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UPR-9022 Réponse immunitaire et développement chez les insectes

THÈSE

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Vincent BARBIER

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Pastrel, a restriction factor for picorna-like viruses in *Drosophila melanogaster*

THÈSE dirigée par :
M. IMLER Jean-Luc Professeur des universités, université de Strasbourg

RAPPORTEURS :
Mme. SALEH Maria-Carla Docteur, Institut Pasteur, Paris
M. KOHL Alain Docteur, université de Glasgow

AUTRES MEMBRES DU JURY :
Mme. SCHUSTER Catherine Directeur de recherches, université de Strasbourg
M. JIGGINS Francis Professeur des universités, université de Cambridge

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(-) ssRNA	negative single-stranded RNA
(+) ssRNA	positive single-stranded RNA
A	Adenine
AA	Amino acid
Ago	Argonaute
AGS	Aicardi-Goutières Syndrome
AIM2	Absent in melanoma 2
AMPs	Antimicrobial peptides
ANV	American Nodavirus
AP-1	Activator protein-1
APOBEC3	Apolipoprotein B mRNA-editing catalytic polypeptide 3
Ars2	Arsenic resistance protein 2
ATPase	Adenosine triphosphatase
BAP	Biotin Acceptor Peptide
BCA2	Breast cancer-associated gene 2
BHK	Baby hamster kidney
BMP2	Bone morphogenetic protein 2
bp	Base pair
C	Cytosine
CARD	Caspase activation and recruitment domain
CARDIF	CARD adapter inducing interferon-β
Cct	Cytidylyltransferase
CD	Cluster of differentiation
cDNA	complementary DNA
cGAMP	cyclic-di-GMP-AMP
cGAS	cGAMP synthetase
CHIKV	Chikungunya virus
CIV	Chilo Iridescent virus
COPI	Coat protein I
CrPV	Cricket Paralysis virus
cxVago	*Culex* Vago
CypA	Cyclophilin A
DAI	DNA-dependent activator of IFN-regulatory factors
DaPKC	*Drosophila* atypical protein kinase C
DAP-PGN	Diaminopimelic acid peptidoglycan
DAV	Drosophila A virus
DBV	Drosophila Birnavirus

Dcr-2	Dicer-2
DCV	Drosophila C virus
DDX	DExD/H-box helicases
DENV	Dengue virus
DFV	Drosophila F virus
dPIAS	*Drosophila* protein inhibitor of activated STAT
dGTP	deoxyguanosine triphosphate
DIF	Dorsal-related immunity factor
dMyd88	drosophila myeloid differentiation factor 88
dN	deoxynucleoside
DNA	Desoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DPV	Drosophila P virus
dsRNA	double stranded RNA
dTak1	drosophila TGF-beta activated kinase 1
DTrV	Drosophila Tetravirus
DTV	Drosophila Totivirus
DUF283	Domain of unknown function 283
DVRF	Dengue virus restriction factors
DXV	Drosophila X virus
E.Coli	Escherichia Coli
EBOV	Ebola virus
EFP	Estrogen-responsive finger protein
EIAV	Equine Infectious Anemia *Virus*
eIF	eukariotic Initiation Factor
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
ERV-L	Endogenous *retrovirus*-like elements
ESCRT	Endosomal sorting complex required for transport
FACS	Fluorescence-Activated Cell Sorting
FADD	Fas-associated-death domain
FHV	Flock House Virus
FIV	Feline immunodeficiency *virus*
FPPS	Farnesyl Diphosphate Synthase
Fv1	Friend-virus susceptibility gene 1
G	Guanine
Gag	Group-specic antigen
GNBP1	Gram-negative binding proteins
GPI	Glycosylphosphatidylinositol
GTPases	Guanosine triphosphatase
HA	Hemagglutinin

HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C Virus
HD	Histidine–aspartic
Herc5	HECT domain and RCC1-like domain containing protein 5
HFV	Human Foamy Virus
HIV-1	Human immunodeficiency virus-1
Hop	Hopscotch
HPV	Human papillomavirus
HRP	HorseRadish Peroxidase
HSP	Heat shock protein
HSV-1	Herpes Simplex Virus type 1
HTLV-1	*Human* T-cell leukemia virus type I
IAV	Influenza A virus
IFI16	gamma-interferon-inductible protein 16
IFITMs	Interferon-inducible transmembrane proteins
IFITs	IFN-induced protein with tetratricopeptide repeats
IFN	Interferon
IGR	Intergenic region
IIV-6	Invertebrate Iridescent Virus 6
IKK	IκB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IMD	Immune deficiency
IPS-1	IFN-β promoter stimulator-1
IRES	Internal Ribosome Entry Site
IRF-9	IFN-regulatory factor 9
ISGs	Interferon-stimulated genes
ISGF-3	IFN-stimulated gene factor-3
IκB	Inhibitor of κB
Jak	Janus kinase
Jak-STAT	Janus kinase -Signal Transducer and Activator of Transcription
JEV	Japanese encephalitis virus
JNK	c-Jun N-terminal kinase
kDa	kilodalton
KSHV	Kaposi's sarcoma-associated herpesvirus
LACV	La Crosse virus
LCMV	Lymphocytic choriomeningitis virus
LGP2	Laboratory of genetics and physiology-2
LGTV	Langat virus
LINE-1	Long interspersed nuclear element-1

Loqs-PD	Loquacious isoform PD
LPS	Lipopolysaccharide
Lv1	Lentivirus susceptibility factor 1
MAPKKK	Mitogen-activated protein kinase kinase kinase
MARV	Marburg virus
MAVS	Mitochondrial antiviral-signaling protein
MDA5	Melanoma differentiation-associated gene 5
mESCs	mouse embryonic stem cells
MHV	Murine GammaHerpes Virus
MHV	Mouse hepatitis *virus*
miRNA	microRNA
MLV	Murine leukemia virus
MOI	Multiplicity of infection
mRNA	Messengers RNA
MV	Measle Virus
Mx	Myxovirus resistance
NBs	Nuclear bodies
Nef	Negative Regulatory Factor
NF-κB	Nuclear factor–κB
NLRs	NOD-like receptors
NOD2	Nucleotide binding oligomerization domain 2
NoV	Nodamura virus
NRAMP	Natural Resistance-Associated Macrophage Protein
NS1	Non-structural
Nts	Nucleotides
OAS	2'-5'-Oligoadenylate synthetase
OASL	OAS-like gene
ONNV	O'nyong-nyong virus
ORF	Open reading frame
OSS	Ovary Somatic Sheet
PAMPs	Pathogen associated molecular patterns (PAMPs)
PAZ	Piwi/Argonaute/Zwille
pDCs	plasmacytoid dendritic cells
PDE	Phosphodiesterase
PFU	Particles Forming Unit
PGRPs	Peptidoglycan receptors proteins
PI3K-Akt-TOR	Phosphatidylinositol 3-kinase-Akt-Target of rapamycine
piRNA	Piwi-associated interfering RNA
PKR	Protein kinase R
PML	Promyelocytic leukaemia
PRR	Pattern-Recognition Receptors

RdRp	RNA-dependent RNA polymerase
Ref(2)P	Refractory for Sigma P virus
REF1	Resistance factor 1
RIG-I	Retinoid acid-inducible gene
RING	Really Interesting New Gene
RIP	Receptor interacting protein
RISC	RNA-induced silencing complex
RLC	RISC loading complex
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RNPs	Ribonucleoproteins
RpS6	Ribosomal protein S6
RRE	Rev response element
rRNAs	Ribosomal RNAs
RRV	Ross River Virus
Rsad2	Radical S-adenosyl methionine domain-containing protein 2
RVFV	Rift Valley fever virus
S2	Schneider 2
SAM	Sterile alpha motif
SAM	S-adenosylmethionine
SAMHD1	SAM domain HD domain-containing protein 1
SARS-CoV	Severe Acute Respiratory Syndrome-Coronavirus
sfRNA	subgenomic flavivirus RNA
SFV	Semliki Forest virus
SIGMAV	Sigma virus
SINV	Sindbis Virus
SIV	Simian immunodeficiency virus
SMUG1	Single-Strand-Selective Monofunctional Uracil-DNA Glycosylase 1
SNP	Single Nucleotide Polymorphism
SREBP	Sterol Regulatory Element Binding Protein
Staf-50	Stimulated Trans-Acting Factor of 50 kDa
STAT1	Signal Transducer and Activator of Transcription 1
STING	Stimulator of IFN genes
SUMO-1	Small Ubiquitin-like MOdifier
SOCS	Suppressor of cytokine signaling
TAP1	Transporter associated with antigen processing 1 gene
Tas	Transactivator
TBEV	Tick-borne encephalitis virus
TBK-1	TANK-binding kinase 1

TEP	Thiol-ester protein
TIR	Toll-IL-1 receptor
TLRs	Toll-like receptors
TMs	Transmembranes
TNFR	Tumour-necrosis factor-receptor
TotM	Turandot M
TPRs	Tetratrico peptide repeats
TRIM5α	Tripartite motif protein isoform 5 alpha
TRIMCyp	TRIM5α-Cyclophilin A
U	Uracil
UbcH8	Ubiquitin-conjugating Enzyme H8
UBE1L	Ubiquitin Activating Enzyme E1 Like Protein
UBP43	Ubiquitin protease 43
UNG	Uracil-N glycosylase
Upd	Unpaired
upd	unpaired
VA1	Viral associated
VAP-A or hVAP-33	Vesicle-associated membrane protein-associated protein subtype A
VEEV	Venezuelan Equine Encephalitis Virus
Vif	Virion infectivity factor
Viperin	Virus-inhibitory protein, endoplasmic reticulum–associated, IFN-inducible
Vir-1	Virus-induced RNA-1
VISA	Virus-induced signaling adapter
VLPs	Virus-like particles
VPg	Viral Protein genome-linked
v-piRNAs	Virus-derived piRNAs
Vpu	Viral protein U
vRNPs	Viral ribonucleoproteins
v-siRNA	virus-derived small interfering RNAs
VSR	Viral suppressors of RNAi
VSV	Vesicular Stomatitis Virus
VSV-G	*G* glycoprotein of the vesicular stomatitis virus
WNV	West Nile virus
YFV	Yellow Fever Virus
ZAP	Zinc-finger antiviral protein

Résumé de thèse en français

Introduction

Les maladies infectieuses d'origine virale sont responsables d'une mortalité importante chez toutes les espèces. La drosophile est un excellent modèle pour l'étude des mécanismes moléculaires de l'immunité innée, y compris les virus. Elle a permis la caractérisation de mécanismes de défense immunitaire conservés au cours de l'évolution, tel que les voies Toll et IMD qui régulent l'expression des peptides antimicrobiens induits en réponse aux infections fongiques et bactériennes. Un certain nombre de maladies virales ou parasitaires infectant l'homme ou le bétail sont en outre transmises par des insectes hématophages, ce qui représente une motivation supplémentaire pour étudier les mécanismes de l'immunité innée chez les insectes. L'objectif de ma thèse est de comprendre les bases de l'immunité antivirale chez la drosophile.

Problématique

Deux types de réponse sont impliqués dans le contrôle des infections virales chez la drosophile. Une réponse inductible et l'ARN interférence qui est un mécanisme global de défense contre les virus à ARN, dont le virus C de la Drosophile (DCV). Le virus DCV est un virus modèle de la famille des *Dicistroviridae*, apparenté aux *Picornaviridae*. Il est couramment utilisé pour étudier les réponses immunitaires chez la drosophile en particulier dans notre laboratoire. Nous avons mis en évidence une différence de sensibilité (charge virale et mortalité accrues) à l'infection par ce virus entre différentes lignées utilisées comme témoins de fond génétique. Une différence de susceptibilité au DCV a également été observée entre deux stocks d'une même lignée sauvage (Oregon-R) maintenus dans deux équipes de notre unité. Plusieurs lignées sensibles au DCV étaient infectées de façon persistante par un virus apparenté aux *Picornaviridae*, le virus Nora, suggérant qu'il était la cause de la susceptibilité à DCV. L'ensemble de ces observations m'ont incité à m'intéresser à trois aspects au cours de ma thèse :

(1) Quelle est la cause de la sensibilité de certaines lignées de Drosophile à l'infection par le virus DCV ?
(2) La présence du virus Nora persistant chez la Drosophile influence-t-elle la susceptibilité des mouches à d'autres infections virales ou bactériennes ?
(3) Quelle est la contribution de l'ARN interférence dans la défense contre un virus à ADN ?

Résultats (1)

Les mouches contrôles présentent une importante variabilité dans la résistance à l'infection par le virus DCV.

De façon surprenante, nous avons observé une grande variabilité dans la susceptibilité à l'infection par le virus DCV entre les mouches contrôles utilisées au laboratoire. Par exemple, les mouches *yw* et w^{A5001} présentent une charge virale et une mortalité accrue à l'infection par le virus DCV comparé aux mouches DD1 *cnbw* et Canton-S. Cette différence de sensibilité ne concerne pas tous les virus puisque les mouches *yw*, w^{A5001}, DD1 *cnbw* et Canton-S présentent la même sensibilité au virus FHV. Nous avons également observé une différence de sensibilité spécifique aux virus DCV et CrPV entre deux stocks d'une même lignée de drosophile (Ore-R^{DF} et Ore-R^{JLI}).

La susceptibilité à l'infection par le virus DCV est dépendante du fond génétique.

J'ai observé que lorsque l'on croise un mâle d'une lignée de drosophile sensible à l'infection par le virus DCV avec une femelle d'une lignée de drosophile résistante, la progéniture est résistante à l'infection par le virus DCV. Cette résistance à l'infection par le virus DCV est donc transmise génétiquement de façon dominante à la descendance. De plus, le croisement réciproque d'une femelle sensible avec un mâle résistant à DCV n'affecte pas la résistance de la progéniture au virus DCV. Ceci indique que la résistance au virus DCV n'est pas portée par le chromosome X. Il était ensuite nécessaire de déterminer quel(s) gène(s) du chromosome 2, 3 ou 4 peut être responsable de cette différence de susceptibilité à l'infection par le virus DCV.

La susceptibilité à l'infection par le virus DCV est corrélée au polymorphisme dans le gène *pst*.

Parallèlement à mes observations, le laboratoire du professeur Francis Jiggins (Cambridge), a associé une région génomique du chromosome 3, comprenant le gène *pastrel (pst)*, avec des phénotypes de sensibilité associés à l'infection par le virus DCV. J'ai donc séquencé le gène *pst* des différentes lignées contrôles du laboratoire et j'ai trouvé trois polymorphismes nucléotidiques simples (SNPs) dont un est présent dans l'exon 6 et induit un changement d'acide aminé. Ce SNP corrèle systématiquement avec la sensibilité ou la résistance au virus DCV. Les deux autres SNPs, présents dans des introns, corrèlent avec la susceptibilité à l'infection par le virus DCV des lignées contrôles à l'exception de la lignée Ore-R^{JLI}. Il était ensuite nécessaire de valider ce gène candidat.

L'expression du gène *pst* limite l'infection par les virus picorna-like DCV et CrPV *in vivo* et *in vitro*.

L'atténuation de l'expression du gène *pst* par ARN interférence *in vivo* accroit la charge virale et la mortalité des mouches infectées par le virus DCV comparé aux mouches contrôles. J'ai effectué les mêmes observations avec le virus de la paralysie du criquet (CrPV), qui comme le virus DCV appartient à la famille des *Dicistroviridae*. De façon consistante, l'atténuation de l'expression du gène *pst in vitro* augmente la charge virale dans les cellules S2 infectées par les virus DCV et CrPV. Afin de tester si une surexpression du gène *pst* limite l'infection par le virus DCV, j'ai établi des lignées stables qui surexpriment sous contrôle du promoteur actine la forme sensible ou résistante de la protéine Pst, couplée en N ou C-terminal avec le fluorochrome RFP. La surexepression de la forme sensible ou résistante de la protéine Pst réduit considérablement la charge virale après infection par les virus DCV et CrPV par rapport à la lignée cellulaire contrôle. De façon consistante, dans le cas d'une infection par les virus DCV et CrPV, mais pas FHV et VSV, le nombre de cellules positives pour le virus est réduit dans les lignées stables surexprimant la forme sensible ou résistante de la protéine Pst par rapport à la lignée contrôle. Cette restriction virale apparait dans les premières heures de l'infection par le virus DCV *in vitro*, sans affecter la fixation du virus sur les cellules. La traduction IRES-dépendante, nécessaire à la synthèse polyprotéique des virus DCV et CrPV, n'est pas affectée par la surexpression de la protéine Pst. Des expériences sont en cours pour tester si la protéine Pst affecte l'entrée ou la réplication du virus.

La région C-terminale de la protéine Pst porte l'activité antivirale.

Afin de déterminer si la région portant le polymorphisme identifié *in vivo* (associé à la sensibilité ou résistance à l'infection par le virus DCV) confère l'activité antivirale à la protéine Pst, j'ai établi des lignées stables surexprimant une forme tronquée de la protéine Pst, depuis le dernier domaine transmembranaire prédit et précédent le polymorphisme. La surexpression de cette forme tronquée de la protéine Pst n'affecte pas l'infection par le virus DCV, indiquant que la région C-terminale de la protéine est nécessaire pour son activité antivirale. L'effet d'autres délétions est en cours d'analyse. De plus, la délétion de la région C-terminale modifie la localisation de la protéine de fusion.

La protéine Pst colocalise avec les gouttelettes lipidiques révélées par le Rouge de Nil, ainsi qu'avec les protéines de capside des virus DCV et CrPV au cours de l'infection.

L'immunomarquage des cellules révèle que la protéine Pst est localisée dans des structures ponctuelles, concentrées dans une zone juxtanucléaire. Le marquage des gouttelettes lipidiques par le Rouge de Nil colocalise avec le marquage de la protéine Pst endogène dans les cellules S2 *in vitro*. Au cours de l'infection, la protéine Pst colocalise avec les protéines de capside des virus DCV et CrPV *in vitro*, mais aussi *in vivo* dans les cellules du corps gras, un tissu analogue au foie chez les mammifères. Cette colocalisation est cohérente avec une activité antivirale de la protéine Pst sur ces virus.

L'extrémité N et C-terminale de la protéine Pst est exposée du côté cytosolique.

Puisque la protéine Pst et les protéines de capside colocalisent dans le cytoplasme, il est probable que la protéine Pst et les particules virales se rencontrent dans le cytosol, permettant à la protéine Pst d'exercer son activité antivirale portée par la région C-terminale. Pour répondre à cette hypothèse, j'ai mis au point une nouvelle méthode basée sur la spécificité d'interaction entre les protéines Biotine et Streptavidine pour déterminer la topologie des régions N et C-terminale de la protéine Pst. J'ai ajouté des sites de biotinylation en N ou C-terminal de la protéine Pst et exprimé ces protéines de fusion dans des cellules exprimant l'enzyme BirA. Si le site de biotinylation est exposé et accessible dans le cytosol, ce site est biotinylé par l'enzyme BirA. La biotinylation est ensuite révélée par western blot

avec la protéine Streptavidine-HRP. La validité de cette technique a été confirmée en utilisant la protéine Toll, dont la topologie est connue, comme contrôle. J'ai observé que les extrémités N et C-terminal de la protéine Pst sont exposées dans le cytosol.

Résultats (2)

L'infection persistante par le virus Nora n'a pas d'effet sur la susceptibilité des mouches à l'infection par le virus DCV, mais affecte la susceptibilité aux infections bactériennes.

La présence du virus persistant Nora a été détectée dans certaines lignées de laboratoire. Le virus Nora appartient à une nouvelle famille des virus de type picorna. J'ai ainsi vérifié si la persistance de ce virus pouvait contribuer à la susceptibilité au virus DCV. En effet, ce virus est détecté par PCR dans toutes les lignées sensibles à DCV alors que les lignées résistantes à DCV ne sont pas infectées par le virus Nora (à l'exception toutefois de la lignée DD1 *cnbw*). La déchorionnation des œufs de la lignée Ore-R^{DF} infectée permet l'élimination du virus Nora, qui n'est pas transmis par la lignée germinale. Cependant, cette lignée présente toujours une sensibilité à l'infection par le virus DCV identique à celle de la lignée Ore-R^{DF} non traitée. Par ailleurs, la contamination de la lignée Ore-R^{JLI}, non infectée par le virus Nora, avec les excréments des mouches de la lignée Ore-R^{DF} infectée, n'induit pas de sensibilité accrue à l'infection par le virus DCV. L'ensemble de ces résultats m'ont permis de conclure que la présence de ce virus persistant n'était pas responsable de la susceptibilité de certaines lignées de drosophiles à l'infection par le virus DCV. Cependant, en collaboration avec l'équipe de Dominique Ferrandon, nous avons observé que la lignée Ore-R^{DF} débarrassée du virus Nora est moins sensible à l'infection par *P. aeruginosa* et *S. marcescens.* Par opposition, la lignée Ore-R^{JLI} contaminée par ce virus devient plus sensible à ces deux infections bactériennes. Le virus Nora affecte la susceptibilité des mouches aux infections bactériennes. De plus, les mouches infectées subissent un renouvèlement important de l'épithélium intestinal.

Résultats (3)

L'ARN interférence est une voie générale de défense antivirale.

Nous avons montré que la voie de l'ARN interférence est une voie générale de défense antivirale, puisqu'elle permet, en plus des virus à ARN, de contrôler l'infection par un virus à ADN. Les mouches mutantes pour le gène *dicer-2* (*dcr-2*^{R416X}) infectées par le virus à ADN IIV-6 présentent une mortalité et une charge virale accrue par rapport aux mouches contrôles *yw*. J'ai également observé une mortalité plus importante chez les mouches mutantes pour un second allèle nul de *dicer-2* (*dcr-2*^{L811fsx}), le phénotype de sensibilité au virus IIV-6 étant restauré chez ces mutants après insertion d'un transgène correspondant à la région génomique de *dicer-2* sauvage. Les mouches mutantes pour les gènes *R2D2* et *AGO2*, deux composants majeurs de cette voie, présentent également une sensibilité accrue au virus IIV-6. J'ai construit une banque de petits ARNs à partir de mouches et de cellules infectées par le virus IIV-6 et identifié la présence de petits ARN, majoritairement de 21 nucléotides, s'alignant avec la séquence du génome viral IIV-6. Ainsi, Dicer-2 produit des ARN interférents contre le génome viral IIV-6. De façon surprenante, à la différence des virus à ARN, ces petits ARN interférents sont produits à partir de régions spécifiques du génome viral puisque leur distribution n'est pas uniforme le long du génome viral. Ces régions correspondent à des régions ou la transcription s'effectue sur les deux brins, conduisant potentiellement à la formation d'ARNdb, substrats de Dicer-2.

Conclusion

J'ai mis en évidence que le gène *pst* est impliqué dans la susceptibilité à l'infection par les virus de type picorna DCV et CrPV. Les expériences de perte-de-fonction et gain-de-fonction indiquent que la protéine Pst est un facteur de restriction antiviral. Son mécanisme d'action reste à éclaircir. Mon étude de l'effet de la présence du virus Nora sur la susceptibilité des mouches aux infections a permis de révéler que cette infection persistante n'affecte pas la sensibilité des mouches à DCV. Cependant, la présence du virus Nora facilite les infections bactériennes et perturbe le renouvellement des cellules épithéliales de l'intestin. Enfin, j'ai également démontré que l'ARN interférence, en plus des virus à ARN, permet de contrôler l'infection par un virus à ADN. Ces travaux ont été publiés dans le *Journal of Immunology*.

Part I

INTRODUCTION

Every species have to face multiple pathogens during their life. In particular, viral infectious diseases are responsible for high lethality in all species. The fruit fly *Drosophila melanogaster* is an excellent model to study the molecular mechanisms of innate immunity in insects (Schneider, 2000). This model allowed the characterization of the evolutionarily conserved Toll and IMD pathways that regulate the expression of antimicrobial peptides in response to fungal and bacterial infections (Hoffmann, 2003). This discovery had a major impact on the understanding of innate immunity in mammals. The necessity to study insect innate immunity is also reinforced by the emergence of human viral diseases transmitted by hematophagous insects, including ticks and mosquitoes (Weaver and Reisen, 2010).

The first part of this introduction will review the natural and non-natural *Drosophila* viruses used in this study. The second part will address the different innate immune pathways mounted in response to pathogens, with a particular focus on *Drosophila* antiviral responses. After a brief comparison with the innate antiviral response orchestrated in mammals, the innate intrinsic immunity will be addressed in mammals and *Drosophila*, with a description of characterized restriction factors.

Chapter 1

Models of *Drosophila* viral infections

Viruses commonly used in research laboratories to study the mechanisms of antiviral immunity in *Drosophila* can be divided in two classes: first, the natural pathogens of *Drosophila*, which include *Drosophila* C virus (*Dicistroviridae*), Sigma virus (*Rhabdoviridae*) and Nora Virus (*unclassified*); secondly, the non-*Drosophila* viruses that were isolated from other hosts but were able to infect *Drosophila* flies and cells in experimental conditions. They include Cricket Paralysis virus (*Dicistroviridae*), Flock House virus (*Nodaviridae*), Invertebrate Iridescent virus 6 (*Iridoviridae*), Sindbis virus (*Togaviridae*) and Vesicular Stomatitis virus (*Rhabdoviridae*). Both classes cover a wide range of virus families with genomes of different nature and polarity, thus enhancing the robustness of the *Drosophila* model to study innate antiviral responses.

