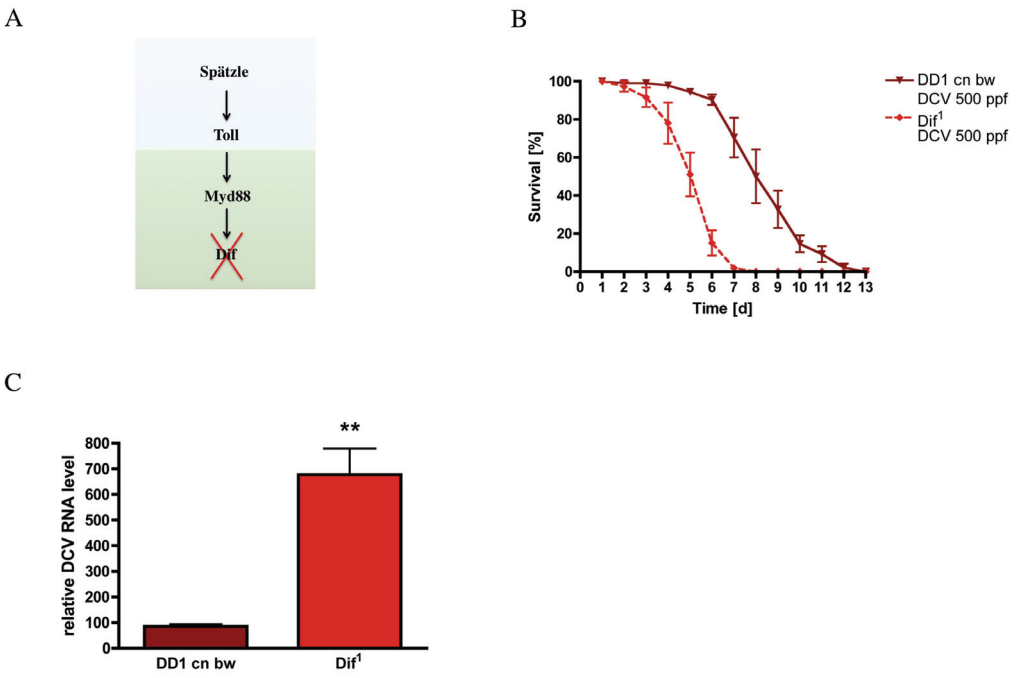


Figure 5.16: *Dif*^{1} **mutant flies are more susceptible to DCV infection.A: The Toll pathway transcription factor Dif functions downstream of the transmembrane receptor Toll and the adaptor molecule MyD88. B: Flies were injected with the indicated dose of virus and survival was monitored daily. Values shown represent the mean and standard deviation of 5 independent experiments. C: Flies were injected with DCV (500 ppf) and viral load was determined 2 days post infection using Q-PCR.** *Dif*^{1} **mutant flies contained significantly more virus (P=0.0043). One representative experiment out of 3 independent experiments is shown. Values shown are the mean and standard deviation of experimental triplicates. Flies were kept at 22°C during the experiment.**

post injection. Similar results were obtained for the Rhabdovirus VSV and the Alphavirus SINV. VSV and SINV did not show any survival phenotype as Dif^1 and $MyD88^{c03881}$ mutant flies resisted like wild-type flies to the infection (Fig. 5.21 and Fig. 5.22 A and C). No difference was observed for either of the mutants concerning VSV titers 1, 2, and 3 days post infection (Fig. 5.21 B and C). Likewise, we could not detect any difference in SINV titers, with the exception of a 3-fold decrease in $MyD88^{c03881}$ mutant flies 1 day post infection, which was not statistically significant (Fig. 5.22 B and C).

To summarize we could only detect a strong involvement of the Toll pathway after infection with DCV. CrPV resulted in a comparable phenotype in Dif^1 mutant flies, which was less pronounced than the phenotype observed with DCV. However, no phenotype for CrPV was detected in $MyD88^{c03881}$ mutant flies. None of the other viruses tested, FHV, VSV and SINV showed any phenotype of Dif^1 or $MyD88^{c03881}$ mutant flies, neither in survival nor viral load.

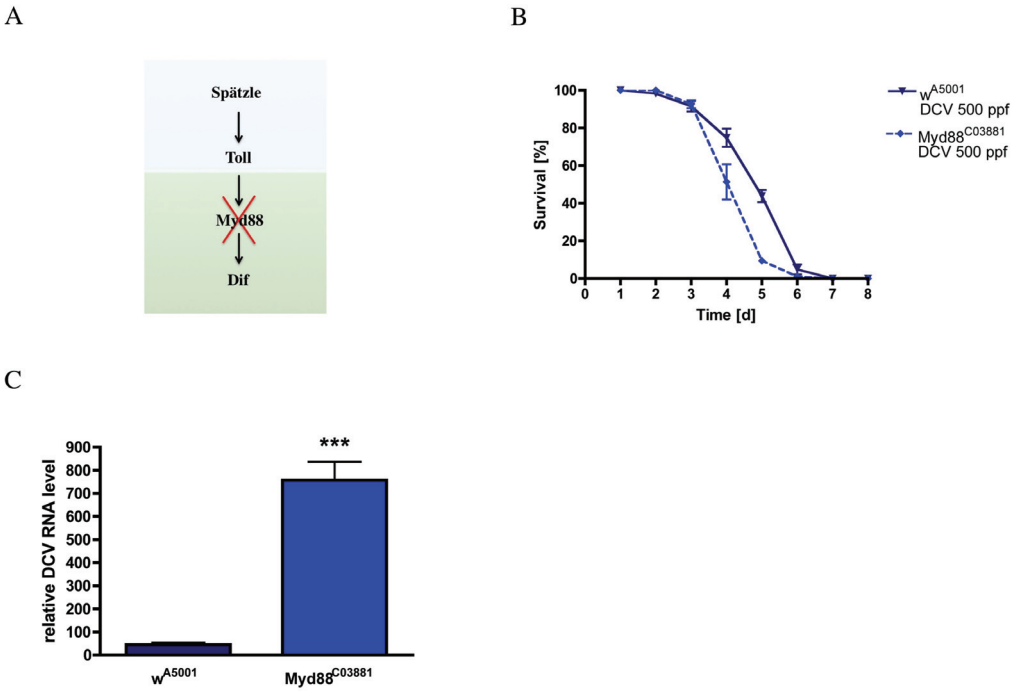


Figure 5.17: *MyD88*^{c03881} **mutant flies are more susceptible to infection with DCV. A: The adaptor molecule** *MyD88*^{c03881} **functions upstream of the transcription factor Dif, and downstream of the transmembrane recptor Toll. B: Flies were injected with the indicated dose of DCV and survival was monitored daily.** *MyD88*^{c03881} **mutant flies died faster from infection than DD1** *cn bw* **wild-type flies and showed a 1 day shift in survival. Values shown represent the mean and standard deviation of 5 independent. Flies were kept at 22°C for the duration of the experiment. C: Flies were injected with 500 paricles of DCV and viral load was determined 2 days post infection using Q-PCR.** *MyD88*^{c03881} **mutant flies showed a significant 16.8-fold increase in viral RNA (P=0.0009). One representative out of 3 independent experiments is shown. Values shown represent the mean and standard deviation of experimental triplicates. Flies were kept at 22°C for the duration of the experiment.**

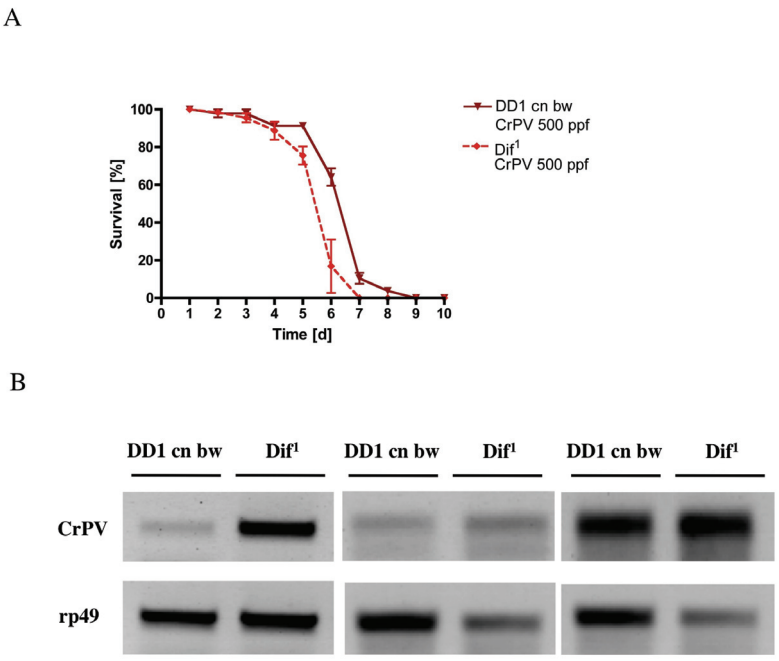
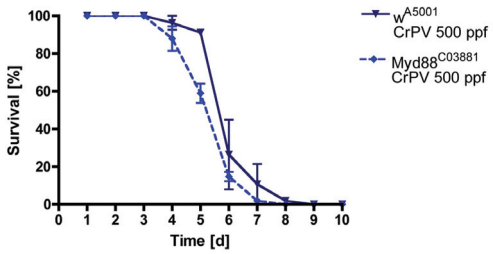


Figure 5.18: *Dif*^{1} **mutant flies are more susceptible to infection with CrPV. A: Flies were injected with the indicated dose of CrPV and survival was monitored daily. Values shown are the mean and standard deviation of 3 independent experiments. Flies were kept at 22°C for the duration of the experiment. B: Flies were injected with 5 particles of CrPV and viral load was determined 2 days post infection using semiquantative RT-PCR. Three independent experiments are shown. Flies were kept at 22°C for the duration of the experiment.**

A



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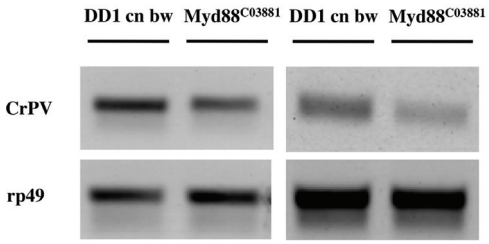


Figure 5.19: *MyD88^{c03881}* **mutant flies show no CrPV phenotype. A: Flies were injected with the indicated dose of CrPV and survival was monitored daily. Values shown are the mean and standard deviation of 3 independent experiments. Flies were kept at 22°C for the duration of the experiment. B: Flies were injected with CrPV (5 ppf) and viral load was determined 2 days post infection. Two independent experiments are shown. Flies were kept at 22°C for the duration of the experiments.**

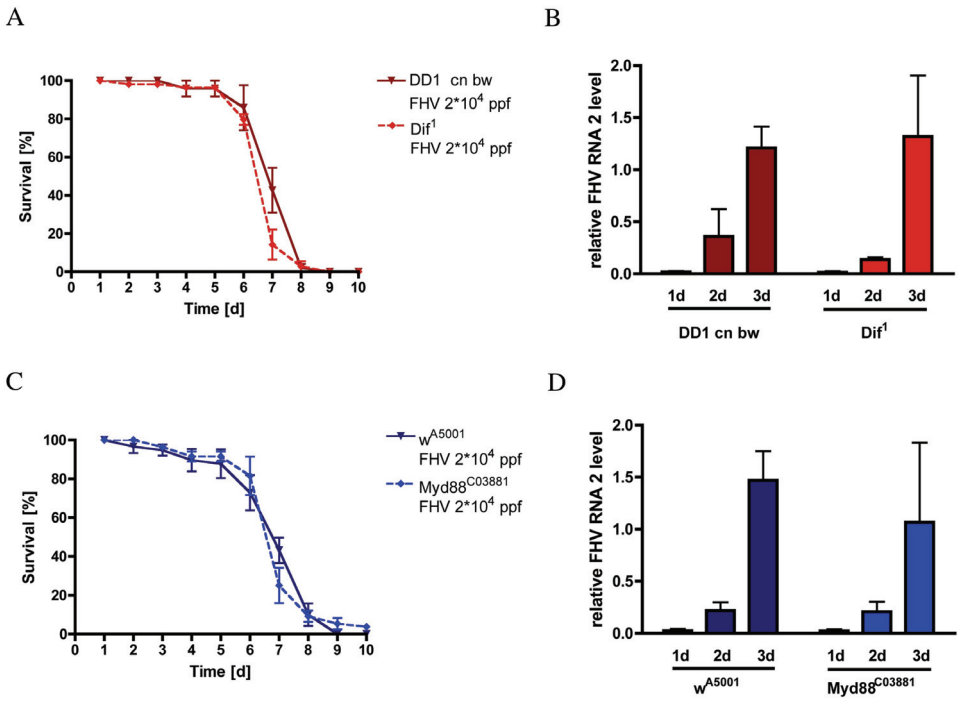


Figure 5.20: Toll pathway mutant flies show no phenotype upon FHV infection. A and C: Flies were injected with the indicated dose of FHV and survival was monitored daily. Values shown are the mean and standard deviation of 3 independent experiments. B and D: Flies were injected with FHV (2*10^4 ppf) and viral load was determined at indicated timepoints. No signifant difference in viral RNA was observed for either *Dif*^{1} (1d P=0.8441; 2d P=0.4858; 3d P=0.8749) or *MyD88*^{c03881} (1d P=0.9599; 2d P= 0.9457; 3d P=0.6702) mutant flies. Values shown represent the mean and standard deviation of 2 independent experiments. Flies were kept at 22°C for the duration of the experiments.

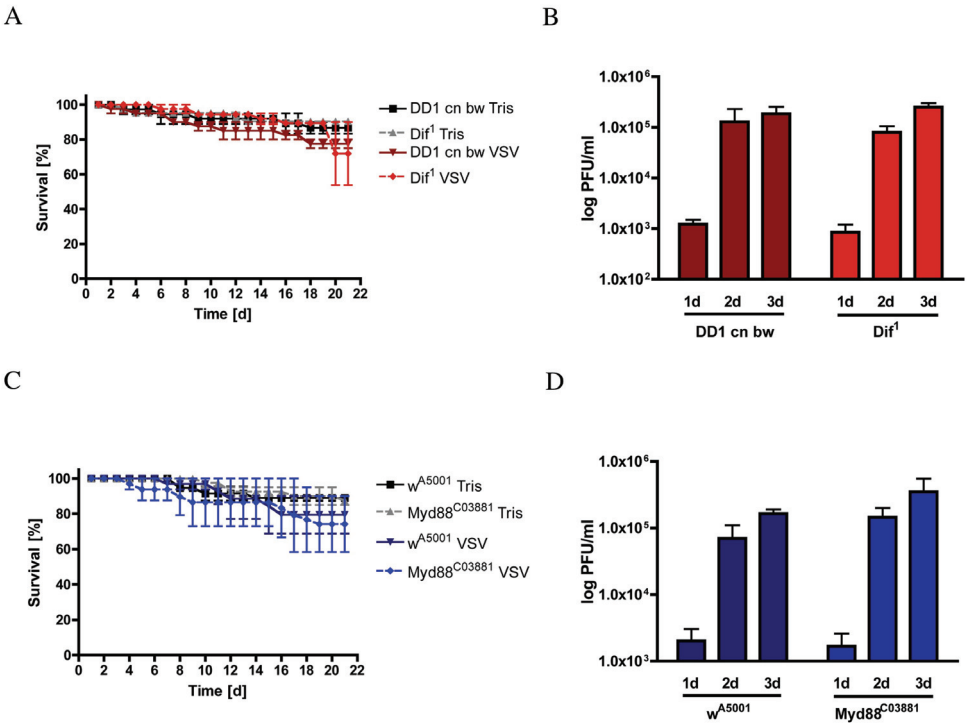


Figure 5.21: Toll pathway mutant flies show no phenotype upon VSV infection. A and C: Flies of the indicated genotypes were injected with 1.8*10^4 particles of VSV and survival was monitored daily. Values shown are the mean and standard deviation of 2 independent experiments. B and D: Flies were injected with 1.8*10^4 particles of VSV and viral load was determined at indicated timepoints. No signifant difference in viral titers was observed for either *Dif^1* (1d P=0.4882; 2d P=0.6857; 3d P=0.5288) or *$MyD88^{c03881}$* (1d P=0.8236; 2d P= 0.3846; 3d P=0.4566) mutant flies. Values shown represent the mean and standard deviation of 2 independent experiments. Flies were kept at 22°C for the duration of the experiments.

