Evaluating antiviral activity of nucleic acid binding proteins across species

Assel Mussabekova

UNIVERSITÉ
DE
STRASBOURG

École Doctorale
des Sciences de la Vie
et de la Santé
STRASBOURG

ÉCOLE DOCTORALE des Sciences de la Vie et de la Santé
UPR9022

THÈSE présentée par :

Assel MUSSABEKOVA

soutenue le : **7 fevrier 2019**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Aspects moléculaires et cellulaires de la biologie

Evaluating antiviral activity of nucleic acid binding proteins across species

THÈSE dirigée par :
Prof. Jean-Luc IMLER

RAPPORTEURS :
Prof. Stefanie BECKER
Dr. Caroline MONTAGNANI

AUTRES MEMBRES DU JURY :
Dr. Sébastien PFEFFER

Acknowledgements

There are many people whom I would like to thank for guiding me through this journey.

To my supervisor Prof Jean-Luc Imler for giving me the chance to work in the team on this exciting research project. I thank you also for the guidance and sharing your expertise during these years. Thank you for your support, especially during my pregnancy, and of course, thank you for the patience and feedback during the process of writing this thesis!

To my co-director Prof Carine Meignin for being an example of a strong woman in science. You are a person of action, a true leader by doing. Thank you for your always precise and practical advice and for your patience in answering all my questions!

To our collaborators, Prof Andreas Pichlmair and Prof Jacques Colinge and their teams, for a great experience of working with you, scientific exchange, and exciting data, that I had a chance to work with.

To Dr Stefanie Blandin and Prof Pascale Romby for your kind advice which helped to advance the project.

To Region Alsace, LabEx NetRNA and FRM for providing funding for this work.

To the heroes of "the" screen: to my dear Stefi, who introduced me to flies, Yasmine, who helped to complete the experiments, and Emilie, whose data and advice helped me to gain confidence. I also thank Claudine for helping me with a tedious task of sorting.

Thanks to three people, who helped me to become friends with the R and computer in general: Laurent, source of zen energy in the lab; Nelson, who taught me to think in columns and Roenick, who knows when he needs to send me to Nelson.

To many friends and colleagues from the lab: Shree, Evelyne, Alex, Katharina, Ludmila, Akira, Gabrielle, Joao, Claire, Juliette, Hua, Loïc, Francesco, Najwa, Laurent D, Miriam, Bechara, Eric, Aurelie and all other members of UPR9022. It is a privilege to work in such a team.

Special thanks to Estelle for all the enormous amount of teaching that she does. Another special thanks to Nathalie, our "maman-poule", the lab should pay you for psychological support!

To my friends from all around the globe: thank you, I feel your support!

To my teacher Mariya Kirillovna, who once shared with me the excitement for biology and taught to learn from beautiful minds!

To all my extended family back home, I can feel your support miles away!

To my mother-in-law Gulnar, your support and love is a true gift!

To my brother Adilet and my sisters Aina and Aliya. You are my best friends and my inspiration!

To my daughters, Amina and Safinur. Thank you for reminding me every day of what is important.

To my dear husband Olzhas. You are the pillar of my strength.

My deepest gratitude goes to my parents. To my mom Zhamal, who sacrificed a lot for me to be here and who is my main motivation. I dedicate this thesis to the loving memory of my dad, Beisenbek, who would be proud of his little girl.

List of Figures and Tables

Figures

Tables

List of abbreviations

2'-5'oA	2'-5'oligoAdenylate	**IFIT**	interferon-induced protein with tetracopeptide repeats	**RIG-I**	retinoic acid-inducible gene-I
ABC	ATP-binding cassette	**IFN**	interferon	**RISC**	RNA-induced silencing complex
ADAR1	adenosine deaminase acting on RNA 1	**IIV6**	invertebrate iridescent virus 6	**RLR**	RIG-like receptor
AGO	argonaute	**IKK**	inhibitor of nuclear factor kappa-B kinase	**RNA**	ribonucleic acid
AIM2	absent in melanoma 2	**IL**	interleukin	**RNAi**	RNA interference
AMP	antimicrobial peptide	**IMD**	immune deficiency	**RP**	ribosomal protein
AP-MS	affinity purification and mass spectrometry	**IRF3**	interferon regulatory factor 3	**RRM**	RNA recognition motif
Atg8/LC3	autophagy-related protein 8/microtubule-associated protein 1A/1B-light chain 3	**ISD**	interferon-stimulating DNA	**rRNA**	ribosomal RNA
ATP	adenosine triphosphate	**Jak/STAT**	Janus kinase/signal transducer and activator of transcription	**RVFV**	Rift Valley fever virus
BRCA1	breast-cancer type 1 susceptibility protein	**KD**	knockdown	**S2**	Schneider 2
CG	computed gene	**Klp**	kinesin-like protein	**SDM**	Schneider's Drosophila medium
cGAS	cyclic GMP-AMP synthase	**KO**	knock-out	**sgRNA**	single guide RNA
Chd1	chromodomain helicase DNA binding protein 1	**LACV**	La Crosse Virus	**sh**	short hairpin
CLIP	Cross-linking immunoprecipitation	**LC-MS/MS**	liquid chromatography followed by tandem mass spectrometry	**SINV**	Sindbis virus
CMTR1	Cap methyltransferase 1	**LGP2**	Laboratory of genetics and physiology 2	**siRNA**	short interfering RNA
CRISPR	clustered regularly interspaced short palindromic repeats	**MAVS**	mitochondrial antiviral-signaling protein	**snRNA**	small non-coding RNA
CrPV	Cricket Paralysis virus	**MDA5**	melanoma differentiation-associated protein 5	**SPNR**	spermatid perinuclear RNA-binding protein
DAffSV	*Drosophila affinis* sigma virus	**miRNA**	microRNA	**ss**	single-stranded
Dcp1	decapping protein 1	**MOI**	multiplicity of infection	**STING**	stimulator of interferon genes
DCV	Drosophila C virus	**mRNA**	messenger RNA	**Tao**	Thousand and one
DDX	DExD/H helicase	**Mst/Ste20**	mammalian serine/threonine protein kinase	**TAP1**	antigen peptide transporter 1
dFOXO	Drosophila forkhead box O	**MVA**	modified vaccinia Ankara	**TATA**	TATA-box binding protein (TBP)-associated factor 11
DIAP1	drosophila inhibitor of apoptosis	**MYD88**	myeloid differentiation primary response protein 88	**TLR**	Toll-like receptor
DIP1	DISCO interacting protein	**NA**	nucleic acid	**TNF**	tumor necroses factor
DMelSV	Drosophila melanogaster Sigma virus	**NF**	nuclear factor	**TRAF**	TNF receptor associated factor
DNA	deoxyribonucleic acid	**NF-kB**	nuclear factor kappa-light-chain-enhancer of activated B cells	**TRAMP**	tyrosine rich acidic matrix protein
DNA-PK	DNA-dependent serine/threonine kinase	**NLRP3**	NACHT, LRR and PYD domains-containing protein 3	**TREX1**	three prime repair exonuclease 1
DNMT2	DNA methyltransferase 2	**NRAMP**	natural resistance-associated macrophage protein	**TRIF**	TIR-domain-containing adapter-inducing interferon-beta
ds	double-stranded	**OAS1**	2'-5'-oligoadenylate synthase 1	**tRNA**	transport RNA
DXV	Drosophila X virus	**PAMP**	pathogen-associated molecular pattern	***Ubc-2EH***	ubiquitin conjugating enzyme E2H
FHV	Flock house virus	**PCR**	polymerase chain reaction	**UDE**	uracil-DNA degrading factor
Gal	galactose	**PGRP-SA**	peptidoglycan recognition protein SA	**UPD**	Unpaired
GD	Golden	**piRNA**	piwi-interacting RNA	**UTR**	upstream activated sequence
GFP	green fluorescent protein	**PKR**	protein kinase R	**VDRC**	Vienna Drosophila Resource Center
GO	gene ontology	**poly(A:U)**	Polyadenylic-polyuridylic acid	**VP**	viral protein
GWAS	genome-wide association study	**poly(I:C)**	polyinosinic:polycytidylic acid	**VSR**	viral suppressor of RNAi
H3K9	histone 3 lysine 9	**PPP**	triphosphate	**VSV**	vesicular stomatitis virus
HA-tag	human influenza hemagglutinin tag	**PRNTase**	polyribonucleotidyltransferase	**WD40**	domain
HCV	Hepatits C virus	**PRR**	pattern-recognition receptor	**WNV**	West Nile virus
HIV	human immunodeficiency virus	**Pst**	pastrel	**Zfr**	Zinc finger-containing protein
IAP	inhibitor of apoptosis	**RACK1**	receptor of activated protein C kinase 1	**Zn72D**	zinc-finger protein at 72 D
IAV	Influenza A virus	**RdRP**	RNA-dependent RNA polymerase	**β-ME**	β-mercaptoethanol
IFI16	Gamma-interferon-inducible protein 16	**RHG**	*reaper, hid and grim*		

Table of Contents

Introduction

In 1880s, Elie Metchnikoff borrowed the word "immunity" from juridico-political terminology to describe the ability of an organism to resist to harmful microorganisms. Now, more than ever, this term resonates with its original meaning. The humanity is extremely vulnerable to the new challenges that the infectious diseases pose. New emerging infections, causing epidemics and pandemics, antibiotic resistance, social issues such as vaccine acceptance and poverty-related diseases, and bioterrorism appear on daily political agenda.

Original model of immunity suggested by Burnet in 1959 defined it as a form of 'self-defence', thus highlighting the importance of discriminating between self and non-self (Burnet 1959). This paved the way to the characterization of the antigen receptors expressed by B and T lymphocytes, the hallmark of the adaptive immune system in vertebrates. Thirty years later, Janeway further developed this model by proposing that the infectious 'non-self' in the form of pathogen-associated molecular patterns (PAMPs), is recognized by pattern-recognition receptors (PRRs) of the host (Janeway 1989). However, this paradigm can be questioned by the existence of many contradictions, such as cancer, autoimmunity, host versus graft disease and pregnancy, as well as the absence of immune response to commensal microbiota. In the attempt to explain these bias in recognition, Matzinger's 'danger model' described immunity as a response to dangerous events through sensing the 'danger signal' (Matzinger 2002; Matzinger 1994). The 'danger model' added another level of complexity to the recognition, involving cell-to-cell communication.

This paradox in the immune recognition of self/non-self or dangerous/safe becomes particularly apparent in viral infection. Viruses are composed of a nucleic acid, encapsulated in a protein or protein/lipid shell and therefore offer few targets for recognition. Indeed, the activation of antiviral pathways largely relies on the sensing of viral nucleic acids (NA). However, the structure of NAs across kingdoms is universal and endogenous NAs are diverse and abundant. Thus, the recognition of viral NAs is a biochemical challenge for the host cell with complex mechanisms involved. Our knowledge of the NA sensing in mammals has dramatically increased during last decades. A number of comprehensive recent reviews are available on different aspects of this topic (Habjan and Pichlmair 2015; Schlee and Hartmann 2016; Gebhardt, Laudenbach, and Pichlmair 2017; Ori, Murase, and Kawai 2017;

Roers, Hiller, and Hornung 2016). In the introduction, I will first discuss the principles of NA recognition in mammals, the characteristics of viral *vs* endogenous NAs and the major sensors and effectors involved. I will then move to the model systems used in my PhD project. I will introduce the host, the fruit fly *Drosophila melanogaster*, with a focus on its antiviral mechanisms, and viral pathogens, used for infection in this study. I will finally present the questions that remain to be answered and the aim of the current study.

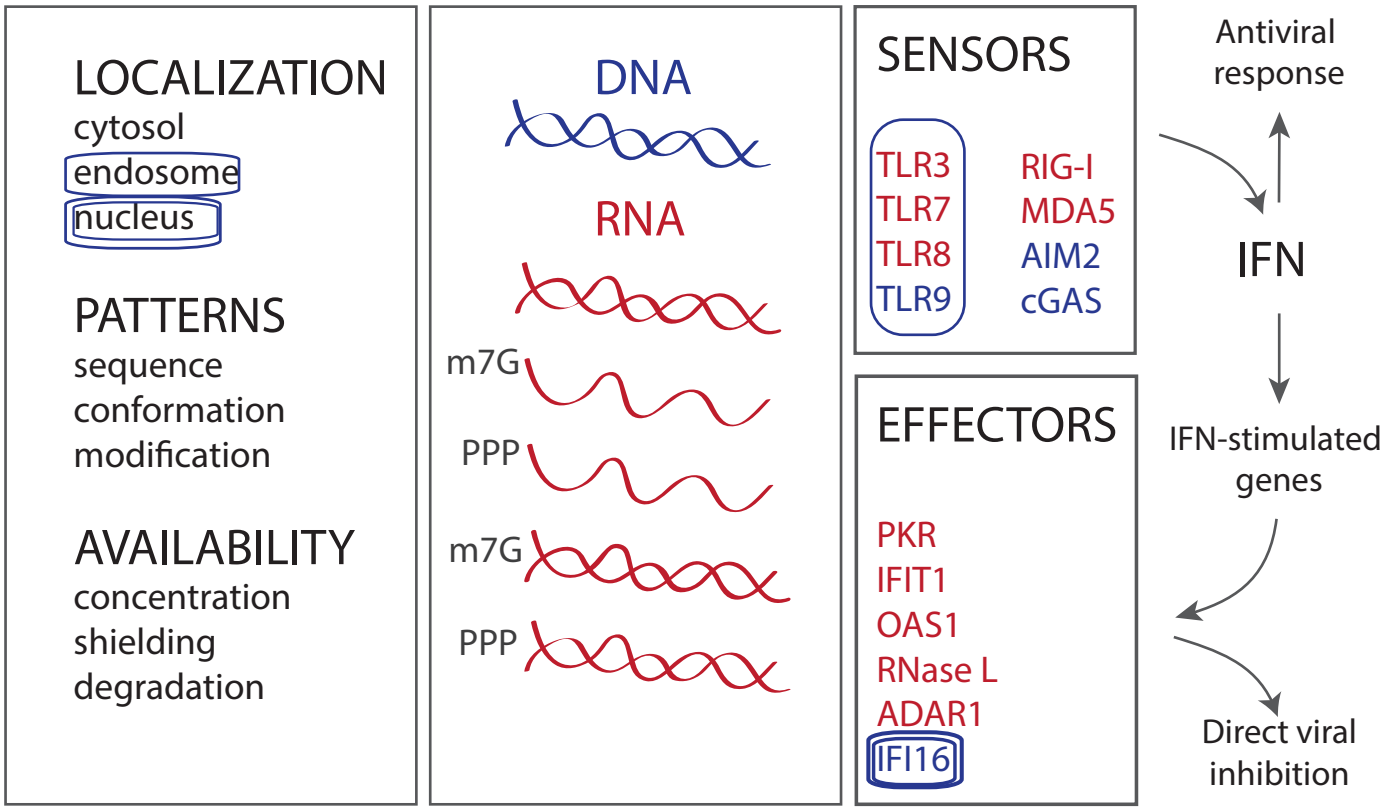


Figure 1. Overview of the principles in mammalian viral NA sensing. Viral NAs are detected on the basis of their localization, patterns and availability. Viral NA products can be present in the cell in the form of DNA (blue) and RNA (red). Viral RNA, eliciting the most studied immune pathways include dsRNA, as well as underprocessed RNA (uncapped 5'PPP, CAP and CAP0 ss- and dsRNA). The molecules detecting viral NAs can be divided into two groups: sensors and effectors. Sensors recognize viral NAs and trigger IFN response, which in turn leads to the activation of effectors, which directly inhibit viruses. Main sensors and effectors with their localization are indicated.

Nucleic acid sensing in mammals: in health, disease and viral infection

RNA interference represents an ancient branch of NA recognition and the main antiviral immune response in most non-vertebrates, including plants, nematodes and insects. This RNA-based system allows sequence-specific recognition of viral genomes and is initially triggered by RNase III enzyme of the Dicer family, which process virus-derived dsRNA. Although several cases of antiviral RNAi in mammalian cells have been described, it remains a topic of debate, as RNAi competes with interferon (IFN)-based antiviral pathways that dominate the response (Ding et al. 2018). Although IFN expression can be induced by dsRNA, there are other features of viral NAs that are being recognized by the immune system.

Principles of NA sensing in mammals

There are several characteristics of viral NAs that can be sensed by mammalian cell and lead to signal transduction. First, the ***localization*** of NAs in specific compartment, such as endosome or cytosol, triggers the recognition by a group of receptors. Secondly, viral NAs are thought to possess certain structural ***patterns***, not otherwise present in the endogenous NAs in normal conditions (Figure 1). Thirdly, a number of complex processes control the ***availability*** of viral-derived NA ligands. This includes the concentration threshold, above which the NAs of any origin are detected, the rate of degradation by cellular nucleases, as well as the mechanisms of shielding.

Localization-based recognition

Inside the cell, the majority of viral NA receptors are found in the cytosol, however, some of them function in the endosomes and even inside the nucleus. Endosomal compartmentalization of some recognition pathways serves two main functions: first, it provides the degradative environment that helps the recognition of shielded ligands, and second, it represents an isolated entity, thereby protecting

the endogenous NAs from recognition. A number of transmembrane Toll-like receptors (TLRs) recognize viral NAs inside the endosomes , namely TLR3, TLR7, TLR8 and TLR9 (Alexopoulou et al. 2001; Hornung et al. 2005; Heil et al. 2004; Diebold et al. 2004; Matsumoto et al. 2003). dsRNAs are sensed by TLR3 and TLR7, ssRNA are detected by TLR7 and TLR8 and DNA is recognized by TLR9. These sensors then act through TIR-domain adaptor proteins myeloid differentiation primary response protein 88 (MYD88) and and/or TIR-domain-containing adapter-inducing interferon-β (TRIF), eventually resulting in type I IFN response and NLRP3 expression. Viral NAs protected by NA-binding proteins from cytosolic nucleases are trafficked into the endosomal compartments and are being recognized as non-self (Majer, Liu, and Barton 2017).

It is known, that DNA viruses hijack the host replication and transcription machineries and therefore viral genome locates to the nucleus of the host to replicate. It is still unclear, how the recognition of non-self NAs might occur in the nucleus. Recently it was shown that mammalian interferon-inducible protein IFI16 binds HSV-1 DNA and inhibits the transcription of both parental and progeny viral genomes inside the nucleus of the host cell (Diner et al. 2016; Merkl, Orzalli, and Knipe 2018). Other putative nuclear viral NA sensors include breast cancer type 1 susceptibility protein (BRCA1), DNA-dependent serine/threonine kinase (DNA-PK) and MRE11 (Schlee and Hartmann 2016).

Patterns of endogenous and microbial NAs

DNA patterns

Several mechanisms protect the host from the adverse consequences of unwanted induction of IFN synthesis by self NAs. One of them is the localization-based process, mentioned above. Another one in the case of DNA is the preferential recognition of unmethylated CpG motifs by TLR9 in the endosome (Hemmi et al. 2000; Hartmann and Krieg 2000; Coch et al. 2009).

DNA-RNA hybrids are also an important hallmark that is recognized by TLR9, RNase H and cGAS. Longer hybrids (cDNA –RNA template) are produced during the reverse transcription of lentiviruses and endogenous retroviruses and are sensed by cyclic GMP-AMP synthase (cGAS) (Mankan et al. 2014). Shorter hybrids arising upon

RNase H deficiency from RNA transcripts and DNA template are detected by TLR9 (Rigby et al. 2014).

RNA patterns

Cellular RNA modifications

Although the generation of cellular RNA occurs in the nucleus and mitochondria, they are most active in the cytosol. And this is where most RNA sensors function. Produced by three types of RNA polymerases (Pol I, Pol II and Pol III), cellular RNA products then undergo a number of modifications at 5' and 3' ends, which facilitate their transport to the cytoplasm. Pol I synthesizes a 5'-triphosphate(PPP) RNA, which is then cleaved to form three types of rRNA (28S, 18S and 8S) with 5'-monophosphates (5'P). Pol II produces mRNA which undergoes capping, or in other words the addition of guanosine nucleotide to the 5'-PPP. These capped transcripts are then methylated to produce CAP0, CAP1 or CAP2 structures. Pol III also produces 5'PPP-transcripts which are then cleaved and methylated to produce 5'P tRNA, 5s rRNA and U6 snRNA.

There are also internal nucleotide modifications of RNA, where a major role is played by RNA-modifying enzyme ADAR1. It modifies dsRNA by converting adenosine to inosine by deamination. It was shown that ADAR1 destabilizes stem-loop structure on the 3'UTR of some mRNAs and thereby prevents excess stimulation of IFN responses (Hartner et al. 2009; George, John, and Samuel 2014; Levanon et al. 2004; Mannion et al. 2015). In addition, the lack of ADAR1 leads to embryonic lethality because of excess dsRNA production (Rice et al. 2012).

Another example of internal RNA modification is the 2'O methylation of tRNA at residues 18 and 34, which decreases the immunogenicity of tRNA (Kaiser et al. 2014; Gehrig et al. 2012; Jöckel et al. 2012).

Overall, RNA biogenesis in the cell involves several modification steps, which prevent the recognition of endogenous RNAs.

Viral RNA modifications

On the other hand, most viral RNAs are produced by simple replication machineries. Therefore, they undergo only partial processing. A well-known

cytoplasmic RNA sensor retinoic acid-inducible gene-I (RIG-I, encoded by DDX58 gene) recognizes uncapped 5'-PPP-RNA (Hornung et al. 2006) (Pichlmair et al. 2006). Many negative strand (-) ssRNA viruses, such as orthomyxo-, paramyxo- and bunyaviruses) produce 5'PPP-RNA as genomic or short subgenomic RNAs (Habjan and Pichlmair 2015; Goubau, Deddouche, and Reis e Sousa 2013; Pichlmair et al. 2009). Viruses encode proteins that help to escape the recognition of such RNAs, for example NS1 protein of Influenza A virus (IAV) and V protein of measles (Versteeg et al. 2010; García-Sastre 2010). Also, some viruses such as Bornaviruses or Bunyaviruses employ 5' trimming strategy during replication, upon which terminal nucleotides are cleaved by viral nucleases (U. Schneider et al. 2007; Habjan et al. 2008)(Schneider, 2007; Habjan 2008). In calciviruses and picornaviruses, covalently bound VPg proteins mask the 5' end (Lee et al. 1977; Nomoto, Detjen, et al. 1977; Nomoto, Kitamura, et al. 1977). Finally, many viruses (eg flavi-, corona-, pox- and reoviruses) encode their own capping enzymes and methyltransferases (Decroly, Ferron, et al. 2011; Decroly, Debarnot, et al. 2011). All this indicates a strong selective pressure for developing shielding strategies. Indeed, it was demonstrated that capped transcripts lacking 2'O methylation are sensed by the immune receptors (Daffis et al. 2010; Habjan et al. 2013) (Schuberth-Wagner et al. 2015). Interestingly, some viruses also developed a cap-snatching strategy, whereby first 10-13 nucleotides of cellular mRNA are fused to the 5' end of viral RNA (Koppstein, Ashour, and Bartel 2015) (Dias et al. 2009). Overall, structural RNA patterns suggest that rather than the recognition of pathogen-specific 'non-self', sensing of the 'missing-self' is key in RNA sensing in mammals. This enables rapid response against a diverse range of viruses, although it can lead to autoimmune response in some cases.

Another structural hallmark of viral RNAs is the double-strandedness. Viral dsRNAs may arise as genomes of dsRNA viruses, replication byproducts of ssRNA viruses and the products of convergent transcription of DNA viruses. Moreover, ds regions of ssRNA forming stem loops and 'panhandles', can be recognized (Schlee et al. 2009; Jie Xu et al. 2015; Moy et al. 2014). Some of these structures are also abundant among cellular RNAs, with a risk of activating autoimmunity.

Availability and control mechanisms

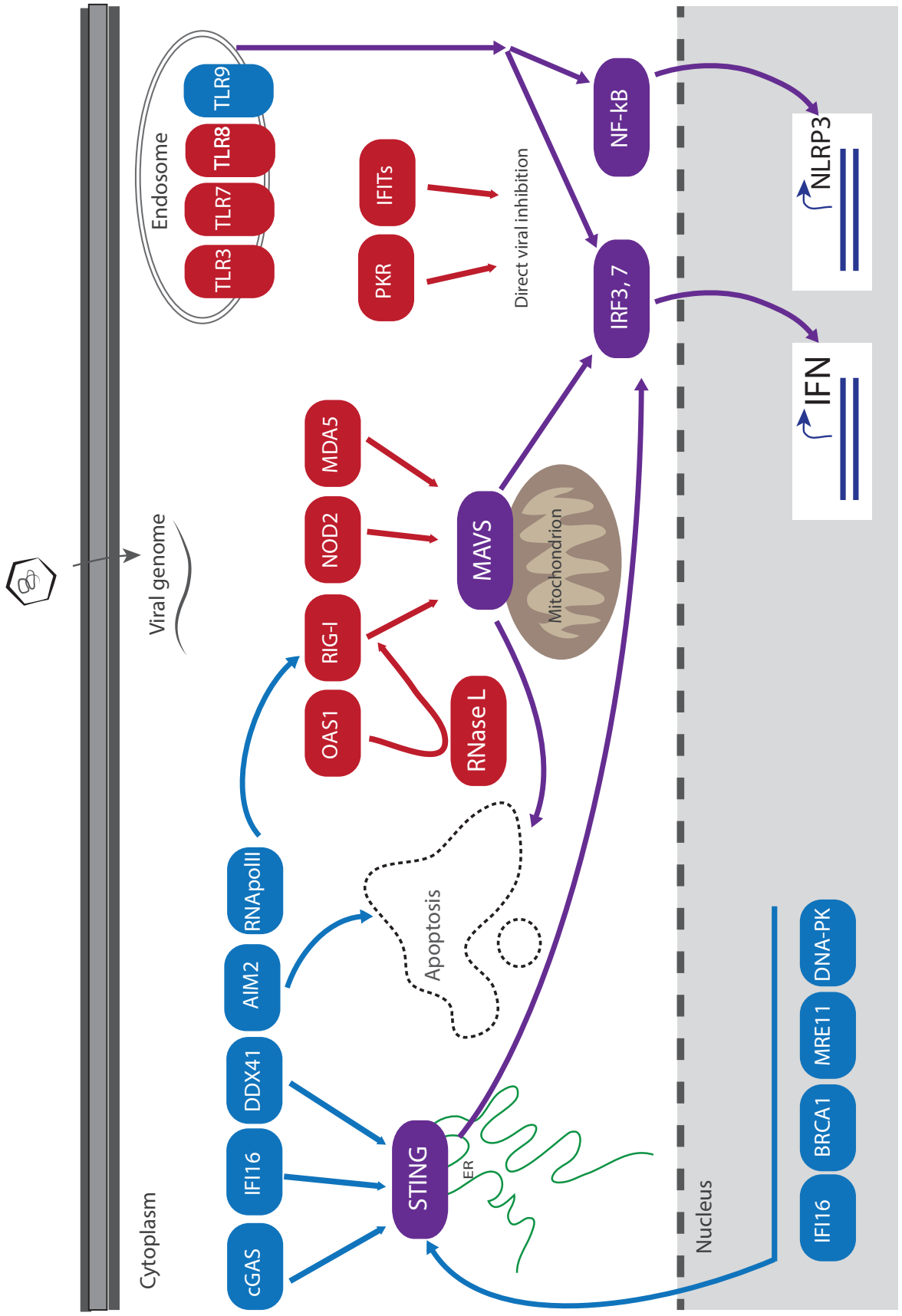


Figure 2. Viral NA sensing in mammals. A number of viral NA sensors are activated upon viral entry. RNA sensors (in red) either activate MAVS signaling (the case of RIG-I, NOD2 and MDA5) which leads to IFN response, or direcly inhibit viral activity (PKR; IFIT; and RNase L activated by OAS pathway). DNA sensors (in blue) may lead to apoptosis (AIM2), activation of RNA sensor RIG-I (RNApolIII), or act through STING pathway leading to IFN response. Endosomal sensors from TLR family also lead to IFN activation and also to the expression of NLRP3 leading to IL-1 production.

Although having specific signatures and localization, some of viral NA products still highly resemble endogenous NAs, raising the question of additional mechanisms making viral NA immunogenic and distinguishable by immune receptors. One such mechanism may be the threshold of abundance of certain NAs. On one hand, it results from the oversupply of the NA ligand, as illustrated in the case of dsRNA sensing. Although cellular dsRNA also is present in the cytosol, upon viral infection the concentration of dsRNA dramatically increases. Furthermore, the increase of dsRNA upon infection with modified vaccinia Ankara (MVA) virus increases immunogenicity (Wolferstätter et al. 2014)(Wolferstatter 2014). On another hand, it may result from the defects in degradation machinery. For example, the lack of TREX1 leads to the accumulation of NA substrate with is detected by cGAS and activates downstream immune pathways. Significantly, TREX1 mutation is a hallmark of many autoimmune disorders (Aicardi-Goutieres syndrome, familial chilblain lupus, systemic lupus erythematosus) (Crow et al. 2006; Lee-Kirsch et al. 2007; Jöckel et al. 2012). Therefore, the recognition of NAs and the activity of NA sensors should be tightly controlled.

Overview of main sensors and effector mechanisms

The overview of viral NA sensors is illustrated on Figure 2. They can be divided into two main groups. The first group encompasses the PRRs that induce transcription factors such as NF-kB and IRF3, which in turn activate the expression of antiviral effector molecules, chemokines and cytokines, dominated by type I IFN signaling. These PRRs are Toll-like receptor family (TLR3, TLR7, TLR8, TLR9), RLRs (RIG-I and MDA5) and DNA sensors (cGAS and AIM2). The second group of proteins possesses direct antiviral activity. They include the dsRNA-activated protein kinase R, 2'-5'-oligoadenylate synthase 1 (OAS1) and IFIT proteins. The majority of RNA sensors then activate MAVS-mediated signaling leading to type I IFN response and apoptosis, whereas the DNA sensors act through STING pathway, which also primarily activates type I IFN response. TLRs in the endosomes activate two major pathways: type I IFN and IL-1 responses (reviewed in (Schlee and Hartmann 2016; Gebhardt, Laudenbach, and Pichlmair 2017) and others).

Fruit flies, as a model for studying antiviral immunity*

*largely based on previously published review (Mussabekova, Daeffler, and Imler 2017)

The fruit fly *Drosophila melanogaster* has been a favorite model organism for biologists since the beginning of the 20th century. Its rapid generation time, small size and the genetic tools created by the pioneering fly geneticists in the 1910s provided a unique model to investigate animal biology during the pre-genome editing era. As a result, studies in *Drosophila* led to many critical advances in developmental biology and neuroscience (Bellen and Yamamoto 2015). Since the 1990s, *Drosophila* has also been used as a model to investigate innate immunity, which contributed greatly to the renewed interest in this field (Hoffmann et al. 1999). Innate immunity is the first line of defense that multicellular organisms deploy to limit pathogen infections. In vertebrates, the innate response also regulates the production of cytokines and co-stimulatory molecules, which shape the subsequent adaptive immune response (Medzhitov and Janeway 2002).

Studies on innate immunity in *Drosophila* initially focused on bacterial and fungal infections, and revealed that the production of antimicrobial peptides (AMPs) plays an important role in the control of infections (Imler and Bulet 2005). Expression of AMPs is controlled by the evolutionarily conserved signaling pathways Toll and IMD (immune deficiency), which regulate the activity of transcription factors of the NF-κB family (Hoffmann 2003; Hultmark 2003). Proteolytic cascades involving sequential activation of serine proteases participate in the activation of the Toll pathway, and in the clotting and melanization responses to wounding (Veillard, Troxler, and Reichhart 2016; Theopold, Krautz, and Dushay 2014). Cellular responses involving both circulating and sessile hemocytes also participate in antimicrobial host defense in flies. In particular via phagocytosis of bacteria by macrophage-like plasmatocytes and encapsulation of parasitic wasp eggs in larvae by lamellocytes (Weavers et al. 2016; Gold and Brückner 2015; Letourneau et al. 2016).

Viruses pose major threats to all organisms, including humans, as illustrated by epidemics such as influenza or HIV. Viruses also have a very significant economic impact through their effect on crops and livestock. Of note, several arboviruses (arthropod borne viruses) are transmitted to mammalian hosts by haematophagous

insect vectors. Recent epidemics associated with these viruses (e.g. Zika virus) are driving interest in the interactions between insect hosts and viruses, which remain poorly understood. As obligate intracellular pathogens, viruses present few targets for sensing or neutralization by the immune system. In addition, viruses evolve rapidly, which makes their control by the immune system a never-ending arms race. Investigating virus-host interactions in a wide set of hosts, including insects, can therefore provide interesting insights into fundamental antiviral strategies (Marques and Imler 2016). Over the last 12 years, a number of groups have started to investigate the genetic basis of antiviral resistance in *Drosophila*. It is now well established that the cell intrinsic mechanism of RNA interference (RNAi) plays a central role in the control of viral infections in flies, as it does in plants and other invertebrates. In addition, inducible responses and restriction factors also contribute to resistance to viral infections. Excellent reviews on the field can be found elsewhere (Bronkhorst and van Rij 2014; Karlikow, Goic, and Saleh 2014; Jie Xu and Cherry 2014; Kingsolver, Huang, and Hardy 2013; Ding and Ding 2010; Lamiable and Imler 2014; Marques and Imler 2016).

RNA interference and nucleic acid-based immunity

RNA interference pathways in drosophila

The term RNA interference (RNAi) includes an array of pathways, in which small non-coding (nc)RNAs (~20- to ~30-nt long) are used to regulate gene expression. First discovered in plants and subsequently in the nematode *Caenorhabiditis elegans* as a way to silence genes with double stranded (ds)RNA, RNAi is conserved across the plant and animal kingdoms (Ratcliff 1997; Fire et al. 1998). RNAi involves the formation of an active RNA-induced silencing complex (RISC), which is formed by a small ncRNA (non-coding RNA) and a protein of the Argonaute (Ago) family. There are three major classes of small silencing RNAs in animals : microRNAs (miRNAs), short interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs). In *Drosophila*, they interact with 5 AGO proteins: Ago1-3, Piwi and Aubergine (Ding and Ding 2010).

miRNAs are involved in the post-transcriptional regulation of gene expression and participate in both developmental pathways and homeostasis. In

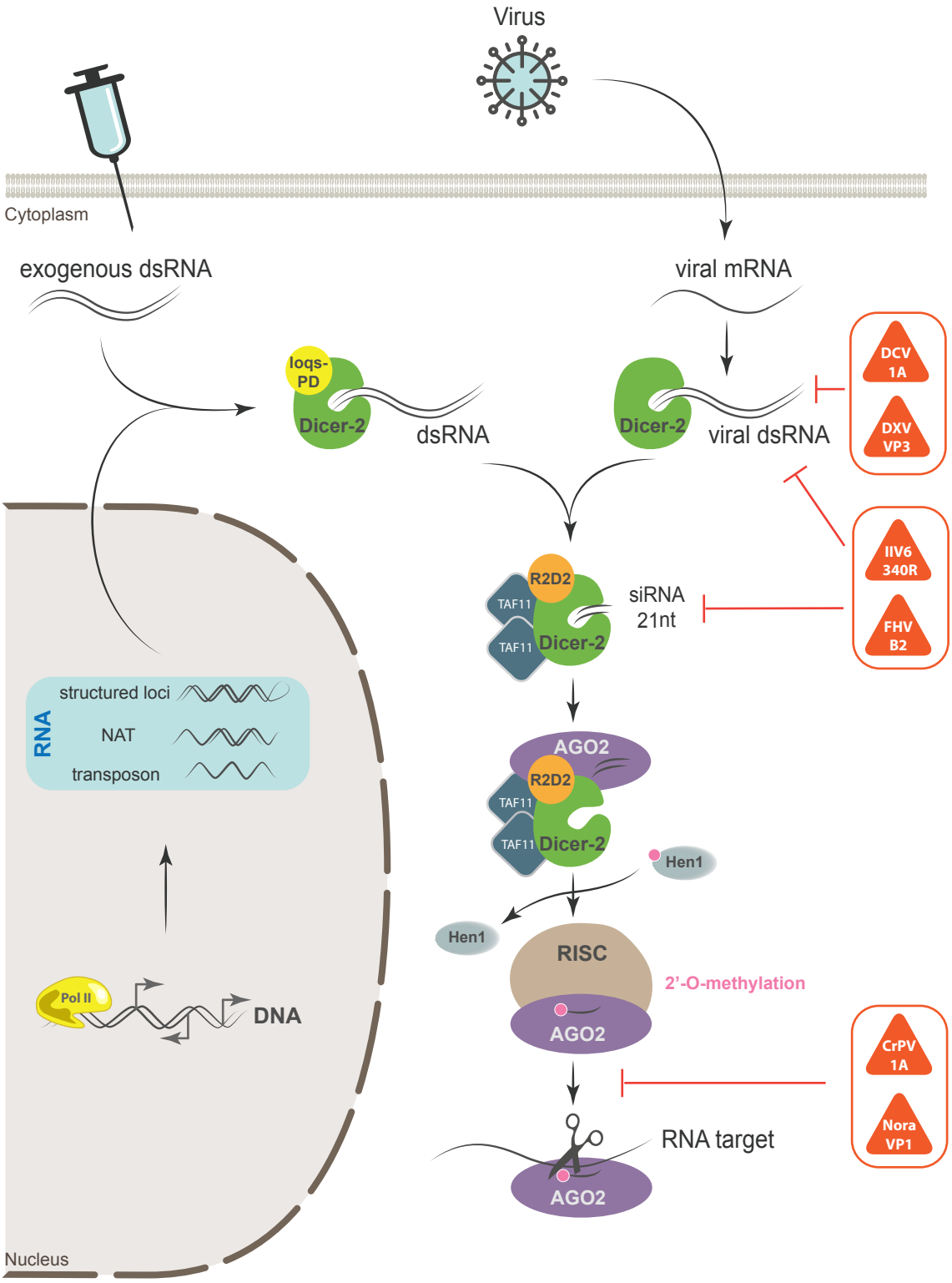


Figure 3. The siRNA pathway in Drosophila. The three core proteins of RNAi machinery (Dicer-2, R2D2 and AGO2) associate with cofactors to sense RNAs and form the RNA induced silencing complex (RISC). The siRNA pathway can be activated by the delivery of exogenous dsRNA in the cytosol or by viruses, but also by the transcription in the nucleus of natural antisense transcripts (NATs) or structured RNA. Virus-encoded suppressor molecules (in red triangles) inhibit the pathway at different steps (see the text for details).

flies, there are hundreds of miRNAs, which might provide regulation of up to half of the coding genome (Berezikov et al. 2011; Carthew, Agbu, and Giri 2016). miRNA biogenesis is a sequential process initiated in the nucleus and continuing in the cytoplasm with the help of several enzymes. Primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II and form a hairpin structure, which is processed by the nuclear RNase III enzyme Drosha and its dsRNA binding co-factor – Pasha (Landthaler, Yalcin, and Tuschl 2004). This initial processing step leads to the formation of precursor miRNAs (pre-miRNAs), which are transported into the cytoplasm. A second RNase III enzyme, Dicer-1, then processes pre-miRNAs into mature 22-nt long RNA duplexes. Again, this processing event is assisted by a dsRNA binding co-factor, the longest isoform of the Loquacious protein, Loqs-PB (Marques et al. 2009; Saito et al. 2005). Of note, the recent solving of the three dimensional structure of Drosha points to a common ancestry with Dicer enzymes (Kwon et al. 2016). The 22-nt duplex miRNA is loaded onto the Ago1 protein, to form the miRNA programmed RNA-induced silencing complex (miRISC) (Förstemann et al. 2007). Binding of miRISC to target mRNAs results in either translation inhibition (when the complementarity with the target is partial) or degradation (when the miRNA is fully complementary to its target RNA). Several DNA viruses express miRNAs to regulate expression of their genome, or the host cell genome (Skalsky and Cullen 2010). Conversely, some viruses are targeted by host-encoded miRNAs (Skalsky and Cullen 2010). The impact of miRNAs on Drosophila viruses remains poorly characterized (Hussain and Asgari 2014). The recent discovery of Kallithea virus, a drosophila DNA virus of the Nudivirus family, which expresses a highly abundant miRNA, provides an opportunity to address its function in the Drosophila model (Webster et al. 2015).

Double stranded RNA (dsRNA) from exogenous and endogenous sources can trigger a second RNAi mechanism – the siRNA pathway. dsRNA is sensed by the second member of the Dicer family in insects, Dicer-2, which processes it into 21nt duplex siRNAs (Figure 3). These are loaded onto the AGO2 protein to form a pre-RISC complex, with the help of the R2D2, a dsRNA binding co-factor of Dicer-2. One of the strands of the duplex, the passenger strand, is then ejected, leading to the formation of mature RISC complex. The remaining strand, known as the guide strand, is stabilized by 2'-O-methylation by Hen1 protein at the 3' nucleotide, and then targets the RNAs containing complementary sequence, which will be cleaved

by the AGO2 slicer activity (Ding and Ding 2010). There is strong genetic evidence that the siRNA pathway, and in particular its three core components, Dcr-2, R2D2 and AGO2, play a major role in antiviral immunity in Drosophila. This is also supported by the production of Dcr-2 dependent virus-derived siRNAs in flies or cells infected by a range of different viruses, irrespective of their type of genome (RNA or DNA) (reviewed in (Karlikow, Goic, and Saleh 2014; Bronkhorst and van Rij 2014)). Virus-derived small RNAs provide a footprint of the action of the fly immune system, giving useful information on the sensing of viral nucleic acids. For example, although immunostaining with antibodies recognizing dsRNA initially failed to detect dsRNA in cells infected by negative-strand RNA viruses (Weber et al. 2006), in the case of the negative RNA virus VSV (Vesicular Stomatitis Virus) the siRNAs profile in infected flies is consistent with viral dsRNA templates (Mueller et al. 2010; Sabin et al. 2013). Indeed, a recent study with more sensitive antibodies has confirmed the presence of dsRNA in VSV-infected cells (Son, Liang, and Lipton 2015). In the case of the DNA virus IIV6 (Invertebrate iridescent virus 6), high-throughput sequencing pinpoints the genomic region whence the dsRNA is produced (Kemp et al. 2013; Bronkhorst et al. 2012). That many insect and plant viruses express suppressors of the siRNA pathway (see below) also attests to the importance of this pathway for the control of viral infection. A major remaining question is the sensing of dsRNA by Dicer-2. Indeed, the mechanism of dsRNA sensing appears to differ depending on the source of the trigger. For example, the short isoform of Loqs, Loqs-PD, is required for the processing of endo-siRNAs or exo-siRNAs by Dicer-2 but, intriguingly, is dispensable in the case of viral RNA (Marques et al. 2009; Marques et al. 2013) (Figure 3).

The third RNAi mechanism in flies, the piRNA pathway, acts in germ cells and in some somatic tissues such as the follicular cells surrounding the ovaries (Malone et al. 2009). piRNAs are small, 24-27nt long ncRNAs, which originate from specific genomic loci, mainly containing defective transposable elements (Brennecke et al. 2007). As implied by their name, piRNAs associate with Piwi and two related proteins named Aubergine and AGO3, which form a separate clade of AGO proteins. Like siRNA, their 3' end is protected by 2'-O-methylation (H. Wang et al. 2016). The synthesis of piRNAs does not depend on Dicer proteins, but rather involves the exonuclease Zucchini and the AGO proteins from the Piwi clade themselves. piRNAs derived from viruses have been observed in mosquito cell line and, for some viruses,

in infected *Aedes* mosquitoes (Vodovar et al. 2012; Morazzani et al. 2012; Léger et al. 2013; Miesen, Girardi, and van Rij 2015; Schnettler et al. 2013). This suggests that the piRNA pathway may participate in antiviral immunity in *Drosophila*, in addition to its well-established role in genomic protection against transposable elements. However, in the case of *Drosophila*, virus-derived piRNAs have only been reported in a cell line derived from ovaries and the piRNA pathway does not appear to participate in antiviral response in flies (Wu et al. 2010; Petit et al. 2016; N. Martins et al. 2018).

Cell biology and regulation of the antiviral siRNA pathway

Besides the core components of the siRNA pathway, namely Dicer-2, R2D2 and AGO2, it is becoming apparent that other factors participate in this central antiviral pathway (Figure 3).

Indeed, loading of the siRNA duplex onto AGO2 to form a pre-RISC complex cannot occur solely in the presence of the Dicer-2/R2D2 complex. Three chaperone proteins, Hsc70, Hsp90 and Hop, are essential for pre-RISC formation, whereas two others (Droj2 and p23) further improve the efficiency of AGO2- RISC assembly (Iwasaki et al. 2015). Overall, the *in vitro* addition of the five chaperone proteins to the core siRNA pathway components reconstituted a fully active RISC complex. The function of the chaperone machinery is ATP-dependent, and ATP hydrolysis provides the energy required to accommodate the siRNA duplex onto AGO2. Hsc70 and Hsp90 also extend the dwell time of the Dicer-2/R2D2/siRNA complex on AGO2, therefore enabling the efficient loading of the siRNA duplex (Iwasaki et al. 2015).

A further essential component of the siRNA pathway was recently identified in an unbiased EMS mutagenesis screen. The screen monitored silencing of the gene *white*, which encodes a transporter for a precursor of an eye color pigment, by a long hairpin RNA expressed from an inverted repeat transgene. In *TAF11* mutant flies, silencing of *white* is significantly reduced, as observed in *r2d2* mutant flies (C. Liang et al. 2015). In agreement with these findings, GFP expression from a recombinant viral replicon derived from FHV (Flock House Virus), which is efficiently silenced by the siRNA pathway in cells from the S2 line, is derepressed after CRISPR/Cas-mediated knock-out of the *TAF11* gene. TAF11 interacts Dicer-2, R2D2 and AGO2 and co-localizes in the cytoplasm with Dicer-2/R2D2 in cytoplasmic

foci known as D2 bodies (Nishida et al. 2013). Although TAF11 does not bind siRNAs itself, it enhances the loading of radiolabeled duplex siRNA on AGO2. Altogether, these results are consistent with a scaffold model in which four TAF11 molecules bind to and facilitate the formation of Dicer-2/R2D2 heterotetramers, which are necessary to bind duplex siRNA (C. Liang et al. 2015). The resulting RISC loading complex is localized to cytoplasmic D2 bodies.

The siRNA pathway is also regulated at the transcriptional level. The *Drosophila* forkhead box O (dFOXO) protein is the single member of the highly conserved family of FOXO transcriptional regulators present in flies (Spellberg, Marr, and Marr 2015). This transcription factor binds the *AGO2* and *Dicer-2* promoters and regulates their activity. Accordingly, dFOXO null flies are more susceptible to CrPV (Cricket Paralysis Virus) and FHV infection and this defect can be rescued by ectopic expression of Dicer-2.

Other genes can have an impact on the siRNA pathway, albeit indirectly. One function of Dicer-2 is to degrade stress induced tRNA fragments (Durdevic, Mobin, et al. 2013). The RNA methyl transferase, DNMT2, limits tRNAs cleavage. In flies mutant for this enzyme, tRNA fragments accumulate and divert Dicer-2 from other substrates e.g. dsRNA. This might account for the increased sensitivity of DNMT2 mutant flies to infections by RNA viruses (Durdevic, Hanna, et al. 2013).

Together, the components of the siRNA pathway exert strong selective pressure on viruses, which have developed countermeasures in the form of suppressor proteins.

Insect viruses express suppressors of RNAi

Many insect viruses, including those of *Drosophila*, encode viral suppressors of RNAi (VSRs). Some of them bind to long viral dsRNAs and prevent binding of Dcr-2. This is the case for the proteins 1A, and 340R from the DCV (Drosophila C virus), and IIV6 viruses, respectively, which contain canonical dsRNA binding domains, dsRBDs (van Rij et al. 2006; van Cleef et al. 2014; Bronkhorst et al. 2014). The proteins B2 of FHV and VP3 of the birnavirus DXV (Drosophila X virus) also bind dsRNA, through a noncanonical domain (Chao et al. 2005). In addition to long dsRNA, 340R and B2 interact with 21nt-long siRNA duplexes, thus also inhibiting efficient loading onto AGO2. Two other VSRs, 1A and VP1 from the CrPV and Nora

viruses, respectively, bind to AGO2 and interfere with its enzymatic activity (van Mierlo et al. 2014; Nayak et al. 2010; Nayak et al. 2018) (Figure 3).

The importance of the VSRs has been particularly well illustrated in the case of FHV. Nodaviruses have small bipartite RNA genomes that are easy to manipulate genetically. RNA1 encodes the replicase, whereas RNA2 encodes the capsid proteins. A third RNA transcript, RNA3, is also produced from RNA1 and encodes the VSR B2. Whereas the wildtype FHV is highly pathogenic upon injection into the body cavity of the fly, viral mutants unable to express B2 are completely attenuated. As expected, the virus regains virulence when injected into *Dcr-2* or *AGO2* mutant flies (Han et al. 2011; Petrillo et al. 2013; N. Martins et al. 2018). Interestingly however, even in the absence of a functional siRNA pathway, the B2 deficient virus exhibits reduced virulence compared to wildtype FHV. Indeed, a second function of B2 is to bind double stranded regions of RNA2 and prevents this RNA being recruited into subcellular structures where its translation would be repressed. As a result, translation of the capsid protein is impaired in the absence of B2, even in RNAi deficient cells (Petrillo et al. 2013).

The intimate relationship between some viral suppressors and components of the siRNA pathway (e.g. the VP1 suppressor of Nora virus and AGO2) exerts a strong selective pressure on the host genes to escape targeting. As a consequence, *AGO2*, *r2d2* and *Dcr-2* are among the fastest evolving genes in *Drosophila* (Obbard et al. 2006). Thus, viral suppressors can have host-specific activities. For example, the VP1 protein from a divergent Nora virus isolated from *D. immigrans* interacts with and suppresses *D. immigrans* AGO2, but not *D. melanogaster* AGO2 (van Mierlo et al. 2014). This provides an excellent example for the co-evolution of the host RNAi machinery and viral suppression mechanisms.

From a cell intrinsic resistance mechanism to systemic immunity?

The host defense against infection engages both cell intrinsic mechanisms and systemic responses. The latter involve communication of immune signals, which alert uninfected cells and amplify the host response. In plants and the worm *Caenorhabditis elegans*, antiviral RNAi includes a systemic component that relies on the spreading of siRNAs (Ding and Ding 2010). This spreading can initiate the production of secondary siRNAs in non-infected cells, upon synthesis of dsRNA by

host encoded RNA-dependent RNA polymerases (RdRPs). In *C. elegans*, transmembrane transporters also participate in a systemic RNAi response. Genes homologous to plant and nematodes RdRP that could trigger systemic RNAi responses have not been identified in insects. Accordingly, RNAi is cell autonomous in *Drosophila* (Roignant et al. 2003), and exposing flies to a low dose of DCV, which will trigger RNAi in infected cells, is not sufficient to induce protection against a challenge with a higher dose of virus (Longdon et al. 2013). However, dsRNA released by infected cells may trigger RNAi in distant cells, upon internalization by the dsRNA uptake pathway (Saleh et al. 2009; Saleh et al. 2006). In addition, viral RNA can be reverse transcribed into DNA in *Drosophila* and transcripts produced by this DNA have been proposed to be processed by the siRNA pathway to inhibit viral replication (Goic et al. 2013; Mondotte et al. 2018). Nanotubes-like structures may also be used to transfer dsRNA and components of the RNAi machinery between cells (Karlikow et al. 2016).

Inducible responses to viral infection in *Drosophila*

Evolutionarily conserved innate immune pathways and antiviral immunity

In *Drosophila*, bacterial and fungal infections activate the Toll and IMD pathways, which contribute to the resolution of the infection. Fungi and most Gram-positive bacteria activate the Toll pathway, in which the kinase Pelle phosphorylates the IκB protein Cactus. This triggers its polyubiquitination and degradation by the proteasome, thus allowing the NF-κB proteins DIF and Dorsal to translocate to the nucleus and activate transcription of AMPs such as Drosomycin. Toll is activated by the neurotrophin-like cytokine Spaetzle. This molecule circulates in the hemolymph as an inactive precursor and is activated by proteolytic processing. The proteolytic cascade acting upstream of Spaetzle is triggered by circulating pattern recognition receptors (PRR) sensing Lysine-type peptidoglycan (e.g. PGRP-SA) or β-glucans (e.g. GNBP3). Alternatively, the pathway can be induced by abnormal proteolytic activity in the hemolymph sensed by the serine-protease Persephone (Issa et al. 2018). Gram-negative bacteria activate the IMD pathway via their cell wall components. In particular, di-aminopimelic acid is sensed by the transmembrane PRR, PGRP-LC,

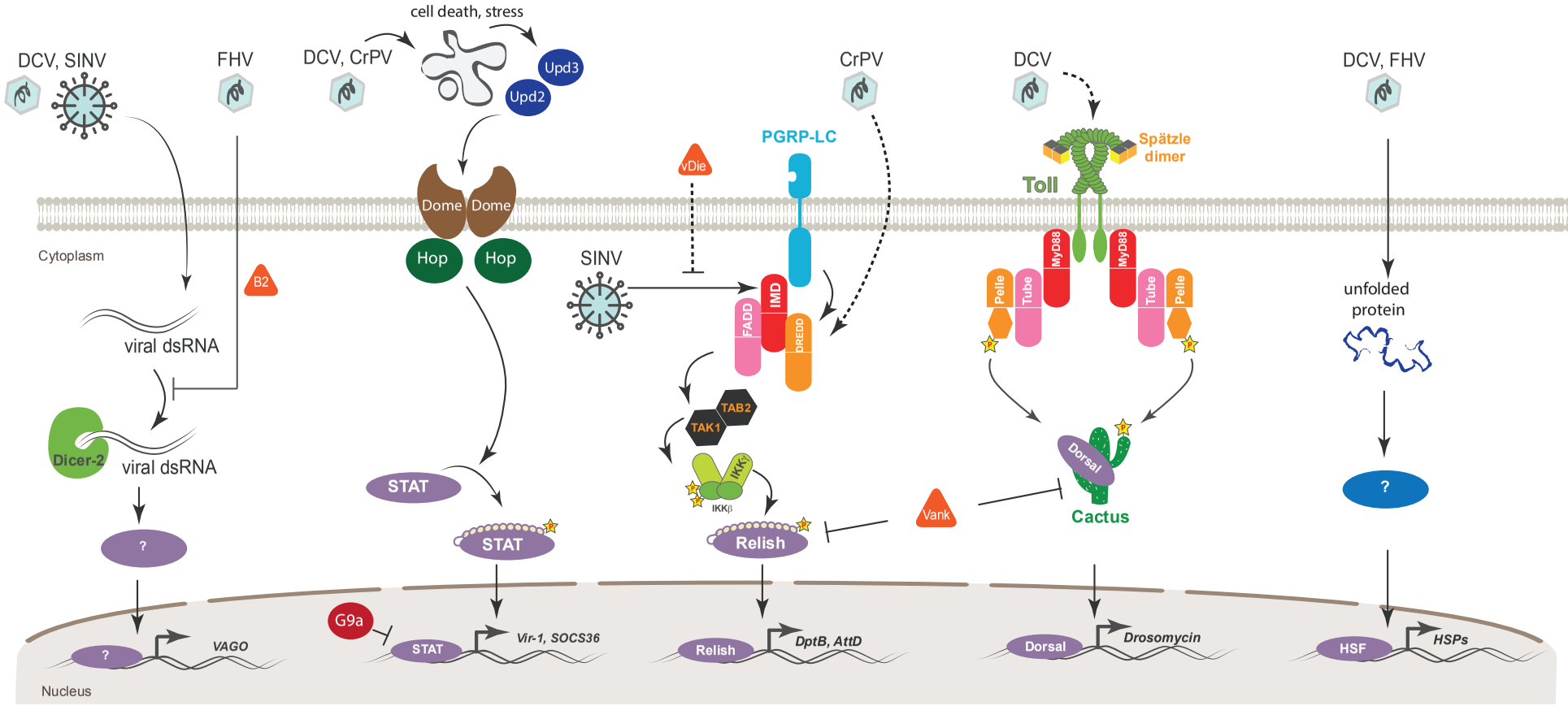


Figure 4. Induced antiviral responses in Drosophila. Examples of signalling pathways activated by viruses in flies are illustrated. Viral suppressors are indicated by red triangles. See the text for details.

which activates a cytoplasmic signaling pathway leading to phosphorylation and activation of another NF-κB protein called Relish. Upon nuclear translocation, Relish transcribes antimicrobial peptide genes (e.g. *diptericin*) (reviewed in (Kleino and Silverman 2014)). The evolutionary ancient Toll and IMD pathways share several similarities with the inflammatory pathways regulated by Toll-like receptors, the interleukin-1 receptor and the TNF receptor in mammals. Activation of the Jak/STAT pathway represents another hallmark of the response to septic injury in *Drosophila*. The *Drosophila* genome encodes a single JAK kinase (Hopscotch) and a single STAT transcription factor (STAT92E), which respond to activation of the gp130-like cytokine receptor Domeless by three cytokines of the Unpaired (UPD) family (Zeidler and Bausek 2013). As described below, the Toll, IMD and Jak/STAT pathways have been proposed to play a role in antiviral immunity (Figure 4).

The Jak/STAT pathway participates in the control of infection by Dicistroviruses and IIV-6

Some 130 genes are upregulated by a factor of at least two in response to DCV infection (Dostert et al. 2005). Characterization of the promoter of a strongly induced gene, *vir-1*, revealed the importance of DNA motifs corresponding to binding sites for the transcription factor STAT92E. Several DCV-induced genes share this property and, like *vir-1*, were no longer induced in flies either mutant for *hopscotch* or expressing a dominant-negative version of Domeless. *Hopscotch* mutant flies have increased viral load and succumb more rapidly than controls to DCV infection, indicating that at least some of the induced genes participate in the control of the infection (Dostert et al. 2005). Like inflammation in mammals, induction of innate immunity pathways is associated with toxicity. This is best illustrated by the phenotype of flies mutant for the histone H3 lysine 9 (H3K9) methyltransferase G9a. These flies are highly susceptible to infection by RNA viruses, including DCV, and lethality is associated with hyperactivation of the Jak/STAT pathway (Merkling, Bronkhorst, et al. 2015). Thus, the epigenetic regulator G9a mediates tolerance to virus infection in *Drosophila* by modulating activation of the Jak/STAT pathway. Importantly, even though *vir-1* and other Jak/STAT regulated genes such as members of the *Turandot* (*Tot*) family are induced by several RNA viruses, *hopscotch* mutant flies appear to be uniquely

susceptible to viruses of the *Dicistroviridae* family (e.g. DCV, CrPV) (Kemp et al. 2013). Probably some aspects of the Jak/STAT-dependent inducible response may be redundant with additional defenses for viruses other than *Dicistroviridae* in flies.

Recently it was reported, that the DNA virus IIV-6 also activates Jak/STAT signaling through induction of p38b-dependent pathway (West and Silverman 2018). This pathway leads to the activation of Turandot proteins and requires the production of reactive oxygen species.

Contribution of the NF-κB family transcription factors Dorsal, DIF and Relish to antiviral immunity.

The Toll pathway has been proposed to play a role in antiviral immunity. An initial report indicated that two mutants of the Toll pathway, affecting the genes *Dif* and *Toll*, die more rapidly than controls following infection by Drosophila X virus (DXV), which belongs to the *Birnaviridae* family (Zambon et al. 2005). Intriguingly, the mutation in *Dif* was a loss of function, whereas the mutation in *Toll* was a gain of function, resulting in a constitutively activated pathway. A possible explanation for this finding could be that, as described above, immunopathology caused by activation of innate immunity pathways in *Drosophila* contributes to the lethality. Indeed, Dif^{1} mutant flies exhibited a significant increase in DXV titer, in agreement with a role of DIF in the activation of an antiviral program, whereas the viral titer was slightly reduced in $Toll^{10b}$ mutant flies. Of note, loss of function mutations in other genes of the pathway (*spaetzle*, *Toll*, *pelle*, *tube*) did not show a phenotype following DXV infection, suggesting that DIF regulates antiviral activity by a non-classical mechanism in this context. An independent study recently reported that the genes *spaetzle*, *Toll*, *pelle* and *dorsal* are required for resistance to oral infection by several RNA viruses (DCV, CrPV, FHV and Nora virus) (Ferreira et al. 2014). Interestingly, although the Toll pathway is activated in the fat body upon oral infection or by direct injection of DCV into the hemocele, only the oral infection route gave a phenotype. This suggests that the antiviral action of the Toll pathway targets a step of the viral cycle specific of the oral infection route, which is bypassed when the virus is directly injected in the body cavity. Of note, polydnaviruses, which are mutualists of parasitic wasps, express vankyrin (vank) genes that encode inhibitors of Dorsal and Dif. Two vankyrins from *Microplitis demolitor* Bracovirus

also bind to and inhibit Relish (Bitra, Suderman, and Strand 2012; Gueguen et al. 2013).

Two studies initially reported that flies mutant for several genes encoding components of the IMD pathway were more susceptible than controls to infection by CrPV or SINV (Avadhanula et al. 2009; Costa et al. 2009). A follow-up study by Hardy and co-workers identified 95 genes differentially expressed in transgenic flies expressing a SINV replicon (Z. Huang et al. 2013). Analysis of the upstream regions of these genes revealed the presence of Rel and STAT binding sites in most of them and the presence of these binding sites correlated with a decreased expression in flies heterozygotes for Relish and STAT92E. Among the genes tested, silencing of *diptericin B* (Relish-dependent) and *attacin C* (STAT-dependent) resulted in increased viral load. Thus, these two antimicrobial peptides appear to participate in the control of SINV infection in *Drosophila*. A completely independent set of experiments recently confirmed that the IMD pathway is involved in antiviral immunity (Lamiable, Kellenberger, et al. 2016). These experiments investigated the function of the gene *Diedel*, which is strongly upregulated by some viruses, including SINV. *Diedel* mutant flies have reduced viability, and succumb more rapidly than controls when infected by SINV, but not the unrelated virus Vesicular Stomatitis Virus (VSV). This increased lethality does not result from uncontrolled viral replication, but rather from over-activation of the IMD pathway. Indeed, a large number of IMD regulated genes are up-regulated in *die* mutant flies, and viability is rescued in flies double mutant for *die* and *imd* or *ikkγ* , two genes encoding key components of the IMD pathway (Lamiable, Kellenberger, et al. 2016). Thus, *die*, which encodes a circulating 12 kDa protein, mediates tolerance to SINV infection by down-regulating activation of the IMD pathway. Most interestingly, homologues of *die* are found in the genome of several insect DNA viruses (e.g. Spodoptera frugiperda Ascovirus 1a), and one of them can partially rescue the phenotype of *die* mutant flies. The hijacking of a negative regulator of the IMD pathway by DNA viruses emphasizes the relevance of this pathway for the control of viral infections (Lamiable, Kellenberger, et al. 2016). Indeed, analysis of the 12 canonical components of the pathway in the S2 cell line revealed that two of them, the kinase Ikkβ and the NF-kB transcription factor Relish, mediate resistance to DCV and CrPV. Further *ex vivo* and *in vivo* experiments revealed that these two factors participate in a novel antiviral pathway, which also involves the orthologue of STING, an

important component of the DNA sensing pathway in mammals (Goto et al. 2018; Y. Liu et al. 2018).

In summary, the genetic data now at hand point to an involvement of the three main evolutionarily conserved "inflammatory" pathways in the control of viral infections in flies. In addition, profiling of the transcriptome of infected cells and RNAi screens revealed that the heat shock pathway as well as transcriptional pausing participate in a rapid response to viral infection in *Drosophila* (Merkling, Overheul, et al. 2015; Jie Xu et al. 2012). In the intestinal epithelium, the cytokine Pvf2 can also trigger an antiviral program upon activating the PVR receptor and the ERK pathway (Jie Xu et al. 2013; Sansone et al. 2015). Altogether, these findings open interesting questions on the nature of the antiviral molecules these pathways up-regulate, and on the mechanisms by which they are activated in the context of viral infections.

Sensing viral infection to trigger inducible responses

How viral infections are sensed in *Drosophila* remains largely unknown. In mammals, several innate immunity receptors sense viral nucleic acids and trigger the interferon response (reviewed in (Goubau, Deddouche, and Reis e Sousa 2013)). The only known sensor for viral RNA in flies is Dicer-2, which triggers RNA interference. Interestingly, Dicer-2 can also trigger an inducible response. Indeed, induction of the gene *Vago* is abolished in flies mutant for Dicer-2 or expressing FHV-B2, a VSR that competes with Dicer-2 for the binding to dsRNA (Deddouche et al. 2008). The induction of an orthologue of *Vago* in *Culex* mosquitoes following infection by West Nile Virus also depends on Dicer-2 (Paradkar et al. 2012). Thus, this cytosolic sensor for viral RNA, which shares an evolutionarily conserved duplex RNA activated ATPase domain with RIG-I like receptors can trigger two types of responses in *Drosophila*, RNAi and induced expression of molecules associated with antiviral immunity (Paro, Imler, and Meignin 2015). The signaling pathway connecting Dicer-2 to induction of Vago has not yet been characterized in flies. In *Culex* however, the Relish ortholog REL2 and a TRAF factor have been associated with the upregulation of CxVago (Paradkar et al. 2014). Thus, at least one component of the IMD pathway, the transcription factor REL2, can be activated by a pathway activated upon sensing dsRNA by Dicer-2 in *Culex* mosquitoes.