1.1. Drosophila viruses: natural pathogens of *Drosophila*

***Drosophila* C virus (DCV)** was firstly identified in 1972 from the *Charolles* strain of *Drosophila melanogaster*. This laboratory stock exhibited an unusual high mortality rate (Jousset et al., 1972). DCV is widely spread in *Drosophila*, infecting about one third of natural and laboratory populations of *Drosophila* (Plus et al., 1975a). Viral particles are non-enveloped, icosahedric, with 30 nm of diameter and share physical and chemical characteristics with the *Picornaviridae* family (Jousset et al., 1977). However, the full sequencing of DCV genome in 1998 revealed clear differences with several *Picornaviridae*, imposing the creation of the new *Dicistroviridae* family to classify this *Drosophila* picorna-like virus (Johnson and Christian, 1998). DCV genome is a positive single stranded ribonucleic acid ((+) ssRNA) of 9,264 nucleotides (nts) length, polyadenylated at the 3' end. The small viral protein genome-linked (VPg) is attached at the 5' extremity of the genome (King and Moore, 1988). The genome consists of two open reading frames (ORF): the 5' ORF encodes non-structural proteins (a viral suppressor of RNA interference (RNAi) DCV-1A, a helicase, a protease, the VPg protein and the viral RNA-dependent RNA polymerase (RdRp)) and the 3' ORF encodes structural proteins (capsid proteins VP1-4). Each ORF is preceded by an internal ribosome entry site (IRES) that initiates translation of non-structural (5' IRES) or structural (Intergenic region (IGR) IRES) polyprotein precursors. Final proteins are obtained after cleavage of the polyproteins by the viral protease. This contrasts with the viral genome from *Picornaviridae* and *Iflaviridae* family which consists of a single ORF with capsid proteins encoded at the 5´ region of the genome and non-structural proteins (helicase,

protease and RdRp) encoded at the 3´ region (**Figure 1**). A single IRES is present at the 5' extremity of the genome and initiates translation.

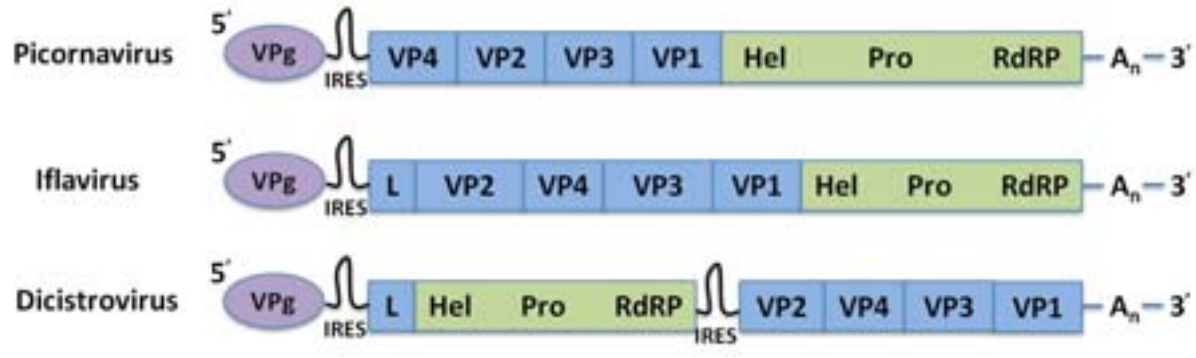


Figure 1. The genomic organisation of dicistroviriruses shares similarities with picornaviruses and iflaviruses. (Leader (L), Helicase (Hel), Protease (Pro), polyA (An)).

DCV is horizontally transmitted, and this transmission is facilitated by females which may act as viral reservoir in nature (Gomariz-Zilber et al., 1995, 1998). The outcome of the infection depends on the infection route. Injection of DCV is highly pathogenic, leading to the death of flies in few days. By contrast, flies infected by oral contamination with DCV appear healthy. However, depending on the dose, the ingestion of DCV can be fatal within days (Gomariz-Zilber et al., 1995; Jousset and Plus, 1975). Surprisingly, some studies reported that DCV induces some beneficial effects on infected flies: DCV decreases the developmental time, increases the mean number of ovarian tubes and the weight of adult females (Gomariz-Zilber and Thomas-Orillard, 1993; Thomas-Orillard, 1984). It also increases the fertility (Thomas-Orillard, 1988) and the daily egg-production (Thomas-Orillard, 1990).

After its injection in adult flies, DCV replicates and spreads to a large number of tissues, including the fat body (Cherry and Perrimon, 2003; Dostert et al., 2003, 2005; Lautié-Harivel and Thomas-Orillard, 1990; Sabatier et al., 2003), the follicular cells (Lautié-Harivel and Thomas-Orillard, 1990), the thoracic muscle fibers, the tracheal cells, the digestive tract (Dostert et al., 2003; Lautié-Harivel and Thomas-Orillard, 1990), the cells of the periovarian sheath (Cherry and Perrimon, 2003; Dostert et al., 2003, 2005; Sabatier et al., 2003), the oenocytes and the blood cells (Dostert et al., 2003).

Little is known about the infectious cycle of DCV and Dicistroviruses in general, except by comparison with Picornaviruses. DCV particles enter in cells through clathrin-mediated endocytosis (Cherry and Perrimon, 2003), but the receptor required for viral entry has not been identified yet. DCV protein synthesis, as for poliovirus, is sensitive to levels of the ribosomal machinery (Cherry et al., 2005). This characteristic seems to be a common feature for IRES-containing RNA viruses. Indeed, the knockdown of ribosomal protein S6 (RpS6) also affects Hepatitis C Virus (HCV) translation and suppresses its replication in Huh7.5 cells (Huang et al., 2012). The depletion of ribosomal proteins RpS6 and RpL19 blocks DCV replication but does not affect the cell growth and viability. However, we cannot rule out that the attenuation of DCV replication is an indirect effect of the depletion of ribosomal subunits. Indeed, a two-fold reduction in host protein synthesis is observed, which may have a physiological significance in the cells. DCV replication takes place in vesicles derived from the Golgi apparatus, and is dependent of coat protein I (COPI), but not COPII vesicles (Cherry et al., 2006). COPI vesicles are responsible of intra-Golgi transport and retrograde transport from the Golgi to the endoplasmic reticulum (ER) (Hsu et al., 2009). It is still unclear whether the vesicles used as viral factories are COPI vesicles since the authors stained these vesicles with an anti-Golgi antibody, rather than a COPI antibody. At least, they showed that viral factories derive from the Golgi apparatus. Authors also reported that fatty acid biosynthesis is required for DCV replication. Flies mutant for the sterol regulatory element binding protein (SREBP) are resistant to DCV infection. SREBP is a major transcriptional regulator of fatty acid metabolism, suggesting that DCV may rely on lipid metabolism for effective replication. Finally, the assembly of DCV particles and their release from infected cells remain poorly characterized.

Other picorna-like viruses were found in *Drosophila melanogaster* but they are much less characterized than DCV (Plus et al., 1976). ***Drosophila* P virus (DPV)** was described in 1969 (Plus and Duthoit, 1969). DPV is largely present in laboratory and wild populations of *Drosophila* (Plus et al., 1975a). DPV is a 25-30 nm non-enveloped virion with a (+) ssRNA genome. As for DCV, DPV appears asymptomatic in naturally infected strains. Injection of DPV in flies reduces their life span and induces female sterility (David and Plus, 1971). The virus mainly targets ovaries and malpighian tubules, and can be vertically transmitted (Teninges and Plus, 1972). ***Drosophila* A virus (DAV)** is a 25-30 nm non-enveloped virus with a (+) ssRNA genome. Unexpectedly, the sequence coding for the viral RdRp shares

characteristics with viruses from *Birnaviridae* and *Tetraviridae* families. In addition, the structure of the virion has unique characteristics (Ambrose et al., 2009). DPV and DAV are not classified yet. Both viruses can be vertically transmitted (Brun and Plus, 1980).

***Drosophila* X virus (DXV)** was first isolated in 1979 in flies that were highly sensitive to oxygen starvation (after exposure to CO_2). Sigma virus was hitherto the only reported virus to induce CO_2 sensitivity in flies. However, flies were free from bullet-shaped Sigma virus infection, but rather infected by an unknown icosahedric virus, thereby named DXV (Teninges et al., 1979). DXV is transmitted horizontally and viral particles are found in many organs, including the gut cells, the trachea cells, the muscle sheath of different organs, the ovaries and the fat body. DXV is a 70 nm non-enveloped virus. The bipartite double stranded RNA (dsRNA) genome indicated that this virus belongs to *Birnaviridae* family (Chung et al., 1996). The replication cycle of DXV is unknown.

***Drosophila* F virus (DFV)** was identified in laboratory stocks of *Drosophila melanogaster* (Plus et al., 1975b). DFV belongs to *Reoviridae* family (Plus et al., 1981). Viral particles are non-enveloped, with a diameter of 60-70 nm. The genome is composed of 10 segments of double stranded RNAs (Huszar and Imler, 2008). The replication cycle of DFV has not been studied.

Sigma virus (SIGMAV) belongs to the family of *Rhabdoviridae*. Viral particles are enveloped, with a bullet shape. The genome is a negative single stranded RNA ((-) ssRNA). SIGMAV is widespread in *Drosophila* populations, its transmission mainly occurs vertically via germ cells (Longdon and Jiggins, 2012). SIGMAV-infected flies are highly sensitive to CO_2 exposure. After CO_2 exposure, SIGMAV replicates rapidly in the nervous tissues, leading to paralysis and death of infected flies (Hogenhout et al., 2003). SIGMAV spreads in all tissues except muscles (Tsai et al., 2008). Interestingly, polymorphisms in the refractory for Sigma P virus (ref(2)P) locus were shown to affect SIGMAV infection in *Drosophila* (Carré-Mlouka et al., 2007), as discussed below in section 1.3.2.

Nora Virus is a picorna-like virus recently identified in laboratory and natural populations of *Drosophila* (Habayeb et al., 2006). Nora virus establishes a persistent infection in flies. Its genome is a (+) ssRNA of 12,333 nts length, ended by a poly(A) tail at the 3'

extremity. The genome has an unusual sequence and organization (**Figure 2,** Ekström et al., 2011). Unlike other picorna-like viruses, the genome encodes fours ORFs (ORF1-4) with unique features: ORF2 encodes a picornavirus-like helicase, a protease (less well conserved) and an iflavirus-like RdRp. The three other ORFs (ORF1, 3 and 4) are not closely related to any viral sequences previously described. ORF4 is proposed to encode capsid proteins. Strikingly, Nora virus titer can vary over 6 orders of magnitude (10^4 and 10^{10} viral genomes per fly in different stocks). Even single flies from a same stock can differ as much as 10^3 in viral titer (Habayeb et al., 2009a). Flies with a high-titer infection establish stable persistent infections, whereas flies with a lower level of infection are able to clear the virus. Nora virus is mainly found in the intestine of infected flies, and is transmitted horizontally via feces (Habayeb et al., 2009b). Nora virus does not cause obvious pathological effects, indicating that the virus is very well adapted to its host. The immune pathway controlling infection is still unclear. Indeed, Nora virus infection is not affected by mutations in the RNAi, Janus kinase-Signal transducer and activator of transcription (Jak-STAT) and Toll pathways (Habayeb et al., 2009a). However, Nora virus small RNAs were detected in infected flies (van Mierlo et al., 2012) and *Drosophila* Ovary Somatic Sheet (OSS) cells (Wu et al., 2010), suggesting that Nora virus is a target of the antiviral RNAi pathway. Furthermore, Nora virus genome encodes a viral suppressor of RNAi (VP1), which inhibits the catalytic activity of Argonaute-2 (Ago-2), a key component of the RNAi pathway (van Mierlo et al., 2012). At present, the role of the RNAi pathway in the control of persistent Nora virus infection is still unclear. As a full length Nora virus infectious clone is available (Ekström et al., 2011), it may be interesting to investigate if deletions in C-terminal region of VP1, resulting in loss of suppressor activity (van Mierlo et al., 2012), could result in better clearance of Nora virus infected wild-type flies. Only a mild effect on life span was reported, but no effect on eggs eclosion and fecundity. Nora virus was proposed as a model to study persistent viral infections. Clearly, the mechanisms controlling the persistence of Nora virus in *Drosophila* should be investigated to highlight how viruses establish persistent infection in their host. Whether the presence of this persistent infection contributes to the pathology caused by other pathogens has not yet been investigated.

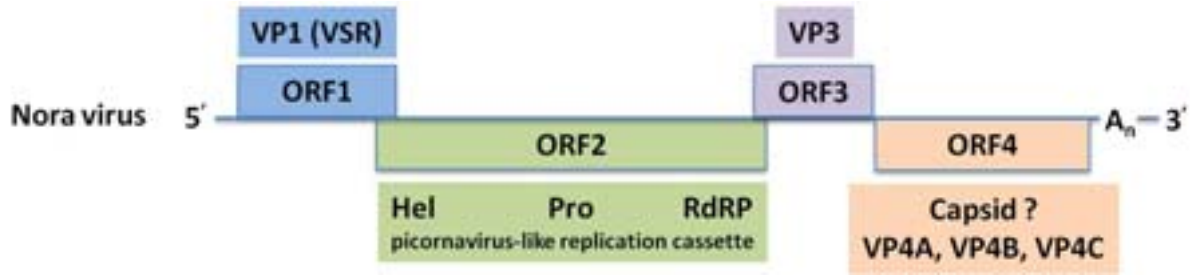


Figure 2. Representation of the Nora virus genome. (adapted from Ekström et al., 2011 and van Mierlo et al., 2012).

Assembly of virus-small interfering RNAs (v-siRNAs): a strategy to discover new viruses in invertebrates

In 2009, the team of Dr. Shou-Wei Ding proposed an original approach to discover new viruses in invertebrates (Wu et al., 2010). Based on the analysis of small RNA libraries, the authors reassembled viral genomes from v-siRNAs. They discovered four previously unknown viruses in *Drosophila* Schneider 2 (S2) cells: the (+) ssRNA viruses American Nodavirus (ANV) and *Drosophila* Tetravirus (DTrV) and the dsRNA viruses *Drosophila* Birnavirus (DBV) and *Drosophila* Totivirus (DTV). The pathogenicity and replication cycle of these viruses are not yet characterized. Viral small RNAs matching with ANV, DBV, DCV, DTrV, DXV and Nora Virus were found in *Drosophila* OSS cells. Overall, these results highlight that *Drosophila* S2 and OSS cells were probably persistently co-infected by five and six RNA viruses, respectively. Their molecular characterization awaits more investigations.

Natural *Drosophila* viruses presented above are summarized in **Table 1**.

Name	Familly	Genome (lentgh)	Diameter (nm)	Transmission	Envelope
DCV	*Dicistroviridae*	(+) ssRNA, 9,264 nts	25-30	Horizontal	No
DPV	Unclassified	(+) ssRNA	25-30	Horizontal and Vertical	No
DAV	Unclassified	(+) ssRNA	25-30	Horizontal and Vertical	No
DXV	*Birnaviridae*	dsRNA, 3,360 bp (Seg.A), 3,243 bp (Seg.B)	70	Horizontal	No

DFV	*Reoviridae*	dsRNA, 10 segments	60-70	Horizontal	No
SIGMAV	*Rhabdoviridae*	(-) ssRNA, 10-15 kb	45-100	Vertical	Yes
Nora Virus	Unclassified	(+) ssRNA, 12,333 nts	27-30	Horizontal	No

Table 1. Overview of viruses naturally infecting *Drosophila melanogaster*.

1.2. Other viruses infecting *Drosophila*

Cricket Paralysis virus (CrPV) was isolated in 1970 from two species of Australian field crickets, *Teleogryllus oceanicus* and *T. commodus*, that rapidly succumbed after paralysis (Reinganum et al., 1970). CrPV is able to infect a broad range of insect species, including *Drosophila melanogaster* flies (Wang et al., 2006) and cell lines (Moore et al., 1980; Scotti, 1975). CrPV injection into flies is highly pathogenic (Wang et al., 2006). The CrPV genome is composed of two ORFs encoding non-structural and structural proteins via IRES mediated translation. Both CrPV IRES were widely used as models to understand cap-independent mechanisms of translation (Deniz et al., 2009; Landry et al., 2009). The sequencing of the genome allowed its classification in the *Dicistroviridae* family, which also includes DCV (Wilson et al., 2000). CrPV genome is a (+) ssRNA with VPg protein attached at the 5' extremity (King and Moore, 1988) and a poly(A) tail at the 3' extremity. The virus is transmitted horizontally (Moore and Tinsley, 1982). CrPV infection leads to a shutoff of host translation in *Drosophila* cells (Garrey et al., 2010). Intriguingly, the infection is increased at higher temperature (37°C instead of 25°C) in *Drosophila* S2 cells (Cevallos and Sarnow, 2010). At higher temperature, CrPV RNA genome and viral proteins production are increased but unexpectedly, viral infectious particles are not. It was proposed that cellular responses at high temperature, including selective expression of heat shock proteins (Hsp) at the expense of other host proteins (Klemenz et al., 1985), may provide a beneficial environment for viruses. At high temperatures, the host protein synthesis is reduced in mammals, allowing some IRES-containing RNA viruses to hijack the translation machinery (Kim and Jang, 2002). Furthermore, several mammalian viruses are able to use Hsp for their replication (Burch and Weller, 2005; Glotzer et al., 2000; López et al., 2006), capsid formation (Chromy et al., 2003), or uncoating (Chromy et al., 2006). In *Drosophila*, it was reported that Hsp90

facilitates the Flock House virus replication (Kampmueller and Miller, 2005). The mechanism facilitating CrPV replication and protein synthesis at high temperature remains to be elucidated.

Flock House Virus (FHV) was originally isolated in 1983 from the grass grub *Costelytra zaelandica*, near the Flock House Agricultural Research Station in New Zealand (Scotti et al., 1983). FHV is able to replicate in plants, insects, yeast and mammalian cells, including different mosquito and *Drosophila* cell lines (Dasgupta et al., 2003, 2007). FHV is not a natural pathogen of *Drosophila*. Intrathoracic injection of FHV in flies is highly pathogenic and the virus spreads to multiple tissues, infecting the fat body, muscles and trachea (Galiana-Arnoux et al., 2006). FHV is a small non-enveloped, bipartite (+) ssRNA virus that belongs to *Nodaviridae* family (Venter and Schneemann, 2008). The genome of FHV consists of two RNAs: RNA1 (3.1 kb) encodes the viral RdRp (FHV protein A) and a subgenomic RNA3 (0.4 kb) containing two overlapping ORFs encoding proteins B1 (unknown function) and B2, a viral suppressor of RNAi (Li et al., 2002). RNA2 (1.4 kb) encodes the precursor protein of the viral capsid. Both RNA1 and RNA2 are capped at their 5' extremity, but are not polyadenylated. FHV replication occurs in viral factories that were visualized by electron microscopy tomography and reconstructed by three dimensional analysis (Kopek et al., 2007). The depletion of two enzymes involved in phosphatidylcholine biosynthesis (cytidylyltransferase (cct) 1 and cct2) affects FHV replication, indicating that glycerophospholipid metabolism positively regulates FHV replication (Castorena et al., 2010). FHV induces the formation of spherule-like vesicles between the inner and outer mitochondrial membranes that support new RNA synthesis by protein A. The two genomic RNAs are transported to the cytoplasm for translation and encapsidation into provirions is performed by the single protein α. Mature virions are produced after the autocatalytic cleavage of protein α into proteins β and γ, which confers physicochemical stability to the viral particle (Venter and Schneemann, 2008). As mentioned previously, the Hsp90 appears to facilitate FHV replication (Kampmueller and Miller, 2005), by promoting efficient synthesis of the viral RdRp in *Drosophila* S2 cells (Castorena et al., 2007). The molecular chaperon Hsp90 plays a role in the replication of a broad spectrum of viruses and appears to have virus-specific functions at unique steps in the viral cycle (Geller et al., 2012). This is one example of the complexity and diversity of the mechanisms employed by viruses to appropriate cellular pathways for their own purposes. FHV infectious cycle ends by the induction of

apoptosis through a caspase-dependent pathway in infected DL-1 cells (Settles and Friesen, 2008). Virus-induced apoptosis may favor the release of infectious particles and their subsequent dissemination to neighboring cells (Best, 2008). **The American Nodavirus (ANV)** is a variant of FHV that was found to persistently infect *Drosophila* S2 cells (Wu et al., 2010). The genome of ANV was assembled from v-siRNAs found in S2 cells and shared 89% and 82% of identity with RNA1 and RNA2 molecules of FHV respectively.

Invertebrate Iridescent virus 6 (IIV-6) is an icosahedral double stranded desoxyribonucleic acid (dsDNA) virus that infects invertebrates, mainly insects and terrestrial isopods. Its name comes from the opalescent hues observed in heavily infected hosts. The surface of paracrystalline arrays of virus particles reflects light that interferes with incident light resulting in diffraction, thus causing the iridescent hues in highly infected hosts. The iridescence was proposed to be a visual indicator of particles size, but this relationship is still debated (Williams, 2008). IIV-6, also named Chilo Iridescent virus (CIV), belongs to *Iridoviridae* family (Williams et al., 2005). Its dsDNA genome is 212,484 base pair (bp) long and encodes for 211 putative ORFs (initially 468 ORF were predicted (Jakob et al., 2001) but a reannotation of the genome was performed by (Eaton et al., 2007). IIV-6 is able to replicate in flies after intrathoracic injection, and iridescence is visualized in the abdomen of infected flies (Bronkhorst et al., 2012; Kemp et al., 2013; Teixeira et al., 2008). The infection is not lethal in wild-type flies, even if the injected dose is high. No DNA viruses that naturally infect *Drosophila melanogaster* have been discovered so far, although a dsDNA virus has recently been identified in wild-caught *Drosophila innubila* (Unckless, 2011). IIV-6, among others, is used in our laboratory as a model to study the antiviral immunity against DNA viruses in *Drosophila*.

Sindbis virus (SINV) is an arthropod-borne virus (arbovirus) first isolated in 1952 from *Culex pipiens* and *Culex univittatus* mosquitoes collected in the Sindbis district, near Cairo in Egypt (Taylor et al., 1955). SINV belongs to the *Togaviridae* family, in the alphavirus genus. Alphaviruses are transmitted by arthropods, typically the mosquitoes, and replicate in both arthropod and vertebrate hosts worldwide (Jose et al., 2009). SINV is the most widely distributed among alphaviruses causing arthritis in humans, which include Chikungunya, o'nyong-nyong, Mayaro and Ross River viruses (Tesh, 1982). However, it is the least dangerous for public health. The wide tropism of alphaviruses and genome

manipulation make them a good tool for applications as vaccine or gene therapy (Atkins et al., 2008). SINV is an enveloped virus containing a (+) ssRNA genome of 11.7 kb length, capped at its 5' end and polyadenylated at its 3' end (Strauss and Strauss, 1994). The genome is composed of a 5' ORF encoding the non-structural proteins and a 3' ORF encoding the structural proteins. The virus binds to the natural resistance-associated macrophage protein (NRAMP) at the host cell surface and enters via clathrin-mediated endocytosis (Rose et al., 2011). SINV activates the phosphatidylinositol 3-kinase (PI3K)-Akt-target of rapamycine (TOR) pathway. The PI3K-Akt pathway is reported to be involved in cell survival and proliferation, and can lead to the activation of the TOR kinase, which phosphorylates eIF4E-BP to facilitate the formation of translation inititiation complexes. Thus, through the activation of this pathway, SINV infection promotes cap-dependent translation of viral messenger RNAs (mRNAs) (Patel and Hardy, 2012). SINV is able to replicate in cells and flies after intrathoracic injection and was found in the fat body and the muscles surrounding the gut (Galiana-Arnoux et al., 2006; Mudiganti et al., 2010; Saleh et al., 2009). The virus is weakly pathogenic in wild-type flies.

Vesicular Stomatitis virus (VSV) is an arbovirus that belongs to *Rhabdoviridae* family. It is able to infect a wide range of species, like rodents, cattle, swine and horses, and causes an acute disease (Letchworth et al., 1999; Lichty et al., 2004). Rare cases of human infection have been reported, but most VSV infections are asymptomatic in humans. The genome is a (-) ssRNA of 11 kb of length, packaged in a bullet-shaped virion. The genome encodes 5 proteins: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the large subunit of the RdRp (L) (Ivanov et al., 2011, includes details of the replication cycle). VSV can replicate in *Drosophila* cells and animals (Mueller et al., 2010; Wyers et al., 1980). By contrast to infection in vertebrates, *Drosophila* cells infected by VSV do not display a shutoff of host protein synthesis and the virus establishes a non-cytopathic persistent infection (Dezélée et al., 1987; Wyers et al., 1980).