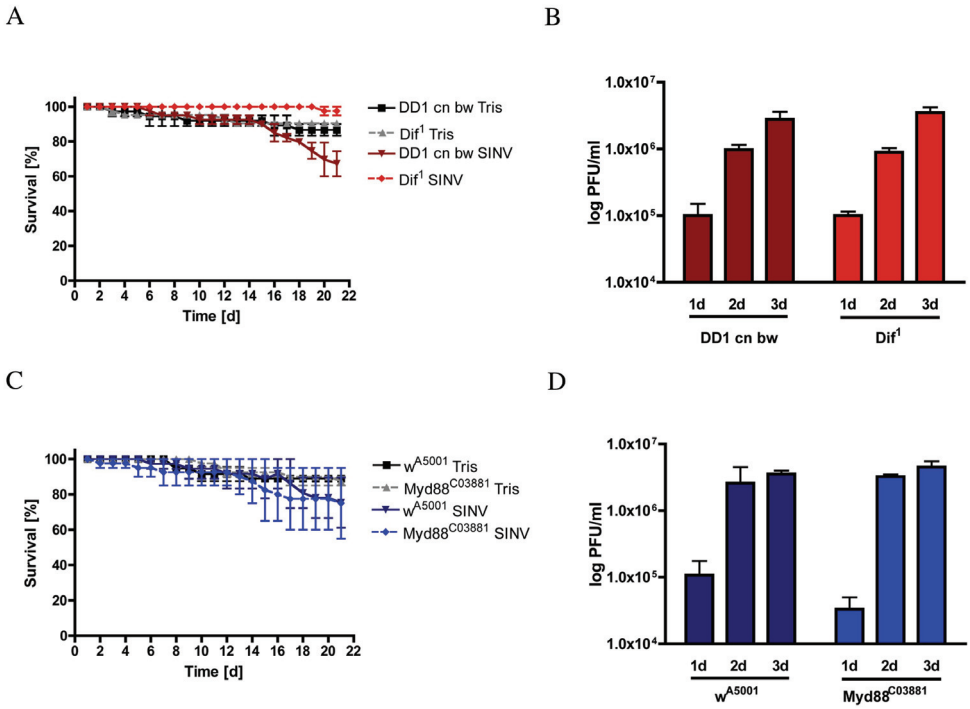


Figure 5.22: Toll pathway mutant flies show no phenotype upon SINV infection. A and C: Flies of the indicated genotypes were injected with 2.5*10^3 particles of SINV and survival was monitored daily. Values shown are the mean and standard deviation of 2 independent experiments. B and D: Flies were injected with 2.5*10^3 particles of SINV and viral titers were determined at indicated timepoints. No signifant difference in viral titers was observed for either *Dif*^{1} (1d P=1.0000; 2d P=0.7497 3d P=0.5761) or *MyD88*^{c03881} (1d P=0.3965; 2d P= 0.7668; 3d P=0.4776) mutant flies. Values shown represent the mean and standard deviation of 2 independent experiments. Flies were kept at 22°C for the duration of the experiments.

5.2.3 Mutation of the IKKγ homologue *key* does not appear to play a major role in the resistance to RNA virus infection

We previously observed a strong reduction in viral load for Rel mutant flies after infection with the Dicistrovirus DCV, suggesting that the Imd pathway promotes DCV replication [153]. We decided to confirm these findings in *key* mutant flies and observed reduced viral loads in mutants 2 days after infection in some experiments (Fig. 5.23 B and T. Huszar, S. Deddouche unpublished data). This reduction was variable ranging from an almost 10-fold reduction in viral RNA up to an 1.67-fold increase compared to DD1 *cn bw* wild-type flies. Injection of 500 particles of DCV, the same dose used for monitoring viral load, did not result in any survival phenotype (Fig. 5.23 A). Hence, although we could observe a highly variable, but still striking reduction of up to 10-fold in viral RNA, this variation in viral load did not manifest in a survival phenotype.

Likewise, no phenotype in survival was found after infection of *key* mutant

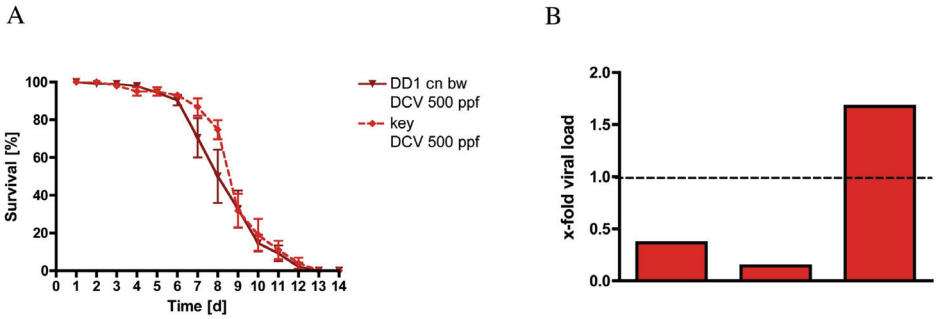


Figure 5.23: The DCV load of *key* mutants is highly variable: A: DD1 *cn bw* wild-type flies and *key* mutant flies were infected with the indicated dose of DCV and survival was monitored daily. Values shown represent the mean and standard deviation of 5 independent experiments. C: Flies were infected with 500 particles of DCV and viral load was determined 2 days post infection using Q-PCR. Values were expressed as x-fold viral load observed in *key* mutants compared to DD1 *cn bw* wild-type flies. 3 independent experiments are shown. Flies were kept at 22°C for the duration of the experiments.

flies with CrPV (Fig. 5.24 A). Concerning the viral load, we observed a trend

towards a reduction in CrPV load in *key* flies of up to 2-fold (Fig. 5.24 B). We next tested the Nodavirus FHV. Infection with FHV did not show any phenotype in survival. Likewise, no significant difference in viral RNA was detected the following 3 days after infection (Fig. 5.25).
For both VSV and SINV we could not observe any difference in survival (Fig. 5.26 A and Fig. 5.27 A). We observed a slight increase in the SINV load for all timepoints, ranging between 2.7-fold for day 2 and 4 and 3.5-fold for day 1 and 3, respectively. However, this modest increase was not reproduced in the second experiment performed where viral titers were similar in mutant and wild-type flies. Thus, no significant difference was detected between the viral titer of mutant and wild-type (Fig. 5.27 B). Similar observations were made after infection with VSV, where we observed a 4.8-fold increase at day 3 of infection. Again, no difference was seen in a further experiment. Hence, like in the case for SINV, no significant difference between *key* mutant and wild-type flies was detected for VSV infection (Fig. 5.26 B).
We conclude that the Imd pathway does not seem to play a major role in the resistane to most RNA viruses. We nevertheless observed in several experiments a reduction in viral load in *key* mutant flies infected with DCV and CrPV.

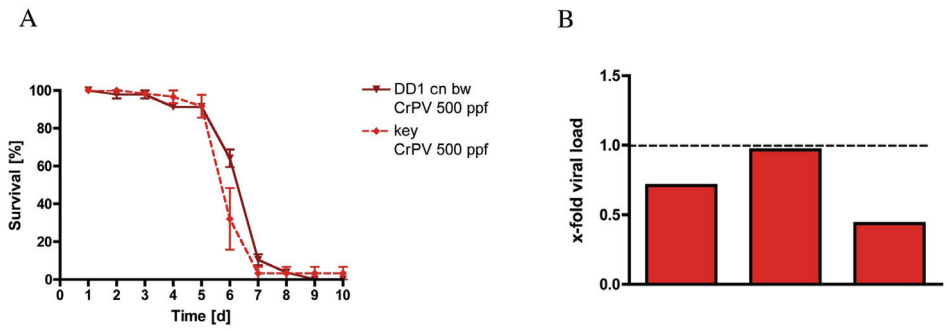


Figure 5.24: *key* **mutant flies resist slightly better after infection with a low dose of CrPV. A: DD1** *cn bw* **wild-type flies and** *key* **mutant flies were infected with the indicated dose of CrPV and survival was monitored daily. Values shown represent the mean and standard deviation of 3 independent experiments. C: Flies were infected with 5 particles of CrPV and viral load was determined 2 days post infection using semiquantitaive RT-PCR. Values were expressed as x-fold viral load observed in** *key* **mutants compared to DD1** *cn bw* **wild-type flies. 3 independent experiments are shown. Flies were kept at 22°C for the duration of the experiments.**

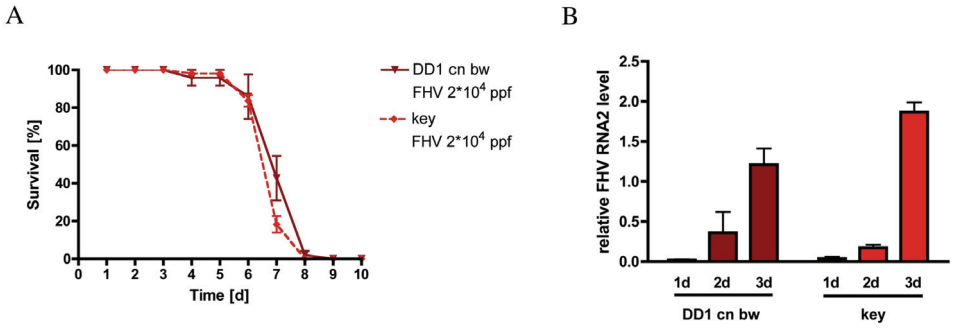


Figure 5.25: No phenotype for *key* **mutant flies upon FHV infection. A: Flies of the indicated genotypes were infected with 2*10^4 particles of FHV and survival was monitored daily. Values shown represent the mean and standard deviation of 3 independent experiments. B: Flies were infected with 2*10^4 particles of FHV and viral load was determined at indicated timepoints. Values shown represent the mean and standard deviation of 2 independent experiments. No significant difference was observed between mutant and wild-type(1d P=0.5468; 2d P=0.5521; 3d P=0.1092). Flies were kept at 22°C for the duration of the experiments.**

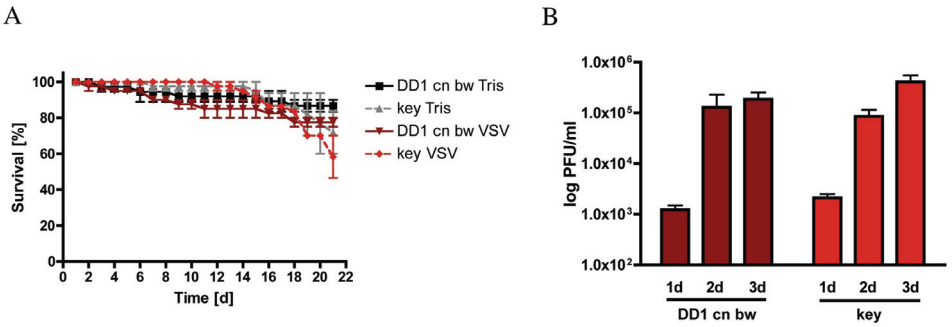


Figure 5.26: No phenotype for *key* mutant flies upon VSV infection. A: Flies of the indicated genotypes were infected with 1.8*10^4 particles of VSV and survival was monitored daily. Values shown represent the mean and standard deviation of 2 independent experiments. B: Flies were infected with 1.8*10^4 particles of VSV and viral load was determined at indicated timepoints. Values shown represent the mean and standard deviation of 2 independent experiments. No significant difference was observed between mutant and wild-type(1d P=0.2132; 2d P=0.7249; 3d P=0.2871). Flies were kept at 22°C for the duration of the experiments.

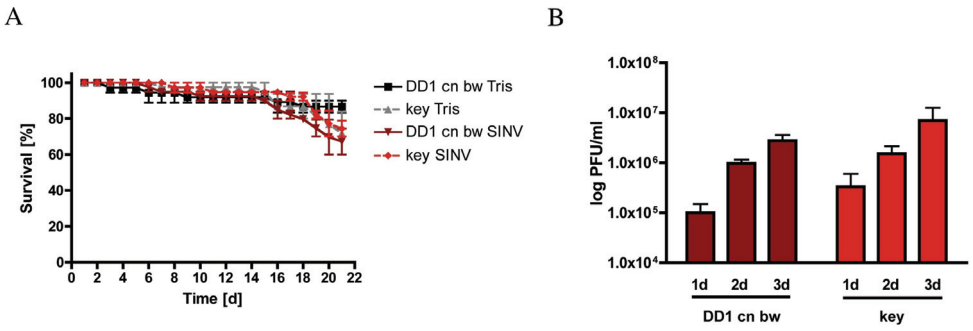


Figure 5.27: No phenotype for *key* mutant flies upon SINV infection. A: Flies of the indicated genotypes were infected with 2.5*10^3 particles of SINV and survival was monitored daily. Values shown represent the mean and standard deviation of 2 independent experiments. B: Flies were infected with 1.8*10^4 particles of VSV and viral load was determined at indicated timepoints. Values shown represent the mean and standard deviation of 2 independent experiments. No significant difference was observed between mutant and wild-type(1d P=0.4829; 2d P=0.4860; 3d P=0.5306). Flies were kept at 22°C for the duration of the experiments.

5.2.4 Summary

We used a set of 5 RNA viruses to investigate a possible involvement of the Jak-STAT, the Toll and the Imd pathway in the antiviral defense of drosophila (Table 5.4). The 5 viruses tested belonged to 4 distinct virus families, the *Dicistroviridae*, with DCV and CrPV as representatives, the *Nodaviridae*, represented by FHV, and the *Rhabdoviridae* and *Togaviridae*, represented by VSV and SINV, respectively.
At the end of this analysis, we can distinguish two groups of viruses, the *Dicistrovirus* DCV and CrPV on one hand, and the three other RNA viruses FHV, SINV and VSV on the other. The Jak-STAT pathway is involved in the resistance to DCV and CrPV, and its role appears to be as important as RNAi. Furthermore, known Jak-STAT pathway activators, namely *upd2* and *upd3*, are induced upon infection with DCV. Clearly, the function of the genes regulated by this pathway in virus infected flies deserves further investigation. By contrast, this pathway does not affect the resistance of the flies to other viruses. Thus, unlike RNAi, the Jak-STAT pathway seems to control specific aspects of *Dicistroviridae* replication.