Excess DNA in the cytosol of *Drosophila* cells can also activate an immune response, suggesting that the sensing of viral DNA could contribute to antiviral immunity. Indeed, mutation of the gene encoding the lysosomal enzyme DNAseII results in the constitutive expression of the IMD-regulated AMPs Diptericin and Attacin A, whereas the Toll-regulated AMP Drosomycin is not affected (Mukae et al. 2002). This suggests that the IMD pathway can be activated upon sensing cytosolic DNA. Although the sensor for DNA remains unknown, this pathway involves the serine phosphatase Eya, which can associate with the kinase IKKβ and the transcription factor Relish (X. Liu et al. 2012). The possible involvement of this pathway in the resistance to DNA virus infection and its connection with the new STING-dependent pathway mentioned above remain to be investigated (Goto et al. 2018).

Besides nucleic acids, other components of viral particles may be sensed by the immune system of the fly. For example, the gene *diedel* (*die*) is strongly upregulated by the enveloped viruses SINV and VSV, but the non-enveloped viruses DCV, CrPV and FHV induce little or no response. Furthermore, UV inactivation of both SINV and VSV does not impair induction of *die*. Altogether, these findings suggest that sensing of molecules from the viral envelope may trigger induction of this host cytokine (Lamiable, Kellenberger, et al. 2016). Interestingly, induction of *die* does not involve Relish, but DIF. However, the signal transducer MyD88 is not required for *die* induction, suggesting that in the context of these viral infections, DIF is not activated by the canonical Toll pathway.

Finally, some genes may be induced by the stress associated with viral infection, or in response to alterations in the host physiology. For example, infection by DCV induces a heat shock response (Merkling, Overheul, et al. 2015). This response may be activated by the accumulation of unfolded viral proteins in the cytosol of infected cells. Activation of the Toll pathway in apoptosis deficient flies may provide another mechanism for induction of immunity genes (Ming et al. 2014). As detailed below, apoptosis is an evolutionarily conserved antiviral mechanism and many DNA viruses have evolved suppressors to escape it (Clem 2015). Virus-induced necrotic death, when apoptosis is inhibited, could result in the release of damage associated molecular patterns activating the cytokine Spaetzle and the Toll pathway (Ming et al. 2014). A third example is provided by viruses such as CrPV, which inhibit the cap-dependent translation of cellular mRNAs, and may thus

promote synthesis of cap-independent mRNAs involved in stress response (Garrey et al. 2010). Finally, altered physiology of the infected host may contribute to the upregulation of immune genes. For example, DCV infects the smooth muscle cells of the crop in the anterior midgut, resulting in intestinal obstruction and a depletion of energy stores (Chtarbanova et al. 2014). This starvation-like condition can lead to activation of the transcription factor FOXO, which participates in the regulation of the expression of AMP genes (Becker et al. 2010) and, as mentioned above, Dicer-2 and AGO2(Spellberg, Marr, and Marr 2015).

Towards the functional characterization of virus-induced Drosophila genes

Only a handful of the genes identified as induced or upregulated by viral infection have been characterized functionally. Some of them do not act directly on the virus, but rather participate in the consolidation of the induced response or the homeostasis of this response. For example, the cytokines Upd2 and Upd3, which activate the Jak/STAT pathway, are induced by DCV infection (Kemp et al. 2013). The nucleoporin Nup98, which is upregulated by SINV and VSV, participates in the induced expression of a subset of putative antiviral genes, together with the transcription factor FoxK (Panda et al. 2014; Panda et al. 2015). The gene *Vago* encodes a 18kDa Cystein-rich peptide containing a single von Willebrand factor type C domain, which probably represents another cytokine, participating in the amplification of the immune response (Deddouche et al. 2008). Indeed, *Vago* restricts DCV infection in the *Drosophila* fat body. Although Vago has not been characterized further in *Drosophila*, in *Culex* mosquitoes it restricts WNV infection by activating the Jak/STAT pathway, suggesting an antiviral cytokine function (Paradkar et al. 2012). The last example is the cytokine Die, which is strongly induced by SINV and VSV and represses activation of the IMD pathway in the context of SINV infection (Lamiable, Kellenberger, et al. 2016). Overactivation of the IMD pathway is associated with deleterious effects on the host. Accordingly, *die* mutant flies succumb more rapidly than wild-type controls following SINV infection. These mutants contain viral loads similar to that of controls, but exhibit an exacerbated induction of IMD regulated genes, which reduces viability.

Other virus-induced genes encode antiviral effectors. As mentioned above, silencing the expression of two antimicrobial peptides induced by SINV infection,

Diptericin B and Attacin C, results in increased SINV replication (Z. Huang et al. 2013). Antimicrobial peptides have also been associated with antiviral activity in vector mosquitoes and mammals (Klotman and Chang 2006; Luplertlop et al. 2011). Activation of the heat shock response in flies improves the control of DCV infection and survival of the flies, indicating that this response is a constituent of antiviral innate immunity in *Drosophila* and that some of the molecules induced are associated with antiviral activity. Indeed, overexpression of the heat shock factor Hsp70 is sufficient to increase survival following DCV infection, suggesting that this factor has antiviral activity (Merkling, Overheul, et al. 2015).

In summary, we are still largely ignorant of the function of most of the host factors induced by viral immune challenge. Even for the few candidate antiviral molecules that have been identified, we have limited understanding of their mode of action. The identification of pathways associated with antiviral resistance (e.g. Toll, IMD, Jak/STAT, transcriptional pausing) by genetic screens opens the way to the characterization of the transcriptome of infected flies mutant for these pathways. This will narrow down the number of candidate antiviral molecules to test functionally. Such approach was successfully used in *Aedes* mosquitoes, where the investigation of 18 genes regulated by the Jak/STAT pathway and induced by Dengue virus infection led to the identification of two anti-Dengue factors (Souza-Neto, Sim, and Dimopoulos 2009).

Induced apoptosis and phagocytosis

Apoptosis is a conserved mechanism of programmed cell death restricting viral replication and dissemination in insects. Caspases, the proteases that trigger apoptosis, are tightly regulated by members of the IAP (inhibitor of apoptosis protein) family (e.g. dIAP1 in *Drosophila*), which are themselves controlled by antagonist proteins (encoded in *Drosophila* by the RHG genes: *reaper*, *hid*, *grim* and *sickle*). Apoptosis can be triggered as a result of depletion of the labile protein dIAP1 when host translation shuts down as a result of viral infection (Settles and Friesen 2008). Alternatively, viral infection can trigger expression of pro-apoptotic RHG genes, following activation of the transcription factor p53 (B. Liu et al. 2013). Programmed cell death can stop the infection before viral replication is completed. Additionally, apoptosis may promote clearance of infected cells by phagocytes, thus

preventing dissemination. Clearly, both the presence hemocytes (blood cells) and active phagocytosis are required to control FHV, DCV and CrPV in infected flies (Lamiable, Arnold, et al. 2016; Nainu et al. 2015; Costa et al. 2009).

Intrinsic antiviral immunity and restriction factors in *Drosophila*

The first line of innate immune defence encountered by infectious viruses is constituted by restriction factors. These proteins are constitutively expressed in host cells already before the infection and target one, or more, steps of the viral replication cycle, resulting in a severe drop in the virus titer. Host proteins must fulfill several criteria to be considered as restriction factors. They are frequently encoded in the germ-line and antiviral activity is often their main function. Their basal expression level can be up-regulated by a viral infection although their function is often antagonized by viral polypeptides. In addition, the direct interaction between rapidly evolving viral proteins and restriction factors exerts a constant pressure on the latter, resulting in a positive selection of these host genes (Kluge, Sauter, and Kirchhoff 2015; Duggal and Emerman 2012).

Multiple mechanisms of viral restriction have been described, with some factors specifically targeting one virus or virus family, while others have a broad spectrum of activity across several viral families. In the last two decades many restriction factors and their mechanisms of action have been discovered in mammals (Harris, Hultquist, and Evans 2012; Duggal and Emerman 2012; van Montfoort, Olagnier, and Hiscott 2014; Smith, Pernstich, and Halford 2014; Simon, Bloch, and Landau 2015; Jia, Zhao, and Xiong 2015; Kluge, Sauter, and Kirchhoff 2015; Zhou 2016). Restriction factors also exist in *Drosophila,* although they remain poorly characterized. Most of them were identified while studying natural viral pathogens of *Drosophila* including the Sigma virus (DMelSV), a rhabdovirus, and the DCV dicistrovirus (Cogni et al. 2016).

The p62 ortholog Ref(2)P restricts the sigma Rhabdovirus (DMelSV)

Five loci involved in resistance to DMelSV infections referred to as *ref(1)H, ref(2)M, ref(2)P, ref(3)D and ref(3)O* were roughly mapped nearly 40 years ago by genetic analysis on the *Drosophila melanogaster* genome (Gay 1978). DMelSV is

transmitted vertically and infects natural fly populations. The infection appears relatively benign but can be easily detected since infected flies succumb following an exposure to CO_2 (Lhéritier 1958). The first and so far best characterized loci at which genetic variation affects virus multiplication in flies is *ref(2)P* which is located on the left arm of the second chromosome. The gene was cloned by P-element tagging and common permissive *ref(2)P^{o}* and restrictive *ref(2)P^{p}* alleles were identified (Contamine, Petitjean, and Ashburner 1989). In fact, *Ref(2)P* is strongly polymorphic, as revealed by 14 different protein sequences obtained from 14 sequenced fly haplotypes (Wayne, Contamine, and Kreitman 1996). Three polymorphisms located in the N-terminal PB1 (*P*hox and *B*em 1) domain contribute to the sensitive or restrictive phenotype (Dru et al. 1993; Wayne, Contamine, and Kreitman 1996). Both restrictive and permissive forms of Ref(2)P can be co-immunoprecipitated with the N and P proteins from DMelSV (Wyers et al. 1993), indicating a direct interaction. Interestingly, some mutations in the virus can overcome the restriction (Contamine 1981; Fleuriet and Periquet 1993), eventually resulting in the invasion of natural populations by variant viruses. Thus, Ref(2)P fulfills most of the criteria of a restriction factor. Surprisingly, however, flies with permissive alleles are more susceptible to DMelSV infection than those carrying a null allele. This suggests that the protein encoded by the permissive allele functions as a dominant-negative, with the virus having co-opted this protein for its replication, and the restrictive allele arisen *a posteriori* (Carré-Mlouka et al. 2007).

How does Ref(2)P affect DMelSV life cycle? This protein forms a complex with the *Drosophila* atypical protein kinase C (daPKC), which positively regulates the Toll-signalling pathway and induces the synthesis of AMPs (Avila et al. 2002; Goto et al. 2003). Activation of the Toll pathway may therefore contribute to the inhibitory effect of Ref(2)P on DMelSV. Intruigingly, Ref(2)P is the *Drosophila* ortholog of the mammalian polyubiquitin binding scaffold protein P62, (aka Sequestosome 1). P62 recognizes autophagic cargos and allows their engulfment into autophagosomes through binding to members of the Atg8/LC3 family (Ktistakis and Tooze 2016). Accordingly, Ref(2)P co-localizes with cytoplasmic protein aggregates induced by aging, reduced proteasomal or autophagic activity, and neurodegenerative diseases in humans (Nezis et al. 2008). Autophagy, which has been associated with antiviral activity in flies (Shelly et al. 2009; Lamiable, Arnold, et al. 2016) (Y. Liu and Cherry 2019) may therefore contribute to the restrictive

activity of Ref(2)P against DMelSV. In this regard, the pro- and anti-viral action of the permissive and restrictive alleles of Ref(2)P may reflect its autophagic functions (e.g. (Joubert et al. 2009; Richetta and Faure 2013; Richetta et al. 2013; Mauthe et al. 2016)). Clearly, it would be interesting to investigate further the role of autophagy in the control of Sigma virus infections as well as the contribution of *Ref(2)P* in the resistance to other rhabdoviruses (e.g. VSV).

Two other restriction factors control susceptibility to DMelSV in Drosophila

Recently, two additional restriction factors for DMelSV, CHKov1 and Ge-1, have been characterized in *Drosophila melanogaster*. The gene *Ge-1* is located on the left arm of the second chromosome. It was identified through high-resolution genetic mapping in the DMelSV refractory locus called *ref(2)M* (Gay 1978; Cao et al. 2016). The Ge-1 protein is composed of an N-terminal WD40 domain and a C-terminal region separated by a serine-rich linker region. A rare polymorphism in *Ge-1* consisting of a deletion of 26 amino acids from the linker region is associated with increased resistance to DMelSV infections in *Drosophila* (Cao et al. 2016). No cross-resistance to DCV or Drosophila A virus (DAV) could be observed. Such specificity may be expected for *bona fide* restriction factors, although proof of an interaction between Ge-1 and a rhabdovirus factor/process is still missing. Of note, silencing of the common sensitive allele of Ge-1 increases susceptibility of flies to the rhabdovirus, indicating that even this allele is endowed with some restrictive activities against DMelSV. Ge-1 is a central component of processing bodies (P-bodies). These cytoplasmic foci are composed of RNA and proteins and are involved in mRNA degradation and posttranscriptional gene regulation. In these cytoplasmic structures Ge-1 bridges Decapping protein 1 (Dcp1) and Decapping protein 2 (Dcp2), which together removes the 5' cap from mRNAs (decapping) leading to their exonuclease-dependent degradation (J. H. Yu et al. 2005; Jun Xu et al. 2006). AGO2, a key effector of the antiviral siRNA pathway has also been localized in P-bodies. However, a link between AGO2 and the *Ge-1* resistance to DMelSV could not be experimentally established (Cao et al. 2016). Alternatively, the antiviral effect of Ge-1 may be mediated by the decapping complex. Indeed, the core component of the decapping complex, Dcp2, restricts the bunyavirus RVFV in Drosophila cells (Hopkins et al. 2013). RNAi-mediated silencing of *Dcp1* led to increased DMelSV

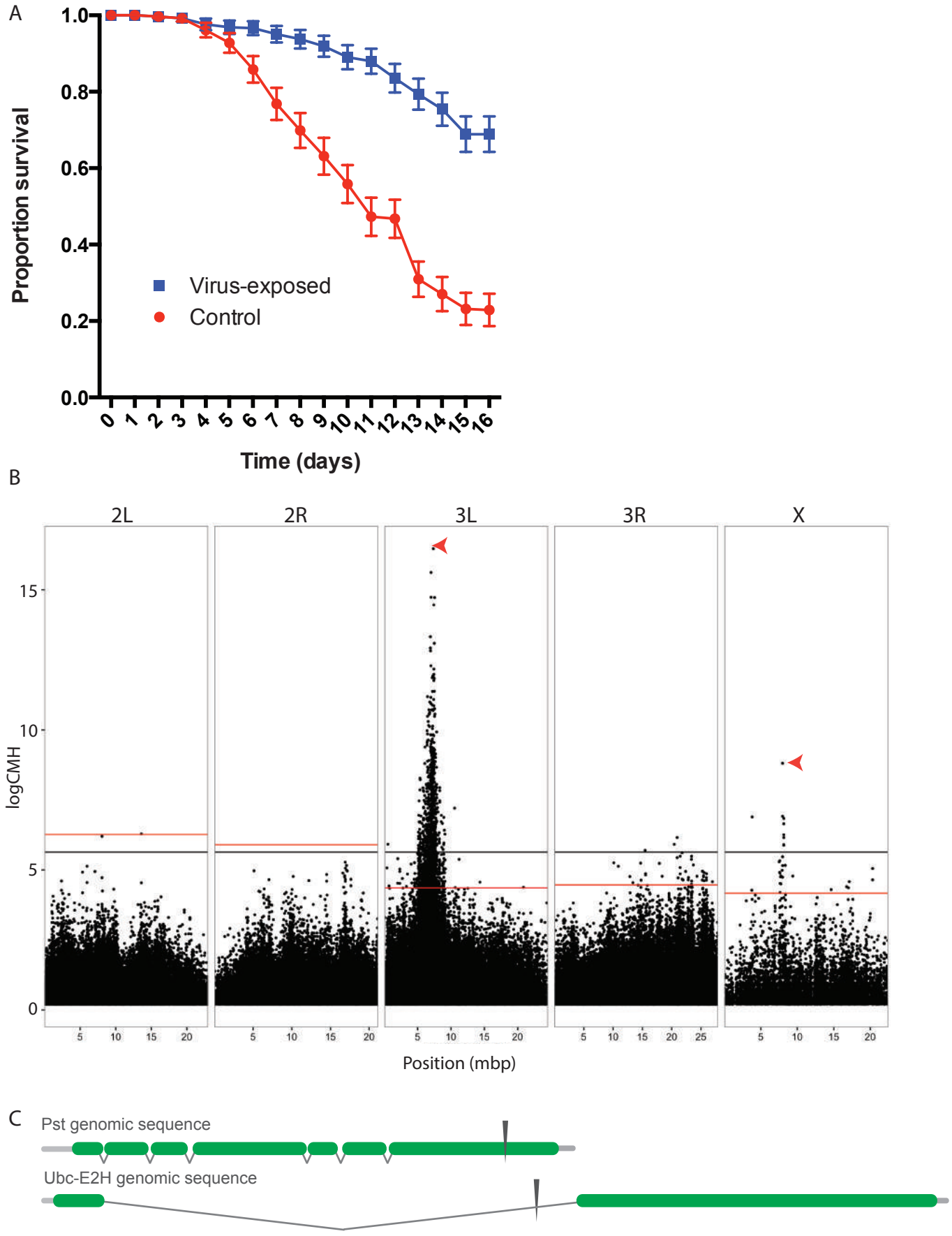


Figure 5. Adaptation of *Drosophila* to DCV highlights the importance of restriction factors. A. Flies grown for 20 generations under DCV infection show increased survival upon infection with the virus compared to controls. B. Comparison of allele frequencies between control and virus-selected populations at generation 20 identified by genome-wide sequencing. The arrowheads indicate the localization of causal polymorphisms on the left arm of chromosome 3 and on the chromosome X. CMH - Cochran–Mantel–Haenszel test (Redrawn and adapted from Martins et al. PNAS 2014;111:5938-5943) C. Schematic representation of the Pst and Ubc-E2H genes, with coding regions in green, and the location of polymorphism indicated in the exon 7 of Pst and in the intron of Ubc-E2H.

loads in flies. However, silencing *Dcp2* had no effect on DMelSV replication, suggesting that the canonical decapping complex is not involved (Cao et al. 2016).

The gene *CHKov1* was identified by linkage mapping as the locus previously called *ref(3)D* (Gay 1978). The insertion of a transposable element (*Doc*) into the coding sequence of *CHKov1* leads to the expression of shorter CHKov1 proteins that are endowed with a higher resistance capacity against DMelSV infections (Magwire et al. 2011). This genotype is the most common in natural populations of *Drosophila*. In few lines, a second allele, even more resistant against this rhabdovirus than the original *Doc* insertion in *CHKov1* was discovered in this region. It is the result of a complex rearrangement, which provides flies with one partial and two full copies of *CHKov2,* a paralog of *CHKov1*, and three full copies of the first transcript of *CHKov1* resulting from the *Doc* insertion. (Magwire et al. 2012; Magwire et al. 2011). A strong decrease in neutral genetic variation was also found in the regions surrounding the *Doc* insertion, probably reflecting a recent selection pressure exerted by the rhabdovirus on the *CHKov1* gene (Magwire et al. 2012). In summary, there is strong genetic evidence indicating that CHKov1 is a third restriction factor for DMelSV, although some criteria, such as an interaction of the polypeptide with a viral factor/process, have not yet been fulfilled. Identifying such interaction would shed light on the mechanism of action of CHKov1.

The gene Pastrel restricts DCV infections in Drosophila

Wild *Drosophila* populations were found to differ in their sensitivity to infection by another natural viral pathogen, DCV. A genome-wide association study (GWAS) identified a cluster of polymorphisms in and around a gene called *pastrel (pst)*, associated with resistance or susceptibility to DCV (Figure 5) (Magwire et al. 2012; Cao et al. 2017). *Pst* is localized on the left arm of the third chromosome. Its restrictive activity seems to be virus-specific since no significant associations with susceptibility to another positive strand RNA virus, the nodavirus FHV, or the rhabdovirus *Drosophila affinis* sigma virus (DAffSV), could be observed in flies. Notably, Pst was also found to restrict infection by another dicistrovirus, namely CrPV (N. E. Martins et al. 2014) (Cao et al. 2017). This implies that the restriction factor may target a conserved step of the replication cycle of these picorna-like viruses. The resistant allele of *pst* codes for Alanine at position 598, whereas the

susceptible variant encodes a Threonine (Figure 5). In this study, the key role of *pst* in DCV restriction was validated by functional RNAi-based analysis. These experiments revealed in addition that the *pst* sensitive allele is also endowed with antiviral effects, although to a lesser extent than its restrictive counterpart (Magwire et al. 2012). Interestingly, an independent study assessing the genetic and phenotypic changes underlying adaptation of *D. melanogaster* to DCV infections also identified the polymorphism affecting position 598 of Pst as a factor exerting major restrictive activities against this virus. (N. E. Martins et al. 2014) (Cao et al. 2017). A second locus involved in the resistance to DCV was identified by genome-wide sequencing in this study. *Ubc-2EH* is located on the X chromosome and two polymorphisms associated with resistance to DCV were identified in introns of the gene. Involvement of *Ubc-2EH* was confirmed by RNAi, and, like *pst*, it was observed to also restrict CrPV. However, *Ubc-2EH* had no impact on FHV, which belongs to a different virus family (N. E. Martins et al. 2014).

Other factors participating in intrinsic immunity in flies

Several other host factors were recently identified as limiting or restricting the multiplication of viruses in *Drosophila*. One example of such factor is the protein Rm62, the fly ortholog of the mammalian DEAD-box helicase DDX17 (Moy et al. 2014). This peptide was identified by an RNAi screen as restricting the replication of bunyaviruses including Rift Valley Fever Virus (RVFV) and La Crosse Virus (LACV), *in vivo* and *in vitro*. In contrast, Rm62 did not impact the replication of VSV, SINV or DCV. Similar results were obtained in infected human cell cultures silenced for DDX17, the human homolog of Rm62. DDX17 is a nuclear protein which participates in the processing of host pri-miRNA via interactions with stem loop structures. In infected cells, it translocates to the cytoplasm where it can interact with viral RNA. Cross-linking immunoprecipitation followed by high-throughput sequencing (CLIP-seq) revealed that DDX17 binds an essential stem loop in bunyaviral RNA, explaining its antiviral action (Moy et al. 2014). RVFV can also be restricted in Drosophila cells by dXPO1, an export receptor, and dRUVBL1, a component of the Tip60 histone acetylase complex (Yasunaga et al. 2014).

The dsRNA-specific endoribonuclease Drosha that processes pri-miRNAs in the nucleus is another example of resistance factor. This enzyme

relocalizes to the cytoplasm in *Drosophila* DL1 cells following infection by SINV, as mentioned above for Rm62 and RVFV. Drosha depletion in both *Drosophila* and mammalian cells results in increased viral RNA expression, demonstrating that the protein functions as an intrinsic antiviral factor upon relocalization to the cytoplasm (Shapiro et al. 2014). Two other nuclear proteins, dMtr4 and dZcch7, were also reported to relocalise to the cytosol upon viral infection. These proteins belong to the exosome cofactor TRAMP complex, and participate in viral RNA degradation after exiting the nucleus (Molleston et al. 2016).

In summary, only a few proteins have been identified in *D. melanogaster* as fulfilling all the criteria of classical restriction factors. This is probably due to the relative lack of interest in *Drosophila* viral diseases and the low number of natural viral pathogens identified for this species to date. The recent discovery of at least 20 new RNA and DNA viruses infecting wild *Drosophila* using metagenomics will provide opportunities to identify additional restriction factors in flies (Webster et al. 2015). Whether these will have mammalian orthologues, or not (like Pst), their functional characterization will provide powerful insights on critical steps of viral replication cycles, or components of the virus that can be targeted for intervention.

New frontiers for antiviral immunity in Drosophila

The choice of the fly *D. melanogaster* as a model to investigate innate immunity in the late 1980s, when the field was largely ignored by the majority of immunologists, was insightful. Indeed, the large range of genetic tools available for this model organism has provided important information on the functioning and regulation of innate immunity. One of the lessons from these studies is that innate immunity is not as unspecific as was formally thought. Indeed, insects and among them *Drosophila* can discriminate between different type of infectious microorganisms and mount somewhat directed responses. The study of antiviral immunity provides another striking example of the degree of specificity that can be achieved by innate immune responses. Indeed, the antiviral siRNA pathway, which relies on base pairing between 21 nucleotide long siRNAs produced by the host and viral RNAs provides a striking example of extreme specificity, and evolutionary adaptability. The studies of the past ten years in the field of *Drosophila* antiviral

immunity have revealed an array of mechanisms. Some of them have been conserved through evolution, while others have not. Until now, the *D. melanogaster* model has largely benefitted from the interest raised by the identification and characterization of evolutionarily conserved molecules and pathways. Arguably, the specificities of insect immunity are as interesting as the evolutionarily conserved aspects. Indeed, they represent species-specific innovations in host-defense. In view of the rapid evolution of microorganisms, these innovations could be enlightening for the design of new therapeutic strategies for infectious diseases.

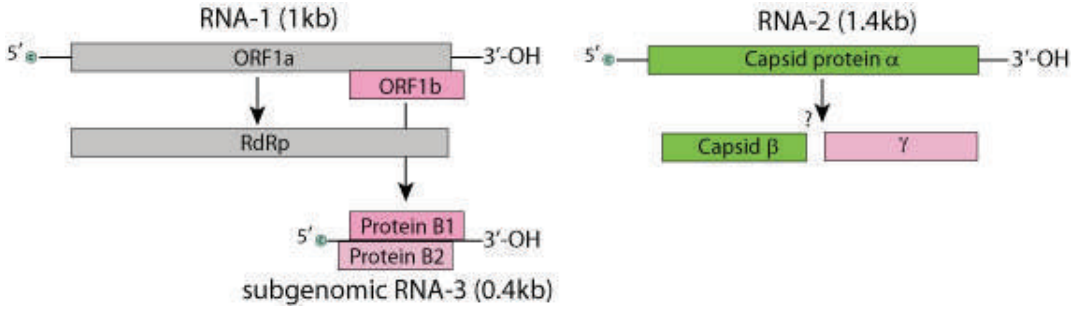
Even though the role of the siRNA pathway in the control of viral infections is well established, important questions remain. They include the mechanism of the sensing of viral RNAs, which appears to differ from the sensing of non-viral dsRNA. Identification and characterization of novel regulators of the core components of the siRNA pathway provide useful information. The exploitation of profiles of virus-derived siRNAs, identified by high throughput sequencing will certainly also provide powerful insights. Questions on the cell biology of antiviral RNAi and on cell-to-cell communication and amplification of responses are also fascinating issues that can be addressed with this model organism.

In addition to antiviral RNAi, several important questions remain regarding the induced antiviral response of *Drosophila*. We are still largely ignorant of the receptors and effector mechanisms involved, but the tools (e.g. mutant strains, differentially expressed transgenic strains, together with markers and reporters of the antiviral response) are now at hand to analyze these interactions. Restriction factors represent a third topic worthy of interest, and one can predict that the structural and functional characterization of the products of the genes that have been identified will provide important insight.

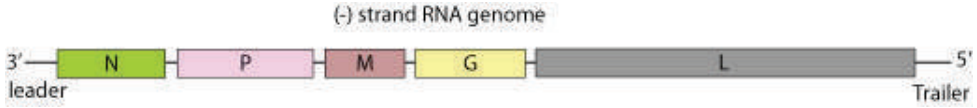
NA sensing host factors are likely to be involved in all three areas functioning either as receptors or as effectors participating in the restriction of viral infection. Identification of these factors was the aim of my PhD.

Dicistroviridae: DCV and CrPV

Nodaviridae: Flock House virus



Vesicular Stomatitis virus



Sindbis virus

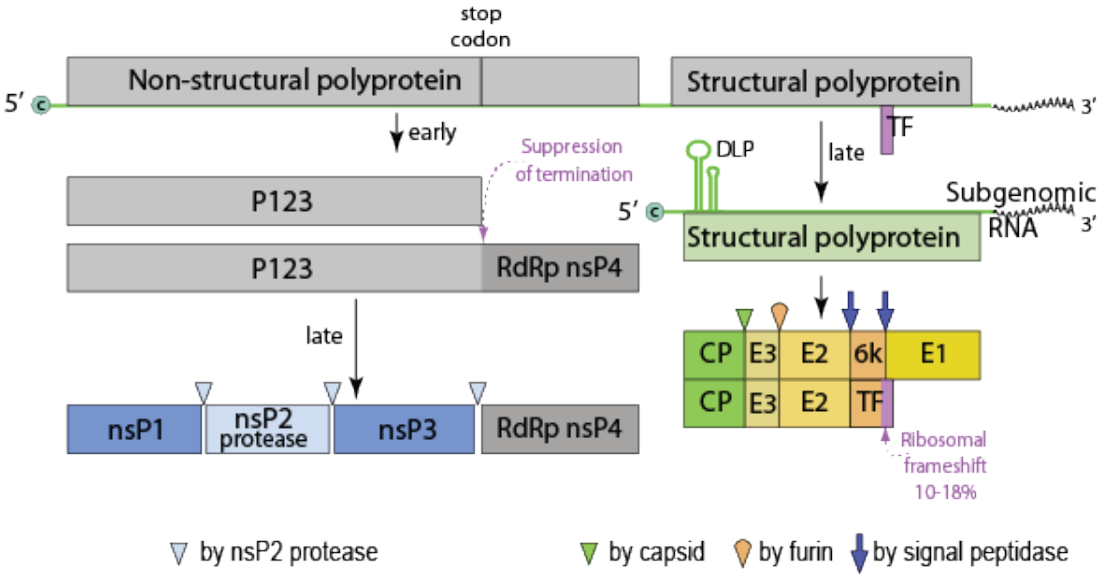


Figure 6. Schematic representation of the genomes of viruses, used in this study. All drawings were copied from Viral Zone (Swiss Institute of Bioinformatics).

Virus models used in Drosophila

Nowadays, a range of viruses is used to study antiviral immunity in Drosophila. They can be divided into three categories. Firstly, there are natural pathogens of Drosophila, only some of which are well characterized – Drosophila C virus of *Dicistroviridae* family (DCV), Nora virus (unclassified) and Sigma virus (*Rhabdoviridae*). Secondly, there are viruses that can infect other insects, such as Cricket Paralysis virus of *Dicistroviridae* (CrPV), Flock House virus of *Nodaviridae* (FHV) and Invertebrate Iridescent Virus 6 of *Iridoviridae* family (IIV-6). And finally, Drosophila has become an important model for studying mammalian arboviruses, which pose important public health concerns, for example Sindbis virus (SINV) and Vesicular stomatitis virus (VSV). These viruses do not naturally infect flies, but very successfully used to infect Drosophila flies and cells in experimental conditions. More details follow on the viruses, which were used in this PhD project with a focus on viral NA products.

Dicistroviridae family: DCV and CrPV

Drosophila C virus (DCV) was discovered in 1972 upon the observation of unusually high mortality rate of laboratory stock of flies (Jousset et al. 1972). First classified as a Picornavirus (Jousset et al. 1972), it revealed many differences with several *Picornaviridae* and led to the formation of a new virus family *Dicistroviridae* (Johnson and Christian 1998). Cricket Paralysis virus (CrPV), isolated from Australian field crickets in 1975 (Reinganum 1975) is able to efficiently infect Drosophila and also is a member of *Dicistroviridae* (X.-H. Wang et al. 2006).

Both DCV and CrPV are positive single stranded RNA ((+)ssRNA) viruses. Their genomes are polyadenylated at the 3' end and protected with a viral protein (VPg) covalently linked at the 5'end of their genome and antigenome. They contain two ORFs each preceded by internal ribosome entry site (IRES): one encoding non-structural proteins on 5' end and another encoding the capsid proteins on 3' end (Figure 6).

DCV virus enters the cell by clathrin-mediated endocytosis (Cherry and Perrimon 2004). Once in the cytosol, viral RNA accesses host ribosomes to express the two ORFs (Cherry et al. 2005). Host factor RACK1 is required to regulate the

IRES-dependent translation of DCV and CrPV polyproteins (Majzoub et al. 2014). The polyproteins are then cleaved into the structural (four VP proteins) and non-structural proteins (VSR, helicase, protease, VPg and RdRP). Once the latter are expressed, DCV replication takes place in Golgi-derived vesicles (Cherry et al. 2006). DCV-encoded viral suppressor of RNAi 1A binds dsRNA substrate and thereby prevents its recognition by Dicer-2 (van Rij et al. 2006; Fareh et al. 2018). It was recently demonstrated in single-molecule fluorescent assay that DCV 1A screens the NA substrates and distinguishes viral-derived dsRNA. It can stably bind to pre-miRNA, and to long dsRNA (>30 bp) (Fareh et al. 2018). On the other hand, VSR encoded by CrPV, which is also called 1A, acts in a different manner. Recent structural studies have shown that CrPV 1A specifically binds to AGO2 to interfere with its antiviral function, and at the same time recruits ubiquitin ligase complex to promote AGO2 degradation (Nayak et al. 2018).

Nodaviridae family: FHV

Flock House virus (FHV) was isolated from grass grub in 1983 (Scotti, Dearing, and Mossop 1983). It has a wide range of hosts, and is able to replicate in plants, insects, yeast and mammalian cells (Dasgupta et al. 2003; Dasgupta et al. 2007). FHV is a small, non-enveloped (+)ssRNA virus of *Nodaviridae* family (Venter and Schneemann 2008). The genome is bipartite: RNA1 encodes for RdRP while RNA2 encodes the precursor of viral capsid. Both of the transcripts are capped but not polyadenylated. Upon replication a subgenomic RNA (RNA3) is produced from RNA1. This transcript encodes the RNAi suppressor B2. During infection, FHV modifies mitochondrial membrane to form spherules, which are used as viral factories (Miller, Schwartz, and Ahlquist 2001; Kopek et al. 2010). Both viral ssRNA and dsRNA can be found in crude mitochondrial extracts from infected cells. dsRNA seems to be protected by the invagination of mitochondrial membrane to escape the recognition by host nucleases (Short et al. 2016).

Rhabdoviridae family: VSV

Vesicular Stomatitis virus (VSV) is one of the best-characterized viral models, infecting virtually all organisms tested. Belonging to *Rhabdoviridae* family, VSV is a (-)ssRNA virus of 11 kb genome, encoding five proteins: G protein, large protein,

phosphoprotein, matrix protein and nucleoprotein (J. Rose and Schubert 1987) (Figure 6). The bullet-shaped virion enters cells by clathrin-mediated endocytosis (Cureton et al. 2009). The genes are transcribed sequentially by RdRP, which binds at the leader region. mRNAs have fully methylated cap (CAP1) and polyadenylated (T. Ogino and Banerjee 2011). The peculiarity of nonsegmented (-)ssRNA viruses such as *Rhabdoviridae* is that the L protein also encodes for specific mRNA capping enzyme GDP polyribonucleotidyltransferase (PRNTase). The mechanism of the function of this enzyme has recently been studied in *in vitro* experiments, which suggests that it has a distinct mode of action comparing to cellular capping mechanisms (M. Ogino and Ogino 2017; T. Ogino and Banerjee 2008).

Togaviridae family: Sindbis virus

The name of the virus comes from Sindbis district near Cairo in Egypt, where it was isolated from Culex mosquitoes (TAYLOR et al. 1955). Sindbis virus (SINV) belongs to the alphavirus subfamily of *Togaviridae*. Some other alphaviruses, such as Chikungunya virus, have important clinical manifestations in humans (Jose, Snyder, and Kuhn 2009).

Alphaviruses are enveloped (+)ssRNA viruses with a genome of around 12 kb, which is capped at 5' end and polyadenyated at 3' end. They encode non-structural proteins at the 5' end and structural (capsid and two envelope) proteins at the 3' end (J. H. Strauss and Strauss 1994). NRAMP was identified as the membrane receptor for viral entry (P. P. Rose et al. 2011).

Paths to the discovery of novel viral NA sensors

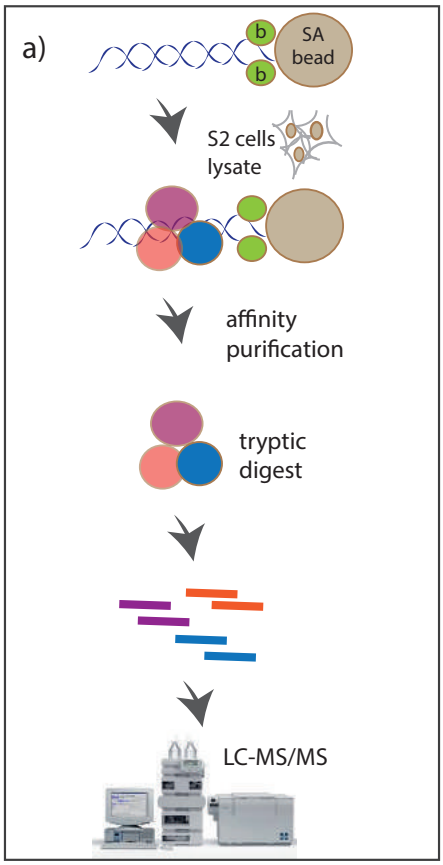
A number of viral NA recognition pathways in mammals have been discovered and discussed above. In flies however, the knowledge of viral recognition is scarce. Main antiviral mechanism RNAi involving Dicer-2 as dsRNA sensor remains most studied in *Drosophila*. Given a wide array of viruses, able to infect *Drosophila*, it is suggested that other sensors and effectors are responsible for controlling viral infection in flies.

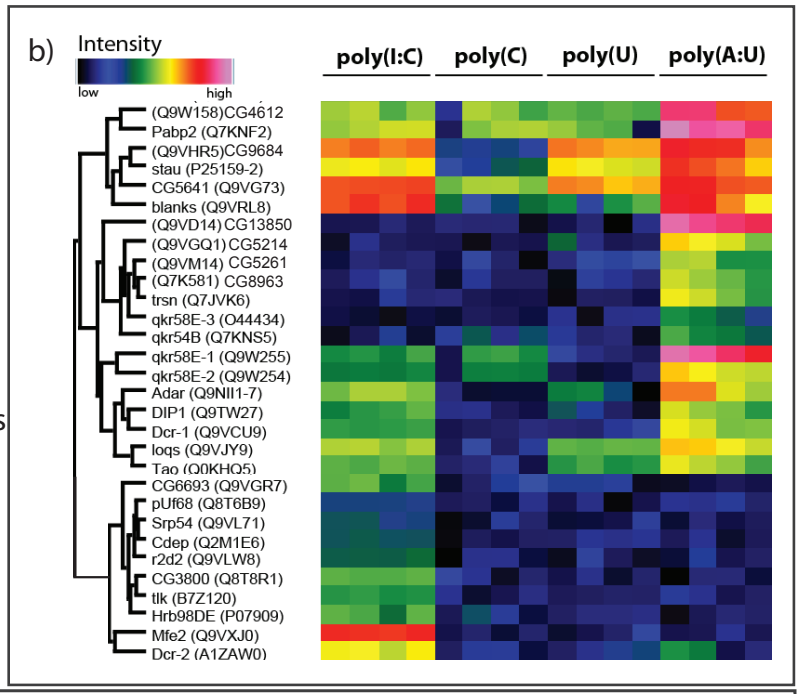
The discovery of novel recognition mechanisms can be undertaken in two directions: identification of NA ligands, binding to known receptors and identification of novel receptors binding to predicted NA ligands. In recent years, next generation sequencing was used to identify NA ligands bound to some of innate immune sensors, such as RLRs. Although the binding affinity does not always reflect the activation of the signaling cascades, it was possible to provide more insight into sequence-specificity of certain receptors. For instance, it was shown upon Measles infection, that both MDA5 and RIG-I prefer to bind to AU-rich sequences (Runge et al. 2014). MDA5, on one hand, shows preference for (+) strand, and RIG-I, on the other hand, binds preferentially to (-) strand of Measles virus. Similarly, the binding affinity of RLRs upon Chikungunya infection was studied (Sanchez David et al. 2016) showing that RIG-I, MDA5 and LGP2 bind to distinct region of viral genome.

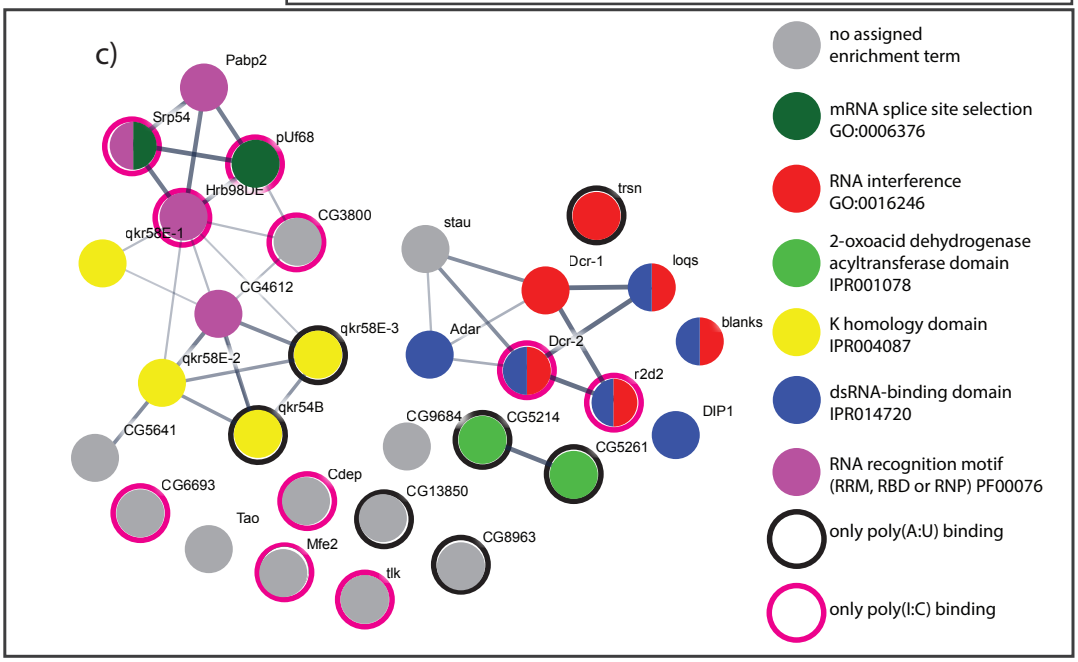
As for the discovery of novel NA sensors, there are unbiased approaches that are being used. One of the examples is the affinity-purification and mass spectrometry approach (AP-MS), using NA bait to precipitate the proteins, preferentially binding to NA ligand. This approach enabled to identify IFIT1 and -5 proteins, which bind 5'PPP-RNA (Pichlmair et al. 2011) providing proof-of-principle for the strategy behind this PhD project.

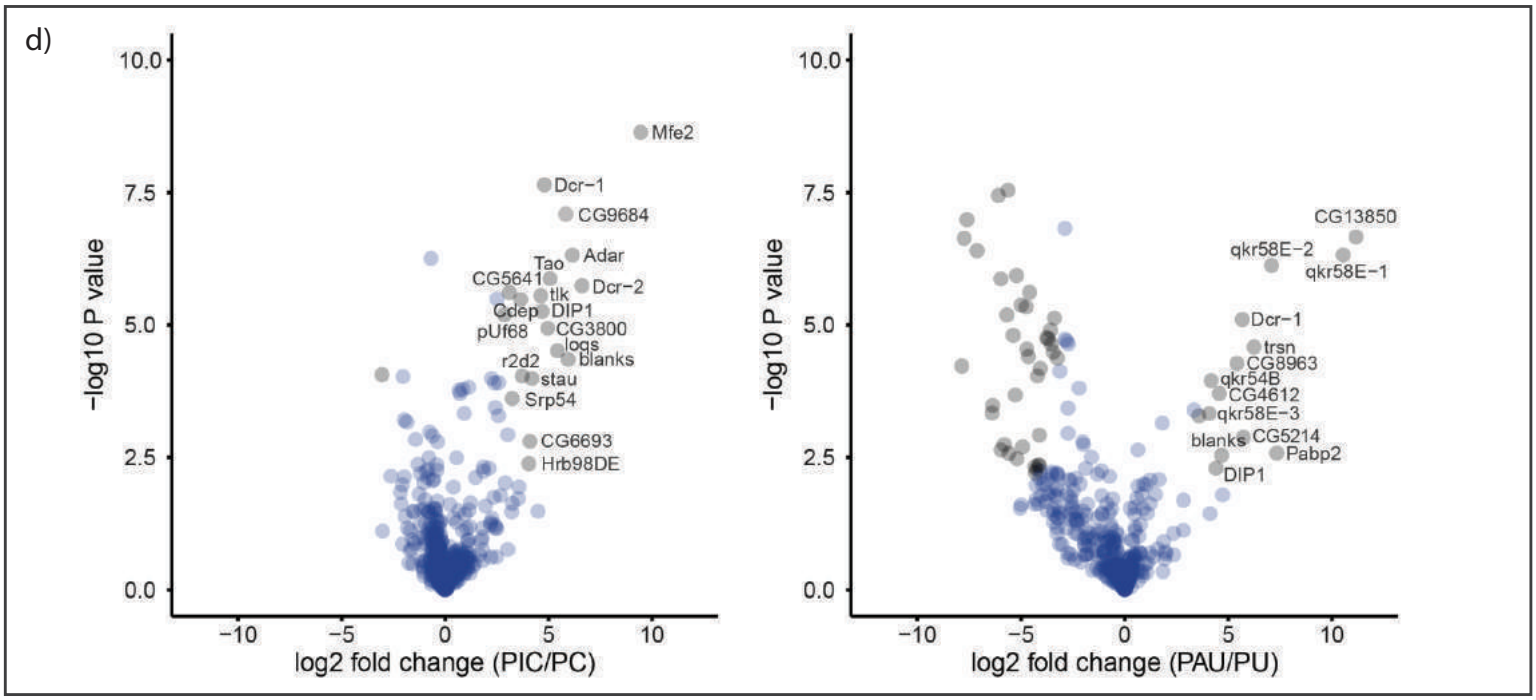
The aim of this study was to identify novel viral NA sensors in *Drosophila*. First, I will present the results of a small proof-of –principle AP-MS screen in *Drosophila* S2 cells, which led to the discovery of novel host factors for CrPV replication, the evolutionary conserved proteins CG5641 and Zn72D. Secondly, I will follow with the analysis of the large cross species AP-MS screen, with attention to tissue-specific vs organismal response and conservation patterns. And finally, I will conclude with the results of the functional screen in flies, which identified some novel antiviral factors in *Drosophila*.

Figure 7. Proof-of-principle AP-MS screen, using poly(I:C) vs poly (C) and to poly(A:U) vs poly(U) as bait vs control and S2 cell lysate as a sample (unpublished data of Habjan M. *et al*) a) schematic representation of the pull-down strategy: biotinylated NAs were coupled to sreptavidin (SA) beads and incubated with S2 cell lysate. Precipitated lysates were digested and analysed by (LC-MS.MS). Biotin - green circles (labelled with (b)) b) Heatmap showing 30 proteins preferentially binding to poly(I:C) and/or poly(A:U) with significant change ($p<0.01$, fold change >8). c) Network and enrichment analysis of 30 significant hits (STRING). Enrichment terms are indicated in colors. Proteins, preferentially binding either to poly(I:C) or poly (A:U) are shown with pink or black edge respectively. All other proteins bind both dsRNA baits. d) volcano plots showing all proteins binding to poly(I:C) vs poly (C) (on the left) and to poly(A:U) vs poly(U) (on the right). Proteins with significant change are indicated in grey.









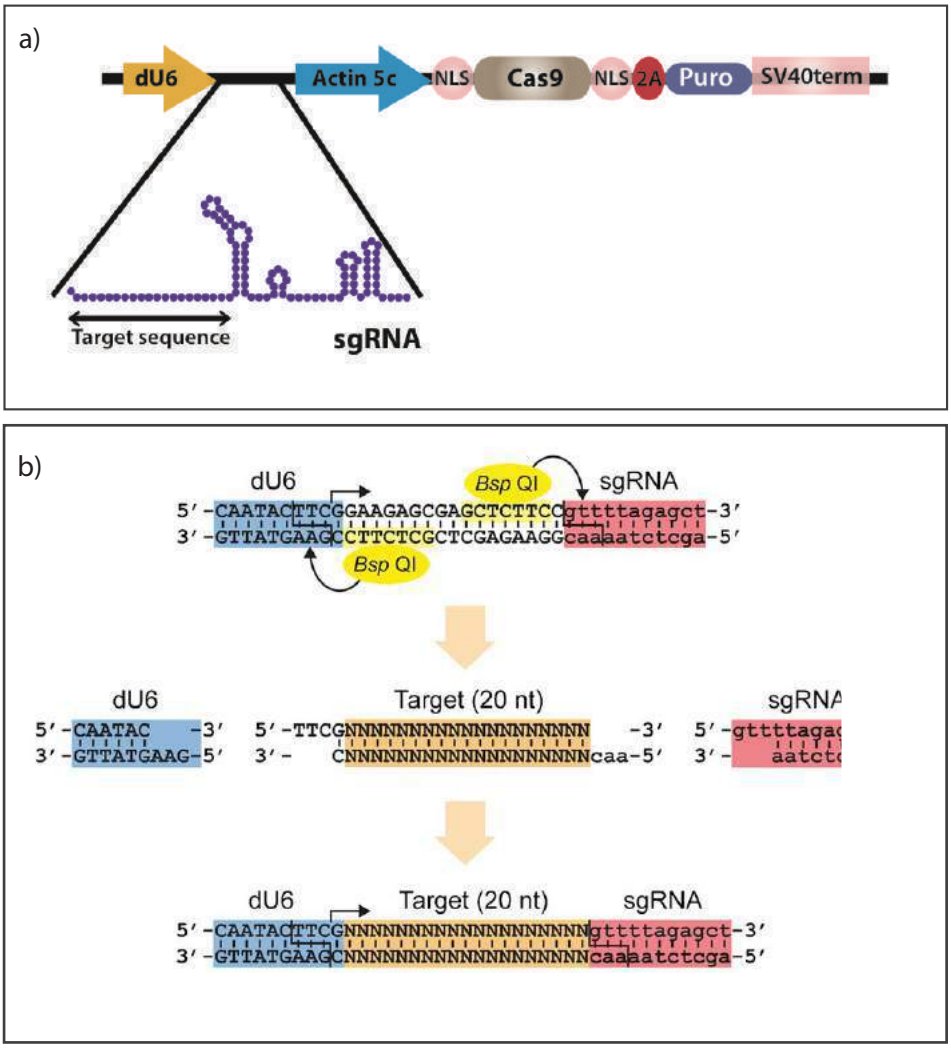
Results

I. Establishment of conditions. Proof-of-principle MS screen.

I.1. Proof-of-principle MS screen

Virus recognition largely relies on sensing viral nucleic acids (NAs). Discrimination between self and non-self is based either on the location of the NA or on the specific features of viral NAs, such as double-strandedness, or presence of specific marks (e.g. methyl groups) (Habjan and Pichlmair 2015). Based on this notion, an affinity purification-mass spectrometry (AP-MS) approach was first developed by Andreas Pichlmair and Giulio Superti-Furga to identify the proteins, which specifically bind to certain NA baits. In this approach, biotinylated NA bait is coupled to streptavidin beads and incubated with cell lysate (Figure 7a). Precipitated lysates are digested by trypsin and analysed by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). Using this approach, they identified IFIT proteins binding to 5'triphosphate RNA and AIM2 binding to viral DNA (Pichlmair et al. 2011; Bürckstümmer et al. 2009).

Our collaboration with A. Pichlmair started with a small AP-MS screen, to establish proof of principle using as a sample the Drosophila S2 cell line. In this initial set of experiments, the bait and control pairs were the mimics of virus-derived long dsRNA – namely polyinosinic-polycytidylic acid (poly(I:C)) vs poly(C) and polyadenylic-polyuridylic acid (poly (A:U)) vs poly(U)(Fig1b, c). Poly (I:C) is the most extensively studied immunogenic agent in mammals. It was historically identified as potent interferon inducer (Field et al. 1967). Later studies showed that poly(I:C) acts as agonist of protein kinase R (PKR) (Clemens and Elia 1997), and a subset of PRRs, namely TLR3 (Alexopoulou et al. 2001) and RIG-like receptor MDA5 (Gitlin et al. 2006). Poly(I:C) was used as an adjuvant in multiple animal models (reviewed in (K. A. O. Martins, Bavari, and Salazar 2015)), however, was not approved for clinical use in humans due to its high toxicity (Robinson et al. 1976). Poly (A:U), which is also a synthetic dsRNA used as an adjuvant, signals through



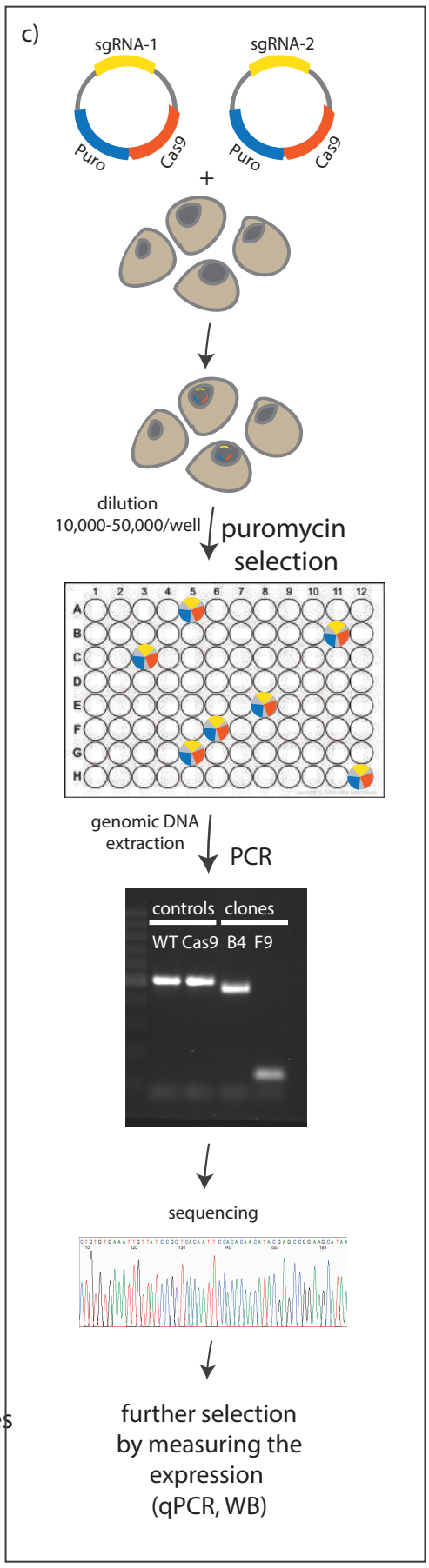


Figure 8. CRISPR/Cas9 knock-out in S2 cells (*a* and *b* adapted from Bassett *et al*, 2013) a) Scheme of the vector used for transfection to introduce the KO. sgRNA is expressed under the control of U6 promoter. Cas9 mRNA (grey oval) with SV40 nuclear localisation signals (NLS) is expressed from actin5C promoter in a bicistronic transcript with the puromycin N-acetyltransferase gene (violet oval). Two ORFs are divided by 2A ribosome skipping site from the insect virus *Thosea asigna* (2A). The transcription is terminated with SV40 terminator. b) Cloning target oligos into the vector. Two complementary DNA oligos with overhangs are annealed to produce the insert (orange). The vector is cut by Bsp QI (yellow) which enables the cloning of the insert. c) Two vectors targeting two different regions of a gene are co-transfected into S2 cells. After 3 days, cells are diluted in three 96-well plates (10,000 cells/well, 20,000 cells/well, 50,000 cells/well) in puromycin-containing medium. In around 3-5 weeks the first cell colonies start to appear. Cell clones are then tested by PCR and sequencing. Mutant clones are further selected according to mRNA and protein expression level.

TLR3 and TLR7, but does not trigger RLR signalling (Perrot et al. 2010; Sugiyama et al. 2008). Of note, the response to these two molecules in Drosophila is largely unknown.

In this experiment, a total of 429 proteins were identified. 30 of them preferentially bind the dsRNA baits (poly(I:C) or poly(A:U)) (Figure 7b). Almost a half of these bind both baits, whereas the others are specific poly(I:C) and poly(A:U) binding proteins (Figure 7b and c). Interestingly, Dcr-2 and R2d2, both important constituents of siRNA pathway, are specifically binding poly (I:C), and not poly(A:U) (Figure 7b,d). In addition, on the volcano plots showing all the proteins binding to baits vs controls it can be seen that although there are almost no proteins preferentially binding to poly(C), there are a great number of hits binding poly(U) with high affinity.

Network and enrichment analysis by STRING showed that there is significant enrichment for three major groups of proteins in terms of interaction, structure and function. Firstly, there is a group of proteins, assigned to the gene ontology (GO) term RNA interference (Dcr-1, Dcr-2, R2D2, loqs, blanks and trsn). These proteins share with the presence of dsRNA-binding domain, together with two other identified factors to DIP1 and Adar. The second major network includes proteins containing two other known RNA recognition motifs: K homology domain and RNA recognition motif (RRM). Of note, two poly (A:U) interacting proteins both contain 2-oxoacid dehydrogenase acyltransferase domains. The rest of the proteins including a few with unknown functions were not part of any of the enriched networks (Figure 7c).

I. 2. Strategy for CRISPR/Cas9 KO in S2 cells

To analyze the function of the new candidates in cells, we chose to exploit the CRISPR/Cas9 technology in order to create knock-out mutants in S2 cells. We used the vector previously described in (Bassett et al. 2014), which contains sequences to express single-guide RNA (sgRNA), Cas9 and puromycin N-acetyltransferase. sgRNA is expressed under the control of Drosophila U6 promoter, which enables the production of uncapped transcript by RNA polymerase III. Human codon-optimized Cas9 and puromycin N-acetyltransferase are expressed as a bicistronic transcript

Figure 9. Generating cell lines deficient for two components of the siRNA pathway: Dicer-2 and AGO2. Western blots of protein extracts from control and mutant cell lines. Protein expression is decreased in four Dicer-2 mutant cell populations (anti-Dicer-2 antibody from Abcam #4732)(a) and in three AGO2 mutant cell populations (F5, B5 and B7) (anti-AGO-2 antibody from Abcam #5072)(b). Asterisks (*) indicate unspecific bands. Actin is used as loading control in Dicer-2 experiment. Molecular weight of AGO-2=137 kDa, of Dicer-2=197 kDa).

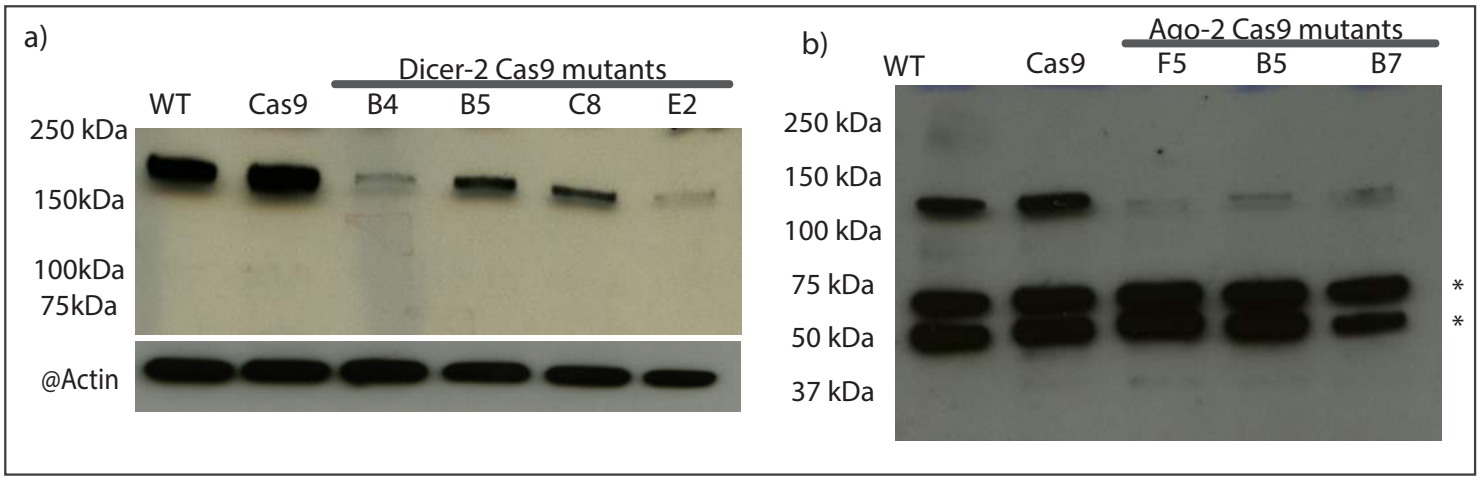
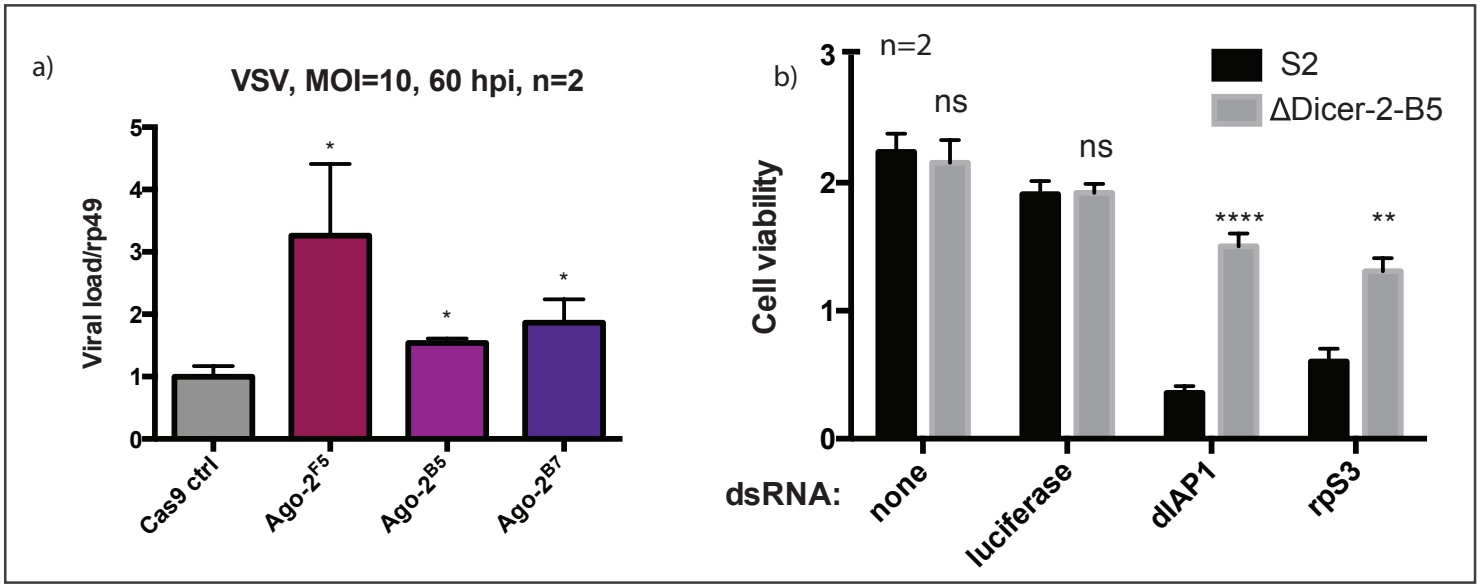


Figure 10. The siRNA pathway is impaired in the Dicer-2 and AGO-2 mutant cells. a) viral load upon infection with VSV (MOI=10, 60 hpi) is significantly increased in AGO-2 mutant cells (*=p<0.05, **=p<0.01, ****=p<0.001, unpaired t-test, 2 independent experiments, each done in triplicates). b) wild-type and Dicer-2 mutant cell line were incubated with dsRNA against essential genes (dIAP1 and RpS3) for 4 days. Then, cell viability was measured by colorimetric MTS assay (CellTiter 96® AQueous). As expected, Dicer-2 mutant cell population B5 has increased viability compared to WT cells.



under the constitutive Actin5C promoter (Figure 8a). This enables direct antibiotic selection of the cells, which express Cas9. BspQI restriction sites in the parental vector are used to clone the sequences complementary to the target gene (Figure 8b).