Other arboviruses have been reported to infect *Drosophila* cells and/or flies after injection, such as Chikungunya virus (CHIKV, *Togaviridae*) (Glaser and Meola, 2010), West Nile virus (WNV, *Flaviviridae*) (Chotkowski et al., 2008; Glaser and Meola, 2010), La Crosse virus (LACV, *Bunyaviridae*) (Glaser and Meola, 2010), Rift Valley fever virus (RVFV, *Bunyaviridae*) (Filone et al., 2010) and Dengue virus (DENV, *Flaviviridae*)

(Mukherjee and Hanley, 2010). A summary of viruses experimentally able to infect *Drosophila* is presented in **Table 2**. A genome wide RNAi screen has even been performed in *Drosophila* S2 cells to identify DENV host factors (Sessions et al., 2009). Overall, it is clear that *Drosophila* remains an extremely useful genetic model to study the interactions between host and insect viruses, including pathogenic human viruses (Hughes et al., 2012).

Name	Familly	Genome (lentgh)	Diameter (nm)	Envelope
CrPV	*Dicistroviridae*	(+) ssRNA, 9,185 nts	27	No
FHV	*Nodaviridae*	(+) ssRNA, bipartite RNA1, 3.1 kb RNA2, 1.4 kb	30	No
IIV-6	*Iridoviridae*	dsDNA, 212,484 kb	185	Yes/No
SINV	*Togaviridae*	(+) ssRNA, 11,703 kb	65-70	Yes
VSV	*Rhabdoviridae*	(-) ssRNA, 11 kb	70x200	Yes
CHIKV	*Togaviridae*	(+) ssRNA, 11.83 kb	65-70	Yes
WNV	*Flaviviridae*	(+) ssRNA, 10.9 kb	50	Yes
DENV	*Flaviviridae*	(+) ssRNA, 10.7 kb	50	Yes
LACV	*Bunyaviridae*	(-) ssRNA, Segment L (6.98 kb) Segment M (4.52 kb) Segment S (0.98 kb)	90-100	Yes
RVFV	*Bunyaviridae*	(-) ssRNA, Segment L (6.4 kb) Segment M (3.88 kb) Segment S (1.69 kb)	90-100	Yes

Table 2. Overview of viruses experimentally able to infect *Drosophila melanogaster*.

Chapter 2

Innate antiviral immunity in *Drosophila melanogaster*

Insects face a wide range of pathogens including bacteria, fungi and viruses. Since the discovery of the evolutionarily conserved Toll and immune deficiency (IMD) pathways, involved in anti-fungal and anti-bacterial immune responses (Hoffmann, 2003), the fruit fly *Drosophila melanogaster* is used as a model to study the molecular mechanisms of innate immunity. The Toll pathway is mainly activated by fungi and Gram-positive bacteria, whereas the IMD pathway is preferentially activated by Gram-negative bacteria. Both Toll and IMD signaling pathways activate members of the Nuclear Factor–κB (NF-κB) family: Dorsal-related immunity factor (Dif) and Relish, conducting a humoral systemic response involving the production of several families of antimicrobial peptides (AMPs). By contrast to anti-bacterial and anti-fungal immunity, which have been the focus of interest since the 1980s, the mechanisms involved in the defense against viruses in *Drosophila* only recently started to be investigated. To defend against viral pathogens, *Drosophila* relies on two main strategies: the RNAi pathway, which plays a broad role in the control of RNA viruses, and the virus specific inducible responses (**Figure 3**). Virus inducible responses are composed of the Toll, IMD, Jak-STAT signaling pathways and autophagy, reported to regulate some viral infections.

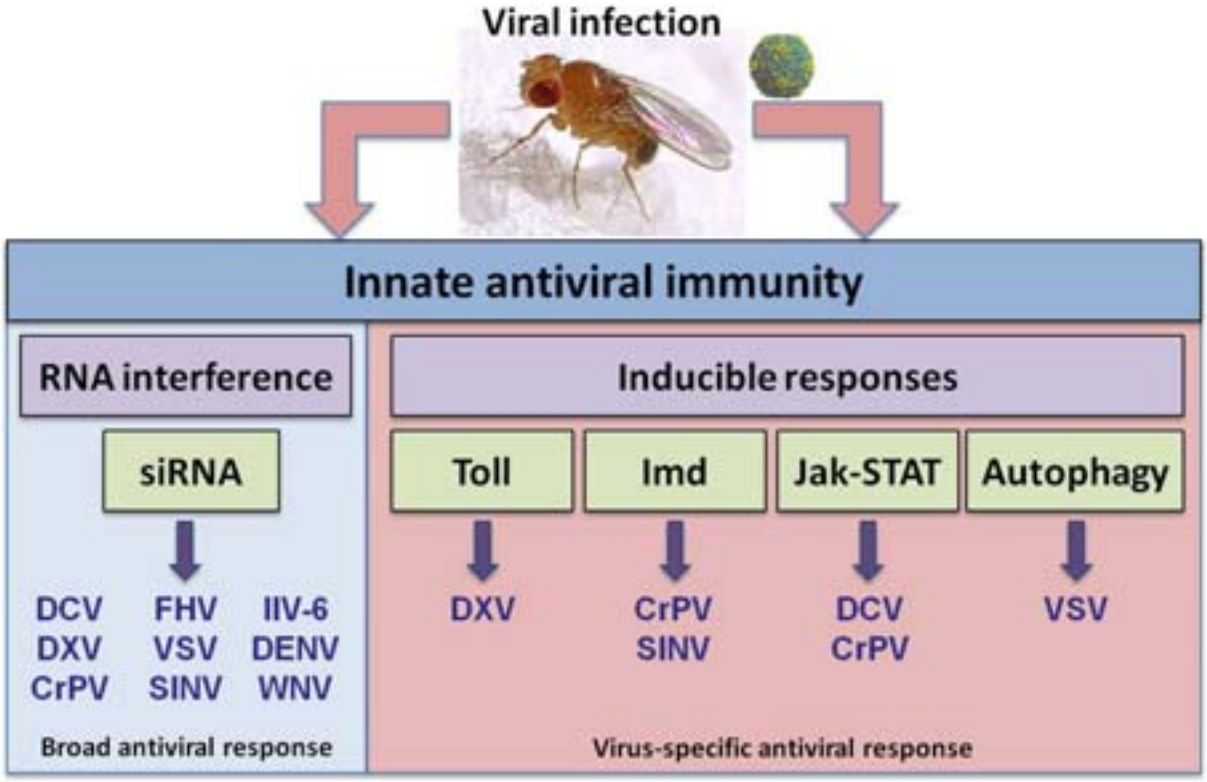


Figure 3. Overview of insect antiviral innate immunity. RNAi is a broad antiviral pathway, active against many viruses from diverse families whereas inducible responses are virus-specific.

2.1. The RNA interference pathway: a broad antiviral defense

Viral sensing

The innate immune sensing relies on the ability of the host to distinguish non-self molecular patterns, occurring during an infection, from self patterns of the host (Janeway, 1989). Accordingly, dsRNAs are a strong non-self pattern because they are generated during the viral cycle in the cytoplasm of infected cells, a place where they are not thought to exist naturally. They are generated either as an intermediate in genome replication by the RNA polymerase of RNA viruses, either as a by-product of converging bidirectional transcription for DNA viruses (Jacobs and Langland, 1996; Kumar and Carmichael, 1998). Other sources of non-self dsRNA signal may be the secondary structures present in the viral RNA genome or in viral transcripts, but also the genome itself for dsRNA viruses. dsRNAs are detected in cells infected by (+) ssRNA, dsRNA or DNA viruses, but not in cells infected by (-) ssRNA viruses (Weber et al., 2006). However, the undetected presence of small amounts of dsRNAs produced by (-) ssRNA viruses cannot be excluded. Indeed, VSV-derived small RNAs produced in *Drosophila* S2 cells and flies equally map the genome and antigenome RNA, indicating that they derive from dsRNAs (Mueller et al., 2010). v-siRNAs produced in cells infected by a trisegmented (-) ssRNA virus, (RVFV), were reported to equally map the genomic and antigenomic strands from L and M segments, but not S segment (Sabin et al., 2013). This suggests that the source of v-siRNAs from segments L and M are dsRNAs replication intermediates. By contrast, v-siRNAs from S segment may be generated from a putative hairpin. The authors also reported that DCV-derived siRNAs preferentially map the genomic (+) strand and proposed that they may derive from structured regions and hairpins within the genome.

RNA interference: the siRNA pathway

Viral dsRNAs trigger an antiviral response mediated by the RNAi pathway in plants, fungi, nematodes and arthropods, including insects *Drosophila melanogaster* (Ding and Voinnet, 2007). RNAi relies on the production of small RNAs that are 21-30 nucleotides in length and are divided in three main classes: siRNAs, microRNAs (miRNAs), and Piwi-associated interfering RNAs (piRNAs). The antiviral defense in *Drosophila* mostly relies on the class of siRNAs. Their generation and mode of action in antiviral defense is well

characterized and will be described hereafter (summarized in **Figure 4**). By contrast, the role of miRNAs and piRNAs in *Drosophila* antiviral defense is poorly characterized so both pathways will be briefly mentioned in this manuscript. (see (Lucas and Raikhel, 2013; Senti and Brennecke, 2010) for a review on miRNAs and piRNAs).

Viral dsRNAs are processed into siRNAs by the ribonuclease (RNase) Dicer-2 (Dcr-2), which belongs to RNaseIII family (Bernstein et al., 2001). Dicer-2 is composed of a DExD/H Adenosine triphosphatase (ATPase) domain, a domain of unknown function (DUF283), a Piwi/Argonaute/Zwille (PAZ) domain, two tandem RNaseIII domains, and a dsRNA-binding domain. The interaction of the PAZ domain with the extremity of the dsRNA allows its good positioning towards the two RNaseIII domains of Dicer-2 (MacRae et al., 2007). Each RNaseIII domain cleaves one strand of the dsRNA molecule, thereby generating 21 nts siRNAs with 2 nts overhang at the 3' end (Zhang et al., 2004). The characteristic 21 nts length of siRNAs is determined by the distance between the PAZ domain and the RNaseIII active site (Macrae et al., 2006). The function of other domains is still unclear. Loquacious isoform PD (Loqs-PD) is a cofactor of Dicer-2 facilitating processing activity, and may enhance the affinity of Dicer-2 to its dsRNA substrate (Marques et al., 2010). Similarly, Arsenic resistance protein 2 (Ars2) may facilitate Dicer-2-mediated cleavage (Sabin et al., 2009). Following dsRNA cleavage, the resulting siRNA is bound by Dicer-2 and its dsRNA-binding protein partner R2D2, generating the RISC loading complex (RLC). Then, the Dcr-2/R2D2 heterodimer loads the siRNA duplex into an Ago-2 containing RISC (Liu et al., 2003; Marques et al., 2010). The 5'-phosphate on the passenger strand of the siRNA duplex enhances R2D2 binding, thereby facilitating the incorporation of the siRNA into the RLC, and consequently into the RNA-induced silencing complex (RISC) (Tomari et al., 2004). The PAZ domain of Ago-2 binds the 3' end of the strand that is retained in the RISC, named the guide strand (Ma et al., 2004). The endoribonuclease Ago-2 cleaves the phosphodiester bond between nucleotides 9 and 10 of the strand that will be excluded from the RISC, named the passenger strand. This cleavage is mediated by the PIWI domain of Ago-2 (Matranga et al., 2005; Miyoshi et al., 2005). The component 3 promoter of RISC (C3PO), a multimeric complex of Translin and Trax, is a key RNAi activator that promotes RISC activity by removing the passenger strand cleavage product (Liu et al., 2009). After the cleavage of the passenger strand and its elimination from the RISC, the guide strand is 2'-O-methylated on the 3' terminal nucleotide by the S-adenosyl-L-methionine-dependent RNA methyltransferase

Hen-1 (Horwich et al., 2007). This methylation increases the stability of the small RNA (Ji and Chen, 2012). The RISC complex is then mature and can bind complementary RNA sequences through the guide strand. The RNase activity of the PIWI domain of Ago-2 cleaves the target RNA (Rand et al., 2004). If the complementarity between the guide strand and the target RNA is not perfect, mismatches may prevent the slicing by Ago-2. In this case, the RISC may act as a translation repressor by competitively blocking the interaction of eukaryotic initiation factor (eIF)-4E with eIF4G (Iwasaki et al., 2009), thereby inhibiting cap-dependent translation. It is questionable if this mechanism may occur for RNA viruses without cap and how the RNAi deals with the genetic diversity of viral populations namely quasispecies.

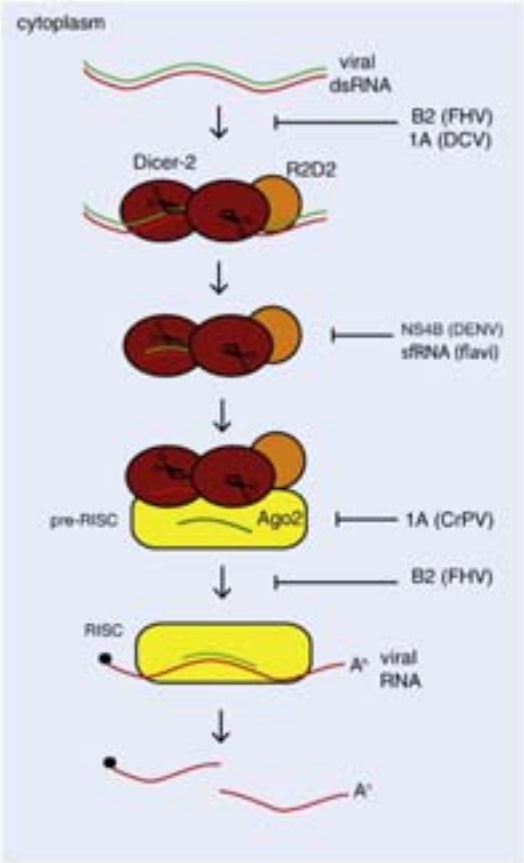


Figure 4. The siRNA pathway in *Drosophila*. (adapted from Kingsolver et al., 2013)

siRNA pathway: the broad antiviral sword of *Drosophila*

The RNAi pathway is a major antiviral defense mechanism in insects, including *Drosophila*. Flies deficient for the three key components of the RNAi pathway (Dcr-2, R2D2 and Ago-2) are more sensitive than wild-type flies to viral infections. The cofactor of Dicer-2,

Loqs-PD, is dispensable for the antiviral siRNA pathway (Marques et al., 2010). The increased sensitivity of Dcr-2, R2D2 and Ago-2 null mutants correlates with a higher viral titer. *Drosophila* RNAi pathway was shown to control infection by (+) ssRNA viruses, such as DCV (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Saleh et al., 2009), CrPV (van Rij et al., 2006; Wang et al., 2006), FHV (Aliyari et al., 2008; Galiana-Arnoux et al., 2006; Han et al., 2011; Wang et al., 2006), SINV (Avadhanula et al., 2009; Galiana-Arnoux et al., 2006; Saleh et al., 2009), WNV (Chotkowski et al., 2008) and DENV (Mukherjee and Hanley, 2010); the (-) ssRNA virus VSV (Mueller et al., 2010), and the dsRNA virus DXV (Zambon et al., 2006). Recently, we and others showed that RNAi controls not only RNA virus infection, but also infection by DNA viruses IIV-6 in *Drosophila* (Bronkhorst et al., 2012; Kemp et al., 2013) and Baculovirus in moth *Helicoverpa* (Jayachandran et al., 2012). Unexpectedly in the case of DXV infection, R2D2 and Ago-2, but not Dcr-2, mutant flies were highly sensitive to DXV compared to wild-type flies. It is proposed that in the absence of Dcr-2, the dicing of dsRNA genome may be supported by Dcr-1, originally involved in the processing of miRNAs precursors, as both enzyme have some overlapping function (Lee et al., 2004). The high sensitivity of Ago-2 mutants, defective for RISC activity, to viral infection (DCV, (van Rij et al., 2006); DXV, (Zambon et al., 2006); VSV, (Mueller et al., 2010)), whereas Dcr-2 is fully functional, suggests that the dicing of viral dsRNAs by Dcr-2 is not sufficient to control viral infection. This additional function of Ago-2 is required. However, it may not be the case for latent infection, as the dicing of dsRNA replication intermediates itself plays a role in the maintenance of FHV latency (Flynt et al., 2009). Moreover, the identification of Vago, an antiviral protein controlling DCV infection in the fat body and whose expression is dependent on Dcr-2, but not R2D2 and Ago-2, also suggests an additional function of Dcr-2, separated from the antiviral function of the RISC (Deddouche et al., 2008). Altogether, the relative contribution of antiviral dicing and slicing in the control of viral infections remains to be elucidated.

Analysis of viral small interfering RNAs (v-siRNAs)

Beside the genetic proof that Dcr-2, R2D2 and Ago-2 are necessary to control viral infections in insects, the generation of viral small interfering RNAs (v-siRNAs) after infection demonstrates that Dcr-2 processes viral RNAs (Wu et al., 2010). The analysis of v-siRNAs profiles reveal that the predominant source of v-siRNAs is viral dsRNA intermediates of replication. The deep sequencing of small RNAs in *Drosophila* S2 cells and OSS cells

persistently infected by positive sense RNA viruses (DAV, DCV, Nora virus and ANV) or dsRNA viruses (DTV, DXV and DBV) revealed that v-siRNAs mapped in equal proportion to both strands of the viral genome. Similarly, the distribution of v-siRNAs derived from positive and negative sense RNA strands of FHV genome are in equal proportion. It indicates that viral dsRNAs replication intermediates or viral genomic dsRNAs are the major substrate of Dcr-2 to trigger an effective antiviral RNAi response. Interestingly, the presence of specific hotspots of v-siRNAs on the viral genome suggests that some regions of the viral genome are accessible and processed by Dcr-2. As mentioned before, these regions may be local secondary structures of the viral genome, as stem-loop structures. In agreement with this hypothesis, a stem-loop structure predicted in FHV genome is a hotspot of v-siRNAs derived from the positive sense (van Rij and Berezikov, 2009; Zhong et al., 1992). Although this source of v-siRNAs is well documented in plants (Molnár et al., 2005), no experimental validation has been performed in *Drosophila*. By contrast to plants, the dicing of structural elements within the viral genome in *Drosophila* appears to be secondary to dicing of viral dsRNAs. Overall, the suggested targeting of replication intermediates is counterintuitive with the observation that RNA viruses replicate in defined membranous structures to protect themselves from ribonucleases or non-self sensors that trigger innate immune responses. As discussed in the chapter I, FHV replicates in spherules vesicles derived from the outer membrane of mitochondria (Kopek et al., 2007) whereas DCV has been proposed to replicate in compartments derived from the Golgi apparatus (Cherry et al., 2006). How and where Dcr-2 accesses to viral dsRNAs are still open questions.

Systemic RNAi response

In addition to the cell-autonomous silencing mediated by RNAi, viral infections trigger a systemic RNAi response that may contribute to antiviral defense in *Drosophila*. It is proposed that virus-mediated cell death or cell lysis release viral dsRNAs that are taken up and processed by neighborhood non-infected cells to protect them from subsequent infection (Saleh et al., 2009). This model is supported by the hypersensitivity of fly mutants for dsRNA uptake pathway. This pathway requires scavenger-like receptors for the entry of exogenous dsRNAs into cells via clathrin-mediated endocytosis (Saleh et al., 2006). A cell-to-cell spread of v-siRNAs and long dsRNAs was also observed in Semliki Forest virus (SFV)-infected mosquito cells (Attarzadeh-Yazdi et al., 2009). In plants and worms, efficiency of the

systemic RNAi response is increased by an amplification mechanism involving the production of secondary siRNAs.

Antiviral RNAi amplification

In plants and *C. elegans*, it is reported that antiviral RNAi pathway requires the amplification of the v-siRNAs by an endogenous RdRp to be effective against viruses (reviewed in Ding, 2010). At present, the production of secondary v-siRNAs by RdRp has not been observed in *Drosophila*. The amplification of antiviral RNAi response may rely on another mechanism. Indeed, it is reported that viral DNAs are generated by a cellular reverse transcriptase during infection by RNA viruses such as FHV and DCV, which are not retroviruses. It is proposed that the transcription of these viral DNAs produce dsRNAs that will amplify the siRNA-mediated antiviral response (Goic et al., 2013). Curiously, a recent report showed that a previous exposure to DCV does not protect flies from subsequent infection (Longdon et al., 2013). It would be interesting to see if viral DNA forms can be produced after the first viral challenge in this experimental setting.

Viral suppressors of RNAi (VSRs)

To counteract the antiviral siRNA pathway, insect viruses encode VSRs. It allows them to replicate even in the presence of an effective antiviral RNAi response. For example, protein B2 of FHV is essential for viral replication in wild-type flies, but not in RNAi-mutant flies (Galiana-Arnoux et al., 2006; Wang et al., 2006). By contrast to plant viruses, only few VSRs have been characterized in insect viruses. The B2 protein of FHV was the first VSR identified in an invertebrate virus (Li et al., 2002). The expression of B2 in plants can also inhibit RNA silencing, indicating that RNAi is evolutionarily conserved between the plant and animal kingdoms. Protein B2 interacts with dsRNAs in infected S2 cells, thereby suppressing dicing (Aliyari et al., 2008; Lu et al., 2005). It also blocks siRNAs incorporation into the RISC by binding siRNAs (Chao et al., 2005; Lu et al., 2005). The VSR of the *Dicistroviridae* DCV, named DCV-1A, also binds dsRNAs, thereby inhibiting the cleavage of dsRNAs by Dcr-2 (van Rij et al., 2006). DCV-1A does not bind siRNAs, by contrast to B2 protein. CrPV, another member of *Dicistroviridae* family, has a suppressor of RNAi that interacts with the endoribonuclease Ago-2 and inhibits its activity (Nayak et al., 2010). It is surprising that two closely related viruses from the same family encode VSRs using different mechanisms to suppress RNAi. However, both DCV-1A and CrPV-1A are unrelated in term

of sequence. SINV does not encode any VSR (mentioned in (van Mierlo et al., 2012). Interestingly, recombinant SINV expressing CrPV-1A replicates faster and causes higher mortality than recombinant SINV expressing DCV-1A (Nayak et al., 2010). This suggests that CrPV-1A is more efficient to suppress RNAi than DCV-1A, and also that VSRs can determine viral pathogenicity. Although not closely related, the protein VP1 of Nora virus inhibits slicer activity of Ago-2 as does protein CrPV-1A (van Mierlo et al., 2012). VSRs are strikingly diverse within and across kingdoms, indicating that they are acquired through fast convergent evolution. The expression of Nora virus VP1 by SINV increases the viral pathogenicity in flies via its RNAi suppressive activity. Similarly, *Aedes aegypti* or *Anopheles gambiae* mosquitoes infected by recombinant SINV expressing B2 protein are hypersensitive to viral infection (Cirimotich et al., 2009; Myles et al., 2008). SFV expressing tombusvirus protein P19, a plant VSR that binds siRNAs, better spread from cell-to-cell in culture (Attarzadeh-Yazdi et al., 2009). Overall, these results highlight that VSRs determine viral pathogenicity in insects.

RNAi is important in another insect model: mosquitoes, vectors of human viral diseases

RNAi controls infection by arboviruses in mosquitoes (Blair, 2011). The knockdown of Dcr-2 and Ago-2 expression in *Aedes aegypti* mosquitoes increases DENV and SINV infection (Campbell et al., 2008; Sánchez-Vargas et al., 2009). Likewise, the viral titre of O'nyong-nyong virus (ONNV) is increased after the knockdown of Ago-2 in *Anopheles gambiae* mosquitoes (Keene et al., 2004). Similarly to *Drosophila*, dsRNA replication intermediates are the major substrate of Dcr-2. For example, the sequencing of v-siRNAs from SINV-infected *Aedes aegypti* or WNV-infected *Culex pipiens quinquefasciatus* mosquitoes reveals an asymmetric distribution of v-siRNAs along the viral genome (Brackney et al., 2009; Myles et al., 2008). Some regions of the viral genome are highly targeted, suggesting that the dicing of structural elements may also contribute to the production of v-siRNAs, as proposed for *Drosophila* viruses. Surprisingly, no VSR has been identified so far in arboviruses, despite VSRs seem to be a common feature in non-arboviral insect viruses (Attarzadeh-Yazdi et al., 2009; Blakqori et al., 2007). Arboviruses cycle between insect vectors and mammalian hosts. To be efficiently transmitted to mammalian hosts, arboviruses have to stay nonpathogenic for insect vectors because killing the vector would be an endpoint for the virus. Transgenic viruses expressing a VSR are highly pathogenic for insects, probably because the RNAi does not control the virus anymore.