The Toll pathway also appears to play a role in the control of DCV and CrPV infection. Further work is required to identify the genes regulated by Dif in infected flies, since neither DCV nor CrPV seem to induce expression of the standard marker of the pathway *drosomycin* (see section 5.1.3).
The involvememt of the other NF-κB pathway in drosophila, the Imd pathway, is not as clear at this stage. Again, the only viruses that may be affected are DCV and CrPV, although the effects observed are variable and restricted to the viral load. Clearly, further experiments are needed in order to clarify the role of this pathway in the antiviral defense.
Finally, none of the three pathways examined appear to contribute to the resistance to FHV, SINV or VSV, even though these viruses induce a response (see section 5.1). This suggests that other pathways, such as the JNK pathway or the MEKK1 pathway, may be involved in the inducible antiviral response.

We conclude that, unlike RNAi which is a general antiviral defense in drosophila, the inducible response to infection appears to be largely virus-specific. Further experiments will be required to determine whether these specificities reflect tissue-specific patterns of infection, differential effects on host and metabolism, or recognition of virus-specific molecular patterns.

Table 5.4: Involvement of the Jak-STAT, Toll and Imd pathway in the defense against RNA viruses. Presented are the phenotypes obtained for survival and viral load. No phenotype is indicated by a slash (/).

Pathway	Mutant	Virus	Survival	Viral load
Jak-STAT	*hop*$^{M38/msv1}$	DCV	susceptible (3-4 d)	12-fold increase (inf. p.)
		CrPV	susceptible (1 d)	7-13-fold increase (viral RNA)
		FHV	/	/
		VSV	/	/
		SINV	/	/
Toll	*Dif*^{1}	DCV	susceptible (4 d)	7.9-fold increase (viral RNA)
		CrPV	susceptible (1 d)	2- to 4-fold increase (viral RNA)
		FHV	/	/
		VSV	/	/
		SINV	/	/
	Myd88^{C03881}	DCV	susceptible (1 d)	16.8-fold increase (viral RNA)
		CrPV	/	/
		FHV	/	/
		VSV	/	/
		SINV	/	/
Imd	*key*	DCV	/	reduction, but highly variable
		CrPV	/	slight reduction, but variable
		FHV	/	/
		VSV	/	/
		SINV	/	/

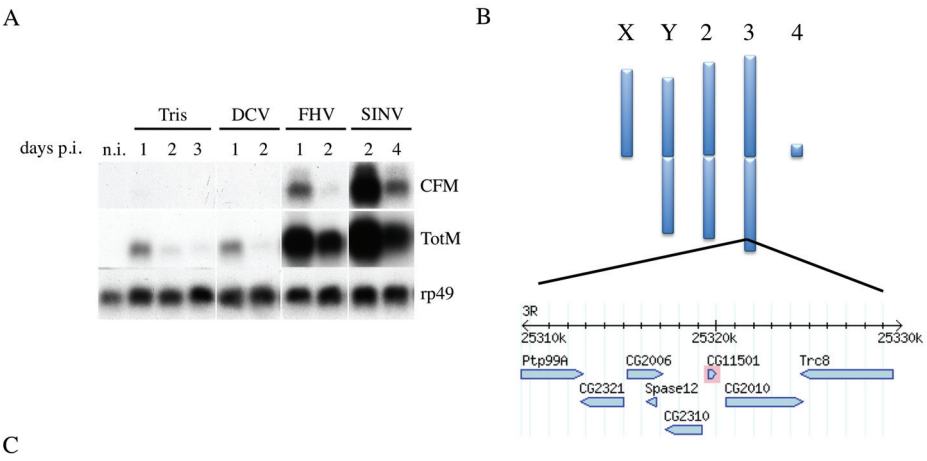
Chapter 6

Characterization of a virus-induced molecule: Chifoumi

Our microarray analysis revealed that FHV was the strongest inducer of gene expression and induced a total of 590 genes (see chapter 5.1). *Turandot M* (*TotM* alias *CG14027*) and *Chifoumi* (*cfm* alias *CG11501*) ranked among the five strongest FHV induced genes and were found to be the two strongest induced genes by SINV. Subsequent northern blot analysis of infected flies confirmed strong induction of *cfm* and *TotM* by both, FHV and SINV, but showed no induction for DCV within the first 2 days of infection (Fig. 6.1 A).

cfm is a poorly characterized gene located on the right arm of chromosome 3. The single exon encodes a small 115 amino acid protein carrying a signal peptide and 10 cysteine residues in the mature peptide (Fig. 6.1 B and C). *cfm* attracted our attention for several reasons.

(i) This gene has been shown to be regulated by the Jak-STAT pathway after bacterial challenge [67]. This finding was of particular interest for us, as we reported an involvement of this pathway in the defense against DCV [142]. Mutants of the Jak-STAT pathway were susceptible to DCV infection and showed higher viral titers than control flies. Thus, virus induced genes