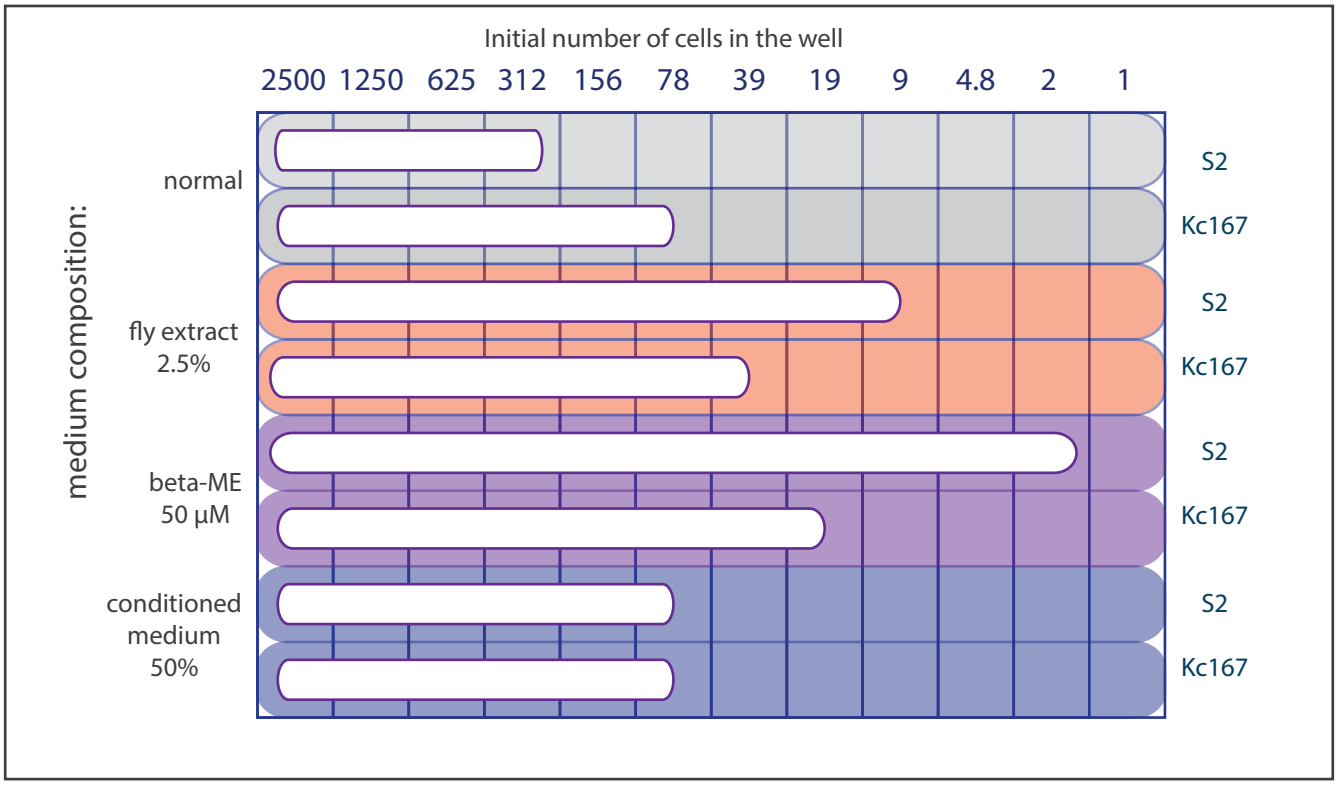
There are several considerations in the design of guide RNAs. First, it should be followed by protospacer adjacent motif (PAM), which corresponds to the sequence NGG. Secondly, the optimal length is 18-20 bp, as longer sequence will increase the chance of off-target effects. In our protocol we aim to target Cas9 to non-coding sequences in order to enable the possibility of cDNA rescue, so the sequence of guide RNA should be at least partially in a non-coding region. Also, we chose to transfect a pair of vectors targeting two different regions of the same gene, aiming to introduce a large deletion.

It was demonstrated previously that even single mismatches can significantly affect the binding of guide RNA and also can lead to off-target effects. Therefore, a first step to proceed with the construction of the vector is to verify the sequence of the gene. Then, following the design of sgRNAs, we can proceed to constructing the vector and transfection. Upon the appearance of the stable cell clones, we can test for the presence of mutation by PCR and sequencing (Figure 8c), and verify the expression level by qPCR and Western blot.

For proof-of-principle CRISPR-Cas9 mediated KO, we first targeted members of the RNAi pathway: Dicer-2, AGO2 and R2D2. I have produced several stable cell populations for Dicer-2 and AGO2. However, I could not obtain any stable cell population expressing Cas9 targeting R2D2. This might indicate that this protein has additional functions and that its expression is essential for the cell. For Dicer-2 and AGO2, we tested the obtained cell populations by PCR, qPCR and eventually Western blot. As shown on Figure 9 a,b, some residual protein was still expressed in all mutant cell lines, meaning that the resulting cell population is mixed, containing both wild-type and mutant cells. Given that S2 cells are 60-80% tetraploid, we cannot exclude that there might be several mutant versions of the gene in the same cell.

In order to verify that the mutant populations are deficient in siRNA pathway, one obvious way is to infect them with a virus. Indeed, I showed that the viral load of VSV is significantly increased in AGO-2 mutant cells, although this increase is modest in some mutant lines (Figure 10a). Another way to verify the

Figure 11. Schematic representation of the experiments aimed at establishing optimal conditions for "clonal" purification of S2 and Kc167 cells. Cells were plated in 96-well plates (U-shaped) with the initial number 2500 cells/well in the leftmost column, followed by serial dilution by two. White bars represent the presence of cell colonies after 4 weeks. Addition of beta-ME (50 µM) to the complete Schneider's medium allows S2 cells to recover cell populations from as low as 2 cells/well (the results are representative of three independent experiments).

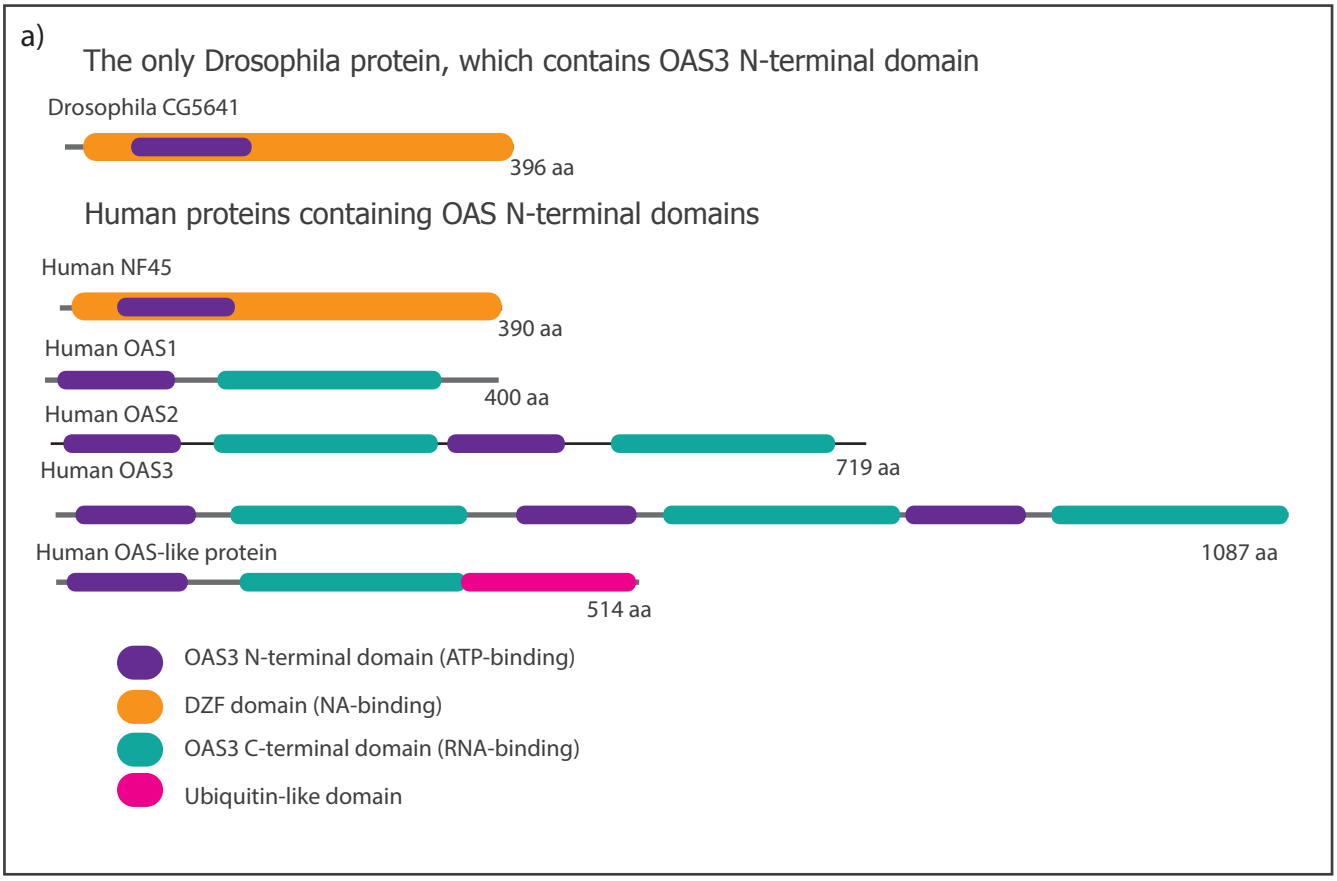


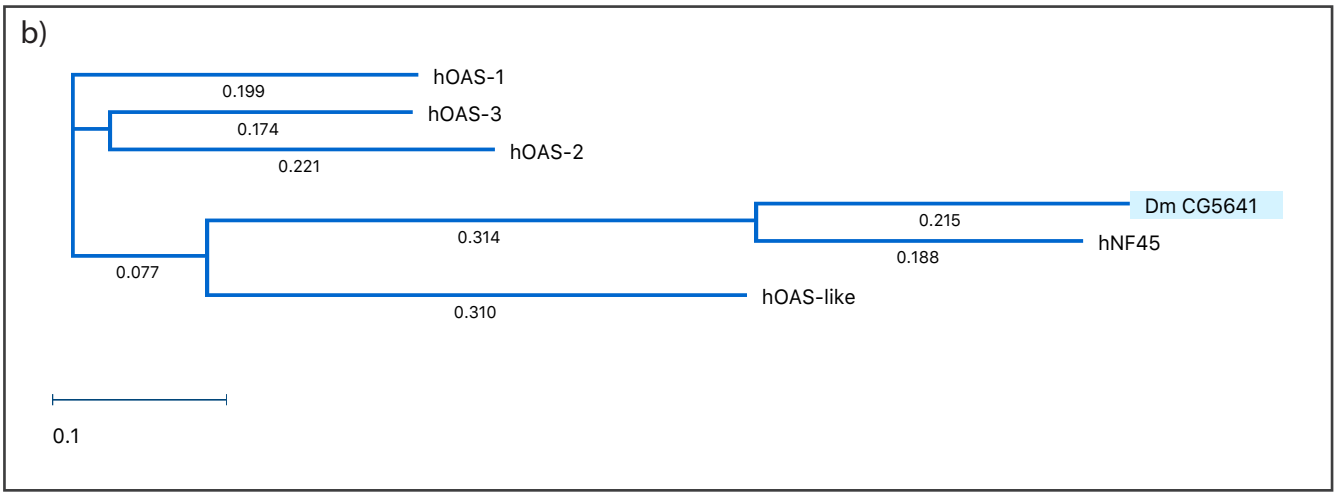
phenotype was to treat the cells with dsRNA against an essential gene (such as DIAP1 or RpS3) and monitor cell viability. As expected, silencing expression of the essential genes DIAP1 and RpS3 in parental S2 cells resulted in strong reduction of cell viability. By contrast, viability of the Dicer-2 mutant line B5, silenced for these genes, was significantly higher, confirming that the siRNA pathway has been impaired in these cells (Figure 10b).

I.3. Isolating clonal population in S2 cells

One serious caveat of using S2 cells is the difficulty to isolate clonal population (Zitzmann et al. 2018; Cherbas and Gong 2014; Schetz and Shankar 2004). Indeed, S2 cells stop proliferating if seeded at low density, which is explained by their need for autocrine growth factors (Moraes et al. 2012). There are some protocols available that enable to obtain more homogeneous population of cells, using either limiting dilution or soft agar to separate the cells. As for the source of growth factors, feeder cells or conditioned medium have been used (protocols summarized in (Zitzmann et al. 2018)). I performed experiments to modify the composition of the media in order to improve cloning ability of Drosophila cells. For this, I used S2 and Kc167 cells and seeded them at initial concentration of 2500 cells/well in 96-well plates (flat- or U-shaped bottom) and 384-well plate, followed by serial dilution by the factor of two, reaching the concentration of approximately 1 cell/well in the rightmost column. I used four different compositions for the culture medium: normal Schneider's Drosophila medium (SDM), SDM with addition of fly extract (2,5%), β-mercaptoethanol (β-ME) (50 μM) or conditioned medium (50%) (Figure 11). As a result, I could observe cell colonies 4 weeks after cell seeding. I observed most efficient cell growth upon dilution in U-shaped 96 well plates, compared to flat-bottom 96-well plates or 384-well plates. The minimum number of initial cells (~ 2 cells) needed in order to grow S2 cells was reached when using in the medium supplemented with of β-ME at 50 μM. β-ME has been routinely used in some mammalian cell cultures as a reducing agent to decrease the oxidative stress and was shown to be important when isolating clonal populations for some cell types (Oshima 1978). However, it was not previously reported as a media constituent in Drosophila cell culture. These findings suggest that the strategies for

Figure 12. Conservation and evolution of OAS N-terminal domain-containing proteins in Drosophila and humans. a) domain organization of CG5641 and related human proteins. b) phylogenetic tree (alignment by Clustal Omega, numbers indicate uncorrected pairwise distance)
CG5641 is the drosophila orthologue of mammalian NF45.





isolating clonal populations of S2 cells should be reconsidered with possible addition of β-ME to the culture medium.

I.4. The poly (I:C) and poly(A:U) binding candidate protein CG5641

Among the proteins with unknown function identified in this preliminary experiment, CG5641 caught our attention because it contains an oligo adenylate synthase 3 (OAS3) N-terminal domain (Figure 12a). OAS1 protein in mammals, which is induced by interferons and mediates synthesis of the secondary messenger oligo 2'5'A, that in turn acts as an agonist for the enzyme RNase L that will target and degrade viral RNAs (Hornung et al. 2014). The product of the gene CG5641 is a highly conserved Drosophila orthologue of mammalian NF45, which also contains OAS N-terminal domain (Figure 12b).

In mammals, NF45 produces a heterodimer with a partner protein, which can be NF90, SPNR or Zfr. This association affects the localization of the proteins as well as the binding affinity (Wolkowicz and Cook 2012). Most of the studies are being done on the NF45/NF90 protein complex, which is well studied on a structural level, binds with high affinity to long dsRNA and is involved in post-translational control of gene expression (Jayachandran, Grey, and Cook 2016). In Drosophila, CG5641 can be immunoprecipitated with Zn72D, which is the orthologue of Zfr (Figure 13)(Guruharsha et al. 2011). Interestingly, Zn72D was also found as a poly(I:C) binding protein in the MS screen.

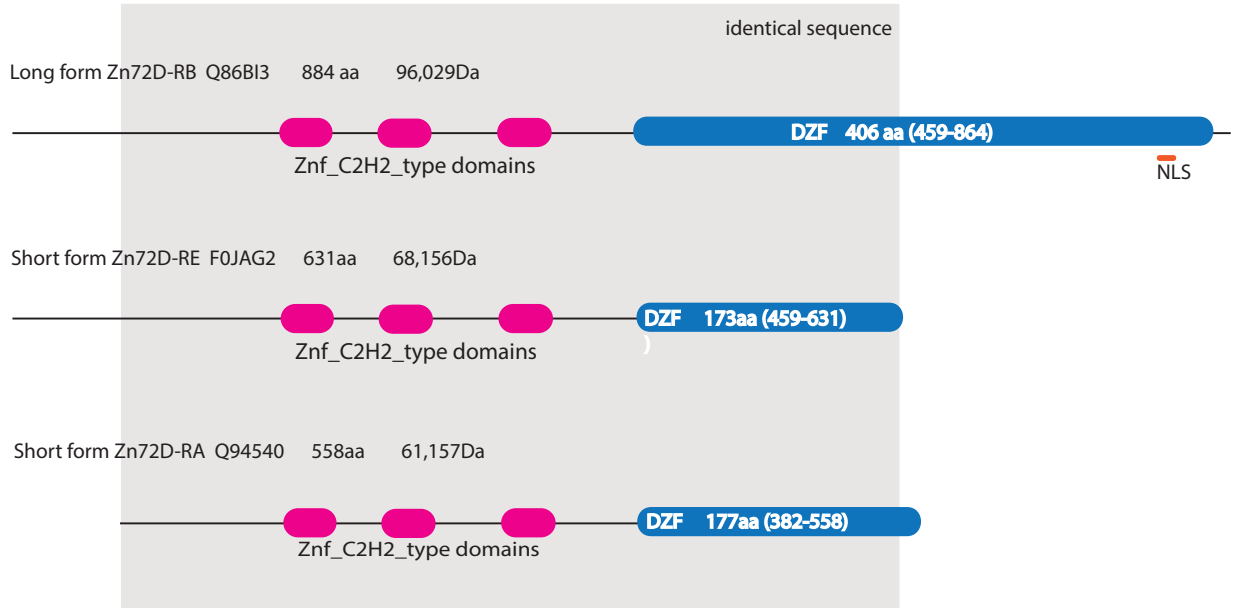
I.5. Generation of KO for CG5641 in S2 cells

The gene CG5641 covers 1792 bp and is organized in three exons and two introns. It produces a single transcript, encoding a protein of 396 amino acids. In order to target the gene by CRISPR/Cas9, I designed three sgRNAs: A – in 5'UTR region, B – partially in the coding sequence and targeting the start codon, and C – in the first intron. I co-transfected two plasmids: containing either A+C or B+C sgRNAs in S2 cells. From these, I have selected 5 cell populations arising from a single colony for PCR and sequencing (Figure 14ab). The sequencing results indicated that the

Figure 13. The DZF partners of NF45. a) domain organization fo the three isoforms of Zn72D in Drosophila b) domain organization of human NF90, Zfr and SPNR.

a)

In flies



b)

In humans

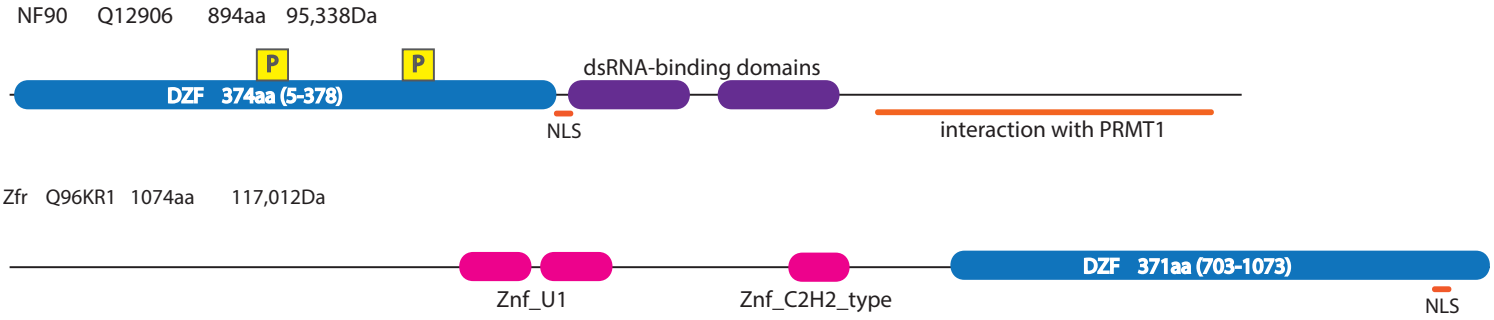
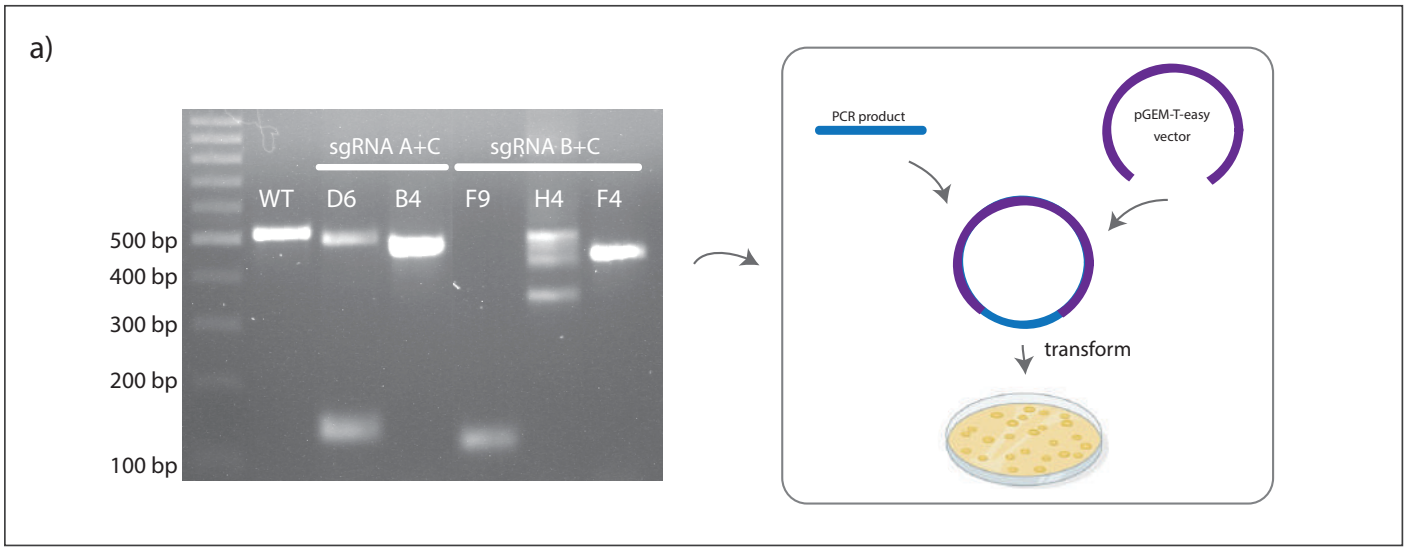


Figure 14. Targeting of CG5641 by CRISPR/Cas9. Two pairs of guide RNAs were used (A and C; B and C) in order to introduce a large deletion. sgRNA A and C are fully complementary to the non-coding sequence, whereas sgRNA B contains the starts codon. a) PCR fragments (wild-type amplification product = 522 bp) from wild-type and mutant cells were subcloned into pGEM-T easy vector. 16 bacterial colonies were chosen for plasmid purification and sequencing for each cell clone. b) Sequences of PCR fragments, indicating the size and location of deletions. Percentage indicates the proportion of sequence product among 16 bacterial subclones. Grey shadow indicates the coding sequence.



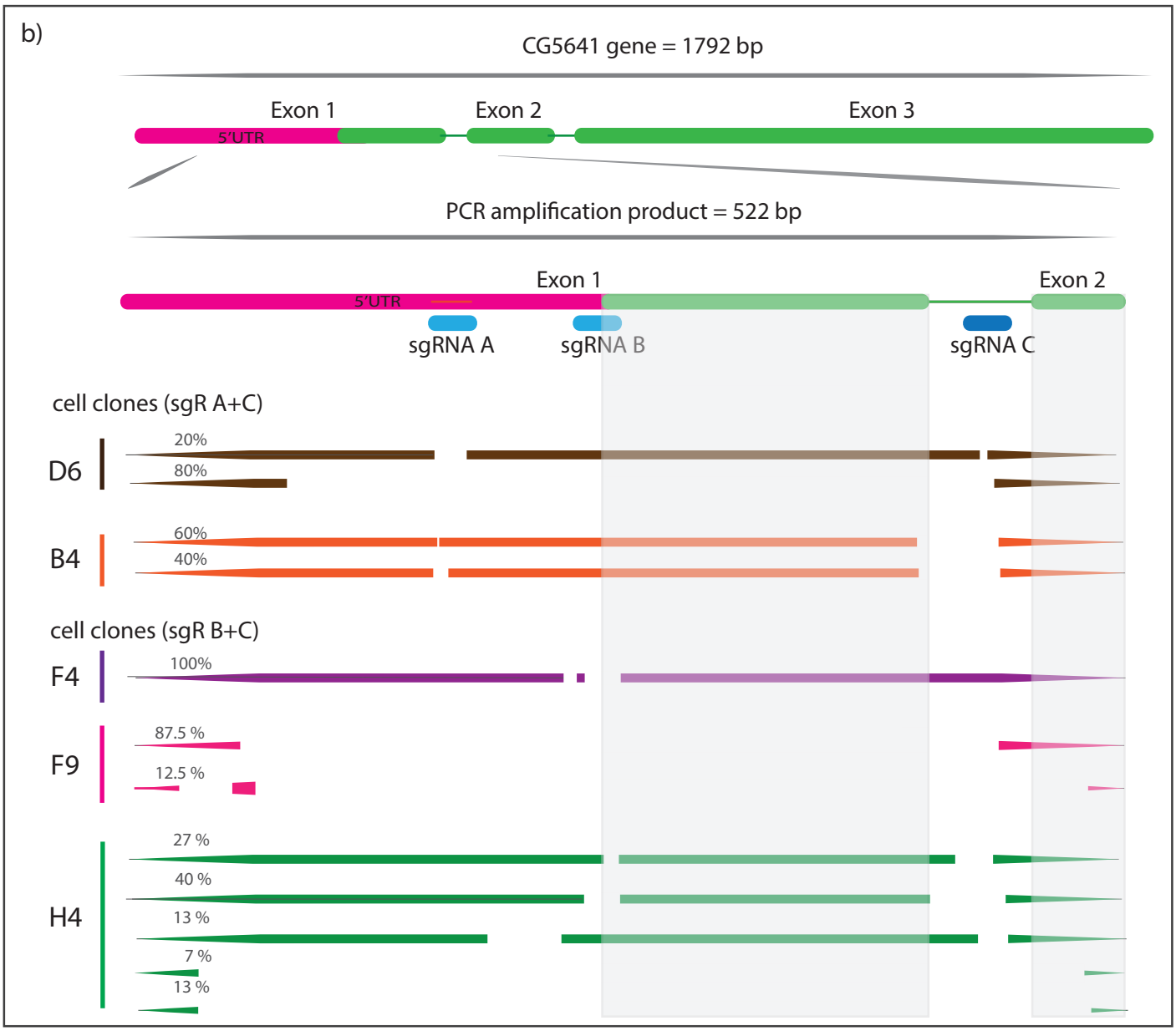


Figure 15. Verification of CRISPR/Cas9 KO of CG5641 in S2 cells by qPCR. Two sets of primers were used are indicated on a schematic of the gene. Expression level is normalised to Rp49. Expression of CG5641 mRNA is significantly reduced in three mutant cell lines (**=p<0.01, ***=p<0.005, ****=p<0.001, unpaired t-test, four independent experiments, each done in triplicates).

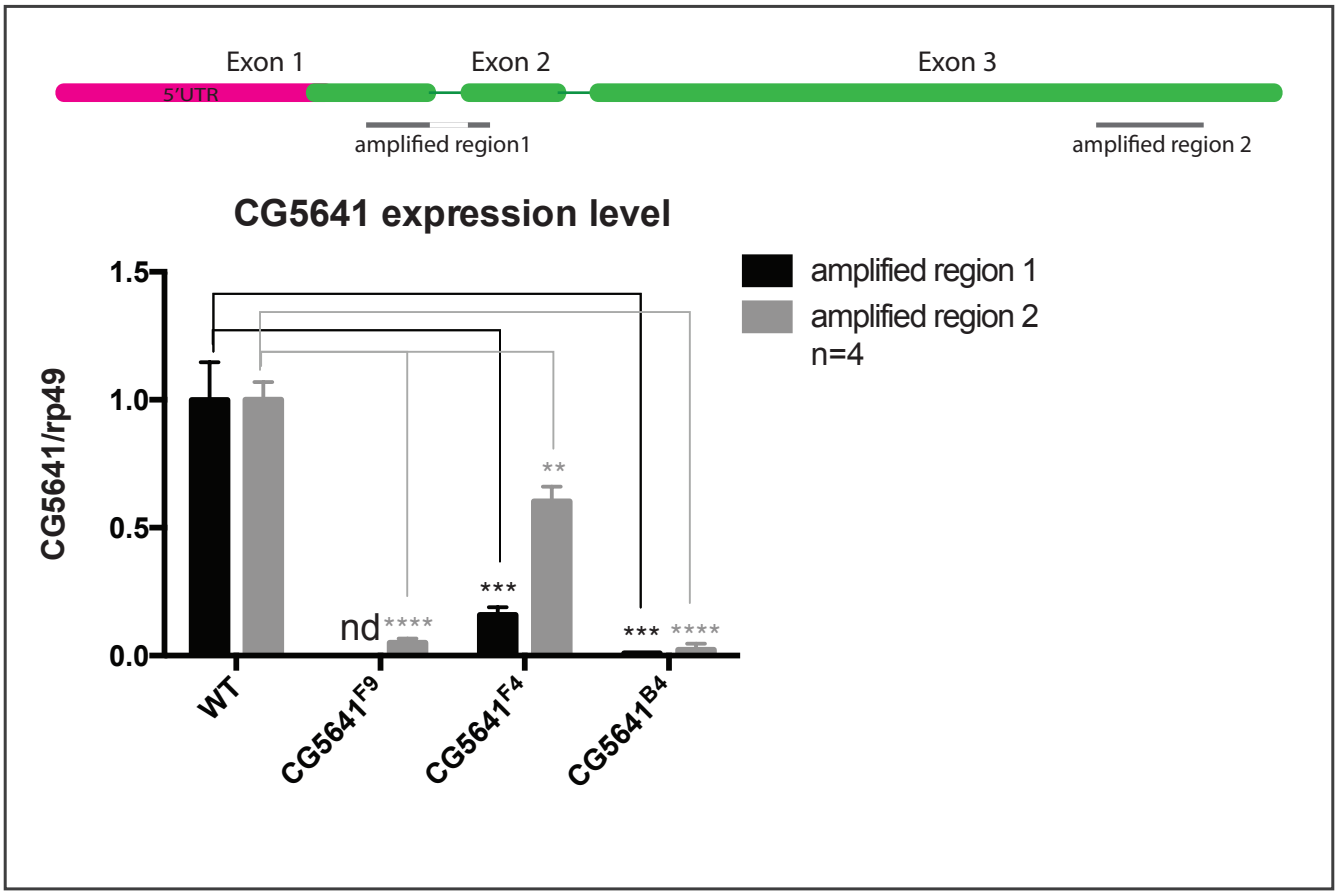


Figure 16. S2 cells mutant for CG5641 have decreased viral load upon the infection with CrPV. Viral load (relative to Rp49) upon infection with four viruses is shown. The change is non-significant compared to Cas9 ctrl unless indicated (***=p<0.005, ****=p<0.001, unpaired t-test, each experiment is done in triplicates). NA=no data

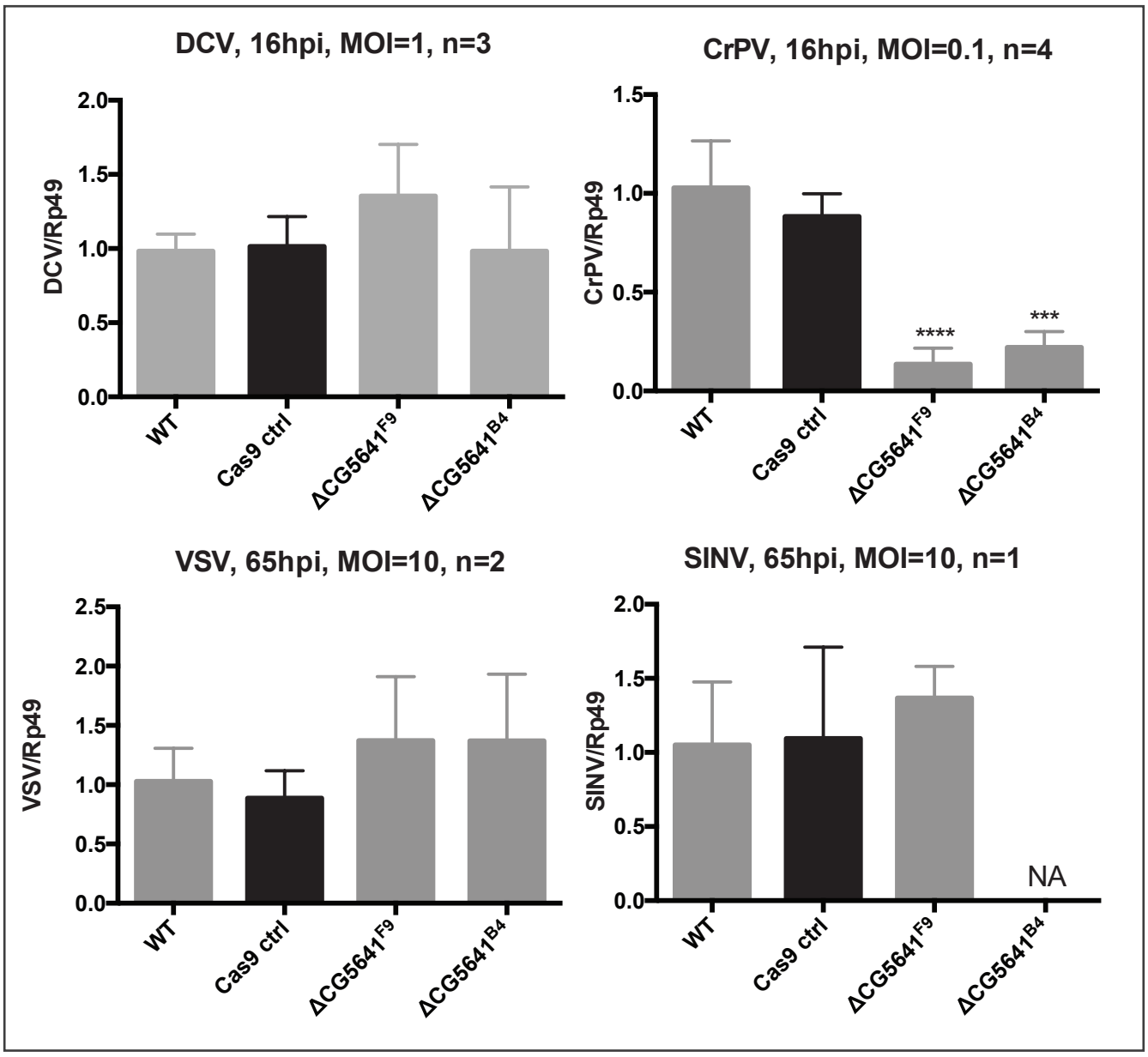
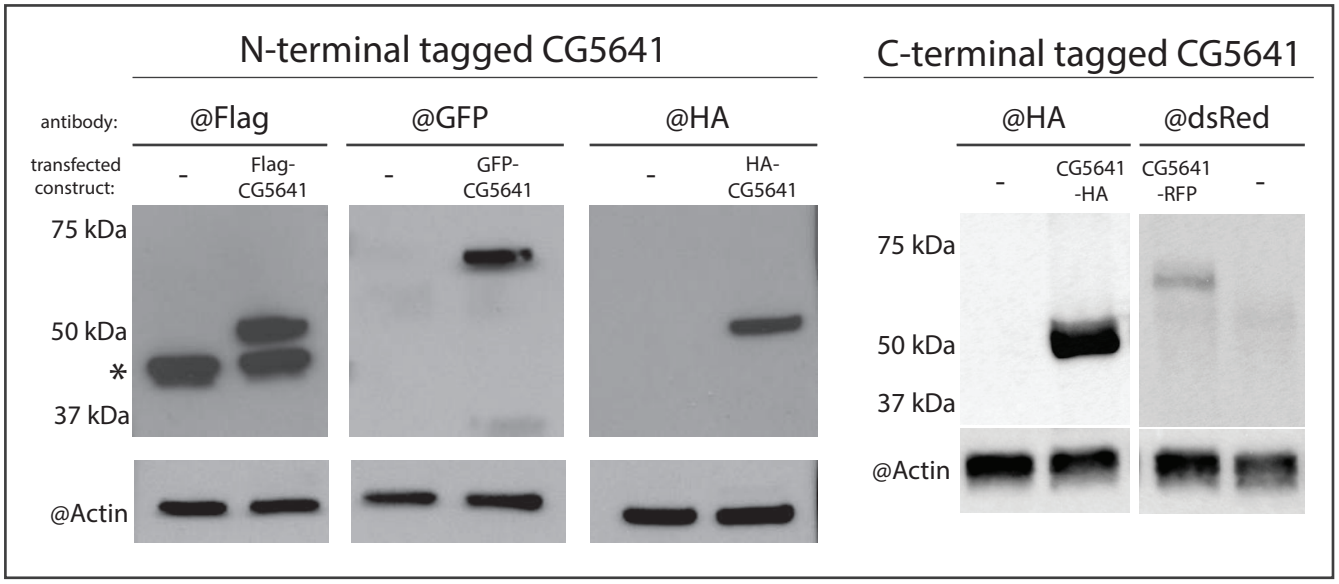


Figure 17. Transfection of tagged CG5641 in S2 cells. WB showing the expression of tagged CG5641 protein at expected size (molecular weight of CG5641=43.5 kDa, of GFP = 26.9 kDa, of RFP = 27 kDa, of HA=3 kDa, of Flag=1 kDa). Asterisk (*) indicates unspecific band.



sgRNA indeed guides Cas9 to cut at the designated places, although the size of the deletion can differ depending on the cell population. Some cells exhibit different deletions, suggesting that they are not clonal, even if they arise from a single colony. Alternatively, given the polyploidy of S2 cells, a clonal cell population could contain multiple version of the gene. I selected three stable cell populations for further analysis. I confirmed that the level of mRNA is significantly reduced in all of them, and selected two (F9 and B4) for subsequent experiments (Figure 15).

I.6. Virus infection in CG5641 KO cells

To test the possible involvement of CG5641 on host-virus interaction, I infected two KO cell lines with several viruses, belonging to different families (Figure 16). Replication of VSV, SINV and DCV was not affected in these cells. CrPV is closely related to DCV and belongs to the same family *Dicistroviridae,* so I expected the same result. Interestingly, however, I observed a significantly reduced viral load upon infection with CrPV in the two mutant cell lines.

I.7. cDNA rescue

In order to attempt to rescue the phenotype, I created plasmids containing the CG5641 cDNA sequence under the control of the Actin5C promoter to express the protein tagged on its N- or C-terminus. The plasmids do not contain the CG5641 introns, and therefore will not be targeted by Cas9, thus allowing rescue in the KO cells. I chose the Gateway strategy for constructing the plasmids and used HA, Flag, GFP and RFP tags. I could confirm the expression of the protein tagged by the FLAG and HA epitopes, or fused with GFP or RFP, at the N- and C-termini (Figure 17).

In order to see whether expression of the rescue plasmid restores the replication of CrPV, I transiently transfected N-terminal tagged constructs (HA and GFP) and after two days infected with CrPV. Firstly, I have confirmed that the expression of CG5641 is increased upon the transfection in control and mutant cell lines (Figure 18a). Also, I showed that the replication of CrPV was increased in control cell line transfected with N-terminal tagged constructs. These results

Figure 18. Tagged CG5641 partially rescues CrPV replication in S2 cells.
S2 cells were transiently transfected with N-tagged CG5641 (60 h), then infected with CrPV (16h hpi, MOI=0.1). a) mRNA level is drastically increased upon transient transfection with tagged cDNA plasmids in control and mutant cell lines. b) CrPV replication is increased in control cell line, transfected with tagged CG5641. Also CrPV replication is partially restored in the mutant cell lines. All changes are statistically not significant, unless otherwise indicated (*=p<0.05, **=p<0.01, ***=p<0.005, unpaired t-test, two independent experiments in triplicates).

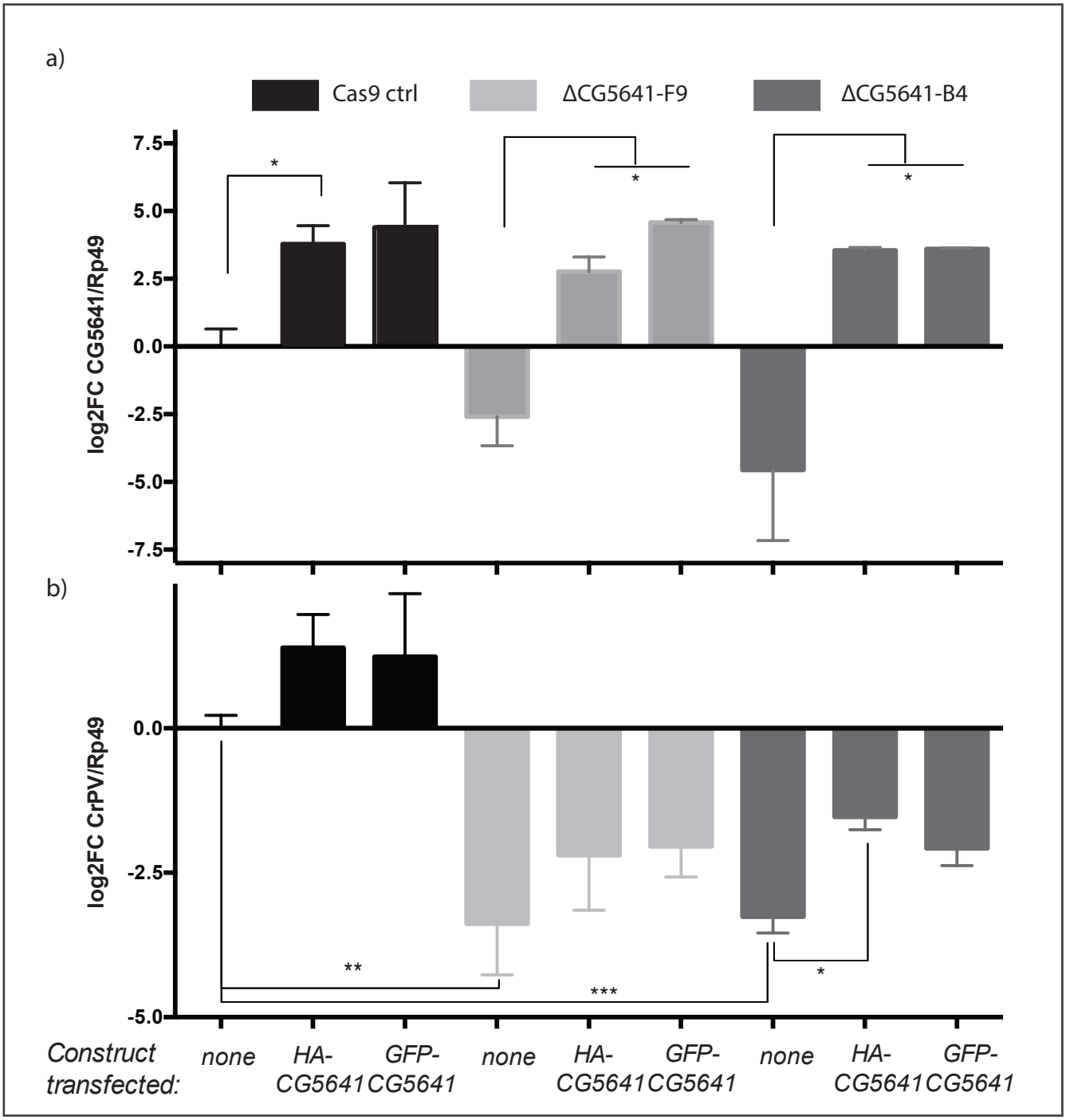


Figure 19. CG5641 and CrPV do not colocalize in infected S2 cells. S2 cells were transiently transfected with HA-CG5641 and three days after transfection infected of CrPV (MOI=0.1). Cells were fixed 16 hpi and stained with antibodies against HA-tag, CrPV and dsRNA. Representatives of 5 captured cells per condition are shown.

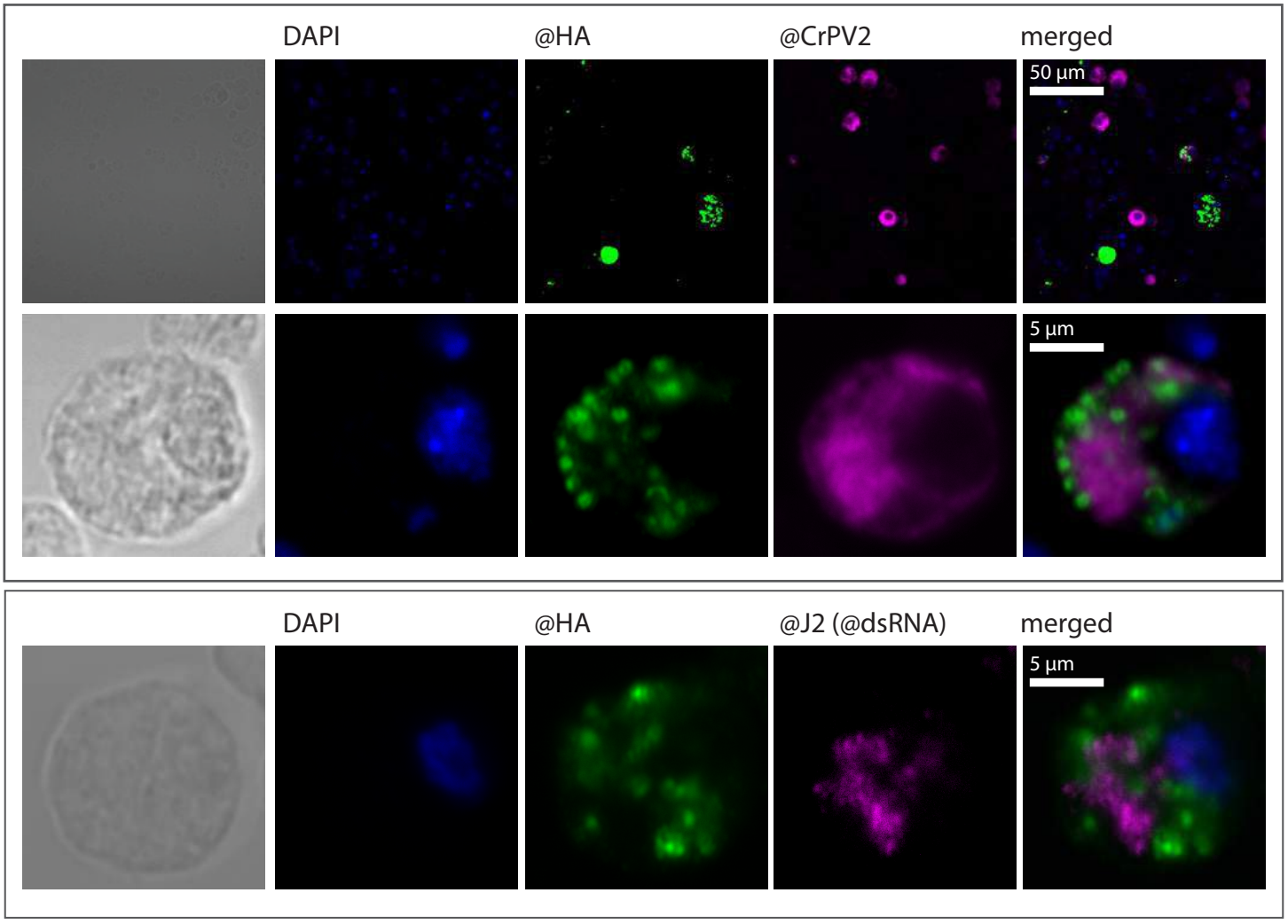


Figure 20. The effect of Zn72D knock-down on the replication of CrPV in WT or CG5641 KO cells. S2 cells were incubated with dsRNA, targeting two different regions of Zn72D for 4 days, then infected with CrPV (MOI=0.1). CrPV viral was monitored by RT-qPCR 16 hpi. CrPV viral load is decreased upon the knock-down of Zn72D. Viral replication is partially restored in CG5641 mutant cells when the KD of Zn72D is induced by dsRNA-1. p values are shown in black (relative to dsGFP in control cells), in blue (relative to dsGFP F9 cells) and in purple (relative to dsGFP B4 cells)(unpaired t-test, n=2, in triplicates).

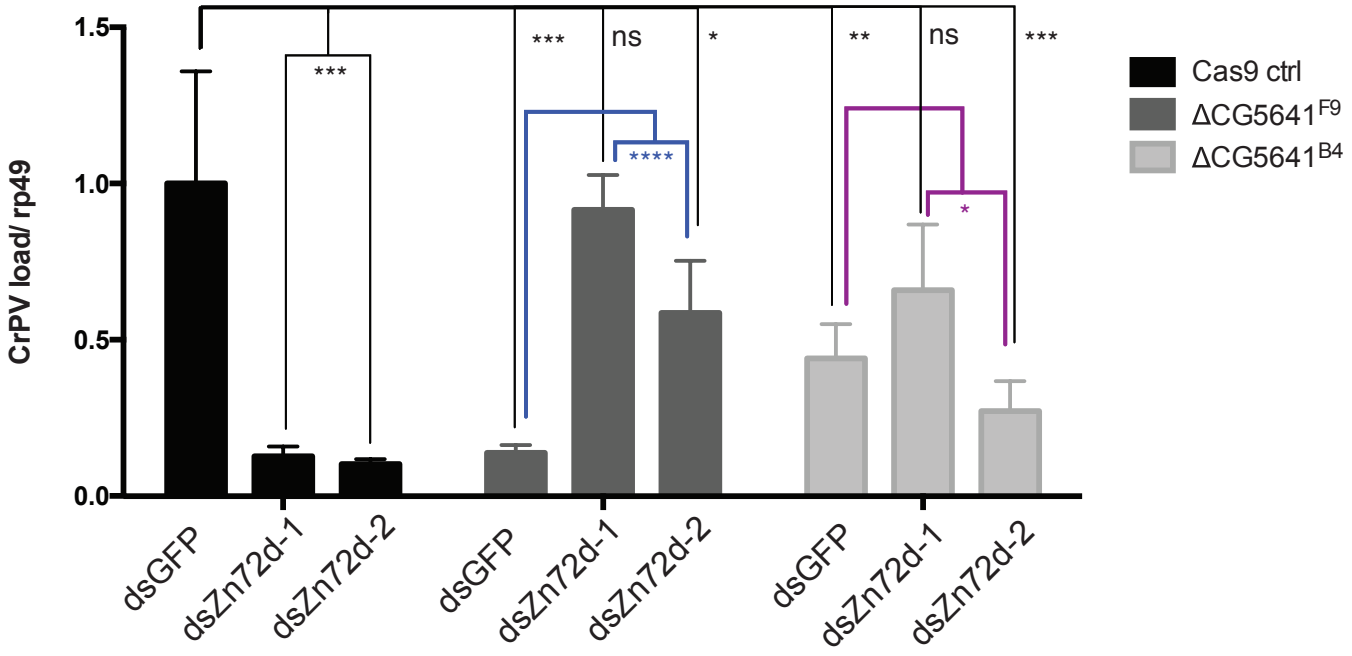
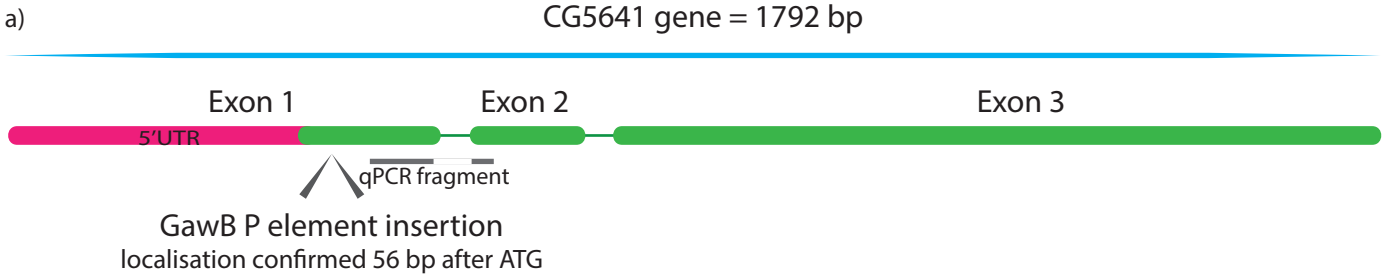
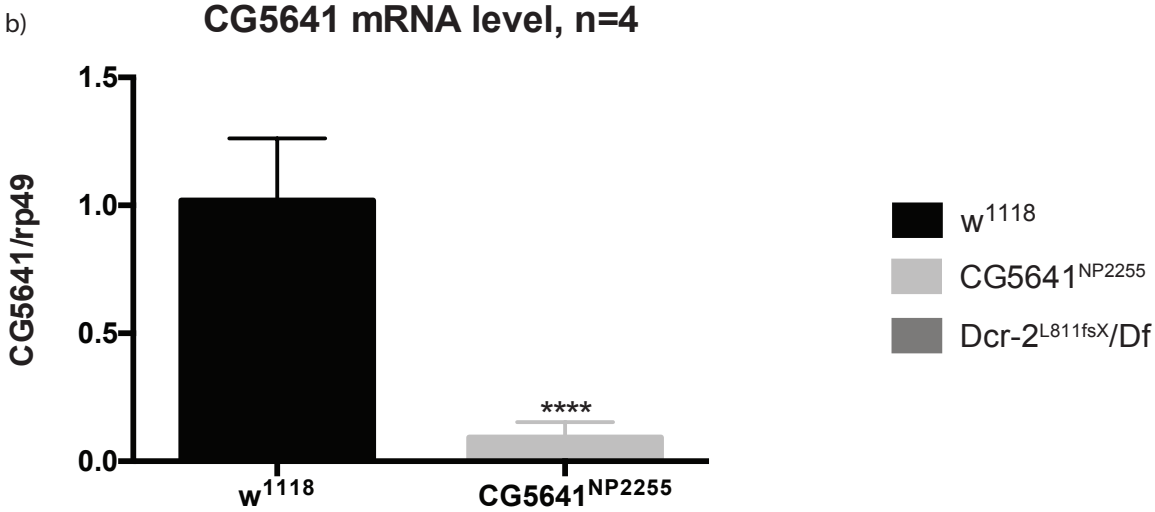
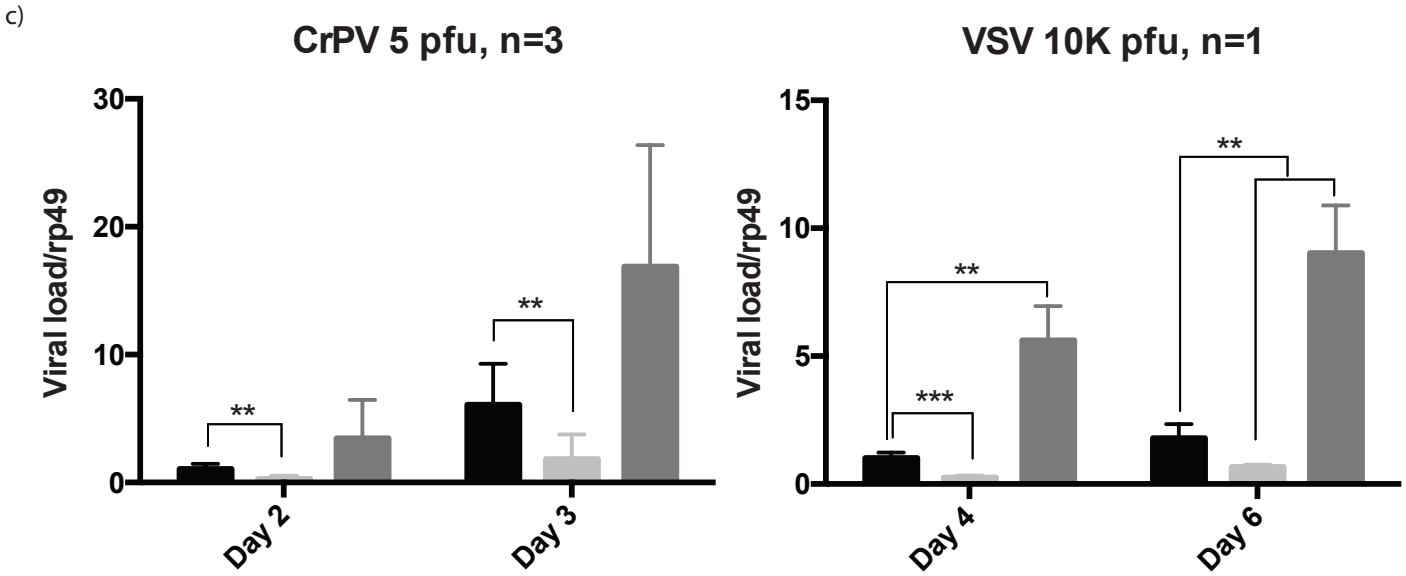


Figure 21. CG5641-mutant flies have reduced replication of CrPV and VSV. a) schematic of the CG5641: the location of the P element(NP2255) is indicated as well as the amplification product for qPCR. b) mRNA level of CG5641 is significantly reduced in the mutant fly line c) the viral load of CrPV and VSV is significantly reduced at two time points upon infection. Dcr-2 mutant line Dcr-$2^{L811fsX}$/Df is used as a positive control.







support the loss-of-function phenotype and confirm that the amount of CG5641 expressed by the cells impacts CrPV replication (Figure 18b). As for mutant cell lines, I observed a trend for rescue in all samples, with statistically significant increase of viral titer in one sample. However, the phenotype was not completely restored. One of the reasons for this could be that the transfection was transient, and therefore not all cells were transfected. In addition, as the MOI of CrPV (0.1) and the duration of infection were low to avoid cytotoxic effect, not all of the cells were infected with the virus.

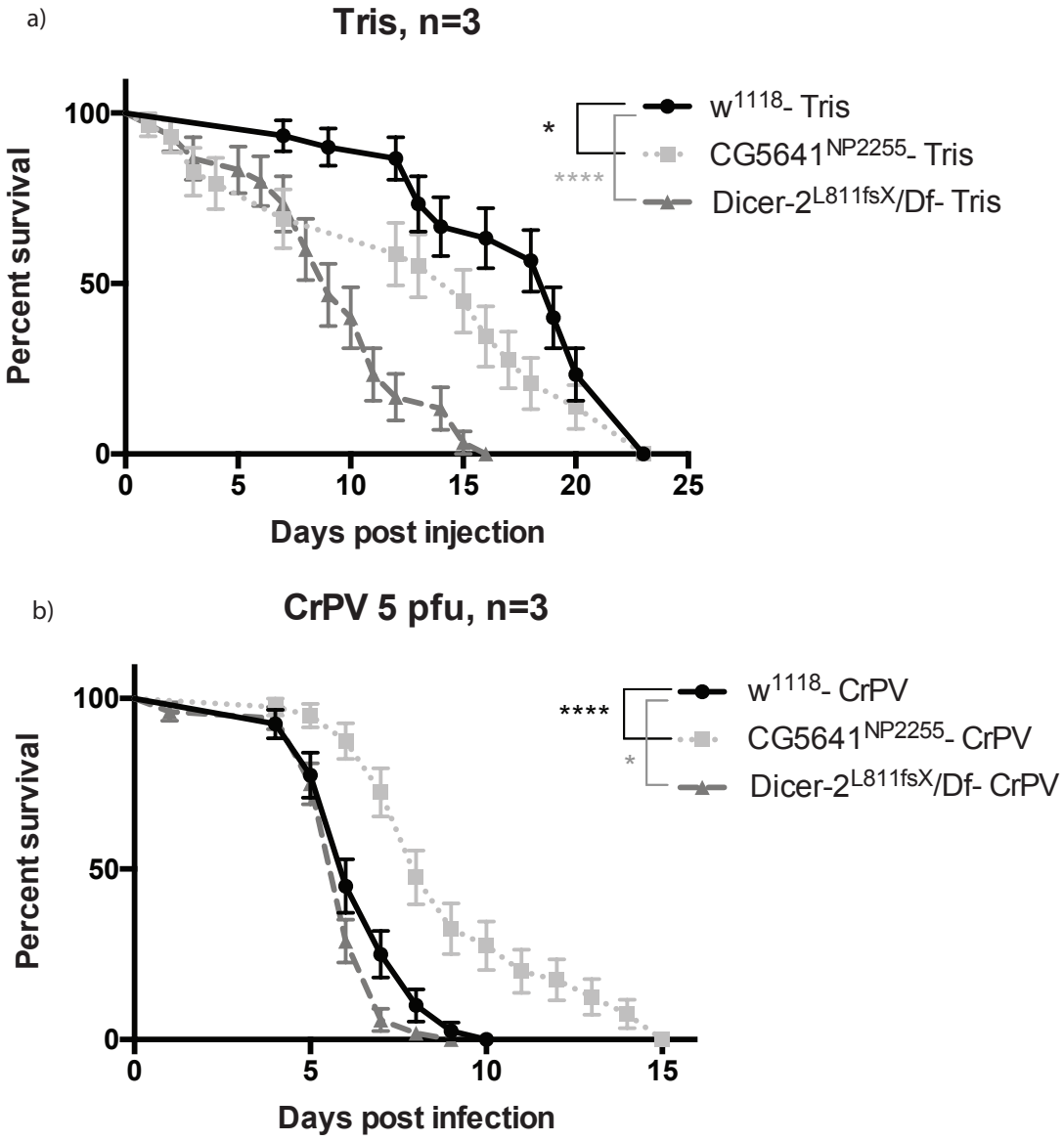
Indeed, I could demonstrate with immunostaining experiment, that only a small proportion of the cells were infected with CrPV in this condition. Moreover, an even smaller number of cells were expressing the tagged protein upon transient transfection (Figure 19). Therefore, the conditions of the infection must be adjusted to achieve greater infectivity. In addition, stable cell lines expressing the plasmid are needed to generate non-ambiguous data in infection experiments. Of note, this preliminary immunofluorescence experiment showed, that in the infected cells expressing the HA-tagged version of CG5641, the virus and protein do not co-localize.

I.8. Zn72D, the partner of CG5641, is also required for CrPV replication

As mentioned above, in mammals NF45 is known to bind three partner proteins: NF90, SPNR and Zfr. The drosophia orthologue of Zfr, Zn72D was pulled down with poly(I:C), like CG5641. We therefore hypothesized, that CG5641 might work in the complex with Zn72D to promote the replication of CrPV.

In order to investigate the function of Zn72D upon viral infection, we decided to exploit RNAi to induce the knock-down of the gene. Interestingly, KD of Zn72D using two dsRNA targeting different regions led to significantly reduced viral load upon infection with CrPV in S2 cells. This result confirms our finding with CG5641 and suggests that the CG5641/Zn72D complex is required for CrPV replication. In line with this hypothesis, silencing of Zn72D in the CG5641-B4 mutant cell line has no or weak impact on CrPV replication. Curiously however, KD of Zn72D in the

Figure 22. CG5641-mutant flies are more resistant to the infection with CrPV. a) flies were injected with Tris, as a negative control. CG5641- and Dicer-2-mutant flies show significantly reduced survival upon injection with Tris 10mM. b) upon the injection with 5 pfu of CrPV, the flies, mutant for CG5641, survived longer than wild-type control. Four tubes of 10 flies were used for each experiment.
Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests were used to analyse the data.



CG5641-F9 mutant cells resulted in significant increase of CrPV replication (Figure 20).

I.9. CG5641 is required to support replication of CrPV *in vivo*

To validate the phenotype *in vivo*, we used *CG5641*^{NP2255} flies, which contain insertion of a P{GawB} element, 56 bp downstream of the start codon. I confirmed the localization of the insert by inverse PCR, and showed that the level of CG5641 expression in these flies is significantly reduced compared to the control *white*^{1118} flies (Figure 21ab). Then, the flies were injected with CrPV, DCV, SINV, VSV. The infection with DCV and SINV resulted in multiple inconclusive experiments. However, the viral load of CrPV and VSV was significantly reduced at the two timepoints post infection (Figure 21c). Of note, CG5641 mutant flies resisted infection better than wild type flies, in agreement with the reduced viral load (Figure 22). Overall, these results tend to confirm our findings in cells, namely that CG5641 is required for CrPV replication.

Conclusion of Part I

In summary, pilot MS screen illustrated that AP-MS approach can be successfully used to identify known and novel NA sensors. I demonstrated that CRISPR/Cas9 system can be successfully used to induce targeted KO in S2 cells. However, I have encountered technical difficulties to isolate homogenous mutant cell populations, due to natural heterogeneity of S2 cells. β-ME was shown to be a useful cell medium constituent in order to achieve maximal dilution. And finally, conserved host factors CG5641 (dNF45) and Zn72D were found to be important for CrPV replication in S2 cells and flies. This first part of project provided a proof-of-principle for subsequent larger scale experiments.

Figure 23. Samples for the large AP-MS screen included cell and whole organism extracts from 5 species.


whole C.elegans


whole fly extract and S2 cells


HD11 cells


RAW264.7 cells


THP1 cells

Table 1. Nucleic acid species used as baits and controls in AP-MS screen. Immune effect in mammals is indi ated, where it is known. Types of NA baits are indicated by different colors.