Accordingly, it is hypothesized that expressing a VSR could be deleterious for the virus, reducing the chance to be transmitted to mammalian hosts. Recently, Schnettler and colleagues showed that WNV proteins do not have any RNAi suppressor activity but rather found that the subgenomic flavivirus RNA (sfRNA) does (Schnettler et al., 2012). This non-coding RNA was already reported to result from incomplete genome degradation by cellular exoribonuclease Xrn1 and to enhance viral pathogenicity in mice (Pijlman et al., 2008), possibly by counteracting the antiviral response mediated by type I interferon (Schuessler et al., 2012). It remains to be determined how sfRNA, by contrast to recombinant arboviruses expressing VSRs, equilibrates the balance between viral replication and vector survival.

A role of miRNAs and piRNAs pathways in antiviral defense ?

In *Drosophila*, five Ago-like proteins have been identified, which raises the question of a functional diversification of antiviral RISCs (Tolia and Joshua-Tor, 2007). We may hypothesize that not only one, but several RISC-like complexes with redundancy and specialization contribute to antiviral defense. The antiviral function of *Drosophila* miRNAs and piRNAs-directed Ago-like proteins is poorly understood. One study reported that Piwi and Aubergine, two slicers involved in the piRNA pathway, control DXV infection in *Drosophila* S2 cells (Zambon et al., 2006). However, the relevance of this finding is questionable because both proteins are normally expressed in germline cells. Moreover, in addition to v-siRNAs, abundant viral piRNAs derived from DCV and ANV genome were identified in persistently infected OSS cells (Wu et al., 2010). Virus-derived piRNAs (v-piRNAs) were also identified for other viruses infecting these cells, but they were much less abundant. Additionally, v-piRNAs were identified in *Aedes albopictus* cells infected by DENV-2 (Scott et al., 2010), SINV-GFP and LACV (Vodovar et al., 2012), but also in *Aedes aegypti* cells infected by SINV-GFP (Vodovar et al., 2012). *In vivo*, it is reported that *Aedes aegypti* and *Aedes albopictus* mosquitoes infected with CHIKV produce v-piRNAs (Morazzani et al., 2012). The authors showed that C6/36 cells are Dicer-2 null mutant cells, harboring a defective siRNA pathway. Interestingly, C6/36 cells infected with a recombinant CHIKV expressing the VSR B2 display a decreased production of v-piRNAs and a strong cytopathic effect, suggesting that B2 increases viral pathogenicity by suppressing the production of v-piRNAs. Additionally, the silencing of components of the piRNA pathway in *Aedes aegypti* cells reduces the production of v-piRNAs and facilitates Semliki Forest virus replication, suggesting that the piRNA pathway contributes to the antiviral defense (Schnettler

et al., 2013). To date, no host miRNAs have been reported to be involved in *Drosophila* antiviral defense and no virus-encoded miRNAs have been identified in *Drosophila*. One study revealed the upregulation of miR-92 and downregulation of miR-989 in WNV-infected *Aedes albopictus* cell line and *Culex quinquefasciatus* mosquitoes (Skalsky et al., 2010). However, the targets of these miRNAs are uncharacterized and their role during WNV infection remains to be elucidated. Recently, an *in silico* screen identified *Drosophila* miRNAs that may target components of innate immune pathways (Fullaondo and Lee, 2012) but the function of these miRNAs in *Drosophila* immune response remains to be clarified experimentally. Overall, the siRNA pathway is a key arm of the antiviral defense mounted by insects, including *Drosophila* and mosquitoes. In addition to siRNA pathway, piRNA pathway may also contribute to antiviral defense.

2.2. The inducible response: a virus-specific antiviral defense

Beside RNAi which is a broad antiviral defense mechanism in insects, including *Drosophila* and mosquitoes, other immune signaling pathways also contribute to viral defense. The hallmark of *Drosophila* immune response against bacteria and fungi is the inducible humoral response (De Gregorio et al., 2001, 2002; Irving et al., 2001). Similarly, several microarrays performed after viral infections reveal the upregulation of hundreds of genes, suggesting that inducible pathways may be important for the control of viruses. For example, some immune-related genes are upregulated in *Drosophila* S2 cells infected by SINV, such as thiol-ester protein II (TEP II) and Gram-negative binding proteins (GNBP1) (Mudiganti et al., 2010). SIGMAV infection upregulates the expression of pattern-recognition receptors (PRRs), such as peptidoglycan receptors proteins (PGRPs) and antimicrobial peptides (AMPs) (Tsai et al., 2008). The microarray analysis of Nora virus infected flies revealed the upregulation of 46 genes (including vago) and the downregulation of 12 genes in response to the infection (Cordes et al., 2013). A recent study also reported the upregulation of 275 genes and the downregulation of 442 genes in early DCV infected S2 cells (Zhu et al., 2013). Some of these genes are PRRs (e.g. PGRPs), AMPs (attacin, cecropin), or components of immune signaling pathways, particularly IMD and Jak-STAT pathways. The function of most of these genes in antiviral defense remains to be addressed.

2.2.a. The Toll pathway

Characterisation of the Toll signaling pathway in the context of bacterial and fungal infections

The Toll pathway is well characterized as the major immune response mounted to defend against fungi and Gram-positive bacteria (Hoffmann, 2003). Pathogen associated molecular patterns (PAMPs) from Gram-positive bacteria, particularly lysine-type peptidoglycan, bind PGRP (-SA, -SD) and GNBP1 (Bischoff et al., 2004; Leulier et al., 2003; Michel et al., 2001). GNBP3 recognizes β-glucans from fungi (Gottar et al., 2006; Mishima et al., 2009). This binding triggers a serine protease cascade that cleaves the extracellular cytokine-like protein Spätzle from pro-protein to functional form. The mature form of Spätzle is the ligand of the transmembrane receptor Toll (Weber et al., 2003), indicating that Toll does not itself function as a recognition receptor of non-self patterns, by contrast to mammalian Toll-like receptors (TLRs). The intracytoplasmic TIR (Toll-IL-1 receptor) domain of Toll interacts with an adaptor complex composed of death-domain proteins dMyd88 (*drosophila* myeloid differentiation factor 88), Tube and Pelle (Sun et al., 2004). The binding of Spätzle to Toll receptor initiates Toll signaling cascade with phosphorylation and degradation of Cactus protein. Cactus is an inhibitor of κB (IκB)-like protein that inhibits the NF-κB-like transcription factors Dorsal and Dif (Lemaitre et al., 1996). The release and degradation of Cactus allows the translocation of Dorsal and Dif in the nucleus where they bind to κB-like sequence motifs to mediate the transcription of AMPs genes (Ip et al., 1993; Rutschmann et al., 2000). A representation of the *Drosophila* Toll pathway is presented in **Figure 5**.

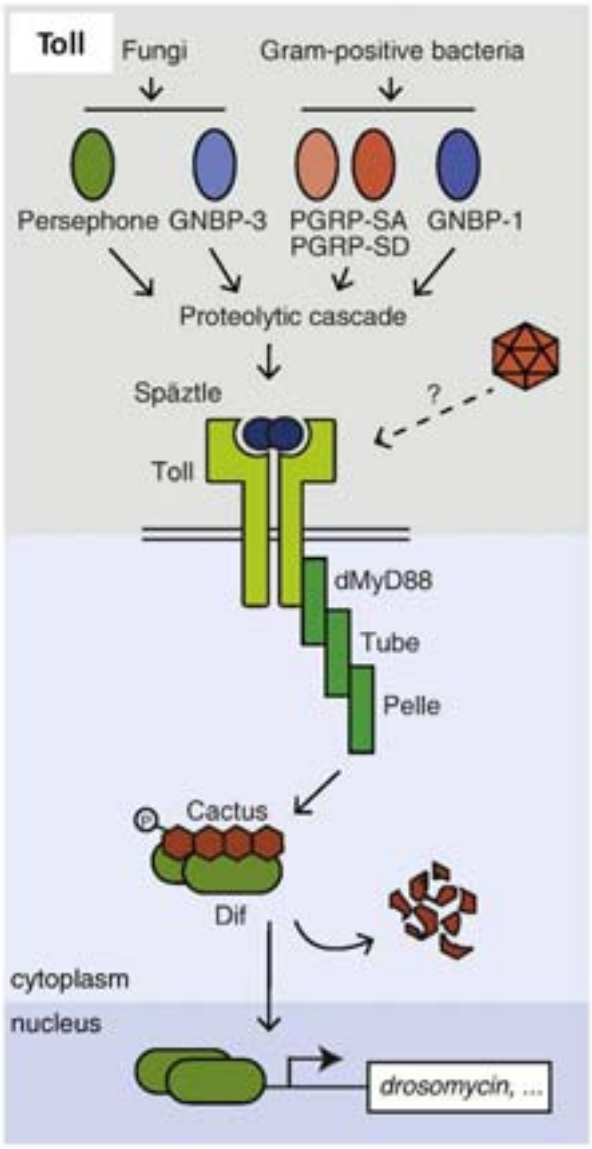


Figure 5. The Toll pathway in *Drosophila*. (adapted from Kingsolver et al., 2013)

Toll pathway and viral infections

Beside the well-characterized role of the Toll pathway in anti-bacterial and anti-fungal immune response, its involvement in the defense against insect viruses is poorly understood. At present, only one article suggests that the *Drosophila* Toll pathway plays a role in the defense against DXV infection (Zambon et al., 2005). One characteristic of this virus is to induce anoxia sensitivity (Teninges et al., 1979). Based on this viral specificity, the authors performed a screen to identify *Drosophila* mutants with altered sensitivity to DXV infection, using anoxia-induced death as readout. Dif mutants have higher DXV titer and succumb more rapidly than wild-type flies to DXV infection. Both DXV and *Escherichia Coli* (*E.coli)*

(Gram-negative bacteria) induce similar expression levels of the Toll and IMD pathway target genes. Since the expression of AMPs is a hallmark of the activation of Toll and IMD pathways, the authors proposed that both pathways are activated in response to DXV infection. However, a null-mutant for Relish displayed similar resistance and viral titre than wild-type flies to DXV infection, suggesting that only the Toll pathway contributes to the defense against DXV infection. A mutant in whom the Toll pathway is constitutively activated (Tl^{10b} mutant) displays a decreased viral titer compared to wild-type flies. As the expression of single AMPs does not have a direct antiviral activity on DXV infection, they propose that the sensitivity of Toll pathway mutants may result of a defect in the Toll-dependent cellular response mediated by hemocytes. However, Tl^{10b} mutant flies were as sensitive as Dif mutants to CO_2 exposure. This suggests that DXV titer may not affect the outcome of the infection. In addition, the Toll pathway is also involved in the control of DENV infection in *Aedes aegypti* mosquitoes (Xi et al., 2008), indicating a potently antiviral role in insects.

Drosophila Toll receptors

In contrast to mammals, only one out of 9 Toll receptors identified in *Drosophila melanogaster* was shown to play an important role in innate immunity (Bilak et al., 2003). Whereas each TLR in mammals is specialized to recognize a specific non-self pattern, this one *Drosophila* Toll is able to respond to broad signals from bacteria, fungi and viral infections. Toll-mutant flies are more susceptible to DXV infection (Zambon et al., 2005). In addition, a recent study showed the involvement of *Drosophila* Toll-7, but not other Toll proteins, in the recognition and restriction of VSV infection *in vitro* and *in vivo* (Nakamoto et al., 2012). Surprisingly, Toll-7 activation by VSV does not seem to signal via the canonical Toll pathway, even if Drosomycin is 2-fold induced by VSV infection in cells, as mutations in Myd88 and Dif do not affect VSV replication *in vivo*. The authors showed that Toll-7 recognizes VSV virions at the plasma membrane and induces autophagy, a pathway reported to be involved in antiviral defense against VSV infection (Shelly et al., 2009). More recently, Toll-8 was shown to negatively regulate the expression of AMPs by antagonizing the IMD pathway in the respiratory epithelium of *Drosophila* (Akhouayri et al., 2011). It remains to be determined whether additional Toll receptors are involved in antiviral response. Four *Drosophila* Toll receptors, including Toll and Toll-7, are transcriptionaly induced upon viral infection (Xu et al., 2012).

2.2.b. The IMD/TNF-R pathway

Characterisation of the IMD signaling pathway in the context of bacterial infection

Another immune signaling pathway, distinct of the Toll pathway, was reported to control *Drosophila* host defense (Lemaitre et al., 1995). The IMD pathway is activated by the diaminopimelic acid peptidoglycan (DAP-PGN) present at the surface of Gram-negative bacteria. DAP-PGN is sensed by the pattern recognition protein PGRP-LE, a secreted PGRP, and the transmembrane receptor PGRP-LC (Choe et al., 2002; Gottar et al., 2002; Hoffmann, 2003; Kaneko et al., 2004; Leulier et al., 2003; Rämet et al., 2002). In addition to PGRP-SA sensor for Toll pathway, PGRP-LC and PGRP-LE, both activating IMD pathway, are the three PGRPs out of thirteen identified in *Drosophila* (Werner et al., 2000), that are involved in the immune response. The binding of DAP-PGN to PGRP-LCs triggers the recruitment of IMD, a death domain adaptor protein similar to mammalian receptor interacting protein (RIP). Then, IMD associates with *drosophila* Fas-associated-death domain (dFADD) protein (Georgel et al., 2001; Naitza et al., 2002) which in turn interacts with the caspase Dredd (Leulier et al., 2000). Dredd subsequently binds and cleaves the phosphorylated form of Relish, a NF-κB-like transcription factor, thereby allowing its translocation in the nucleus to induce the expression of AMP genes (Ertürk-Hasdemir et al., 2009; Stöven et al., 2000; Stoven et al., 2003). Relish is phosphorylated by the *Drosophila* IκB kinase complex (Kenny and Ird5), which is activated by the mitogen-activated protein kinase kinase kinase (MAPKKK) *drosophila* TGF-beta activated kinase 1 (dTak1) (Lu et al., 2001; Vidal et al., 2001). A representation of the IMD signaling pathway is presented in **Figure 6**. Null-mutant flies for most of the components of the IMD pathway are hypersensitive to Gram-negative bacterial infections. This immune deficiency phenotype is the result of an inability to induce the expression of AMP genes, and thereby to control bacterial infections (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). Interestingly, the IMD pathway can induce the expression of cytosqueletal proteins through activation of the c-Jun N-terminal kinase (JNK) signaling pathway, suggesting that both antimicrobial defenses and tissue repair processes may act in concert to recover from a pathogen infection (Boutros et al., 2002).

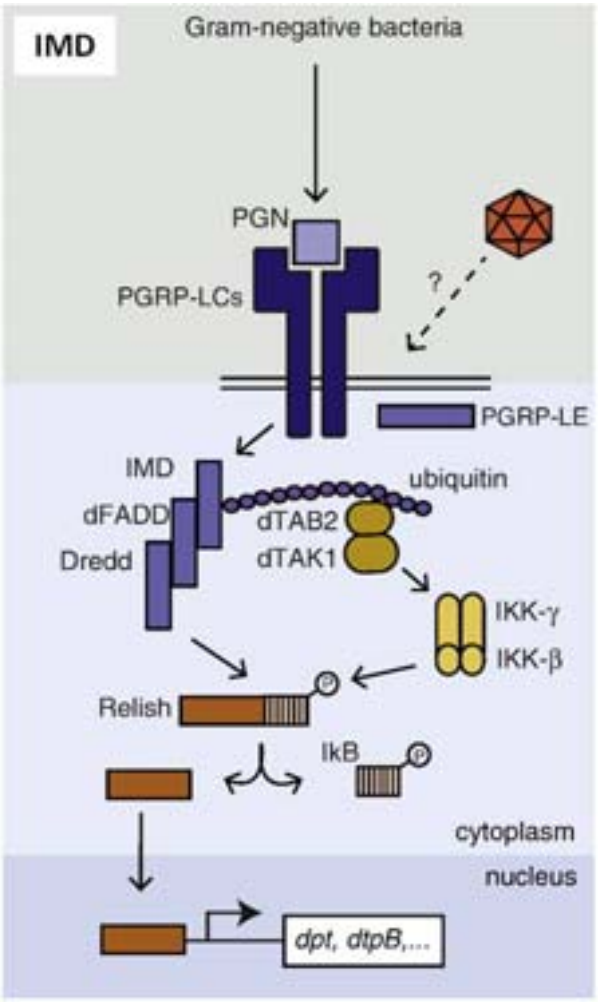


Figure 6. The IMD pathway in *Drosophila*. (adapted from Kingsolver et al., 2013)

IMD pathway and viral infections

An antiviral role for the IMD pathway is suggested for CrPV infection (Costa et al., 2009). Fly mutants for the core components of the IMD pathway, except Imd-deficient flies, are more sensitive to CrPV infection and display higher CrPV RNA levels. To explain the discrepancy observed with Imd-mutants, it is proposed that Imd gene itself is dispensable for the defense against CrPV infection. CrPV infection does not induce the expression of Toll or IMD-mediated AMPs genes, suggesting that AMPs do not play a role in the IMD-mediated antiviral response. Other groups including ours reported that null-mutant flies for the IMD-mediated NF-κB transcription factor Relish are resistant to DCV, SINV and DXV infections (data not shown, Deddouche et al., 2008; Zambon et al., 2005). It contrasts with results from Costa and colleagues that show the importance of IMD pathway to control this infection. However, all flies from their study were infected by Wolbachia that is known to affect the

susceptibility of flies to CrPV infection (Hedges et al., 2008). Therefore, we cannot rule out that the presence of Wolbachia could have affected the results presented in this study.

Overall, it is clear that there is a gap between our knowledge of the role of IMD and Toll pathways in anti-bacterial and anti-fungal defense, and their role in antiviral immune response. Moreover, viral activators and antiviral effectors of these pathways need to be characterized.

2.2.c. AMPs

Characterization of AMPs

AMPs are a key component of the innate defenses in vertebrates and invertebrates (Hancock and Scott, 2000). Insects fight against pathogens by synthesizing a broad spectrum of AMPs targeting bacteria and fungi (Hoffmann et al., 1996). They are mostly synthesized by the fat body, an analogue of the mammalian liver, and secreted into the hemolymph to kill invading microorganisms. Around one hundred AMPs have been characterized in insects, and at least seven types of AMPs, plus isoforms, have been identified in *Drosophila* (Hoffmann and Reichhart, 1997; Hoffmann et al., 1996). They are divided in two classes: the cyclic peptides and the linear peptides. The cyclic AMPs include insect Defensins (anti-Gram-positive bacteria; (Dimarcq et al., 1994)) and Drosomycin (anti-fungi; (Fehlbaum et al., 1994). The second class of linear AMPs is divided into three families: the Cecropins, the proline-rich peptides and the glycine-rich polypeptides. Cecropins lyse predominantly Gram-negative bacteria by perforing membrane cell wall, but they also have anti-fungal activity (Ekengren and Hultmark, 1999). The proline-rich peptides include Drosocin, an anti-Gram-negative bacteria peptide (Charlet et al., 1996), and Metchikowin that is active against Gram-positive bacteria and fungi (Levashina et al., 1995). The glycine-rich family is composed mostly of anti-Gram negative AMPs such as Attacin and Diptericin (Hedengren et al., 2000).

Toll and IMD-dependent AMPs expression

Immune-inducible peptides are expressed early after immune challenge, in a range of 15 to 30 min, and persist for one to several days (Gross et al., 1996; Hoffmann et al., 1996). Their expression depends on *Drosophila* NF-κB transcription factors activated by Toll signaling pathway (Dif and Dorsal) and IMD signaling pathway (Relish). Both Dorsal and Dif

can induce the expression of diptericin and cecropin genes (Ip et al., 1993; Petersen et al., 1995). Without the presence of Dorsal, Dif still mediates the induction of drosomycin and defensin (Meng et al., 1999). However, both Dorsal and Dif may have redundant functions for the induction of AMPs (Manfruelli et al., 1999).

Importance of AMPs in bacterial versus viral defense

Fly mutants for the IMD or Toll pathway, which do not express AMP genes, are highly sensitive to bacterial or fungal infections, supporting a key role of AMPs to face pathogens (Hedengren et al., 1999; Lemaitre et al., 1996). To address their functional relevance in the immune response of *Drosophila,* AMP genes were expressed under the control of the UAS-Gal4 system in immune deficient flies (Tzou et al., 2002). The constitutive expression of a single AMP such as Drosomycin or Defensin is sufficient to rescue the sensitivity of flies deficient for both the IMD and Toll pathways to some microbial infections. Tzou and colleagues were the first to clearly demonstrate *in vivo* the role of each AMP in host defense. However, the diversity of AMP-encoding genes suggests that they may act in cooperation to clear infections. This hypothesis has not been addressed experimentally. In 2005, another group reproduced this experiment in the context of a viral infection (DXV, (Zambon et al., 2005). However, none of the seven *Drosophila* AMPs tested was alone sufficient to confer resistance to DXV infection. Finally, even if the Toll and IMD pathways were shown to be activated after DXV infection, AMPs may not be antiviral effectors in *Drosophila* or, as suggested before, may act in cooperation together. By contrast to DXV infection, SIGMAV and DCV infection significantly but only weakly induced the expression of AMPs genes (Dostert et al., 2005; Tsai et al., 2008). Moreover, no AMPs were detected in the hemolymph of DCV-infected flies (Sabatier et al., 2003), nor in CrPV-infected flies (Costa et al., 2009). It suggests that flies may not use AMPs to counter Dicistroviruses infection.

Transcriptional profiles were performed in *Drosophila* after infection by DCV (oral infection, (Roxström-Lindquist et al., 2004); intrathoracic injection (Dostert et al., 2005) and DXV infection (Zambon et al., 2005). As can be expected, the fly immune response to DCV infection differs depending on the infection route. For example, virus-induced RNA-1 (vir-1) is highly induced after intrathoracic injection of DCV but not in the case of an oral infection. A comparison of the profile of immune response genes in SIGMAV, DCV and DXV infected

flies has been reported (table 3, (Tsai et al., 2008)). However, it does not bring a clear view of immune genes required for viral defense. Only Attacin-A was clearly upregulated by the three viruses. Upregulation of other AMPs seemed virus-specific, as defensin for DXV. The role of other genes induced after viral infections remains to be characterized.

2.2.d. The Jak-STAT pathway

The Jak-STAT pathway is important for *Drosophila* development (Hou et al., 2002). The unpaired (upd) family of secreted factors comprises three members (upd1, upd2 and upd3) that are able to activate the receptor Domeless (Agaisse et al., 2003; Harrison et al., 1998; Hombría et al., 2005), which controls the activation of the kinase Hopscotch (Brown et al., 2001; Chen et al., 2002). The kinase Hopscotch (Hop) recruits and phosphorylates STATs proteins that are translocated in the nucleus to induce the transcription of STAT-responsive genes. The activation of the Jak-STAT pathway is negatively regulated by proteins such as suppressor of cytokine signaling (SOCS) and *Drosophila* protein inhibitor of activated STAT (dPIAS) (Betz et al., 2001; Callus and Mathey-Prevot, 2002). The *Drosophila* Jak-STAT signaling pathway is represented in **Figure 7**. Its role in the defense against bacterial infections in insects has been reported. The expression of Jak-STAT-induced genes is observed after microbial challenges in *Drosophila,* as Turandot M (TotM) and CG11501 (Boutros et al., 2002). Septic injury also induces the expression of the cytokine-like Upd3 by hemocytes to activate the Jak-STAT pathway in the fat body (Agaisse et al., 2003). The thiol-esther protein 1 (Tep1), a complement-like protein in *Drosophila*, is upregulated through the activation of the Jak-STAT and Toll pathway after bacterial infection (Lagueux et al., 2000). *Anopheles* mosquitoes may also activate the Jak-STAT pathway in response to microbial infection (Barillas-Mury et al., 1999). For viruses, it is the transcriptional profile of DCV infected flies that allowed the identification of this third innate antiviral pathway in *Drosophila* (Dostert et al., 2005). Around 90 genes were upregulated during DCV infection, but not microbial infection, such as *vir-1* gene. Interestingly, the expression of *vir-1* was also induced in FHV-infected flies, but was unaffected during stress responses (Ekengren et al., 2001), indicating that this induction is a signature of viral infection. The expression of vir-1 is dependent on the Jak-STAT pathway, as its promoter contains an active STAT-binding site. Accordingly, Jak-STAT-deficient flies are more sensitive to viral infection and display higher viral titre. However, loss and gain-of-function experiments indicate that vir-1 is not an

antiviral effector because the knockdown or overexpression of vir-1 does not affect viral infection (data not shown, Dostert et al., 2005). This result was consistent with the absence of vir-1 promoter induction in the tissues infected by DCV, as the fat body, but rather in the neighboring tissues (ventral epidermis). The antiviral effectors mediating the antiviral activity of the Jak-STAT pathway remain to be elucidated. By comparison to *Drosophila*, the Jak-STAT pathway is also important to control DENV infection in *Aedes aegypti* mosquitoes (Souza-Neto et al., 2009). Moreover, two putative Jak-STAT-induced Dengue virus restriction factors (DVRF1 and DVRF2) were reported to control DENV infection.