C

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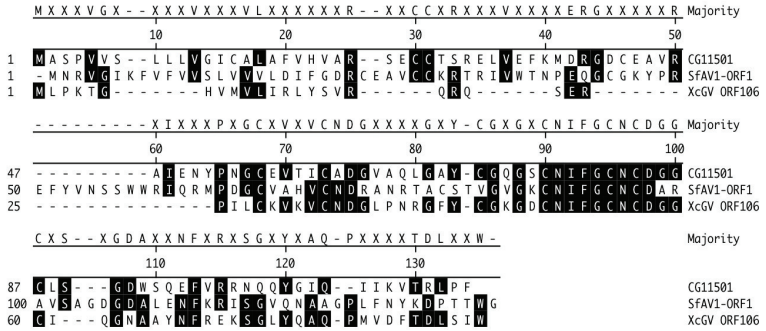
1  maspvvslll vgicalafvh varsecctsr elvefkmdrg dceavraien ypngcevtic
61 adgvaqlgay cgqgscnifg cncdggclsg dwsqefvrrn qqygiqiikv trlpf
```

Figure 6.1: *cfm* **codes for a small peptide induced after viral infection. A: Northern Blot analysis of** *cfm* **and** *TotM* **gene expression following injection of Tris buffer or viruses. B:** *cfm* **(***CG11501***) is located on the right arm of chromosome 3. C:** *cfm* **codes for a small 115 aa protein that contains a signal peptide and 10 cystein residues.**

regulated by this pathway might have immuno-related functions.
(ii) Furthermore, *cfm* was identified in a screen for Jak-STAT pathway components as a putative negative regulator of this pathway [68], although it was not identified in a similar independent screen [169].
(iii) We identified orthologs of *cfm* in the genome of 2 insect DNA viruses, Spodoptera frugiperda ascovirus 1 (SfAV1) and Xestia c-nigrum granulevirus (XcGV) (percentage of identity 40% and 49%, respectively, Fig. 6.2 A). SfAV1 belongs to the *Ascoviridaea*. It is an enveloped virus, which possesses a circular double-stranded DNA genome and it infects the lepidopteran *Spodoptera frugiperda*. XcGV is a member of the *Baculoviridae*. XcGV is an enveloped virus with a circular dsDNA genome that infects the spotted cutworm *Xestia c-nigrum*. Interestingly, DNA viruses (e.g. poxviruses) are known to hijack host genes in order to modulate the host's immune response [168]. Phylogenetic analysis based on the viral DNA polymerase showed, that the *Baculoviridae* and *Ascoviridae* are not closely related, indicating that the *cfm* orthologues were acquired independently by the two viruses, suggesting that these sequences confer an advantage to these viruses (Fig. 6.2 B). Overall, *cfm* was a very promising candidate for further characterization.

A



B

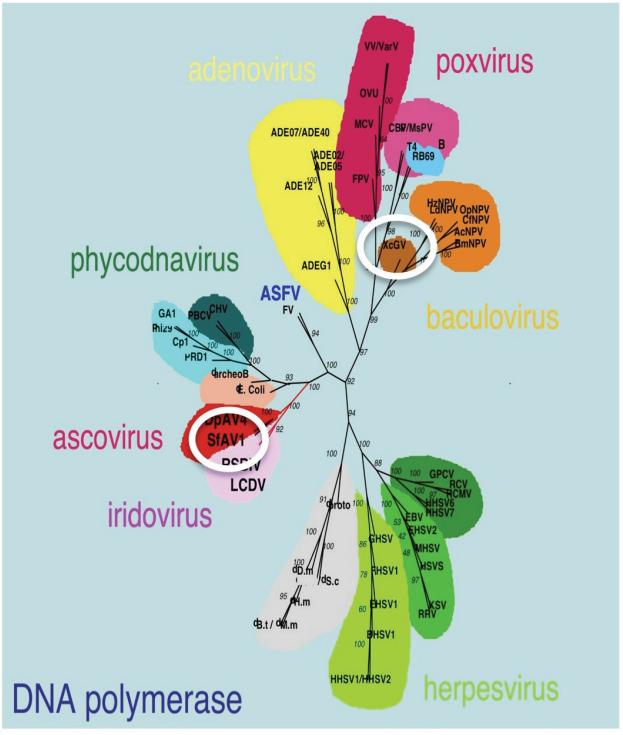


Figure 6.2: CFM orthologues in insect DNA virus.A: CFM orthologues were found in the genome of SfAV1 and XcGV with a percentage of identity of 40% and 49%, respectively. B: SfAV1 and XcGV belong to two distinct virus families. Phylogenetic analysis based on the DNA polymerase sequence showed that Asco- and Bacullovirus are not closely related families. Adapted from Y. Bigot, University F. Rabelais, Tours.

6.1 Induction of *cfm* gene expression

6.1.1 Bacteria are minor inducers of *cfm* gene expression compared to viruses

cfm was initially reported to be induced upon septic injury with a mix of Gram-positive and Gram-negative bacteria [67]. As we observed induction of the *cfm* transcript upon infection with several viruses (see chapter 5), we wanted to compare the induction strength of bacteria with the strongest viral inducer, SINV. OR wild-type flies were therefore pricked with either a mix of Gram-positive and Gram-negative bacteria or injected with 250 particles of SINV. As a control for the injury effect, flies were as well pricked with a clean needle. *Attacin A* expression was monitored to confirm the presence of bacteria upon septic injury (Fig. 6.3 A). Infection with SINV led to a strong induction of *cfm* expression 3h, 6h and 24 h after injection (Fig. 6.3 B). However, induction of the *cfm* transcript by bacteria was only minor and restricted to 24 h post pricking (Fig. 6.3 B).

We furthermore monitored the presence of the CFM peptide in flies upon septic injury. OR wild-type flies were either pricked with a mix of Gram-positive and Gram-negative bacteria or injected with 250 particles of SINV. The CFM peptide was detected in whole flies 1 days and 2 days post infection with SINV using western blot analysis. However, we failed to detect CFM on the protein level upon septic injury (Fig. 6.4).

Taken together, we could only observe an increase of *cfm* expression on the transcription level upon septic injury. This induction was only minor when compared to flies infected with SINV and restricted to one timepoint, indicating that viruses are much stronger inducers of *cfm* expression than bacteria. Moreover this minor induction by bacteria was not observed in all experiments performed (see Fig. 5.6).

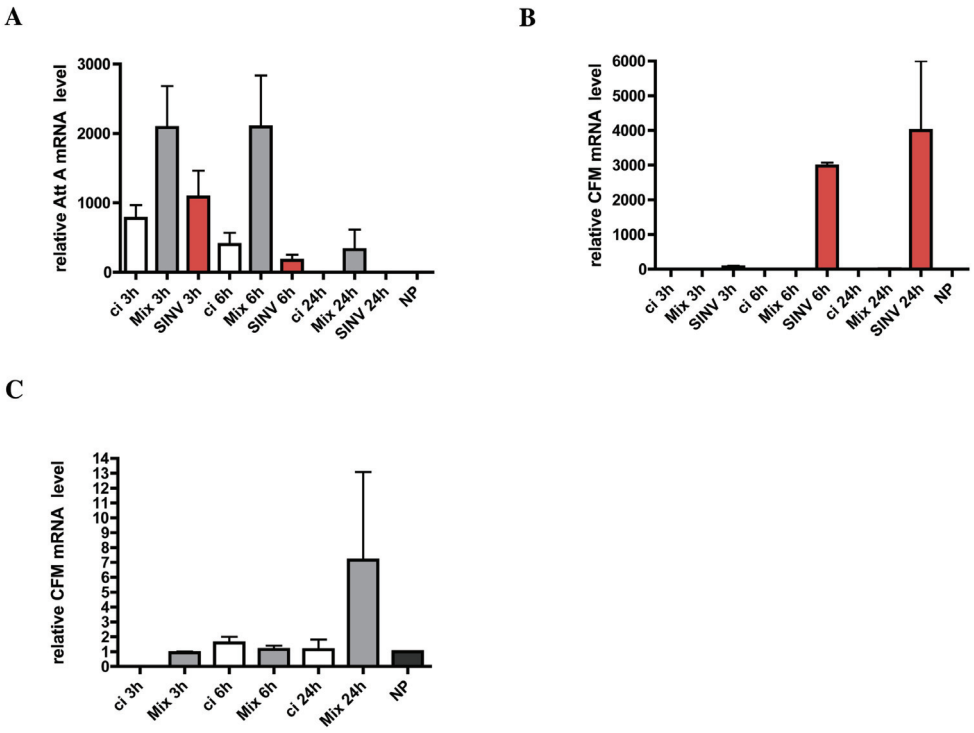


Figure 6.3: *cfm* **is not induced by bacteria. OR flies were pricked with a mix of Gram-positive and Gram-negative bacteria or infected with 250 particles of SINV, and expression level of** *Attacin A* **(A) and** *cfm* **(B) were determined at indicated timepoints. Values shown are the mean and standard deviation of 2 independent experiments (ci: clean injury). C: SINV was removed from the data set.**

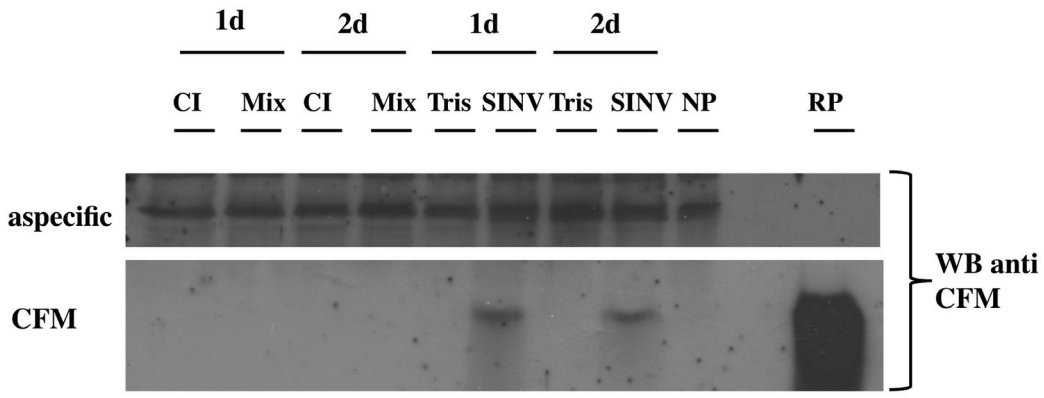


Figure 6.4: The CFM peptide is not induced by bacteria. OR flies were pricked with a mix of Gram-positive and Gram-negative bacteria or infected with 250 particles of SINV, and the presence of the CFM peptide was determined at indicated timepoints. One blot out of 2 experiments is show.

6.1.2 *cfm* remains inducible in *hopscotch* mutant flies

cfm was reported to be regulated in response to septic injury by the Jak-STAT pathway [68]. As we could only observe a minor induction of *cfm* by bacteria, we were interested to know whether *cfm* induction by viruses is indeed regulated by this pathway. *Wolbacchia* cured $hop^{M38/msv1}$ mutant flies were infected with $2.5*10^3$ particles of SINV and *cfm* transcript level were monitored using QPCR 6 h and 24 h post injection (Fig. 6.5). Infection with SINV led to a strong *cfm* induction in both, OR wild-type and $hop^{M38/msv1}$ mutant flies, when compared to Tris injected control flies. Hence, *cfm* expression is not abolished in *hopscotch* mutant flies, indicating that the Jak-STAT pathway is not necessary to regulate *cfm* expression in response to SINV infection.

Surprisingly, *cfm* transcript numbers were generally much higher in $hop^{M38/msv1}$

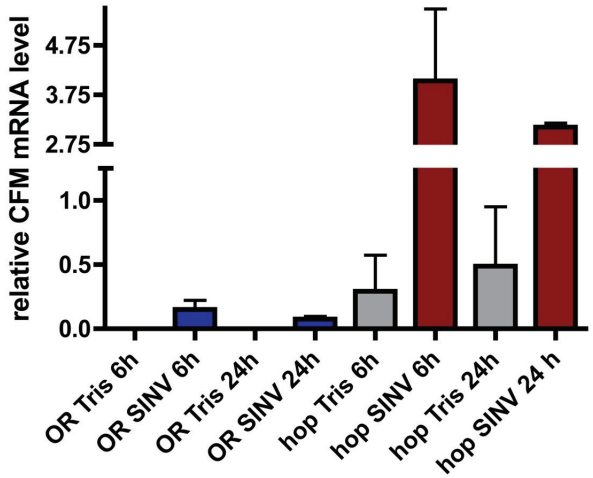


Figure 6.5: *cfm* **remains inducible in** *hopscotch* **mutant flies. Flies of the indicated genotype were infected with 2.5*10^3 particles of SINV and** *cfm* **transcript level determined using QPCR. Values shown are the mean and standard deviation of 2 independent experiments.**

mutant flies than in OR control flies (Fig. 6.5). Currently, we are not able to explain this observation and further experiments are needed to clarify these findings.

6.1.3 Enveloped viruses induce *cfm* early after infection

We showed in chapter 5, that *cfm* is induced upon infection with DCV, FHV, VSV and SINV. Although these 4 viruses commonly triggered *cfm* expression, we noted considerable differences in the induction kinetic. VSV and SINV, which are both enveloped viruses, both seemed to induce *cfm* very early, only a few hours post injection (see chapter 5). We further investigated this early induction by injecting OR wild-type flies with either DCV, FHV, VSV and SINV and monitored *cfm* level 3 h and 6 h post infection (Fig. 6.6). Neither DCV nor FHV induced *cfm* expression at the chosen timepoints, whereas VSV and SINV infection led to a robust increase in *cfm* transcript numbers 6 h post injection. Moreover, *cfm* expression could be detected as early as 3 h post infection using these two viruses. These data suggest, that the signal leading to *cfm* induction does not involve an active replication of either VSV or SINV.

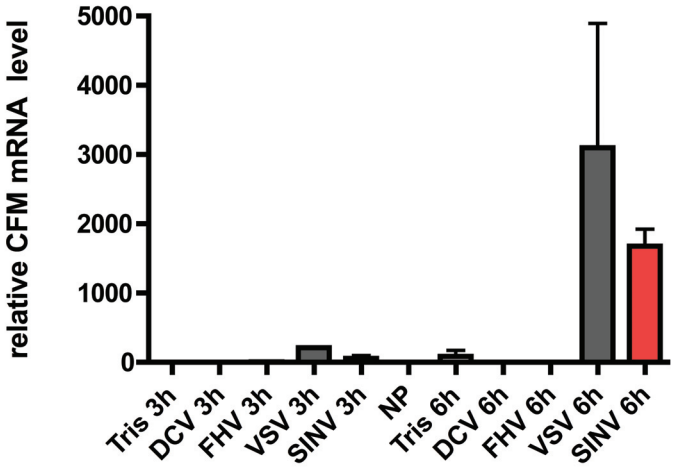


Figure 6.6: Enveloped viruses induce *cfm* early after infection. OR wild-type flies were injected either with Tris, DCV (500ppf), FHV (2*10^4 ppf), SINV (250 ppf) or VSV (1.8*10^4 ppf). Flies were frozen at indicated timepoints, RNA was extracted and *cfm* expression determined using Q-PCR. Values shown represent the mean and standard deviation of 2 independent experiments.

6.1.4 Early *cfm* induction by SINV does not require active virus replication

Given the fact that we could detect *cfm* expression as early as 3 h post infection with VSV and SINV, we decided to investigate if the signal triggering *cfm* induction involves active replication of the virus. To address this question, we produced a replication deficient SINV using UV inactivation of viral particles. SINV that was UV treated for 20 min, failed to replicate when injected into OR wild-type flies, proving the effectiveness of the treatment (Fig. 6.7 A). We next injected OR flies with either replication potent or UV inactivated SINV and monitored *cfm* transcript numbers 6 h and 48 h post infection. Injection of both viruses led to an induction of *cfm* 6 h post infection when compared to Tris injected control flies. However, although same amounts of viral particles were injected, *cfm* expression was slightly reduced in the case of UV-inactivated SINV (Fig. 6.7 B). 48 h after injection, *cfm* expression was largely diminished in flies infected with UV-inactivated SINV, reflecting the fact that no new viral particles were generated. These data indicate that the signal leading to *cfm* expression might not involve viral replication but rather viral patterns such as envelope proteins. However, we found that *cfm* expression was triggered to similar levels as SINV infection did, when flies were injected with conditioned cell culture medium (GMEM, data not shown). We therefore can not exclude, that induction of *cfm* is triggered by a range of mediators that possibly include growth factors, membrane components and/ or cell debris. Hence, the signal leading to *cfm* expression might not involve the sensing of a virus-specific molecular pattern.

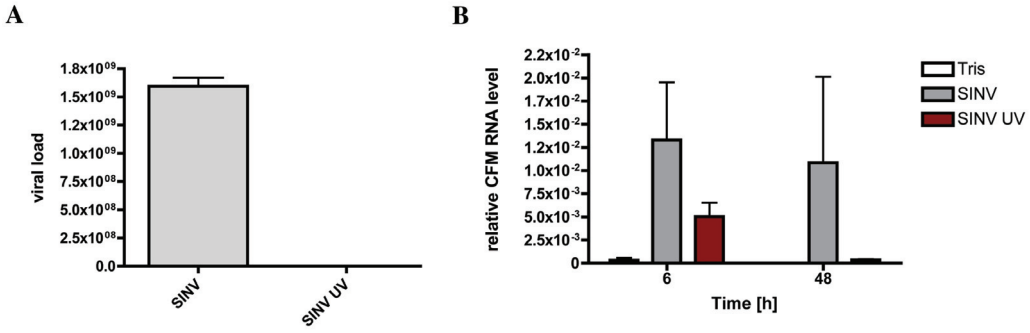


Figure 6.7: Early *cfm* induction by SINV does not require active virus replication. A: OR wild-type flies were infected with either SINV or UV-inactivated SINV and viral titers were determined 2 days post infection. Values shown are the mean and standard deviation of measurement duplicates B: OR wild-type flies were infected with either SINV or UV-inactivated SINV (2.5*10^3 ppf) and *cfm* mRNA levels were determined at indicated timepoints. Values shown are the mean and standard deviation of two independent experiments.

6.2 CFM is a circulating protein secreted by the fat body

We next adressed the question, in which tissues *cfm* is induced upon SINV infection. Flyatlas recorded *cfm* mRNA signals in various tissues of adult flies, including the head, eye, heart, fat body and adult carcass (Table 6.1). The strongest signal was obtained in virgin and mated spermatheca. As

Table 6.1: Gene expression results for *cfm* (*CG11501*) from flyatlas.

Tissue	mRNA Signal	Present Call	Enrichment
Head	305 ± 135	4 of 4	7.80
Eye	106 ± 35	4 of 4	2.73
Heart	34 ± 7	4 of 4	0.88
Fat body	183 ± 126	4 of 4	4.73
Virgin spermatheca	582 ± 287	4 of 4	14.97
Mated spermatheca	472 ± 363	4 of 4	12.16
Adult carcass	104 ± 21	4 of 4	2.7
Whole fly	38 ± 17	3 of 4	