Type	Affinity bait	Description of ligand	Effect in mammals
single-stranded RNA	ssRNA-OH	control 20-mer ligand, chemically synthesised	Control
	ssRNA-PPP	RNA bearing triphosphate end, chemically synthesised, 20-mer	Cytokine induction
	ssRNA-CAP	Capped ssRNA, non-methylated, chemically synthesised	Impaired viral growth
	ssRNA-CAP0	Capped ssRNA, N7-methylated, chemically synthesised	Impaired viral growth
	ssRNA-CAP1	Capped ssRNA, methylated at N7 of CAP and 2'O of first ribose	Normal mRNA
	ssRNA-CAP2	Capped ssRNA, methylated at N7 of CAP and 2'O of first and second ribose	Normal mRNA
	poly(C)	ssRNA polycytidine, enzymatically synthesised	Control
double-stranded RNA	poly(I:C)	heterogeneous dsRNA polyinosine:polycytidine, enzymatically synthesised	Cytokine induction
	dsRNA-PPP	dsRNA bearing triphosphate end, chemically synthesised, 20-mer	Cytokine induction
	dsRNA-CAP0	dsRNA capped, N7-methylated, chemically synthesised, 20-mer	Unknown
	dsRNA-OH	control dsRNA, chemically synthesised, 20-mer	Control
DNA	dsISD	Interferon stimulatory dsDNA, synthetic ligand	Cytokine induction
	ssISD	Interferon stimulatory ssDNA, synthetic ligand	Control
	RNA-ISD	RNA-DNA hybrid	Cytokine induction
secondary messenger	2'5'oA	2'5' oligoadenylate	Activates RNase L
	ATP	ATP	Control

II. Large cross species AP-MS screen identified novel conserved and specific NA sensors.

Following the results of a proof-of-concept experiment in S2 cells, our collaborators proceeded to the larger AP-MS screen. In this screen, total of six samples from five species were used: whole organism extracts of *C.elegans* and *D. melanogaster*, Drosophila S2 cells, chicken HD11 cells, mouse RAW264.7 cells and human THP1 cells (Figure 23). In addition to the organismal diversity, a total of 11 different comparisons between bait and control NA species were used, in order to cover the wide repertoire of possible viral NA products (Table 1). For some of the baits, such as poly(I:C) and triphosphate-RNA, the immunogenic effect and subsequent signaling pathways are already extensively studied in mammals. For others, such as RNA-ISD, the effect is unknown. In all cases, there was little research on the effect of these molecules in Drosophila.

A total of 10751 proteins were identified. All protein numbers by sample and bait vs control are shown in Table 2. As expected, more hits were identified from whole organism samples (*C. elegans* and *D. melanogaster*) as compared to cell lines. This suggests, that the range of the NA sensing molecules in a cell line is limited due to tissue specificity. It is clear that most of the hits in all samples (up to 89% in whole fly) bind poly(C) and poly(I:C). This is not surprising given high immunogenicity of these synthetic molecules. For some of the baits, experiments were not done for all samples. For example, when we compare the data in whole flies and S2 cells, both sample lysates were used for the experiments with 11 out of 18 baits. Further analysis was done analyzing primarily whole fly and S2 cells data and separated for each bait vs control pair. Because of time constraints, cross species analysis was only performed for the experiments with 2’5’oA as bait.

Table 2. Numbers of proteins identified in the cross-species AP-MS screen
by bait vs control pair and by type of lysate

Bait	C. elegans	Fly	S2	HD11	RAW264.7	THP1
total	2235	2495	1317	1153	1942	1609
poly(I:C) vs poly(C)	2093	2208	735	793	1498	1075
poly(A:U) vs poly(U)	*ND*	*ND*	433	*ND*	528	444
dsISD vs ssISD	612	997	437	463	601	495
RNA-ISD vs ssISD	602	987	ND	475	590	450
2'5'oA vs ATP	446	839	630	166	194	250
ssRNA-CAP vs ssRNA-CAP1	684	878	577	407	613	627
ssRNA-CAP0 vs ssRNA-CAP1	690	875	577	407	619	629
ssRNA-CAP2 vs ssRNA-CAP1	682	877	*ND*	408	*ND*	*ND*
ssRNA-PPP vs ssRNA-OH	519	836	527	381	583	612
dsRNA-CAP0 vs dsRNA-OH	574	841	*ND*	373	350	422
dsRNA-PPP vs dsRNA-OH	553	800	*ND*	351	323	377

II.1. Poly(I:C) vs poly(C) binding proteins in whole fly and S2 cell extracts

A total of 2342 proteins were found binding to poly(I:C) and poly(C) in Drosophila samples, out of a total of 2495 identified proteins (Figure 24a,b). This pair of bait and control was therefore the most “sticky”. This can highlight the immunogenicity of the molecules, but also indicates the possibility of fishing many unspecific hits. As expected, more proteins were identified in whole fly sample as opposed to S2 cells (about 3 fold). This supports the notion that by selecting a certain cell line, we are limited only to a tissue-specific response. 601 of them were identified both in S2 cells and whole fly samples. 134 were S2 specific, whereas 1607 were found only in the experiment with whole fly as a sample. I selected significant hits, binding preferentially to poly(I:C) with fold change more than four. While there were 40 significant hits found in S2 cells, there were 1410 proteins found in whole fly sample. Only 21 of them were common with S2 cells. Then, I analyzed these 21 common proteins using STRING network and enrichment analysis (Figure 24c). As a result, there were several networks enriched in this group of proteins. First, there are 7 proteins, which belong to a network where all of them interact with each other (caz, U2A, Rpb10, pUf68, cyp33, CG9667, CG6197). Most of these proteins are assigned to the GO term catalytic step 2 spliceosome. CG3800 presumably binds pUf68, however its putative function or molecular localization is unknown. A second group of proteins is related to RNAi pathway (Dcr-2, loqs, blanks). Stau is also shown to bind to Dcr-2. Although not assigned to a GO term, the proteins DIP1 is related to R2D2, loqs and blanks, and has been associated with antiviral immunity in drosophila (Zhang et al. 2015). Also, there are two interacting proteins (Chd1, Iswi), which play role in chromatin remodeling. Other proteins were not assigned to any enriched GO term or network. All of the 21 proteins have orthologue(s) in human. In many cases fly protein has several probably redundant human orthologues (Table 3).

poly(I:C) vs poly(C)

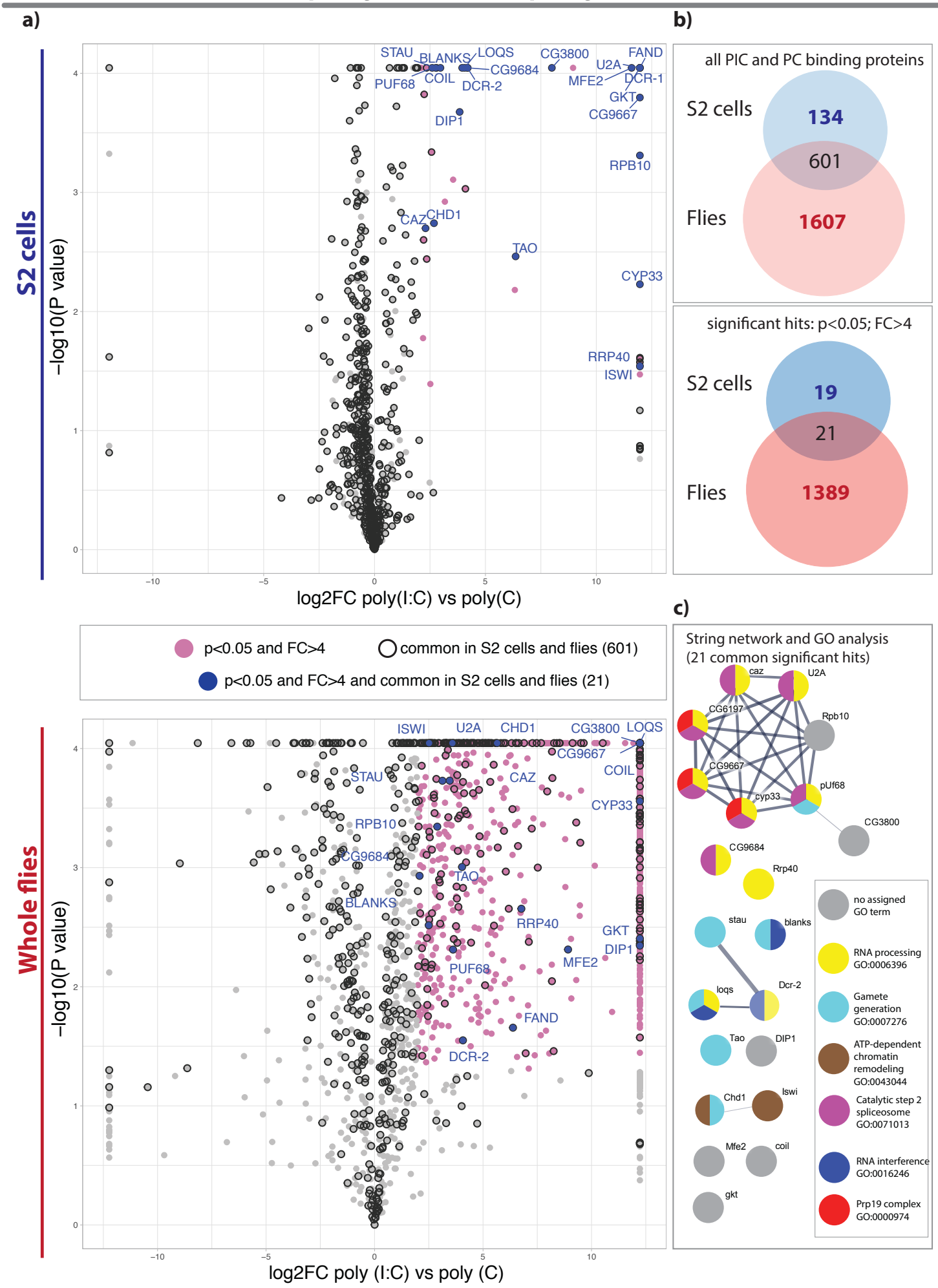


Figure 24. Drosophila proteins, binding to poly (I:C) vs poly(C). **a)** Volcano plots of AP-MS/MS of individual poly(I:C) vs poly(C)-interacting proteins. S2 cells (top) and whole fly (bottom) extracts were used as samples. Each volcano plot displays all identified proteins (mean log2(fold change) of bait (poly(I:C)) protein enrichment (to the right) to the control (poly(C))(to the left) against the corresponding -log10(P value). Cut-offs used to identify the significant hits: log2(fold change)>2 and P value<0.05. Significant hits, specific for S2 or whole fly samples are shown in pink circles. Significant hits, which are found in data with both samples, are indicated in blue and are labeled with gene names. All interacting proteins, common for S2 and whole fly samples, are indicated by black edge of the circle. n = 3 independent experiments, significance testing (P values) results from multiple t-test analysis (FDR(Q)=1%). **b)** Venn diagrams showing the specific and common interacting proteins: all binders (top) and significant hits (bottom). Number of proteins found in S2 cells (blue) and whole flies (red) samples are shown.**c)** Network and gene ontology analysis of 21 common significant hits, preferentially binding poly (I:C) in S2 cells and flies. Protein interaction is indicated by grey lines (thickness corresponds to the confidence measure of data indicating the interaction). GO terms are indicated by colors. PPI enrichment p-value: 3.11e-11, meaning that the obtained network has significantly more interactions compared to the whole genome. Analysis performed in STRING v.10.5.

Table 3. Human orthologues of 21 proteins, identified in S2 cells and whole flies, interacting with poly(I:C). Analysis performed using DIOPT v7.1 - DRSC Integrative Ortholog Prediction Tool.

Fly Symbol	FlyBase ID	Human ortholog(s)	Rank
stau	FBgn0003520	STAU1, STAU2	high
Iswi	FBgn0011604	SMARCA1, SMARCA5	high
DIP1	FBgn0024807	ADARB1	moderate
cyp33	FBgn0028382	PPIE	high
hfp	FBgn0028577	PUF60	high
Mfe2	FBgn0030731	HSD17B4	high
Tao	FBgn0031030	TAOK1, TAOK2, TAOK3	high
loqs	FBgn0032515	PRKRA, TARBP2, STAU1, STAU2	high
U2A	FBgn0033210	SNRPA1	high
coil	FBgn0033265	COIL	low
fand	FBgn0033859	XAB2	high
Dcr-2	FBgn0034246	DICER1	moderate
CG3800	FBgn0034802	CNBP, ZCCHC13, ZCCHC9	high
blanks	FBgn0035608	ADARB1, ADARB2	low
CG9667	FBgn0037550	ISY1-RAB43	high
CG9684	FBgn0037583	TDRD1, SMYD1, SMYD3	low
Rpb10	FBgn0039218	POLR2L	high
Chd1	FBgn0250786	CHD2, CHD1	high
Rrp40	FBgn0260648	EXOSC3	high
gkt	FBgn0260817	TDP1	high
caz	FBgn0285954	FUS, EWSR1	high

II.2. ssRNA-PPP vs ssRNA-OH binding proteins in whole fly and S2 cell extracts

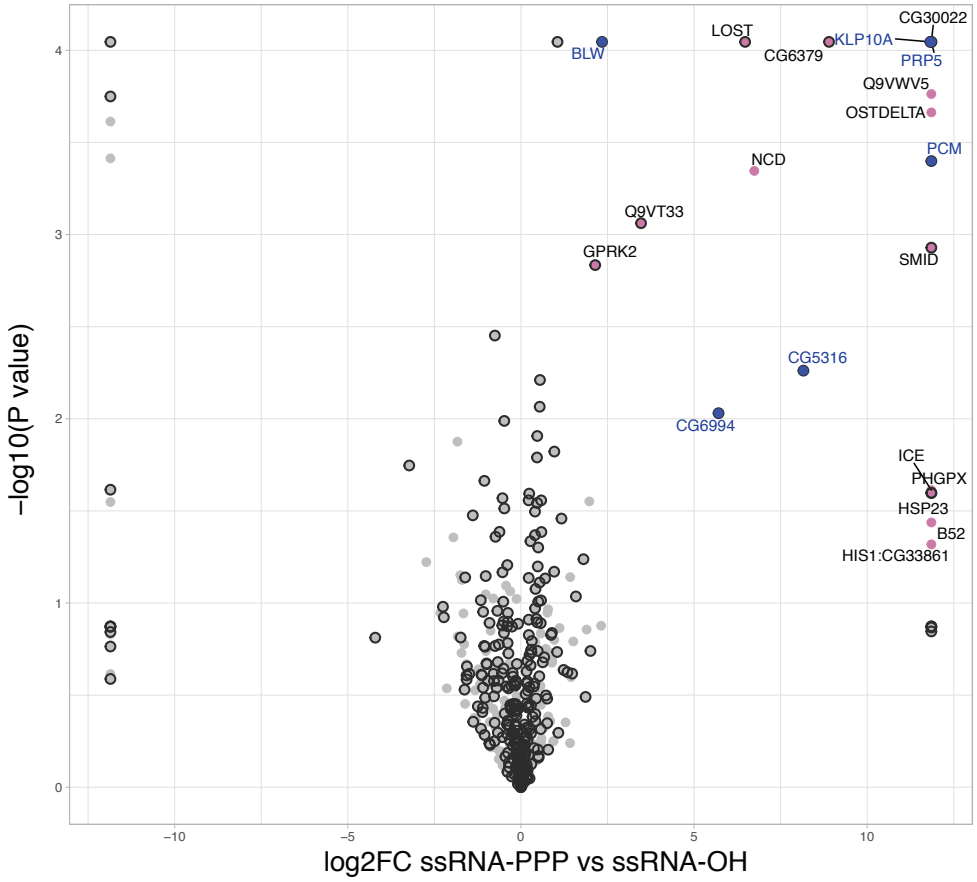
A total of 1029 proteins were found binding to ssRNA-PPP and ssRNA-OH in Drosophila samples (Figure 25a,b). As expected, more proteins were identified in whole fly sample as opposed to S2 cells. 334 of them were identified both in S2 cells and whole fly samples. 193 were S2 specific, whereas 502 were found only in the experiment with whole fly as a sample. I selected significant hits, binding preferentially to ssRNA-PPP with fold change more than four. While there were 20 significant hits found in S2 cells, there were 47 proteins found in whole fly sample. Only 6 of them were common with S2 cells. All of these six proteins have predicted orthologues in human (Table 4). Interestingly, two of these proteins are DEXD/H helicase proteins, which are known to be involved in several aspects of RNA metabolism and viral RNA sensing (Q. Zheng et al. 2017; Milek et al. 2017). Then, I analyzed the 61 significant hits using STRING network and enrichment analysis (Figure 25c). As a result, there were several networks enriched in this group of proteins.

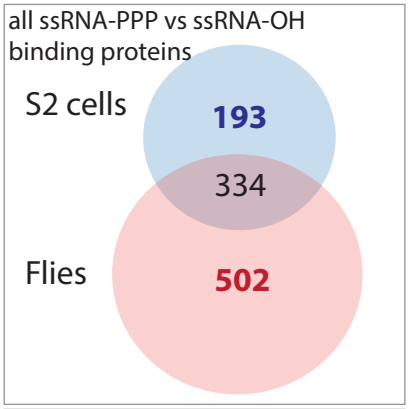
First, there are 10 proteins tightly interacting in one network mostly assigned to two GO terms: ATP synthesis-coupled proton transport and phosphate-containing compound metabolic process (sun, I(1)G0230, mt:ATPase8, ATPsyn-d, blw, ATPsyn-b, ATPsyn-Cf6,Oscp, CG4692, I(2)06225). CG7920 and CG8360 were not assigned to any enriched term, however, they bind to the network members. CG32549 and CG6767 do not bind any of the proteins within the network, but are also assigned to one of the two GO terms (phosphate-containing compound metabolic process). Of these 14 proteins, only blw was found in both S2 cells and whole fly samples. CG6767 and CG32549 were specific for S2 cells. Other proteins were specific for whole fly.

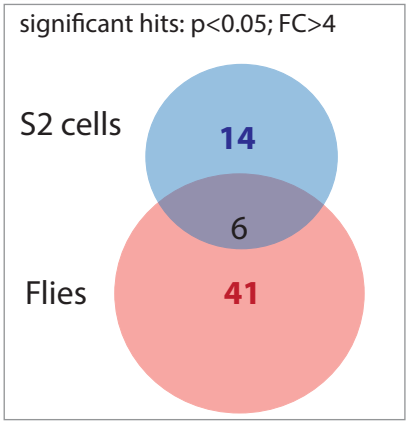
A second group of proteins contains 8 nodes, which are interconnected, and other 27 hits, which bind some of the members of the network. In terms of function, these proteins are enriched for cell development, mRNA processing and response to stress. As for the structure, there are ten proteins with RNA recognition motif, four proteins with DEAD/DEAH box helicase domain, four interconnected proteins with kinesin motor domain and two interacting with XRN 5-3 exonuclease domain.

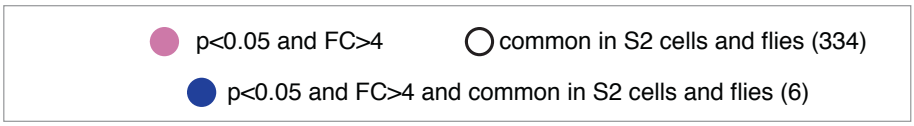
ssRNA-PPP vs ssRNA-OH

S2 cells

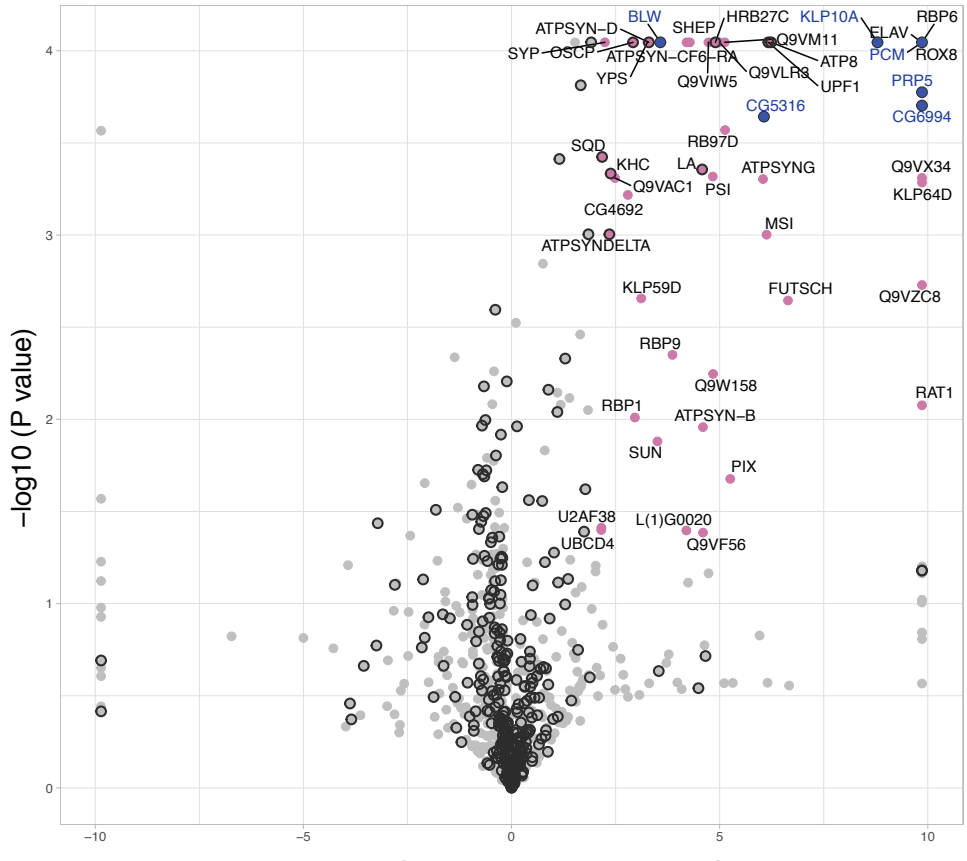








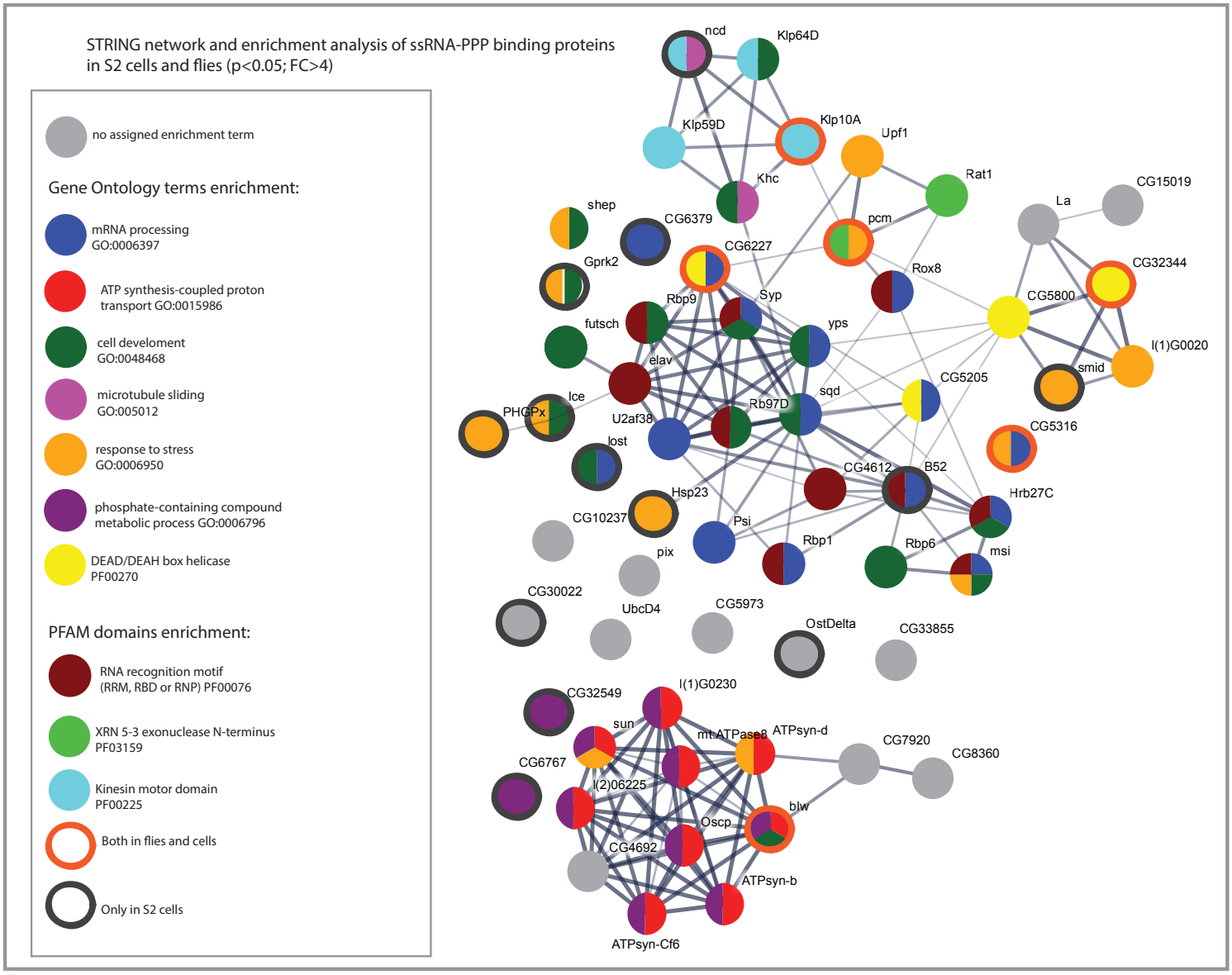
Whole flies



Fly Symbol	Human Symbol	Rank
blw	ATP5F1A	high
Klp10A	KIF2A	high
CG6227	DDX46	high
pcm	XRN1	high
CG5316	APTX	high
CG32344	DDX54	high

Table 4. Human orthologues of 6 proteins, identified in S2 cells and whole flies, interacting with ssRNA-PPP. Analysis performed using DIOPT v7.1.

Figure 25. Drosophila proteins, binding to ssRNA-PPP vs ssRNA-OH. **a)** Volcano plots of AP-MS/MS of individual ssRNA-PPP vs ssRNA-OH-interacting proteins. S2 cells (top) and whole fly (bottom) were used as samples. Each volcano plot displays all identified proteins (mean log2(fold change) of bait (ssRNA-PPP) protein enrichment (to the right) to the control (ssRNA-OH)(to the left) against the corresponding -log10(P value). Cut-offs used to identify the significant hits: log2(fold change)>2 and P value<0.05. Significant hits, specific for S2 or whole fly samples are shown in pink circles. Significant hits, which are found in data with both samples, are indicated in blue and are labeled with gene names. All interacting proteins, common for S2 and whole fly samples, are indicated by black edge of the circle. n = 3 independent experiments, significance testing (P values) results from multiple t-test analysis (FDR(Q)=1%). **b)** Venn diagrams showing the specific and common interacting proteins: all binders (top) and significant hits (bottom). Number of proteins found in S2 cells (blue) and whole flies (red) samples are shown.**c)** Network and gene ontology analysis of 61 significant hits, preferentially binding ssRNA-PPP in S2 cells and flies. Protein interaction is indicated by grey lines (thickness corresponds to the confidence measure of data indicating the interaction). GO terms are indicated by colors. Obtained network has significantly more interactions compared to the whole genome. Analysis performed in STRING v.10.5. The proteins found significant only in S2 cells, are outlined black; in both samples - in orange. The rest of proteins are found specifically in whole flies sample.



The remaining eight proteins are not predicted to bind to any of the stated networks.

As already stated, there are only six significant binders in common in S2 cells and flies, and they represent each five different networks. The enrichment that gives the information on possible pathways implicated in binding to ssRNA-PPP comes mostly from the proteins found exclusively in whole flies.

II.3 ssRNA-CAP and ssRNA-CAP0 vs ssRNA-CAP1 binding proteins in whole fly and S2 cell extracts

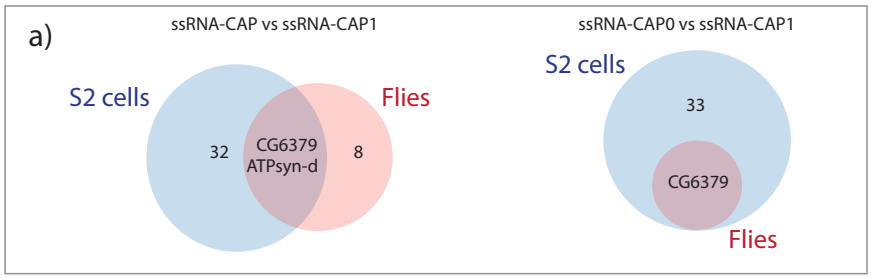
Newly synthesized cellular RNA is marked by RNA polymerases at the 5' end to enable further processing and export into the cytoplasm for translation. This modification includes the addition of inverted guanine nucleotide cap, which is methylated at N7-position, as well as methylation of the first ribose at 2'O position (CAP1) (Topisirovic et al. 2010). Therefore, the lack of N7 and/or 2'O methylation makes viral RNAs vulnerable to host recognition (Habjan et al. 2013; Daffis et al. 2010).

ssRNA-CAP (lacking both N7 and 2'O methylation) and ssRNA-CAP0 (lacking 2'O methylation) were therefore used as bait and compared to normal mRNA mimic ssRNA-CAP1. Another bait of the same group is ssRNA-CAP2, which in addition to two mentioned methylation marks also has 2'O methyl group on the second ribose. Surprisingly, however, the comparison of ssRNA-CAP2 to ssRNA-CAP1 did not yield in any significant hits (data not plotted).

Another intriguing observation is that for comparison of ssRNA-CAP or ssRNA-CAP0 with ssRNA-CAP1, the number of significant proteins in whole fly sample was lower than in cells (Figure 26a). When we combine the 4 conditions (cells vs flies), we found one common protein – CG6379 (Figure 26ab). This protein is an S-adenosyl-L-methonine-dependent methyltransferase that mediates 2'O-ribose methylation of mRNA ((Gaudet et al. 2011). It is a Drosophila orthologue of Cap methyltransferase 1 (CMTR1) (Table 5).

Another significant common hit found in three of the four conditions is ATPsyn-delta, which is involved in ATP synthesis-coupled proton transfer and is an orthologue of human ATP5F1D.

Significant proteins binding ssRNA-CAP and ssRNA-CAP0 vs CAP1 in S2 cells and flies



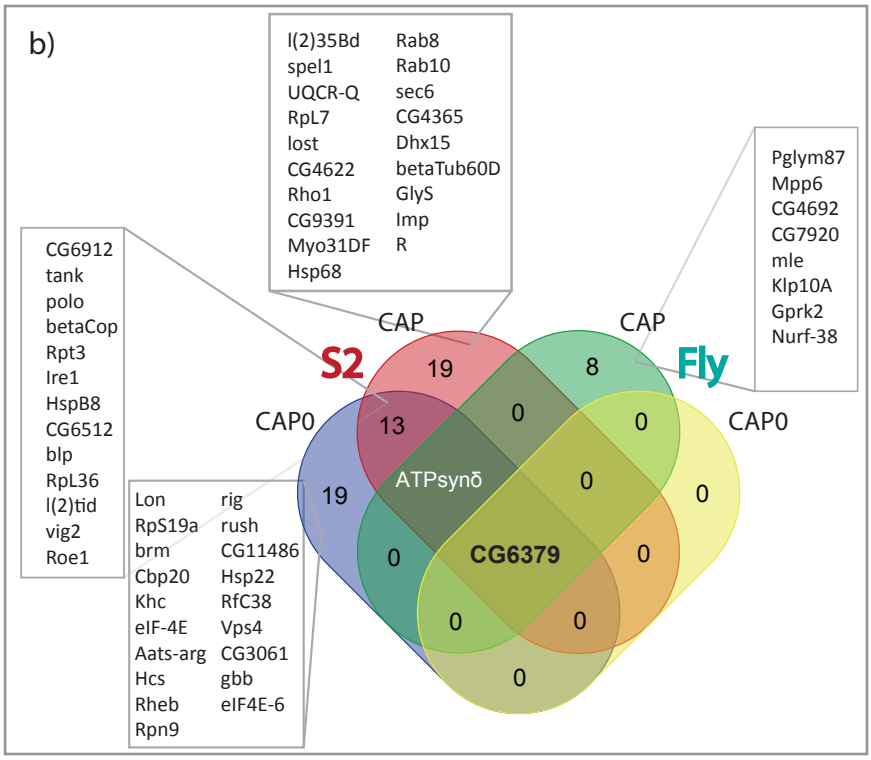


Figure 26. Drosophila proteins, preferentially binding to ssRNA-CAP and ssRNA-CAP0 vs ssRNA-CAP1 (only significant hits: p value<0.05, fc>2)a) Venn diagrams showing the specific and common interacting proteins in S2 cells (blue) and whole flies (red). Names of common proteins are indicated. b) Venn diagram combining the data for ssRNA-CAP and ssRNA-CAP0 binding proteins in S2 and flies. CG6379 is the only common protein for all four conditions. **c)** Network and gene ontology analysis of 61 significant hits, preferentially binding ssRNA-CAP and ssRNA-CAP0 in S2 cells and flies. Protein interaction is indicated by grey lines (thickness corresponds to the confidence measure of data indicating the interaction). GO terms are indicated by colors. Obtained network has significantly more interactions compared to the whole genome. Analysis performed in STRING v.10.5.

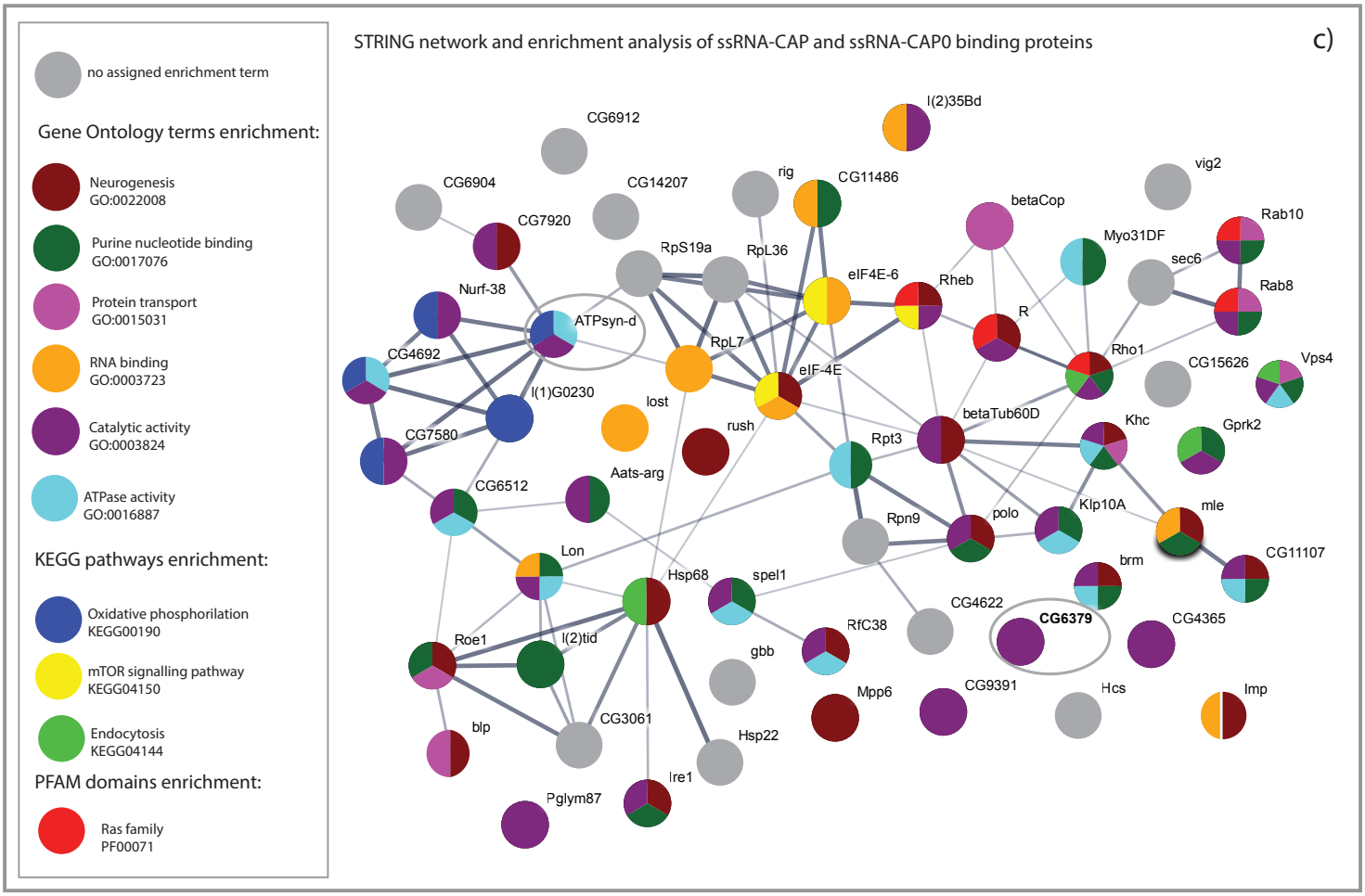


Table 5. Human orthologues of 61 proteins, identified in S2 cells and whole flies, interacting preferentially with ssRNA-CAP and ssRNA-CAP0 vs ssRNA-CAP1.
Analysis performed using DIOPT v7.1 - DRSC Integrative Ortholog Prediction Tool.

Fly GeneID	Human Symbol
CG6379	CMTR1
ATPsyndelta	ATP5F1D
CG6912	orthologues in insects
tank	EI24
polo	PLK1
betaCop	COPB1
Rpt3	PSMC4
Ire1	ERN1, ERN2
HspB8	CRYAB, HSPB1
CG6512	AFG3L2, SPG7
blp	PAM16
RpL36	RPL36
l(2)tid	DNAJA3
vig2	SERBP1, HABP4
Roe1	GRPEL1,GRPEL2
Lon	LONP1
RpS19a	RPS19
brm	SMARCA2
Cbp20	NCBP2
Khc	KIF5B
eIF-4E	EIF4E
Aats-arg	RARS
Hcs	HLCS
Rheb	RHEB
Rpn9	PSMD13
rig	GEMIN5
rush	PLEKHF2
CG11486	PAN3
Hsp22	HSPB6
RfC38	RFC3
Vps4	VPS4A

Fly GeneID	Human Symbol
CG3061	DNAJB12
gbb	BMP7
eIF4E-6	EIF4E1B
l(2)35Bd	RNMT
spel1	MSH2
UQCR-Q	UQCRQ
RpL7	RPL7
lost	MTHFSD
CG4622	ZCCHC8
Rho1	RHOA
CG9391	IMPA1
Myo31DF	MYO1D
Hsp68	HSPA1B
Rab8	RAB8A
Rab10	RAB10
sec6	EXOC3
CG4365	HAGH
Dhx15	DHX15
betaTub60D	TUBB6
GlyS	GYS1
Imp	IGF2BP2
R	OPN4
Pglym87	PGAM2
Mpp6	MPHOSPH6
CG4692	ATP5MF
CG7920	orth-gs in Arthropods
mle	DHX9
Klp10A	KIF2A
ATPsyn-d	ATP5PD
Gprk2	GRK5
Nurf-38	PPA1

In addition, there are 13 proteins in common preferentially binding ssRNA-CAP and ssRNA-CAP0 in S2 cells.

A total number of 61 significant proteins were identified from all four conditions. Analysis of these proteins in STRING network and enrichment analysis revealed that most of the proteins form a large network with some enriched ontology terms. Five of them are interconnected and implicated in oxidative phosphorylation (including ATPsyn-delta). Nine proteins are related to RNA binding. There are also five proteins of the Ras family and three proteins involved in the mTOR signaling pathway. (Figure 26c)

Most of the 61 proteins have orthologues in human (Table 5). However, two proteins do not: CG6912, which is specific for the insect lineage and CG7920, which is found in other Arthropod species. These two proteins may represent a phyla-specific innovation in recognition of CAP structures.

II.4. dsISD vs ssISD binding proteins in S2 cells and flies

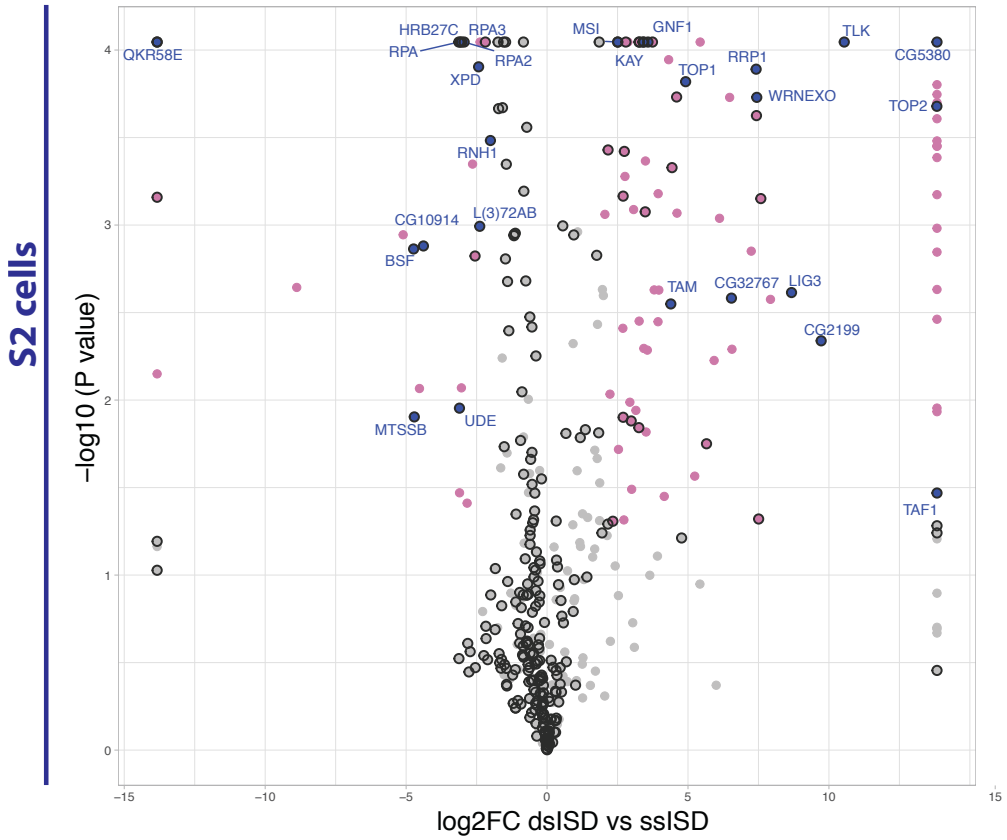
Single of double-stranded interferon stimulating DNA transfection has been widely used to study the response to DNA viral infection (Stetson and Medzhitov 2006). It was also shown, that both ssISD and dsISD induce metabolic stress, independent of the presence of known DNA sensors cGAS and STING (M. Zheng et al. 2015).

Our AP-MS experiments comparing dsISD and ssISD revealed a total of 1167 proteins were found. Most of them were identified in whole fly sample with the overlap of 267 proteins in S2 cells and flies. It was shown that some DNA viruses, such as HIV, may have viral-derived DNA in both ss and ds form inside the host cell. Therefore, I have used the two cut offs: 4 fold enrichment and 4 fold depletion. This way we can see both the proteins specific for ssISD and for dsISD. The number of significant hits ($p<0.05$ and fold change>4 and <0.25) was bigger in S2 cells sample compared to whole fly, with the intersection of 26 proteins (Figure 27ab).

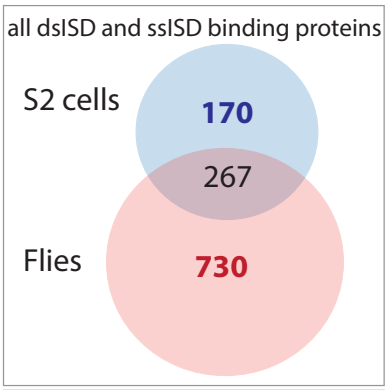
I further analyzed these 26 common significant hits for the presence of enriched networks and ontology terms (Figure 27c). Most of them are grouped in one large network, related to DNA repair, DNA replication and stress response. On the other hand, there are some proteins, not binding to other hits and with unknown function. All of the 26 proteins have one or several orthologues in human except

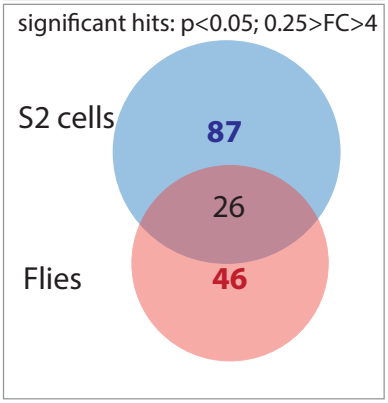
a)

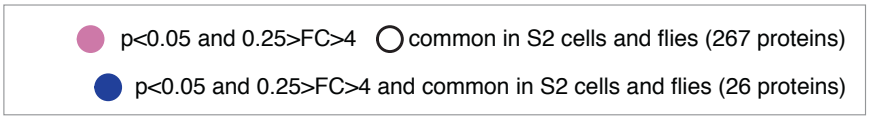
dsISD vs ssISD binding proteins

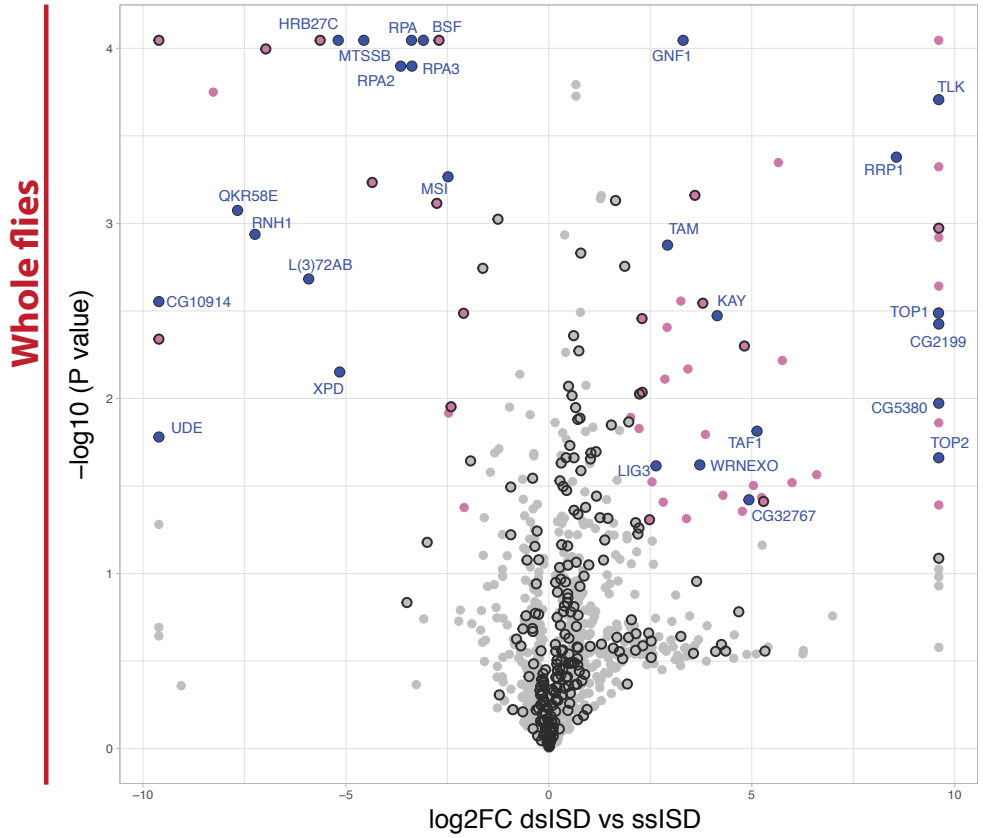


b)









c)

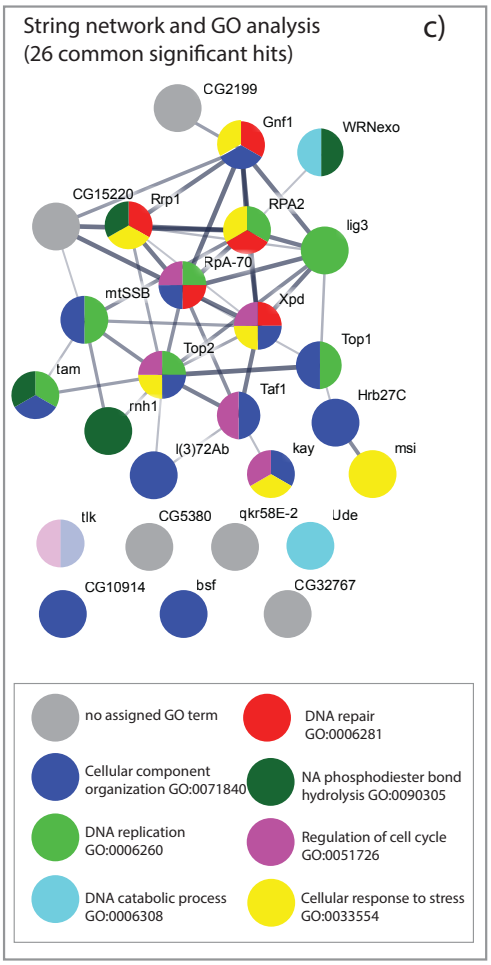


Figure 27. Drosophila proteins, binding to dsISD vs ssISD. **a)** Volcano plots of AP-MS/MS of individual dsISD and ssISD-interacting proteins. S2 cells (top) and whole fly (bottom) extracts were used as samples. Each volcano plot displays all identified proteins (mean log2(fold change) of bait (dsISD) protein enrichment (to the right) to the control (ssISD)(to the left) against the corresponding -log10(P value). Cut-offs used to identify the significant hits: 0.25>fold change>4 and P value<0.05. Significant hits, specific for S2 or whole fly samples are shown in pink circles. Significant hits, which are found in data with both samples, are indicated in blue and are labeled with gene names. All interacting proteins, common for S2 and whole fly samples, are indicated by black edge of the circle. n = 3 independent experiments, significance testing (P values) results from multiple t-test analysis (FDR(Q)=1%). **b)** Venn diagrams showing the specific and common interacting proteins: all binders (top) and significant hits (bottom). Number of proteins found in S2 cells (blue) and whole flies (red) samples are shown.**c)** Network and gene ontology analysis of 26 common significant hits, binding dsISD and ssISD in S2 cells and flies. Protein interaction is indicated by grey lines (thickness corresponds to the confidence measure of data indicating the interaction). GO terms are indicated by colors. PPI enrichment p-value: 3.95e-13, meaning that the obtained network has significantly more interactions compared to the whole genome. Analysis performed in STRING v.10.5.

Table 6. Orthologue analysis of 26 significant hits, binding to dsISD and ssISD. Proteins UDE does not have predicted orthologues in mammals, but has orthologues in Arthropod species. Human orthologues of 25 other proteins are shown in the table. Analysis performed using DIOPT v7.1 - DRSC Integrative Ortholog Prediction Tool.

Gene ID	FlyBaseID	Human Symbol	Rank
TLK	FBgn0283657	TLK2, TLK1	high
CG2199	FBgn0035213	TP63	low
CG5380	FBgn0038951	POLR3F	high
TOP2	FBgn0284220	TOP2A, TOP2B	high
TOP1	FBgn0004924	TOP1, TOP1MT	high
RRP1	FBgn0004584	APEX1, APEX2	high
TAF1	FBgn0010355	TAF1	high
CG32767	FBgn0052767	ZNF485	low
KAY	FBgn0001297	FOSL2, FOS, FOSL1	high
WRNEXO	FBgn0038608	WRN	high
GNF1	FBgn0004913	RFC1	high
TAM	FBgn0004406	POLG, TJP2, TJP1	high
LIG3	FBgn0038035	LIG3	high
MSI	FBgn0043025	ADA2, MSI1, MSI2, DAZAP1	moderate
BSF	FBgn0284256	LRPPRC	high
RPA3	FBgn0266421	RPA3	high
RPA	FBgn0010173	RPA1	high
RPA2	FBgn0032906	RPA2, RPA4	high
MTSSB	FBgn0010438	SSBP1	high
XPD	FBgn0261850	ERCC2	high
HRB27C	FBgn0004838	DAZAP1, MSI2, MSI1, HNRNPA3	high
L(3)72AB	FBgn0263599	SNRNP200	high
RNH1	FBgn0023171	RNASEH1	high
CG10914	FBgn0034307	NOA1	high
UDE	FBgn0039226	none, orthologues in Arthropods	
qkr58E-2	FBgn0022985	KHDRBS3	moderate

uracil-DNA degrading factor (UDE), which is conserved in insects and sea urchin (Table 6).

II.5. 2'-5'oligoAdenylate binding proteins across species

The OAS pathway is one of the key mammalian pathways for recognition of viral dsRNA. 2'5'oligoAdenylates are formed as intermediate messenger and activate RNase L dimers to degrade viral RNAs. In drosophila, there are no OAS proteins and no RNase L (Hornung et al. 2014). Therefore, we were curious to see what molecules could be found to specifically bind 2'5'oA and how it would compare with the mammalian system. ATP was used as control ligand.

Overall, 1114 proteins were identified in S2 cells and whole flies, with 355 of them in common for both samples (Figure 28ab). After selection of significant hits ($p<0.05$ and fold change > 4) 44 proteins were identified. The majority was found from the whole fly sample with only 2 proteins common in flies and cells. STRING analysis of the 44 proteins revealed very specific enrichment for only 4 proteins (CG1703, CG9330, Pixie and CG3164), belonging to the same network and also the same family of proteins: ATP-binding cassette (ABC) transporters (Figure 28c). It is striking that both of the common hits - CG9330 and Pixie - belong to the same family.

Experiments with the same bait in human THP1 and mouse RAW2 cells have, as expected, identified RNase L as the major hit. Interestingly, members of ABC transporter family were also found: ABCF1 (orthologue of CG1703) and ABCF3 (orthologue of CG9330) and TAP1 (Figure 29). In chicken HD11 cells the major hits are also Rnase L and ABCF2, and in *C. elegans* (system lacking OAS pathway) among 2'5'oA binding proteins there are also abcf-2, abcf-3 (data not plotted).

ATP transporters are a large and ancient superfamily of transporters, which is conserved from prokaryotes to humans. They share ATP-binding cassette domain and are divided into three major group: importers and exporters implicated in transport of various substrates, and a third group playing role in translation and DNA repair (Davidson et al. 2008). They are also known to contribute to individual variability in drug resistance, and therefore a known target of pharmaceutical development (Choi and Yu 2014). The candidate proteins that preferentially bind 2'5'oA belong to ABCF and ABCE subfamily, which do not encode transmembrane domains, but are implicated in translation regulation (Paytubi et al. 2009; Barthelme et al. 2011). Of note, ABCE1 has been long known to inhibit the function of RNase L

2’5’oA vs ATP

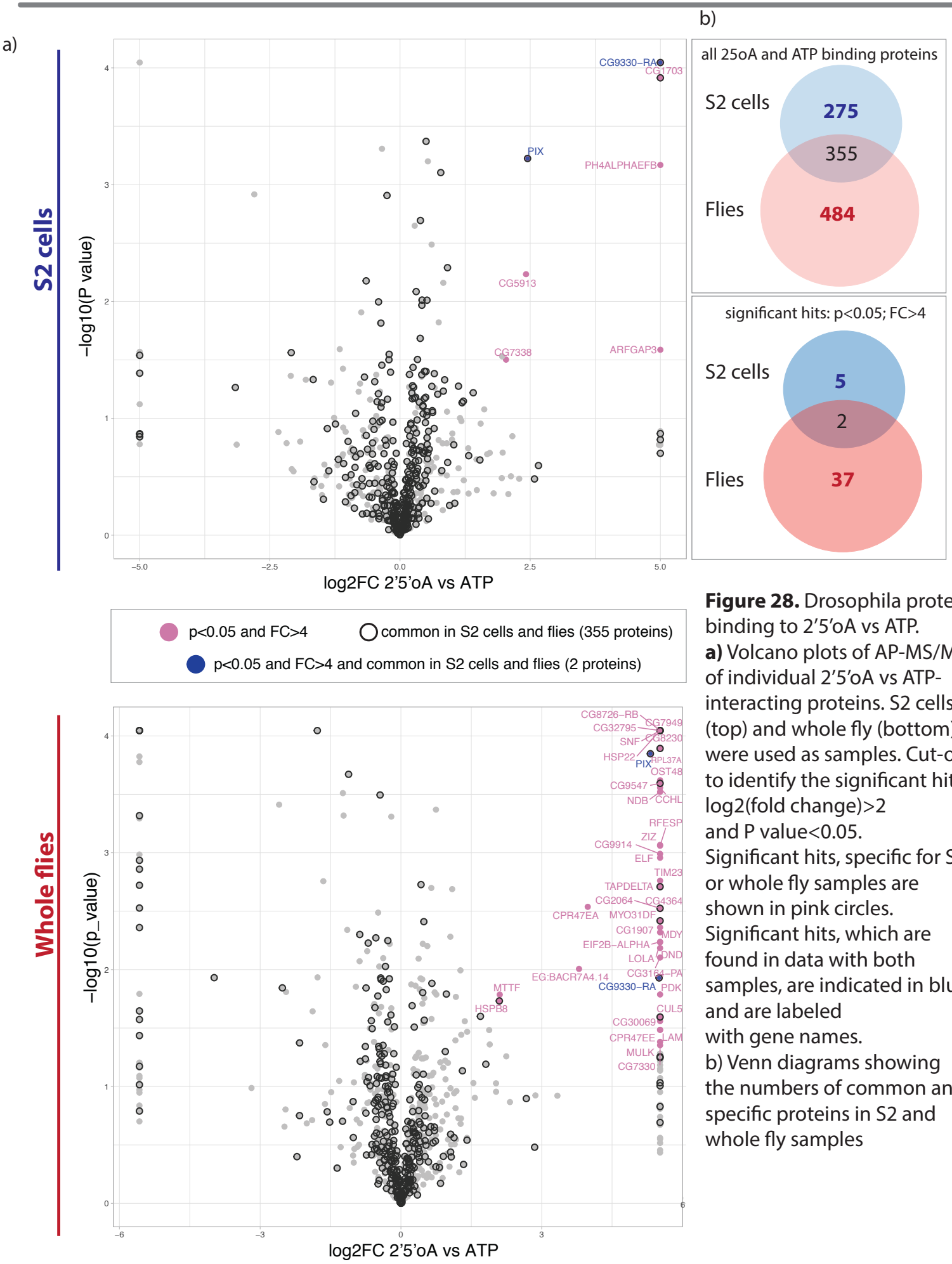
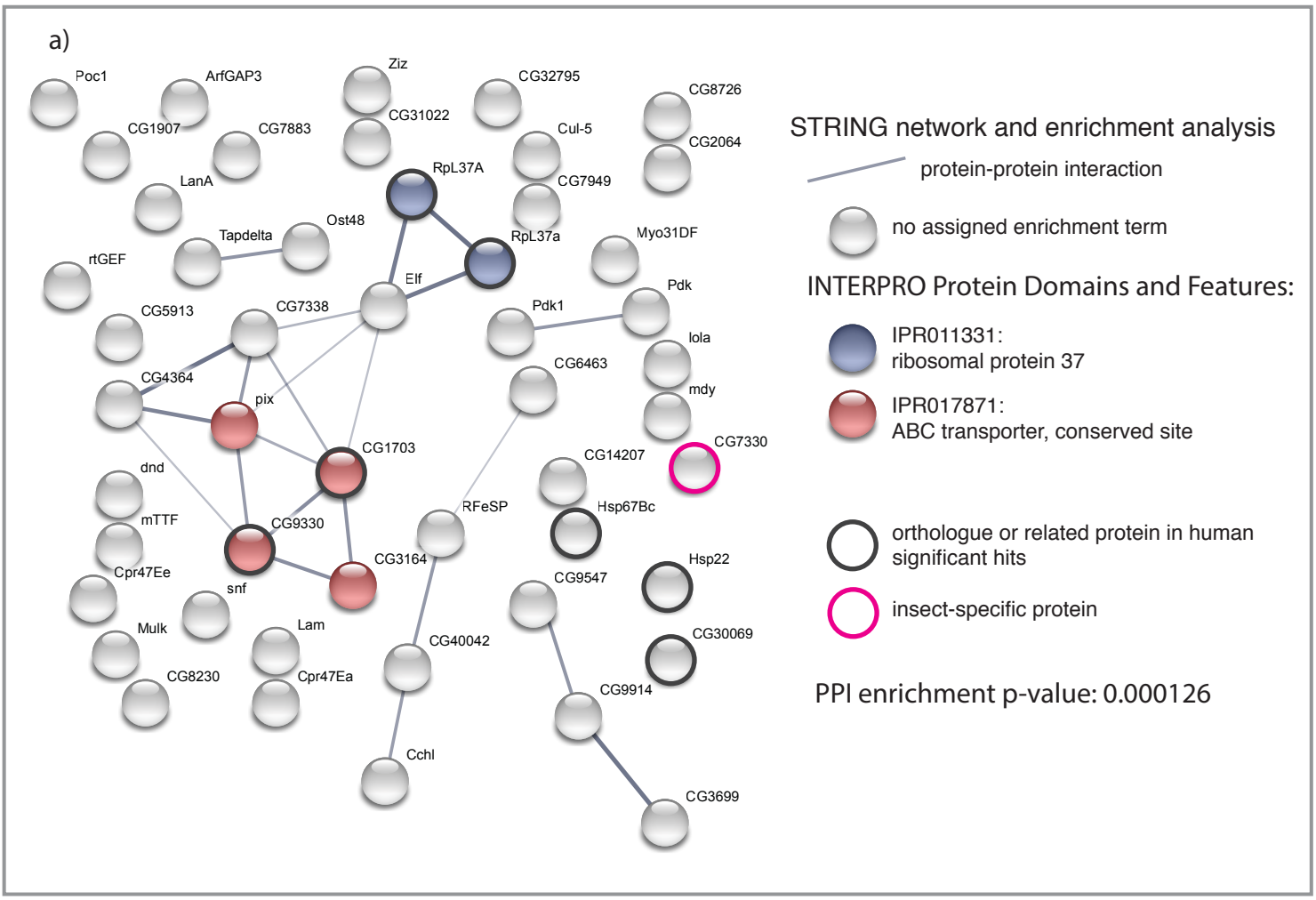
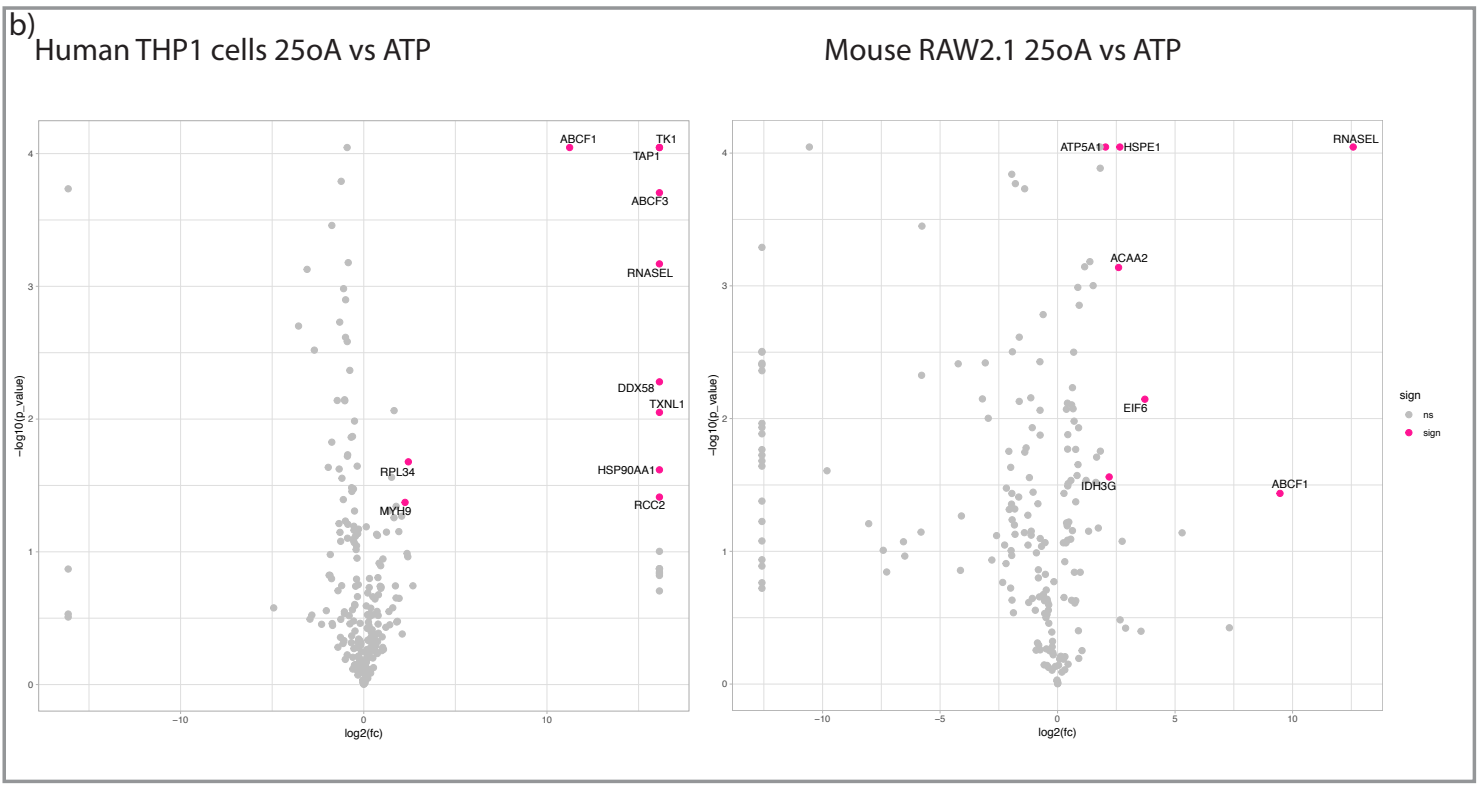


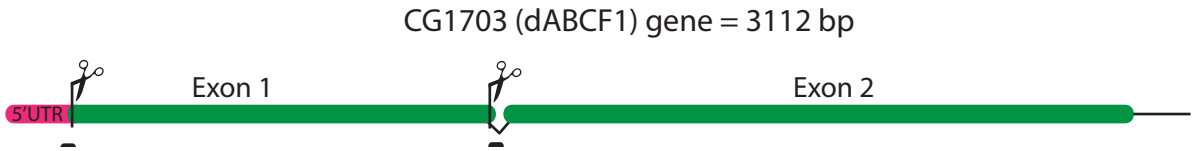
Figure 28. Drosophila proteins, binding to 2’5’oA vs ATP.
a) Volcano plots of AP-MS/MS of individual 2’5’oA vs ATP-interacting proteins. S2 cells (top) and whole fly (bottom) were used as samples. Cut-offs to identify the significant hits: log2(fold change)>2 and P value<0.05. Significant hits, specific for S2 or whole fly samples are shown in pink circles. Significant hits, which are found in data with both samples, are indicated in blue and are labeled with gene names.
b) Venn diagrams showing the numbers of common and specific proteins in S2 and whole fly samples

Figure 29. ABC transporter family is implicated in 2′5′oA binding across species. a) STRING analysis of 44 significant binding proteins in Drosophila. 4 members of ABC transporter family are shown in red. CG7330 is the only protein, not conserved in humans. b) Volcano plots showing 2′5′oA binding proteins in human and mouse cells. RNase L, known to be activated by 2′5′oA, is found as a significant hit. Among other candidates there are ABCF1 and ABCF3.





a)



b)

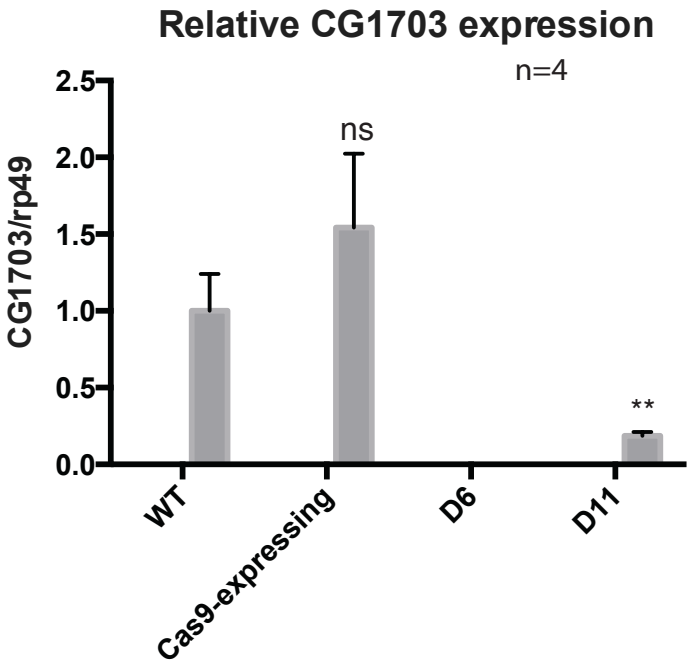


Figure 30. CG1703 CRISPR/Cas9-mediated KO in S2 cells. a) schematic of CG1703 gene, the location of sgRs is indicated. b) CG1703 mRNA level is significanty reduced in KO cell lines.

by preventing its binding to 2'5'oA (Bisbal et al. 1995). In flies, most of the ABC transporter members are conserved. Pixie (ABCE1 orthologue) was also shown to play role in translation and is essential for the development (Andersen and Leevers 2007). CG3164 belongs to ABCG subfamily, together with known genetic markers *white, brown, scarlet* etc., which are known functional transporters (phylogeny of ABC transporters in arthropods is reviewed in (Dermauw and Van Leeuwen 2014)).