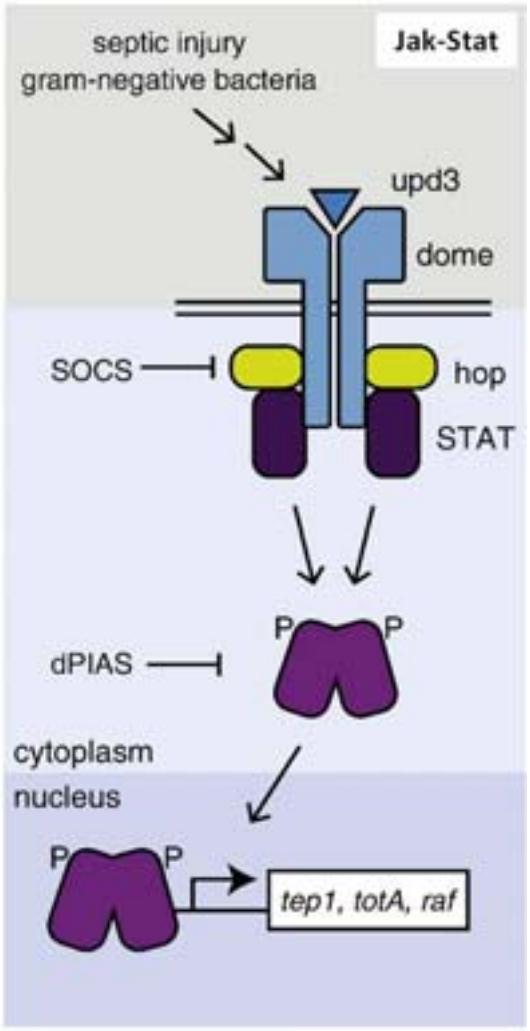


Figure 7. The Jak-STAT pathway in *Drosophila.* (adapted from Kingsolver et al., 2013)

2.2.e. Autophagy

Autophagy is an evolutionarily conserved pathway by which damaged organelles are recycled in a lysosomal-dependent process. Recent studies have involved this mechanism as a component of the antiviral innate immunity, allowing the elimination of multiple pathogens, including bacteria, parasites and viruses (Richetta and Faure, 2013). The hallmark of autophagy is the presence of the double-membrane autophagosome but its turnover is rapid so that it can be difficult to observe (Deretic and Levine, 2009). This autophagic organelle rises with the envelopment of cytoplasmic material by an isolation membrane, also named phagophore. Its maturation into autolysosome is driven by fusion with lysosome, thereby allowing degradation of the engulfed content by lysosomal enzymes. Autophagy genes and regulators are conserved from mammals to insects, including *Drosophila* (Moy and Cherry, 2013; Zirin and Perrimon, 2010). The bacteria *Listeria monocytogenes* is recognized by the pattern recognition receptor PGRP-LE that activates autophagy to control bacteria growth in a Toll and IMD-independent manner (Yano et al., 2008). Similarly, activation of autophagy (by starvation or rapamycin treatment) also controls Wolbachia infection in *Aedes albopictus* mosquito cells and *Drosophila* cells and flies (Voronin et al., 2012). In *Drosophila*, autophagy was recently reported to be essential for antiviral defense against VSV (Shelly et al., 2009). The knockdown of core autophagy genes increased viral infection in *Drosophila* S2 cells and flies and also the mortality in flies. The PI3K/Akt signaling pathway, which normally regulates autophagy depending on nutrients availability, also controls this antiviral response. Unexpectedly, the glycoprotein G of VSV was sufficient to activate the antiviral autophagy process. This unusual PAMP is recognized by the PRR Toll-7 to initiate autophagy (Nakamoto et al., 2012). The signaling pathway linking this PRR to the activation of autophagy remains to be elucidated.

An overall picture of the inducible antiviral pathways in *Drosophila* is showed in **Figure 8**.

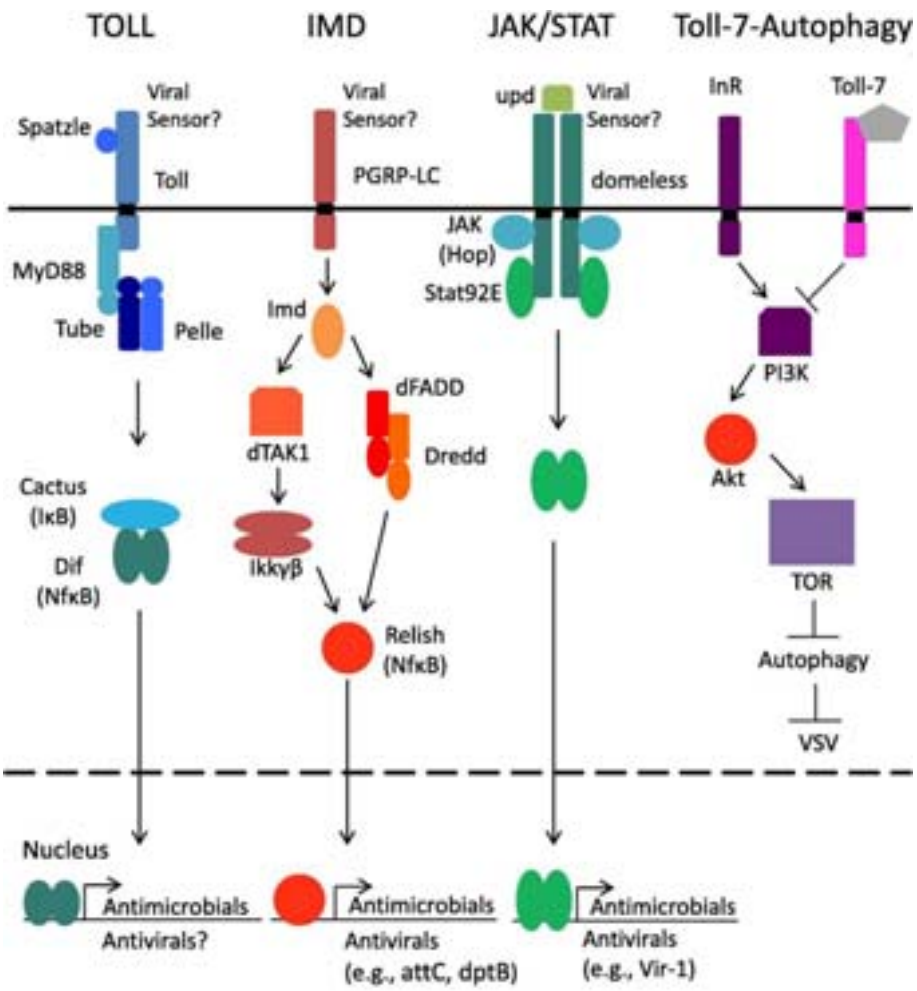


Figure 8. Inducible antiviral pathways in *Drosophila*. (adapted from Xu and Cherry, 2014).

2.3. Parallel with innate antiviral immunity in mammals

Toll in *Drosophila* / IL-1R-TLR in mammals

There are remarkable differences between immune responses in *Drosophila* and mammals. First, Toll does not directly sense invading pathogens in insects by contrast to TLRs in mammals. Indeed, the cytokine spätzle binds the Toll receptor to activate the Toll pathway whereas mammalian TLRs are activated directly by the pathogen. Secondly, two distinct signaling pathways are required in *Drosophila* to defend against Gram-positive and Gram-negative bacteria whereas a single TLR-dependent pathway is sufficient in mammals. In both insects and mammals, immune signaling pathways lead to the activation of NF-κB family members that induce the expression of immune-response genes. However, NF-κB activation in *Drosophila* is either Toll dependent (Dif and Dorsal) or IMD dependent (Relish), depending on the nature of the bacteria. Interestingly, the cytokine IL-1 binds and activates

interleukin-1 receptor (IL-1R) in mammals, leading to the activation of NF-κB and promoting inflammatory responses involved in antiviral defense. This strategy can be compared to the situation in *Drosophila*, where the cytokine Spätzle activates the Toll pathway (Hoffmann, 2003). Overall, the Toll signaling pathway shares some features with the mammalian signaling cascades downstream of IL-1R and the TLRs.

IMD in *Drosophila* / TNFR in mammals

The IMD pathway is similar to the tumour-necrosis factor-receptor (TNFR) pathway in mammals. They also display some differences. The first one is the activation of the pathway. PGRPs activate *Drosophila* IMD pathway and they are conserved from insects to mammals. At present, four PGRPs have been identified in different tissues in humans (Liu et al., 2001), and 12 PGRPs in *Drosophila*. Interestingly, one PGRP from the moth *Trichoplusia ni* is conserved in humans and binds peptidoglycans (Kang et al., 1998). However, its role in human innate immunity is still unexplored. In insects, PGRPs circulate in the hemolymph, recognize peptidoglycans present on the bacterial cell wall and activate the IMD and Toll pathways. A mouse PGRP was reported to be expressed in neutrophils and inhibit the growth of Gram-positive bacteria, but also some functions of neutrophils and macrophages (phagocytosis, cytokine induction). By contrast to the role of PGRPs in *Drosophila*, it is proposed that mammalian PGRP may function as an antibacterial intracellular protein present in neutrophils (Liu et al., 2000). Secondly, as mentioned above, the *Drosophila* IκB kinase (IKK) complex acts on Relish in the IMD pathway but not on Dif-Dorsal in the Toll pathway. By contrast, the mammalian IKK complex is required for most NF-κB activations.

Overall, the activation of NF-κB in *Drosophila* after pathogen infection relies on two distinct pathways (Toll and IMD) that are composed of molecules that are homologous or related to ones of TLR and TNFR mammalian signaling pathways activated during innate immune response.

RNAi in *Drosophila* versus interferon in mammals

Even if some immune signaling pathways have homologies with their mammalian counterparts, one of the most important vertebrate innate antiviral pathways, the type I interferon (IFN-I) system, is clearly unique to vertebrates. The RNAi pathway is the major antiviral arm in insects to defend against a broad panel of RNA and DNA viruses. RNAi

pathway is functional in mammalian cells (Elbashir et al., 2001) but it has long been assumed to have little or no role in vertebrate innate immunity (Cullen, 2006). Whereas v-siRNAs are easily detected in infected plants and invertebrates, no viral siRNAs were identified after cloning and sequencing of small RNAs from viral infected mammalian cells, but several viral miRNAs have been identified after DNA virus infection (Pfeffer et al., 2005). By contrast, a single viral siRNA was identified in human immunodeficiency virus-1 (HIV-1)-infected cells and its sequence targets the Rev response element (RRE) of viral mRNAs, involved in their nuclear export (Bennasser et al., 2005). However, this finding was strongly criticized as *in vitro* experiments were performed with a too much perfect artificial substrate for Dicer, compared to the natural HIV-1 RRE RNA substrate. More recently, small RNAs with some features of v-siRNAs were identified in mammalian cells infected by diverse viruses but their abundance was remarkably lower compared to invertebrates (Parameswaran et al., 2010). Whether these small RNAs mediate an antiviral response in mammalian cells remained to be determined. Some studies reported that RNAi controls retrotransposition of long interspersed nuclear element-1 (LINE-1) in human cells (Soifer et al., 2005; Yang and Kazazian, 2006). Moreover, the transfection of siRNAs protects mammalian cells against viral infections, suggesting that RNA silencing can be an antiviral mechanism in mammalian cells (Gitlin et al., 2002). Artificially induced RNAi responses in mammals can confer protection against pathogenic viruses (McCaffrey et al., 2002), but it has long been debated whether mammalian cells can mount a protective RNAi response naturally. Two recent studies now provide a support for an antiviral role of RNAi in mammals (Li et al., 2013c; Maillard et al., 2013). Maillard and colleagues identified v-siRNAs in mouse embryonic stem cells (mESCs) infected with encephalomyocarditis virus (EMCV). The depletion of Dicer inhibits the production of v-siRNAs but surprisingly, it does not increase viral replication. The production of v-siRNAs is decreased after differentiation of mESCs cells. The higher ability of undifferentiated mESCs cells to produce v-siRNAs compared to somatic cells remains unclear. Nodamura virus (NoV) deficient for B2 VSR, but not wild-type virus, triggered the production of v-siRNAs in mESCs cells (Maillard et al., 2013) and baby hamster kidney (BHK) fibroblasts cells, plus in newborn mice (Li et al., 2013c), leading to the control of the infection. Indeed, the replication of B2-deficient NoV was rescued by expression of B2, but also of VSR from Ebola virus (VP35). This suggests that VSRs mask the effective role of RNAi in antiviral response, which may explain that previous studies did not reveal the antiviral role of RNAi in mammalian somatic cells. The presence of VSRs in mammalian

viruses or the generation of viral RNAs with an RNAi suppressor activity suggests an antiviral function of RNAi in mammals. Indeed, the sfRNA of WNV and DENV suppresses the RNAi pathway in both insects and mammals (Schnettler et al., 2012). Some mammalian viruses encode protein with a VSR activity: the non-structural NS1 protein of human Influenza A Virus (IAV) binds siRNAs and suppresses RNA silencing in plants and insects *Drosophila* (Bucher et al., 2004; Li et al., 2004). However, even if the authors hypothesize that NS1 may inhibit RNAi during influenza infection in mammalian cells, there is no experimental evidence. Moreover, proteins that randomly bind dsRNAs such as *E. Coli* RNase III can also suppress RNAi in plants (Lichner et al., 2003). This is surely not their primary aim, as NS1 only requires the dsRNA binding domain for its VSR activity (Li et al., 2004). This raises the question whether the VSR activity from NS1 comes from a non-specific mechanism. It is even more intriguing that the binding of dsRNAs is a common characteristic of all VSRs proteins identified in mammalian viruses. Moreover, Influenza A mutants lacking NS1 protein replicate better in IFN-deficient cells or mice, but not when IFN response is effective in wild-type conditions (García-Sastre et al., 1998). This suggests that the main function of NS1 is to antagonize the IFN response and that the VSR activity of NS1 is dispensable for viral pathogenicity. The same criticism can be applied in regard to VSR activity of the dsRNA binding protein E3L of Vaccinia virus (Li et al., 2004). Adenovirus viral associated (VA1) RNA, highly expressed during infection, acts as a competitive inhibitor of Dicer (Andersson et al., 2005). Overall, the contribution of the RNAi pathway in mammals in regard to the strong antiviral response mediated by IFN-I remains to be elucidated. At present, it is proposed that RNAi may be complementary to IFN in undifferentiated ESCs cells, which are deficient for IFN inducibility and sensitivity (Burke et al., 1978). It seems that RNAi does not play a major role in antiviral defense in mammals compared to plants and invertebrates. Despite this contrast between insect and mammal immune systems, both RNAi and IFN response are triggered by the same non-self pattern, the cytoplasmic viral dsRNAs.

Toll and autophagy

Several mammalian TLRs can trigger autophagy in macrophages (Delgado et al., 2008; Shi and Kehrl, 2008). For example, TLR4 activation by its ligand Lipopolysaccharide (LPS) induces autophagy in murine and human macrophages (Xu et al., 2007). TLR8 activates autophagy in a vitamine-D-dependent manner to inhibit HIV infection in

macrophages (Campbell and Spector, 2012). In mammals, viral infections can trigger autophagy after induction by several PRRs such as TLR binding, dsRNA binding on protein kinase R (PKR), viral ssRNA binding on nucleotide oligomerization domain 2 (NOD2), and maybe DNA sensors (Richetta and Faure, 2013). However, the contribution of autophagy in antiviral defense remains to be elucidated in mammals. In *Drosophila*, Toll-7 activation triggers autophagy that protects against VSV infection (Nakamoto et al., 2012; Shelly et al., 2009). In plasmacytoid dendritic cells (pDCs), VSV RNAs are recognized by TLR7 upon autophagosome formation (Lee et al., 2007). There is no genetic evidence that autophagy restricts viral replication in mammals but the connection between TLRs activation and autophagy is conserved in *Drosophila* and mammals. The signaling pathway from PRR recognition to autophagy activation is currently unknown in both *Drosophila* and mammals.

2.3.a. Nucleic acid sensors: pattern recognition receptors

An arsenal of sensor proteins is present at the plasma membrane, in endosomes or in the cytosol to recognize viruses invading host cells (Shayakhmetov et al., 2010). In mammalian cells, three classes of sensors are involved in virus recognition: TLRs, retinoid acid-inducible gene (RIG)-I-like receptors (RLRs), NOD-like receptors (NLRs) and cytosolic DNA sensors. The host immune recognition system is based on a feature that is shared among all viruses: a genome composed of nucleic acids. The binding of viral nucleic acids with these sensors activate the major innate antiviral defense system in mammals: the IFN-I pathway (**Figure 9**).

Toll-like receptors comprise a family of at least 11 members (Takeda and Akira, 2005). TLR2, TLR3, TLR4, TLR7 and TLR9 are involved in the recognition of different types of viral components, and together they provide enough coverage to detect most types of viruses. TLRs are expressed on key sentinel cells of the innate immune system: macrophages and dendritic cells. TLR2 and TLR4 are present on the plasma membrane and can recognize viral envelope protein on the cell surface. TLR3, TLR7 and TLR9 are located in endosomes and recognize viral dsRNA, ssRNA and unmethylated CpG DNA respectively. Activation of all TLRs converge towards induction of IFN-I, through a signaling cascade involving IFN regulatory factors, and the synthesis of pro-inflammatory cytokines such as IL-1β via NF-κB activation (Shayakhmetov et al., 2010).

(RIG)-I-like receptors family comprises the cytoplasmic viral RNA sensors RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology-2 (LGP2) (Kang et al., 2002; Rothenfusser et al., 2005; Yoneyama et al., 2004). This class of sensors is expressed ubiquitously and is localized in the cytosol where it detects viral nucleic acids upon infection. They are strongly induced by IFNs upon viral infection. The DExD/H box RNA helicase domain of RLRs is highly similar to the one of mammalian Dicer. RIG-I binds short dsRNAs and 5'triphosphate-ssRNAs whereas MDA5 binds long dsRNAs (review on RLRs in (Goubau et al., 2013; Shayakhmetov et al., 2010; Takeuchi and Akira, 2009)). RIG-I and MDA5 detect distinct spectrum of viruses (Kato et al., 2006; Yoneyama et al., 2008). Mice deficient for RIG-I and MDA5 are highly susceptible to viral infections, underlying the importance of these molecules for host antiviral response (Kato et al., 2006). Both RIG-I and MDA5 rely on the adaptor protein IFN-β promoter stimulator-1 (IPS-1), that triggers a signaling cascade leading to the activation of type I IFN. IPS-1 is also known by the names virus-induced signaling adapter (VISA), mitochondrial antiviral-signaling protein (MAVS) and CARD adapter inducing interferon-β (CARDIF). The role of LGP2 in cytosolic RNA sensing remains unclear. By contrast to RIG-I and MDA5, LGP2 does not have the caspase activation and recruitment domain (CARD) in N-terminal, normally required to couple downstream signaling adaptors. LGP2 has no signaling capacity. It is proposed that LGP2 is a negative regulator of RIG-I and MDA5 signaling by competing with these molecules for the engagement with viral RNAs. Others DExD/H-box helicases have been involved in DNA sensing such as DDX41 (Zhang et al., 2011), DHX9 and DHX36 (Kim et al., 2010).

NOD-like receptors family comprises a large number of cytosolic proteins. NLR activation leads to MAPK and NF-κB activation, and also to inflammasome formation that can activate the IL-1R signaling pathway. NOD-like receptor family protein 3 (NLRP3) can sense viral DNA in macrophages. DNA-dependent activator of IFN-regulatory factors (DAI) senses viral dsDNA to activate type I IFN. (reviewed in Shayakhmetov et al., 2010)

DNA sensors also initiate the IFN-I system because the presence of DNA in aberrant location such as the cytoplasm and endosomes is recognized as a non-self or danger signal and triggers immune activation (Paludan and Bowie, 2013). In addition to TLR9 on

endosomes, many other DNA sensors are present in the cytosol such as DAI, IFI16 (gamma-interferon-inductible protein 16) (Unterholzner et al., 2010) and AIM2 (Absent in melanoma 2). Loss- and gain-of-function experiments revealed that DAI is a dsDNA sensor that activates innate immune responses, including IFN-I (Takaoka et al., 2007). The intracellular signaling triggered by cytosolic DNA sensing depends on the central adaptator STING (stimulator of IFN genes) to activate IFN-I (Burdette and Vance, 2013; Ishikawa and Barber, 2008). STING-deficient mice are hypersensitive to HSV-1 infection (Ishikawa and Barber, 2008). Recently, the group of Z. Chen observed that DNA sensing was coupled with the synthesis of cyclic-di-GMP-AMP (cGAMP) by the enzyme cGAMP synthetase (cGAS), leading to the induction of IFN in a STING-dependent manner (Sun et al., 2013b; Wu et al., 2013). They suggest that the key DNA sensor is the cytosolic cyclase cGAS that generates the second messenger cGAMP that binds and activates STING to induce IFN-I and other cytokines. They further demonstrated that cGAS is essential to trigger innate antiviral responses against retroviruses such as HIV, simian immunodeficiency virus (SIV) and murine leukemia virus (MLV) (Gao et al., 2013). Interestingly, it was reported that cGAMP can be transferred from producing cells to naïve neighboring cells through gap junctions to trigger STING activation and promote antiviral immunity in these cells (Ablasser et al., 2013).

2.3.b. Type I interferon pathway

The viral nucleic acid sensors RLRs and TLRs trigger an intracellular signaling cascade, leading to the secretion of IFN-I and also of pro-inflammatory cytokines and chemokines. IFN-I are produced by all cells in the organism: innate immune cells (dendritic cells (strong producer), macrophages) and non immune cells as fibroblasts. Pro-inflammatory cytokines and chemokines lead to inflammation and recruitment of cells involved in adaptive immunity such as lymphocytes. IFN-I response attracts and activates natural killer cells ant cytotoxic T cells, both major effector cell populations that eliminate infected cells (Stetson and Medzhitov, 2006). Thereby, IFN-I is among the first line of defense during viral infections. The secretion of IFNs amplifies and spreads the response to surrounding uninfected cells (**Figure 9**). The binding of IFN-I on their receptor activates the Jak-STAT signaling pathway. The dimerization of IFN receptor at the plasma membrane initiates a tyrosine phosphorylation cascade involving the Janus kinase (Jak) family, resulting in the dimerisation of the phosphorylated STAT1 and STAT2. Then, activated STATs interact with

IFN-regulatory factor 9 (IRF-9) to form the IFN-stimulated gene factor-3 (ISGF-3) complexe that translocates in the nucleus to activate the transcription of hundreds of genes, named Interferon-stimulated genes (ISGs). They have pleiotropic functions and include antiviral effectors such as PKR and the 2'5'-oligoadenylate synthetase (Der et al., 1998). Many of these genes encode proteins that have a direct antiviral activity and participate to intrinsic innate antiviral immunity.

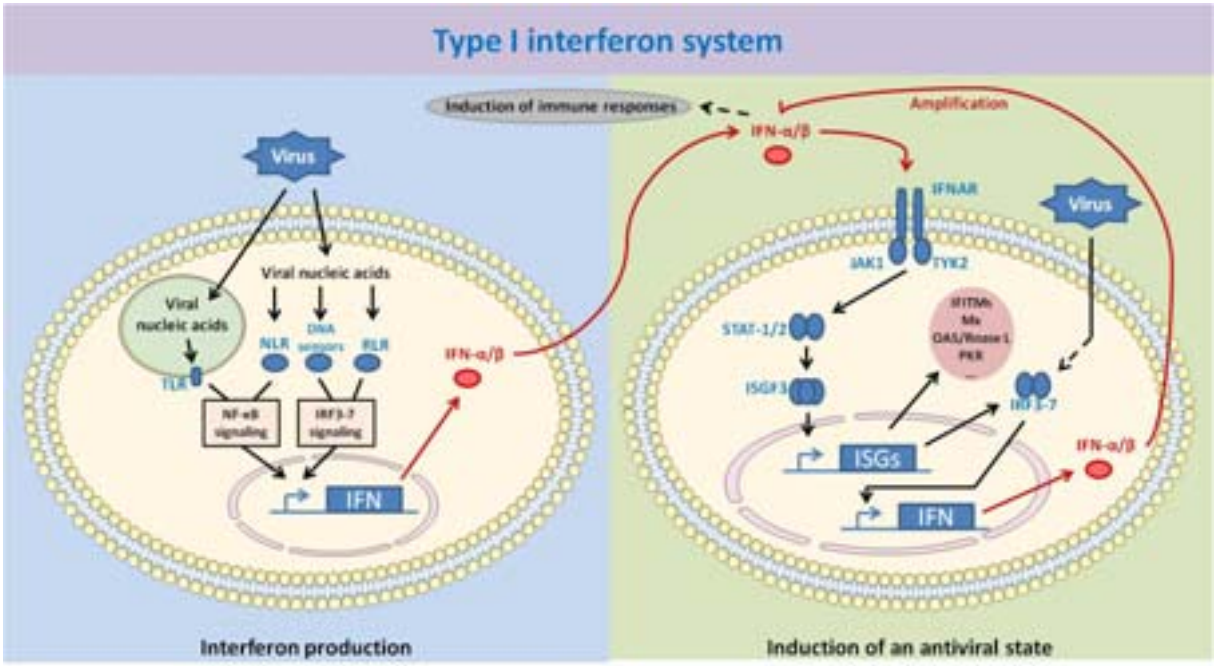


Figure 9. The type I interferon response.