these data was derived from uninfected flies, we decided to perform *in situ* hybridization upon infection with SINV (Fig. 6.8). We could detect a weak background staining in the fat body of Tris injected flies 18 hours post injection. Infection with SINV, however, resulted in a much stronger signal in fat body cells. Strong, but aspecific labelling was detected in the spermatheca, as the same staining was observed using the sense probe. No staining was observed in ovaries or the digestive tract. Hence, the *cfm* transcript is specifically expressed in the fat body upon immune challenge.

We used the recombinant protein carrying a V5-tag to produce a CFM specific antibody in mice. The obtained antiserum gave a nice dose dependent signal in Western Blot for a non-tagged version of recombinant CFM. No signal was detected using the pre-immune serum (Fig. 6.9 A). Likewise,

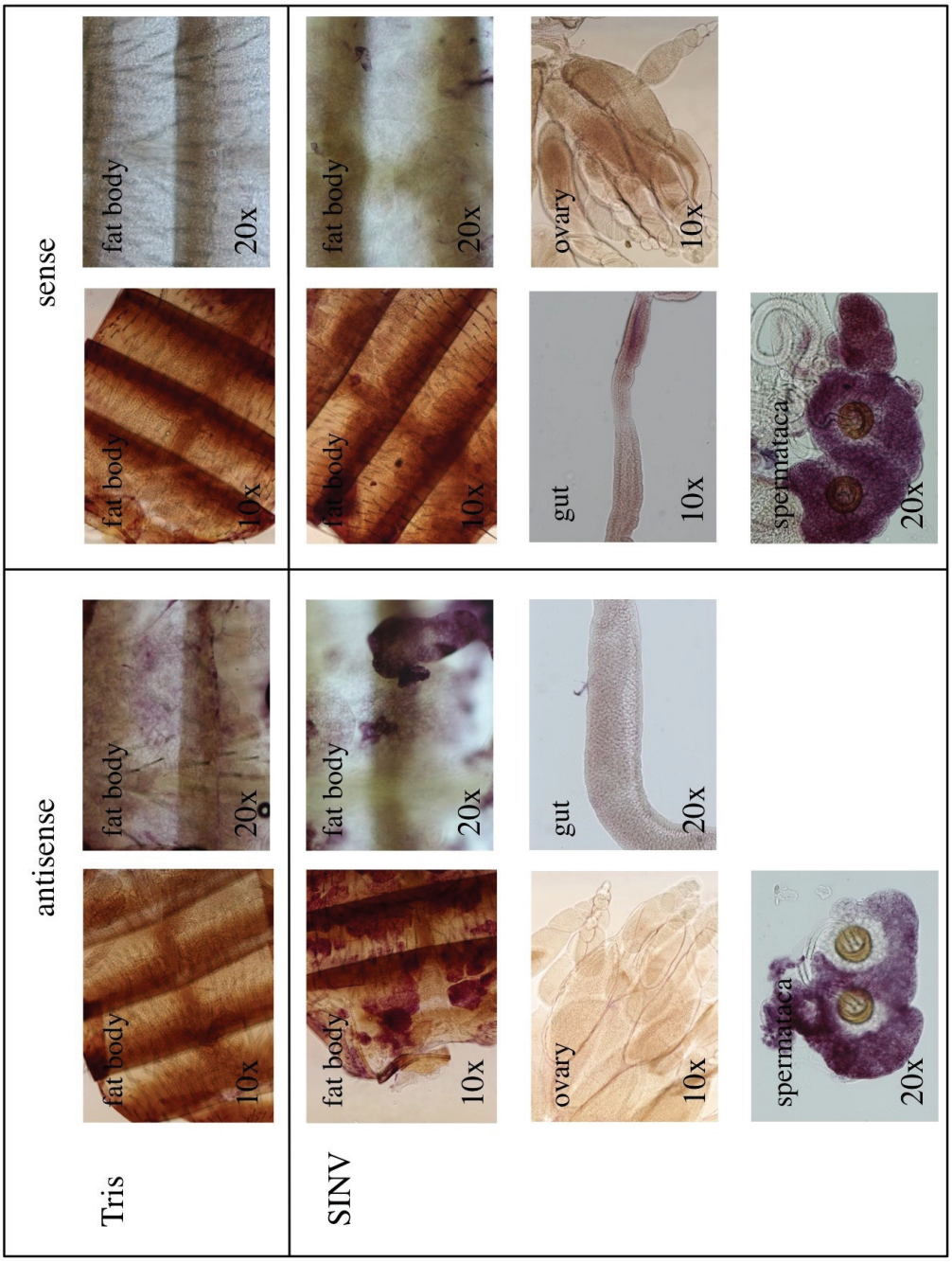


Figure 6.8: *cfm* **is expressed in the fat body upon viral challenge. Strong signals were detected in the fat body of SINV infected flies, 18 h post injection.**

no signal was obtained using a tagged version of recombinant Vago, proving the specificity of the antibody for the CFM peptide (Fig. 6.9 B). The *cfm* transcript is induced in the fat-body upon immune challenge and carries a signal peptide, suggesting secretion of the peptide into the hemolymph. Indeed, we could detect the presence of CFM in the hemolymph 4 days after infection with SINV (Fig. 6.9 C). We applied to the gel 1 μl of serially diluted recombinant CFM and compared the resulting band intensities to the intensity obtained in the hemolymph at indicated time-points after infection. The strong induction observed in RNA level was reflected by a μM concentration of CFM in the hemolymph of SINV infected flies. The band intensity of SINV infected flies 1 and 2 days after infection was around 12 μM (0.12 g/l) CFM. Taking into account, that around 3 μl of recovered hemolymph was applied to the gel, we found a final concentration of CFM in the hemolymph of SINV infected flies of around 4 μM (Fig. 6.9 D). We next tried to detect the presence of CFM protein after infection with DCV and FHV, as we observed an induction of *cfm* transcript by these two viruses. However, the x-fold induction was lower than observed with VSV and SINV and followed a slower kinetic. In agreement with the reduced amount of transcripts we could not detect the CFM protein in the hemolymph of DCV and FHV infected flies either 2 or 5 days post infection (Fig. 6.10).

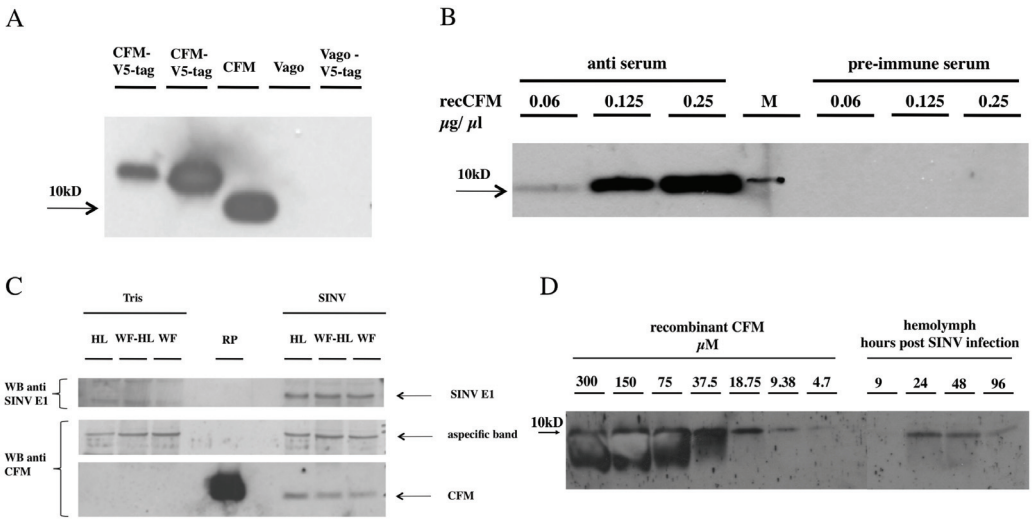


Figure 6.9: CFM is secreted in the hemolymph of SINV infected flies. A and B: Validation of the anti-CFM antibody by western blot on recombinant proteins. C: The CFM peptide is present in the hemolymph of infected flies 4 days post injection. Aspecific bands are shown as loading control (HL: hemolymph, WF: whole fly, WF-HL: whole fly after hemolymph was taken). D: The CFM concentration in the hemolymph is around 4 μM. One μl of recombinant CFM dilutions were loaded on a gel together with 3 μl of hemolymph extracted from SINV infected flies.

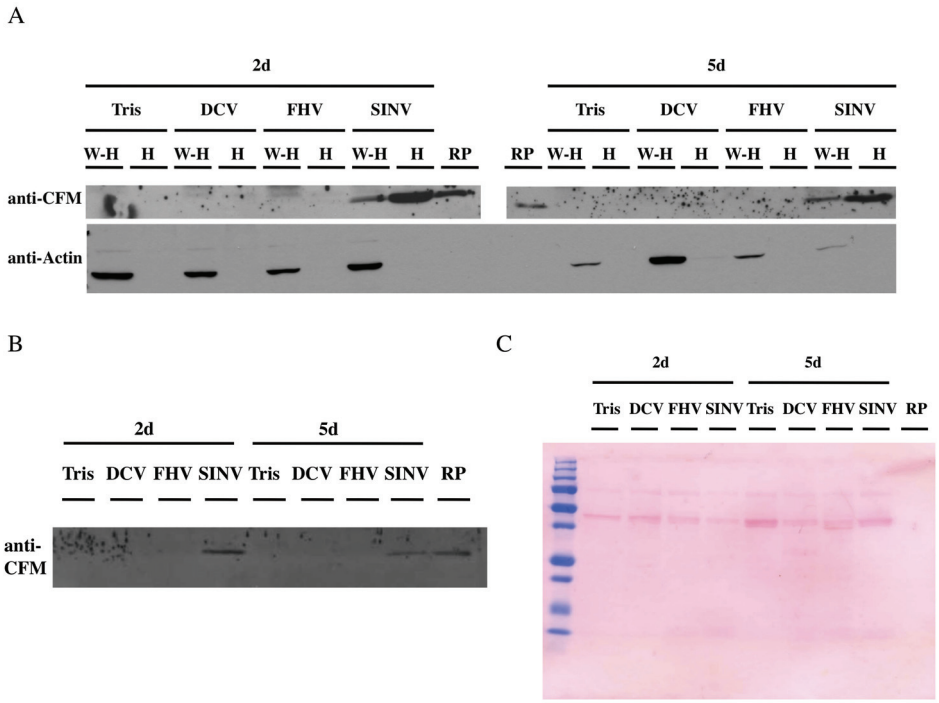


Figure 6.10: The CFM peptide is not detectable in the hemolymph of DCV and FHV infected flies. A: CFM is not detected in the hemolymph after 2 and 5 days of infection with DCV or FHV (W-H: whole fly after hemolymph was taken, H: hemolymph, RP: recombinant CFM). B: Western blot of the hemolymph extracted from virus infected flies. Equal amounts of hemolymph samples were verified by Ponceau Red staining (C).

6.3 Searching for a function: *in vitro*

6.3.1 CFM does not function as a negative regulator of the Jak-STAT pathway

One of the reasons CFM attracted our attention was that it was identified as a putative negative regulator in a cell based RNAi screen for Jak-STAT pathway components [68]. In order to further test this hypothesis *in vitro*, we used quantitative assays for Jak-STAT signaling activity in cultured drosophila cells. (Fig. 6.11). We used reporter plasmids that contained fragments of either the *TotM* or the *Draf* promotor upstream of the firefly luciferase gene. For the *TotMLuc* reporter, the region -1141 upstream of the transcription start containing three STAT92E binding sites was cloned in front of the reporter gene. The *6x2xDrafLuc* reporter was constructed by multimerizing a STAT92E binding site from the *Draf* promotor and is described in [68].
Transfection of the *TotMLuc* reporter in S2 cells together with a plasmid that constitutively expressed the pathway ligand Upd led to a robust induction of reporter gene activity (Fig. 6.12 A). We assessed the effect of dsRNA targeting the mRNA of the gene *hop*, as well as dsRNA directed against the negative regulator *PtP61F* [68]. *knock-down* of the pathway component *hop* resulted in a significant decrease of Jak-STAT reporter activity when compared to cells treated with dsRNA directed against *GFP*. *Vice versa*, depletion of the negative regulator *PtP61F*, resulted in a significant increase in luciferase activity. *knock-down* of *cfm* however, did not interfere with reporter activity.
Similar results were obtained when using Kc 167 cells in combination with the *6x2xDrafLuc* reporter (Fig. 6.12 B). Taken together, our data indicates that *cfm* is not functioning within the Jak-STAT pathway and conflict with the work of Mueller and colleagues, who identified *cfm* as a negative regulator of Jak-STAT signaling [68].

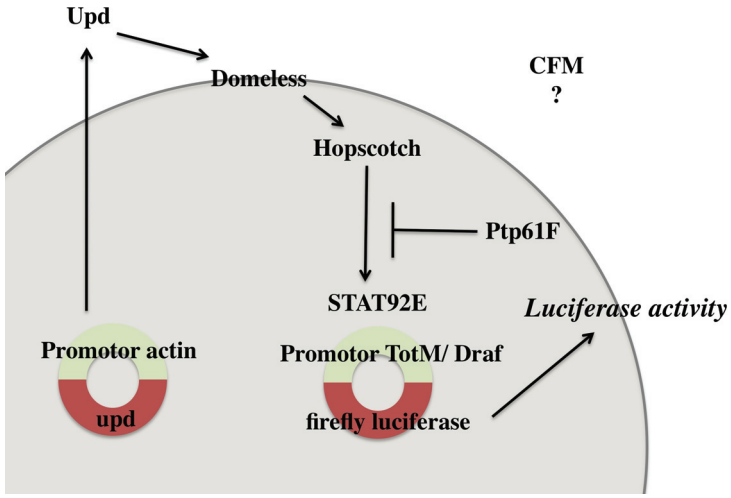


Figure 6.11: Cell-based reporter assay for Jak-STAT activity. Co-transfection of the reporter and a plasmid constitutively expressing Upd will lead to reporter gene acvtivation. Depletion of pathway components, such as the kinase hopscotch or the negative regulator Ptp61F, using dsRNA will alter Jak-STAT signaling activity.

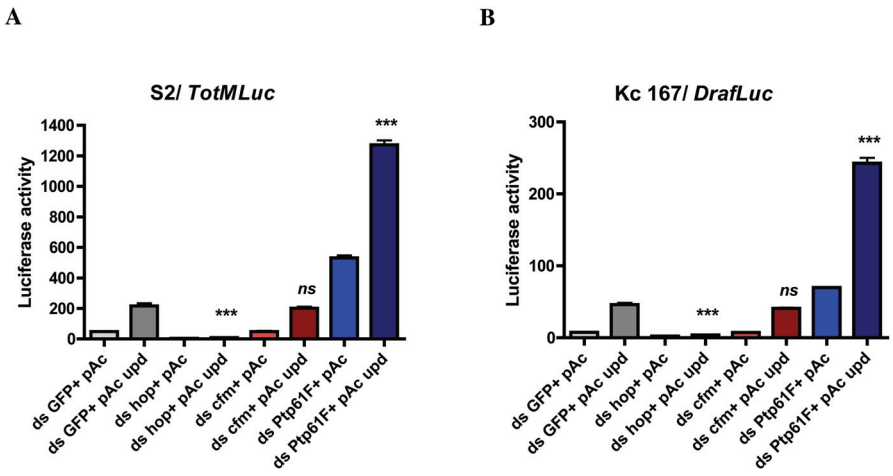


Figure 6.12: CFM does not function as a negative regulator of the Jak-STAT pathway. S2 (A) or Kc 167 (B) cells were transfected with the *TotMLuc* reporter or the *6x2xDrafLuc* reporter (B). *knock-down* of known pathway components significantly affects pathway induction by Upd when compared to samples treated with dsRNA directed against GFP. One representative out of three independent experiments is shown.Values shown represent the mean and standard deviation of measurement triplicates. A: dsGFP pAc upd vs dshop pAc upd P=0.0002; dsGFP pAc upd vs dscfm pAc upd P=0.4841; dsGFP pAc upd vs dsPtp61F pAc upd P<0.0001. B: dsGFP pAc upd vs ds hop pAc upd P<0.0001; dsGFP pAc upd vs dscfm pAc upd P=0.1256; dsGFP pAc upd vs dsPtp61F pAc upd P<0.0001.

6.3.2 CFM does not possess antibacterial or antiviral properties

One characteristic of the drosophila innate immune response is the induction of AMPs in the fat body and their secretion into the hemolymph. These AMPs are mostly small (around 10 kDa), cationic peptides. Several AMPs contain disulfide bridges, which stabilize them in the hemolymph, where their concentration ranges from 0.5 μM for Diptericin up to 100 μM for Drosomycin. Although CFM does not possess any surface charge, it shares several of those characteristics. It is a small peptide (10 kD), which is highly stable due to 5 intramolecular disulfide bridges. It is induced in the fat body and secreted into the hemolymph where it accumulates in a μM concentration upon SINV infection. As a consequence of these undeniable parallels we decided to investigate if CFM functions as an effector molecule.

Although we could only detect a minor induction of the CFM transcript after septic injury, we performed growth inhibition assays using a gram positive (*M. luteus*) and a gram negative (*E. coli*) bacteria. Incubation of bacteria with Ampicillin showed, dependent on the bacterium and antibiotic dose, a dramatic reduction in bacterial growth. Incubation with up to 50 μM recCFM, however, did not result in growth inhibation, suggesting no antibacterial properties of the recombinant protein (Fig. 6.13).

In a collaboration with Friedemann Weber (Freiburg, Germany) we further investigated if CFM was able to directly or indirectly interfere with viral growth. For these experiments, three new viruses were included. Semliki Forest virus (SFV), which is like SINV a Togavirus, Encephalomyocarditis Virus (EMCV), which is a Picornavirus and belongs to the Picornavirales order, like DCV, and finally Rift Valley Fever Virus (RVFV) which is a Bunyavirus (Table 6.2).

We first tested if recCFM could directly interfere with the infectivity of the virus. As a positive control, Interferon-β (1000 U/ml) was added to the cells 1 d prior to infection. Pre-incubation of 2.5 mM recCFM with EMCV, SFV and RVFV 1.5 h prior to infection of Vero cells did not show any interference with viral growth (Fig. 6.14). Similar results were obtained for SINV.

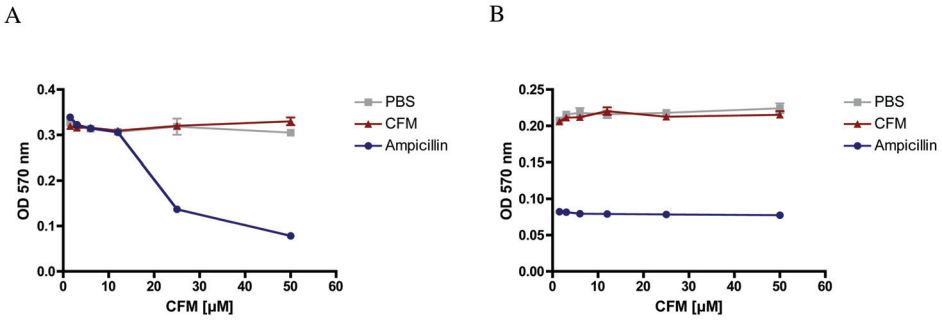


Figure 6.13: Recombinant CFM showed no direct antibacterial properties. A and B: Incubation of up to 50 μM CFM did not show any inhibitory effect on *E. coli* (A) or *M. luteus* (B) growth. Serial diluted Ampicillin was used as a positive control.

Table 6.2: Characteristics of the viruses.

Virus	Family	Genome	Envelope
Rift Valley Fever Virus (RVFV Cl13)	Bunyaviridae	(-) ssRNA segmented	enveloped
Semliki Forest Virus (SFV)	Togaviridae	(+) ssRNA	enveloped
Encephalo-myocarditis Virus (EMCV)	Picornaviridae	(+) ssRNA	/

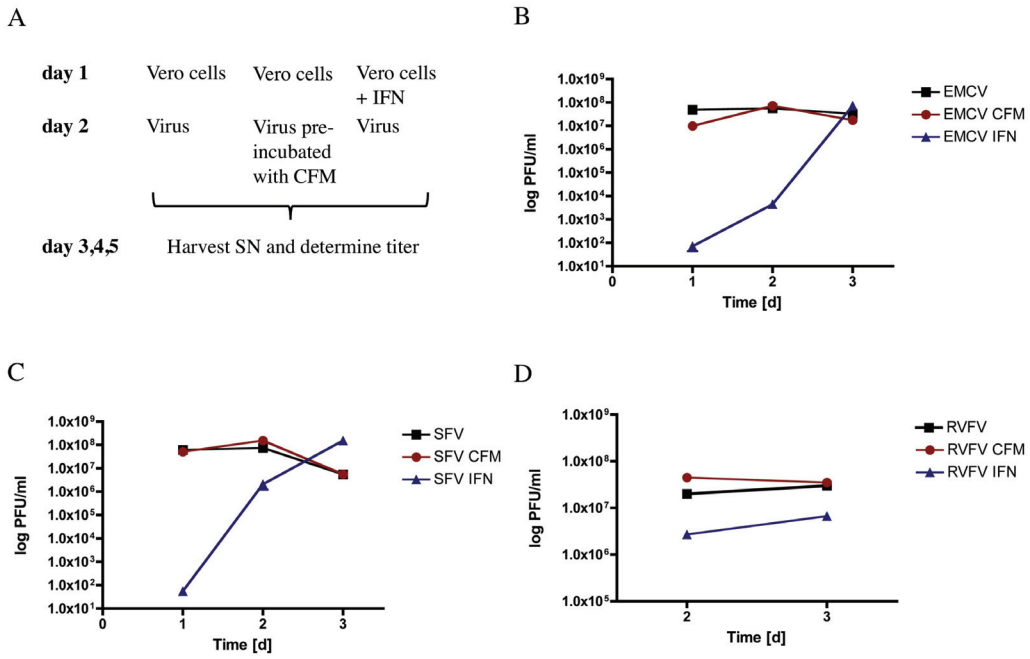


Figure 6.14: Recombinant CFM shows no direct antiviral effect. A: Outline of the experiment. Cells were treated with 1000 U/ml IFN-β as a positive control. B-D: The indicated viruses were incubated with 2.5 mM CFM for 1.5 h at RT prior to infection.

SINV was incubated with either 100 μM recCFM or BSA for 1 h. Following incubation, serial dilutions were made and directly titrated. Treatment with recCFM did not interfere with the infectivity of the virus and showed similar titers as BSA treated SINV (Fig. 6.15). In summary, no direct interference of recCFM with viral growth was observed.
We next investigated if CFM functions as a cytokine. First, we excluded

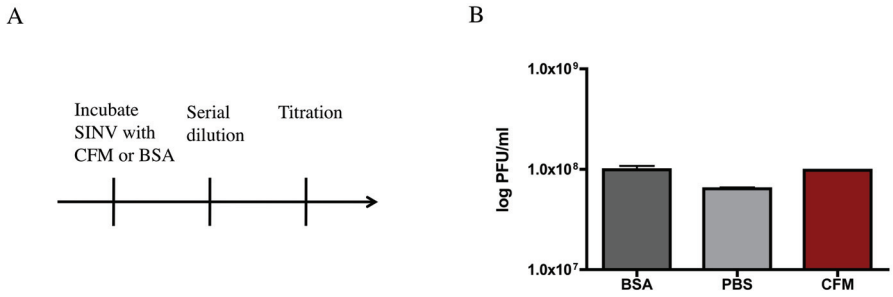


Figure 6.15: Recombinant CFM shows no direct inhibitory effect on SINV. A: Outline of the experiment. SINV was incubated with either 100 μM CFM or BSA for 1 h at RT, serially diluted and directly titrated on Vero cells. Values shown represent the mean and standard deviation of experimental duplicates. No statistically significant difference was observed between CFM or BSA treated samples (P=0.9217).