Another similarity between mammalian and Drosophila samples is the presence of heat shock proteins and ribosomal proteins in the significant candidate list. Among other candidates in human cells there is a known cytoplasmic viral RNA sensor RIG-I. As for conservation, only one of 44 proteins is not conserved in humans: CG7330, which is specific for insects and has not been studied (Table 7).

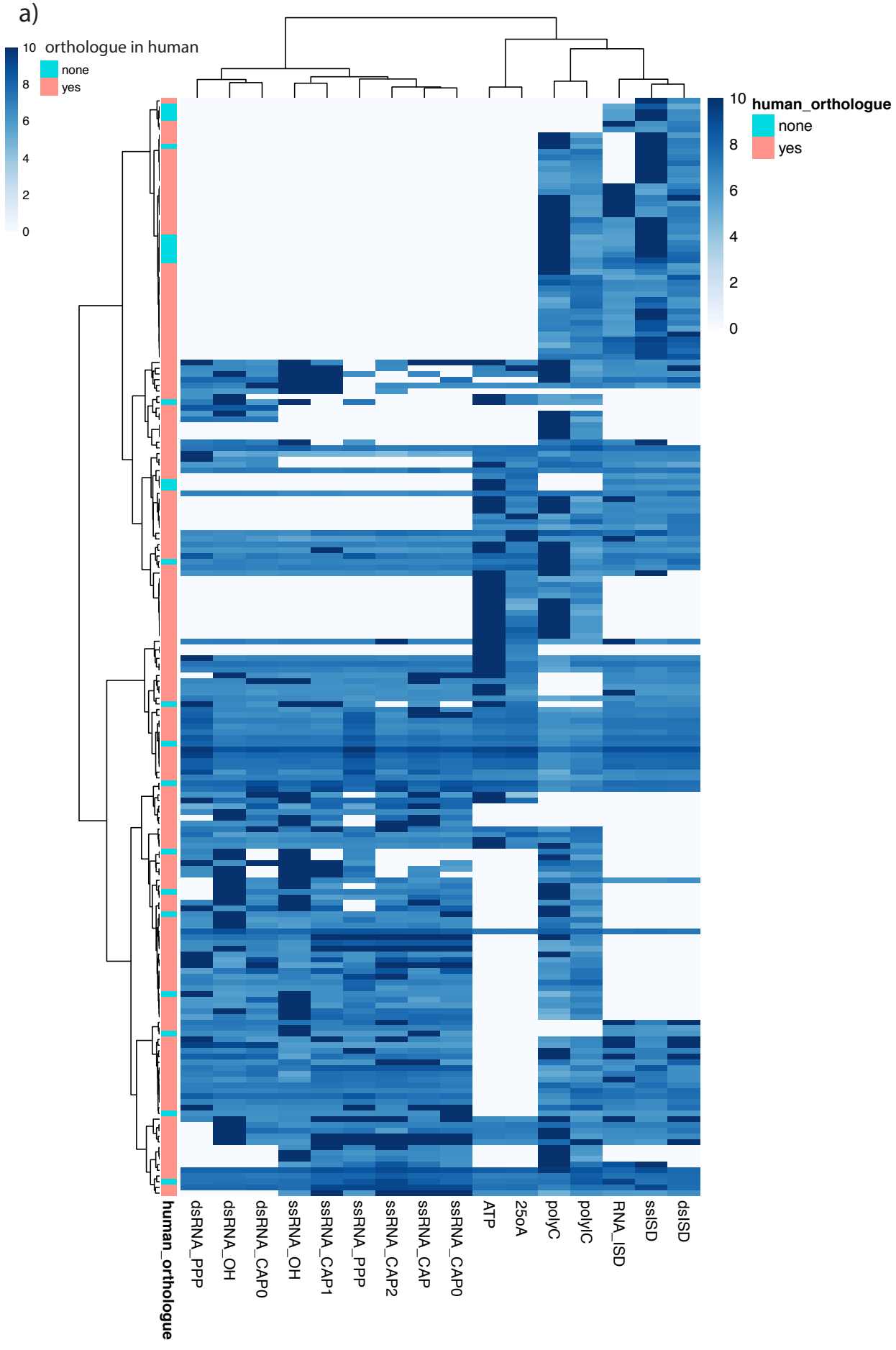
II.6. CRISPR-Cas9 KO of ABC transporters in S2 cells

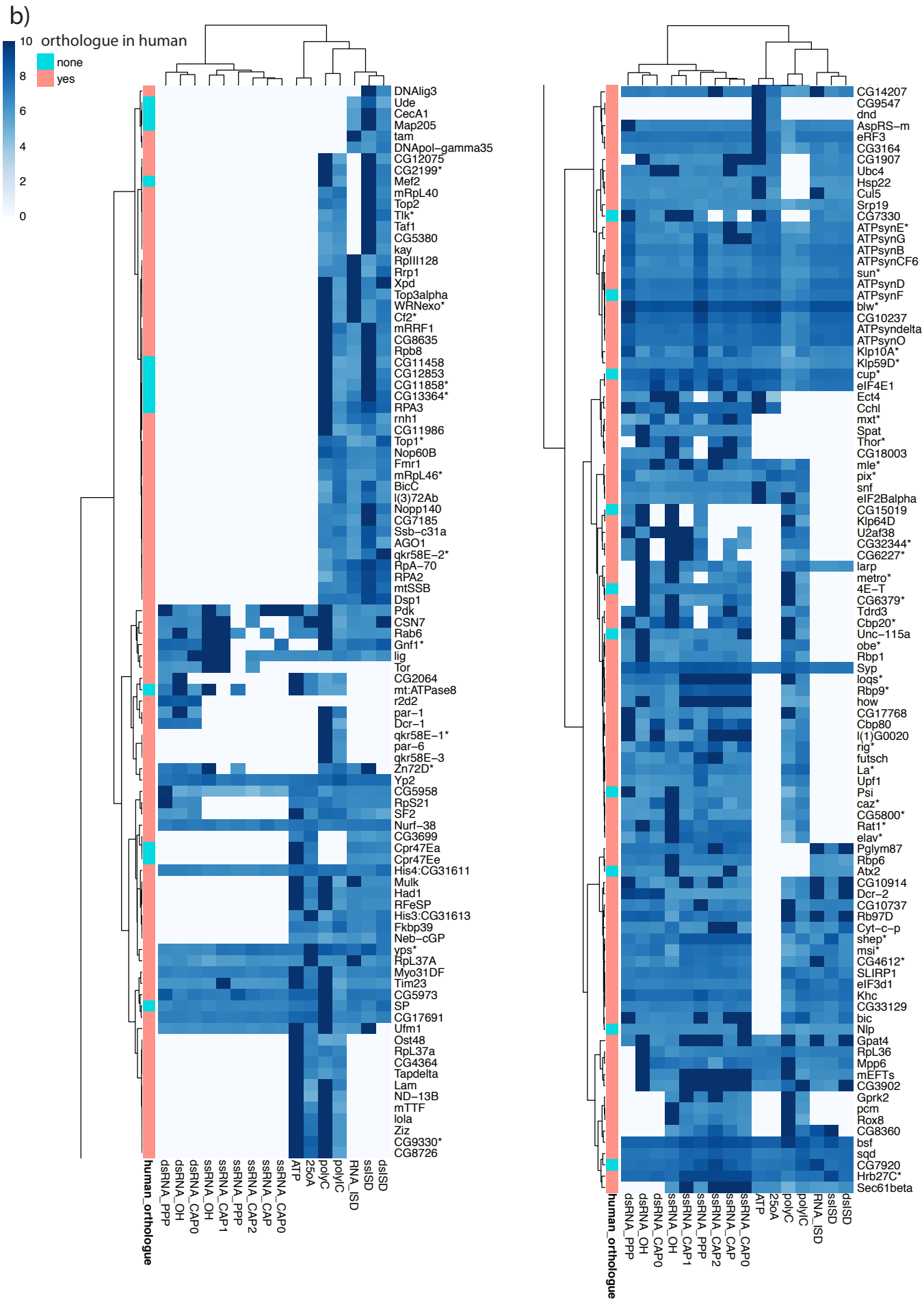
The genes CG1703, CG9330 and CG5651 (Pixie) were identified in the screen with 2'5'oligoadenylate as bait. They encode the orthologues of mammalian proteins ABCF1, ABCF3 and ABCE1 respectively. In order to study their function in antiviral response, I attempted to create CRISPR/Cas9 KO cells of these three proteins, as described previously. I did not recover any cell line for CG9330 and Pixie. This is not surprising, as the proteins are most likely essential in S2 cells. As for CG1703, I have established two mutant cell lines with significantly reduced level of mRNA (Figure 30). In the preliminary experiment, I infected them with DCV, CrPV, VSV and SiNV, however, did not see any phenotype (not shown). More experiments need to be performed using these knock-out cell lines to elucidate possible function of this protein.

II.7. Overview of significant hits identified in whole flies

Next, I aimed to compare different baits and identify the proteins, which bound to several baits. For this, I included all significant proteins found in whole fly sample, except the 1322 proteins which only bind to poly (I:C). This resulted in 203 proteins, which were plotted in a heatmap, showing the LFQ intensity for every bait. As it can be seen, similar baits (such as ssRNAs with different CAP structures) were

Figure 31. Heatmap showing the 203 significant proteins, binding to all baits (except the proteins specific for poly(I:C)).Blue color gradation from 0 to 10 indicates LFQ intensity for each bait. Rows and columns are clustered: tree on the left side shows the proteins with similar binding specificity; tree on the top shows similarity of baits based on the proteins binding to them. a) heatmap without names of proteins b) (next page) the same heatmap, but split in two, showing the names of proteins. Names with * indicate the proteins that were used in the functional screen.

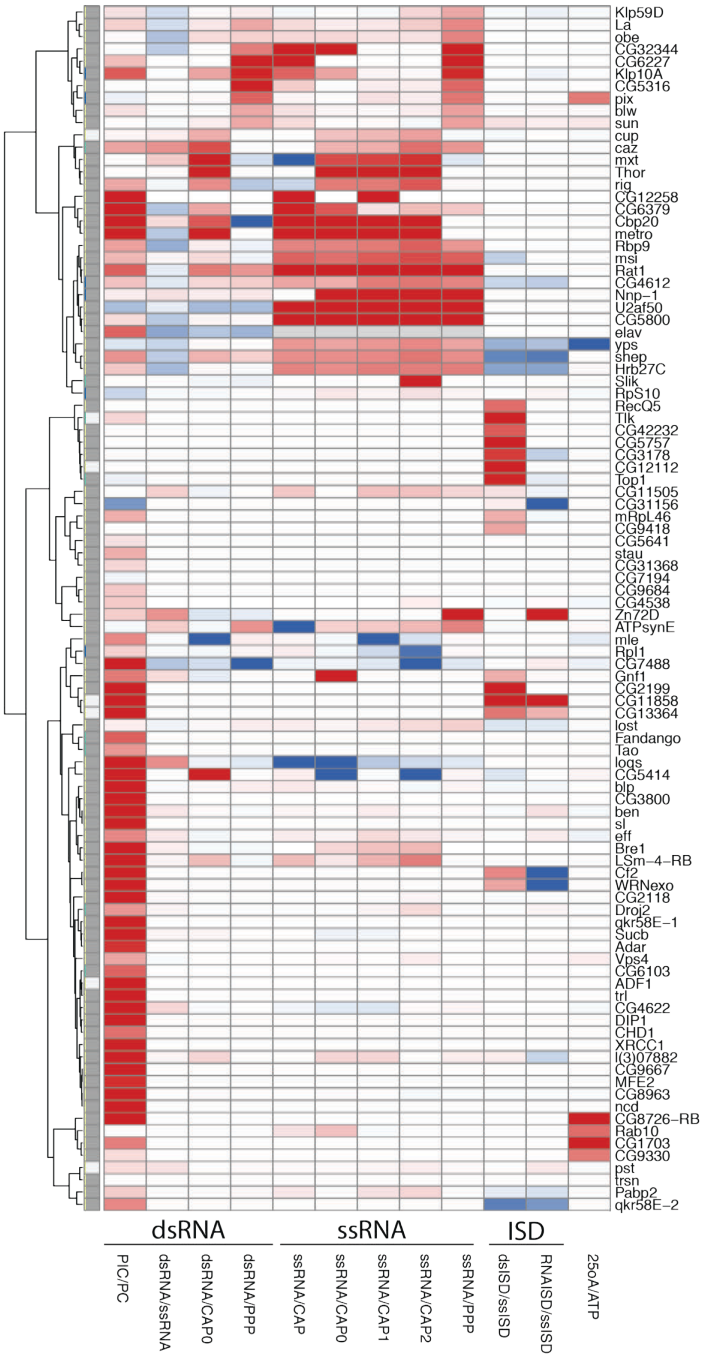



b)
orthologue in human
10
8
6
4
2
0
none
yes
DNAlig3
Ude
CecA1
Map205
tam
DNApol−gamma35
CG12075
CG2199*
Mef2
mRpL40
Top2
Tlk*
Taf1
CG5380
kay
RpIII128
Rrp1
Xpd
Top3alpha
WRNexo*
Cf2*
mRRF1
CG8635
Rpb8
CG11458
CG12853
CG11858*
CG13364*
RPA3
rnh1
CG11986
Top1*
Nop60B
Fmr1
mRpL46*
BicC
l(3)72Ab
Nopp140
CG7185
Ssb−c31a
AGO1
qkr58E−2*
RpA−70
RPA2
mtSSB
Dsp1
Pdk
CSN7
Rab6
Gnf1*
lig
Tor
CG2064
mt:ATPase8
r2d2
par−1
Dcr−1
qkr58E−1*
par−6
qkr58E−3
Zn72D*
Yp2
CG5958
RpS21
SF2
Nurf−38
CG3699
Cpr47Ea
Cpr47Ee
His4:CG31611
Mulk
Had1
RFeSP
His3:CG31613
Fkbp39
Neb−cGP
yps*
RpL37A
Myo31DF
Tim23
CG5973
SP
CG17691
Ufm1
Ost48
RpL37a
CG4364
Tapdelta
Lam
ND−13B
mTTF
lola
Ziz
CG9330*
CG8726
human_orthologue
dsRNA_PPP
dsRNA_OH
dsRNA_CAP0
dsRNA_CAP1
ssRNA_OH
ssRNA_CAP1
ssRNA_PPP
ssRNA_CAP2
ssRNA_CAP
ssRNA_CAP0
ATP
25oA
polyC
polyIC
RNA_ISD
ssISD
dsISD
CG14207
CG9547
dnd
AspRS−m
eRF3
CG3164
CG1907
Ubc4
Hsp22
Cul5
Srp19
CG7330
ATPsynE*
ATPsynG
ATPsynB
ATPsynCF6
sun*
ATPsynD
ATPsynF
blw*
CG10237
ATPsyndelta
ATPsynO
Klp10A*
Klp59D*
cup*
eIF4E1
Ect4
Cchl
mxt*
Spat
Thor*
CG18003
mle*
pix*
snf
eIF2Balpha
CG15019
Klp64D
U2af38
CG32344*
CG6227*
larp
metro*
4E−T
CG6379*
Tdrd3
Cbp20*
Unc−115a
obe*
Rbp1
Syp
logs*
Rbp9*
how
CG17768
Cbp80
l(1)G0020
rig*
futsch
La*
Upf1
Psi
caz*
CG5800*
Rat1*
elav*
Pglym87
Rbp6
Atx2
CG10914
Dcr−2
CG10737
Rb97D
Cyt−c−p
shep*
msi*
CG4612*
SLIRP1
eIF3d1
Khc
CG33129
bic
Nlp
Gpat4
RpL36
Mpp6
mEFTs
CG3902
Gprk2
pcm
Rox8
CG8360
bsf
sqd
CG7920
Hrb27C*
Sec61beta
human_orthologue
dsRNA_PPP
dsRNA_OH
dsRNA_CAP0
dsRNA_CAP1
ssRNA_OH
ssRNA_CAP1
ssRNA_PPP
ssRNA_CAP2
ssRNA_CAP
ssRNA_CAP0
ATP
25oA
polyC
polyIC
RNA_ISD
ssISD
dsISD

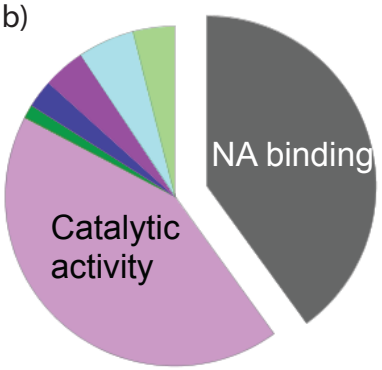
clustered together. Interestingly, however, poly(I:C) clustered with other synthetic baits (dsISD etc) and not with other dsRNA (Figure 31).

Figure 32. 100 candidate proteins, selected for the functional screen, as a result of bioinformatic analysis. a) heatmap, showing the binding to different bait vs control NAs. b) GO terms for the 100 proteins are dominated by NA binding (analysis done in PANTHER).

a)

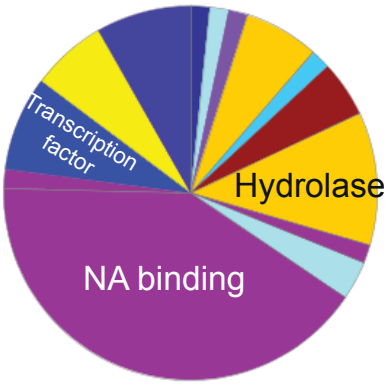


b)



GO molecular function

Signal transduction
Receptor activity
Translation
Transporter



GO protein class

Part III. Functional screen *in vivo* identifies novel players in Drosophila antiviral immunity

III.1. Selection of 100 candidate proteins for the functional screen

Because of time constraints, the AP-MS analysis described above, which involved collaboration with the group of J. Colinge (Montpellier), was done in parallel with the functional screen. Initially, the data from large cross species AP-MS screen was processed by the laboratory of J. Colinge in order to rank the candidates based on enrichment factor and specificity of binding. All hits were also analyzed in terms of evolutionary conservation and available transcriptomics data. As a result of this extensive analysis, the list of top 10% of the candidates was produced for each type of lysate sample (Boulos et al, unpublished).

Out of the total 2495 hits from whole fly and S2 samples, the ranking resulted in 221 proteins in top 10% of the ranked candidates. We have decided to select 100 proteins from these 221 to proceed to the functional screen *in vivo* (Figure 32). A first criterion for the selection was the identification of the protein in both whole flies and S2 cells (20 proteins). Then, comparing the lists of top 10% for different animal species, I chose the proteins, which were conserved in more than two species (another 21 proteins). The remaining proteins were selected according to the GO terms (mostly selecting for the proteins related to nucleic acid binding) and network enrichment (members of the same network would be preferred).

III.2. *In vivo* validation pipeline for the AP-MS analysis

We have developed a functional screen to characterize the candidate proteins. In order to select the approach to examine the function of candidates, we have come up with several criteria. First, the genetic background of the fly lines should be the same or similar. It has been shown that allelic variation largely affects the phenotype, as it arises from complex interactions of the mutation with the genetic environment (reviewed in (Chandler, Chari, and Dworkin 2013) and (Cogni et al. 2016)). Therefore, genetic background check should become a routine measure in fly genetics. For a screen such as ours, with 100 candidate genes, it is therefore not possible to rely on the mutants, generated by different techniques (eg. chemical mutagenesis, X-ray induced chromosomal rearrangements, transposon

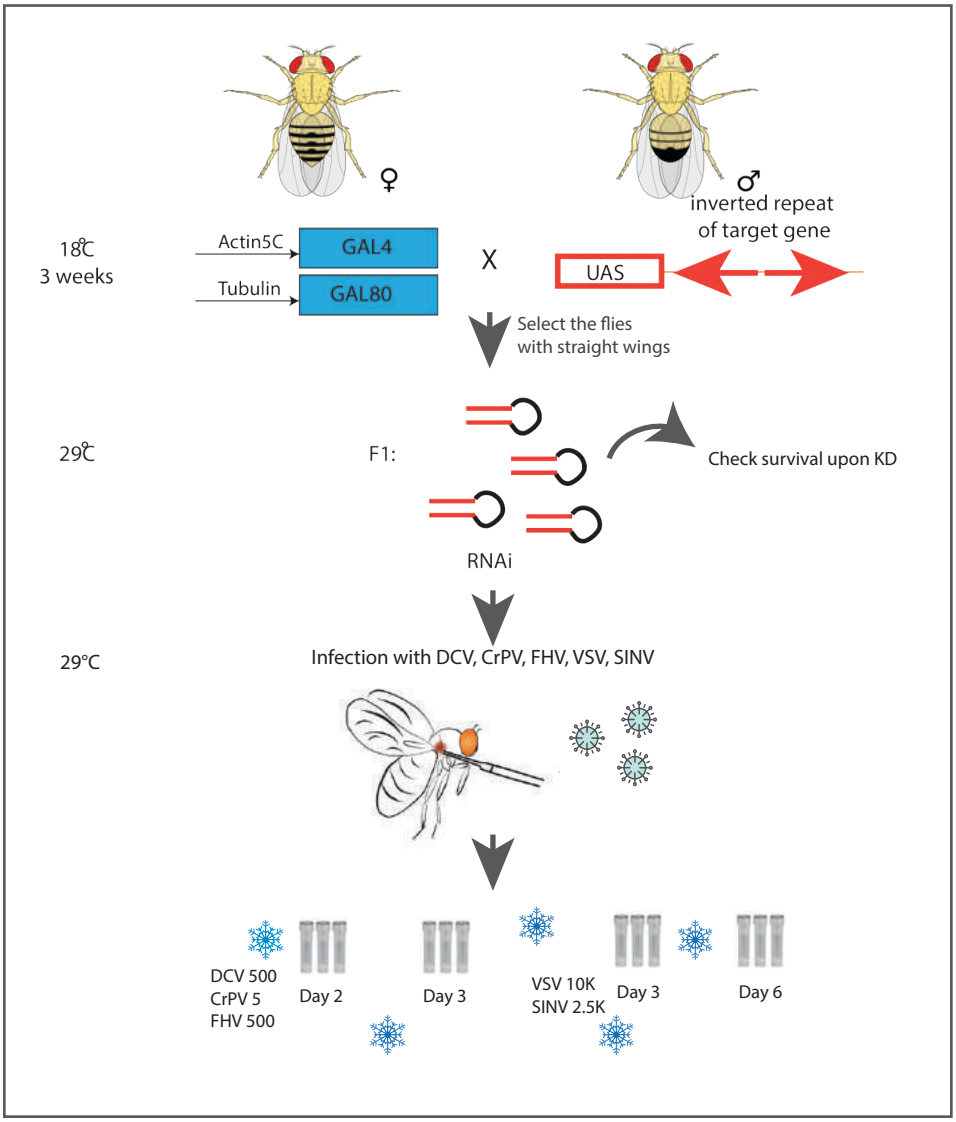


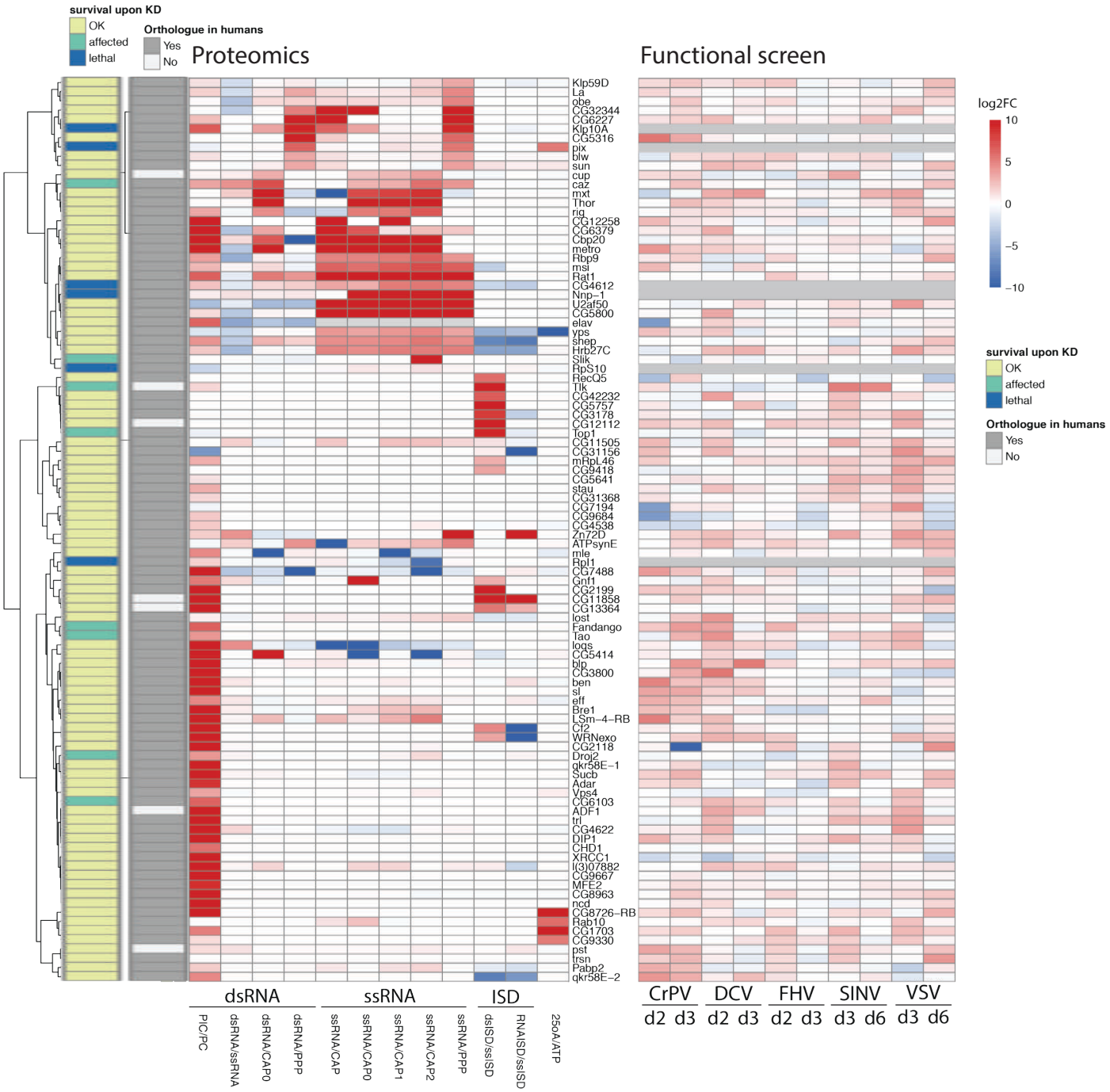
Figure 33. Experimental pipeline for the functional screen in flies. Females with Actin-Gal4/Tubulin-Gal80 are crossed with the males that encode UAS-IR of the target gene at 18°C. As soon as the flies hatch, they are sorted and moved to 29°C. The survival at 29°C is monitored during 3 weeks. After 5-7 days the KD is considered to be induced and flies are injected with 5 viruses (concentrations in pfu are indicated by numbers on the right of the virus). The flies (three tubes of 3 males and 3 females each) are collected at two time points.

insertions). One possibility is to use RNAi lines, with transgenic fragment inserted on the same location to minimize any positional effects. A second criterion is based on the notion that many of the NA binding proteins can be important during the development, but at the same time also play a role in immunity. That is why we need to be able to induce the knockdown only in the adult stage to minimize the developmental effects. Here comes a very versatile and useful tool of Drosophila genetics – Gal4 system. Gal4 was discovered in 1980s in baker's yeast as gene regulator induced by galactose (Laughon et al. 1984; Laughon and Gesteland 1984). Gal4-mediated transcriptional activation relies on binding to defined 17 bp regions in the promoter of target genes, called Upstream Activating Sequences (UAS). Shortly afterwards it was shown that Gal4 is able to activate the expression of a reporter gene with UAS sequence in Drosophila (Fischer et al. 1988). These discoveries led to the development of bipartite system where the driver is expressed in one parent and the UAS with the target sequence in another (Brand and Perrimon 1993), so that the target is expressed only in the progeny. Further development of a number of GAL4 drivers enabled targeted gene expression in almost every tissue type of the fly. Another feature of this system is the temperature dependence. At low temperatures (16°C-18°C) the activity of Gal4 in flies is minimal, whereas at 29°C flies are fully viable and the activity of the driver is maximal. On the other hand, a thermo-sensitive version of the Gal4 repressor called Gal80 ($Gal80^{ts}$) inhibits the action of Gal4 at 18°C and cannot do it at 29°C, thus allowing to efficiently control the activity of the driver with temperature (reviewed in (Duffy 2002)).

Taking all this into account, we chose to exploit temperature-sensitive inducible RNA interference-mediated gene silencing in adult flies as the strategy to survey the loss-of-function phenotypes of candidate genes. Females with ubiquitously expressed Actin5C-Gal4/Tubulin-$Gal80^{ts}$ were crossed with the males, expressing UAS with inverted repeat of the target gene (Figure 33).

The fly lines used for the screen were produced in VDRC and mostly are from KK stock, with the exception of a few from GD stock (https://stockcenter.vdrc.at/control/library_rnai). For these lines VDRC suggests two control lines 60100 (for KK) and 60000 (for GD), containing template plasmids with no inverted repeats. However, after having done several injection experiments using these lines as controls, I observed their increased sensitivity, especially upon injection. Other lab members also confirmed this problem. Therefore, we decided to

Figure 34. 100 candidate proteins in AP-MS and the functional screen. The heatmap on the left shows the log2fc of the binding affinity to NA baits in the AP-MS screen. The heatmap on the right shows the log2FC of viral load compared to shmCherry control. Two side bars on the left show the survival of the fly lines upon the KD and the presence of human orthologues of the genes. Viral titer was monitored by the RT-qPCR for each virus at the indicated time points post-infection.



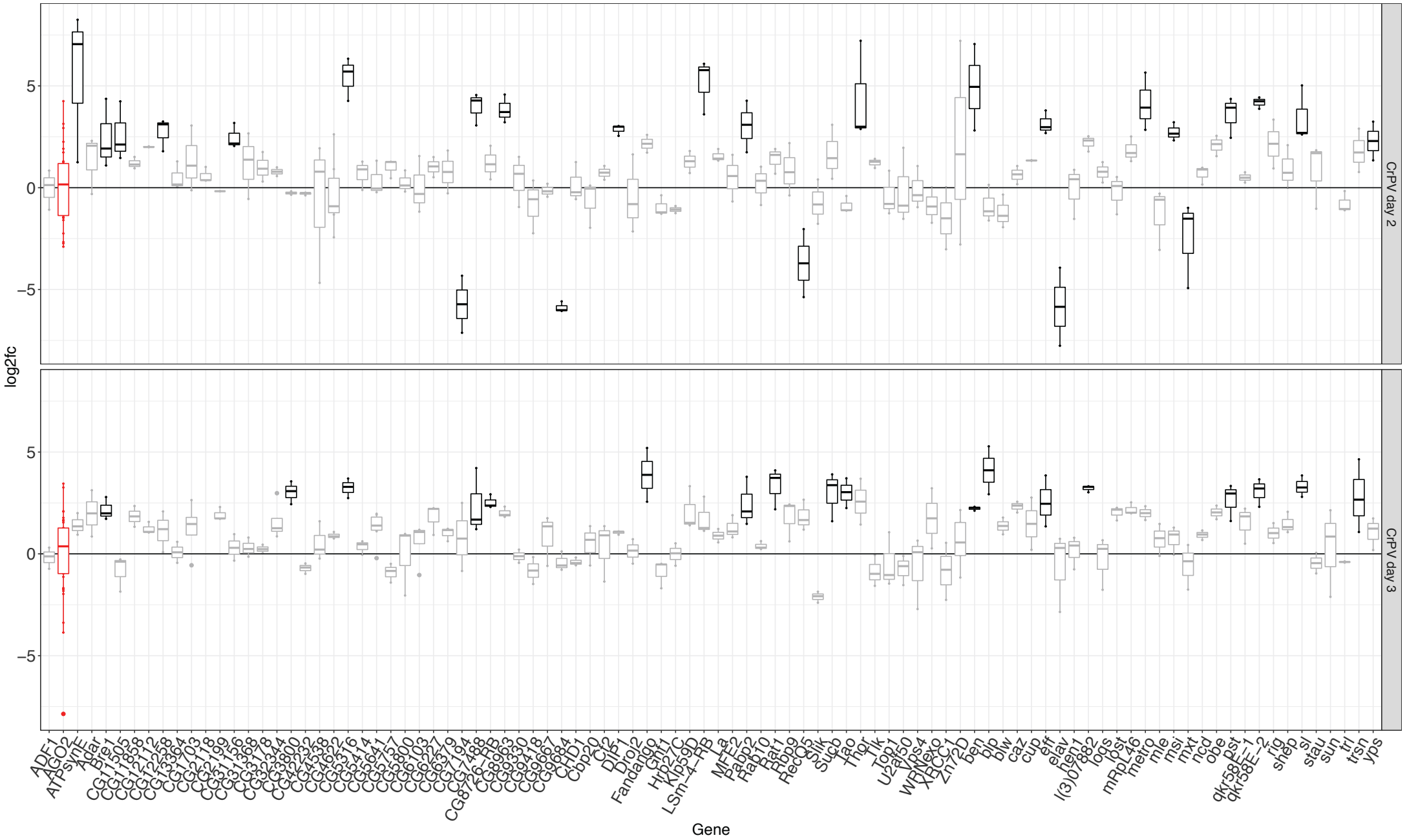


Figure 35. Boxplot showing the log2FC of CrPV viral load normalised to mCherry control per experiment. Day post injection are indicated on the right side of each graph. Results for the positive control AGO2 are shown in red. Significant values are indicated in black. Significance was calculated using least square means (lsmeans) function using Dunnet's adjustment for the p-values in R.

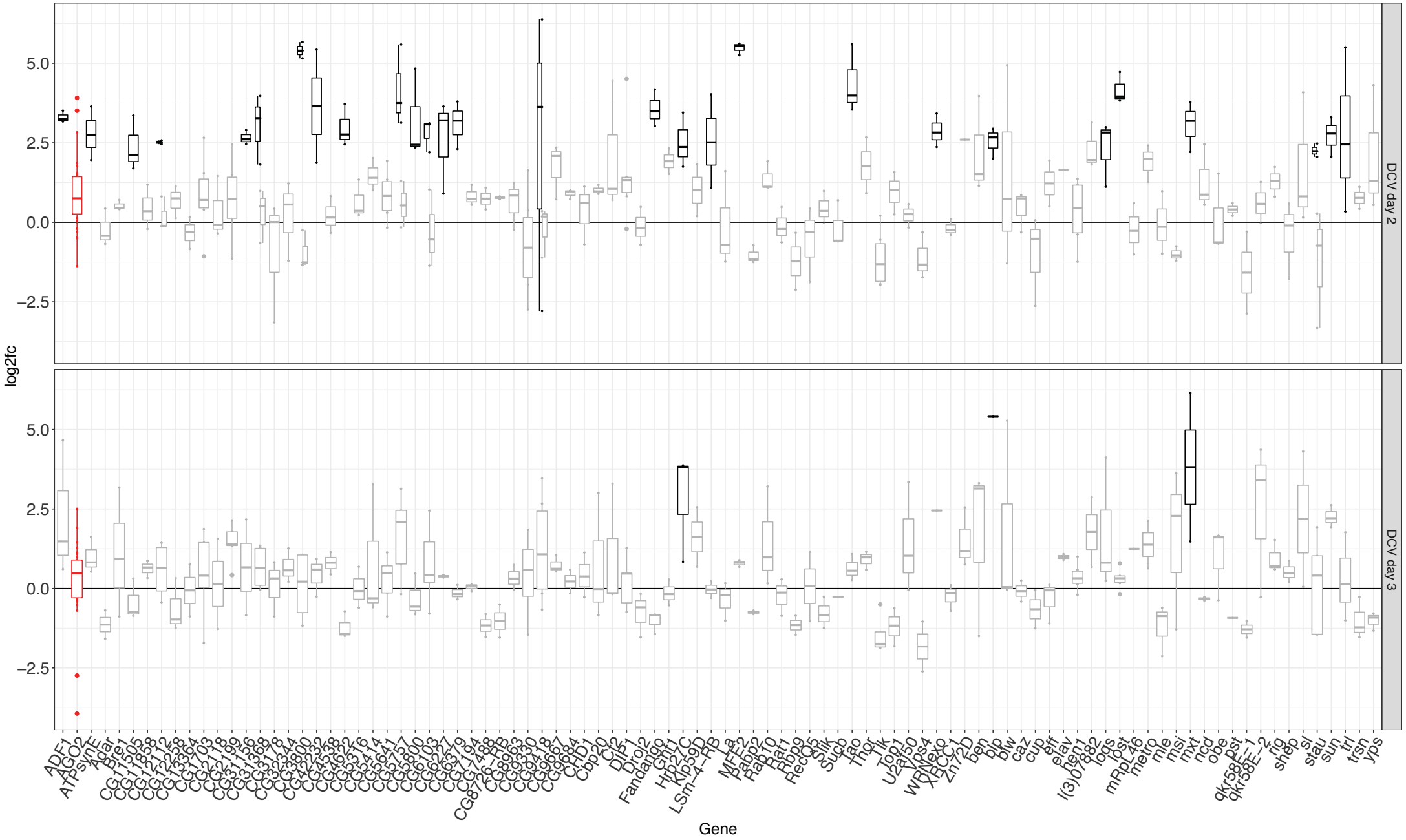


Figure 36. Boxplot showing the log2FC of DCV viral load normalised to mCherry control per experiment. Day post injection are indicated on the right side of each graph. Results for the positive control AGO2 are shown in red. Significant values are indicated in black. Significance was calculated using least square means (lsmeans) function using Dunnet's adjustment for the p-values in R.

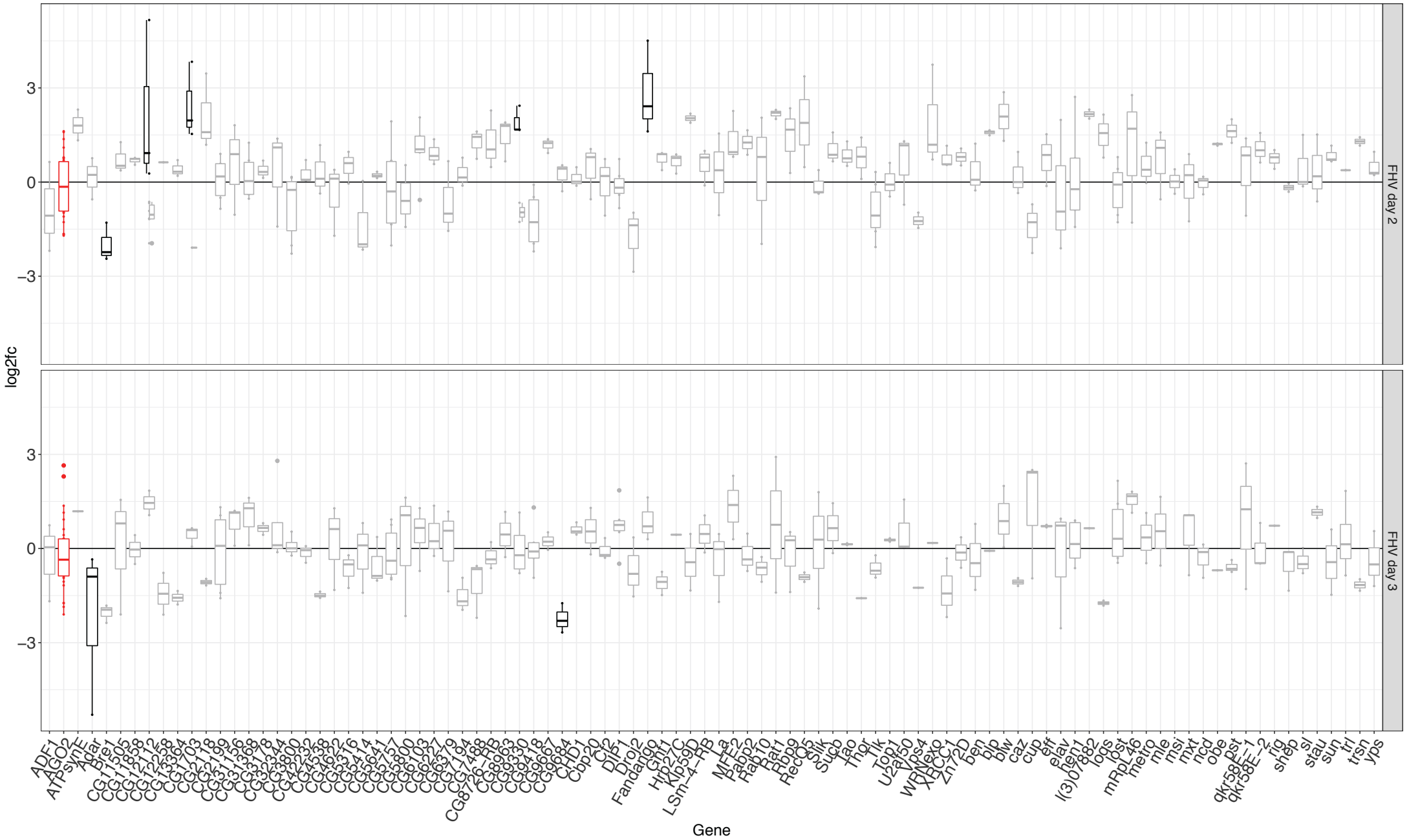


Figure 37. Boxplot showing the log2FC of FHV viral load normalised to mCherry control per experiment. Day post injection are indicated on the right side of each graph. Results for the positive control AGO2 are shown in red. Significant values are indicated in black. Significance was calculated using least square means (lsmeans) function using Dunnet's adjustment for the p-values in R.

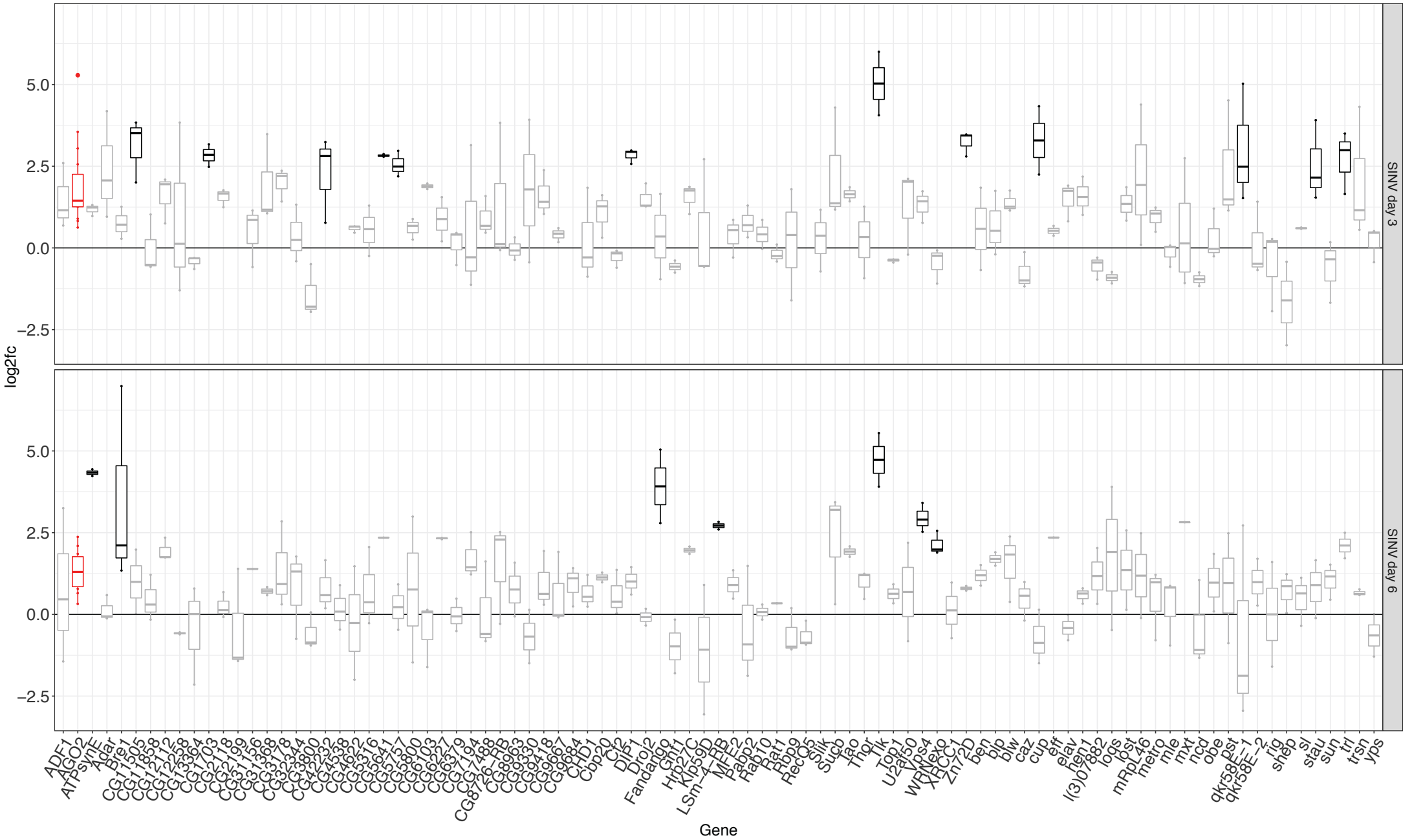


Figure 38. Boxplot showing the log2FC of SINV viral load normalised to mCherry control per experiment. Day post injection are indicated on the right side of each graph. Results for the positive control AGO2 are shown in red. Significant values are indicated in black. Significance was calculated using least square means (lsmeans) function using Dunnet's adjustment for the p-values in R.

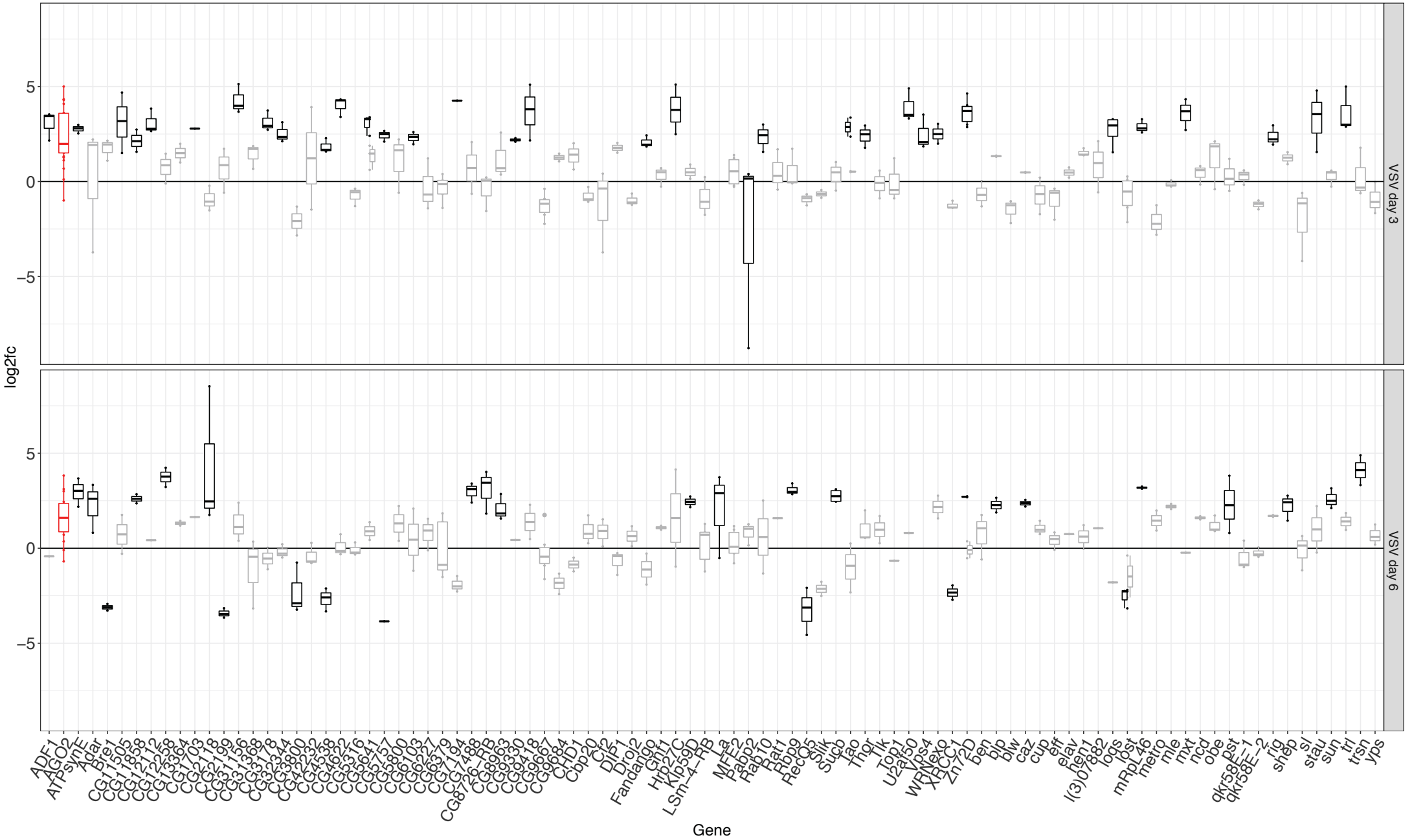


Figure 39. Boxplot showing the log2FC of VSV viral load normalised to mCherry control per experiment. Day post injection are indicated on the right side of each graph. Results for the positive control AGO2 are shown in red. Significant values are indicated in black. Significance was calculated using least square means (lsmeans) function using Dunnet's adjustment for the p-values in R.

change the negative control for the line, which also induces Gal4-mediated KD, but using short hairpin RNA against mCherry (shmCherry) and therefore exploits miRNA pathway. As for the positive control, we have used shAGO2 line, which also induces AGO2 KD by miRNA pathway at 29°C. All fly lines were treated against Wolbachia and confirmed to have Pst-sensitive allele (Faria et al. 2016; N. E. Martins et al. 2014).

Flies develop for three weeks at 18°C and as soon as adults hatch, flies with the desired genotype are sorted and moved to 29°C incubator in order to induce the knockdown of a target gene.

All 100 fly lines yielded viable adult progeny at 18°C. However, when switched to the restrictive temperature of 29°C, six out of 100 fly lines exhibited considerable mortality upon the knockdown (Klp10A, Pixie, CG4612, Nnp-1, RpS10, RpI1) (Figure 34). For some of them, carrying essential housekeeping functions, such as Pixie and ribosomal proteins Rps10 and RpI1, it was expected. Klp10A is implicated in chromosome segregation during mitosis (Afonso et al. 2014). Nuclear protein Nnp-1 is presumably involved in rRNA processing. These results confirm that the knockdown of the target proteins was induced and validates our strategy to generate loss of function drosophila lines.

Another eight fly lines had significant reduction in survival upon the knockdown. However, a substantial amount of flies were still alive at 7 days after hatching at the time of the injection (Figure 34), so these flies were used for virus injection. These candidates are caz, Slik, Tlk, Top1, Fand, Tao, Droj2, CG6103. Of note, the positive control line shAGO2 also had compromised survival at 29°C, which may reflect the involvement of the siRNA pathway in stress response (Lim et al. 2011).

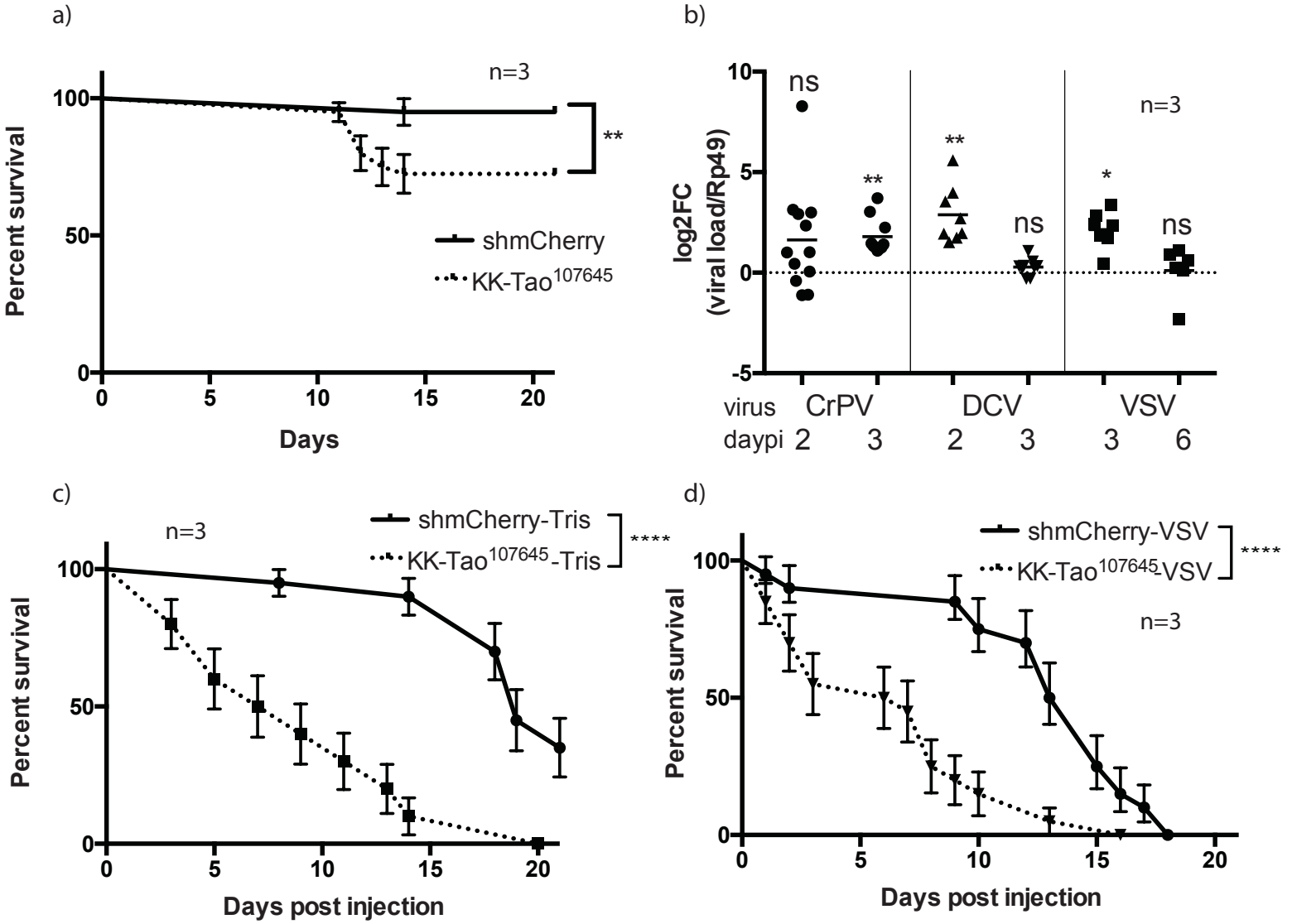
Thus, 94 fly lines were selected for virus injection. After five to seven days at 29°C, the flies were infected with five RNA viruses and the level of viral RNA was monitored in triplicates (containing 3 males and 3 females per sample) at two time points post infection.

The time points for flies collection were chosen to address the dynamics of viral replication. However, I observed high mortality for the second time point for some viruses (day 3 for DCV; day 6 for VSV and SINV). Considering that only flies, which were alive at the time of collection, were taken for RNA analysis, it is not surprising that the phenotype for the second time point was often lost. For instance,

for the flies on three days post infection with DCV, there are only three candidates with significant change in viral load (Figure). This is also related to the fact that the time points that were used are usually mentioned in the experiments at 25°C. The screen is based on the temperature-sensitive KD system, and therefore all flies before and after injection were put at 29°C, which may increase viral replication (Cevallos and Sarnow 2010).

Another important observation is that shAGO2 flies do not have significantly increased viral load compared to the control when infected with DCV, CrPV and FHV. But they do have a significant increase of replication upon injection with VSV and SINV. This might be due to the fact that neither VSV nor SINV express VSRs. Alternatively, it could be related to the rate of viral replication since SINV and VSV have slow replication kinetics. The dynamic replication of DCV, CrPV and FHV in Drosophila tissues may facilitate their escape from the antiviral effect of AGO2. In addition, shAGO2 had compromised survival even without injection upon the induction of the KD. All this suggests, that another control could be used for the injection. One of the options is to use AGO2 mutant flies (*AGO2*^{414}), however, here the flies have a knock-out of the gene and cannot be compared to the candidate lines.

The analysis that was done on the qPCR data compared viral load in reference to housekeeping gene Rp49 in all samples to the shmCherry control per experiment. This decision was taken because of high variability of the control measures between different dates of injection. Here it is important to discuss the possible sources of variability in viral replication. As it was already mentioned, the temperature at which the flies are kept plays a role in viral replication. The flies were kept at 29°C in the incubator 5-7 days before and 2-6 days after the injection, while the injection itself was done at 20°C. It was noted that the incubator itself experienced the fluctuations in temperature. As the experimental part of the screen was done during 18 months, it is possible that temperature fluctuations could take place. Another source of variation is the fly medium. It was observed by several members of the lab that the change of medium batch affected the phenotypes related to gut microbiota (unpublished). Again, a more profound analysis is needed to validate if there are any changes in viral response due to media composition and viscosity. Another source of variation is the virus that was used. All virus aliquots were done from the same stock of a certain virus. However, the injection, which uses



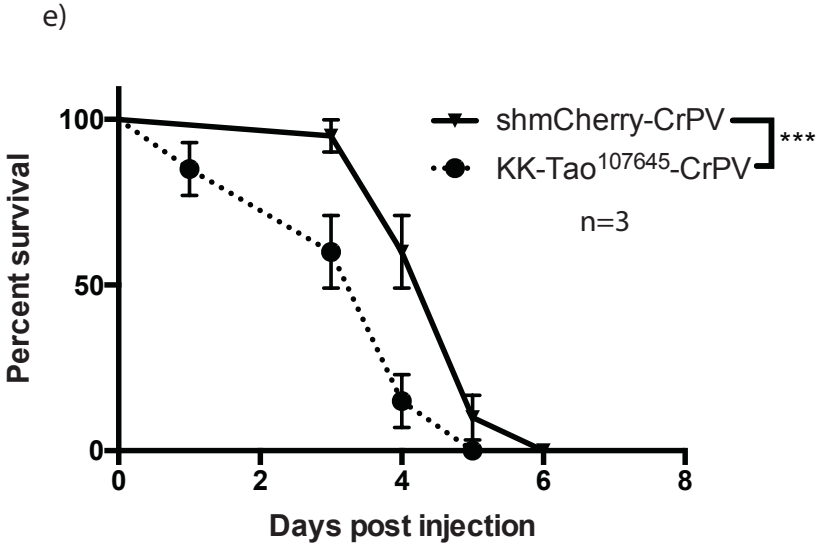


Figure 40. Tao is putative antiviral factor in Drosophila. a) At day 0 flies (1-3 days old) are put at 29°C to induce the expression of inverted repeats of the target gene. KD is considered to be induced at day 5-7. Survival was monitored for 21 days. Flies showed descreased survival upon the KD of Tao. b) KD of Tao leads to increased viral load of CrPV, DCV and VSV. Three samples of 6 flies (3 males, 3 females) were used for RNA extraction per experiment. All values are normalized to shmCherry control. Upon the induction of the KD, flies were injected with Tris, VSV and CrPV and the survival was monitored. Tao-107645 flies are more sensitive to Tris (c), VSV (d) and CrPV (e) injection. shmCherry survival curve is indicated by black line, KK-Tao107645 - by dotted line. Two tubes of 10 flies were used for each survival experiment. Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests were used to analyse survival data.

a minuscule amount of viral suspension cannot always guarantee the same amount of viral particles injected into every fly. It is particularly apparent for CrPV. Due to high replication efficiency and subsequent toxicity of the virus, only five plaque-forming units per 4.6 nl are injected into each fly. This small number can result in higher rate of variation between the injected samples.