Chapter 3

Intrinsic antiviral immunity

Intrinsic antiviral immunity relies on the action of proteins that recognize specific viral components and block viral infection immediately and directly. These proteins are named restriction factors or intrinsic antiviral factors. More generally, the term restriction factor could be attributed to any protein whose the major biological function is to inhibit the infectious cycle of a virus. The cell-intrinsic antiviral defense creates an antiviral state prior to infection, and so before the onset of the IFN response in vertebrates. Indeed, restriction factors display a constitutive basal level of expression in most types of cells and tissues as observed by microarray analysis for Apolipoprotein B mRNA-editing catalytic polypeptide 3 (APOBEC3) genes (Refsland et al., 2010) or Tetherin (Erikson et al., 2011). Additionally, they can be induced by viral infection through IFN signaling to amplify their antiviral activity and further establish the cell-intrinsic antiviral immunity (Yan and Chen, 2012). Many of the restriction factors that have been characterized in mammals are induced by IFN, which is somehow expected as their major activity is to fight viruses. It is probably necessary for the host that some restriction factors are upregulated and active only upon infection, such as PKR and RNase L that display a broad and strong antiviral restriction against a large spectrum of viruses, but also a detrimental effect for the host. The antiviral activity of restriction factors is demonstrated by loss- and gain-of-function experiments: the knockdown or overexpression of a restriction factor respectively increases or decreases viral infectivity. In vertebrates, a large panel of restriction factors has been characterized, covering inhibition of all steps of viral infection (**Figure 10**). Most characterized viral restriction factors were classified depending on the step of the viral life cycle that they target and will be described hereafter.

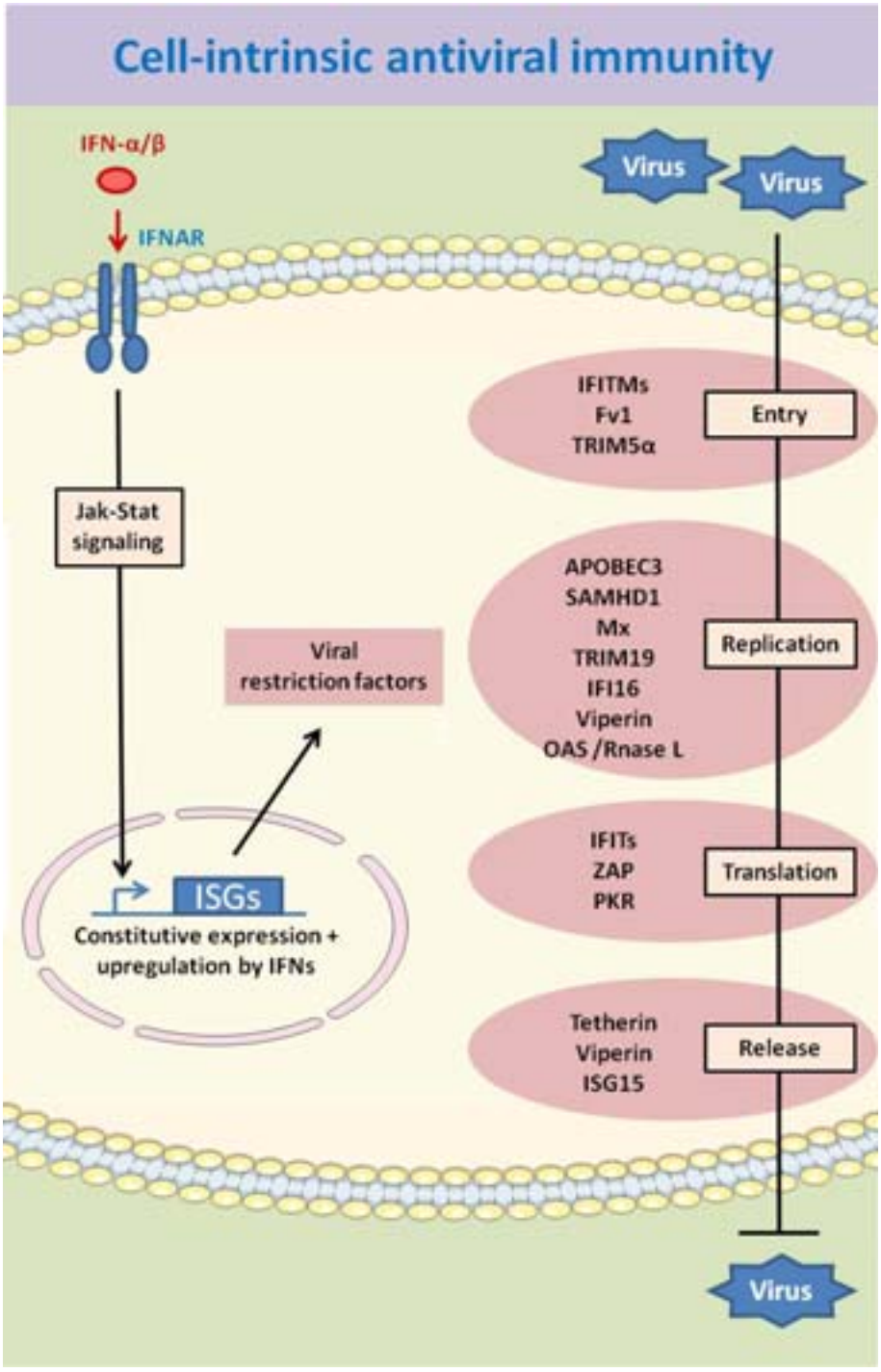


Figure 10. Cell-intrinsic antiviral immunity in mammals.

3.1. Viral restriction factors in mammals

3.1.a. Inhibition of viral entry

Interferon-inducible transmembrane proteins (IFITMs)

IFITMs are IFN inducible genes encoding intrinsic plasma membrane proteins that restrict viral entry (reviewed in (Diamond and Farzan, 2013; Perreira et al., 2013). IFITMs possess two Transmembranes (TMs) domains. The IFITMs are small proteins (around 130 amino acids) composed of two TMs domains separated by a highly conserved intra-cellular loop. The first TM domain and the intracellular loop define the cluster of differentiation (CD) 225 domain that is important for antiviral activity (John et al., 2013). The human IFITM locus is composed of four functional genes (*IFITM1*, *IFITM2*, *IFITM3* and *IFITM5*) and a pseudogene (*IFITM4p*). IFITMs display a basal expression level in cells. IFITM1, IFITM2 and IFITM3 were first identified in a siRNA genomic screen for IAV-dependency factors (Brass et al., 2009). They inhibit the early steps of IAV, but also of flaviviruses DENV and WNV replication, but not MLV replication (Brass et al., 2009). The depletion or overexpression of IFITM3 respectively increases and inhibits IAV replication. Hemagglutinin (HA)-pseudotyped retroviruses are inhibited by IFITMs whereas wild-type retrovirus are not, indicating that IFITMs target the IAV entry. The entry processes of Marburg virus (MARV), Ebola virus (EBOV) and severe acute respiratory syndrome-coronavirus (SARS-CoV) are restricted by IFITMs (Huang et al., 2011). Infection by MLV is not affected by IFITMs but MLV-pseudotyped with the entry proteins of EBOV, MARV, IAV is antagonized by IFITMs. Furthermore, viral fusion assays indicated that HA and VSV-G pseudoparticles are unable to release their content in the cytosol of cells overexpressing IFITM3 (Feeley et al., 2011). These results were the first to indicate that IFITMs prevent viral-host membrane fusion. The depletion and overexpression of IFITM2 and IFITM3, but not IFITM1, respectively enhances and restricts RVFV infection (Mudhasani et al., 2013). IFITMs also restrict other members of *Bunyaviridae* family, including LACV. In the case of RVFV infection, IFITM2 and IFITM3 do not affect the viral binding and entry into cells but a step before the replication, suggesting an inhibition of viral membrane fusion and release of ribonucleoproteins (RNPs) into the cytoplasm. Accordingly, another recent study claimed that IFITMs restrict viral membrane fusion (Li et al., 2013a). The authors used the properties of diverse viral fusion proteins to look at the effect of IFITM overexpression on the formation of syncytia and cell-cell fusion process. IFITM3 antiviral activity is negatively regulated by monomethylation on Lysine 88, directed by the lysine methyltransferase SET7 (Shan et al., 2013). Interestingly, this methylation is promoted by VSV and IAV infection, probably as a counteracting strategy.

Friend-virus susceptibility gene 1 (Fv1)

The murine Fv1 gene was shown to confer resistance to MLVs (Wolf and Goff, 2008). MLVs were classified depending of their ability to cause disease in different strains of mice: **N**-tropic MLVs are able to infect **N**IH mice but not BALB/C mice, whereas **B**-tropic MLVs infect **B**ALB/C mice but not NIH mice. Thus, two major alleles of Fv1 were described: $Fv1^{n}$ that restricts B-tropic MLV but not N-tropic MLV and $Fv1^{b}$ allele that restricts N-tropic MLV but not B-tropic MLV. $Fv1^{n/b}$ heterozygote mice are resistant to both N and B-tropic viruses. Surprisingly, this gene has similarities with the Gag (Group-specic antigen) protein of a member of the endogenous retrovirus-like elements (ERV-L) family (Best et al., 1996). Fv1 gene encodes a capsid-like protein. The restriction of MLV replication by Fv1 may require its interaction with the viral capsid protein (Bishop et al., 2001; Bock et al., 2000). To date, there is no direct evidence for binding of Fv1 to capsid, except genetic studies that determined amino acids (AAs) positions required for tropism-specific Fv1 restriction. The Fv1 tropism is determined by the nature of the AA at position 110 in the viral capsid of retroviruses (Kozak and Chakraborti, 1996).

Tripartite motif protein isoform 5 alpha (TRIM5α)

An Fv1-like restriction to N-tropic virus infection was observed in some non-murine cells, including human cells (Towers et al., 2000). Similarly to the situation in mice, a virus mutated at the position 110 in the viral capsid is able to bypass this Fv1-like restriction in non-murine cells, indicating that the restriction is determined by sequences within the capsid protein. This unknown restriction factor was first named Resistance factor 1 (REF1) in humans and Lentivirus susceptibility factor 1 (Lv1) in monkeys and then identified as the gene TRIM5α in a screen for factors mediating HIV-1 resistance (Keckesova et al., 2004; Stremlau et al., 2004). TRIM5α has no sequence homology to the Fv1 gene but binds to the same region of the capsid, around position 110. TRIM5α is a member of tripartite motif family (TRIM). This motif is present at the N-terminus and contains a Really Interesting New Gene (RING) domain, a B-box domain and a coiled-coil domain (Nisole et al., 2005). The C-terminus of the protein contains the B30.2 domain that binds the viral capsid of HIV-1 to restrict viral infection (Stremlau et al., 2004; Yap et al., 2005). Its sequence determines the specificity of TRIM5α for retroviruses. The variation of TRIM5α sequences between species, specifically in the B30.2 domain, leads to differences in the ability to restrict different retroviruses (Hatziioannou et al., 2004). TRIM5α restricts retrovirus infection early, prior to

reverse transcription, probably during the process of uncoating (Stremlau et al., 2004; Yan and Chen, 2012). The presence of an E3 ubiquitin ligase domain in TRIM5α (the RING domain) suggested that this protein mediates the proteasomal degradation of viral proteins, but mutations in this domain or the inhibition of proteasome activity do not abolish TRIM5α-mediated restriction (Javanbakht et al., 2005; Stremlau et al., 2006; Wu et al., 2006). It was proposed that TRIM5α binds the HIV-1 capsid and accelerates the uncoating of the viral capsid, which may affect viral infectivity (Stremlau et al., 2006). TRIM5α is also proposed to be a sensor of HIV-1 capsid that promotes innate immune signaling leading to Activator protein-1 (AP-1) and NF-κB activation (Pertel et al., 2011). The mechanism of TRIM5α-mediated restriction remains to be fully elucidated, even if all the mechanisms described here may contribute to this restriction. Even if it is clearly admitted that TRIM5α binds the capsid protein, this has not been demonstrated experimentally due to technical limitations. This binding seems important according to the discovery of TRIM5α-Cyclophilin A (CypA) (TRIMCyp) fusion protein in old world monkeys to resist HIV-1 infection (Sayah et al., 2004). Indeed, CypA has been reported to interact with the HIV-1 capsid and to facilitate HIV infection (Luban et al., 1993). TRIMCyp uses the ability of CypA to bind the capsid, thereby replacing the B30.2 domain, and inhibits HIV-1 replication. Interestingly, this fusion protein seems to have appeared twice independently among old world monkeys (Virgen et al., 2008; Wilson et al., 2008).

3.1.b. Inhibition of viral replication

Apolipoprotein B mRNA-editing catalytic polypeptide 3 (APOBEC3) family

The APOBEC3 family of cytidine deaminases (cytidine to uridine conversion) contains 7 members in primates (APOBEC3A, B, C, D/E, F, G and H). Surprisingly, only one gene is homolog to this family in mouse (mApobec3), indicating that the APOBEC3 locus has been under strong positive selection during evolution of primates (Sawyer et al., 2004). The discovery of this factor resulted from the observation that some cells were either permissive, either non permissive to an HIV mutant strain defective for the virion infectivity factor (Vif) (Gabuzda et al., 1992). The fusion of permissive and non permissive cells resulted in a non-permissive phenotype, suggesting that a cellular factor only expressed in non permissive cells was able to restrict infection by Vif-deficient HIV but not wild-type HIV (Madani and Kabat, 1998). This factor named CEM15 (and then renamed APOBEC3G) was one of the first

intrinsic antiviral factor identified for HIV-1 (Sheehy et al., 2002). The expression of this factor in permissive cells confers resistance to Vif-deficient HIV infection. Vif counteracts this restriction factor by preventing its incorporation into progeny virions and promoting its ubiquitination, leading to the degradation of APOBEC3G by the proteasome in infected cells (Mehle et al., 2004; Sheehy et al., 2003). Vif may also inhibit the packaging of APOBEC3G into HIV-1 virions (Kao et al., 2003; Mariani et al., 2003). APOBEC3G deaminates cytosine to uracil (C to U) during reverse transcription in the nascent retroviral DNA, generating a high level of Guanine to Adenine (G to A) mutations in the newly synthesized HIV genome, deleterious for the fitness of the virus (Mangeat et al., 2003; Zhang et al., 2003). In addition, the presence of U in the viral complementary DNA (cDNA) may be recognized and excised by Uracil-DNA glycosylase generating an abasic site that may be targeted by endonucleases (Harris et al., 2003). However, other studies have challenged this hypothesis as the antiviral effect of APOBEC3G on HIV-1 infectivity is not affected in cell lines deficient for the Uracil-DNA glycosylases UNG (Uracil-N glycosylase) and SMUG1 (Single-Strand-Selective Monofunctional Uracil-DNA Glycosylase 1) (Kaiser and Emerman, 2006; Langlois and Neuberger, 2008). Furthermore, there is mounting evidence that hypermutations may be dispensable for APOBEC-mediated antiviral activity (Holmes et al., 2007a). Indeed, APOBEC proteins mutated in their cytidine deaminase domains are still able to prevent the accumulation of reverse transcripts (Bishop et al., 2006; Holmes et al., 2007b). However, the relevance of this viral restriction mediated by deaminase-defective APOBEC3G is a matter of discussion *in vivo*, in physiological conditions, as the restriction mediated by the mutants is lower than ones of the full length APOBEC3G (Miyagi et al., 2007). APOBEC3G may also have other antiviral function than deamination, as inhibiting the provirus integration (Mbisa et al., 2007) or viral DNA synthesis (Bishop et al., 2008). APOBEC3G interacts with the nucleocapsid domain of the HIV-1 Gag polyprotein, triggering its incorporation into the HIV-1 particle (Alce and Popik, 2004; Cen et al., 2004). APOBEC3G is also able to restrict Hepatitis B virus (HBV) replication (Turelli et al., 2004). This restriction seems completely independent of the cytidine deaminase activity of APOBEC3G as deaminase-defective APOBEC3G conserve a wild-type level of antiviral activity against HBV and the HBV genome is no edited. The restriction may rather affect the packaging of the viral pregenomic RNA. However, other teams made contradictory observations, revealing that APOBEC3B, 3C, 3F and 3G can edit HBV genome and thereby may affect its replication (Suspène et al., 2005). Overall, the mechanism underlying HIV-1 and HBV restriction by APOBECs proteins

is still under debate. For a review on APOBECs, see (Vieira and Soares, 2013). Additionally, Human Papillomavirus (HPV) and Herpesvirus genomes are edited by APOBEC3 enzymes (Suspène et al., 2011; Vartanian et al., 2008).

SAM domain HD domain-containing protein 1 (SAMHD1)

SAMHD1 was identified as responsible of the non-permissiveness of dendritic cells and macrophages to HIV-1 infection (Hrecka et al., 2011; Laguette et al., 2011). Accordingly, permissive cell lines such as Jurkat do not express SAMHD1 whereas dendritic and other myeloid cells highly express SAMHD1. The lentiviral accessory protein Vpx binds and antagonizes SAMHD1-mediated restriction by inducing its ubiquitination by E3 ligases (DCAF1 and DDB-CUL4) and subsequent proteasomal degradation (Hrecka et al., 2011; Laguette et al., 2011). The depletion of SAMHD1 enhances HIV-1 replication whereas its overexpression restricts the infection (Hrecka et al., 2011; Laguette et al., 2011). SAMHD1 comprises a sterile alpha motif (SAM) and a histidine–aspartic (HD) domain. It is proposed that the HD domain displays a deoxyguanosine triphosphate (dGTP)-stimulated triphosphohydrolase activity that converts deoxynucleoside triphosphates (dNTPs) to deoxynucleoside (dN) and inorganic triphosphate. Thus, the depletion of dNTPs pool may restrict HIV-1 infection by inhibiting reverse transcription and viral cDNA synthesis (Goldstone et al., 2011). The expression of the sole HD domain of SAMHD1 is sufficient to restrict HIV-1 infection (White et al., 2013). By contrast, the SAM domain of SAMHD1 is dispensable for its antiviral activity. SAMHD1 has a broad anti-retroviral activity in myeloid cells (macrophages and dendritic cells) (Gramberg et al., 2013). Loss- and gain-of-function experiments indicate that SAMHD1 restricts Herpes Simplex Virus type 1 (HSV-1) replication and this restriction is partially reversed with supply of exogenous deoxynucleosides (Kim et al., 2013). Phosphorylation of SAMHD1 negatively regulates its antiviral function, at least for HIV-1 (Welbourn et al., 2013). Indeed, HSV-1 restriction is not affected by SAMHD1 phosphorylation, by contrast to HIV-1 restriction. Mutations in the *SAMHD1* gene are associated with Aicardi-Goutières Syndrome (AGS), an immune disorder characterized by an increased production of IFNs (Rice et al., 2009). Thus, a role of SAMHD1 as negative regulator of the innate immune response has been proposed.

Myxovirus resistance (Mx) protein

The IFN-inducible Mx proteins are large Guanosine triphosphatases (GTPases) that have protective effects against a large variety of viruses. Humans have two Mx genes, *MxA* and *MxB* (*Mx1* and *Mx2* in mice) (Aebi et al., 1989). The MxA protein is only cytoplasmic and the MxB protein exhibits different cellular localization depending on the cell type (Haller and Kochs, 2011). The antiviral mechanism of MxA protein remains to be elucidated. At present, it is proposed that MxA protein oligomerizes to form rings around the ribonucleocapsids of different viruses in order to block viral replication (Gao et al., 2011; von der Malsburg et al., 2011). A recent study reported that MxA prevents the transport of viral RNPs (vRNPs) to the nucleus, and thereby blocks IAV replication, which normally occurs in the nucleus (Xiao et al., 2013). However, if the depletion of MxA prevented the retention of vRNPs in the cytoplasm, gain-of-function experiments were not very convincing. It seems that MxA overexpression is not sufficient to retain vRNPs in the cytoplasm in absence of IFN pre-treatment, but rather only if cells are pre-treated with IFN. This may indicate that another ISG is required for this antiviral function of MxA. This strategy of trapping viral components in a place where they become unavailable for the viral infectious cycle seems common to other viruses. MxA blocks the transport of virus nuclecapsids from Thogoto virus into the nucleus, thereby preventing viral transcription (Kochs and Haller, 1999). Similarly, MxA binds the nucleocapsid protein of LACV that is redistributed in perinuclear areas (Kochs et al., 2002). MxA inhibits HBV replication by interacting with hepatitis B core antigen protein that is redistributed in perinuclear compartments (Li et al., 2012). The overexpression of human MxA protein confers resistance to VSV and IAV in a mouse cell line and Measle Virus (MV) and VSV in a human cell line (Schnorr et al., 1993). The inhibitory effect of MxA on VSV infection is directed at the level of viral transcription but the inhibition of MV is post-transcriptional, indicating a virus-specific effect of MxA protein. Mx1-loss-of function and Mx1-gain-of-function experiments in mice indicate that Mx1 plays a role in resistance to an Influenza-like virus (the Dhori virus) (Thimme et al., 1995). Surprisingly, mice and cells overexpressing the human MxA protein are protected from Thogoto virus, but not Dhori virus, infection (Frese et al., 1995; Thimme et al., 1995). The explanation for the difference in MxA, but not Mx1, sensitivity between two tick-borne orthomyxoviruses remains unclear. The overexpression of human MxA protein in Vero cells confers protection against members of *Bunyaviridae* family (Bunyaviruses, Phleboviruses, and Hantaviruses), blocking an early step of the viral cycle, probably viral RNA synthesis (Frese et al., 1996). Mx proteins are also able to inhibit dsRNA viruses and some DNA viruses (Mundt, 2007; Netherton et al., 2009).

According to the model proposed, it is still unclear how such diversity of viruses with divergent RNPs in sequence can all be antagonized by MxA. Interestingly, adaptive mutations in the nucleoproteins were found in pandemic strains of IAV and are correlated with an escape of MxA-mediated restriction (Mänz et al., 2013). Recently, an antiviral function was also reported for the MxB protein. MxB, but not MxA, inhibits HIV-1 replication and integration of HIV-1 DNA (Liu et al., 2013). Accordingly, the knockdown of MxB attenuates the antiviral effect of IFN on HIV-1 infection. Mutations in HIV-1 capsid allow the virus to escape MxB restriction and CypA-MxA fusion protein restricts HIV-1 infection, indicating that Mx proteins target the viral capsid to exert their antiviral activity. Intriguingly, the authors did not detect an interaction between MxB protein and Gag protein nor the mature capsid by co-immunoprecipitation. Concomitantly, another team confirmed that MxB suppresses HIV-1 infection by inhibiting the nuclear accumulation and integration of HIV-1 provirus and that viral capsid is a target of MxB (Goujon et al., 2013). The spectrum of MxB restriction seems to extend only to primate lentiviruses (HIV-1 and SIVs) and does not affect non-primate lentiviruses (MLV, Equine Infectious Anemia Virus (EIAV) and Feline immunodeficiency virus (FIV) or IAV. This contrasts with MxA, which represses IAV but not HIV-1 infection. Both Mx proteins have different viral specificity. This species-specific antiviral activity is determined by the loop L4 of MxA protein (Mitchell et al., 2012). Mx proteins are atypical restriction factors because they have a broad antiviral activity against RNA and DNA viruses whereas their antiviral activity seems to require the specific recognition of viral proteins from diverse family of viruses. This ambiguity remains to be elucidated. Complementary informations on MxA antiviral activity can be found in Mitchell et al., 2013.

TRIM19 or promyelocytic leukaemia (PML)

Another member of the TRIM family, named TRIM19 or PML, interferes with the infectious cycle of many DNA and RNA viruses (Nisole et al., 2005). PML localizes both in the nucleoplasm and in nuclear bodies (NBs), the latter localization is dependent of post-translational modifications by Small Ubiquitin-like MOdifier (SUMO-1) (Müller et al., 1998). PML is induced by IFN-I (Lavau et al., 1995). The overexpression of PML in CHO cells confers resistance to VSV and IAV, but not EMCV, infection (Chelbi-Alix et al., 1998). After Lassa virus and LCMV infection, PML has been shown to relocalize in the cytoplasm where it interacts with the Z protein of these viruses (Borden et al., 1998). Surprisingly, the

overexpression of PML in Hela cells does not affect Lassa virus and lymphocytic choriomeningitis virus (LCMV) replication whereas PML-deficient MEF cells or knockout mice display increased LCMV replication (Asper et al., 2004; Djavani et al., 2001). The authors propose that the basal expression of PML is sufficient to restrict infection by these two viruses, explaining why its overexpression does not affect infection whereas the depletion of basal expression impairs the antiviral response mediated by PML. The authors suggest that PML acts as a mediator of antiviral pathways rather than as a direct antiviral effector. This is supported by other groups reporting that PML knockout has positive effects on rabies virus and HSV replication but PML overexpression has no negative effects (Blondel et al., 2002; Chee et al., 2003; Lopez et al., 2002). PML also restricts retrovirus replication. HIV infection induces a cytoplasmic redistribution of PML that colocalizes with the incoming HIV-1 preintegration complex and thereby may inhibit HIV-1 replication (Turelli et al., 2001). This evidence is indirect, as it consists in arsenic treatment of cells, which sequesters PML in the nucleus and correlates with an increased HIV transduction efficiency. In contradiction with this observation, other groups did not observe changes in PML bodies after HIV-1 infection, nor any PML effect on HIV-1 infectivity (Berthoux et al., 2003). The overexpression of PML was shown to inhibit Human Foamy Virus (HFV) replication, and also transcription by interacting with the transcriptional transactivator (Tas) protein of HFV (Regad et al., 2001). Moreover, IFN treatment failed to inhibit HFV replication in PML-deficient MEF cells, suggesting that PML mediates the IFN-induced antiviral response against HFV. As mentioned above for HIV-1, the effect of PML on HFV is still debated (Meiering and Linial, 2003). Some viruses have developed strategies to counteract PML. Arenaviruses encode the Z protein that interacts with PML and induces its relocation from NBs to the cytoplasm (Borden et al., 1998). Both PML and Z protein are able to bind eIF4E to inhibit host translation (Kentsis et al., 2001).