toxiticity of the recombinant protein for S2 cells. A high dose of recCFM (50 μM) did not show any influence on cell viability and growth. (Fig. 6.16 A). We therefore decided to use this dose in the following experiments. Pretreatment of S2 cells with CFM 15 h prior to infection with DCV did not interfere with the infectivity (Fig. 6.16 B). Similar results were obtained in the case of VSV, and pretreatment of S2 cells with CFM did not strongly affect the viral load. We noted however a 1.3-fold decrease in VSV titer in CFM treated cells 1 and 2 days post infection, which was statistically significant (Fig. 6.16 C). The biological significance of this observation is not clear at present, since the effect is weak, virus specific, and not supported by the *in vivo* experiments with *cfm* mutant flies (see next section).
However, S2 cells pretreated with recCFM showed around 4-fold higher titers after infection with RVFV 2 and 3 days post infection, but again as an increase in viral particles was observed, it would not argue for an IFN-like

function of CFM (Fig. 6.16 D). Taken together, we did not observe any

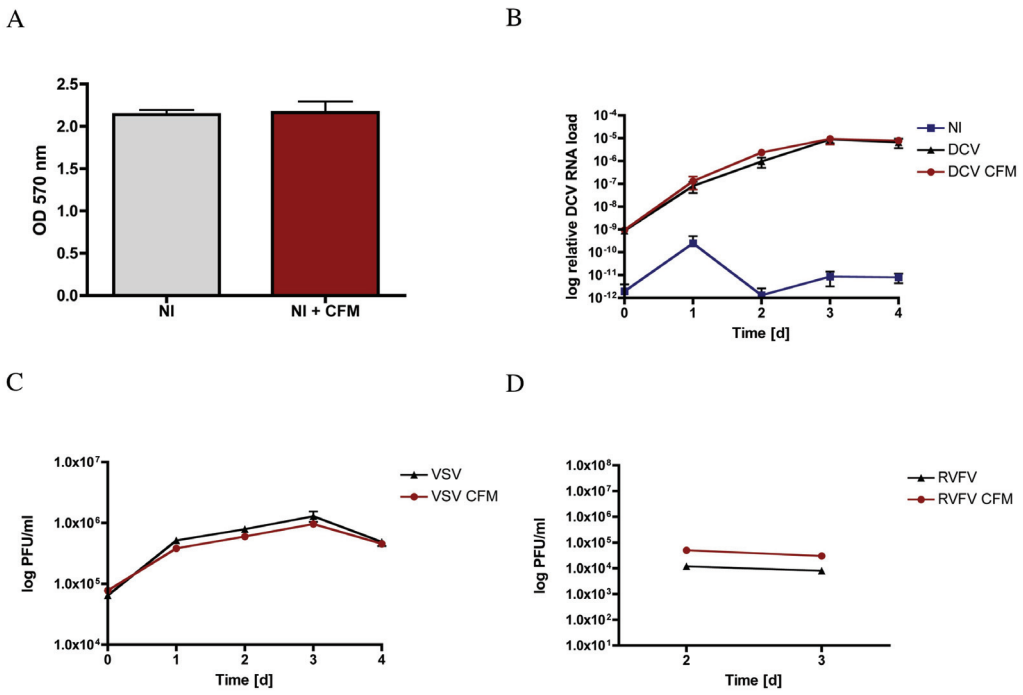


Figure 6.16: CFM is not inducing an antiviral state in S2 cells. A: Incubation of 50 μM CFM with S2 cells for 15 h did not show any influence on cell growth and viability. Cell viability was determined after a growth period of 4d using MTT test. B and C: CFM does not induce an antiviral state in S2 cell as viral loads for DCV and VSV were similar to that of untreated cells. A significant but only 1.3-fold difference was observed 2 and 3 days after VSV infection. Values shown represent the mean and standard deviation of experimental triplicates (P=0.1809 VSV 0 d, P=0.0194 VSV 1d, P=0.027 VSV 2d, P=0.2919 VSV 3d, P=0.7103 VSV 4d, P=0.8164 DCV 0 d, P=0.5867 DCV 1 d, P=0.1129 DCV 2d, P=0.9308 DCV 3d, P=0.6903 DCV 4 d). D: Pre-treated cells showed a mild increase in RVFV titers.

convincing evidence that CFM could directly or indirectly function as an antiviral effector molecule.

6.4 Searching for a function: *in vivo* studies

In order to investigate CFM function *in vivo* we obtained a CFM mutant (CFMex) that was created by P-element excision by Nadege Pelte and Michael Boutros (DKFZ Heidelberg, Germany). The excision spans the whole *cfm* gene plus the 5' region of the adjacent gene *CG2310* (Fig. 6.17 A). Very few homozygous mutant flies were observed in this stock, suggesting either that *cfm* is required during drosophila development, or that the stock contains secondary mutations on the chromosome, which affect development. To clarify the issue, we decided to cross mutant flies with flies carrying a deficiency, covering the *cfm* gene (Df(3R)ED6316 in the following referred to as Def8925). As a control, we used another deficiency on the third chromosome, that was not covering *CG11501* (Df(3R)Ptp99A[R3] in the following referred to as Def5091) and therefore should complement the mutation. We first verified by PCR that CFMex flies crossed with Def8925 were actually mutants. The position of the oligos used is shown in Fig. 6.17 A. No amplification was observed in CFMex/Def8925 flies, confirming that they were indeed null mutants for *cfm*, and lost both copies of the gene (Fig. 6.17 B). Indeed, we could no longer detect the CFM protein in the hemolymph of SINV infected CFMex/Def8925 flies 4 d after infection, whereas the protein was present in flies carrying either 2 copies (w1118/Def5091) or 1 copy (w1118/Def8925 and CFMex/Def5091) of the *cfm* gene (Fig. 6.17 C).

We first challenged *cfm* mutant flies with SINV and VSV, the strongest inducers of *cfm*. Surprisingly no difference in survival was observed, as mutant flies resisted to the infection like control flies (Fig. 6.18 A and 6.19 A). Likewise, no significant differences were observed in viral titers, between *cfm* mutant flies and w1118/Def5091 control flies the following 3 days after SINV infection (Fig. 6.18 B). Similarly, we could not detect significant differences after VSV infection, with the exception of day 2, where *cfm* mutants showed a minor increase (2.6-fold) in viral titer compared to w1118/Def5091, which was statistically significant (Fig. 6.19 B). However, the biological significance is questionable since we did not observe differences in viral load at day 1 and day 3. To summarize we could not observe any consistent phenotype, neither

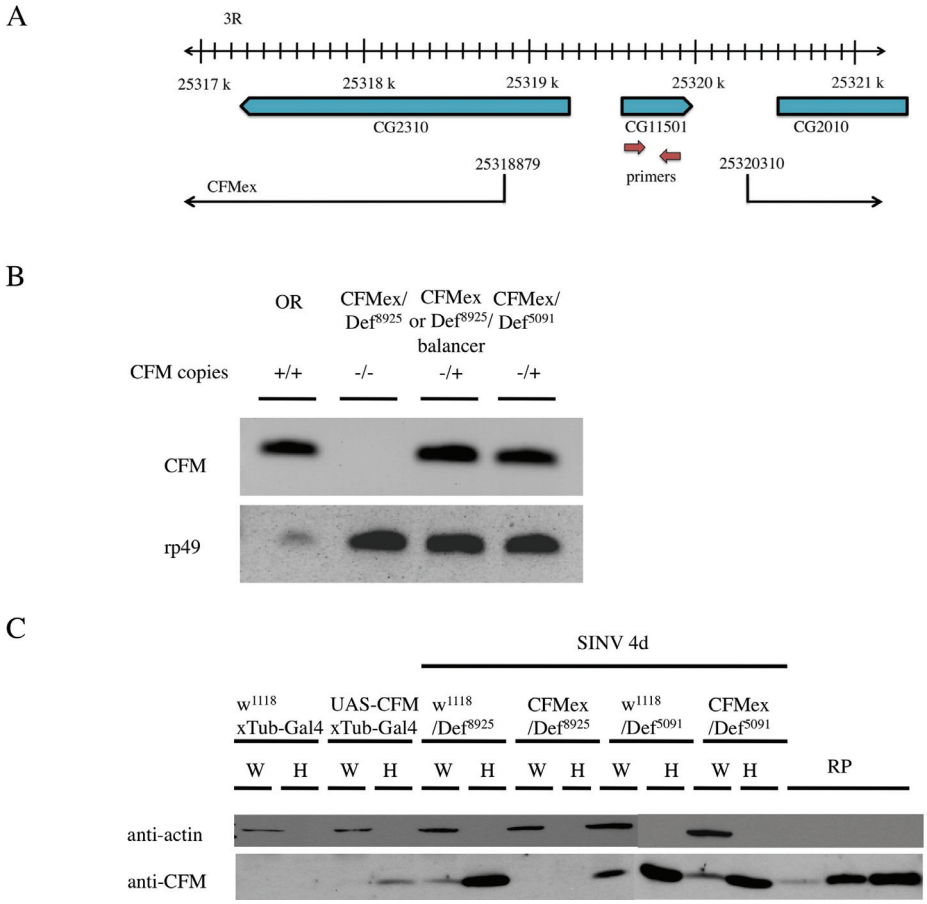


Figure 6.17: Validation of CFM null mutants and overexpressing flies. A: Position of the deletion and position of primers. B: The deletion of the *cfm* gene was verified by PCR. C: Null mutant flies for CFM (CFMex/Def8925) do not induce the CFM peptide after SINV infection. UAS-CFM flies show a detectable amount of CFM in the hemolymph using a Tub-Gal4 driver. W: whole fly, H: hemolymph, RP: recombinant CFM (0.06, 0.125 and 0.25 μg/ μl).

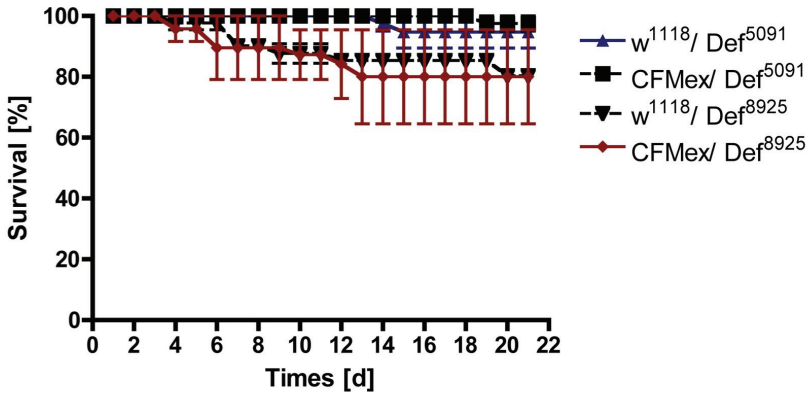
in survival, nor viral load after infection with SINV or VSV.

We next tested our second strongest inducer FHV. *cfm* mutant flies showed weak resitance in survival compared to w1118/Def5091, which died 10 and 9 days after infection respectively. Flies carrying only one copy of *cfm* showed the expected intermediate phenotype, however, overall the observed differences in survival were weak (Fig. 6.20 A). Concerning viral load, no significant difference was observed 1 and 2 days post infection between mutant and w1118/Def5091 flies (Fig. 6.20 B), suggesting that the absence of CFM does not interfere with FHV replication.

Finally, we tested DCV and strikingly, *cfm* mutant flies resisted notably better than w1118/Def5091 control flies after infection with this virus. Mutant flies died up to 2 days later, with 97% surviving mutant flies compared to 31% w1118/Def5091 flies 5 days post infection. The observed difference in survival correlated with the DCV load 1 and 2 days post infection. Mutant flies showed a significant reduction in viral RNA compared to w1118/Def5091 of up to 6.8-fold.

Although unexpected, these results are rather interesting, as we identified a candidate for the first host-encoded factor in drosophila that actually propagates viral replication. However, a genetic rescue of the phenotype is required to prove that *CG11501* does indeed code for this factor. Crosses are currently ongoing and preliminary results were encouraging.

A



B

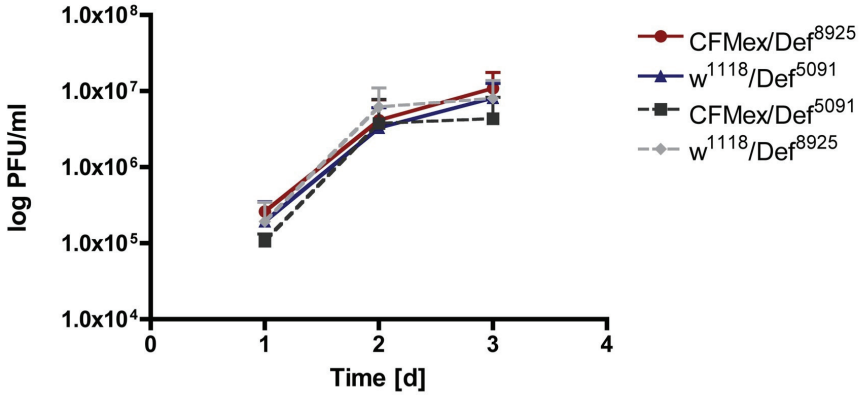
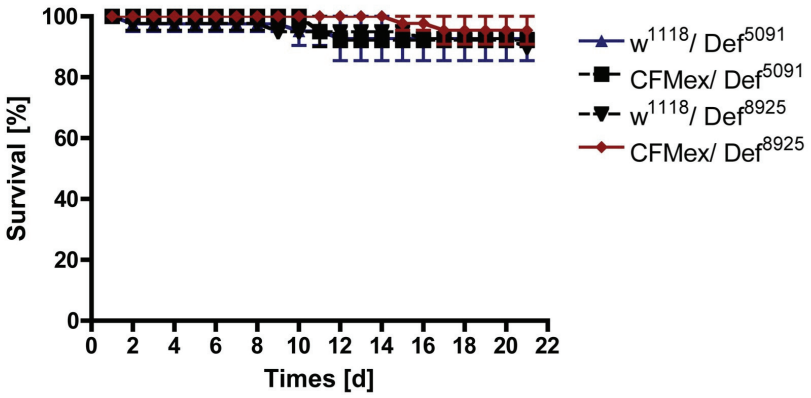


Figure 6.18: *cfm* **null mutants show no phenotype upon SINV infection. A: Flies of the indicated phenotypes were injected with 2.5*10^3 particles of SINV and survival was monitored daily. Values shown represent the mean and standard deviation of 2 independent experiments. B: Flies were injected with 2.5*10^3 particles of SINV and viral titers were determined at indicated timepoints. No significant difference was observed between CFMex/Def8925 and w1118/Def5091 flies (1d P=0.7364, 2d P=0.8663, 3dP=0.7658). Values shown represent the mean and standard deviation of 2 independent experiments.**

A



B

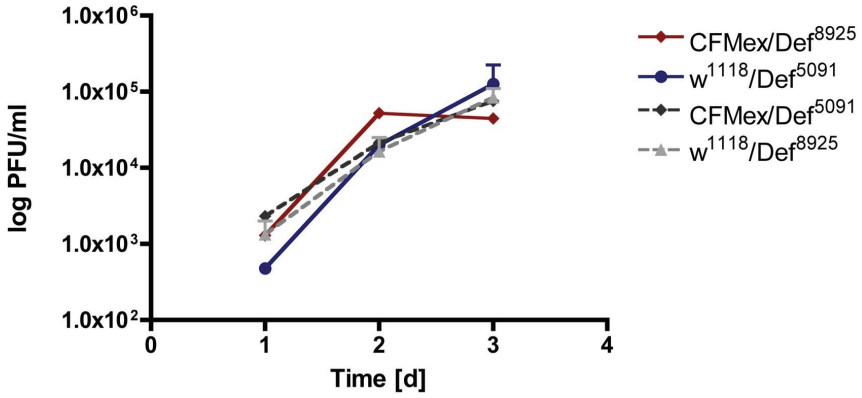


Figure 6.19: *cfm* **null mutant flies show no phenotype upon VSV infection. A: Flies of the indicated genotypes were injected with 1.8*10^4 particles of VSV and survival was mintored daily. Values shown represent the mean and standard deviation of 2 independent experiments. B: Flies were injected with 1.8*10^4 particles of VSV and viral titers were determined at indicated timepoints. No significant difference was observed between CFMex/Def8925 and w1118/Def5091 flies, with the exception of day 2 (1d P=0.0583, 2d P=0.0111, 3dP=0.4932). Values shown represent the mean and standard deviation of 2 independent experiments.**

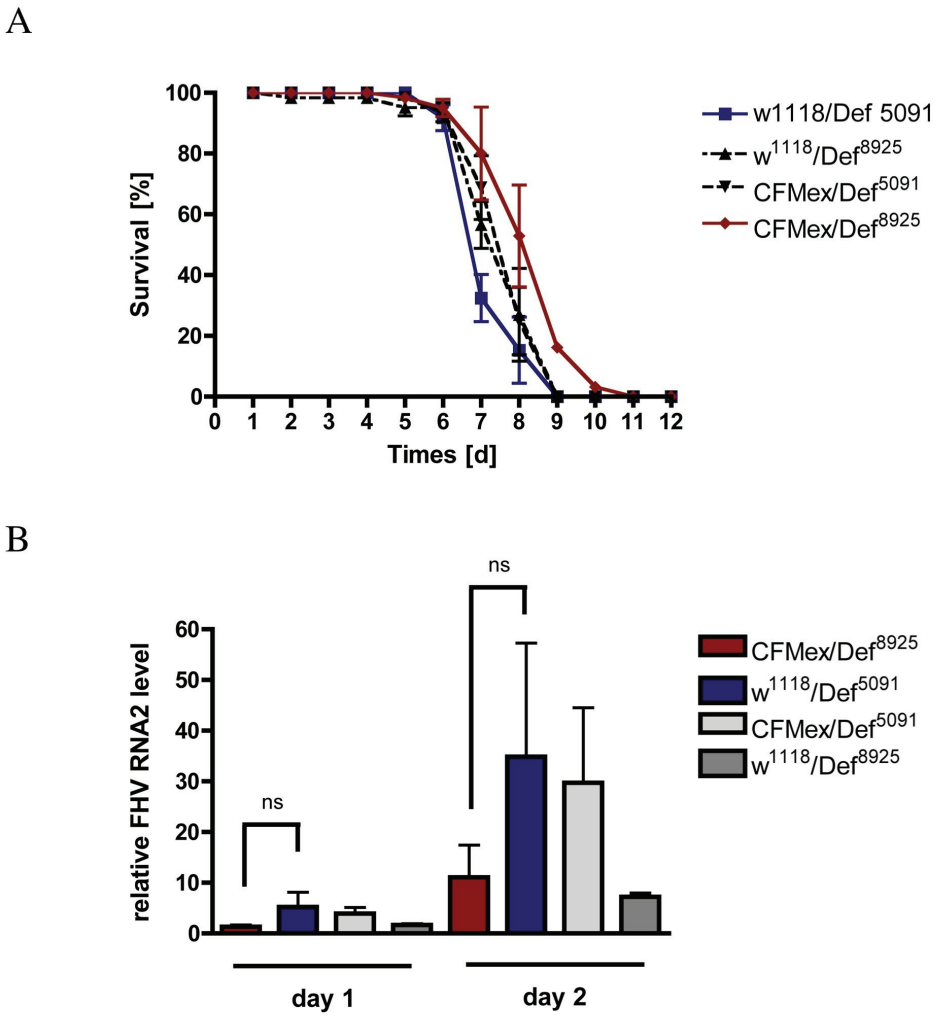


Figure 6.20: *cfm* **null mutant flies show no phenotype upon FHV infection. Flies of the indicated genotypes were infected with 2*10^4 particles of FHV and survival was monitored daily. Values shown represent the mean and standard deviation of 3 independent experiments. B: Flies of the indicated genotypes were infected with 2*10^4 particles of FHV and the viral load was determined at indicated timepoints. Although variability between genotypes were observed, no significant difference was detected between null mutants and w1118/Def5091 flies (1d P=0.235, 2d P=0.3464). Values shown represent the mean and standard deviation of 4 independent experiments.**

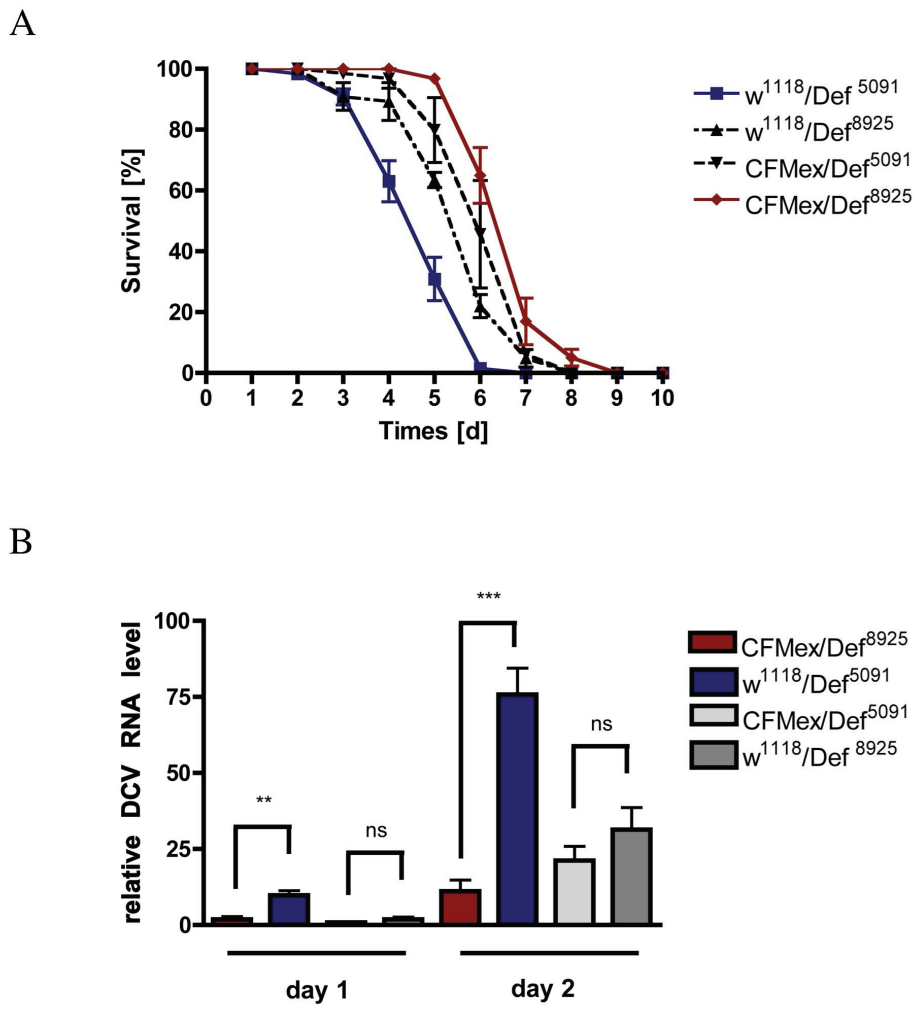


Figure 6.21: *cfm* **null mutant flies show no phenotype upon DCV infection. Flies of the indicated genotypes were infected with 500 particles of DCV and survival was monitored daily. Values shown represent the mean and standard deviation of 3 independent experiments. B: Flies of the indicated genotypes were infected with 500 particles of DCV and the viral load was determined at indicated timepoints. Significant differences were detected between null mutants and w1118/Def5091 flies (1d P=0.0043, 2d P=0.0004). Values shown represent the mean and standard deviation of 4 independent experiments.**

6.5 Summary

In this chapter we presented a detailed characterization of the virus induced gene *cfm* (*CG11501*).

We showed that the *cfm* transcript is induced upon infection with 4 distinct RNA viruses, DCV, FHV, SINV and VSV. SINV and VSV were by far the strongest inducers of *cfm* expression, and induction by bacteria was only minor when compared to viruses. Upon infection with SINV, induction of *cfm* was independent of the Jak-STAT pathway, demonstrating that this pathway is not sufficient to induce *cfm* in the context of virus infection. Although induced by four distinct RNA viruses, we observed considerable differences in the *cfm* expression kinetics. *cfm* expression level were transient for FHV, SINV and VSV, whereas *cfm* expression upon DCV infection increased with time (see chapter 5). Notably, induction of the *cfm* transcript was observed as early as 3 h following infection with the enveloped viruses SINV and VSV. We further showed, that this early induction is not dependent on an active replication of the virus, as a replication deficient SINV was still able to trigger infection 6 h post infection. All together, these data suggest, that the signal leading to *cfm* expression differs between viruses and might involve in the case of SINV and VSV the sensing of viral envelope proteins. However, we can not exclude that *cfm* expression is triggered by secondary effects caused by the infection, such as tissue damage. Indeed, *cfm* expression level increased during the course of DCV infection, and conditioned cell culture media, which is expected to contain cell debris, was found to be an as potent inducer as SINV.

We next characterized the tissue distribution of the *cfm* transcript and the resulting gene product, and showed that CFM is a circulating protein that is secreted by the fat body, the major immune responsive tissue of drosophila. Upon infection with SINV, the CFM level in the hemolymph was around 4 μM, whereas we could not detect the presence of the protein in the hemolymph upon infection with DCV and FHV.