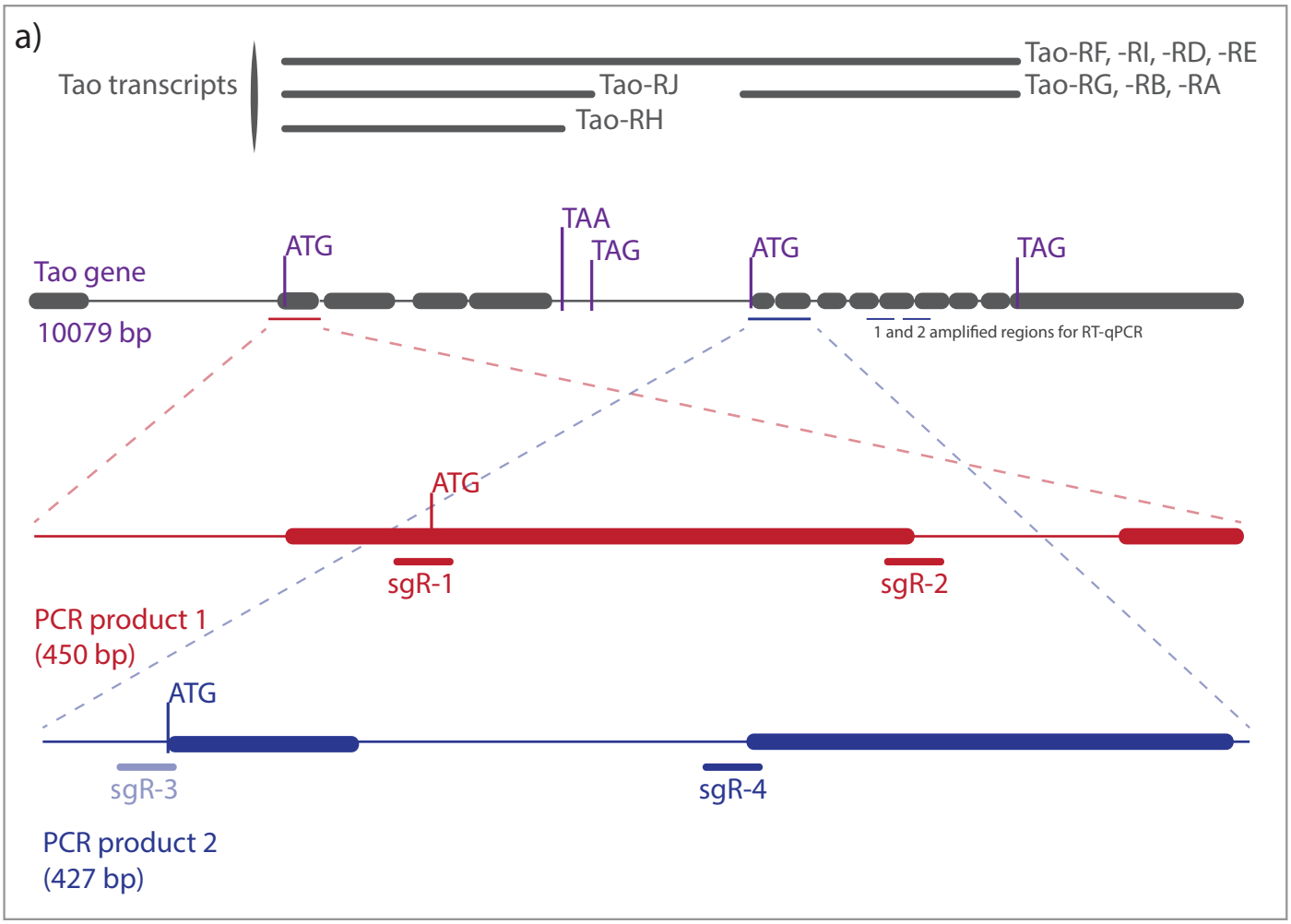
III.3. Candidate proteins

The functional screen in flies has resulted in a number of important candidate proteins. As a result of the screen, 78 of 94 fly lines had significant change in viral load for at least one condition (virus type and time point) (Figure 34-39). Most of the hits come from the infection with CrPV, DCV and VSV. The major overlap in phenotype is, as expected, between CrPV and DCV, which belong to the same family of viruses. Eight of the proteins had a significant phenotype upon the infection with three viruses DCV, CrPV and VSV (mxt, lost, blp, Tao, Fandango, CG3800, CG31156, CG11505).

III. 4. Tao is a putative antiviral factor in flies

One of the 8 candidates, exhibiting phenotype for DCV, CrPV and VSV, Tao, was of particular interest. Thousand and one (Tao) kinases belong to the family of Mst/Ste20 kinases and are conserved across species. In humans, there are three Tao kinases (TAOK1, TAOK2, TAOK3), while in Drosophila there is single ancestral gene Tao (or Tao-1). Tao kinase plays an important role in Hippo pathway (Boggiano, Vanderzalm, and Fehon 2011; Poon et al. 2011; Poon et al. 2016; Poon et al. 2018). This pathway controls organs size in animals, is highly conserved and was extensively studied in Drosophila. Tao activates Hippo kinase by phosphorylating. It also activates related Sterile 20-like kinase Misshapen in Drosophila midgut (X. Huang et al. 2014). Tao also was found to play role in the control of microtubule dynamics, ethanol-stimulated behavior, neuronal development and cell proliferation (T. Liu et al. 2010; King and Heberlein 2011; King et al. 2011; Poon et al. 2016).

It was observed that in flies, the KD of Tao induced in the adults causes sex-specific survival defect (Figure 40). Upon 10 days at 29°C (around 3-5 days after the



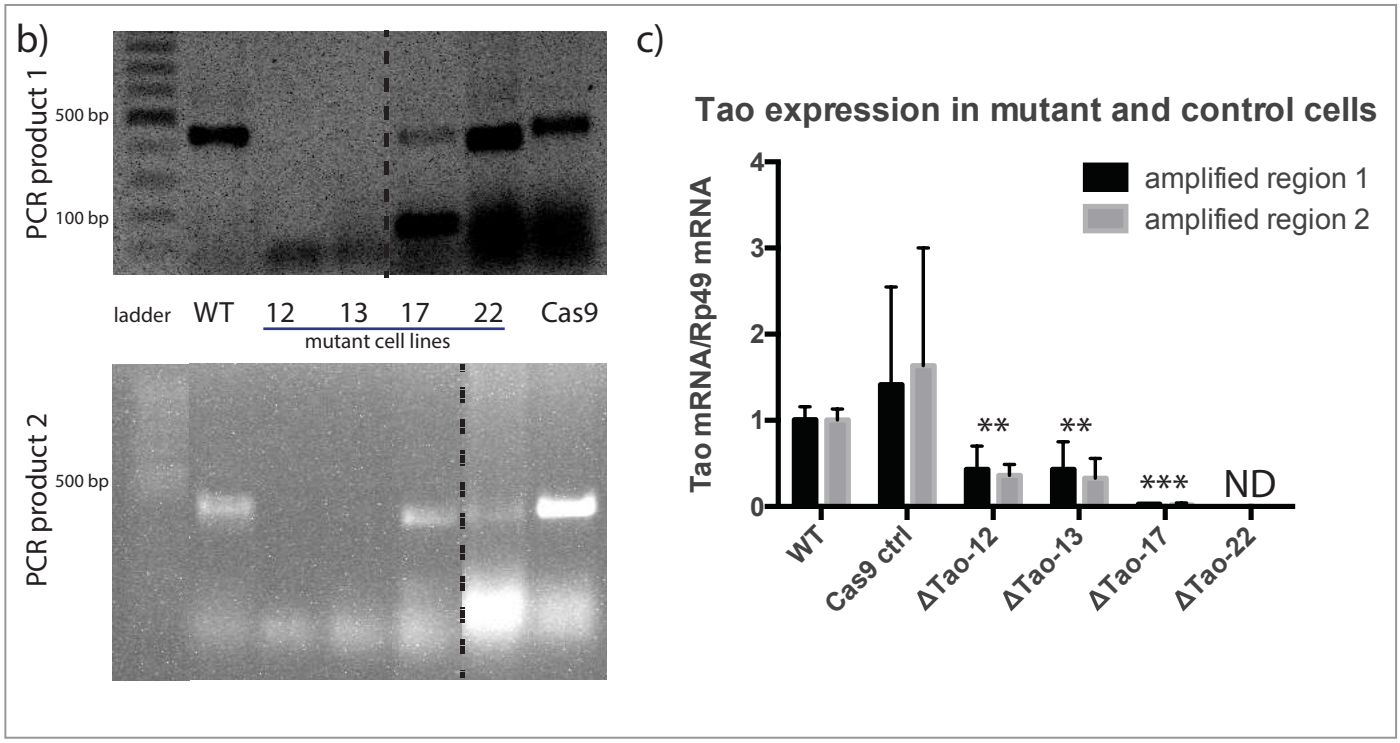


Figure 41. Targeting drosophila Tao for CRISPR/Cas9-mediated KO. a) The products of drosophila Tao gene (10079 bp) include several forms (long and three short), produced from 9 transcripts (shown on the top in grey). Four sgRs were designed in order to target both start codons. The localization of sgR-1 and sgR-2 can be seen in the PCR product 1 containing the second exon with the first ATG and surrounding sequence. The localization of sgR-3 and sgR-4 can be seen in the PCR product 2 containing the sixth exon with the second ATG and surrounding sequence. All sgRs, except sgR-3, were successfully inserted into Cas9-expressing vector and transfected into S2 cells. b) PCR fragments from control and mutant cell lines Dotted line indicate where gel pictures were cut. c) Tao expression is significantly diminished in four mutant cell lines. RT-qPCR was performed using two pairs of primers, targeting different regions. ND=not detected

induction of the KD), males start to die reaching 60% mortality rate at day 20. This phenotype becomes more profound upon injection. On the day 5-7 at 29°C (at the time of the induction of the KD) the flies were injected with virus suspensions or Tris control. Again, males show increased mortality rate, reaching 100% at day 7 after injection with Tris (Figure 40). This phenotype was confirmed by another Tao-KD fly line (data not plotted). The females, although more resistant than males, also die faster than the control shmCherry flies. Upon the infection with CrPV and VSV, the survival of Tao-KD flies is reduced. However, this might be because of general survival deficiency of these flies. As for viral load, several independent experiments confirm the phenotype observed in the functional screen, namely the increase of viral load for CrPV at day 3, DCV at day 2 and VSV at day 3.

Unfortunately, the efficiency of the KD in S2 cells using 2 different dsRNAs was not sufficient (data not shown). Therefore, I have decided to proceed to the creating of KO cell lines.

III. 5. CRISPR/Cas9-mediated KO of Tao in S2 cells

In drosophila Tao gene results in the production of one long and two short isoforms, because of two start codon sites in the same frame. According to previous studies, the long isoform is expressed throughout the life, whereas short form containing C-terminal part is expressed during early development (King et al. 2011). I designed four sgRNAs in order to target both start codons (Figure 41). All sgRNAs, except sgR-3, were successfully inserted into Cas9-expressing vector (as described above) and transfected into S2 cells. I have tested 41 stable cell lines by PCR and selected four cell lines, where the expression of Tao is significantly diminished (Figure 41).

It was previously shown, that knockdown of Tao in Drosophila S2R+ cells affects cell morphology and leads to “spiky” phenotype due to impaired microtubule dynamics (T. Liu et al. 2010). I observed similar phenotype in the KO cell lines.

Discussion

AP-MS screen identified candidate proteins binding to viral-like NA species

Patterns in NA recognition across species

Given the diversity of the viruses infecting different organisms, a number of novel viral sensing mechanisms are yet to be discovered. The array of NA baits and the range of species used in the large AP-MS screen has produced a lot of data. There are many ways to organize and analyze this multitude of data. Upon the initial analysis, we can make several conclusions. Firstly, the number of proteins identified in whole organism samples (*C. elegans* and *D. melanogaster*) was higher than in any cells. This supports the notion, that cell lines mostly represent a tissue-specific response, and might miss out many pathways. When comparing significant proteins in S2 cells and whole flies, this is true for most baits, except dsISD, ssRNA-CAP, and ssRNA-CAP0.

A second immediately apparent conclusion is that poly(I:C) was found to be the most "sticky" bait. The difference in numbers of significant proteins binding preferentially to poly(I:C) is striking when comparing S2 cells and flies (40 vs 1410, with only 21 overlapping). Although in the 21 common proteins, we can see the important known hits such as Dcr-2 and promising candidates such as Tao, fly and cell-specific hits should also be studied. In the heatmap showing significant hits, poly(I:C) clustered together with dsISD, RNA-ISD and ssISD, but not with dsRNA baits. Poly(I:C) also had more binding partners than similarly synthesized poly(A:U). This might be explained by the fact that poly(I:C) although regarded as dsRNA mimic, might actually have a web-like structure. It was suggested that branched structures in RNA could be specifically immunogenic and sensed by MDA5 (Pichlmair et al. 2009). More studies should be implemented to specify the response to synthetic immunogenic molecules, especially in Drosophila.

Another interesting group of baits comprise ss- and dsRNA with different CAP structures (CAP, CAP0, CAP1, CAP2). ssRNA-CAP and ssRNA-CAP0 were compared to ssRNA-CAP1, which represents normal cellular mRNA upon its transport to the cytoplasm. However, it was suggested that the systems lacking

interferon response, such as yeast, lack 2'O methylation on capped RNA (Byszewska et al. 2014). One protein that repeatedly comes up from all analyzed comparison involving different CAP structures is CG6379. This is the Drosophila orthologue of mammalian CMTR1, which is involved in 2'O methylation of CAP0 mRNA. Also, CMTR1 cooperates with DHX15 helicase to modify structured 5'termini of some RNAs (Toczydlowska-Socha et al. 2018). CG6379 was one of the 100 candidates included in the functional screen. The KD of CG6379 did not cause viability defect, although the efficiency of the KD was not verified. Therefore, either the KD of CG6379 was not efficient, or the gene is not essential for adult fly. Upon the infection with DCV, the flies with the KD of CG6379 had higher viral load two days post infection.

Evolutionary perspective

One of the recurring themes when analyzing the AP-MS screen results is the cross-species conservation. It was not surprising to see some conserved known sensors binding to the same NA baits, such as Adar or Dicer. More cross species analysis needs to be done, as illustrated by some interesting novel conservation patterns that were found as a result of the AP-MS screen. Some most important candidates binding to 2'-5' oligoadenylates in all tested samples belong to the ABC transporter family, playing a role in translation (Paytubi et al. 2009; Barthelme et al. 2011). And although ABCE1 was some while ago implicated in preventing the binding of RNase L to 2'-5'oA (Bisbal et al. 1995), the putative function of ABC transporters in viral infection has not been studied. Our preliminary results in S2 cell do not indicate any phenotype upon viral infection. However, all three CG1703 (ABCF1), Pixie (ABCE1) and CG9330 (ABCF3) were included in the functional screen. Whereas the knockdown of Pixie even at the adult stage caused severe mortality and could not be tested for resistance to viruses, the flies with the knockdown of CG1703 and CG9330 were infected with the six viruses. Upon the infection with FHV, VSV and SINV both lines had increased viral load. Interestingly, the phenotype of the two related proteins was similar, suggesting that they carry similar functions. CG3164, which is an ABCG orthologue and preferentially binds 2'-5'oA in S2 cells and flies, could be the next target to study in the context of viral infection. In connection with the previous studies performed in our lab, I have noted

that among the candidate genes that were induced upon the infection with FHV and SINV, there were two ABC transporters CG31793 and CG17646 (Kemp et al, 2013). Therefore, I believe, that the ABC transporters' contribution in viral infection and response is worth exploring, especially in concert with more detailed studies in other species, used in the AP-MS screen.

On the other hand, there is growing interest in species-specific innovations in immunology, the field otherwise called "evo-immuno". From this point of view, the multispecies AP-MS screen provides a lot of new insights. I have only started to explore the data on specific candidates by looking which of the significant binders does not have an orthologue in human. Several proteins were identified: the arthropod-specific UDE protein, which preferentially binds dsISD; the products of genes CG6912 and CG7920, binding ssRNA-CAP; the product of the insect-specific CG7330 which binds to 2'-5'oA and others. Among the proteins, non-conserved in humans, that were tested in the functional screen, there are CG11858 (more VSV upon KD), ADF1 (more DCV and VSV), cup (more SINV), Tlk (more SINV), CG12112 (more VSV), pst (more CrPV and VSV), CG13364 (no phenotype). Further studies on these proteins may uncover novel phyla-specific recognition and effector pathways.

Advantages and limitations of the AP-MS approach

There are several advantages to the AP-MS approach. Firstly, it is unbiased and solely based on the binding of proteins to NA baits. Secondly, the variety of sample lysates allowed identification of the evolutionary patterns. However, there might be also limitations to this approach. Firstly, the binding affinity does not always reflect the function: there are proteins, that can recognize certain bait, however not to produce any signal upon binding; and on the other hand, there are also proteins which recognize and bind only transiently, but still able to transmit the signal to elicit the response. These latter may not be identified in the interactome. Second possible confusion might arise from the fact, that whole cells were used for sample lysates. Therefore, not only cytoplasmic sensors, but also nuclear proteins, binding NAs were precipitated. This could "pollute" the results, although on the other hand it can give more insight into nuclear mechanisms of sensing. Multispecies approach might also have certain limitations, as bait vs control samples were designed based on the mammalian pathways, whereas there is a possibility of

phyla-specific features. For instance, it was argued that animals devoid of IFN response do not produce CAP1-mRNA transcripts (Byszewska et al. 2014), therefore, this feature cannot be used as a control bait. And finally, the synthetic ligands, used for the screen, may not represent actual viral analogs. To overcome this limitation, the studies directed to look for the proteins, which bind specific viral products, might give more insight into virus-specific responses. For instance, recent study performed in the lab of A.Pichlmair described proteomic and phosphoproteomic screen, which identified host proteins which bind to Zika virus proteins (Scaturro et al. 2018).

Functional screen identified a number of important candidates

The selection of the 100 candidates for the functional screen was mostly based on the unbiased bioinformatic selection of the top 10% of binding proteins from the AP-MS screen. Some of them are specific for certain bait, others bind many different baits.

Having stated a number of possible technical limitations and comparing to mammals, it is nevertheless clear, that Drosophila remains an important and relatively simple model for studying the breadth and depth of organismal antiviral response.

Upon the infection with six viruses, the majority of the candidates had significant change in viral load for at least one virus and time point. It is very reassuring that the largest overlap in the candidates is between two viruses of the same family: DCV and CrPV. Another virus with many candidate proteins exhibiting an antiviral phenotype is VSV. This is also not surprising, as VSV is a popular virus to investigate virus-host interactions, possibly because it lacks efficient suppressors of innate immune defenses, at least in flies.

The KD of 8 genes caused significant change in viral load upon the infection with DCV, CrPV and VSV (mxt, lost, blp, Tao, Fandango, CG3800, CG31156, CG11505). Some of these proteins have already come up in the studies of our lab. CG3800 was found to interact with the members of RNAi pathway AGO2 and R2D2 (Majzoub et al, University of Strasbourg, thesis, 2013). It also binds RACK1, ribosomal protein, responsible for IRES-mediated translation of *Dicistroviridae*

(Majzoub et al. 2014; L. Kuhn et al. 2017). CG3800 seems to be antiviral for CrPV and DCV, and proviral in the case of VSV. Another hit, CG11505 (Larp4B), was found to bind to Dicer-2 (Rousseau and Meignin, personal communication). It showed antiviral phenotype for all three viruses. Its orthologue in humans, LARP1, as well as the orthologues of Fandango (XAB2 in humans) and Tao are promising candidates in the mammalian loss-of-function screen (Pennemann and Pichlmair, personal communication).

Suggestions for the experimental design

As it was mentioned in the Results section, the functional screen uncovered some of the technical issues associated with RNAi screens and viral infection in flies. As a result, I have come across the problem of variability of samples between different experiments. Although temperature-sensitivity of Gal4/Gal80 drivers is a useful tool, the change in temperature during the lifetime of the fly considerably changes infection rates. The increase in temperature stimulates the replication of the viruses, and at the same time causes stress in flies. It is therefore suggested, the time points for this kind of experiments could be reconsidered and the mortality should be carefully monitored.

KD of the genes was induced only in the adult stage, in order to minimize the developmental defects, which can be expected, as the majority of candidates are NA binding proteins. However, this might pose a problem, if the KD is incomplete and still leaves substantial amount of the protein. This could be addressed by the verification of KD efficiency, which was not performed for all proteins in my experiment due to important number of samples.

It was shown, that around 25% of KK RNAi fly lines result in off-target effects, more specifically enhancing Hippo pathway by ectopic production of Tiptop transcription pathway (Vissers et al. 2016). This is due to the location of transgenic insert. This might affect related pathways. In our study this is worth considering, as one of the main candidate proteins is Tao, which is the member of Hippo pathway. This suggests that the results first have to be confirmed by exploiting an independent RNAi line or/and mutant fly lines.

There are a number of new techniques for performing *in vivo* screens in flies. A wide array of possible genetic changes is achievable with emerging CRISPR/Cas9-

mediated gene editing techniques. These can be used separately or in combination with RNAi approaches. Another improvement is that there are techniques available to rapidly detect CRISPR/Cas9-introduced mutations (Kane et al. 2017). However, one must be careful using new genome editing approaches, as it is not yet clear, what are the molecular consequences of the presence of transgenic editing enzymes (Mohr et al. 2014). Another improvements might help to detect virus more rapidly, compared to measuring RNA levels. For example, a recent study described a transgenic reporter, which, when cleaved by viral protease, could be used to detect live virus infection (Ekström and Hultmark 2016).

Overall, the functional screen resulted in several important candidate proteins, whose function is yet to be elucidated. One way to immediately address the question of the mechanisms involved is to use the cDNA from the infected flies from the screen and check for the readout of the induction of certain immune pathways, such as Imd, Toll, dSTING. As for the long-term perspectives, the results for some interesting candidate proteins first have to be confirmed by exploiting an independent RNAi line or/and mutant fly lines.

CG5641 and Zn72D: novel CrPV replication factors in Drosophila

CG5641 and Zn72d are conserved proteins, orthologues of mammalian NF45 and Zfr, respectively. NF45 is known to form a dimer with NF90, SPNR and Zfr, all are closely related proteins. Most of the studies in mammals were done on the NF45/NF90 protein complex, which is involved in post-translational control of gene expression (Jayachandran, Grey, and Cook 2016). Of note, several studies in mammalian cells show that either NF45/NF90 or NF90 on its own positively regulate the replication of diverse viruses such as HCV, Dengue virus and HIV-1. Recently, the NF45/NF90 complex was found to have an RNA chaperone activity: upon binding, it can specifically facilitate a first step of replication of HCV virus RNA (Schmidt et al. 2017). Overall, these studies establish the relevance of the complex to the process of virus replication, although the mechanism of action is still fairly elusive.

The results obtained in this study in Drosophila S2 cells also support the model, where CG5641 (dNF45) and Zn72D form a complex, which is required for

CrPV replication. The results from the functional screen, on the contrary, suggest that both CG5641 and Zn72D are antiviral against VSV and SINV. Based on the structural studies on mammalian orthologues, and on the binding to the NA baits, it can be hypothesized that the two proteins bind dsRNA with high affinity and mask it from the recognition by Dicer-2. This hypothesis is supported by the fact that the binding of CG5641 and Zn72D was confirmed by immunoprecipitation (Worringer, Chu, and Panning 2009). However, my preliminary results of immunostaining show that tagged CG5641 and dsRNA do not colocolize in infected cells. As it was emphasized in the recent study by (Schmidt et al. 2017), the NF45-NF90 complex may modify RNA by different mechanisms, such as RNA annealing and strand displacement. Therefore, it is possible that the action of the host factors is virus-specific and depends on the NA substrate, and thus can generate different effect on viral replication. One of the important functions of the NA-binding proteins is to mark the NAs for trafficking to endosomes where they will be subsequently degraded (Diebold et al. 2004). To sum up, binding to the viral NAs can lead to both proviral and antiviral effect and, in the case of CG5641-Zn72D complex, depending on structural features of the viral NA. As a result of proteomic screen, it was found that CG5641 binds Dicer-2 both in infected and non-infected cells, suggesting that the interaction does not depend on the presence of viral dsRNA (Rousseau and Meignin, personal communication). This might indicate, that the mechanism of action also may depend on the RNAi pathway.

Intriguingly, the phenotype in cells differs between DCV and CrPV, both members of *Dicistroviridae*. One the major differences between DCV and CrPV is the mechanisms of action of their VSRs. While CrPV 1A acts through inhibiting the AGO2, while DCV 1A masks dsRNA to prevent its recognition by Dicer-2 (Fareh et al. 2018; Nayak et al. 2018). If the CG5641-Zn72D complex performs similar function as DCV1A, it will be interesting to find out whether DCV1A can recover CrPV replication in CG5641 KO cells.

A number of other experiments can be proposed to investigate the hypotheses suggested above. First, the viral NA substrates binding to both proteins can be identified using RNA-immunoprecipitation upon tagged protein expression. More specifically, the affinity of the binding of these NAs can be assessed by mobility shift assay, as previously described for DCV 1A protein (van Rij et al. 2006). Also, the proteins partners of both proteins can be studied in infected and non-infected cells.

This will confirm the binding of CG5641 to Dicer-2, and probably uncover new insights.

Tao kinase in Drosophila antiviral immunity

Tao kinase, which was identified in the AP-MS screen, specifically binding to poly(I:C) in flies and S2 cells, is a conserved protein with several described functions, mainly related to the control of organ size and development. As for proteomic screen, the preferential binding to poly(I:C) was also confirmed for all three Tao orthologues in mouse and human cells. The fact that it binds specifically to poly(I:C) and not to other dsRNA baits suggests that structured features of NAs are probably recognized. Considering many functions of Tao, it is not surprising, that its KD led to survival defects, even when the KD is only induced in the adult stage. This creates a difficulty to assess the antiviral function of Tao, as it is not clear whether the phenotype is directly related to viral infection or can be explained by the sensitivity of loss-of-function flies.

Therefore, more detailed characterization of Tao function should follow in S2 cells, using CRISPR/Cas9 KO cell lines I generated. Firstly, these cells need to be tested in viral infection experiments. Secondly, binding to poly(I:C) should be confirmed *in vitro*, along with testing other NA species. A next question is the binding partners of Tao in infected and non-infected cells, which could be studied using MS. One of the important perspectives for studying the function of Tao kinases in antiviral immunity is to analyze how phosphorylation signatures change when Tao is absent or ectopically expressed. This can be done using emerging phosphoproteome techniques, such as the recently developed EasyPhos (Humphrey et al. 2018), which was already used in an innate immunity study (Scaturro et al. 2018).

As for *in vivo* studies, more precise tissue-specific depletion of Tao could be used to decrease the survival issues. Also, the phenotype can be studied according to tissue tropism of virus. Ectopic expression using transgenic flies can also be helpful in elucidating the function of Tao in antiviral immunity.

Conclusion

It seems unfair to a drosophilist, that the studies on the pathways of innate immune recognition, once initiated in flies, have concentrated so much on the mammalian system in the last decades. Yet, fly has a lot to offer to an immunologist, and not only as an alternative model for human diseases, but also as a model of novel, previously unstudied host-pathogen interactions.

Overall, the data acquired in this study provides a first glance on possible recognition pathways in flies and may give rise to new projects. Eventually, finding new mechanisms of recognition will help us to understand the complexity of antiviral response in Drosophila.

Materials and methods

Experiments on Drosophila cells

Cell culture

Schneider 2 (S2) cells are derived from a primary culture of late stage Drosophila embryos and are likely from macrophage-like lineage (I. Schneider 1972). Cells were grown in Schneider's medium (Biowest) supplemented with 10% fetal calf serum, Glutamax (Invitrogen) and Penicillin/Streptomycin (100x mix, 10 mg/mL/ 10000 U, Invitrogen).

CRISPR/Cas9-mediated KO

Vector assembly

The protocol was adapted from Bassett et al. (Bassett et al. 2014). Backbone vector used for cloning was ordered from Addgene (#49330: pAc-sgRNA-Cas9). Genomic DNA from wild-type S2 cells was extracted by standard phenol-chloroform extraction. The PCR product covering the sgRNAs was sequenced (primers in Table M1). sgRNA amplicons were synthesized and the vector was assembled as previously described in Bassett et al. (Bassett et al. 2014). The plasmid was transformed in chemically competent *E. coli* DH5α cells and grown on Ampicilline plates. The colonies were tested by PCR using U6F and specific reverse sgR primers or by restriction digestion using BglII and SacI in Tango2x buffer. Minipreps (GE healthcare) of selected colonies were sequenced with U6F primer and subsequently selected for midiprep (Qiagen).

Transfection

On day 0, S2 cells were seeded in a 6-well plate (2 mln cells/well). Day 1: total of 2µg of purified vectors (1µg for each sgR amplicon) in 100µl of serum-free medium were mixed with 6µl of Fugene HD ® reagent for each well. 2µg of

pActin5C-GFP vector was used as a control for transfection and Puromycin selection. The solution then was added to the cells. On day 2, medium was changed (no washing required). On day 4, cells were diluted in Puromycin-containing medium (5μg/ml, Sigma) to achieve the final concentration of 10,000 cells, 20,000 cells and 50,000 cells/well in 96-well plate. Half medium change was done once a week. Typically in around four weeks, one could observe the apparition of first colonies. Single spot colonies were preferred, amplified and selected for further testing. The selected cell populations were tested by PCR, qPCR (for primers see Table M1) and where available, Western blot (antibodies in Table M3).

dsRNA synthesis

cDNAs were prepared from S2 cells or DNA templates (Table M4) were obtained by RT-PCR using sets of primers containing the T7 polymerase recognition sequence at their 5′end (see Table M1). dsRNAs were then synthesized using the T7 MegaScript® (Ambion) and annealed. Product lengths for both DNA and dsRNA were verified by agarose gel electrophoresis. 10^6 of S2 cells were bathed in 250μL of serum-free medium containing 12μg of dsRNA per well of a 24-well plate for 3h. The soaked cells were then supplemented with 250μL complete medium and incubated for 4-5 days at 25°C (Boutros et al. 2004). The efficiency of knockdown was checked by RT-qPCR (see primers in Table M1).

Clonal cell population isolation

S2 and Kc167 cells were diluted in Schneider's medium with different composition. To prepare fly extract containing medium, 250ul of fly extract (DGRC, Bloomington) was added to 10ml complete medium. For conditioned medium, complete medium was diluted by half with medium, filtered after 2-3 days of S2 cell culture. For bME-containing medium, 35ml of bME (Sigma-Aldrich) were added to 10ml of MilliQ water. 10μl of the solution was dissolved in 10mL of complete medium to achieve the concentration of 50μM. All media types were filtered (0.22μm). Cells were plated in 96-well plates (U-shaped) with the initial number 2500 cells/well in the leftmost column, followed by serial dilution by two.

Virus infection

S2 cells were infected with DCV (MOI 1), CrPV (MOI 0.1), FHV (MOI 1), VSV (MOI 10), SINV (MOI 10). 1 mln cells per well were seeded in 24-well plate. Upon attachment, medium was removed and 150µl of virus-containing medium were added and incubated at 25°C for 1 hour (swirling every 15 min) to allow the entry of viral particles. Afterwards, the medium was removed and 500µl of complete medium were added to each well. Cells then were incubated for either 16h (DCV, CrPV and FHV) or 48h (VSV, SINV).

Gateway® cloning for producing expression plasmids

cDNA sequences of target genes were amplified with attB overhangs and subcloned into pGEM-T easy ® vector. For BP reaction, 75ng of pDONR221® donor vector, 25fmol of PCR product and 1µl of 5xBP-clonaseTMII in a total volume of 5µl were mixed and left for 3h at room temperature. 10 µg of proteinase K (Invitrogen) were added and incubated for 10min at 37°C. The solution was transformed in chemically competent *E. coli* DH5α cells and grown on Kanamycin (50 µg/ml) plates. Colonies were sequenced with M13 primers. For LR reaction, 25ng of resulting donor vector, 50ng of destination vector and 1µl of 5xLR-Clonase Enzyme II Mix were mixed in a total of 5µl and incubated for 1h at room temperature. 0.5µl of proteinase K (Invitrogen) were added and incubated for 10 min at 37°C. The solution was transformed in chemically competent *E. coli* DH5α cells and grown on Ampicillin plates. Resulting expression vectors were sequenced with Actin5C and SV40 primers. Each vector was transiently transfected into S2 cells by CaPO4 precipitation method.

Experiments on flies

Fly cultures were grown on standard cornmeal agar medium at 25°C, unless otherwise stated. All fly lines were tested for *Wolbachia* infection and cured whenever necessary by treating with tetracycline (5µg/ml food) for at least two generations.

For CG5641 KO experiments, w^{1118} flies were used as wild-type controls; Dicer-2 mutant flies Dcr-$2^{L811fsX}$/Df – as positive control (Girardi et al. 2015). We used null mutant flies of CG5641 (Kyoto Stock center, DGRC number 104141, *w*; P{w+mW.hs=GawB}CG5641^{NP2255}/TM3,Sb1 Ser1*) - CG5641^{NP2255}. These flies contain P element, it was localized by inverse PCR and cycle sequencing (as described by Jay

Rehm E., Berkeley Drosophila Genome Project). As a weak stock, $CG5641^{NP2255}$ were backcrossed to w^{1118} flies during 6 generations before proceeding to virus injection.

Knock down gene expression in flies

KK and GD RNAi fly lines from VDRC were used to induce the knockdown of candidate genes (Table M2). shmCherry (BDSC no. 35787) and shAGO2 (BDSC no. 34799) lines were used as controls. Males expressing UAS-IR of the target gene were crossed with virgin females expressing {Actin-Gal4/CyO; $Tubulin\text{-}Gal80^{TS}$} at 18°C. In 1 week, adults were removed. In another 2 weeks, when the F1 generation appears, the flies are moved to 29°C for 5-7 days to induce RNAi. All experiments were subsequently done at 29°C.

Virus injection

Viral stocks were prepared in 10mM Tris-HCl, pH7.5. Infections were performed with 6-8 days old adult flies by intrathoracic injection (Nanoject II apparatus, Drummond Scientific) with 4.6nL of viral particle solution (500pfu/fly for DCV and FHV, 5pfu/fly for CrPV, 2,500pfu/fly for SINV, 10,000pfu/fly for VSV, 5000pfu/fly for IIV-6). Injection of the same volume of 10mM Tris-HCl, pH7.5, was used as a control. Infected flies were monitored daily for survival, or were frozen for RNA isolation at the indicated time points.

Molecular biology techniques

RNA analysis

Total RNA from flies was isolated using a Nucleuspin®96 kit or manually using Trizol Reagent RT bromoanisole solution (MRC), according to the manufacturer's instructions. Total RNA from S2 cells was isolated using Nucleospin®RNA kit. 1µg total RNA was reverse transcribed using an iScript™ cDNA synthesis kit (Biorad). 100ng of cDNA was used for quantitative real time PCR (RT-qPCR), using iQ™ Custom SYBR Green Supermix Kit (Biorad) for fly samples, according to the manufacturer's instructions, on a CFX384 Touch Real-Time PCR

platform (Bio-Rad). The list of primers used and their sequence is presented in Table M1. Primers, targeting viral sequences are listed in (Goto et al. 2018). Normalization was performed with the housekeeping gene *Rp49*.

Protein analysis and immunofluorescence

Cells were lysed in protein extraction buffer (30mM Tris-HCl, pH7.5, 150mM NaCl, 2mM $MgCl_2$, 1%NP40 2xComplete Protease Inhibitor Cocktail EDTA free from Roche). Then lysates were kept on ice for 30 min and span at 13,000rpm for 30 min at 4°C. The concentration of the supernatants was measured with Bradford reagent (Bio-Rad), mixed with LDS sample buffer (Life Technologies) and run on SDS-PAGE ready-to-use gels (Bio-Rad). Separated proteins were then transferred on nitrocellulose membrane and blotted with the appropriate antibody.

Immunofluorescence

S2 cells were transiently transfected with N-terminally HA-tagged CG5641 expression plasmid. Three days post transfection cells were infected with DCV and CrPV for 16h. After that, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature followed by blocking in PBS containing 10% FBS and 0.1% Triton X-100 for 30 min. The cells were then incubated overnight with primary antibody (see Table M3) in blocking buffer. Then cells were washed with PBST (PBS containing 0.1% Triton X-100) two times, and then incubated with Alexa Fluor 488 anti-rabbit or Alexa 594 anti-mouse IgG (500-fold dilution in PBT, Molecular Probes) for 1 h, then again washed two times with PBST. Vectashield mounting medium with DAPI was used to visualize nuclei (Vector Laboratories). Specimens were observed under Zeiss LSM780 confocal microscope.

Data analysis

Unpaired two-tailed Student's t test and multiple t-tests were used for statistical analysis of data with GraphPad Prism (GraphPad Software). Survival curves were plotted and analyzed by log-rank analysis (Kaplan-Meier method) using

GraphPad Prism (GraphPad Software). *p* values lower than 0.05 were considered statistically significant.

Analysis of AP-MS screen

Label-free quantification (LFQ) intensity data for all baits and biological samples was provided by A. Pichlmair's lab. The ranking of the candidates to produce the list of top 10% of candidates were performed by J. Colinge's lab.

To calculate the significance values for the volcano plots of the proteins binding to a given bait vs control, multiple t-tests (one per row, desired FDR(Q)=1%) were used (GraphPad Prism). Volcano plots were drawn using *ggplot2* package on R. Heatmaps were done using *pheatmap* function.

STRING v10.5 was used to perform network and enrichment analysis of significant hits. PPI enrichment p-value shows whether obtained network has more interactions than the whole genome.

DIOPT v7.1 - DRSC Integrative Ortholog Prediction Tool was used for orthologue prediction analysis.

Functional screen analysis

All analysis was done on R (version 1.1.447). Delta Cq was calculated by subtracting Cq_{virus} from Cq_{Rp49}. Log2FC was calculated against mCherry control per experiment (separated by virus, day post infection and date of injection), to account for variability between the controls on different dates of injection. Boxplots were plotted using *ggplot2* package and show log2FC change where mCherry values correspond to 0. Significance was calculated using *lsmeans* function with Dunnet's adjustment for p values. Heatmaps were done using *pheatmap* function. All scripts for analysis and raw data files are available in Supplementary Data.

Table M1. Primers table.			
Primer no.	Gene	Description	Sequence
Primers for sgRNA synthesis			
OJL3697	AGO2	sgRNA 1	TTCGTATATTTGAATAGCTGTA
OJL3698	AGO2	sgRNA 1	AACTACAGCTATTCAAATATAC
OJL3699	AGO2	sgRNA 2	TTCGGCAACTTACAGTCACTTC
OJL3700	AGO2	sgRNA 2	AACGAAGTGACTGTAAGTTGCC
OJL3685	CG1703	sgRNA 1	TTCGTGTTCACAGACATGTCCA
OJL3686	CG1703	sgRNA 1	AACTGGACATGTCTGTGAACAC
OJL3687	CG1703	sgRNA 2	TTCGACTCACCCTTGGCTGAGA
OJL3688	CG1703	sgRNA 2	AACTCTCAGCCAAGGGTGAGTC
OJL3489	CG5641	sgR-A Fw	TTCGGTGTGACCGAAGCTGGAC
OJL3490	CG5641	sgR-A Rev	AACGTCCAGCTTCGGTCACACC
OJL3495	CG5641	sgR-B Fw	TTCGCCACAGaAATATGGTTCG
OJL3496	CG5641	sgR-B Rev	AACCGAACCATATTtCTGTGGC
OJL3487	CG5641	sgR-C Fw	TTCGTATCCAAGGTGTCTGCAA
OJL3488	CG5641	sgR-C Rev	AACTTGCAGACACCTTGGATAC
OJL3689	CG9330	sgRNA 1	TTCGAAAGCGAATTTGCGATCA
OJL3690	CG9330	sgRNA 1	AACTGATCGCAAATTCGCTTTC
OJL3691	CG9330	sgRNA 2	TTCGTTGGAGTGGTTCGTCAAG
OJL3692	CG9330	sgRNA 2	AACCTTGACGAACCACTCCAAC
OJL3701	Dicer-2	sgRNA 3	TTCGTGACAAGTTAGTAGCGAA
OJL3702	Dicer-2	sgRNA 3	AACTTCGCTACTAACTTGTCAC
OJL4028	Lost	sgRNA Lost F1 (ATG site) Fw	ttcgTGGCGAGCGATTCGATGG
OJL4029	Lost	sgRNA Lost F1 (ATG site) Rev	aacCCATCGAATCGCTCGCCAc
OJL4030	Lost	sgRNA Lost F2 Fw	ttcgTGAATACCCATGTTCCAG
OJL4031	Lost	sgRNA Lost F2 Rev	aacCTGGAACATGGGTATTCAc
OJL3693	Pixie	sgRNA 1	TTCGACAGCATGTCGCGCAGAA
OJL3694	Pixie	sgRNA 1	AACTTCTGCGCGACATGCTGTC
OJL3695	Pixie	sgRNA 2	TTCGTCTGGGGAATTGAAAGCA
OJL3696	Pixie	sgRNA 2	AACTGCTTTCAATTCCCCAGAC
OJL3497	R2d2	sgRNA_R1_F	TTCGCTCCAATGTTATTAGAGA
OJL3498	R2d2	sgRNA_R1_R	AACTCTCTAATAACATTGGAGC
oJL5141	Tao	sgR, first start Fw	TTCGTGAAAGACAGCCATGCCTT
oJL5142	Tao	sgR, first start Rev	AACAAGGCATGGCTGTCTTTCAC
oJL5143	Tao	sgR, intron 1 Fw	TTCGAAAGAGATACGGTGAGCAC
oJL5144	Tao	sgR, intron 1 Rev	AACGTGCTCACCGTATCTCTTTC
oJL5147	Tao	sgR, intron 3 Fw	TTCGCCATCCGCGATCCGGCAGG
oJL5148	Tao	sgR, intron 3 Rev	AACCCTGCCGGATCGCGGATGGC
oJL5145	Tao	sgR, second start Fw	TTCGGGCCAAAACATTGGCTCCT
oJL5146	Tao	sgR, second start Rev	AACAGGAGCCAATGTTTTGGCCC
Primers for PCR and sequencing			
OJL3664	Pixie	Pix Rev	CGTTGTGTGCTTCTCCAGA
OJL3663	Pixie	Pix Fw	ATATTTCAGCACTCTGTCAACG
OJL4205	Lost	Lost Rev	CAGCTCCCAGTAAACGCCAT
OJL4204	Lost	Lost Fw	TGCAGGTGGTGGACTCACTG

OJL3277	CG5641	Genomic DNA, Rev	GCTGGGAGTGGGACTCAGG
oJL5155	Tao	Genomic DNA, Rev	CTCACCTCAAGCCGATCGAGGTC
OJL3461	CG5641	Genomic DNA, Fw	GCAGATTAAAAACACCAGTCTCCG
oJL5154	Tao	Genomic DNA, before 2nd ATG Fw	CTTAGAATCCGTTGGCGTGCCG
oJL5152	Tao	Genomic DNA, 5'UTR Fw	CGCACAGAACTTCAAGACAG
oJL5153	Tao	Genomic DNA, 2nd exon Rev	CATCCCTTGTATTCTATGGTGTT
OJL3794	Dicer-2	Dcr-2 Rv	CTTTCCGCCGCTTTCGAT
OJL3660	CG9330	CG9330 Rv	CCGCCTTTCCTAGTTTCTTGG
OJL3659	CG9330	CG9330 Fw	TGATGGTGCTAAAACTGCTGG
OJL3658	CG1703	CG1703 Rv Exon 1, Intron	CAATCAATAGGTTGGCGTTCA
OJL3656	CG1703	CG1703 Rv 3'UTR	CTATGCTCTCCAGATCCTCGTT
OJL3657	CG1703	CG1703 Fw Exon 1, Intron	GATGTCACGACCAGGAATCG
OJL3655	CG1703	CG1703 Fw 3'UTR	TTGCAAGTAAGTGTGTGTGTGG
Primers for dsRNA production			
OJL3272	CG5641	RNAi 1 Fw	taatacgactcactatagggGAGGAAGTGCGTCAGGTTGGC
OJL3273	CG5641	RNAi 1Rv	taatacgactcactatagggGATGGCCAAATGGGCGATCAGG
OJL3274	CG5641	RNAi 2 Fw	taatacgactcactatagggCCCGTCGCGACAGGCACTGCCC
OJL3275	CG5641	RNAi 2 Rv	taatacgactcactatagggCTCGGCGCCATCGTCGCTGCCC
OJL3907	Zn72D	dsRNA 1; 5 prime	taatacgactcactatagggCGATTACGCTGCGGTCACACT
OJL3908	Zn72D	dsRNA 1; 5 prime	taatacgactcactatagggCTGACCGAGGCTGTCGAAC
OJL3909	Zn72D	dsRNA 2; 3 prime	taatacgactcactatagggTTACCCTCACCTCGCCTTTGC
OJL3910	Zn72D	dsRNA 2; 3 prime	taatacgactcactatagggGCTCCATGCCAAGCACTTTGT
OJL4024	Lost	dsLost 5' Fw	taatacgactcactatagggGAGGACCAAAGCAACGCAGC
OJL4025	Lost	dsLost 5' Rev	taatacgactcactatagggTCCGCGTCCAATGCGGTAAC
OJL4026	Lost	dsLost middle Fw	taatacgactcactatagggTCTCGTGATATTCGCGTCAAGG
OJL4027	Lost	dsLost middle Rev	taatacgactcactatagggCGACCGTTCATTGGCTGCG
oJL4956	Tao	DRSC19573 Fw	taatacgactcactatagggCAGCAGGATGTGGAGAGG
oJL4957	Tao	DRSC19573 Fw	taatacgactcactatagggGCGCAGCTGCTGATCC
oJL5088	Tao	Fw	taatacgactcactatagggGATGCTCACTGCCTACCAG
oJL5089	Tao	Rev	taatacgactcactatagggCTTAGCGGCGATTTCTGTTGCTTG
OJL3511	DIAP1	dsDIAP1/th.F	TAATACgACTCACTATAgggAgACCACCCAACGACTCGACGCT
OJL3512	DIAP1	dsDIAP1/th.R	TAATACgACTCACTATAgggAgACCACGCCACCGTATCGATATAGAG
oJL5077	Zn72D	dsZn72D Fw DRSC11318	taatacgactcactatagggGGAGGAGCCCAAAAAGATG
oJL5078	Zn72D	dsZn72D Rv DRSC11319	taatacgactcactatagggCGACATCCAGTTGTTGTAGTCA
oJL5139	Zn72D	Zn72D dsRNA n4 Fw	taatacgactcactatagggCAGTCAAGAATCAGGTCAAGGGC
oJL5140	Zn72D	Zn72D dsRNA n4 Rev	taatacgactcactatagggCACCTTCTGATGCTTGGCTCC
OJL 2685	RpS3	Fw	taatacgactcactatagggTTCGTTTCCGATGGCATCTT
OJL 2686	RpS3	Rev	taatacgactcactatagggAGTCCTCCGGTGAGCTTGTA
Primers for qPCR			
OJL3276	CG5641	Fw	GACATTCGTGCCGCGCCAC
OJL3277	CG5641	Rev	GCTGGGAGTGGGACTCAGG
OJL3750	CG5641	Exon2 Fw	TAACCAGGACCTGAGTCCCA
OJL3751	CG5641	Exon2-3 Rev	GCACTTCCTCCAGTTGACAAG
OJL3752	CG5641	Exon2-3 Fw	CAACTTGTCAACTGGAGGAAGTG
OJL3753	CG5641	Exon 3 Rev	GTGGGCAGGGTTTTGAGGAT

OJL3703	CG5641	Fw	GCAACACCAGCGTTGTGCGCG
OJL3704	CG5641	Rev	CATGTCGATGTCCTCCTCGAGG
oJL4978	Tao	PA60276 Fw	AGACACAGGAGCTGGAGTAC
oJL4979	Tao	PA60276 Rv	TCGTGTTGCTTGTTTATCTGCTC
oJL5090	Tao	qPCR Fw	CAAGCATGCGGTTGAGCTG
oJL5091	Tao	qPCR Rev	CGTTTGTATTGCTTCGTCTGC
OJL3780	CG1703	Cg1703	CCATCTCAGCCAAGGGTAACG
OJL3781	CG1703	Cg1703	CAATATGCCGGAGCAGGGT
OJL3823	Pixie	Pixie CG5651	CTTGACCATTCGATCACTCCTG
OJL3824	Pixie	Pixie CG5651	CATAGTAACCACACCGTAGCAG
OJL3825	CG9330	CG9330	TGGAGCGAAAGGTTCTCAACG
OJL3826	CG9330	CG9330	CCACTTTCATTGGCGCCATCTT
oJL5079	Zn72D	Zn72D Fw	GGCGACTATCGAAGTGCCAT
oJL5080	Zn72D	Zn72D Rev	GAGCCGAGTAGTGCGTCTT
OJL3960	Zn72D	Zn72d rev for qPCR to pair with OJL3909	CTCGAGCCTGGAACCATTTGG
OJL3961	Zn72D	Zn72d rev for qPCR	TTACCCTCACCTCGCCTTTGC
oJL3992	Zn72D	Zn72d	ATGCCGGAGCGGCCAATAAG
oJL3993	Zn72D	Zn72d	CTCGCATCACTCCCTTCAGG
OJL4018	Lost	Lost fw	TGGAAGCCAGCGGAGAACG
OJL4019	Lost	Lost rv	CAGTGCCGCGAGCGTGTAG
Primers for Gateway® cloning			
oJL5071	Zn72D	Zn72D attB Fw	ggggacaagtttgtacaaaaaagcaggcttcATGGCCAACAACAACTACGCC
oJL5072	Zn72D	Zn72D attB Rv N-ter	ggggaccactttgtacaagaaagctgggtcTTAGGTGCTGGCAGGAGTG
oJL5073	Zn72D	Zn72D attB Rv C-ter	ggggaccactttgtacaagaaagctgggtcGGTGCTGGCAGGAGTGCC
OJL3761	CG5641	attB	ggggacaagtttgtacaaaaaagcaggcttcATGGTTCGCGGAGCTCTC
OJL3762	CG5641	attB	ggggaccactttgtacaagaaagctgggtcTTACTCGGCGCCATCGTC
OJL3763	CG5641	attB	ggggaccactttgtacaagaaagctgggtcCTCGGCGCCATCGTCGCT

Table M2. RNAi fly lines from VDRC Drosophila stock center for the candidate proteins used in functional screen.

No.	Gene name	VDRC ID	Collection		No.	Gene name	VDRC ID	Collection
1	CG9684	24090	GD		**51**	shep	37863	GD
2	CG4538	17250	GD		**52**	rig	107777	KK
3	Klp10A	41534	GD		**53**	Thor	100739	KK
4	RecQ5	13363	GD		**54**	Zn72D	105954	KK
5	XRCC1	30506	GD		**55**	Sucb	101554	KK
6	CG2199	20839	GD		**56**	Adar	105612	KK
7	CG7194	24723	GD		**57**	CG2118	110398	KK
8	CG7194	24724	GD		**58**	CG7488	106677	KK
9	Fandango	104186	KK		**59**	Pabp2	106466	KK
10	CG9667	104484	KK		**60**	pst	107243	KK
11	MFE2	108880	KK		**61**	Klp59D	100530	KK
12	CG3800	108342	KK		**62**	CG12258	104460	KK
13	Tao	107645	KK		**63**	trsn	108456	KK
14	CG6379	103723	KK		**64**	CG8726	109451	KK
15	lost	110736	KK		**65**	Rbp9	101412	KK
16	CHD1	103640	KK		**66**	qkr58E-1	26332	GD
17	DIP1	108186	KK		**67**	La	2988	GD
18	CG31368	110348	KK		**68**	cup	18179	GD
19	stau	106645	KK		**69**	Hrb27C	101555	KK
20	Rps10	106323	KK		**70**	loqs	108358	KK
21	Tlk	105732	KK		**71**	CG11505	105949	KK
22	CG12112	104224	KK		**72**	mRpL46	110327	KK
23	hen1	103400	KK		**73**	Vps4	105977	KK
24	CG42232	108471	KK		**74**	CG31156	107269	KK
25	CG5757	110460	KK		**75**	U2af50	107723	KK
26	CG9418	108979	KK		**76**	Nnp-1	106204	KK
27	CG6103	101512	KK		**77**	CG4622	106312	KK
28	trl	106433	KK		**78**	Rab10	101454	KK
29	CG3178	108661	KK		**79**	ADF1	102176	KK
30	CG9330	105156	KK		**80**	elav	37915	GD
31	CG1703	105998	KK		**81**	mxt	21763	GD
32	mle	19691	GD		**82**	RpI1	110680	KK
33	ncd	22570	GD		**83**	pix	109630	KK
34	Gnf1	10942	GD		**84**	Droj2	104880	KK
35	Top1	330246	KK		**85**	CG6227	110778	KK
36	Slik	43783	GD		**86**	CG5316	108346	KK
37	l(3)07882	105967	KK		**87**	metro	110814	KK
38	caz	100291	KK		**88**	Cbp20	107112	KK
39	sun	23685	GD		**89**	qkr58E-2	106944	KK
40	CG13364	28464	GD		**90**	Cf2	103664	KK
41	Rat1	105380	KK		**91**	blw	34664	GD
42	CG8963	110576	KK		**92**	yps	27472	GD
43	CG4612	52497	GD		**93**	msi	11784	GD
44	CG5641	101343	KK		**94**	LSm-4-RB	34752	GD
45	ATPsynE	46764	GD		**95**	CG5414	100000	KK
46	obe	107282	KK		**96**	CG5800	103769	KK
47	blp	13794	GD		**97**	ben	109638	KK
48	WRNexo	44595	GD		**98**	eff	110767	KK
49	CG11858	49686	GD		**99**	sl	108593	KK
50	CG32344	110441	KK		**100**	Bre1	108206	KK

Table M3. List of antibodies used for SDS-PAGE and immunofluorescence.

Antibody	Source	Conditions
Anti-DCV	Chtarbanova et al., 2014; Dostert et al., 2005	1:1000 for IF
Anti-CrPV	Chtarbanova et al., 2014	1:1000 for IF
Anti-Actin5C	Millipore, clone 4, RRID: AB_2223041	1:5000 for WB
Anti-Dicer2	Abcam #4732	1:500 for WB
Anti-AGO2	Abcam #5072	1:1000 for WB
Anti-HA	Abcam #9110	1:1000 for IF
Anti-Flag	Sigma. RRID: AB_259529	1:1000 for WB
Anti-GFP	A6455 zymed	1:1000 for WB
Anti-dsRed	Clontech 632496 Ozyme	1:1000 for WB
Anti-J2	Scicons J2 tech	1:500 for IF

Table M4. List of vectors used and produced during the study.

pJL824	U6-Dicer-2-F1-sgRNA Actin5C-Cas9-Puro
pJL825	U6-Dicer-2-R1-sgRNA Actin5C-Cas9-Puro
pJL826	U6-Dicer-2-F2-sgRNA Actin5C-Cas9-Puro
pJL827	U6-Dicer-2-R2-sgRNA Actin5C-Cas9-Puro
pJL828	U6-Dicer-2-3-sgRNA Actin5C-Cas9-Puro
pJL829	U6-AGO2-1-sgRNA Actin5C-Cas9-Puro
pJL830	U6-AGO2-2-sgRNA Actin5C-Cas9-Puro
pJL831	U6-R2D2-F1-sgRNA Actin5C-Cas9-Puro
pJL832	U6-R2D2-R1-sgRNA Actin5C-Cas9-Puro
pJL833	U6-CG5641-F1-sgRNA Actin5C-Cas9-Puro
pJL834	U6-CG5641-R1-sgRNA Actin5C-Cas9-Puro
pJL835	U6-CG5641-F2-sgRNA Actin5C-Cas9-Puro
pJL836	U6-CG8368-F1-sgRNA Actin5C-Cas9-Puro
pJL837	U6-CG8368-R1-sgRNA Actin5C-Cas9-Puro
pJL838	U6-CG1703-1-sgRNA Actin5C-Cas9-Puro
pJL839	U6-CG1703-2-sgRNA Actin5C-Cas9-Puro
pJL840	U6-CG9330-1-sgRNA Actin5C-Cas9-Puro
pJL841	U6-CG9330-2-sgRNA Actin5C-Cas9-Puro
pJL842	U6-CG5651(Pixie)-1-sgRNA Actin5C-Cas9-Puro
pJL843	U6-CG5651(Pixie)-2-sgRNA Actin5C-Cas9-Puro
pJL847	p-DONR for N-terminal CG5641
pJL848	N-GFP-CG5641 for cells
pJL849	N-HA-CG5641 for cells
pJL850	N-FLAG-CG5641 for cells
D-E8	cDNA clone Pixie RE71924
D-E9	cDNA CG1703 LD04461
D-F1	cDNA CG5641 GH25564
D-F2	cDNA CG9330 FI01412

Bibliography

Afonso, Olga, Irina Matos, António J Pereira, Paulo Aguiar, Michael A Lampson, and Helder Maiato. 2014. "Feedback Control of Chromosome Separation by a Midzone Aurora B Gradient.." *Science* 345 (6194). American Association for the Advancement of Science: 332–36. doi:10.1126/science.1251121.

Alexopoulou, L, A C Holt, R Medzhitov, and R A Flavell. 2001. "Recognition of Double-Stranded RNA and Activation of NF-kappaB by Toll-Like Receptor 3.." *Nature* 413 (6857). Nature Publishing Group: 732–38. doi:10.1038/35099560.

Andersen, Ditte S, and Sally J Leevers. 2007. "The Essential DrosophilaATP-Binding Cassette Domain Protein, Pixie, Binds the 40 S Ribosome in an ATP-Dependent Manner and Is Required for Translation Initiation." *The Journal of Biological Chemistry* 282 (20): 14752–60. doi:10.1074/jbc.M701361200.

Avadhanula, Vasanthi, Brandon P Weasner, Gail G Hardy, Justin P Kumar, and Richard W Hardy. 2009. "A Novel System for the Launch of Alphavirus RNA Synthesis Reveals a Role for the Imd Pathway in Arthropod Antiviral Response.." Edited by Shou-Wei Ding. *PLoS Pathog* 5 (9). Public Library of Science: e1000582. doi:10.1371/journal.ppat.1000582.

Avila, Antonia, Neal Silverman, María T Diaz-Meco, and Jorge Moscat. 2002. "The Drosophila Atypical Protein Kinase C-Ref(2)P Complex Constitutes a Conserved Module for Signaling in the Toll Pathway.." *Molecular and Cellular Biology* 22 (24). American Society for Microbiology (ASM): 8787–95. doi:10.1128/MCB.22.24.8787-8795.2002.

Barthelme, D, S Dinkelaker, S V Albers, P Londei, U Ermler, and R Tampe. 2011. "Ribosome Recycling Depends on a Mechanistic Link Between the FeS Cluster Domain and a Conformational Switch of the Twin-ATPase ABCE1." *Proceedings of the National Academy of Sciences* 108 (8): 3228–33. doi:10.1073/pnas.1015953108.

Bassett, Andrew R, Charlotte Tibbit, Chris P Ponting, and Ji-Long Liu. 2014. "Mutagenesis and Homologous Recombination in Drosophila Cell Lines Using CRISPR/Cas9.." *Biology Open* 3 (1). The Company of Biologists Ltd: 42–49. doi:10.1242/bio.20137120.

Becker, Thomas, Gerrit Loch, Marc Beyer, Ingo Zinke, Anna C Aschenbrenner, Pilar Carrera, Therese Inhester, Joachim L Schultze, and Michael Hoch. 2010. "FOXO-Dependent Regulation of Innate Immune Homeostasis.." *Nature* 463 (7279). Nature Publishing Group: 369–73. doi:10.1038/nature08698.

Bellen, Hugo J, and Shinya Yamamoto. 2015. "Morgan's Legacy: Fruit Flies and the Functional Annotation of Conserved Genes." *Cell* 163 (1): 12–14. doi:10.1016/j.cell.2015.09.009.

Berezikov, E, N Robine, A Samsonova, J O Westholm, A Naqvi, J H Hung, K Okamura, et al. 2011. "Deep Annotation of Drosophila Melanogaster microRNAs Yields Insights Into Their Processing, Modification, and Emergence." *Genome Research* 21 (2): 203–15. doi:10.1101/gr.116657.110.

Bisbal, Catherine, Camille Martinand, Michelle Silhol, Bernard Lebleu, and Tamim Salehzada. 1995. "Cloning and Characterization of a RNase L Inhibitor.." *The Journal of Biological Chemistry* 270 (22). American Society for Biochemistry and Molecular Biology: 13308–17. doi:10.1074/jbc.270.22.13308.

Bitra, Kavita, Richard J Suderman, and Michael R Strand. 2012. "Polydnavirus Ank Proteins Bind NF-κB Homodimers and Inhibit Processing of Relish.." Edited by

David S Schneider. *PLoS Pathog* 8 (5). Public Library of Science: e1002722. doi:10.1371/journal.ppat.1002722.
Boggiano, Julian C, Pamela J Vanderzalm, and Richard G Fehon. 2011. "Tao-1 Phosphorylates Hippo/MST Kinases to Regulate the Hippo-Salvador-Warts Tumor Suppressor Pathway.." *Developmental Cell* 21 (5): 888–95. doi:10.1016/j.devcel.2011.08.028.
Boutros, Michael, Amy A Kiger, Susan Armknecht, Kim Kerr, Marc Hild, Britta Koch, Stefan A Haas, Renato Paro, Norbert Perrimon, Heidelberg Fly Array Consortium. 2004. "Genome-Wide RNAi Analysis of Growth and Viability in Drosophila Cells.." *Science* 303 (5659). American Association for the Advancement of Science: 832–35. doi:10.1126/science.1091266.
Brand, A H, and N Perrimon. 1993. "Targeted Gene Expression as a Means of Altering Cell Fates and Generating Dominant Phenotypes.." *Development (Cambridge, England)* 118 (2): 401–15.
Brennecke, J, A A Aravin, A Stark, M Dus, M Kellis, R Sachidanandam, and G J Hannon. 2007. "Discrete Small RNA-Generating Loci as Master Regulators of Transposon Activity in Drosophila." *Cell* 128 (6): 1089–1103. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17346786.
Bronkhorst, Alfred W, and Ronald P van Rij. 2014. "The Long and Short of Antiviral Defense: Small RNA-Based Immunity in Insects." *Current Opinion in Virology* 7 (August): 19–28. doi:10.1016/j.coviro.2014.03.010.
Bronkhorst, Alfred W, Koen W R van Cleef, Hanka Venselaar, and Ronald P van Rij. 2014. "A dsRNA-Binding Protein of a Complex Invertebrate DNA Virus Suppresses the Drosophila RNAi Response.." *Nucleic Acids Research* 42 (19). Oxford University Press: 12237–48. doi:10.1093/nar/gku910.
Bronkhorst, Alfred W, Koen W R van Cleef, Nicolas Vodovar, Ikbal Agah Ince, Hervé Blanc, Just M Vlak, Maria-Carla Saleh, and Ronald P van Rij. 2012. "The DNA Virus Invertebrate Iridescent Virus 6 Is a Target of the Drosophila RNAi Machinery.." *Proceedings of the National Academy of Sciences of the United States of America* 109 (51). National Acad Sciences: E3604–13. doi:10.1073/pnas.1207213109.
Burnet, F M. 1959. *The Clonal Selection Theory of Acquired Immunity.* Nashville,: Vanderbilt University Press. doi:10.5962/bhl.title.8281.
Bürckstümmer, Tilmann, Christoph Baumann, Stephan Blüml, Evelyn Dixit, Gerhard Dürnberger, Hannah Jahn, Melanie Planyavsky, et al. 2009. "An Orthogonal Proteomic-Genomic Screen Identifies AIM2 as a Cytoplasmic DNA Sensor for the Inflammasome.." *Nat Immunol* 10 (3): 266–72. doi:10.1038/ni.1702.
Byszewska, Magdalena, Mirosław Śmietański, Elzbieta Purta, and Janusz M Bujnicki. 2014. "RNA Methyltransferases Involved in 5' Cap Biosynthesis.." *RNA Biology* 11 (12): 1597–1607. doi:10.1080/15476286.2015.1004955.
Cao, Chuan, Michael M Magwire, Florian Bayer, and Francis M Jiggins. 2016. "A Polymorphism in the Processing Body Component Ge-1 Controls Resistance to a Naturally Occurring Rhabdovirus in Drosophila.." Edited by David S Schneider. *PLoS Pathog* 12 (1). Public Library of Science: e1005387. doi:10.1371/journal.ppat.1005387.
Cao, Chuan, Rodrigo Cogni, Vincent Barbier, and Francis M Jiggins. 2017. "Complex Coding and Regulatory Polymorphisms in a Restriction Factor Determine the Susceptibility of Drosophila to Viral Infection.." *Genetics* 206 (4): 2159–73. doi:10.1534/genetics.117.201970.
Carré-Mlouka, A, S Gaumer, P Gay, A M Petitjean, C Coulondre, P Dru, F Bras, S

Dezélée, and D Contamine. 2007. “Control of Sigma Virus Multiplication by the Ref(2)P Gene of Drosophila Melanogaster: an in Vivo Study of the PB1 Domain of Ref(2)P..” *Genetics* 176 (1). Genetics: 409–19. doi:10.1534/genetics.106.063826.
Carthew, Richard W, Pamela Agbu, and Ritika Giri. 2016. “MicroRNA Function in Drosophila Melanogaster.” *Seminars in Cell & Developmental Biology*, March. doi:10.1016/j.semcdb.2016.03.015.
Cevallos, R C, and P Sarnow. 2010. “Temperature Protects Insect Cells From Infection by Cricket Paralysis Virus.” *Journal of Virology* 84 (3). American Society for Microbiology Journals: 1652–55. doi:10.1128/JVI.01730-09.
Chandler, Christopher H, Sudarshan Chari, and Ian Dworkin. 2013. “Does Your Gene Need a Background Check? How Genetic Background Impacts the Analysis of Mutations, Genes, and Evolution..” *Trends in Genetics : TIG* 29 (6): 358–66. doi:10.1016/j.tig.2013.01.009.
Chao, J A, J H Lee, B R Chapados, E W Debler, A Schneemann, and J R Williamson. 2005. “Dual Modes of RNA-Silencing Suppression by Flock House Virus Protein B2.” *Nat Struct Mol Biol* 12 (October): 952–57. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16228003.
Cherbas, Lucy, and Lei Gong. 2014. “Cell Lines..” *Methods (San Diego, Calif.)* 68 (1): 74–81. doi:10.1016/j.ymeth.2014.01.006.
Cherry, Sara, Amit Kunte, Hui Wang, Carolyn Coyne, Robert B Rawson, and Norbert Perrimon. 2006. “COPI Activity Coupled with Fatty Acid Biosynthesis Is Required for Viral Replication..” *PLoS Pathog* 2 (10). Public Library of Science: e102. doi:10.1371/journal.ppat.0020102.
Cherry, Sara, and Norbert Perrimon. 2004. “Entry Is a Rate-Limiting Step for Viral Infection in a Drosophila Melanogaster Model of Pathogenesis..” *Nat Immunol* 5 (1): 81–87. doi:10.1038/ni1019.
Cherry, Sara, Tammy Doukas, Susan Armknecht, Sean Whelan, Hui Wang, Peter Sarnow, and Norbert Perrimon. 2005. “Genome-Wide RNAi Screen Reveals a Specific Sensitivity of IRES-Containing RNA Viruses to Host Translation Inhibition..” *Genes & Development* 19 (4). Cold Spring Harbor Lab: 445–52. doi:10.1101/gad.1267905.
Choi, Young, and Ai-Ming Yu. 2014. “ABC Transporters in Multidrug Resistance and Pharmacokinetics, and Strategies for Drug Development.” *Current Pharmaceutical Design* 20 (5): 793–807. doi:10.2174/13816128200514021416521 2.
Chtarbanova, Stanislava, Olivier Lamiable, Kwang-Zin Lee, Delphine Galiana, Laurent Troxler, Carine Meignin, Charles Hetru, Jules A Hoffmann, Laurent Daeffler, and Jean-Luc Imler. 2014. “Drosophila C Virus Systemic Infection Leads to Intestinal Obstruction..” *J Virol* 88 (24). American Society for Microbiology: 14057–69. doi:10.1128/JVI.02320-14.
Clem, Rollie J. 2015. “Viral IAPs, Then and Now..” *Seminars in Cell & Developmental Biology* 39 (March): 72–79. doi:10.1016/j.semcdb.2015.01.011.
Clemens, M J, and A Elia. 1997. “The Double-Stranded RNA-Dependent Protein Kinase PKR: Structure and Function..” *Journal of Interferon & Cytokine Research : the Official Journal of the International Society for Interferon and Cytokine Research* 17 (9): 503–24. doi:10.1089/jir.1997.17.503.
Coch, Christoph, Nicolas Busch, Vera Wimmenauer, Evelyn Hartmann, Markus Janke, Mona Mohamed Ahmed Abdel-Mottaleb, Alf Lamprecht, et al. 2009. “Higher Activation of TLR9 in Plasmacytoid Dendritic Cells by Microbial DNA Compared with Self-DNA Based on CpG-Specific Recognition of Phosphodiester DNA..”