Other members of the TRIM family restrict viral infection. For example, **TRIM1** restricts N-tropic MLV (Yap et al., 2005). **TRIM22** (also named Staf-50 for Stimulated Trans-Acting Factor of 50 kilodalton (kDa)) is an IFN-inducible factor that restricts HIV-1 by down regulating transcription mediated by the LTR promoter of HIV-1 (Tissot and Mechti, 1995). TRIM22 interacts and ubiquitinates the viral nucleoprotein of IAV, leading to its degradation by the proteasome and thereby inhibiting IAV replication (Di Pietro et al., 2013). TRIM22 confers protection against EMCV by interacting with 3C protease and inducing its

degradation (Eldin et al., 2009). Viral restriction by TRIM22 was recently reviewed in (Hattlmann et al., 2012). **TRIM79α** is an IFN-inducible gene that restricts Langat virus (LGTV) and tick-borne encephalitis virus (TBEV) replication by binding the NS5 protein and promoting its degradation (Taylor et al., 2011). It does not bind nor affect the replication of WNV, a member of the same *Flaviviridae* family, indicating that its action is virus-specific. A recent study reported that most members of E3 ligase TRIM family positively regulate signaling pathways triggering innate antiviral responses (Versteeg et al., 2013).

IFN-inducible IFI16 protein

The human IFI16 protein, initially reported as a viral DNA sensor (Unterholzner et al., 2010), is also a restriction factor for human cytomegalovirus (HCMV) (Gariano et al., 2012). The depletion of IFI16 increases HCMV replication whereas the overexpression of IFI16 strongly inhibits HCMV replication by affecting viral DNA synthesis.

3.1.c. Inhibition of viral translation

IFN-induced protein with tetratricopeptide repeats (IFITs)

The IFIT family comprises four members in humans: IFIT1 (ISG56), IFIT2 (ISG54), IFIT3 (ISG60) and IFIT5 (ISG58). Their particular tetratrico peptide repeats (TPRs) may be involved in protein-protein interactions and complex assembly (Yan and Chen, 2012). IFIT1 and IFIT2 interact with a subunit of the mammalian translation initiation factor eIF-3, thereby inhibiting overall cellular protein synthesis (Guo et al., 2000; Terenzi et al., 2006). This may contribute to the antiviral response triggered by IFN. The binding of IFIT1 on eIF3 inhibits HCV IRES translation (Wang et al., 2003). Both IFN-induced proteins PKR and IFIT1 participate in the blocking of initiation of viral RNA translation, by targeting eIF2 and eIF3, respectively. IFIT1 and IFIT2 expression is induced in the central nervous system after LCMV or WNV infection (Wacher et al., 2007). However, their antiviral function against these two viruses has not been directly addressed. IFIT1 binds to the viral protein E1 of HPV and inhibits its replication (Terenzi et al., 2008). The depletion of IFIT1 abrogates the restriction mediated by IFN on HPV DNA replication. Accordingly, the overexpression of IFIT1 inhibits HPV DNA replication. Another role of IFIT proteins is to recognize viral RNAs with 5'PPP at their extremity or that lack 2'-O-methyl (Daffis et al., 2010; Pichlmair et

al., 2011). Cellular mRNAs or self-RNAs usually contain a 5'-guanosine cap and are 2'-O-methylated whereas viral RNAs or non-self RNAs are not capped or methylated. In this sense, many RNA viruses encode a methyltransferase to methylate their viral RNA at the 2'-O position to mimic host mRNAs (Züst et al., 2011). WNV, poxvirus and coronavirus mutants lacking 2'-O-methyl transferase activity are more sensitive to the antiviral response mediated by IFN, and specifically by IFITs proteins (IFIT1 and IFIT2). The 2'-O-methylation of viral RNA is a strategy to escape IFIT-mediated antiviral activity (Daffis et al., 2010). Loss of IFIT family members in HeLa cells by RNAi increases VSV and RVFV viral titer, but does not affect EMCV titer. Among these three viruses, only EMCV does not generate 5'PPP-RNA during replication, suggesting that IFIT family contributes to the antiviral response to viruses generating 5'-PPP-RNAs (Pichlmair et al., 2011). Accordingly *in vivo*, IFIT1-deficient mice are hypersensitive to VSV, but not EMCV, infection. A recent study showed that IFIT1 preferentially binds 5' capped viral mRNAs without 2'-O methylation and inhibits their translation, thereby restricting infection by Japanese encephalitis virus (JEV) mutant in 2'-O methyltransferase (Kimura et al., 2013). Overall, in addition to RIG-I that senses 5'-PPP-RNA and induces the production of IFN-I, the induction of IFIT family expression by IFN creates a feedback mechanism for the sensing of 5'-PPP viral RNAs. By contrast, IFIT1 also interacts with the adaptor STING and prevents its interaction with IPS-1 or TANK-binding kinase 1 (TBK-1), thereby inhibiting the signaling cascade triggered by viral sensing (Li et al., 2009). It is proposed that IFIT1 plays the role of negative regulator of the viral-induced IFN-I response. Overall, the exact mechanism by which IFIT proteins restrict viral infection remains to be elucidated. Viral restriction by IFITs was recently reviewed in (Diamond and Farzan, 2013; Zhou et al., 2013).

Zinc-finger antiviral protein (ZAP)

ZAP was identified in a screen for mammalian cDNAs able to inhibit MLV infection (Gao et al., 2002). This protein displays a cluster of four CCCH-type zinc fingers, found in some RNA-binding proteins (Lai et al., 2000). The expression of ZAP is induced by IFN (Zhang et al., 2007) and was first shown to reduce viral RNA level in the cytoplasm (Gao et al., 2002). Further studies demonstrated that ZAP interacts via its zinc finger motifs with the 3'-LTR of MLV RNAs or SINV mRNAs and that this interaction is necessary for its antiviral function against both viruses (Guo et al., 2004). ZAP-antiviral activity is not restricted to retroviruses. The overexpression of ZAP affects members of the alphavirus genus, including

SINV, SFV, Ross River Virus (RRV) infection and Venezuelan Equine Encephalitis Virus (VEEV) replicon, but not HSV, Yellow Fever Virus (YFV), VSV, and Poliovirus replicon (Bick et al., 2003). The expression of ZAP does not affect SINV binding onto the cell surface but inhibits SINV replication and the translation of a SINV-luciferase reporter defective for replication. ZAP is also reported to inhibit filoviruses replication (EBOV and MARV) by decreasing the level of viral mRNAs (Müller et al., 2007). These studies suggest that ZAP targets viral RNA for degradation. This hypothesis is supported by the demonstration that ZAP interacts with components of the exosome (Rrp40p and Rrp46p), an RNA processing complex containing multiple 3'-5' exoribonucleases (Guo et al., 2007; Mitchell et al., 1997). The depletion of Rrp40p and Rrp46p by RNAi reduced the antiviral activity of ZAP against MLV, suggesting that the exosome is required for ZAP-mediated antiviral activity (Guo et al., 2007). Only recently, it was shown that ZAP localizes in RNA granules where it recruits MLV transcripts and the exosome component EXOSC5 to induce the degradation of MLV transcripts (Lee et al., 2013).The authors show that ZAP antiviral activity is independent of RLRs in MEF cells, whereas a previous study showed that ZAP is a positive regulator of RIG-I signaling in human cells (Hayakawa et al., 2011). The DEXH box RNA helicase DHX30 is a cellular factor required for ZAP antiviral activity (Ye et al., 2010). The crystal structure of Rat ZAP has been resolved and showed that ZAP forms a dimer to bind one RNA molecule (Chen et al., 2012). However, the motifs or structures on viral transcripts that are recognized by ZAP are still unknown.

Protein kinase R (PKR)

Another well characterized IFN-induced antiviral effector, the protein kinase PKR, is activated by viral dsRNAs and phosphorylates the initiation factor eIF2α, resulting in a rapid inhibition of translation of viral and host mRNAs (Meurs et al., 1990; Pindel and Sadler, 2011). The overexpression of PKR limits EMCV, but not VSV, replication in cells (Meurs et al., 1992). In addition, PKR-deficient cells are more sensitive to EMCV infection compared to control cells, and display an impaired induction of IFNs after EMCV infection (Der and Lau, 1995). NS1-deficient Influenza virus can replicate in PKR-deficient mice, but not in wild-type mice, indicating that PKR controls Influenza virus infection and its antiviral activity is counteracted by the NS1 protein (Bergmann et al., 2000). PKR also contributes to the protection against WNV infection (Samuel et al., 2006), Human T-cell leukemia virus type I (HTLV-1) and HTLV-2 infection (Cachat et al., 2013).

3.1.d. Inhibition of viral release

Tetherin (or BST-2 or CD317)

Viral protein U (Vpu) is required for efficient HIV-1 particle release in HeLa cells but not in COS-7 cells. Heterokaryons resulting from the fusion of both cell lines exhibit similar restriction of viral particles release than Hela cells, suggesting that Hela cells, but not COS-7 cells, express a dominant acting intrinsic antiviral factor (Varthakavi et al., 2003). This restriction factor is effective only against Vpu-defective HIV-1, and does not affect wild-type HIV-1 replication. The overexpression of Vpu in Hela cells or heterokaryon reverses the restriction. Tetherin is an IFN-inducible protein that was identified by microarray analysis of permissive and non-permissive cells as a factor that restricts HIV-1 budding from infected cells (Van Damme et al., 2008; Neil et al., 2008). The accessory protein Vpu counteracts its antiviral function. Tetherin is constitutively expressed but is also induced by IFN (Neil et al., 2008). The protein is localized at the plasma membrane, in endosomes and in vesicles of the trans-Golgi network (Habermann et al., 2010), and co-localize with budding virions. It is proposed that the unique topology of Tetherin with one TM domain and a glycosylphosphatidylinositol (GPI) anchor could tether cellular and viral membranes through their cholesterol-enriched lipid rafts (Evans et al., 2010; Kupzig et al., 2003). Accordingly, many envelope viruses including HIV-1, IAV or EBOV bud from lipid rafts (Nguyen and Hildreth, 2000; Panchal et al., 2003; Scheiffele et al., 1999). This hypothesis is challenged by a recent study on HIV-1 where the depletion of cellular cholesterol does not affect the antiviral activity of Tetherin (Grover et al., 2013). In addition, HIV-1 Gag protein does not colocalize with lipid raft markers, suggesting that lipid rafts are not involved, at least for HIV-1, in Tetherin-mediated inhibition of viral release. The unusual topology of Tetherin and its ability to dimerize suggest two possibilities of action : Tetherin could hold virions at the cell surface either by inserting a GPI anchor into the virion envelope or by dimerization, with one Tetherin molecule anchored in the cell membrane and the other one in the virion envelope (Evans et al., 2010). Retained virions are either internalized by endocytosis and the association of Tetherin with the E3 ubiquitin ligase BCA2 (Breast cancer-associated gene 2) directs tethered virions to lysosomes for degradation, or they remain at the cell surface (Miyakawa et al., 2009; Neil et al., 2006). These models of restriction suggest that Tetherin has a broad antiviral activity against enveloped viruses. Indeed, it was reported to restrict

retroviruses, filoviruses, arenaviruses and herpesviruses (Evans et al., 2010). The team of Bieniasz originally demonstrated by designing an artificial Tetherin lacking sequence homology but with the same structural features than native Tetherin that the protein displays autonomously and directly its antiviral activity (Perez-Caballero et al., 2009). The overexpression of Tetherin decreases DENV release from Huh-7 cells (Pan et al., 2012). CHIKV virus-like particles (VLPs) are retained by Tetherin at the cell surface and both CHIKV E1 glycoprotein and nsP1 protein colocalize and interact with Tetherin (Jones et al., 2013). They also show that nsP1 antagonizes Tetherin restriction by reducing the expression of Tetherin. Tetherin-mediated restriction is antagonized by the K5 protein of Kaposi's sarcoma-associated herpesvirus (KSHV), which ubiquitinates Tetherin to induce its degradation by the proteasome. The glycoprotein of EBOV also inhibits its antiviral function. The mechanism by which Vpu antagonizes Tetherin is unknown. One group observed by flow cytometry a down-regulation of Tetherin from the cell surface in cells expressing HIV-1 Vpu (Van Damme et al., 2008). A recent paper also suggests that Vpu expression directs internalized Tetherin preferentially to lysosomal degradation rather than recycling at the cell surface (Rollason et al., 2013). Independently of this mechanism, Vpu also interacts directly with Tetherin to displace it from sites of virion assembly (McNatt et al., 2013). Recent studies have highlighted that Tetherin can also act as a viral sensor that induces proinflammatory response through activation of NF-κB (Galão et al., 2012; Hotter et al., 2013; Tokarev et al., 2013). Viral restriction by Tetherin was recently reviewed in (Neil, 2013; Swiecki et al., 2013).

IFN-stimulated gene 15 (ISG15)

ISG15 protein is a 15 kDa ubiquitin-like protein (Narasimhan et al., 2005). Like ubiquitin, ISG15 conjugates with proteins via its C-terminal motif (LRLRGG). This conjugation is called ISGylation. ISG15 conjugation cascade is catalyzed by a serie of enzymes: E1 (Ubiquitin Activating Enzyme E1 Like Protein (UBE1L)), E2 (Ubiquitin-conjugating Enzyme H8 (UbcH8)) and two E3 ligases (estrogen-responsive finger protein (EFP) and HECT domain and RCC1-like domain containing protein 5 (Herc5)) as well as a deconjugating enzyme (Ubiquitin protease 43 (UBP43)) (Lenschow et al., 2007). It is unclear if ISG15 conjugation, like ubiquitin, results in the degradation of the targeted proteins. These ISGylated proteins were identified by mass spectrometry and cover a large spectrum of biological processes such as translation or stress response (Giannakopoulos et al., 2005).

Interestingly, IFN-induced antiviral effectors (MxA, PKR, RNase L) and components of immune signaling pathways (RIG-I, Jak1, STAT1) are also suggested to be ISGylated (Malakhov et al., 2003; Zhao et al., 2005). The treatment of cells with proteasome inhibitor does not accumulate ISGylated proteins in the cells, indicating that conjugation of ISG15 onto proteins does not lead to proteasomal-degradation. ISG15 has an antiviral activity, but its mechanism of action is unclear. ISG15-deficient mice are highly sensitive to both RNA and DNA virus infections, including Influenza A and B virus, HSV-1, SINV (Lenschow et al., 2007). In addition, this susceptibility is rescued after infection by recombinant SINV expressing wild-type ISG15, but not an ISG15 mutant for conjugation. Mice deficient for the E1 ligase UBE1L, necessary for ISG15 conjugation to targeted proteins, are hypersensitive to Influenza B virus suggesting that ISG15 conjugation is important for its antiviral activity (Lai et al., 2009). In agreement with this hypothesis, ISG15 is conjugated on NS1 protein of IAV and inhibits its function (Tang et al., 2010; Zhao et al., 2010). Similarly, the HPV capsid protein is ISGylated and incorporated in the released virus that is less infectious, perhaps because ISGylation destabilizes the viral particle (Durfee et al., 2010). It is not clear if ISGylation of host proteins, viral proteins (NS1, IAV) or both contribute to antiviral activity. Interestingly, the overexpression of ISG15 in cells blocks the release of HIV-1 retrovirus and EBOV from cells by either ISGylating components of the Endosomal sorting complex required for transport (ESCRT) pathway, or inhibiting the ubiquitination of HIV-1 Gag or Ebola VP40 proteins, which are both processes required for virus-budding (Seo and Leis, 2012). Thus, the current model is that ISG15 inhibits virus release by affecting the virus-budding events but the exact mechanism of its antiviral activity remains to be clarified. A recent study revealed that ISGylation of PKR by ISG15 activates PKR in gain-of-function experiments (Okumura et al., 2013), but the physiological relevance of this finding remains to be determined. ISG15-deficient mice, but not UBE1L-deficient mice, are highly sensitive to CHIKV (Werneke et al., 2011). This sensitivity is not associated with an increased viral titer (indicating that ISG15 does not control viral replication) but rather correlated with a high level of proinflammatory cytokines and chemokines. By contrast with previous studies, this finding suggests that the antiviral activity of ISG15 is independent from conjugation, but may rather play the role of an immunomodulatory cytokine. Indeed, it has been reported that ISG15 can be secreted and function like a cytokine (D'Cunha et al., 1996a, 1996b). Viral restriction by ISG15 was recently reviewed in Morales and Lenschow, 2013.

3.1.e. Multiple step antiviral function

Virus-inhibitory protein, endoplasmic reticulum–associated, IFN-inducible (Viperin)

Viperin is composed of three domains: an amphipathic α-helix domain at its N-terminus, involved in the targeting of Viperin to the cytosolic face of the ER and lipid droplets; a conserved central domain containing a CxxxCxxC motif, and related to domain present in S-adenosylmethionine (*SAM*) enzymes; and a conserved C-terminal domain (Seo et al., 2011a). Viperin is an IFN-inducible protein that localizes in the ER where it interacts with Farnesyl Diphosphate Synthase (FPPS) to inhibit isoprenoid-derived lipids biosynthesis. This inhibition leads to changes in membrane fluidity and lipid raft microdomains, thus affecting the budding of Influenza virus, but not VSV, particles from infected cells (Wang et al., 2007). The overexpression of FPPS reverses the inhibition of IAV release and restores normal membrane fluidity whereas its knockdown inhibits Influenza replication and release. Accordingly, VSV does not bud from lipid rafts and therefore its replication is not affected by Viperin expression. Viperin restricts WNV pathogenesis as Viperin-deficient mice display an increased lethality and the viral replication is enhanced in central nervous system tissues (Szretter et al., 2011). The overexpression of Viperin in cells restricts CHIKV infection and the antiviral activity is conferred by the N-terminal amphipathic α-helical domain (Teng et al., 2012). Accordingly, mice deficient for *Rsad2* (Radical S-adenosyl methionine domain-containing protein 2), the gene encoding Viperin, display an increased viremia and joint inflammation. If the colocalization between Viperin and ER marker is clear, the colocalization with the virus is not obvious and remains to be reevaluated. Loss- and gain-of-function experiments indicate that Viperin confers protection against DENV-2 infection, and its C-terminal region display its antiviral activity (Helbig et al., 2013). DENV-2 infection induces Viperin that colocalizes with lipid droplets and also the viral capsid and NS3 protein. Viperin inhibits DENV-2 replication and interacts with viral RNAs, but also the viral capsid and NS3 protein. HIV-1 induces Viperin expression in macrophages and its depletion increases HIV-1 replication (Nasr et al., 2012). The overexpression of full-length Viperin, but not Viperin mutants in *SAM* domain, decreases HIV-1 production and release from HEK293T cells. HIV-1 infection redistributes Viperin from ER into foci which colocalize with two markers of HIV-1 replication and assembly, p24 antigen and CD81. *SAM* mutants do not colocalize with HIV-1 anymore. As for IAV, farnesol treatment reverses Viperin-mediated restriction of HIV-1, indicating that Viperin may inhibit FPPS, thereby disrupting lipid raft and virus budding.

Another study showed that the expression of Viperin does not seem to restrict lentiviruses, except the LAI strain of HIV-1 and more significantly HIV-1 Negative Regulatory Factor (Nef) mutant. This indicates that the HIV-1 Nef protein abrogates this restriction, but Viperin depletion does not affect HIV-1 LAI infection (Lim et al., 2012). The N-terminal amphipathic alpha-helix of Viperin and HCV NS5A protein directs their localization to lipid droplets and this region is sufficient to direct dsRed to lipid droplets (Hinson and Cresswell, 2009a). This region of Viperin is also important for its localization to ER and Viperin-mediated inhibition of protein secretion, suggesting that it may interfere with the transport of viral proteins (Hinson and Cresswell, 2009b). Viperin depletion increases HCV replication in IFN-stimulated Huh-7 cells and its overexpression decreases HCV replication (Helbig et al., 2011). Viperin interacts with HCV core and NS5A proteins on the surface of lipid droplets. The N-terminal region of Viperin is important for its localization to lipid droplets and the C-terminal region confers its antiviral activity. Viperin interacts with VAP-A (vesicle-associated membrane protein-associated protein subtype A also named hVAP-33), a cellular cofactor that normally interacts with NS5A and NS5B to facilitate the formation of replication complexes (Gao et al., 2004). This suggests that Viperin may affect HCV RNA replication. A recent study confirmed that Viperin affects HCV RNA replication by using HCV replicon system and that Viperin binds hVAP-33 via its C-terminal region, probably by competition with NS5A (Wang et al., 2012b). Intriguingly, Viperin enhances HCMV infectivity (Seo et al., 2011b). A recent study reported that lipogenesis induced during HCMV infection is dependent on Viperin expression on mitochondria and required for the formation of HCMV envelope and the production of infectious particles (Seo and Cresswell, 2013). Viperin is also reported to be involved in the signaling between TLR7 and TLR9 activation and production of type I IFN (Saitoh et al., 2011). Loss- and gain-of-function experiments indicate that Viperin does not affect flavivirus JEV infection but the treatment of infected cells with proteasome inhibitor restores high level of Viperin expression and reveals its antiviral effect on JEV (Chan et al., 2008). JEV counteracts the antiviral function of Viperin through its degradation. To summarize, Viperin targets two steps of virus life cycle. It disrupts lipid rafts to block virus budding and release, and also interferes with replication of some viruses. Viral restriction by Viperin was recently reviewed (Mattijssen and Pruijn, 2012; Seo et al., 2011a).

3.1.f. OAS/RNase L: Intrinsic antiviral pathway

2'-5'-Oligoadenylate synthetase (OAS) / RNase L system

The IFN-inducible protein OAS is activated by viral dsRNAs and produces 2'-5' oligoadenylates that bind to the inactive monomeric form of the endoribonuclease RNase L. This binding induces RNase L dimerization and activation, leading to the degradation of viral RNAs and also host ribosomal RNAs (rRNAs) to stop viral replication (Carroll et al., 1996; Dong and Silverman, 1995). Humans encode three OAS genes (OAS1, OAS2 and OAS3), and one OAS-like gene (OASL). In mice, OAS family is composed of seven OAS1-type genes, OAS2, OAS3 and 2 OASL-type genes (Kakuta et al., 2002). The OAS/RNase L system has a broad antiviral activity. The overexpression of OAS protein confers resistance to a Picornavirus (Mengo virus), but not VSV, infection in a hamster cell line (Chebath et al., 1987). Similarly, a human cell line overexpressing OAS protein is resistant to EMCV infection, and displays a reduced cell proliferation rate (Rysiecki et al., 1989). In addition, loss-of-function experiments by overexpression of a dominant negative RNase L mutant in cells increases the susceptibility to EMCV, but not VSV, infection and the antiproliferative effect is counterbalanced (Hassel et al., 1993). RNase L has specific antiviral activity and may also be involved in the regulation of cell growth. The overexpression of OAS and RNase L proteins blocks Vaccinia Virus replication (Díaz-Guerra et al., 1997). RNase L-deficient mice are more susceptible to EMCV and WNV infection (Samuel et al., 2006; Zhou et al., 1997). The susceptibility of mice to WNV infection is correlated with a nonsense mutation in the gene encoding the isoform L1 of 2′-5′-oligoadenylate synthetases, suggesting that this enzyme restricts WNV infection (Mashimo et al., 2002; Perelygin et al., 2002). The OAS/RNase L system contributes to the antiviral defense in many ways, in addition to its RNAs degradation activity (Chakrabarti et al., 2011). For example, it can induce apoptosis in virus-infected cells (Castelli et al., 1997; Zhou et al., 1997). Moreover, the small RNA products resulting from the cleavage by RNase L can be used as substrates by the RIG-I sensor to amplify the production of IFNs (Malathi et al., 2007, 2010). The OAS/RNase L pathway is negatively regulated by a 2'-phosphodiesterase (2'-PDE) which degrades 2'-5' oligoadenylates (Kubota et al., 2004). The depletion of 2'-PDE reduces Vaccinia Virus replication. Interestingly, this strategy is also used by viruses to counteract the activation of RNase L. The NS2 protein of mouse hepatitis virus (MHV) (coronavirus) and VP3 protein of rotavirus display PDE activity, which cleaves and inhibits the accumulation of 2'-5' oligoadenylates, preventing the activation of RNase L (Zhang et al., 2013; Zhao et al., 2012).