Our functional *in vitro* studies showed no evidence that CFM functions as a negative regulator of the Jak-STAT pathway. Experiments involving re-

combinant CFM gave no indication that CFM might possess antibacterial or antiviral properties.
Surprisingly, CFM null mutant flies behaved like wild-type control flies upon infection with SINV, VSV and FHV. Mutant flies, however, survived notably better after DCV infection and showed a significant decrease in viral titers. Hence, we may have discovered the first host-encoded factor in drosophila that actually seems to propagate viral replication. These findings are supported by the fact that we identified orthologues of *cfm* in the genome of two insect DNA viruses. However, a genetic rescue of the phenotype is required to confirm that *cfm* does indeed encode for this factor.

DISCUSSION

Chapter 7

Discussion

7.1 A comparative study of the drosophila immune response towards infection with RNA viruses

The data presented in this thesis clearly demonstrate that virus infection does indeed trigger an inducible response in drosophila. Our results further show that this inducible response is largely dependent on the virus type. To our knowledge, this work is unique as it involved the use of five distinct RNA viruses instead of being restricted to a single virus. Furthermore we showed that the inducible response can be as potent as RNAi, a commonly accepted major defense mechanism of drosophila against various viruses.

7.1.1 Evidence for a virus-specific inducible response

Our microarray analysis revealed profound differences in the induction pattern of distinct viruses. Although 45 genes were shared among the three viruses tested, numerous induced genes were specific to certain viruses. Interestingly, DCV was not found to be the strongest inducer of gene expression, although its replication was found to be affected by all three signaling pathways tested. Indeed, FHV triggered expression of almost 4-fold as many genes than DCV did whereas SINV infection was found to result in the induc-

tion of 250 genes, 101 genes more than induced by DCV, which is a natural pathogen of drosophila that co-evolved with its host. Clearly, it would be useful to perform genome-wide microarrays on flies infected with CrPV or VSV in order to further understand where these virus-specific induction patterns originate from.
VSV is the only negative-sense RNA virus among the five viruses tested. To date, genome-wide microarray analysis has only been performed with one negative-sense RNA virus, the sigma virus, which belongs like VSV to the *Rhabdoviridae*. No activation of the Toll, Imd or Jak-STAT pathway was found, suggesting that these known immune pathways do not participate to the host defense against sigma virus [146]. It would be certainly interesting to compare gene expression profiles of VSV and sigma virus infected flies, as we found no involvement of these three pathways in the control of VSV. Furthermore, VSV, like SINV, is an enveloped virus, which replicates to high levels in drosophila without killing its host. It would therefore be interesting to compare the VSV induced expression pattern with the genes specifically induced by SINV.
Microarrays on CrPV infected flies should be performed due to its close relation with DCV and the observed similarities between these two viruses in our comparative study. Hence, genes specifically induced by DCV and CrPV represent potential candidates for antiviral effector molecules.

7.1.2 Dicistroviruses and the inducible response - what makes them special?

Genetic analysis revealed two distinct groups among the tested viruses, the Dicistroviruses DCV and CrPV on one hand and FHV, SINV and VSV on the other hand. We found that the Jak-STAT and the Toll pathway do participate to the host-defense against DCV and CrPV, whereas they do not appear to be involved in the defense against the other viruses examined. This observation is very interesting and provokes the question where do these differences originate from.
Interestingly, although all 5 viruses tested replicate to high levels when in-

jected into adult flies, DCV is the only natural pathogen among this group. DCV was first isolated from a french drosophila strain in 1972 and has since been detected from geographically isolated natural populations of *Drosophila melanogaster*. Hence and in contrast to the other viruses, DCV co-evolved with its host and drosophila might have developed specific and unique defense strategies to counteract this virus.

However, the observed specificities might as well reflect tissue-specific patterns of infection or differential effects on host and metabolism. Indeed, work from our group on DCV and FHV infected flies revealed significant differences in the pathophysiology caused by these two viruses (personal communication S. Chtarbanova and I. Eleftherianos). Although both viruses do target common tissues such as the fat body and periovarian sheet upon intrathorical injection, other tissues seem to be specifically affected by either DCV or FHV. FHV specifically infects the heart, whereas DCV infection seems to target the digestive tract interfering with the nutrient uptake of the fly. Hence, these specific impacts on fly physiology and metabolism may well account for the observed differences and would support the idea of a tissue-specific function of the investigated pathways.

Furthermore, and in contrast to FHV, SINV and VSV, the genome of Dicistroviruses is not capped and possesses internal ribosome entry sites (IRES) that allow translation in a cap-independent manner. DCV and CrPV are therefore able to shut down the translation of cellular genes so that the host's translational machinery will operate to translate viral RNA. This very intimate interference with basic host cell functions represents yet another difference between the two virus groups. Moreover, given the existing differences in viral genome structures, drosophila is possibly able to sense distinct viral molecular patterns, such as the 5' linked VpG protein, or the secondary structure of the IRES, that will result in differential responses.

Although we identified the Jak-STAT and the Toll pathway as components of the antiviral defense machinery against Dicistroviruses, we found no evidence for a major role of the Imd pathway. Mutants of the IKKγ homologue *key* behaved like wildtype flies upon infection with SINV, VSV or FHV and even showed a reduction in viral titers for DCV and to a lower extent CrPV. Thus,

although Imd pathway mutant flies were affected upon Dicistrovirus infection, the observed phenotype did not suggest an antiviral function of this pathway as we observed a reduction rather than an increase in viral load. These data conflict with the work of other groups, who found that loss-of-function mutants of the Imd pathway, including *key* mutant flies, showed increased viral titers for both CrPV and SINV [147, 148]. In the case of CrPV infection, the increase in viral load correlated with reduced survival to infection [148]. At this point we can not explain this discrepancy and further experiments involving additional mutants of Imd pathway components are needed to clarify the situation, as we only investigated one gene of this pathway. It should be noted, however, that the fact that all flies used in the study involving CrPV were infected with the endosymbiont *Wolbachia* [148], which strongly interferes with viral replication, may provide some explanation fort the discrepancies between the two studies. Indeed, all of our flies were tested for and free of *Wolbachia*.

7.1.3 Identification of novel immune pathways and relative importance of the inducible response

The observation that none of the investigated pathways seem to participate to the host defense against FHV, SINV and VSV, points to the existence of additional signaling pathways.

Indeed, a fourth pathway, the JNK Mitogen Activated Protein Kinase (MAPK) pathway has been described to be implicated in the regulation of genes induced after bacterial challenge [67]. Microarray analysis of S2 cells showed, that the JNK pathway is activated in response to bacteria by TAK1 [67]. These data are consistant with a model where Imd signaling bifurcates downstream of TAK1, activating both JNK and IKK signaling (reviewed in [7], for a scheme of the Imd pathway see Fig. 1.2). Thus, the possible involvement of the JNK pathway in the regulation of FHV, SINV and VSV induced genes should be investigated. Moreover, the MAPK Kinase Kinase (MAPKKK) Mekk1 was shown to regulate the expression of *Turandot* genes in response to septic injury [66], including *TotM* which is strongly induced upon FHV,

SINV and VSV infection.
Furthermore, work from our group showed, that the virus-induced expression of *Vago* was dependent on the heliase Dicer-2 [153]. Dicer-2 belongs to the same DExD/H-box helicase family as do the RIG-I-like receptors in mammals, which sense viral presence and mediate interferon induction. Hence, Dicer-2 has a dual function in drosophila as it regulates in addition to its function in RNAi the induction of molecules such as Vago that control viral load in some tissues. Definitely, it would be of interest to identify further genes that are regulated by Dicer-2.
Finally, we wanted to evaluate the relative contribution of the inducible response to the antiviral defense machinery compared to RNAi. We found that the Jak-STAT pathway was not only involved in the defense against DCV and CrPV, but appeared to be as important for fly survival and virus control as RNAi. It would certainly be interesting to evaluate as well the relative importance of the second pathway found, the Toll pathway. Both, the Jak-STAT and the Toll pathway were identified to function within the antiviral defense in mosquitos [149, 170], highlighting the importance of an inducible response in insects in general. Taken together, these findings clearly demonstrate, that drosophila antiviral innate immunity does indeed rely on two axes of defense, RNAi on the one hand and an inducible response on the other hand.
However, the identification and characterization of virus-induced effector molecules remains a difficult but necessary task in order to fully understand the nature of this inducible response.

7.2 Function of virus-induced genes

The findings of this thesis demonstrate that virus-infection does trigger an inducible response, which can be as important as RNAi. Besides identifying further pathways, a clear emphasis should be made on the functional characterization of these virus-induced genes.
Although we found evidence, that the Toll pathway is implicated in the defense against Dicistroviruses, the question of the nature of the corresponding