Journal of Leukocyte Biology 86 (3): 663–70. doi:10.1189/jlb.0509314.
Cogni, Rodrigo, Chuan Cao, Jonathan P Day, Calum Bridson, and Francis M Jiggins. 2016. "The Genetic Architecture of Resistance to Virus Infection in Drosophila.." *Molecular Ecology*, July. doi:10.1111/mec.13769.
Contamine, D. 1981. "Role of the Drosophila Genome in Sigma Virus Multiplication. I. Role of the Ret(2)P Gene; Selection of Host-Adapted Mutants at the Nonpermissive Allele Pp.." *Virology* 114 (2): 474–88.
Contamine, D, A M Petitjean, and M Ashburner. 1989. "Genetic Resistance to Viral Infection: the Molecular Cloning of a Drosophila Gene That Restricts Infection by the Rhabdovirus Sigma.." *Genetics* 123 (3). Genetics Society of America: 525–33.
Costa, Alexandre, Eric Jan, Peter Sarnow, and David Schneider. 2009. "The Imd Pathway Is Involved in Antiviral Immune Responses in Drosophila.." Edited by Peter Sommer. *PLoS ONE* 4 (10). Public Library of Science: e7436. doi:10.1371/journal.pone.0007436.
Crow, Yanick J, Bruce E Hayward, Rekha Parmar, Peter Robins, Andrea Leitch, Manir Ali, Deborah N Black, et al. 2006. "Mutations in the Gene Encoding the 3'-5' DNA Exonuclease TREX1 Cause Aicardi-Goutières Syndrome at the AGS1 Locus.." *Nature Genetics* 38 (8). Nature Publishing Group: 917–20. doi:10.1038/ng1845.
Cureton, David K, Ramiro H Massol, Saveez Saffarian, Tomas L Kirchhausen, and Sean P J Whelan. 2009. "Vesicular Stomatitis Virus Enters Cells Through Vesicles Incompletely Coated with Clathrin That Depend Upon Actin for Internalization.." Edited by John A T Young. *PLoS Pathog* 5 (4): e1000394. doi:10.1371/journal.ppat.1000394.
Daffis, Stephane, Kristy J Szretter, Jill Schriewer, Jianqing Li, Soonjeon Youn, John Errett, Tsai-Yu Lin, et al. 2010. "2′-O Methylation of the Viral mRNA Cap Evades Host Restriction by IFIT Family Members." *Nature* 468 (7322). Nature Publishing Group: 452–56. doi:10.1038/nature09489.
Dasgupta, Ranjit, Heather M Free, Suzanne L Zietlow, Susan M Paskewitz, Serap Aksoy, Lei Shi, Jeremy Fuchs, Changyun Hu, and Bruce M Christensen. 2007. "Replication of Flock House Virus in Three Genera of Medically Important Insects.." *Journal of Medical Entomology* 44 (1): 102–10.
Dasgupta, Ranjit, Li-Lin Cheng, Lyric C Bartholomay, and Bruce M Christensen. 2003. "Flock House Virus Replicates and Expresses Green Fluorescent Protein in Mosquitoes.." *The Journal of General Virology* 84 (Pt 7). Microbiology Society: 1789–97. doi:10.1099/vir.0.18938-0.
Davidson, A L, E Dassa, C Orelle, and J Chen. 2008. "Structure, Function, and Evolution of Bacterial ATP-Binding Cassette Systems." *Microbiology and Molecular Biology Reviews* 72 (2): 317–64. doi:10.1128/MMBR.00031-07.
Decroly, Etienne, Claire Debarnot, François Ferron, Mickael Bouvet, Bruno Coutard, Isabelle Imbert, Laure Gluais, et al. 2011. "Crystal Structure and Functional Analysis of the SARS-Coronavirus RNA Cap 2'-O-Methyltransferase Nsp10/Nsp16 Complex.." Edited by Félix A Rey. *PLoS Pathog* 7 (5): e1002059. doi:10.1371/journal.ppat.1002059.
Decroly, Etienne, François Ferron, Julien Lescar, and Bruno Canard. 2011. "Conventional and Unconventional Mechanisms for Capping Viral mRNA.." *Nature Reviews. Microbiology* 10 (1). Nature Publishing Group: 51–65. doi:10.1038/nrmicro2675.
Deddouche, Safia, Nicolas Matt, Aidan Budd, Stefanie Mueller, Cordula Kemp, Delphine Galiana-Arnoux, Catherine Dostert, Christophe Antoniewski, Jules A Hoffmann, and Jean-Luc Imler. 2008. "The DExD/H-Box Helicase Dicer-2 Mediates the Induction of Antiviral Activity in Drosophila.." *Nat Immunol* 9 (12).

Nature Publishing Group: 1425–32. doi:10.1038/ni.1664.
Dermauw, Wannes, and Thomas Van Leeuwen. 2014. “The ABC Gene Family in Arthropods: Comparative Genomics and Role in Insecticide Transport and Resistance..” *Insect Biochemistry and Molecular Biology* 45 (February): 89–110. doi:10.1016/j.ibmb.2013.11.001.
Dias, Alexandre, Denis Bouvier, Thibaut Crépin, Andrew A McCarthy, Darren J Hart, Florence Baudin, Stephen Cusack, and Rob W H Ruigrok. 2009. “The Cap-Snatching Endonuclease of Influenza Virus Polymerase Resides in the PA Subunit..” *Nature* 458 (7240). Nature Publishing Group: 914–18. doi:10.1038/nature07745.
Diebold, Sandra S, Tsuneyasu Kaisho, Hiroaki Hemmi, Shizuo Akira, and Caetano Reis e Sousa. 2004. “Innate Antiviral Responses by Means of TLR7-Mediated Recognition of Single-Stranded RNA..” *Science* 303 (5663). American Association for the Advancement of Science: 1529–31. doi:10.1126/science.1093616.
Diner, Benjamin A, Krystal K Lum, Jared E Toettcher, and Ileana M Cristea. 2016. “Viral DNA Sensors IFI16 and Cyclic GMP-AMP Synthase Possess Distinct Functions in Regulating Viral Gene Expression, Immune Defenses, and Apoptotic Responses During Herpesvirus Infection..” *mBio* 7 (6): 363. doi:10.1128/mBio.01553-16.
Ding, S W, and Ding, Shou-Wei. 2010. “RNA-Based Antiviral Immunity.” *Nature Reviews Immunology* 10 (9): 632–44. doi:10.1038/nri2824.
Ding, Shou-Wei, Qingxia Han, Jinyan Wang, and Wan-Xiang Li. 2018. “Antiviral RNA Interference in Mammals..” *Curr Opin Immunol* 54 (October): 109–14. doi:10.1016/j.coi.2018.06.010.
Dostert, Catherine, Emmanuelle Jouanguy, Phil Irving, Laurent Troxler, Delphine Galiana-Arnoux, Charles Hetru, Jules A Hoffmann, and Jean-Luc Imler. 2005. “The Jak-STAT Signaling Pathway Is Required but Not Sufficient for the Antiviral Response of Drosophila..” *Nat Immunol* 6 (9). Nature Publishing Group: 946–53. doi:10.1038/ni1237.
Dru, P, F Bras, S Dezélée, P Gay, A M Petitjean, A Pierre-Deneubourg, D Teninges, and D Contamine. 1993. “Unusual Variability of the Drosophila Melanogaster Ref(2)P Protein Which Controls the Multiplication of Sigma Rhabdovirus..” *Genetics* 133 (4). Genetics Society of America: 943–54.
Duffy, Joseph B. 2002. “GAL4 System in Drosophila: a Fly Geneticist's Swiss Army Knife..” *Genesis (New York, N.Y. : 2000)* 34 (1-2): 1–15. doi:10.1002/gene.10150.
Duggal, Nisha K, and Michael Emerman. 2012. “Evolutionary Conflicts Between Viruses and Restriction Factors Shape Immunity.” *Nature Reviews Immunology* 12 (10). Nature Publishing Group: 687–95. doi:10.1038/nri3295.
Durdevic, Zeljko, Katharina Hanna, Beth Gold, Tim Pollex, Sara Cherry, Frank Lyko, and Matthias Schaefer. 2013. “Efficient RNA Virus Control in Drosophila Requires the RNA Methyltransferase Dnmt2..” *EMBO Reports* 14 (3). EMBO Press: 269–75. doi:10.1038/embor.2013.3.
Durdevic, Zeljko, Mehrpouya Balaghy Mobin, Katharina Hanna, Frank Lyko, and Matthias Schaefer. 2013. “The RNA Methyltransferase Dnmt2 Is Required for Efficient Dicer-2-Dependent siRNA Pathway Activity in Drosophila..” *Cell Reports* 4 (5): 931–37. doi:10.1016/j.celrep.2013.07.046.
Ekström, Jens-Ola, and Dan Hultmark. 2016. “A Novel Strategy for Live Detection of Viral Infection in Drosophila Melanogaster..” *Scientific Reports* 6 (1). Nature Publishing Group: 26250. doi:10.1038/srep26250.
Fareh, Mohamed, Jasper van Lopik, Iason Katechis, Alfred W Bronkhorst, Anna C Haagsma, Ronald P van Rij, and Chirlmin Joo. 2018. “Viral Suppressors of RNAi

Employ a Rapid Screening Mode to Discriminate Viral RNA From Cellular Small RNA.." *Nucleic Acids Research* 46 (6): 3257–57. doi:10.1093/nar/gky042.
Faria, Vítor G, Nelson E Martins, Sara Magalhães, Tânia F Paulo, Viola Nolte, Christian Schlötterer, Élio Sucena, and Luis Teixeira. 2016. "Drosophila Adaptation to Viral Infection Through Defensive Symbiont Evolution.." Edited by Harmit S Malik. *PLoS Genetics* 12 (9). Public Library of Science: e1006297. doi:10.1371/journal.pgen.1006297.
Ferreira, Álvaro Gil, Huw Naylor, Sara Santana Esteves, Inês Silva Pais, Nelson Eduardo Martins, and Luis Teixeira. 2014. "The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral Infection in Drosophila.." Edited by David S Schneider. *PLoS Pathog* 10 (12). Public Library of Science: e1004507. doi:10.1371/journal.ppat.1004507.
Field, A K, A A Tytell, G P Lampson, and M R Hilleman. 1967. "Inducers of Interferon and Host Resistance. II. Multistranded Synthetic Polynucleotide Complexes.." *Proceedings of the National Academy of Sciences* 58 (3). National Academy of Sciences: 1004–10.
Fire, Andrew, Fire, A, SiQun Xu, S Xu, Mary K Montgomery, M K Montgomery, S A Kostas, et al. 1998. "Potent and Specific Genetic Interference by Double-Stranded RNA in Caenorhabditis Elegans.." *Nature* 391 (6669). Nature Publishing Group: 806–11. doi:10.1038/35888.
Fischer, J A, E Giniger, T Maniatis, and M Ptashne. 1988. "GAL4 Activates Transcription in Drosophila.." *Nature* 332 (6167): 853–56. doi:10.1038/332853a0.
Fleuriet, A, and G Periquet. 1993. "Evolution of the Drosophila Melanogaster-Sigma Virus System in Natural Populations From Languedoc (Southern France).." *Archives of Virology* 129 (1-4): 131–43.
Förstemann, Klaus, Michael D Horwich, LiangMeng Wee, Yukihide Tomari, and Phillip D Zamore. 2007. "Drosophila microRNAs Are Sorted Into Functionally Distinct Argonaute Complexes After Production by Dicer-1." *Cell* 130 (2): 287–97. doi:10.1016/j.cell.2007.05.056.
García-Sastre, Adolfo. 2010. "Influenza Virus Receptor Specificity: Disease and Transmission.." *The American Journal of Pathology* 176 (4): 1584–85. doi:10.2353/ajpath.2010.100066.
Garrey, Julianne L, Yun-Young Lee, Hilda H T Au, Martin Bushell, and Eric Jan. 2010. "Host and Viral Translational Mechanisms During Cricket Paralysis Virus Infection.." *J Virol* 84 (2). American Society for Microbiology: 1124–38. doi:10.1128/JVI.02006-09.
Gaudet, Pascale, Michael S Livstone, Suzanna E Lewis, and Paul D Thomas. 2011. "Phylogenetic-Based Propagation of Functional Annotations Within the Gene Ontology Consortium.." *Briefings in Bioinformatics* 12 (5): 449–62. doi:10.1093/bib/bbr042.
Gay, Pierre. 1978. "Drosophila Genes Which Intervene in Multiplication of Sigma Virus." *MGG Molecular & General Genetics* 159 (3): 269–83. doi:10.1007/BF00268263.
Gebhardt, Anna, Beatrice T Laudenbach, and Andreas Pichlmair. 2017. "Discrimination of Self and Non-Self Ribonucleic Acids.." *Journal of Interferon & Cytokine Research : the Official Journal of the International Society for Interferon and Cytokine Research* 37 (5). Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA: 184–97. doi:10.1089/jir.2016.0092.
Gehrig, Stefanie, Mariel-Esther Eberle, Flavia Botschen, Katharina Rimbach, Florian Eberle, Tatjana Eigenbrod, Steffen Kaiser, et al. 2012. "Identification of

Modifications in Microbial, Native tRNA That Suppress Immunostimulatory Activity.." *The Journal of Experimental Medicine* 209 (2). Rockefeller University Press: 225–33. doi:10.1084/jem.20111044.
George, Cyril X, Lijo John, and Charles E Samuel. 2014. "An RNA Editor, Adenosine Deaminase Acting on Double-Stranded RNA (ADAR1).." *Journal of Interferon & Cytokine Research : the Official Journal of the International Society for Interferon and Cytokine Research* 34 (6). Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA: 437–46. doi:10.1089/jir.2014.0001.
Girardi, Erika, Mathieu Lefèvre, Béatrice Chane-Woon-Ming, Simona Paro, Bill Claydon, Jean-Luc Imler, Carine Meignin, and Sebastien Pfeffer. 2015. "Cross-Species Comparative Analysis of Dicer Proteins During Sindbis Virus Infection.." *Scientific Reports* 5 (1). Nature Publishing Group: 10693. doi:10.1038/srep10693.
Gitlin, Leonid, Winfried Barchet, Susan Gilfillan, Marina Cella, Bruce Beutler, Richard A Flavell, Michael S Diamond, and Marco Colonna. 2006. "Essential Role of Mda-5 in Type I IFN Responses to Polyriboinosinic:Polyribocytidylic Acid and Encephalomyocarditis Picornavirus.." *Proceedings of the National Academy of Sciences* 103 (22). National Academy of Sciences: 8459–64. doi:10.1073/pnas.0603082103.
Goic, Bertsy, Nicolas Vodovar, Juan A Mondotte, Clément Monot, Lionel Frangeul, Hervé Blanc, Valérie Gausson, Jorge Vera-Otarola, Gael Cristofari, and Maria-Carla Saleh. 2013. "RNA-Mediated Interference and Reverse Transcription Control the Persistence of RNA Viruses in the Insect Model Drosophila." *Nat Immunol* 14 (4): 396–403. doi:10.1038/ni.2542.
Gold, Katrina S, and Katja Brückner. 2015. "Macrophages and Cellular Immunity in Drosophila Melanogaster." *Seminars in Immunology* 27 (6): 357–68. doi:10.1016/j.smim.2016.03.010.
Goto, Akira, Kiyoshi Okado, Nelson Martins, Hua Cai, Vincent Barbier, Olivier Lamiable, Laurent Troxler, et al. 2018. "The Kinase IKKβ Regulates a STING- and NF-κB-Dependent Antiviral Response Pathway in Drosophila.." *Immunity* 49 (2): 225–234.e4. doi:10.1016/j.immuni.2018.07.013.
Goto, Akira, Stéphanie Blandin, Julien Royet, Jean-Marc Reichhart, and Elena A Levashina. 2003. "Silencing of Toll Pathway Components by Direct Injection of Double-Stranded RNA Into Drosophila Adult Flies.." *Nucleic Acids Research* 31 (22). Oxford University Press: 6619–23. doi:10.1093/nar/gkg852.
Goubau, Delphine, Safia Deddouche, and Caetano Reis e Sousa. 2013. "Cytosolic Sensing of Viruses.." *Immunity* 38 (5): 855–69. doi:10.1016/j.immuni.2013.05.007.
Gueguen, Gwenaelle, Marta E Kalamarz, Johnny Ramroop, Jeffrey Uribe, and Shubha Govind. 2013. "Polydnaviral Ankyrin Proteins Aid Parasitic Wasp Survival by Coordinate and Selective Inhibition of Hematopoietic and Immune NF-Kappa B Signaling in Insect Hosts.." Edited by David S Schneider. *PLoS Pathog* 9 (8). Public Library of Science: e1003580. doi:10.1371/journal.ppat.1003580.
Guruharsha, K G, Jean-François Rual, Bo Zhai, Julian Mintseris, Pujita Vaidya, Namita Vaidya, Chapman Beekman, et al. 2011. "A Protein Complex Network of Drosophila Melanogaster." *Cell* 147 (3): 690–703. doi:10.1016/j.cell.2011.08.047.
Habjan, Matthias, and Andreas Pichlmair. 2015. "Cytoplasmic Sensing of Viral Nucleic Acids.." *Current Opinion in Virology* 11 (April): 31–37. doi:10.1016/j.coviro.2015.01.012.
Habjan, Matthias, Ida Andersson, Jonas Klingström, Michael Schümann, Arnold

Martin, Petra Zimmermann, Valentina Wagner, et al. 2008. “Processing of Genome 5' Termini as a Strategy of Negative-Strand RNA Viruses to Avoid RIG-I-Dependent Interferon Induction..” Edited by Peter Sommer. *PLoS ONE* 3 (4). Public Library of Science: e2032. doi:10.1371/journal.pone.0002032.

Habjan, Matthias, Philipp Hubel, Livia Lacerda, Christian Benda, Cathleen Holze, Christian H Eberl, Angelika Mann, et al. 2013. “Sequestration by IFIT1 Impairs Translation of 2′O-Unmethylated Capped RNA.” Edited by Michael S Diamond. *PLoS Pathog* 9 (10): e1003663. doi:10.1371/journal.ppat.1003663.

Han, Y H, Y J Luo, Q Wu, J Jovel, X H Wang, R Aliyari, C Han, W X Li, and S W Ding. 2011. “RNA-Based Immunity Terminates Viral Infection in Adult Drosophila in the Absence of Viral Suppression of RNA Interference: Characterization of Viral Small Interfering RNA Populations in Wild-Type and Mutant Flies.” *J Virol* 85 (24): 13153–63. doi:10.1128/JVI.05518-11.

Harris, Reuben S, Judd F Hultquist, and David T Evans. 2012. “The Restriction Factors of Human Immunodeficiency Virus..” *The Journal of Biological Chemistry* 287 (49). American Society for Biochemistry and Molecular Biology: 40875–83. doi:10.1074/jbc.R112.416925.

Hartmann, G, and A M Krieg. 2000. “Mechanism and Function of a Newly Identified CpG DNA Motif in Human Primary B Cells..” *The Journal of Immunology* 164 (2): 944–53.

Hartner, Jochen C, Carl R Walkley, Jun Lu, and Stuart H Orkin. 2009. “ADAR1 Is Essential for the Maintenance of Hematopoiesis and Suppression of Interferon Signaling..” *Nat Immunol* 10 (1). Nature Publishing Group: 109–15. doi:10.1038/ni.1680.

Heil, Florian, Hiroaki Hemmi, Hubertus Hochrein, Franziska Ampenberger, Carsten Kirschning, Shizuo Akira, Grayson Lipford, Hermann Wagner, and Stefan Bauer. 2004. “Species-Specific Recognition of Single-Stranded RNA via Toll-Like Receptor 7 and 8..” *Science* 303 (5663): 1526–29. doi:10.1126/science.1093620.

Hemmi, Hiroaki, Osamu Takeuchi, Taro Kawai, Tsuneyasu Kaisho, Shintaro Sato, Hideki Sanjo, Makoto Matsumoto, et al. 2000. “A Toll-Like Receptor Recognizes Bacterial DNA.” *Nature* 408 (6813). Nature Publishing Group: 740–45. doi:10.1038/35047123.

Hoffmann, J A. 2003. “The Immune Response of Drosophila.” *Nature* 426: 33–38.

Hoffmann, J A, F C Kafatos, C A Janeway, and R A Ezekowitz. 1999. “Phylogenetic Perspectives in Innate Immunity..” *Science* 284 (5418): 1313–18.

Hopkins, Kaycie C, Laura M McLane, Tariq Maqbool, Debasis Panda, Beth Gordesky-Gold, and Sara Cherry. 2013. “A Genome-Wide RNAi Screen Reveals That mRNA Decapping Restricts Bunyaviral Replication by Limiting the Pools of Dcp2-Accessible Targets for Cap-Snatching..” *Genes & Development* 27 (13). Cold Spring Harbor Lab: 1511–25. doi:10.1101/gad.215384.113.

Hornung, Veit, Jana Ellegast, Sarah Kim, Krzysztof Brzózka, Andreas Jung, Hiroki Kato, Hendrik Poeck, et al. 2006. “5'-Triphosphate RNA Is the Ligand for RIG-I..” *Science* 314 (5801). American Association for the Advancement of Science: 994–97. doi:10.1126/science.1132505.

Hornung, Veit, Margit Guenthner-Biller, Carole Bourquin, Andrea Ablasser, Martin Schlee, Satoshi Uematsu, Anne Noronha, et al. 2005. “Sequence-Specific Potent Induction of IFN-Alpha by Short Interfering RNA in Plasmacytoid Dendritic Cells Through TLR7..” *Nature Medicine* 11 (3). Nature Publishing Group: 263–70. doi:10.1038/nm1191.

Hornung, Veit, Rune Hartmann, Andrea Ablasser, and Karl-Peter Hopfner. 2014. “OAS Proteins and cGAS: Unifying Concepts in Sensing and Responding to

Cytosolic Nucleic Acids.." *Nature Reviews Immunology* 14 (8). Nature Publishing Group: 521–28. doi:10.1038/nri3719.
Huang, Xudong, Lai Shi, Jun Cao, Fangfei He, Renling Li, Yan Zhang, Shuang Miao, et al. 2014. "The Sterile 20-Like Kinase Tao Controls Tissue Homeostasis by Regulating the Hippo Pathway in Drosophila Adult Midgut.." *Journal of Genetics and Genomics = Yi Chuan Xue Bao* 41 (8): 429–38. doi:10.1016/j.jgg.2014.05.007.
Huang, Zhijing, Megan B Kingsolver, Vasanthi Avadhanula, and Richard W Hardy. 2013. "An Antiviral Role for Antimicrobial Peptides During the Arthropod Response to Alphavirus Replication.." *J Virol* 87 (8). American Society for Microbiology: 4272–80. doi:10.1128/JVI.03360-12.
Hultmark, D. 2003. "Drosophila Immunity: Paths and Patterns." *Curr Opin Immunol* 15 (1): 12–19. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12495727.
Humphrey, Sean J, Ozge Karayel, David E James, and Matthias Mann. 2018. "High-Throughput and High-Sensitivity Phosphoproteomics with the EasyPhos Platform.." *Nature Protocols* 13 (9). Nature Publishing Group: 1897–1916. doi:10.1038/s41596-018-0014-9.
Hussain, Mazhar, and Sassan Asgari. 2014. "MicroRNAs as Mediators of Insect Host–Pathogen Interactions and Immunity." *Journal of Insect Physiology* 70 (November): 151–58. doi:10.1016/j.jinsphys.2014.08.003.
Imler, Jean-Luc, and Philippe Bulet. 2005. "Antimicrobial Peptides in Drosophila: Structures, Activities and Gene Regulation.." *Chemical Immunology and Allergy* 86. Basel: KARGER: 1–21. doi:10.1159/000086648.
Issa, Najwa, Nina Guillaumot, Emilie Lauret, Nicolas Matt, Christine Schaeffer-Reiss, Alain Van Dorsselaer, Jean-Marc Reichhart, and Florian Veillard. 2018. "The Circulating Protease Persephone Is an Immune Sensor for Microbial Proteolytic Activities Upstream of the Drosophila Toll Pathway.." *Molecular Cell* 69 (4): 539–550.e6. doi:10.1016/j.molcel.2018.01.029.
Iwasaki, Shintaro, Hiroshi M Sasaki, Yuriko Sakaguchi, Tsutomu Suzuki, Hisashi Tadakuma, and Yukihide Tomari. 2015. "Defining Fundamental Steps in the Assembly of the Drosophila RNAi Enzyme Complex." *Nature* 521 (7553): 533–36. doi:10.1038/nature14254.
Janeway, C A. 1989. "Approaching the Asymptote? Evolution and Revolution in Immunology." *Cold Spring Harbor Symposia on Quantitative Biology* 54 (0). Cold Spring Harbor Laboratory Press: 1–13. doi:10.1101/SQB.1989.054.01.003.
Jayachandran, Uma, Heather Grey, and Atlanta G Cook. 2016. "Nuclear Factor 90 Uses an ADAR2-Like Binding Mode to Recognize Specific Bases in dsRNA.." *Nucleic Acids Research* 44 (4): 1924–36. doi:10.1093/nar/gkv1508.
Jia, Xiaofei, Qi Zhao, and Yong Xiong. 2015. "HIV Suppression by Host Restriction Factors and Viral Immune Evasion.." *Current Opinion in Structural Biology* 31 (April): 106–14. doi:10.1016/j.sbi.2015.04.004.
Johnson, K N, and P D Christian. 1998. "The Novel Genome Organization of the Insect Picorna-Like Virus Drosophila C Virus Suggests This Virus Belongs to a Previously Undescribed Virus Family.." *The Journal of General Virology* 79 (Pt 1) (1). Microbiology Society: 191–203. doi:10.1099/0022-1317-79-1-191.
Jose, Joyce, Jonathan E Snyder, and Richard J Kuhn. 2009. "A Structural and Functional Perspective of Alphavirus Replication and Assembly.." *Future Microbiology* 4 (7). Future Medicine Ltd London, UK: 837–56. doi:10.2217/fmb.09.59.
Joubert, Pierre-Emmanuel, Grégory Meiffren, Isabel Pombo Grégoire, Guillemette

Pontini, Clémence Richetta, Monique Flacher, Olga Azocar, et al. 2009. "Autophagy Induction by the Pathogen Receptor CD46.." *Cell Host & Microbe* 6 (4): 354–66. doi:10.1016/j.chom.2009.09.006.
Jousset, F X, N Plus, G Croizier, and M Thomas. 1972. "[Existence in Drosophila of 2 Groups of Picornavirus with Different Biological and Serological Properties].." *Comptes Rendus Hebdomadaires Des Seances De l'Academie Des Sciences. Serie D: Sciences Naturelles* 275 (25): 3043–46.
Jöckel, Stefanie, Gernot Nees, Romy Sommer, Yang Zhao, Dmitry Cherkasov, Hiroyuki Hori, Gundi Ehm, et al. 2012. "The 2'-O-Methylation Status of a Single Guanosine Controls Transfer RNA-Mediated Toll-Like Receptor 7 Activation or Inhibition.." *The Journal of Experimental Medicine* 209 (2): 235–41. doi:10.1084/jem.20111075.
Kaiser, Steffen, Katharina Rimbach, Tatjana Eigenbrod, Alexander H Dalpke, and Mark Helm. 2014. "A Modified Dinucleotide Motif Specifies tRNA Recognition by TLR7.." *RNA (New York, N.Y.)* 20 (9): 1351–55. doi:10.1261/rna.044024.113.
Kane, Nanci S, Mehul Vora, Krishna J Varre, and Richard W Padgett. 2017. "Efficient Screening of CRISPR/Cas9-Induced Events in Drosophila Using a Co-CRISPR Strategy.." *G3 (Bethesda, Md.)* 7 (1): 87–93. doi:10.1534/g3.116.036723.
Karlikow, Margot, Bertsy Goic, and Maria-Carla Saleh. 2014. "RNAi and Antiviral Defense in Drosophila: Setting Up a Systemic Immune Response." *Developmental & Comparative Immunology* 42 (1): 85–92. doi:10.1016/j.dci.2013.05.004.
Karlikow, Margot, Bertsy Goic, Vanesa Mongelli, Audrey Salles, Christine Schmitt, Isabelle Bonne, Chiara Zurzolo, and Maria-Carla Saleh. 2016. "Drosophila Cells Use Nanotube-Like Structures to Transfer dsRNA and RNAi Machinery Between Cells.." *Scientific Reports* 6. Nature Publishing Group: 27085. doi:10.1038/srep27085.
Kemp, Cordula, Stefanie Mueller, Akira Goto, Vincent Barbier, Simona Paro, François Bonnay, Catherine Dostert, et al. 2013. "Broad RNA Interference-Mediated Antiviral Immunity and Virus-Specific Inducible Responses in Drosophila.." *Journal of Immunology (Baltimore, Md. : 1950)* 190 (2). American Association of Immunologists: 650–58. doi:10.4049/jimmunol.1102486.
King, Ian, and Ulrike Heberlein. 2011. "Tao Kinases as Coordinators of Actin and Microtubule Dynamics in Developing Neurons.." *Communicative & Integrative Biology* 4 (5): 554–56. doi:10.4161/cib.4.5.16051.
King, Ian, Linus T-Y Tsai, Ralf Pflanz, Aaron Voigt, Seongsoo Lee, Herbert Jäckle, Bingwei Lu, and Ulrike Heberlein. 2011. "Drosophila Tao Controls Mushroom Body Development and Ethanol-Stimulated Behavior Through Par-1.." *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience* 31 (3). Society for Neuroscience: 1139–48. doi:10.1523/JNEUROSCI.4416-10.2011.
Kingsolver, Megan B, Zhijing Huang, and Richard W Hardy. 2013. "Insect Antiviral Innate Immunity: Pathways, Effectors, and Connections." *Journal of Molecular Biology* 425 (24): 4921–36. doi:10.1016/j.jmb.2013.10.006.
Kleino, Anni, and Neal Silverman. 2014. "The Drosophila IMD Pathway in the Activation of the Humoral Immune Response.." *Developmental & Comparative Immunology* 42 (1): 25–35. doi:10.1016/j.dci.2013.05.014.
Klotman, Mary E, and Theresa L Chang. 2006. "Defensins in Innate Antiviral Immunity.." *Nature Reviews Immunology* 6 (6). Nature Publishing Group: 447–56. doi:10.1038/nri1860.
Kluge, Silvia F, Daniel Sauter, and Frank Kirchhoff. 2015. "SnapShot: Antiviral Restriction Factors.." *Cell* 163 (3): 774–774.e1. doi:10.1016/j.cell.2015.10.019.
Kopek, B G, E W Settles, P D Friesen, and P Ahlquist. 2010. "Nodavirus-Induced

Membrane Rearrangement in Replication Complex Assembly Requires Replicase Protein a, RNA Templates, and Polymerase Activity." *Journal of Virology* 84 (24): 12492–503. doi:10.1128/JVI.01495-10.
Koppstein, David, Joseph Ashour, and David P Bartel. 2015. "Sequencing the Cap-Snatching Repertoire of H1N1 Influenza Provides Insight Into the Mechanism of Viral Transcription Initiation.." *Nucleic Acids Research* 43 (10): 5052–64. doi:10.1093/nar/gkv333.
Ktistakis, Nicholas T, and Sharon A Tooze. 2016. "Digesting the Expanding Mechanisms of Autophagy.." *Trends in Cell Biology* 26 (8): 624–35. doi:10.1016/j.tcb.2016.03.006.
Kuhn, Lauriane, Karim Majzoub, Evelyne Einhorn, Johana Chicher, Julien Pompon, Jean-Luc Imler, Philippe Hammann, and Carine Meignin. 2017. "Definition of a RACK1 Interaction Network in Drosophila Melanogaster Using SWATH-MS.." *G3 (Bethesda, Md.)* 7 (7). G3: Genes, Genomes, Genetics: 2249–58. doi:10.1534/g3.117.042564.
Kwon, S Chul, Tuan Anh Nguyen, Yeon-Gil Choi, Myung Hyun Jo, Sungchul Hohng, V Narry Kim, and Jae-Sung Woo. 2016. "Structure of Human DROSHA." *Cell* 164 (1-2). Elsevier: 81–90. doi:10.1016/j.cell.2015.12.019.
Lamiable, Olivier, and Jean-Luc Imler. 2014. "Induced Antiviral Innate Immunity in Drosophila." *Current Opinion in Microbiology* 20 (August): 62–68. doi:10.1016/j.mib.2014.05.006.
Lamiable, Olivier, Christine Kellenberger, Cordula Kemp, Laurent Troxler, Nadège Pelte, Michael Boutros, João Trindade Marques, et al. 2016. "Cytokine Diedel and a Viral Homologue Suppress the IMD Pathway in Drosophila.." *Proceedings of the National Academy of Sciences of the United States of America* 113 (3). National Acad Sciences: 698–703. doi:10.1073/pnas.1516122113.
Lamiable, Olivier, Johan Arnold, Isaque João da Silva de Faria, Roenick Proveti Olmo, Francesco Bergami, Carine Meignin, Jules A Hoffmann, João Trindade Marques, and Jean-Luc Imler. 2016. "Analysis of the Contribution of Hemocytes and Autophagy to Drosophila Antiviral Immunity." Edited by R M Sandri-Goldin. *J Virol* 90 (11): 5415–26. doi:10.1128/JVI.00238-16.
Landthaler, Markus, Abdullah Yalcin, and Thomas Tuschl. 2004. "The Human DiGeorge Syndrome Critical Region Gene 8 and Its D. Melanogaster Homolog Are Required for miRNA Biogenesis.." *Curr Biol* 14 (23): 2162–67. doi:10.1016/j.cub.2004.11.001.
Laughon, A, and R F Gesteland. 1984. "Primary Structure of the Saccharomyces Cerevisiae GAL4 Gene.." *Molecular and Cellular Biology* 4 (2). American Society for Microbiology (ASM): 260–67.
Laughon, A, R Driscoll, N Wills, and R F Gesteland. 1984. "Identification of Two Proteins Encoded by the Saccharomyces Cerevisiae GAL4 Gene.." *Molecular and Cellular Biology* 4 (2). American Society for Microbiology (ASM): 268–75.
Lee, Y F, A Nomoto, B M Detjen, and E Wimmer. 1977. "A Protein Covalently Linked to Poliovirus Genome RNA.." *Proceedings of the National Academy of Sciences* 74 (1). National Academy of Sciences: 59–63.
Lee-Kirsch, Min Ae, Maolian Gong, Dipanjan Chowdhury, Lydia Senenko, Kerstin Engel, Young-Ae Lee, Udesh de Silva, et al. 2007. "Mutations in the Gene Encoding the 3'-5' DNA Exonuclease TREX1 Are Associated with Systemic Lupus Erythematosus.." *Nature Genetics* 39 (9). Nature Publishing Group: 1065–67. doi:10.1038/ng2091.
Letourneau, Manon, Francois Lapraz, Anurag Sharma, Nathalie Vanzo, Lucas Waltzer, and Michèle Crozatier. 2016. "Drosophilahematopoiesis Under Normal

Conditions and in Response to Immune Stress." *FEBS Letters*, August. doi:10.1002/1873-3468.12327.
Levanon, Erez Y, Eli Eisenberg, Rodrigo Yelin, Sergey Nemzer, Martina Hallegger, Ronen Shemesh, Zipora Y Fligelman, et al. 2004. "Systematic Identification of Abundant a-to-I Editing Sites in the Human Transcriptome.." *Nature Biotechnology* 22 (8). Nature Publishing Group: 1001–5. doi:10.1038/nbt996.
Léger, P, E Lara, B Jagla, O Sismeiro, Z Mansuroglu, J Y Coppée, E Bonnefoy, and M Bouloy. 2013. "Dicer-2- and Piwi-Mediated RNA Interference in Rift Valley Fever Virus-Infected Mosquito Cells.." *J Virol* 87 (3). American Society for Microbiology: 1631–48. doi:10.1128/JVI.02795-12.
Lhéritier, Ph. 1958. "The Hereditary Virus of Drosophila." In *Advances in Virus Research Volume 5*, 5:195–245. Advances in Virus Research. Elsevier. doi:10.1016/S0065-3527(08)60674-0.
Liang, Chunyang, Yibing Wang, Yukiko Murota, Xiang Liu, Dean Smith, Mikiko C Siomi, and Qinghua Liu. 2015. "TAF11 Assembles the RISC Loading Complex to Enhance RNAi Efficiency." *Molecular Cell* 59 (5). Elsevier: 807–18. doi:10.1016/j.molcel.2015.07.006.
Lim, Do-Hwan, Chun-Taek Oh, Langho Lee, Jae-Sang Hong, Su-Hyun Noh, Seungwoo Hwang, Sungchan Kim, Sung-Jun Han, and Young Sik Lee. 2011. "The Endogenous siRNA Pathway in Drosophila Impacts Stress Resistance and Lifespan by Regulating Metabolic Homeostasis.." *FEBS Letters* 585 (19). John Wiley & Sons, Ltd: 3079–85. doi:10.1016/j.febslet.2011.08.034.
Liu, Bo, Susanta K Behura, Rollie J Clem, Anette Schneemann, James Becnel, David W Severson, and Lei Zhou. 2013. "P53-Mediated Rapid Induction of Apoptosis Conveys Resistance to Viral Infection in Drosophila Melanogaster." Edited by Edward Mocarski. *PLoS Pathog* 9 (2): e1003137. doi:10.1371/journal.ppat.1003137.
Liu, Tao, Jennifer L Rohn, Remigio Picone, Patricia Kunda, and Buzz Baum. 2010. "Tao-1 Is a Negative Regulator of Microtubule Plus-End Growth.." *Journal of Cell Science* 123 (Pt 16). The Company of Biologists Ltd: 2708–16. doi:10.1242/jcs.068726.
Liu, Xi, Teruyuki Sano, Yongsheng Guan, Shigekazu Nagata, Jules A Hoffmann, and Hidehiro Fukuyama. 2012. "Drosophila EYA Regulates the Immune Response Against DNA Through an Evolutionarily Conserved Threonine Phosphatase Motif.." Edited by Justin Kumar. *PLoS ONE* 7 (8). Public Library of Science: e42725. doi:10.1371/journal.pone.0042725.
Liu, Yuan, and Sara Cherry. 2019. "Zika Virus Infection Activates Sting-Dependent Antiviral Autophagy in the Drosophila Brain.." *Autophagy* 15 (1). Taylor & Francis: 174–75. doi:10.1080/15548627.2018.1528813.
Liu, Yuan, Beth Gordesky-Gold, Michael Leney-Greene, Nathan L Weinbren, Matthew Tudor, and Sara Cherry. 2018. "Inflammation-Induced, STING-Dependent Autophagy Restricts Zika Virus Infection in the Drosophila Brain.." *Cell Host & Microbe* 24 (1): 57–68.e3. doi:10.1016/j.chom.2018.05.022.
Longdon, Ben, Chuan Cao, Julien Martinez, and Francis M Jiggins. 2013. "Previous Exposure to an RNA Virus Does Not Protect Against Subsequent Infection in Drosophila Melanogaster.." Edited by Nicole M Gerardo. *PLoS ONE* 8 (9). Public Library of Science: e73833. doi:10.1371/journal.pone.0073833.
Luplertlop, Natthanej, Pornapat Surasombatpattana, Sirilaksana Patramool, Emilie Dumas, Ladawan Wasinpiyamongkol, Laure Saune, Rodolphe Hamel, et al. 2011. "Induction of a Peptide with Activity Against a Broad Spectrum of Pathogens in the Aedes Aegypti Salivary Gland, Following Infection with Dengue Virus."

Edited by Michael S Diamond. *PLoS Pathog* 7 (1). Public Library of Science: e1001252. doi:10.1371/journal.ppat.1001252.
Magwire, Michael M, Daniel K Fabian, Hannah Schweyen, Chuan Cao, Ben Longdon, Florian Bayer, and Francis M Jiggins. 2012. "Genome-Wide Association Studies Reveal a Simple Genetic Basis of Resistance to Naturally Coevolving Viruses in Drosophila Melanogaster." Edited by Marta L Wayne. *PLoS Genetics* 8 (11). Public Library of Science: e1003057. doi:10.1371/journal.pgen.1003057.
Magwire, Michael M, Florian Bayer, Claire L Webster, Chuan Cao, and Francis M Jiggins. 2011. "Successive Increases in the Resistance of Drosophila to Viral Infection Through a Transposon Insertion Followed by a Duplication.." Edited by David J Begun. *PLoS Genetics* 7 (10). Public Library of Science: e1002337. doi:10.1371/journal.pgen.1002337.
Majer, Olivia, Bo Liu, and Gregory M Barton. 2017. "Nucleic Acid-Sensing TLRs: Trafficking and Regulation.." *Curr Opin Immunol* 44 (February): 26–33. doi:10.1016/j.coi.2016.10.003.
Majzoub, Karim, Mohamed Lamine Hafirassou, Carine Meignin, Akira Goto, Stefano Marzi, Antonina Fedorova, Yann Verdier, et al. 2014. "RACK1 Controls IRES-Mediated Translation of Viruses.." *Cell* 159 (5): 1086–95. doi:10.1016/j.cell.2014.10.041.
Malone, C D, Malone, Colin D, J Brennecke, Julius Brennecke, Monica Dus, M Dus, Alexander Stark, et al. 2009. "Specialized piRNA Pathways Act in Germline and Somatic Tissues of the Drosophila Ovary." *Cell* 137 (3): 522–35. doi:10.1016/j.cell.2009.03.040.
Mankan, Arun K, Tobias Schmidt, Dhruv Chauhan, Marion Goldeck, Klara Höning, Moritz Gaidt, Andrew V Kubarenko, Liudmila Andreeva, Karl-Peter Hopfner, and Veit Hornung. 2014. "Cytosolic RNA:DNA Hybrids Activate the cGAS-STING Axis.." *The EMBO Journal* 33 (24). EMBO Press: 2937–46. doi:10.15252/embj.201488726.
Mannion, Niamh, Fabiana Arieti, Angela Gallo, Liam P Keegan, and Mary A O'Connell. 2015. "New Insights Into the Biological Role of Mammalian ADARs; the RNA Editing Proteins.." *Biomolecules* 5 (4). Multidisciplinary Digital Publishing Institute: 2338–62. doi:10.3390/biom5042338.
Marques, João T, and Jean-Luc Imler. 2016. "The Diversity of Insect Antiviral Immunity: Insights From Viruses.." *Current Opinion in Microbiology* 32 (May): 71–76. doi:10.1016/j.mib.2016.05.002.
Marques, João Trindade, Ji-Ping Wang, Xiaohong Wang, Karla Pollyanna Vieira de Oliveira, Catherine Gao, Eric Roberto Guimaraes Rocha Aguiar, Nadereh Jafari, and Richard W Carthew. 2013. "Functional Specialization of the Small Interfering RNA Pathway in Response to Virus Infection.." Edited by Shou-Wei Ding. *PLoS Pathog* 9 (8). Public Library of Science: e1003579. doi:10.1371/journal.ppat.1003579.
Marques, João Trindade, Kevin Kim, Pei-Hsuan Wu, Trevis M Alleyne, Nadereh Jafari, and Richard W Carthew. 2009. "Loqs and R2D2 Act Sequentially in the siRNA Pathway in Drosophila." *Nat Struct Mol Biol* 17 (1): 24–30. doi:10.1038/nsmb.1735.
Martins, Karen A O, Sina Bavari, and Andres M Salazar. 2015. "Vaccine Adjuvant Uses of Poly-IC and Derivatives.." *Expert Review of Vaccines* 14 (3): 447–59. doi:10.1586/14760584.2015.966085.
Martins, Nelson E, Vítor G Faria, Viola Nolte, Christian Schlötterer, Luis Teixeira, Élio Sucena, and Sara Magalhães. 2014. "Host Adaptation to Viruses Relies on Few Genes with Different Cross-Resistance Properties.." *Proceedings of the National*

Academy of Sciences of the United States of America 111 (16). National Acad Sciences: 5938–43. doi:10.1073/pnas.1400378111.
Martins, Nelson, Aurélie Lemoine, Estelle Santiago, Simona Paro, Jean-Luc Imler, and Carine Meignin. 2018. "A Transgenic Flock House Virus Replicon Reveals an RNAi Independent Antiviral Mechanism Acting in Drosophila Follicular Somatic Cells.." *G3 (Bethesda, Md.)*, December. G3: Genes, Genomes, Genetics, g3.200872.2018. doi:10.1534/g3.118.200872.
Matsumoto, Misako, Kenji Funami, Masako Tanabe, Hiroyuki Oshiumi, Masashi Shingai, Yoshiyuki Seto, Akitsugu Yamamoto, and Tsukasa Seya. 2003. "Subcellular Localization of Toll-Like Receptor 3 in Human Dendritic Cells.." *The Journal of Immunology* 171 (6): 3154–62.
Matzinger, P. 1994. "Tolerance, Danger and the Extended Family." *Annual Review of Immunology* 12 (1). Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA: 991–1045. doi:10.1146/annurev.immunol.12.1.991.
Matzinger, Polly. 2002. "The Danger Model: a Renewed Sense of Self.." *Science* 296 (5566): 301–5. doi:10.1126/science.1071059.
Mauthe, Mario, Martijn Langereis, Jennifer Jung, Xingdong Zhou, Alex Jones, Wienand Omta, Sharon A Tooze, et al. 2016. "An siRNA Screen for ATG Protein Depletion Reveals the Extent of the Unconventional Functions of the Autophagy Proteome in Virus Replication.." *The Journal of Cell Biology* 214 (5). Rockefeller University Press: 619–35. doi:10.1083/jcb.201602046.
Medzhitov, R, and C A Jr Janeway. 2002. "Decoding the Patterns of Self and Nonself by the Innate Immune System." *Science* 296 (5566): 298–300. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11951031.
Merkl, Philipp E, Megan H Orzalli, and David M Knipe. 2018. "Mechanisms of Host IFI16, PML, and Daxx Protein Restriction of Herpes Simplex Virus 1 Replication." Edited by Jae U Jung. *J Virol* 92 (10): 829. doi:10.1128/JVI.00057-18.
Merkling, Sarah H, Alfred W Bronkhorst, Jamie M Kramer, Gijs J Overheul, Annette Schenck, and Ronald P van Rij. 2015. "The Epigenetic Regulator G9a Mediates Tolerance to RNA Virus Infection in Drosophila.." Edited by Pedro F Vale. *PLoS Pathog* 11 (4). Public Library of Science: e1004692. doi:10.1371/journal.ppat.1004692.
Merkling, Sarah H, Gijs J Overheul, Joel T van Mierlo, Daan Arends, Christian Gilissen, and Ronald P van Rij. 2015. "The Heat Shock Response Restricts Virus Infection in Drosophila.." *Scientific Reports* 5. Nature Publishing Group: 12758. doi:10.1038/srep12758.
Miesen, Pascal, Erika Girardi, and Ronald P van Rij. 2015. "Distinct Sets of PIWI Proteins Produce Arbovirus and Transposon-Derived piRNAs in Aedes Aegypti Mosquito Cells.." *Nucleic Acids Research* 43 (13). Oxford University Press: 6545–56. doi:10.1093/nar/gkv590.
Milek, Miha, Koshi Imami, Neelanjan Mukherjee, Francesca De Bortoli, Ulrike Zinnall, Orsalia Hazapis, Christian Trahan, et al. 2017. "DDX54 Regulates Transcriptome Dynamics During DNA Damage Response.." *Genome Research* 27 (8). Cold Spring Harbor Lab: 1344–59. doi:10.1101/gr.218438.116.
Miller, D J, M D Schwartz, and P Ahlquist. 2001. "Flock House Virus RNA Replicates on Outer Mitochondrial Membranes in Drosophila Cells.." *Journal of Virology* 75 (23): 11664–76. doi:10.1128/JVI.75.23.11664-11676.2001.
Ming, Ming, Fumiaki Obata, Erina Kuranaga, and Masayuki Miura. 2014. "Persephone/Spätzle Pathogen Sensors Mediate the Activation of Toll Receptor Signaling in Response to Endogenous Danger Signals in Apoptosis-Deficient

Drosophila.." *The Journal of Biological Chemistry* 289 (11). American Society for Biochemistry and Molecular Biology: 7558–68. doi:10.1074/jbc.M113.543884.
Mohr, Stephanie E, Jennifer A Smith, Caroline E Shamu, Ralph A Neumüller, and Norbert Perrimon. 2014. "RNAi Screening Comes of Age: Improved Techniques and Complementary Approaches.." *Nature Reviews. Molecular Cell Biology* 15 (9). Nature Publishing Group: 591–600. doi:10.1038/nrm3860.
Molleston, Jerome M, Leah R Sabin, Ryan H Moy, Sanjay V Menghani, Keiko Rausch, Beth Gordesky-Gold, Kaycie C Hopkins, et al. 2016. "A Conserved Virus-Induced Cytoplasmic TRAMP-Like Complex Recruits the Exosome to Target Viral RNA for Degradation.." *Genes & Development* 30 (14). Cold Spring Harbor Lab: 1658–70. doi:10.1101/gad.284604.116.
Mondotte, Juan A, Valérie Gausson, Lionel Frangeul, Hervé Blanc, Louis Lambrechts, and Maria-Carla Saleh. 2018. "Immune Priming and Clearance of Orally Acquired RNA Viruses in Drosophila.." *Nature Microbiology* 3 (12). Nature Publishing Group: 1394–1403. doi:10.1038/s41564-018-0265-9.
Moraes, Angela M, Soraia A C Jorge, Renato M Astray, Claudio A T Suazo, Camilo E Calderón Riquelme, Elisabeth F P Augusto, Aldo Tonso, et al. 2012. "Drosophila Melanogaster S2 Cells for Expression of Heterologous Genes: From Gene Cloning to Bioprocess Development.." *Biotechnology Advances* 30 (3): 613–28. doi:10.1016/j.biotechadv.2011.10.009.
Morazzani, Elaine M, Michael R Wiley, Marta G Murreddu, Zach N Adelman, and Kevin M Myles. 2012. "Production of Virus-Derived Ping-Pong-Dependent piRNA-Like Small RNAs in the Mosquito Soma.." Edited by Shou-Wei Ding. *PLoS Pathog* 8 (1). Public Library of Science: e1002470. doi:10.1371/journal.ppat.1002470.
Moy, Ryan H, Brian S Cole, Ari Yasunaga, Beth Gold, Ganesh Shankarling, Andrew Varble, Jerome M Molleston, Benjamin R tenOever, Kristen W Lynch, and Sara Cherry. 2014. "Stem-Loop Recognition by DDX17 Facilitates miRNA Processing and Antiviral Defense.." *Cell* 158 (4): 764–77. doi:10.1016/j.cell.2014.06.023.
Mueller, Stefanie, Valérie Gausson, Nicolas Vodovar, Safia Deddouche, Laurent Troxler, Jonathan Perot, Sebastien Pfeffer, Jules A Hoffmann, Maria-Carla Saleh, and Jean-Luc Imler. 2010. "RNAi-Mediated Immunity Provides Strong Protection Against the Negative-Strand RNA Vesicular Stomatitis Virus in Drosophila.." *Proceedings of the National Academy of Sciences of the United States of America* 107 (45). National Acad Sciences: 19390–95. doi:10.1073/pnas.1014378107.
Mukae, Naomi, Hideki Yokoyama, Takakazu Yokokura, Yasuhiko Sakoyama, and Shigekazu Nagata. 2002. "Activation of the Innate Immunity in Drosophila by Endogenous Chromosomal DNA That Escaped Apoptotic Degradation.." *Genes & Development* 16 (20). Cold Spring Harbor Lab: 2662–71. doi:10.1101/gad.1022802.
Mussabekova, Assel, Laurent Daeffler, and Jean-Luc Imler. 2017. "Innate and Intrinsic Antiviral Immunity in Drosophila.." *Cellular and Molecular Life Sciences : CMLS* 74 (11). Springer International Publishing: 2039–54. doi:10.1007/s00018-017-2453-9.
Nainu, F, Nainu, Firzan, Yumiko Tanaka, Y Tanaka, Akiko Shiratsuchi, A Shiratsuchi, Yoshinobu Nakanishi, and Y Nakanishi. 2015. "Protection of Insects Against Viral Infection by Apoptosis-Dependent Phagocytosis." *The Journal of Immunology* 195 (12): 5696–5706. doi:10.4049/jimmunol.1500613.
Nayak, Arabinda, Bassam Berry, Michel Tassetto, Mark Kunitomi, Ashley Acevedo, Changhui Deng, Andrew Krutchinsky, John Gross, Christophe Antoniewski, and Raul Andino. 2010. "Cricket Paralysis Virus Antagonizes Argonaute 2 to

Modulate Antiviral Defense in Drosophila.." *Nat Struct Mol Biol* 17 (5). Nature Publishing Group: 547–54. doi:10.1038/nsmb.1810.
Nayak, Arabinda, Dong Young Kim, Michael J Trnka, Craig H Kerr, Peter V Lidsky, David J Stanley, Brianna Monique Rivera, et al. 2018. "A Viral Protein Restricts Drosophila RNAi Immunity by Regulating Argonaute Activity and Stability.." *Cell Host & Microbe* 24 (4): 542–49. doi:10.1016/j.chom.2018.09.006.
Nezis, Ioannis P, Anne Simonsen, Antonia P Sagona, Kim Finley, Sébastien Gaumer, Didier Contamine, Tor Erik Rusten, Harald Stenmark, and Andreas Brech. 2008. "Ref(2)P, the Drosophila Melanogaster Homologue of Mammalian P62, Is Required for the Formation of Protein Aggregates in Adult Brain.." *The Journal of Cell Biology* 180 (6). Rockefeller Univ Press: 1065–71. doi:10.1083/jcb.200711108.
Nishida, Kazumichi M, Keita Miyoshi, Akiyo Ogino, Tomohiro Miyoshi, Haruhiko Siomi, and Mikiko C Siomi. 2013. "Roles of R2D2, a Cytoplasmic D2 Body Component, in the Endogenous siRNA Pathway in Drosophila." *Molecular Cell* 49 (4). Elsevier: 680–91. doi:10.1016/j.molcel.2012.12.024.
Nomoto, A, B Detjen, R Pozzatti, and E Wimmer. 1977. "The Location of the Polio Genome Protein in Viral RNAs and Its Implication for RNA Synthesis.." *Nature* 268 (5617): 208–13.
Nomoto, A, N Kitamura, F Golini, and E Wimmer. 1977. "The 5'-Terminal Structures of Poliovirion RNA and Poliovirus mRNA Differ Only in the Genome-Linked Protein VPg.." *Proceedings of the National Academy of Sciences* 74 (12). National Academy of Sciences: 5345–49.
Obbard, D J, F M Jiggins, D L Halligan, and T J Little. 2006. "Natural Selection Drives Extremely Rapid Evolution in Antiviral RNAi Genes." *Curr Biol* 16 (6): 580–85. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16546082.
Ogino, Minako, and Tomoaki Ogino. 2017. "5'-Phospho-RNA Acceptor Specificity of GDP Polyribonucleotidyltransferase of Vesicular Stomatitis Virus in mRNA Capping.." Edited by Stanley Perlman. *J Virol* 91 (6): 277. doi:10.1128/JVI.02322-16.
Ogino, Tomoaki, and Amiya K Banerjee. 2008. "Formation of Guanosine(5')Tetraphospho(5')Adenosine Cap Structure by an Unconventional mRNA Capping Enzyme of Vesicular Stomatitis Virus.." *J Virol* 82 (15): 7729–34. doi:10.1128/JVI.00326-08.
Ogino, Tomoaki, and Amiya K Banerjee. 2011. "An Unconventional Pathway of mRNA Cap Formation by Vesiculoviruses.." *Virus Research* 162 (1-2): 100–109. doi:10.1016/j.virusres.2011.09.012.
Ori, Daisuke, Motoya Murase, and Taro Kawai. 2017. "Cytosolic Nucleic Acid Sensors and Innate Immune Regulation.." *International Reviews of Immunology* 36 (2). Taylor & Francis: 74–88. doi:10.1080/08830185.2017.1298749.
Oshima, R. 1978. "Stimulation of the Clonal Growth and Differentiation of Feeder Layer Dependent Mouse Embryonal Carcinoma Cells by Beta-Mercaptoethanol.." *Differentiation; Research in Biological Diversity* 11 (3): 149–55.
Panda, Debasis, Beth Gold, Michael A Tartell, Keiko Rausch, Sergio Casas-Tinto, and Sara Cherry. 2015. "The Transcription Factor FoxK Participates with Nup98 to Regulate Antiviral Gene Expression.." *mBio* 6 (2). American Society for Microbiology: e02509–14. doi:10.1128/mBio.02509-14.
Panda, Debasis, Pau Pascual-Garcia, Margaret Dunagin, Matthew Tudor, Kaycie C Hopkins, Jie Xu, Beth Gold, Arjun Raj, Maya Capelson, and Sara Cherry. 2014. "Nup98 Promotes Antiviral Gene Expression to Restrict RNA Viral Infection in

Drosophila.." *Proceedings of the National Academy of Sciences of the United States of America* 111 (37). National Acad Sciences: E3890–99. doi:10.1073/pnas.1410087111.
Paradkar, Prasad N, Jean-Bernard Duchemin, Rhonda Voysey, and Peter J Walker. 2014. "Dicer-2-Dependent Activation of Culex Vago Occurs via the TRAF-Rel2 Signaling Pathway." Edited by Ken E Olson. *PLoS Neglected Tropical Diseases* 8 (4). Public Library of Science: e2823. doi:10.1371/journal.pntd.0002823.
Paradkar, Prasad N, Lee Trinidad, Rhonda Voysey, Jean-Bernard Duchemin, and Peter J Walker. 2012. "Secreted Vago Restricts West Nile Virus Infection in Culex Mosquito Cells by Activating the Jak-STAT Pathway.." *Proceedings of the National Academy of Sciences of the United States of America* 109 (46). National Acad Sciences: 18915–20. doi:10.1073/pnas.1205231109.
Paro, Simona, Jean-Luc Imler, and Carine Meignin. 2015. "Sensing Viral RNAs by Dicer/RIG-I Like ATPases Across Species.." *Curr Opin Immunol* 32 (February): 106–13. doi:10.1016/j.coi.2015.01.009.
Paytubi, Sonia, Xuemin Wang, Yun W Lam, Luis Izquierdo, Mairi J Hunter, Eric Jan, Harinder S Hundal, and Christopher G Proud. 2009. "ABC50 Promotes Translation Initiation in Mammalian Cells." *The Journal of Biological Chemistry* 284 (36): 24061–73. doi:10.1074/jbc.M109.031625.
Perrot, Ivan, Florence Deauvieau, Catherine Massacrier, Nicola Hughes, Pierre Garrone, Isabelle Durand, Olivier Demaria, et al. 2010. "TLR3 and Rig-Like Receptor on Myeloid Dendritic Cells and Rig-Like Receptor on Human NK Cells Are Both Mandatory for Production of IFN-Gamma in Response to Double-Stranded RNA.." *Journal of Immunology (Baltimore, Md. : 1950)* 185 (4). American Association of Immunologists: 2080–88. doi:10.4049/jimmunol.1000532.
Petit, Marine, Vanesa Mongelli, Lionel Frangeul, Hervé Blanc, Francis Jiggins, and Maria-Carla Saleh. 2016. "piRNA Pathway Is Not Required for Antiviral Defense in Drosophila Melanogaster.." *Proceedings of the National Academy of Sciences of the United States of America* 113 (29). National Acad Sciences: E4218–27. doi:10.1073/pnas.1607952113.
Petrillo, J E, Petrillo, Jessica E, P A Venter, P Arno Venter, J R Short, James R Short, R Gopal, et al. 2013. "Cytoplasmic Granule Formation and Translational Inhibition of Nodaviral RNAs in the Absence of the Double-Stranded RNA Binding Protein B2." *J Virol* 87 (24): 13409–21. doi:10.1128/JVI.02362-13.
Pichlmair, Andreas, Caroline Lassnig, Carol-Ann Eberle, Maria W Górna, Christoph L Baumann, Thomas R Burkard, Tilmann Bürckstümmer, et al. 2011. "IFIT1 Is an Antiviral Protein That Recognizes 5'-Triphosphate RNA.." *Nat Immunol* 12 (7). Nature Publishing Group: 624–30. doi:10.1038/ni.2048.
Pichlmair, Andreas, Oliver Schulz, Choon-Ping Tan, Jan Rehwinkel, Hiroki Kato, Osamu Takeuchi, Shizuo Akira, Michael Way, Giampietro Schiavo, and Caetano Reis e Sousa. 2009. "Activation of MDA5 Requires Higher-Order RNA Structures Generated During Virus Infection.." *J Virol* 83 (20). American Society for Microbiology Journals: 10761–69. doi:10.1128/JVI.00770-09.
Pichlmair, Andreas, Oliver Schulz, Choon-Ping Tan, Tanja I Näslund, Peter Liljeström, Friedemann Weber, and Caetano Reis e Sousa. 2006. "RIG-I-Mediated Antiviral Responses to Single-Stranded RNA Bearing 5'-Phosphates.." *Science* 314 (5801): 997–1001. doi:10.1126/science.1132998.
Poon, Carole L C, Jane I Lin, Xiaomeng Zhang, and Kieran F Harvey. 2011. "The Sterile 20-Like Kinase Tao-1 Controls Tissue Growth by Regulating the Salvador-Warts-Hippo Pathway.." *Developmental Cell* 21 (5): 896–906.