effectors remains. AMPs as known targets of the Toll pathway are an obvious choice as putative antiviral agents. Indeed, defensins and cathelecins have been reported for their antiviral activity in mammals (reviewed in [109]). DXV was claimed to induce expression of several AMPs, including *defensin*, *drosomycin* and *metchnikowin*, to a similar level than *E. coli* [117]. However, *E. coli* as a Gram-negative bacterium is a poor inducer of Toll pathway activity [41]. Overexpression of AMPs did not alter viral infection or titers, suggesting that AMPs do not act as antiviral effectors against DXV [117].
No AMPs were found to be present in the hemolymph of DCV infected flies [140] and we did not observe expression of the *drosomycin* transcript within the first 4 days of infection. Likewise, AMP expression was not observed upon infection with CrPV [148], and although we observed slightly altered *diptericin* expression upon infection with CrPV and SINV, the induction strength was much weaker then of bacteria infected flies. However, AMPs should not be discarded as putative antiviral effectors as viral infection might not trigger a humoral but rather a local immune response. An in-depth characterization of Dicistrovirus-induced pathophysiology would help to answer that question by identifying putative tissues of local AMP expression.
As for the Toll pathway, no Jak-STAT controlled effector molecule has been described in drosophila to date. *vir-1* is an established marker of virus-induced Jak-STAT activity and is induced specifically upon infection with DCV, FHV and CrPV, but not by pathogenic or apathogenic bacteria (see chapter 5 and [142]). However, there is no formal proof that this gene participates in the immune control of viral infection.
Another promising candidate for a Jak-STAT pathway regulated putative effector molecule was *Chifoumi* alias *CG11501*. However, we showed in this thesis that *Chifoumi* expression upon virus infection is regulated in a Jak-STAT independent manner (see section 7.3).
Thus, further candidates for Jak-STAT regulated effectors need to be characterized. *GNBP-L1* (*CG12780*) might be an interesting choice as (1) it was induced in our microarray analysis by all three viruses, (2) its induction was considerably reduced or abolished in Jak-STAT mutant flies [142] and (3) it belongs to the GNBP family of PRR receptors in drosophila. Furthermore,

GNBP-L1 does not possess a signal peptide, indicating that it might function as an intracellular receptor.
Taken together, the functional characterization of virus-induced molecules is of crucial importance, but has proven to be rather difficult. One main drawback lies in the fact that functional studies, which are commonly focused on one candidate gene, are very time consuming. The genetic tools available in drosophila can help facilitate the choice of suitable candidate genes for further characterization. Transgenic lines carrying a hairpin construct against most of the genes of the drosophila genome are available, allowing the controlled *knock-down* of its target sequences. The existence of these lines allow fast screening for antiviral functions of the targeted genes.
Furthermore, microarray analysis of DCV and CrPV infected Toll and Jak-STAT mutant flies would enable us to reduce candidate gene numbers by correlating the observed phenotypes with pathway specific gene induction patterns.

7.3 *Chifoumi* - an example of a virus-induced gene

We have characterized the gene *cfm*, which may encode a host factor involved in the propagation of DCV replication in drosophila. Our preliminary data indicate that CFM null mutant flies have a significant decrease in viral titers and survive better upon infection with DCV, suggesting that the presence of CFM does actually confer an advantage to the virus.
Indeed, experiments using recombinant CFM showed no evidence for CFM being an antiviral molecule, which would either directly or indirectly reduce the virulence of the viruses tested. Moreover, the observed phenotype fits perfectly with the identification of CFM orthologues in the genome of two insect DNA viruses, SfAV1 and XcGV. DNA viruses, such as poxviruses, are known to hijack host genes in order to manipulate the host's immune response in their favour. Poxviruses evolved together with their host by employing host and tissue specific strategies. This adaptation process is reflected by

the aquisition of cellular host genes. Viral homologues of these cellular genes display a broad functional spectrum, ranging from TNF receptor homologues to Interleukin, IFN or growth factor homologues and allow the virus to very efficiently manipulate the host [168]. Hence, the identification of viral CFM orthologues does directly support the observed phenotype.
Although these results are encouraging, the question about the function of this virus-induced molecule remains unanswered.

7.3.1 Searching for a function of CFM

The observed phenotype of the CFM null mutant resembles the expected phenotype of a negative regulator of the Jak-STAT pathway and would support the work of Mueller and colleagues [68]. However, we were unable to confirm their findings and found no evidence *in vitro* that CFM does indeed function within or as a negative regulator of the Jak-STAT pathway. Furthermore, CFM was not identified in an independent screen for Jak-STAT pathway components, that was carried out using a different drosophila cell line and reporter gene [169]. Overall, there is no proof that CFM functions as a negative regulator of Jak-STAT signaling. Thus, the precise function of CFM can only be speculated at this stage.
CFM is a circulating peptide that is secreted by the fat body upon virus infection. The fact that we could not detect the peptide in the hemolymph upon infection with DCV and that overexpression of CFM did not enhance DCV replication (data not shown) suggests that CFM functions in a low concentration below the μM range. Taken together, these observations indicate that CFM might act as a cytokine and provokes the question of potential interacting partners.
The fact, that *cfm* expression does increase over time upon DCV infection and that infection induced tissue damage possibly mediates *cfm* induction might argue for a role of CFM in tissue repair. Either case, the potential receptor, as well as the signaling network in which CFM is embedded needs to be identified in order to fully understand the biological processes CFM is involved in.

Furthermore, our efforts to characterize its function might well have been hampered by existing redundency or the possibility of CFM acting in a complex. Indeed, we observed striking similarities in the expression pattern of *TotM* and *cfm*, confirming previous reports that suggested co-regulation of these two genes [67]. Theoretically, however, there are numerous other potential interacting partners. A hallmark of the drosophila immune system is the secretion of peptides and proteins by the fat body in response to septic injury. Among those peptides, AMPs constitute the best characterized group of induced molecules. However, there is an additional group of putative immune factors that includes the 8 members of the Turandot as well as 17 members of the DIM (Drosophila Immune Molecule) family. As for members of the Turandot family, we found DIMs among the induced genes of our microarray analysis. Thus, we can not exclude that CFM might function in synergy with any of these or yet unidentified molecules. It should be mentioned at this point, that although Turandot and DIM genes were discovered more than 10 years ago, their function remains unclear. This fact highlights once more the difficulties involved in the functional characterization of immune-induced molecules.

7.3.2 Regulation of virus-induced *cfm* expression

One of the reasons we became interested in *cfm* was that it was reported to be induced in a Jak-STAT dependent manner upon septic injury. In the context of virus infection, however, *cfm* remained fully inducible in *hopscotch* mutant flies, indicating that virus-induced expression of this gene was independent of the Jak-STAT pathway. Furthermore, we confirmed previous findings that showed co-regulation of *cfm* and *TotM* expression. The regulation of Turandot genes in response to septic injury is known to be complex and involves input from the Jak-STAT, the Imd and the MAPK pathway. Clearly, it should be investigated if virus-induced *cfm* expression is dependent on the Imd or the MAPK pathway.

Another remaining question is the nature of the signal that mediates *cfm* induction. We found that enveloped viruses such as SINV and VSV triggered

a very rapid and robust increase in *cfm* transcript numbers. Moreover, an active replication of SINV was not required to mediate this early induction, suggesting that a viral protein component might be sensed. However, we found that injection of conditioned cell culture medium triggered *cfm* expression to a similar level than SINV, indicating that *cfm* induction might also be mediated by factors such as tissue damage (data not shown). Indeed, we observed an increase in *cfm* transcript numbers as late as three and four days post infection with DCV.
At this stage, we can neither explain these striking differences in the induction strength caused by different viruses, nor the fact, that only DCV replication seemed to be affected in *cfm* mutants. Nevertheless, the results obtained are very interesting and require further investigation.

7.4 Concluding remarks

The results of this thesis clearly demonstrate that drosophila relies on two axes of defense to counteract viral invaders. RNAi, as a viral nucleic acid based defense, functions against a broad range of viruses, whereas the inducible response seems to be specific for certain virus groups. However, the identification of inducible effectors is generally a difficult task. Indeed, although IFN was discovered some fifty years ago, only a few of the 300 IFN induced genes are well characterized for their antiviral activities [102]. Making use of the powerful genetic tools of the drosophila model will largely help to solve this problem. Based on present and past findings, one can hope that this understanding will lead to new concepts that will be helpful for unravelling the genetic mechanisms of antiviral resistance in other organisms. Furthermore, these studies are also likely to be of particular relevance for understanding the interactions between insect vectors and arboviruses, a major challenge for years to come.

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APPENDIX

Eidesstattliche Erklärung

Ich erkläre an Eides Statt, dass die vorliegende Dissertation in allen Teilen von mir selbständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben worden sind.

Veröffentlichungen von irgendwelchen Teilen der vorliegenden Dissertation sind von mir nicht vorgenommen worden, mit Ausnahme von Abbildung 3.3, welche in Current Opinion in Immunology 2009 veröffentlicht wurde.

Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Datum, Ort, Unterschrift