doi:10.1016/j.devcel.2011.09.012.
Poon, Carole L C, Katrina A Mitchell, Shu Kondo, Louise Y Cheng, and Kieran F Harvey. 2016. "The Hippo Pathway Regulates Neuroblasts and Brain Size in Drosophila Melanogaster.." *Curr Biol* 26 (8): 1034–42. doi:10.1016/j.cub.2016.02.009.
Poon, Carole L C, Weijie Liu, Yanjun Song, Marta Gomez, Yavuz Kulaberoglu, Xiaomeng Zhang, Wenjian Xu, et al. 2018. "A Hippo-Like Signaling Pathway Controls Tracheal Morphogenesis in Drosophila Melanogaster.." *Developmental Cell* 47 (5): 564–65. doi:10.1016/j.devcel.2018.09.024.
Ratcliff, F. 1997. "A Similarity Between Viral Defense and Gene Silencing in Plants." *Science* 276 (5318). American Association for the Advancement of Science: 1558–60. doi:10.1126/science.276.5318.1558.
Reinganum, C. 1975. "The Isolation of Cricket Paralysis Virus From the Emperor Gum Moth, Antheraea Eucalypti Scott, and Its Infectivity Towards a Range of Insect Species.." *Intervirology* 5 (1-2). Karger Publishers: 97–102. doi:10.1159/000149886.
Rice, Gillian I, Paul R Kasher, Gabriella M A Forte, Niamh M Mannion, Sam M Greenwood, Marcin Szynkiewicz, Jonathan E Dickerson, et al. 2012. "Mutations in ADAR1 Cause Aicardi-Goutières Syndrome Associated with a Type I Interferon Signature.." *Nature Genetics* 44 (11). Nature Publishing Group: 1243–48. doi:10.1038/ng.2414.
Richetta, Clémence, and Mathias Faure. 2013. "Autophagy in Antiviral Innate Immunity.." *Cellular Microbiology* 15 (3): 368–76. doi:10.1111/cmi.12043.
Richetta, Clémence, Isabel P Grégoire, Pauline Verlhac, Olga Azocar, Joël Baguet, Monique Flacher, Frédéric Tangy, Chantal Rabourdin-Combe, and Mathias Faure. 2013. "Sustained Autophagy Contributes to Measles Virus Infectivity." Edited by Vojo Deretic. *PLoS Pathog* 9 (9). Public Library of Science: e1003599. doi:10.1371/journal.ppat.1003599.
Rigby, Rachel E, Lauren M Webb, Karen J Mackenzie, Yue Li, Andrea Leitch, Martin A M Reijns, Rachel J Lundie, et al. 2014. "RNA:DNA Hybrids Are a Novel Molecular Pattern Sensed by TLR9.." *The EMBO Journal* 33 (6). EMBO Press: 542–58. doi:10.1002/embj.201386117.
Robinson, R A, V T DeVita, H B Levy, S Baron, S P Hubbard, and A S Levine. 1976. "A Phase I-II Trial of Multiple-Dose Polyriboinosic-Polyribocytidylic Acid in Patieonts with Leukemia or Solid Tumors.." *Journal of the National Cancer Institute* 57 (3): 599–602.
Roers, Axel, Björn Hiller, and Veit Hornung. 2016. "Recognition of Endogenous Nucleic Acids by the Innate Immune System.." *Immunity* 44 (4): 739–54. doi:10.1016/j.immuni.2016.04.002.
Roignant, Jean-Yves, Clément Carré, Bruno Mugat, Dimitri Szymczak, Jean-Antoine Lepesant, and Christophe Antoniewski. 2003. "Absence of Transitive and Systemic Pathways Allows Cell-Specific and Isoform-Specific RNAi in Drosophila.." *RNA (New York, N.Y.)* 9 (3). Cold Spring Harbor Laboratory Press: 299–308. doi:10.1261/rna.2154103.
Rose, John, and Manfred Schubert. 1987. "Rhabdovirus Genomes and Their Products." In *The Rhabdoviruses*, 129–66. Boston, MA: Springer US. doi:10.1007/978-1-4684-7032-1_4.
Rose, Patrick P, Sheri L Hanna, Anna Spiridigliozzi, Nattha Wannissorn, Daniel P Beiting, Susan R Ross, Richard W Hardy, Shelly A Bambina, Mark T Heise, and Sara Cherry. 2011. "Natural Resistance-Associated Macrophage Protein Is a Cellular Receptor for Sindbis Virus in Both Insect and Mammalian Hosts.." *Cell*

Host & Microbe 10 (2): 97–104. doi:10.1016/j.chom.2011.06.009.
Runge, Simon, Konstantin M J Sparrer, Charlotte Lässig, Katharina Hembach, Alina Baum, Adolfo García-Sastre, Johannes Söding, Karl-Klaus Conzelmann, and Karl-Peter Hopfner. 2014. "In Vivo Ligands of MDA5 and RIG-I in Measles Virus-Infected Cells.." Edited by Karen L Mossman. *PLoS Pathog* 10 (4). Public Library of Science: e1004081. doi:10.1371/journal.ppat.1004081.
Sabin, Leah R, Qi Zheng, Pramod Thekkat, Jamie Yang, Gregory J Hannon, Brian D Gregory, Matthew Tudor, and Sara Cherry. 2013. "Dicer-2 Processes Diverse Viral RNA Species.." Edited by Sebastien Pfeffer. *PLoS ONE* 8 (2). Public Library of Science: e55458. doi:10.1371/journal.pone.0055458.
Saito, Kuniaki, Akira Ishizuka, Haruhiko Siomi, and Mikiko C Siomi. 2005. "Processing of Pre-microRNAs by the Dicer-1–Loquacious Complex in Drosophila Cells." Edited by James C Carrington. *PLoS Biology* 3 (7). Public Library of Science: e235. doi:10.1371/journal.pbio.0030235.
Saleh, Maria-Carla, Michel Tassetto, Ronald P van Rij, Bertsy Goic, Valérie Gausson, Bassam Berry, Caroline Jacquier, Christophe Antoniewski, and Raul Andino. 2009. "Antiviral Immunity in Drosophila Requires Systemic RNA Interference Spread.." *Nature* 458 (7236). Nature Publishing Group: 346–50. doi:10.1038/nature07712.
Saleh, Maria-Carla, Ronald P van Rij, Armin Hekele, Amethyst Gillis, Edan Foley, Patrick H O'Farrell, and Raul Andino. 2006. "The Endocytic Pathway Mediates Cell Entry of dsRNA to Induce RNAi Silencing.." *Nature Cell Biology* 8 (8). Nature Publishing Group: 793–802. doi:10.1038/ncb1439.
Sanchez David, Raul Y, Chantal Combredet, Odile Sismeiro, Marie-Agnès Dillies, Bernd Jagla, Jean-Yves Coppée, Marie Mura, et al. 2016. "Comparative Analysis of Viral RNA Signatures on Different RIG-I-Like Receptors.." *eLife* 5 (March). eLife Sciences Publications Limited: e11275. doi:10.7554/eLife.11275.
Sansone, Christine L, Jonathan Cohen, Ari Yasunaga, Jie Xu, Greg Osborn, Harry Subramanian, Beth Gold, Nicolas Buchon, and Sara Cherry. 2015. "Microbiota-Dependent Priming of Antiviral Intestinal Immunity in Drosophila.." *Cell Host & Microbe* 18 (5): 571–81. doi:10.1016/j.chom.2015.10.010.
Scaturro, Pietro, Alexey Stukalov, Darya A Haas, Mirko Cortese, Kalina Draganova, Anna Płaszczyca, Ralf Bartenschlager, Magdalena Götz, and Andreas Pichlmair. 2018. "An Orthogonal Proteomic Survey Uncovers Novel Zika Virus Host Factors.." *Nature* 561 (7722). Nature Publishing Group: 253–57. doi:10.1038/s41586-018-0484-5.
Schetz, John A, and Eswar P N Shankar. 2004. "Protein Expression in the Drosophila Schneider 2 Cell System.." *Current Protocols in Neuroscience* Chapter 4 (September). Hoboken, NJ, USA: John Wiley & Sons, Inc.: Unit4.16. doi:10.1002/0471142301.ns0416s27.
Schlee, Martin, and Gunther Hartmann. 2016. "Discriminating Self From Non-Self in Nucleic Acid Sensing.." *Nature Reviews Immunology* 16 (9). Nature Publishing Group: 566–80. doi:10.1038/nri.2016.78.
Schlee, Martin, Andreas Roth, Veit Hornung, Cristina Amparo Hagmann, Vera Wimmenauer, Winfried Barchet, Christoph Coch, et al. 2009. "Recognition of 5' Triphosphate by RIG-I Helicase Requires Short Blunt Double-Stranded RNA as Contained in Panhandle of Negative-Strand Virus.." *Immunity* 31 (1): 25–34. doi:10.1016/j.immuni.2009.05.008.
Schmidt, Tobias, Susann Friedrich, Ralph Peter Golbik, and Sven-Erik Behrens. 2017. "NF90-NF45 Is a Selective RNA Chaperone That Rearranges Viral and Cellular Riboswitches: Biochemical Analysis of a Virus Host Factor Activity.." *Nucleic*

Acids Research 45 (21): 12441–54. doi:10.1093/nar/gkx931.
Schneider, I. 1972. "Cell Lines Derived From Late Embryonic Stages of Drosophila Melanogaster.." *Journal of Embryology and Experimental Morphology* 27 (2): 353–65.
Schneider, U, A Martin, M Schwemmle, and P Staeheli. 2007. "Genome Trimming by Borna Disease Viruses: Viral Replication Control or Escape From Cellular Surveillance?." *Cellular and Molecular Life Sciences : CMLS* 64 (9). Birkhäuser-Verlag: 1038–42. doi:10.1007/s00018-007-6545-9.
Schnettler, Esther, Claire L Donald, Stacey Human, Mick Watson, Ricky W C Siu, Melanie McFarlane, John K Fazakerley, Alain Kohl, and Rennos Fragkoudis. 2013. "Knockdown of piRNA Pathway Proteins Results in Enhanced Semliki Forest Virus Production in Mosquito Cells.." *The Journal of General Virology* 94 (Pt 7). Microbiology Society: 1680–89. doi:10.1099/vir.0.053850-0.
Schuberth-Wagner, Christine, Janos Ludwig, Ann Kristin Bruder, Anna-Maria Herzner, Thomas Zillinger, Marion Goldeck, Tobias Schmidt, et al. 2015. "A Conserved Histidine in the RNA Sensor RIG-I Controls Immune Tolerance to N1-2'O-Methylated Self RNA.." *Immunity* 43 (1): 41–51. doi:10.1016/j.immuni.2015.06.015.
Scotti, P D, S Dearing, and D W Mossop. 1983. "Flock House Virus: a Nodavirus Isolated From Costelytra Zealandica (White) (Coleoptera: Scarabaeidae).." *Archives of Virology* 75 (3): 181–89.
Settles, E W, and P D Friesen. 2008. "Flock House Virus Induces Apoptosis by Depletion of Drosophila Inhibitor-of-Apoptosis Protein DIAP1." *J Virol* 82 (3): 1378–88. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17989181.
Shapiro, Jillian S, Sonja Schmid, Lauren C Aguado, Leah R Sabin, Ari Yasunaga, Jaehee V Shim, David Sachs, Sara Cherry, and Benjamin R tenOever. 2014. "Drosha as an Interferon-Independent Antiviral Factor.." *Proceedings of the National Academy of Sciences of the United States of America* 111 (19). National Acad Sciences: 7108–13. doi:10.1073/pnas.1319635111.
Shelly, Spencer, Nina Lukinova, Shelly Bambina, Allison Berman, and Sara Cherry. 2009. "Autophagy Is an Essential Component of Drosophila Immunity Against Vesicular Stomatitis Virus.." *Immunity* 30 (4): 588–98. doi:10.1016/j.immuni.2009.02.009.
Short, James R, Jeffrey A Speir, Radhika Gopal, Logan M Pankratz, Jason Lanman, and Anette Schneemann. 2016. "Role of Mitochondrial Membrane Spherules in Flock House Virus Replication.." Edited by D S Lyles. *J Virol* 90 (7). American Society for Microbiology Journals: 3676–83. doi:10.1128/JVI.03080-15.
Simon, Viviana, Nicolin Bloch, and Nathaniel R Landau. 2015. "Intrinsic Host Restrictions to HIV-1 and Mechanisms of Viral Escape.." *Nat Immunol* 16 (6). Nature Research: 546–53. doi:10.1038/ni.3156.
Skalsky, Rebecca L, and Bryan R Cullen. 2010. "Viruses, microRNAs, and Host Interactions." *Annual Review of Microbiology* 64 (1). Annual Reviews: 123–41. doi:10.1146/annurev.micro.112408.134243.
Smith, R M, C Pernstich, and S E Halford. 2014. "TstI, a Type II Restriction-Modification Protein with DNA Recognition, Cleavage and Methylation Functions in a Single Polypeptide." *Nucleic Acids Research* 42 (9). Oxford University Press: 5809–22. doi:10.1093/nar/gku187.
Son, Kyung-No, Zhiguo Liang, and Howard L Lipton. 2015. "Double-Stranded RNA Is Detected by Immunofluorescence Analysis in RNA and DNA Virus Infections,

Including Those by Negative-Stranded RNA Viruses." Edited by S Perlman. *J Virol* 89 (18): 9383–92. doi:10.1128/JVI.01299-15.
Souza-Neto, Jayme A, Shuzhen Sim, and George Dimopoulos. 2009. "An Evolutionary Conserved Function of the JAK-STAT Pathway in Anti-Dengue Defense.." *Proceedings of the National Academy of Sciences of the United States of America* 106 (42). National Acad Sciences: 17841–46. doi:10.1073/pnas.0905006106.
Spellberg, Michael J, Michael T Marr, and Michael T Marr II. 2015. "FOXO Regulates RNA Interference in Drosophilaand Protects From RNA Virus Infection." *Proceedings of the National Academy of Sciences* 112 (47). National Acad Sciences: 14587–92. doi:10.1073/pnas.1517124112.
Stetson, Daniel B, and Ruslan Medzhitov. 2006. "Recognition of Cytosolic DNA Activates an IRF3-Dependent Innate Immune Response." *Immunity* 24 (1): 93–103. doi:10.1016/j.immuni.2005.12.003.
Strauss, J H, and E G Strauss. 1994. "The Alphaviruses: Gene Expression, Replication, and Evolution.." *Microbiological Reviews* 58 (3). American Society for Microbiology (ASM): 491–562.
Sugiyama, Takahiro, Katsuaki Hoshino, Masuyoshi Saito, Takahiro Yano, Izumi Sasaki, Chihiro Yamazaki, Shizuo Akira, and Tsuneyasu Kaisho. 2008. "Immunoadjuvant Effects of Polyadenylic:Polyuridylic Acids Through TLR3 and TLR7.." *International Immunology* 20 (1): 1–9. doi:10.1093/intimm/dxm112.
TAYLOR, R M, H S HURLBUT, T H WORK, J R KINGSTON, and T E FROTHINGHAM. 1955. "Sindbis Virus: a Newly Recognized Arthropodtransmitted Virus.." *The American Journal of Tropical Medicine and Hygiene* 4 (5): 844–62.
Theopold, Ulrich, Robert Krautz, and Mitchell S Dushay. 2014. "The Drosophila Clotting System and Its Messages for Mammals.." *Developmental & Comparative Immunology* 42 (1): 42–46. doi:10.1016/j.dci.2013.03.014.
Toczydlowska-Socha, Diana, Magdalena M Zielinska, Malgorzata Kurkowska, Astha, Catarina F Almeida, Filip Stefaniak, Elzbieta Purta, and Janusz M Bujnicki. 2018. "Human RNA Cap1 Methyltransferase CMTr1 Cooperates with RNA Helicase DHX15 to Modify RNAs with Highly Structured 5' Termini.." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 373 (1762). The Royal Society: 20180161. doi:10.1098/rstb.2018.0161.
Topisirovic, Ivan, Yuri V Svitkin, Nahum Sonenberg, and Aaron J Shatkin. 2010. "Cap and Cap-Binding Proteins in the Control of Gene Expression." *Wiley Interdisciplinary Reviews: RNA* 2 (2). John Wiley & Sons, Ltd: 277–98. doi:10.1002/wrna.52.
van Cleef, Koen W R, Joel T van Mierlo, Pascal Miesen, Gijs J Overheul, Jelke J Fros, Susan Schuster, Marco Marklewitz, Gorben P Pijlman, Sandra Junglen, and Ronald P van Rij. 2014. "Mosquito and Drosophila Entomobirnaviruses Suppress dsRNA- and siRNA-Induced RNAi.." *Nucleic Acids Research* 42 (13). Oxford University Press: 8732–44. doi:10.1093/nar/gku528.
van Mierlo, Joel T, Gijs J Overheul, Benjamin Obadia, Koen W R van Cleef, Claire L Webster, Maria-Carla Saleh, Darren J Obbard, and Ronald P van Rij. 2014. "Novel Drosophila Viruses Encode Host-Specific Suppressors of RNAi." Edited by David S Schneider. *PLoS Pathog* 10 (7): e1004256. doi:10.1371/journal.ppat.1004256.
van Montfoort, Nadine, David Olagnier, and John Hiscott. 2014. "Unmasking Immune Sensing of Retroviruses: Interplay Between Innate Sensors and Host Effectors." *Cytokine & Growth Factor Reviews* 25 (6): 657–68. doi:10.1016/j.cytogfr.2014.08.006.
van Rij, Ronald P, Maria-Carla Saleh, Bassam Berry, Catherine Foo, Andrew Houk, Christophe Antoniewski, and Raul Andino. 2006. "The RNA Silencing

Endonuclease Argonaute 2 Mediates Specific Antiviral Immunity in Drosophila Melanogaster.." *Genes & Development* 20 (21). Cold Spring Harbor Lab: 2985–95. doi:10.1101/gad.1482006.
Veillard, Florian, Laurent Troxler, and Jean-Marc Reichhart. 2016. "Drosophila Melanogaster Clip-Domain Serine Proteases: Structure, Function and Regulation." *Biochimie* 122 (March): 255–69. doi:10.1016/j.biochi.2015.10.007.
Venter, P A, and A Schneemann. 2008. "Recent Insights Into the Biology and Biomedical Applications of Flock House Virus.." *Cellular and Molecular Life Sciences : CMLS* 65 (17): 2675–87. doi:10.1007/s00018-008-8037-y.
Versteeg, Gijs A, Benjamin G Hale, Sander van Boheemen, Thorsten Wolff, Deborah J Lenschow, and Adolfo García-Sastre. 2010. "Species-Specific Antagonism of Host ISGylation by the Influenza B Virus NS1 Protein.." *J Virol* 84 (10). American Society for Microbiology Journals: 5423–30. doi:10.1128/JVI.02395-09.
Vissers, Joseph H A, Samuel A Manning, Aishwarya Kulkarni, and Kieran F Harvey. 2016. "A Drosophila RNAi Library Modulates Hippo Pathway-Dependent Tissue Growth.." *Nature Communications* 7 (1). Nature Publishing Group: 10368. doi:10.1038/ncomms10368.
Vodovar, Nicolas, Alfred W Bronkhorst, Koen W R van Cleef, Pascal Miesen, Hervé Blanc, Ronald P van Rij, and Maria-Carla Saleh. 2012. "Arbovirus-Derived piRNAs Exhibit a Ping-Pong Signature in Mosquito Cells." Edited by Sebastien Pfeffer. *PLoS ONE* 7 (1): e30861. doi:10.1371/journal.pone.0030861.
Wang, Hui, Zaijun Ma, Kongyan Niu, Yi Xiao, Xiaofen Wu, Chenyu Pan, Yun Zhao, Kai Wang, Yaoyang Zhang, and Nan Liu. 2016. "Antagonistic Roles of Nibbler and Hen1 in Modulating piRNA 3' Ends in Drosophila.." *Development (Cambridge, England)* 143 (3). Oxford University Press for The Company of Biologists Limited: 530–39. doi:10.1242/dev.128116.
Wang, Xiao-Hong, Roghiyh Aliyari, Wan-Xiang Li, Hong-Wei Li, Kevin Kim, Richard Carthew, Peter Atkinson, and Shou-Wei Ding. 2006. "RNA Interference Directs Innate Immunity Against Viruses in Adult Drosophila.." *Science* 312 (5772). American Association for the Advancement of Science: 452–54. doi:10.1126/science.1125694.
Wayne, M L, D Contamine, and M Kreitman. 1996. "Molecular Population Genetics of Ref(2)P, a Locus Which Confers Viral Resistance in Drosophila.." *Molecular Biology and Evolution* 13 (1): 191–99.
Weavers, Helen, Iwan R Evans, Paul Martin, and Will Wood. 2016. "Corpse Engulfment Generates a Molecular Memory That Primes the Macrophage Inflammatory Response.." *Cell* 165 (7): 1658–71. doi:10.1016/j.cell.2016.04.049.
Weber, F, V Wagner, S B Rasmussen, R Hartmann, and S R Paludan. 2006. "Double-Stranded RNA Is Produced by Positive-Strand RNA Viruses and DNA Viruses but Not in Detectable Amounts by Negative-Strand RNA Viruses." *J Virol* 80 (10): 5059–64. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16641297.
Webster, Claire L, Fergal M Waldron, Shaun Robertson, Daisy Crowson, Giada Ferrari, Juan F Quintana, Jean-Michel Brouqui, et al. 2015. "The Discovery, Distribution, and Evolution of Viruses Associated with Drosophila Melanogaster." Edited by Harmit S Malik. *PLoS Biology* 13 (7): e1002210. doi:10.1371/journal.pbio.1002210.
West, Cara, and Neal Silverman. 2018. "P38b and JAK-STAT Signaling Protect Against Invertebrate Iridescent Virus 6 Infection in Drosophila.." Edited by Sara Cherry. *PLoS Pathog* 14 (5). Public Library of Science: e1007020.

doi:10.1371/journal.ppat.1007020.
Wolferstätter, Michael, Marc Schweneker, Michaela Späth, Susanne Lukassen, Marieken Klingenberg, Kay Brinkmann, Ursula Wielert, et al. 2014. "Recombinant Modified Vaccinia Virus Ankara Generating Excess Early Double-Stranded RNA Transiently Activates Protein Kinase R and Triggers Enhanced Innate Immune Responses.." *J Virol* 88 (24): 14396–411. doi:10.1128/JVI.02082-14.
Wolkowicz, Urszula M, and Atlanta G Cook. 2012. "NF45 Dimerizes with NF90, Zfr and SPNR via a Conserved Domain That Has a Nucleotidyltransferase Fold.." *Nucleic Acids Research* 40 (18): 9356–68. doi:10.1093/nar/gks696.
Worringer, Kathleen A, Feixia Chu, and Barbara Panning. 2009. "The Zinc Finger Protein Zn72D and DEAD Box Helicase Belle Interact and Control Maleless mRNA and Protein Levels.." *BMC Molecular Biology* 10 (1). BioMed Central: 33. doi:10.1186/1471-2199-10-33.
Wu, Q, Y Luo, R Lu, N Lau, E C Lai, W X Li, and S W Ding. 2010. "Virus Discovery by Deep Sequencing and Assembly of Virus-Derived Small Silencing RNAs." *Proceedings of the National Academy of Sciences* 107 (4): 1606–11. doi:10.1073/pnas.0911353107.
Wyers, F, P Dru, B Simonet, and D Contamine. 1993. "Immunological Cross-Reactions and Interactions Between the Drosophila Melanogaster Ref(2)P Protein and Sigma Rhabdovirus Proteins.." *J Virol* 67 (6). American Society for Microbiology (ASM): 3208–16.
Xu, Jie, and Sara Cherry. 2014. "Viruses and Antiviral Immunity in Drosophila." *Developmental & Comparative Immunology* 42 (1): 67–84. doi:10.1016/j.dci.2013.05.002.
Xu, Jie, Gregory Grant, Leah R Sabin, Beth Gordesky-Gold, Ari Yasunaga, Mathew Tudor, and Sara Cherry. 2012. "Transcriptional Pausing Controls a Rapid Antiviral Innate Immune Response in Drosophila.." *Cell Host & Microbe* 12 (4): 531–43. doi:10.1016/j.chom.2012.08.011.
Xu, Jie, Kaycie Hopkins, Leah Sabin, Ari Yasunaga, Harry Subramanian, Ian Lamborn, Beth Gordesky-Gold, and Sara Cherry. 2013. "ERK Signaling Couples Nutrient Status to Antiviral Defense in the Insect Gut.." *Proceedings of the National Academy of Sciences of the United States of America* 110 (37). National Acad Sciences: 15025–30. doi:10.1073/pnas.1303193110.
Xu, Jie, Xiomara Mercado-López, Jennifer T Grier, Won-keun Kim, Lauren F Chun, Edward B Irvine, Yoandris Del Toro Duany, et al. 2015. "Identification of a Natural Viral RNA Motif That Optimizes Sensing of Viral RNA by RIG-I.." *mBio* 6 (5): e01265–15. doi:10.1128/mBio.01265-15.
Xu, Jun, Jun-Yi Yang, Qi-Wen Niu, and Nam-Hai Chua. 2006. "Arabidopsis DCP2, DCP1, and VARICOSE Form a Decapping Complex Required for Postembryonic Development.." *The Plant Cell* 18 (12). American Society of Plant Biologists: 3386–98. doi:10.1105/tpc.106.047605.
Yasunaga, Ari, Sheri L Hanna, Jianqing Li, Hyelim Cho, Patrick P Rose, Anna Spiridigliozzi, Beth Gold, Michael S Diamond, and Sara Cherry. 2014. "Genome-Wide RNAi Screen Identifies Broadly-Acting Host Factors That Inhibit Arbovirus Infection.." Edited by Abraham L Brass. *PLoS Pathog* 10 (2). Public Library of Science: e1003914. doi:10.1371/journal.ppat.1003914.
Yu, Jiang Hong, Wei-Hong Yang, Tod Gulick, Kenneth D Bloch, and Donald B Bloch. 2005. "Ge-1 Is a Central Component of the Mammalian Cytoplasmic mRNA Processing Body.." *RNA (New York, N.Y.)* 11 (12). Cold Spring Harbor Lab: 1795–1802. doi:10.1261/rna.2142405.

Zambon, Robert A, Madhumitha Nandakumar, Vikram N Vakharia, and Louisa P Wu. 2005. "The Toll Pathway Is Important for an Antiviral Response in Drosophila.." *Proceedings of the National Academy of Sciences* 102 (20). National Acad Sciences: 7257–62. doi:10.1073/pnas.0409181102.

Zeidler, Martin P, and Nina Bausek. 2013. "The Drosophila JAK-STAT Pathway.." *Jak-Stat* 2 (3). Taylor & Francis: e25353. doi:10.4161/jkst.25353.

Zhang, Qi, Liqin Zhang, Xinlei Gao, Shuishui Qi, Zhaoxia Chang, and Qingfa Wu. 2015. "DIP1 Plays an Antiviral Role Against DCV Infection in Drosophila Melanogaster.." *Biochemical and Biophysical Research Communications* 460 (2): 222–26. doi:10.1016/j.bbrc.2015.03.013.

Zheng, Min, Linna Xie, Yaoji Liang, Suqin Wu, Huijuan Xu, Yuedong Zhang, Hekun Liu, Dexin Lin, Jiahuai Han, and Kunping Lu. 2015. "Recognition of Cytosolic DNA Attenuates Glucose Metabolism and Induces AMPK Mediated Energy Stress Response.." *International Journal of Biological Sciences* 11 (5): 587–94. doi:10.7150/ijbs.10945.

Zheng, Qingliang, Jin Hou, Ye Zhou, Zhenyang Li, and Xuetao Cao. 2017. "The RNA Helicase DDX46 Inhibits Innate Immunity by Entrapping m6A-Demethylated Antiviral Transcripts in the Nucleus.." *Nat Immunol* 18 (10). Nature Publishing Group: 1094–1103. doi:10.1038/ni.3830.

Zhou, Li-Ya. 2016. "Host Restriction Factors for Hepatitis C Virus." *World Journal of Gastroenterology* 22 (4): 1477. doi:10.3748/wjg.v22.i4.1477.

Zitzmann, Jan, Christine Schreiber, Joel Eichmann, Roberto Otmar Bilz, Denise Salzig, Tobias Weidner, and Peter Czermak. 2018. "Single-Cell Cloning Enables the Selection of More Productive Drosophila Melanogaster S2 Cells for Recombinant Protein Expression.." *Biotechnology Reports (Amsterdam, Netherlands)* 19 (September): e00272. doi:10.1016/j.btre.2018.e00272.

Résumé de thèse

Évaluation de l'activité antivirale des protéines de liaison aux acides nucléiques : une analyse multi-espèces

CONTEXTE ET SIGNIFICATION

Les virus impactent notre vie de nombreuses manières, causant des maladies graves et répandues. Le système immunitaire permet à l'hôte de lutter contre les infections virales. Chez les mammifères, le système immunitaire comprend une branche innée conservée au cours de l'évolution et une branche adaptative acquise plus récemment, basées sur des récepteurs immunitaires hautement spécifiques exprimés par les lymphocytes. L'immunité innée joue un rôle majeur dans la détection et la limitation de la croissance des agents pathogènes, en particulier au cours des premières étapes de l'infection, mais entraîne également la production de cytokines et de molécules co-stimulantes, qui déterminent la réponse immunitaire adaptative. En conséquence, l'immunité innée et adaptative sont fortement interconnectées chez les mammifères. En revanche, les invertébrés ne s'appuient que sur des mécanismes immunitaires innés pour lutter contre les infections.

L'immunité innée est activée par ce que l'on appelle les récepteurs de reconnaissance de formes (Pattern recognition receptor ou PRR), qui détectent les caractéristiques moléculaires partagées par de grandes classes de micro-organismes. Les virus sont composés, dans leur forme la plus simple, d'une capside protéique entourant une molécule d'acides nucléiques et offrent peu de cibles pour la reconnaissance. En effet, l'activation des voies antivirales repose en grande partie sur la détection des acides nucléiques (AN) viraux, par exemple la détection d'ARN et d'ADN viraux par les TLR ou les RLR chez les mammifères [1]. La discrimination entre soi et non-soi de AN est basée sur des caractéristiques spécifiques des AN viraux, telles que la présence d'ARN double-brin viraux ou des modifications des ARN viraux (par exemple, des groupes méthyl) [2]. Il est à noter que plusieurs maladies auto-immunes (par exemple, le

lupus érythémateux systémique) sont causées ou exacerbées par une détection anormale des AN [3].

L'objectif de mon projet de thèse était d'identifier de nouvelles protéines de liaison aux AN viraux. Une approche de biologie intégrative des systèmes a été choisie et a été réalisée en collaboration avec les groupes de A. Pichlmair à Munich, M. Superti-Furga à Vienne et J. Colinge à Montpellier. Il comprend une procédure de validation fonctionnelle, largement basée sur le modèle Drosophile. Une série d'appâts composés d'AN de type viraux a permis d'identifier des protéines par spectrométrie de masse à la suite d'une purification par affinité (AP-MS). Les protéines candidates sont identifiées à partir d'extraits provenant de cellules humaines, de souris, de poulet et de drosophile, ainsi que d'extraits de mouches adultes et du ver *Caenorhabditis elegans*. Au total, 20 appâts différents d'ARN et d'ADN, correspondant à un répertoire de marques et de contrôles spécifiques au virus, ont été utilisées comme appâts [2].

RÉSULTATS

I. Protéomique et sélection de protéines candidates

Pour apporter une preuve de principe, le projet a débuté avec un crible pilote dans le laboratoire de A. Pichlmair avec des extraits de cellules S2 de Drosophile, en utilisant de l'ARN double-brin synthétique, polyI:C, comme appât et le polyC comme appât témoin. Nous avons identifié les protéines de liaison à l'ARN attendues, pertinentes dans le contexte d'une infection virale, telles que Dicer-2 ou Adar. Nous avons également identifié d'autres protéines, parmi lesquelles nous avons sélectionné CG5641 pour une analyse plus poussée (voir ci-dessous). Par la suite, le criblage MS a été étendu avec des extraits de cellules THP1 humaines et RAW264.7 de souris. De plus, huit appâts d'AN ont été utilisés. Les résultats montrent des protéines conservées au cours de l'évolution, spécialement dans les voies de l'immunité antivirale.

Sur la base de ces résultats encourageants, le criblage AP-MS a été étendu à d'autres appâts ainsi que des extraits protéiques de d'autres espèces (lignée cellulaire de poulet HD11, extraits de drosophiles adultes ainsi que *C. elegans*). Des extraits d'organismes entiers ont été ajoutés afin d'intégrer la complexité tissulaire, par opposition à la limitation d'une lignée cellulaire donnée. Les résultats ont été traités par le laboratoire de J. Colinge afin de classer les candidats en fonction du facteur d'enrichissement et de la spécificité de liaison. Tous les résultats ont également été analysés en termes de conservation évolutive. À la suite de cette analyse approfondie, une liste des 10% de candidats les plus significatifs a été produite pour chaque type d'extraits protéiques utilisés.

Dans le cas des extraits de drosophile, 221 protéines constituent 10% des protéines candidates les plus significatives. Afin de procéder à la validation fonctionnelle, j'ai sélectionné 100 candidats sur la base de : (i) l'identification dans les extraits de drosophiles adultes et cellules S2; (ii) de la conservation évolutive et, par la suite, (iii) de l'ontologie des gènes et de l'analyse des réseaux d'interaction protéiques. Parmi les 100 protéines sélectionnées, 95 ont des orthologues connus chez l'homme. Elles peuvent être regroupées en groupes de préférence pour se lier à certains appâts ou à un groupe d'appâts donnés.

II. Caractérisation fonctionnelle

1. Pipeline de validation *in vivo* pour l'analyse AP-MS

Nous avons développé un crible fonctionnel afin de valider les protéines sélectionnées. Ce crible est basé sur l'extinction de l'expression génique par ARN interference (ARNi) des gènes cibles, en utilisant des lignées transgéniques exprimant une construction répétée inversée sous le contrôle d'un promoteur sensible à la température. Cela me permet de réprimer l'expression du gène d'intérêt uniquement au stade adulte et d'éviter tout défaut au cours du développement. Les croisements sont réalisés à 18°C (température restrictive), les adultes éclosent après trois semaines de développement et sont transférés à

29°C afin d'induire l'inactivation d'un gène cible. L'effet de l'inactivation du gène cible est contrôlé en mesurant la survie des individus à 29°C sans traitement. Cinq lignées testées sur 100 montrent une mortalité des individus lors du passage à 29°C. Ainsi, 95 lignées ont été sélectionnées pour l'injection de virus pour la suite des expériences. Après cinq jours à 29°C, les lignées ont été infectées par cinq virus à ARN et le niveau d'ARN viral a été contrôlé en triplicat, à deux temps après l'infection.

À la suite du crible, 78 des 95 lignées présentent une modification significative de la charge virale dans au moins une condition (type de virus et temps post-infection). La plupart des hits concernent l'infection par les virus CrPV (Cricket paralysis virus), DCV (Drosophila C virus) et VSV (Vesicular stomatitis virus). Comme attendu, une corrélation est observée entre le CrPV et le DCV, qui appartiennent à la même famille de virus ; les Dicistrovirus. Parmi les candidats qui présentent une augmentation de la charge virale pour CrPV, DCV et VSV, la protéine kinase Tao (une Ser/Thr kinase de la famille Mst/Ste20) a été sélectionée pour une analyse plus poussée. Ce candidat présente un intérêt particulier, car ses orthologues chez l'homme (TAOK1, TAOK2 et TAOK3) ont également été identifiés comme participant au contrôle des infections virales dans le criblage fonctionnel utilisant une approche CRISPR/Cas9 dans des cellules humaines.

2. Identification de deux facteurs d'hôte nécessaires à la réplication d'un virus de type picorna

La protéine CG5641 a été identifiée à la suite du crible pilote utilisant les cellules S2 de drosophile avec l'AN polyI:C comme appât. Ce candidat a attiré notre attention, car il contient un domaine oligo adénylate synthase (OAS), caractéristique de certaines protéines impliquées dans l'immunité antivirale chez les mammifères [4].

La protéine codée par le gène CG5641 est un orthologue de la protéine NF45 de mammifère. Chez les mammifères, NF45 lie l'ARN en tant

qu'hétérodimère à une protéine partenaire, qui peut être NF90, SPNR ou Zfr. Cette association affecte la localisation des protéines ainsi que son affinité de liaison [5]. La plupart des études portent sur le complexe protéique NF45/NF90, impliqué dans le contrôle post-traductionnel de l'expression des gènes [6]. Il est à noter que plusieurs études sur des cellules de mammifère montrent que NF45/NF90 ou NF90 à lui seul régule de manière positive la réplication de divers virus tels que le virus de l'hépatite C (VHC), le virus de la dengue et le virus de l'immunodéficience humain (VIH-1) [7-9]. Récemment, il a été découvert que le complexe NF45/NF90 a une activité chaperone sur l'ARN. En effet, lors de sa liaison, le complexe NF45/NF90 peut spécifiquement faciliter une première étape de réplication de l'ARN du virus du VHC [10]. Globalement, ces études montrent la pertinence du complexe pour le processus de réplication du virus, bien que le mécanisme d'action soit encore a éludier.

Afin de comprendre l'implication de la protéine codée par le gène CG5641 dans l'immunité antivirale chez la drosophile, j'ai généré des lignées cellulaires KO CRISPR/Cas9 pour CG5641 (dNF45) et infectées par plusieurs virus appartenant à différentes familles. La réplication de la plupart des virus testés n'est affectée dans ces cellules. De façon inattendue, j'ai cependant observé une charge virale réduite lors de l'infection par le virus CrPV, qui fait partie de la famille des Dicistroviridae, des virus apparentés aux picornavirus. Il est intéressant de noter que l'orthologue Drosophila de ZFR, le partenaire de NF45 chez les mammifères, appelé Zn72D, a également été identifié lors du crible AP-MS utilisant le polyI:C comme appât. En outre, j'ai pu montrer que cette protéine est également nécessaire pour la réplication de CrPV.

Afin de valider l'implication de NF45 *in vivo*, nous avons utilisé une lignée mutante CG5641$^{[NP2255]}$, contenant l'insertion d'un élément P{GawB} 56pb en aval du codon d'initiation du gène invalidant ainsi le gène. J'ai confirmé la mutation par PCR inverse et j'ai montré que le niveau d'expression de CG5641 chez ces lignées est significativement réduit (10x) par rapport à une souche témoin w^{1118}. Ensuite, les mutants NF45 ont été infectés avec CrPV, DCV ou VSV. Alors que le DCV se réplique normalement chez les lignées mutantes, la charge

virale de CrPV et de VSV sont significativement réduite aux deux temps post-infection examinés. Ces résultats confirment nos découvertes dans les cellules, à savoir que CG5641 est requis pour la réplication de CrPV, et suggèrent que ce facteur pourrait aussi réguler le VSV.

Conclusion

À ce jour, nos connaissances sur la reconnaissance virale chez la drosophile se limitent à la voie de l'ARNi et à la protéine Dicer-2 en tant que principal récepteur d'ARN viraux. Dans cette étude, nous avons utilisé une approche de protéomique afin d'identifier de nouveaux facteurs susceptible d'être impliqués dans la détection des virus chez plusieurs espèces animales.

La première protéine candidate - CG5641 (orthologue de drosophile de NF45 de mammifère) - ainsi que sa protéine partenaire Zn72D s'est révélée être un régulateur positif de la réplication du CrPV. Ce résultat est inattendu pour deux raisons: d'une part, l'effet de la protéine est pro-viral, et d'autre part, sa spécifique de virus. Bien qu'il existe plusieurs hypothèses de travail, d'autres expériences sont nécessaires pour élucider le mécanisme d'action de ces protéines.

Un large crible MS a également conduisant à un certain nombre de conclusions. Premièrement, nous avons démontré que l'AP-MS utilisant des AN imitant des produits viraux peut être utilisée avec succès pour identifier les protéines impliquées dans la reconnaissance des ARN viraux. Deuxièmement, nous montrons qu'un grand nombre de protéines, conservées d'une espèce à l'autre, porte une signature distincte de la liaison à certains AN appâts. Ceci est important lorsque l'on étudie le développement de voies immunitaires au cours de l'évolution. Troisièmement, il y a aussi une place pour l'innovation spécifique à une espèce. Davantage d'études doivent être menées sur les protéines spécifiques à la drosophile identifiées dans le crible AP-MS qui a été réalisé.

Le crible fonctionnel a permis une analyse plus poussée d'un certain nombre de candidats. Il a mis en évidence un schéma de conservation, comme illustré par l'exemple des kinases Tao, dont l'importance a été révélée à la fois dans le crible *in vivo* chez la drosophile et le crible avec la lignée cellulaire humaine THP1. D'autres protéines dont le rôle antiviral semblent avoir été conservé au cours de l'évolution ont aussi été identifiées.

Globalement, les données acquises dans le cadre de cette étude donnent un premier aperçu du répertoire des récepteurs des acides nucléiques viraux chez la drosophile et pourraient donner lieu à de nouveaux projets. À terme, trouver de nouveaux mécanismes de reconnaissance nous aidera à comprendre la complexité de la réponse antivirale chez la drosophile.

Discussion

Le criblage AP-MS a permis d'identifier des protéines candidates se liant à des espèces d'ARNs caractéristiques de différents virus

Profils de reconnaissance de NA entre les espèces

Étant donné la diversité des virus qui infectent différents organismes, un certain nombre de nouveaux mécanismes de détection virale n'ont pas encore été découverts. L'éventail d'appâts NA et la gamme d'espèces utilisées dans le grand crible AP-MS ont produit beaucoup de données. Il existe de nombreuses façons d'organiser et d'analyser cette multitude de données. L'analyse initiale nous permet de tirer plusieurs conclusions. Premièrement, le nombre de protéines identifiées dans les échantillons d'organismes entiers (C. elegans et D. melanogaster) était plus élevé que dans toute cellule. Cela confirme l'idée que les lignées cellulaires représentent surtout une réponse tissulaire spécifique et qu'elles peuvent passer à côté de nombreuses voies d'exposition. Lorsque l'on compare les protéines significatives dans les cellules S2 et les mouches entières, cela est vrai pour la plupart des appâts, sauf dsISD, ssRNA-CAP et ssRNA-CAP0.

Une deuxième conclusion immédiatement apparente est que le poly(I:C) s'est avéré être l'appât le plus "collant". La différence dans le nombre de protéines significatives se liant préférentiellement au poly(I:C) est frappante lorsqu'on compare les cellules S2 et les mouches (40 contre 1410, avec seulement 21 se chevauchant). Bien que dans les 21 protéines communes, nous pouvons voir les hits importants connus tels que Dcr-2 et les candidats prometteurs tels que Tao, les hits spécifiques aux mouches et aux cellules devraient également être étudiés. Dans la carte thermique montrant les occurrences significatives, les poly(I:C) se sont regroupés avec dsISD, RNA-ISD et ssISD, mais pas avec les appâts dsRNA. Le poly(I:C) avait aussi des partenaires plus liants que le poly(A:U) synthétisé de façon similaire. Cela pourrait s'expliquer par le fait que le poly(I:C), bien que considéré comme une imitation de l'ARNdb, pourrait en fait avoir une structure de type web. Il a été suggéré que les structures ramifiées de l'ARN pourraient être spécifiquement immunogènes et détectées par MDA5 (Pichlmair et al. 2009). D'autres études devraient être menées pour préciser la réponse aux molécules immunogènes synthétiques, en particulier chez la drosophile.
Un autre groupe intéressant d'appâts comprend des ss- et des dsARNdb avec différentes structures CAP (CAP, CAP0, CAP1, CAP2). ssRNA-CAP et ssRNA-CAP0 ont été comparés à ssRNA-CAP1, qui représente le mRNA cellulaire normal lors de son transport vers le cytoplasme. Cependant, il a été suggéré que les systèmes manquant de réponse à l'interféron, comme la levure, manquent de méthylation 2'O sur ARN coiffé (Byszewska et al. 2014). Le CG6379 est une protéine qui ressort à maintes reprises de toutes les comparaisons analysées portant sur différentes structures de PAC. Il s'agit de l'orthologue drosophile du CMTR1 mammifère, qui est impliqué dans la méthylation 2'O de l'ARNm CAP0. De plus, le CMTR1 coopère avec l'hélicase DHX15 pour modifier les terminaisons 5'structurées de certains ARN (Toczydlowska-Socha et al. 2018). CG6379 était l'un des 100 candidats inclus dans le crible fonctionnel. Le KD du CG6379 n'a pas causé de défaut de viabilité, bien que l'efficacité du KD n'ait pas été vérifiée. Par conséquent, soit le KD du CG6379 n'était pas efficace, soit le gène n'est pas essentiel pour la mouche adulte. Lors de l'infection par DCV, les mouches avec le KD du CG6379 avaient une charge virale plus élevée deux jours après l'infection.

Perspective évolutive

L'un des thèmes récurrents dans l'analyse des résultats du crible AP-MS est la conservation inter-espèces. Il n'était pas surprenant de voir certains capteurs connus et conservés se fixer aux mêmes appâts de NA, comme Adar ou Dicer. Davantage d'analyses interespèces doivent être effectuées, comme l'illustrent certains nouveaux modèles de conservation intéressants qui ont été trouvés à la suite de l'analyse de l'AP-MS. Certains des candidats les plus importants se liant aux oligoadénylates 2'-5' dans tous les échantillons testés appartiennent à la famille des transporteurs ABC et jouent un rôle dans la traduction (Paytubi et al. 2009 ; Barthelme et al. 2011). Bien que l'ABCE1 ait été impliqué il y a quelque temps dans la prévention de la liaison de la RNase L à la 2'-5'oA (Bisbal et al. 1995), la fonction présumée des transporteurs ABC dans les infections virales n'a pas été étudiée. Nos résultats préliminaires dans la cellule S2 n'indiquent aucun phénotype lors d'une infection virale. Cependant, les trois CG1703 (ABCF1), Pixie (ABCE1) et CG9330 (ABCF3) étaient inclus dans le crible fonctionnel. Alors que le knockdown de Pixie, même au stade adulte, a causé une mortalité sévère et n'a pas pu être testé pour la résistance aux virus, les mouches avec le knockdown de CG1703 et CG9330 étaient infectées par les six virus. Lors de l'infection par le virus de l'hépatite C, le VSV et le SINV, les deux lignées présentaient une charge virale accrue. Fait intéressant, le phénotype des deux protéines apparentées était similaire, ce qui suggère qu'elles ont des fonctions similaires. Le CG3164, qui est un orthologue de l'ABCG et se lie de préférence en 2'-5'oA dans les cellules S2 et les mouches, pourrait être la prochaine cible à étudier dans le contexte de l'infection virale. Dans le cadre des études précédentes réalisées dans notre laboratoire, j'ai noté que parmi les gènes candidats qui ont été induits lors de l'infection par le FHV et le SINV, il y avait deux transporteurs ABC CG31793 et CG17646 (Kemp et al, 2013). Par conséquent, je crois que la contribution des transporteurs de l'ABC à l'infection virale et à la réponse mérite d'être explorée, en particulier de concert avec des études plus détaillées sur d'autres espèces.

D'autre part, on s'intéresse de plus en plus aux innovations spécifiques aux espèces dans le domaine de l'immunologie, un champs d’investigation émergent qu’on pourrait appeler "evo-immuno". De ce point de vue, le crible AP-MS multi-

espèces apporte beaucoup de nouvelles perspectives. J'ai seulement commencé à explorer les données sur des candidats spécifiques en regardant lequel des interactants significatifs n'a pas d'orthologue en humain. Plusieurs protéines ont été identifiées : la protéine UDE spécifique des arthropodes, qui se lie de préférence au dsISD ; les produits des gènes CG6912 et CG7920, se liant au ssRNA-CAP ; le produit du CG7330 spécifique des insectes qui se lie au 2'-5'oA et autres. Parmi les protéines non conservées chez l'homme, qui ont été testées dans le crible fonctionnel, il y a CG11858 (plus de VSV dans les cellules KD), ADF1 (plus de DCV et VSV), cup (plus de SINV), cup (plus de SINV), Tlk (plus de SINV), CG12112 (plus de VSV), pst (plus de CrPV et VSV), CG13364 (aucun phénotype). D'autres études sur ces protéines pourraient mettre au jour de nouvelles voies de reconnaissance et d'effecteur spécifiques de ce phylum.

Avantages et limites de l'approche AP-MS

L'approche AP-MS présente plusieurs avantages. Premièrement, elle est non biasée et repose uniquement sur la liaison des protéines aux appâts NA. Deuxièmement, la variété des échantillons de lysats a permis de dégager des perspectives évolutives. Toutefois, cette approche pourrait aussi présenter des limites. Premièrement, l'affinité de liaison ne reflète pas toujours la fonction : il y a des protéines qui peuvent reconnaître certains appâts, mais qui ne produisent aucun signal lors de la liaison ; et d'autre part, il y a aussi des protéines qui reconnaissent et se lient de façon transitoire, mais qui sont toujours capables de transmettre le signal pour déclencher la réponse. Ces derniers peuvent ne pas être identifiés dans l'interactome. Une deuxième confusion possible pourrait découler du fait que des cellules entières ont été utilisées pour les échantillons de lysats. Par conséquent, non seulement les capteurs cytoplasmiques, mais aussi les protéines nucléaires qui lient les NA ont été précipités. Cela pourrait "polluer" les résultats, mais d'un autre côté, cela peut donner plus d'informations sur les mécanismes nucléaires de détection. L'approche multispécifique pourrait également avoir certaines limites, car les échantillons d'appât par rapport aux échantillons témoins ont été conçus en fonction des voies de migration des mammifères, alors qu'il est possible que des caractéristiques propres aux phylactères existent. Par exemple, on a fait valoir que les animaux dépourvus de

réponse IFN ne produisent pas de transcriptions d'ARNm CAP1 (Byszewska et al. 2014) ; par conséquent, cette caractéristique ne peut être utilisée comme appât témoin. Enfin, les ligands synthétiques, utilisés pour le crible peuvent ne pas représenter des analogues viraux réels. Pour surmonter cette limitation, les études visant à rechercher les protéines qui se lient à des produits viraux spécifiques pourraient permettre de mieux comprendre les réponses spécifiques des virus. Par exemple, une étude récente réalisée dans le laboratoire d'A. Pichlmair a décrit le criblage protéomique et phosphoprotéomique, qui a identifié des protéines hôtes qui se lient aux protéines du virus Zika (Scaturro et al. 2018).

Le crible fonctionnel a permis d'identifier un certain nombre de candidats importants

La sélection des 100 candidats pour l'examen fonctionnel était principalement fondée sur la sélection bioinformatique impartiale des 10 % de protéines de liaison les plus importantes de l'examen AP-MS. Certains d'entre eux sont spécifiques à certains appâts, d'autres lient de nombreux appâts différents. Après avoir énoncé un certain nombre de limites techniques possibles et comparé aux mammifères, il est néanmoins clair que la drosophile demeure un modèle important et relativement simple pour étudier l'ampleur et la profondeur de la réponse antivirale de l'organisme.
Lors de l'infection par six virus, la majorité des candidats présentaient un changement significatif de la charge virale pour au moins un virus et un point de cinétique. Il est très rassurant de constater que le plus grand chevauchement dans les candidats est entre deux virus de la même famille : DCV et CrPV. Un autre virus avec de nombreuses protéines candidates présentant un phénotype antiviral est le VSV. Cela n'est pas surprenant non plus, car le VSV est un virus populaire pour étudier les interactions virus-hôte, peut-être parce qu'il manque de suppresseurs efficaces des défenses immunitaires innées, du moins chez les mouches.

Le KD de 8 gènes a provoqué un changement significatif de la charge virale lors de l'infection par DCV, CrPV et VSV (mxt, lost, blp, Tao, Fandango, CG3800, CG31156, CG11505). Certaines de ces protéines sont déjà apparues dans les études de notre laboratoire. Le CG3800 interagit avec les membres des voies AGO2 et R2D2 de l'ARNi (Majzoub et al, Université de Strasbourg, thèse, 2013). Il se lie également à RACK1, protéine ribosomale, responsable de la traduction des Dicistroviridae par IRES (Majzoub et al. 2014 ; L. Kuhn et al. 2017). Le CG3800 semble être un antiviral pour le CrPV et le DCV, et proviral dans le cas du VSV. Un autre succès, CG11505 (Larp4B), a été trouvé lié à Dicer-2 (Rousseau et Meignin, communication personnelle). Il a montré un phénotype antiviral pour les trois virus. Son orthologue chez l'homme, LARP1, ainsi que les orthologues de Fandango (XAB2 chez l'homme) et Tao sont des candidats prometteurs dans le dépistage de la perte fonctionnelle chez les mammifères (Pennemann et Pichlmair, communication personnelle).

Suggestions pour la conception expérimentale

Comme il a été mentionné dans la section Résultats, le crible fonctionnel a permis de mettre au jour certains des problèmes techniques associés aux cribles ARNi et à l'infection virale chez les mouches. Par conséquent, j'ai rencontré le problème de la variabilité des échantillons entre les différentes expériences. Bien que la sensibilité à la température des pilotes Gal4/Gal80 soit un outil utile, le changement de température pendant la durée de vie de la mouche modifie considérablement les taux d'infection. L'augmentation de la température stimule la réplication des virus et provoque en même temps un stress chez les mouches. Il est donc suggéré de reconsidérer les points dans le temps pour ce type d'expériences et de surveiller attentivement la mortalité.

Le KD des gènes n'a été induit qu'au stade adulte, afin de minimiser les défauts de développement, qui peuvent être attendus, car la majorité des candidats sont des protéines de liaison NA. Cependant, cela peut poser un problème si le KD est incomplet et laisse encore une quantité importante de la protéine. Cela pourrait être résolu par la vérification de l'efficacité du KD, qui n'a pas été effectué pour toutes les protéines de mon expérience en raison du nombre important d'échantillons.

Il a été démontré qu'environ 25 % des lignées de mouches KK RNAi ont des effets hors cible, et plus particulièrement l'amélioration de la voie Hippo par la production ectopique de la voie de transcription Tiptop (Vissers et al. 2016). Ceci est dû à l'emplacement de l'insert transgénique. Cela pourrait avoir une incidence sur les voies d'exposition connexes. Dans notre étude, cela vaut la peine d'être considéré, car l'une des principales protéines candidates est le Tao, qui est le membre de la voie Hippo. Ceci suggère que les résultats doivent d'abord être confirmés par l'exploitation d'une lignée d'ARNi indépendante et/ou de lignées de mouches mutantes.
Il existe un certain nombre de nouvelles techniques pour effectuer des criblages in vivo dans les mouches. Un large éventail de changements génétiques possibles est réalisable avec les nouvelles techniques d'édition de gènes à médiation CRISPR/Cas9. Ils peuvent être utilisés séparément ou en combinaison avec des approches ARNi. Une autre amélioration est qu'il existe des techniques permettant de détecter rapidement les mutations introduites par CRISPR/Cas9 (Kane et al. 2017). Cependant, il faut être prudent en utilisant de nouvelles approches de vérification du génome, car il n'est pas encore clair, quelles sont les conséquences moléculaires de la présence d'enzymes transgéniques de vérification (Mohr et al., 2014). D'autres améliorations pourraient aider à détecter le virus plus rapidement que la mesure des taux d'ARN. Par exemple, une étude récente a décrit un rapporteur transgénique qui, lorsqu'il est clivé par une protéase virale, pourrait être utilisé pour détecter une infection virale vivante (Ekström et Hultmark 2016).

Dans l'ensemble, l'examen fonctionnel a donné lieu à plusieurs protéines candidates importantes, dont la fonction n'a pas encore été élucidée. Une façon d'aborder immédiatement la question des mécanismes impliqués est d'utiliser l'ADNc des mouches infectées pendant le crible et de vérifier la lecture de l'induction de certaines voies immunitaires, telles que Imd, Toll, dSTING. Quant aux perspectives à long terme, les résultats pour certaines protéines candidates intéressantes doivent d'abord être confirmés par l'exploitation d'une lignée d'ARNi indépendante et/ou de lignées de mouches mutantes.

CG5641 et Zn72D : nouveaux facteurs de réplication du CrPV chez la drosophile

CG5641 et Zn72d sont des protéines conservées, orthologues des protéines NF45 et Zfr, respectivement, chez les mammifères. Le NF45 est connu pour former un dimère avec le NF90, le SPNR et le Zfr, toutes des protéines étroitement apparentées. La plupart des études chez les mammifères ont porté sur le complexe protéique NF45/NF90, qui participe au contrôle post-traductionnel de l'expression génétique (Jayachandran, Grey et Cook 2016). Il est à noter que plusieurs études sur des cellules de mammifères montrent que NF45/NF90 ou NF90 régulent positivement la réplication de divers virus tels que le VHC, le virus de la dengue et le VIH-1. Récemment, le complexe NF45/NF90 s'est avéré avoir une activité de chaperon d'ARN : en se liant, il peut spécifiquement faciliter une première étape de réplication de l'ARN du VHC (Schmidt et al. 2017). Dans l'ensemble, ces études établissent la pertinence du complexe pour le processus de réplication du virus, bien que le mécanisme d'action soit encore assez insaisissable.

Les résultats obtenus dans cette étude sur des cellules de drosophile S2 soutiennent également le modèle où CG5641 (dNF45) et Zn72D forment un complexe nécessaire à la réplication du CrPV. Les résultats du crible fonctionnel, au contraire, suggèrent que le CG5641 et le Zn72D sont tous deux des antiviraux contre le VSV et le SINV. D'après les études structurelles sur les orthologues mammifères et sur la liaison aux appâts NA, on peut supposer que les deux protéines lient l'ARNdb avec une grande affinité et le masquent de la reconnaissance par Dicer-2. Cette hypothèse est appuyée par le fait que la liaison du CG5641 et du Zn72D a été confirmée par immunoprécipitation (Worringer, Chu et Panning 2009). Cependant, mes résultats préliminaires d'immunomarquage montrent que le CG5641 et l'ARNdb marqués ne se colocalisent pas dans les cellules infectées. Comme il a été souligné dans l' récente étude de (Schmidt et al. 2017), le complexe NF45-NF90 peut modifier l'ARN par différents mécanismes, tels que le recuit de l'ARN et le déplacement des brins. Par conséquent, il est possible que l'action des facteurs hôtes soit spécifique du virus et dépende du substrat de l'AN, ce qui peut générer un effet différent sur la réplication virale. L'une des fonctions importantes des protéines

de liaison aux AN est de marquer les AN pour le trafic vers les endosomes où elles seront ensuite dégradées (Diebold et al. 2004). En résumé, la liaison aux AN virales peut avoir un effet à la fois proviral et antiviral et, dans le cas du complexe CG5641-Zn72D, selon les caractéristiques structurelles de l'AN virale. À la suite d'un dépistage protéomique, on a découvert que le CG5641 se lie à Dicer-2 dans les cellules infectées et non infectées, ce qui suggère que l'interaction ne dépend pas de la présence de l'ARNdb viral (Rousseau et Meignin, communication personnelle). Cela pourrait indiquer que le mécanisme d'action peut aussi dépendre de la voie de l'ARNi.

Curieusement, le phénotype des cellules diffère entre DCV et CrPV, deux membres de Dicistroviridae. L'une des principales différences entre le ces deux virus apparentés réside dans les mécanismes d'action de leurs VSR. Alors que le CrPV 1A agit en inhibant l'AGO2, le DCV 1A masque l'ARNdb pour empêcher sa reconnaissance par Dicer-2 (Fareh et al. 2018 ; Nayak et al. 2018). Si le complexe CG5641-Zn72D remplit une fonction similaire à celle du DCV1A, il sera intéressant de savoir si le DCV1A peut récupérer la réplication du CrPV dans les cellules KO CG5641.

Un certain nombre d'autres expériences peuvent être proposées pour étudier les hypothèses suggérées ci-dessus. Tout d'abord, les substrats NA viraux se liant aux deux protéines peuvent être identifiés à l'aide de l'ARN-immunoprécipitation lors de l'expression des protéines marquées. Plus précisément, l'affinité de la liaison de ces AN peut être évaluée au moyen d'un test de mobilité par déplacement, tel que décrit précédemment pour la protéine DCV 1A (van Rij et al. 2006). De plus, les protéines partenaires des deux protéines peuvent être étudiées dans des cellules infectées et non infectées. Ceci confirmera la liaison du CG5641 à Dicer-2, et permettra probablement de découvrir de nouvelles idées.

Tao kinase dans l'immunité antivirale de la drosophile

La Tao kinase, qui a été identifiée dans le crible AP-MS, se liant spécifiquement au poly(I:C) dans les mouches et les cellules S2, est une protéine conservée avec plusieurs fonctions décrites, principalement liées au contrôle de la taille et du développement des organes. Quant au crible protéomique, la liaison

préférentielle au poly(I:C) a également été confirmée pour les trois orthologues Tao dans les cellules de souris et humaines. Le fait qu'il se lie spécifiquement au poly(I:C) et non à d'autres appâts à ARNdb suggère que les caractéristiques structurées des AN sont probablement reconnues. Considérant de nombreuses fonctions du Tao, il n'est pas surprenant que son KD ait conduit à des défauts de survie, même lorsque le KD n'est induit qu'au stade adulte. Il est donc difficile d'évaluer la fonction antivirale du Tao, car il n'est pas clair si le phénotype est directement lié à une infection virale ou s'il peut être expliqué par la sensibilité des mouches à perte de fonction.

Par conséquent, une caractérisation plus détaillée de la fonction Tao devrait suivre dans les cellules S2, en utilisant les lignées cellulaires CRISPR/Cas9 KO que j'ai générées. Premièrement, ces cellules doivent être testées dans des expériences d'infection virale. Deuxièmement, la liaison au poly(I:C) doit être confirmée in vitro, en même temps que l'essai d'autres espèces de NA. Une autre question est celle des partenaires de liaison du Tao dans les cellules infectées et non infectées, qui pourraient être étudiées en utilisant la SEP. Une des perspectives importantes pour l'étude de la fonction des kinases de Tao dans l'immunité antivirale est d'analyser comment les signatures de phosphorylation changent quand Tao est absent ou ectopiquement exprimé. Cela peut se faire à l'aide de techniques émergentes de phosphoprotéome, comme la récente EasyPhos (Humphrey et al. 2018), qui a déjà été utilisée dans une étude sur l'immunité innée (Scaturro et al. 2018).

Comme pour les études in vivo, une déplétion tissulaire spécifique plus précise du Tao pourrait être utilisée pour diminuer les problèmes de survie. De plus, le phénotype peut être étudié en fonction du tropisme tissulaire du virus. L'expression ectopique à l'aide de mouches transgéniques peut également être utile pour élucider la fonction du Tao dans l'immunité antivirale.

Conclusion

Il semble injuste que les études sur les voies de la reconnaissance immunitaire innée, bien qu'initiée chez les mouches, se soient autant concentrées sur le système mammifère au cours des dernières décennies. Pourtant, la mouche a beaucoup à offrir à un immunologiste, non seulement comme modèle alternatif pour les maladies humaines, mais aussi comme modèle d'interactions hôte-pathogène inédites.

Dans l'ensemble, les données acquises dans cette étude donnent un premier aperçu des voies de reconnaissance possibles chez les mouches dont la caractérisation pourrait donner lieu à de nouveaux projets. Éventuellement, trouver de nouveaux mécanismes de reconnaissance nous aidera à comprendre la complexité de la réponse antivirale de la drosophile.

Assel MUSSABEKOVA

Évaluation de l'activité antivirale des protéines de liaison aux acides nucléiques : une analyse multi-espèces

Résumé en français suivi des mots-clés en français

Le projet a permis d'identifier des répertoires de protéines interagissant avec différentes espèces d'acides nucléiques caractéristiques des virus chez cinq espèces animales (Homme, souris, poulet, drosophile, nématode). Ces protéines représentent des candidats pour remplir des fonctions de récepteurs de l'immunité innée ou de molécules antivirales. Certaines d'entre elles ont été conservées au cours de l'évolution, ce qui m'a permis de tester leur fonction dans la drosophile. J'ai réalisé un crible impliquant des infections avec cinq virus différents sur 100 protéines conservées. Ce crible m'a permis d'identifier huit protéines dont l'inhibition impacte la réplication virale. Deux d'entres elles, CG5641 et Zn72D, sont nécessaires pour la réplication des virus de type picornavirus (CrPV). Le candidat le plus intéressant identifié est cependant la protéine Tao, dont l'inhibition entraîne une augmentation de la réplication de virus appartenant à plusieurs familles, chez la drosophile et dans les cellules de mammifères.

Mots clés : virus, acide nucléique, Drosophile, CrPV, Tao kinase

Résumé en anglais suivi des mots-clés en anglais

Antiviral response largely relies on the recognition of viral nucleic acids. The aim of the project was to characterize the range of nucleic acid binding proteins in the context of viral infection in flies. We identified a wide repertoire of proteins, which recognize viral nucleic acids in five species (human, mouse, chicken, fruit fly and roundworm). Among these proteins, there are ones, which are conserved in insects and humans, and therefore their function can be easily studied in the fruit fly model. Afterwards, we have performed a large screen in flies to study more precisely the function of 100 proteins in infection with 5 different viruses. We have found eight promising candidates as a result of this screen. We identified two Drosophila proteins CG5641 and Zn72D, which are also present in humans, as proviral factors. We also identified a protein Tao, which is conserved in humans, and is antiviral against several types of viruses.

Keywords : virus, nucleic acid, Drosophila, immune recognition, CrPV, Tao kinase