Overall, restriction factors can have a broad or virus-specific antiviral activity. For example, TRIM5α seems to be only active against retroviruses, because its antiviral activity relies on the specific interaction with the capsid of retroviruses. By contrast, the unique feature of Tetherin, which creates a bridge between the envelope of virus particles and the plasma membrane, can prevent viral release of enveloped viruses independently of their family of origin. It is possible that not a single but several viral restriction factors act in concert to face viral infections in cells (Jiang et al., 2010). Indeed, four ISGs that target different steps of the virus cycle (IFITM2, IFITM3, Viperin and ISG20) were shown to inhibit both WNV and DENV infections. Some restriction factors may have other cellular functions than viral restriction, such as TRIM5α that may have a role in intracellular signaling (Pertel et al., 2011). Most viruses have evolved to counteract restriction factors activity. For example, the accessory genes of retroviruses are required only for effective replication in cells expressing a restriction factor (Malim and Emerman, 2008). One strategy used by Vpx and Vif accessory proteins of lentiviruses is to direct the proteasomal degradation of the restriction factor, SAMHD1 and APOBEC3G, respectively (Laguette et al., 2011; Yu et al., 2003). Vpu protein uses another strategy to antagonize Tetherin by altering its normal localization from the plasma membrane to endosomes (Kueck and Neil, 2012). Viruses can also escape viral restriction by mutations in the protein targeted by the restriction factor, as exemplified by mutations in the capsid of lentivirus, which allow evasion from TRIM5α restriction (Hatziioannou et al., 2006). Another strategy used by Poxvirus is to express the K3L protein, a mimic of eIF2α that compete with normal eIF2α for the binding to PKR (Dar and Sicheri, 2002). K3L prevents the phosphorylation of eIF2α and the host translation shutoff. The genes encoding restriction factors evolve under positive selection, displaying an excess of non-synonymous mutations compared with synonymous mutations (reviewed in Duggal and Emerman, 2012). The duplication and diversification of genes encoding restriction factors allow to adapt to many viruses, as observed for the primate APOBEC family (Sawyer et al., 2004) and IFITM family (Siegrist et al., 2011).

Identification of novel viral restriction factors

Two studies aimed to uncover new viral restriction factors by performing a screen to examine the antiviral activity of hundreds of ISGs after infection by several viruses including HCV, YFV, WNV, CHIKV, VEEV, HIV-1 (Schoggins et al., 2011) and VSV and Murine GammaHerpes Virus (MHV) (Liu et al., 2012). These studies revealed new ISGs with

hitherto unknown antiviral properties such as C6orf150 that inhibits alphaviruses CHIKV and VEEV, and TAP1 (Transporter associated with antigen processing 1 gene), BMP2 (Bone morphogenetic protein 2) that inhibit MHV. Cell-based screening strategies were recently reviewed in (Panda and Cherry, 2012). Recently, the group of Dr. Tenoever used an original approach to identify new viral restriction factors *in vivo* (Varble et al., 2013). They infected mice with a library of engineered RNA viruses (SINV) expressing siRNAs to screen for host genes restricting viral infection. After 48h of infection, the analysis of small RNAs from the spleen of infected animals reveals which siRNA allowed viruses to take precedence over the others, suggesting a role for the targeted gene in antiviral response. They identified and characterized Mga and Zfx, two novel genes involved in antiviral signaling.

3.2. Viral restriction factors in *Drosophila ?*

Refractory for Sigma P virus (Ref(2)P)

The control of SIGMAV infection was reported to involve 5 loci on different chromosomes (Gay, 1978). Among the identified loci, Ref(2)p is the best characterized. Initially, two alleles were described: a permissive allele (ref(2)Po) and a restrictive allele (ref(2)Pp) (Contamine et al., 1989). Flies carrying a ref(2)Po allele are infected by SIGMAV whereas flies with ref(2)Pp allele are refractory to SIGMAV infection. Transgenic flies carrying the genomic sequence of Ref(2)P restrictive allele are resistant to SIGMAV infection (mentioned in Carré-Mlouka et al.,2007). These results suggest that Ref(2)P is a viral restriction factor for SIGMAV in *Drosophila*. The analysis of restrictive and permissive alleles of Ref(2)P allowed to determine that polymorphisms in the PB1 domain are associated with the control of SIGMAV infection (Carré-Mlouka et al., 2007; Wayne et al., 1996). Surprisingly, flies with permissive alleles are more susceptible to SIGMAV infection than flies with null alleles (Carré-Mlouka et al., 2007), which is counterintuitive with the role of Ref(2)P as a restriction factor, except if permissive alleles have a dominant negative effect on SIGMAV infection. The Ref(2)P protein interacts with N and P proteins of SIGMAV by co-immunoprecipitation (Wyers et al., 1993). The antiviral mechanism is not known. Ref(2)P is the *Drosophila* homolog of mammalian P62 and localizes to protein aggregates in autophagy-deficient flies and in models of human neurodegenerative diseases (Nezis et al., 2008). Given the homology of Ref(2)P with autophagic protein, it would be interesting to test if autophagy

is involved in Ref(2)P-mediated restriction of SIGMAV infection. Additionally, Ref(2)P was shown to form a complex with the *Drosophila* atypical protein kinase C (DaPKC) that positively regulates the Toll-signaling pathway and induces Drosomycin expression (Avila et al., 2002; Goto et al., 2003). This raises the hypothesis that Ref(2)P may restrict SIGMAV infection through the activation of the Toll pathway.

Vago

The expression of Vago is dependent of Dicer-2, but not Ago-2, suggesting that Dicer-2 may have additional antiviral functions than the generation of RISC in the canonical RNAi pathway (Deddouche et al., 2008). Vago is specifically upregulated in the fat body after viral infection (DCV and SINV), but not infection by FHV or a bacterial mixture. Vago-mutant flies accumulate more DCV RNA in the fat body, consistent with a role of Vago in antiviral defense. An antiviral function of a mosquito ortholog of *Drosophila* Vago, named *Culex* Vago (CxVago), has been reported in *Culex* mosquito cells (Paradkar et al., 2012). WNV infection induces the expression of CxVago but the depletion of Dcr-2 (and not R2D2) by RNAi reduces its up-regulation, indicating that CxVago is up-regulated in a Dcr-2 dependent manner. The overexpression of CxVago reduces WNV replication and viral particles secretion. Loss- and gain-of-function experiments indicate that Vago has antiviral activity in mosquito cells. Furthermore, this restriction is mediated by the secretion of CxVago. The treatment of cells with the supernatant of CxVago-overexpressing cells protects from WNV infection in mosquito cells but not African green monkey kidney cells (Vero). This result suggests that secreted CxVago does not directly target WNV but may activate an insect-specific signaling pathway to mediate its antiviral activity. Interestingly, the upregulation of the Jak-STAT marker vir-1, but not the NF-κB marker defensin-A, was observed after treatment of mosquito cells with the supernatant of CxVago-overexpressing cells, indicating that secreted CxVago activates the Jak-STAT pathway. These observations do not fit with their counterparts in *Drosophila* because vir-1 expression was still induced in Vago-mutant flies, suggesting that either vir-1 expression can be induced by another protein than Vago, or that the function of Vago as a circulating cytokine in mosquitoes is not conserved in *Drosophila*. Furthermore, the depletion of CxSTAT2 and CxJak by RNAi abolishes the upregulation of vir-1 and increases the viral titer. However, the CxVago-mediated activation of the Jak-STAT pathway is independent of the *Culex* ortholog of *Drosophila* Domeless, the receptor activating the Jak-STAT pathway after binding of the ligand Unpaired (Upd). This

activation by an unknown receptor may induce an antiviral response to WNV. It is tempting to assimilate the role of Vago in insects to IFNs in mammals, both activating the Jak-STAT pathway to initiate an antiviral response.

Vir-1

In *Drosophila* S2 cells, the expression of the gene vir-1 is under the control of the Jak-STAT pathway in response to DCV infection. The first evidence is the presence of STAT-binding sites in the promoter of vir-1 gene and the second evidence is that flies deficient for the Jak kinase Hopscotch display a reduced expression of vir-1 (Dostert et al., 2005). However, the knockdown or overexpression of vir-1 did not affect DCV infection. Accordingly, the overexpression of CxVir-1 in mosquito cells does not affect the WNV titer (Paradkar et al., 2012).

Overall, the intrinsic antiviral immunity in *Drosophila* clearly remains to be elucidated. Although many transcriptional profiles obtained after viral infection reveal the upregulation of genes specifically after viral infection, their role in the antiviral response has not been addressed. *Drosophila* Vago and vir-1 were thought to be good candidate for the identification of viral effectors in *Drosophila* but do not appear to have a direct antiviral function. Viral restriction factors in *Drosophila*, and also in other insects, remains to be discovered and characterized.

Aim of the study

Two types of responses are involved in the control of viral infections in *Drosophila*. An inducible response and RNAi that is a broad antiviral defense against RNA viruses, including the picorna-like DCV. This virus is broadly used to study antiviral responses in *Drosophila*, particularly in our laboratory. We observed a difference of susceptibility between laboratory control flies, characterized by an increased viral titer and lethality after challenge with this virus. A difference of susceptibility to DCV infection was also observed between two stocks from the same wild-type strain (*Oregon-R*), maintained independently in two teams from our laboratory. The major aim of my PhD thesis was to study the cause of the sensitivity of some *Drosophila* strains to DCV infection. This work is presented in **Chapter 4**. Additionaly, we noticed that some strains maintained in the laboratory were persistently infected by Nora virus, another related picorna-like virus. Surprisingly, many of these strains were sensitive to DCV, suggesting that Nora virus may be the cause of this susceptibility. We decided to investigate if the presence of Nora virus affects the susceptibility of flies to other pathogens. This part of my work is discussed in **Chapter 5**. During my thesis, I also participated to the characterization of the role of the siRNA pathway in the defense against a DNA virus (IIV-6). This work led to a publication in Journal of Immunology and is summarized in **Chapter 6**.

Part II

MATERIAL AND METHODS

II.1. Fly strains and infection

Wild-type flies (*Oregon-R*, *Canton-S*) and mutant flies carrying phenotypically visible genetic markers (*yw*, w^{1118}, w^{A5001}, *DD1 cnbw*) were used in this study. These flies are generally used as control in experiments performed in the laboratory. For RNAi experiments, we used flies with *Actin-Gal4;Tubulin-Gal80, Cg-Gal4*, and *Np-Gal4* driver, the Pst-RNAi line purchased from the Vienna *Drosophila* RNAi Center collection (ID107243) and a UAS-GFP-RNAi line constructed in the laboratory. Dr. Stéphane Noselli provided UAS-Pst-GFP fly line. All flies used were Wolbachia-free. Flies were fed on standard cornmeal–agar medium at 25°C. Crossings were performed at 25°C, except ones with *Actin-Gal4;Tubulin-Gal80* driver that were done at 18°C, and then the progeny was transferred for 4 days at 29°C.

For viral infections, viral stocks were prepared in 10 mM Tris-HCl, pH 7.5 with the exception of VSV, which was used directly from Vero R cells culture supernatant. Infections were performed with 4–8 days old adult flies by intrathoracic injection (Nanoject II apparatus, Drummond Scientific) with viral particles indicated in the figure legends. Injection of the same volume of 10 mM Tris-HCl, pH 7.5, was used as a control. Infected flies were then incubated at 22°C or 25°C and monitored each day for survival, or frozen for RNA isolation and virus titration at indicated time points.

Contamination of flies with Nora virus was performed by putting 200 males naturally infected by Nora virus, in a big vial for 5 days at 25°C. Flies were then removed and replaced by 50 males and 50 females of non infected flies. After 5 days, the tube was emptied and flies were monitored for Nora virus titer (sample “parents”). Once the progeny emerged, 0-4 days old flies were transferred to a fresh tube for 4 days and then monitored for virus titer or used for experiments (sample “F1”).

II.2. Dechorionation of eggs

The dechorionation of eggs to cure flies from Nora Virus was performed as follows: Nora virus infected flies were put in a cage on apple juice agar plate with a yeast paste on the middle, for them to lay eggs. The cages were transferred overnight at 25°C. Eggs were collected, washed with water, and dechorionated with a solution of 50% bleach for 3 min with

constant flowing by pipetting the solution up and down. Then, eggs were abundantly rinsed with water, aligned under the microscope on a piece of agar medium and transferred by capillarity on a coverslip. One drop of mineral oil was applied to cover the eggs and the coverslip was deposited on a petri dish with normal *Drosophila* food. After 2-3 days, larvae were transferred to normal food vial. Once flies emerged, they were tested for Nora Virus.

II.3. Cell lines and virus infection

Drosophila S2 cells and Kc167 cells were grown in Schneider's medium (Biowest) supplemented with 10% fetal calf serum, 1x Glutamax (100x, 200 mM, Invitrogen), 1x Penicillin/Streptomycin (100x mix, 10 mg/ml/ 10000 U, Invitrogen).

Stable cell lines were established as follows: Day 1: plasmid expressing RFP fusion (1µg), plasmid expressing puromycin (0.2µg), salmon sperm (4µg) and $CaCl_2$ (2M) were mixed together and added on a solution of 2x HEPES-buffered saline (HEBS). After 30 min incubation at room temperature, the mixture was deposited drop by drop on *Drosophila* S2 cells in a 6-cm petri dish. Day 2: cells were washed once with PBS and complete media was added. Day 3: Cells were counted, resuspended in complete medium supplemented with puromycin antibiotic, and distributed in three 96-well plates at different concentrations (1000 cells/well, 10000 cells/well, 20000 cells/well). Then, fresh medium with puromycin was added every two days to maintain selection. Wells where clones appeared were selected and clones were then transferred to 24-well plate. Clones were screened by FACS analysis thanks to RFP (FACS ARIA II) and the ones harboring an homogenous expression of the fusion protein were amplified in cell culture for viral experiments, and backed up in liquid nitrogen.

Viral infections were performed as follows. The volume of viral suspension was calculated depending on the multiplicity of infection (MOI) desired (indicated in the figure legends) and then added to cells for 2 hours. Cells were then washed twice with Schneider media and complete medium was added. Incubation times depended on the experiment (see figure legends).

II.4. Virus binding and entry assays

Cells were infected at a MOI of 20 for DCV or 0.5 for CrPV at 4°C for 2 h to allow virus binding at the cell surface but not the internalization of viral particles. At 4°C, endocytosis mechanism is blocked. Next, cells were washed twice with PBS and proceed for RNA analysis (binding assay) or transferred to 25°C for entry assay. Cells were incubated at different time points, as indicated in the figure legends. Then, cells were washed twice with PBS and proceed for RNA analysis.

II.5. Transfection, Luciferase assay, secretion assay

Transfection

Drosophila S2 cells were transfected with plasmid DNAs either by the CaPO4 precipitation method, or by Effecten transfection reagent (Qiagen). This is indicated in the figure legends.

Luciferase assay

Drosophila S2 cells treated with dsRNAs or stable cell lines were transfected by Effecten with plasmid reporters to monitor the activity of the two CrPV IRES. The day after, reporter expression was induced by addition of $CuSO_4$ (500 µM). After 48 hours, cells were lysed and luciferase activity was measured by luminometer with Dual-Luciferase® Reporter Assay System (Promega).

Secretion assay

Cells were transfected with vector expressing secretion signal (ss) fused to Horseradish peroxidase (ss-HRP), under the control of methallothionein promoter (). Thus, cells were treated with 500 µM $CuSO_4$ for 16h. Then, 10µl of supernatant were transferred into a 96-wells black plate containing 50µl of ECL reagent per well. The luminescence was measured by luminometer.

II.6. Gene silencing: dsRNA synthesis and treatment

Template DNAs were generated from *Drosophila* cDNAs by PCR with primers carrying the T7 polymerase sequence (**Table 3**). Their size was verified on agarose gel and they were purified with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). Then, *in vitro* transcription by T7 polymerase from T7 megascript kit (Ambion®) allowed to generate dsRNAs targeting our genes of interest. For precipitation, RNAs were mixed with sodium acetate/ ethanol and incubated for 15 min at -20°C. After centrifugation for 20 min at 14000 rpm and 4°C, RNAs were washed with ethanol 70%, centrifuged 5 min at 14000 rpm and 4°C and then, the pellet was dried and RNAs were reconstituted in nuclease free water. dsRNAs were verified on agarose gel and quantified. dsRNAs (3µg/well) were mixed with S2 cells without serum in 96-well plate. After 2 hours incubation, complete medium was added and cells were incubated for 5 days at 25°C. After this treatment, cells were challenged with viruses.

Primers	Sequence
Pst A Fwd	5'-taatacgactcactatagggGATACTCATTCGCACCCGAT-3'
Pst A Rev	5'-taatacgactcactatagggAGTTGCACGTCCTCGAAGTT-3'
Pst B Fwd	5'-taatacgactcactatagggGGCAGTAGTGGCACCAGCAGC-3'
Pst B Rev	5'-taatacgactcactatagggCACCTCGCGTCCAGCATTTGTG-3'
Syntaxin 5 Fwd	5'-taatacgactcactatagggAAAAAGAAGAGCTTATTTGATGA-3'
Syntaxin 5 Rev	5'-taatacgactcactatagggCTCAACGATGGTAGATTCTATAT-3'
beta-cop Fwd	5'-taatacgactcactatagggTTTCGGAGTGCGTCAAAAC-3'
beta-cop Rev	5'-taatacgactcactatagggCTCTTTAACCAGAGACATGTTG-3'
Bmm A Fwd	5'- taatacgactcactatagggCTGCGGATTCCTGGGCAT -3'
Bmm A Rev	5'- taatacgactcactatagggGCAAATCGCAGAGGAGACAG -3'
Bmm B Fwd	5'- taatacgactcactatagggAAGCGGGTGAATGGACGACTG -3'
Bmm B Rev	5'- taatacgactcactatagggCGCACGAAGCGATTGATGTTCT -3'
Mdy A Fwd	5'-taatacgactcactatagggAACCGCAAGTCAACACAAAA-3'
Mdy A Rev	5'-taatacgactcactatagggCAAACGCAGACCTCCAATG-3'
Mdy B Fwd	5'-taatacgactcactatagggGGAGTTTTGAATGGAGGTGAA-3'
Mdy B Rev	5'-taatacgactcactatagggGCTCAGTGGCTAAGGCCAC-3'

Lsd-1 A Fwd	5'-taatacgactcactatagggCGGCAGTGGACTCCATCT-3'
Lsd-1 A Rev	5'-taatacgactcactatagggCGGCAGATAGACCAGTGGTA-3'
Lsd-2 A Fwd	5'-taatacgactcactatagggAAATCGTGTCTTCGAATGGG-3'
Lsd-2 A Rev	5'-taatacgactcactatagggTCTTCCTCGACATCCGACTC-3'
Lsd-2 B Fwd	5'-taatacgactcactatagggGGCATTATCGATAGGGAACTC-3'
Lsd-2 B Rev	5'-taatacgactcactatagggCATGTGGCAAGGCAACAACG-3'

Table 3. List of primers used for dsRNA synthesis.

II.7. Virus titration

The supernatant of DCV infected cells was titrated on Kc167 cells. Cells were seeded in 96-well plates (Falcon). The supernatant was serially 10-fold diluted with full Schneider media. The media was removed from the seeded cells and 50 µl of the serially diluted virus inoculum was added (each dilution in quadruplicate). After 2 days of incubation at 25°C, free virus was removed. Next, cells were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature. Afterwards, cells were washed twice with PBT solution. Then blocking solution (1x PBT, 10% fetal calf serum (PerBio)) was added and incubated for 30 min at room temperature. The primary antibody was added for 2 h at room temperature (Rabbit anti-DCV F3 1:500). Cells were then washed twice as described above, before the secondary antibody (goat-anti-rabbit-Alexa488) was added for 1 h at room temperature. Cells were again washed twice with 1x PBT and dried. Positive and negative stained wells were identified using a fluorescent microscope. Then, the titer was determined by the Reed–Muench method to calculate 50% tissue culture–infective dose and converted to PFU with a conversion factor of 0.7.

II.8. Cloning by Gateway system

Pastrel cDNAs were amplified from *DD1 cnbw* flies by standard PCR with primers containing AttB sites (**Table 4**). PCR products were cut on electrophoresis gel and purified with NucleoSpin® Gel and PCR Clean-up kit (Machery-Nagel). PCR products flanked by

AttB sites were then inserted in an entry vector (pDONR-221, Invitrogen) containing AttP sites by recombination with Gateway® BP Clonase® II enzyme (Invitrogen). DH5-α bacteria were transformed and selected on LB agar mixed with kanamycine (50 µg/ml). Minipreps were verified by restriction enzymes and selected minipreps were sequenced. The sequencing revealed that it is the sequence of the isoform Pst-PE that was cloned for overexpression experiments. It seems to be the predominant isoform of Pst because most entry vectors that we sequenced corresponded to this isoform. The entry vector containing the insert of interest was recombined with a destination vector (pARW or pAWR, from *Drosophila* Gateway Vector Collection) by using Gateway® LR Clonase® II enzyme (Invitrogen). These destination vectors contain actin promoter, a cassette containing the ccdB gene flanked by AttR sites and the RFP tag in N-terminal (pARW) or C-terminal (pAWR). DH5-α bacteria were transformed and selected on LB agar mixed with antibiotics. The vectors expressing Pastrel sensitive version (3L:7350895 T (Thr)) were constructed by site directed mutagenesis using the QuickChange® Site-Directed Mutagenesis Kit (Stratagen). Vectors used for Pastrel topology were constructed by amplifying cassettes HA-Pst or Pst-HA (FLAG-Toll for control) with primers containing AttB sites (**Table 4**) and proceeded for gateway cloning with destination vectors containing the Biotin Acceptor Peptide (BAP, GLNDIFEAQKIEWHE) in N-terminal (pHF6 vector) or C-terminal (pHF7 vector) of insert cassette. Cassettes containing HA-Pst-BAP, BAP-Pst-HA, BAP-FLAG-Toll and FLAG-Toll-BAP were then cloned in pAc5.1/V5-His A vector (Invitrogen).

Primers	Sequence
Pst N-ter fusion Fwd	5'-GGGGacaagtttgtacaaaaaagcaggctTCGCGAATAGATTAGGAAACC-3'
Pst N-ter fusion Rev	5'-GGG GaccactttgtacaagaaagctgggtCTTATCACTATTACAAGGGACAAACGCTGAAG-3'
Pst C-ter fusion Fwd	5'-GGGGacaagtttgtacaaaaaagcaggctGCCACCATGGCGAATAGATTAGGAAACCATGA-3'
Pst C-ter fusion Rev	5'-GGGGaccactttgtacaagaaagctgggtCCAAGGGACAAACGCTGAAGTGG-3'
Flag-Toll N-ter fusion Fwd	5'-GGGGacaagtttgtacaaaaaagcaggctTCTCTGCACTTCTGATCCTAGCTCTTGTTG-3'
Flag-Toll N-ter fusion Rev	5'-GGGGaccactttgtacaagaaagctgggtCTTATCACTATACGTCGCTCTGTTTGGCATTCGTG-3'
Flag-Toll C-ter fusion Fwd	5'-GGGGacaagtttgtacaaaaaagcaggctGCCACCATGTCTGCACTTCTGATCCTAGCTCTTGTTG-3'
Flag-Toll C-ter fusion Rev	5'-GGGGaccactttgtacaagaaagctgggtCTACGTCGCTCTGTTTGGCATTCGTG-3'
Pst-HA N-ter fusion Rev	5'-GGGGaccactttgtacaagaaagctgggtCTTATCACTACGTGGACCGGTGTCCGCCATGAGCA-3'

HA-Pst C-ter fusion Fwd	5'-GGGGacaagtttgtacaaaaaagcaggctGCCACCATGGATCTCCACCGCGGTGGAGGCCGCATC-3'
Pst$_{\Delta C\text{-}ter}$ N-ter fusion Rev	5'-GGGGaccactttgtacaagaaagctgggtCTTATCACTAGATTTTTCCGTATTTCTCCAGTACG-3'
Pst T521 mutagenesis Fwd	5'-catataatgctattgaccagaaccttcatggacaccatgc-3'
Pst T521 mutagenesis Rev	5'-gcatggtgtccatgaaggttctggtcaatagcattatatg-3'
2bases+KpnI+pHF6/7 Fwd	5'-GGGGTACCGCATCTGTTCGAATTTAAAGC-3'
10bases+NotI+PHF7 Rev	5'-TTTTCCTTTTGCGGCCGCTTATCACTATTCGTGCCATTC-3'
10bases+NotI+PHF6 Rev	5'-TTTTCCTTTTGCGGCCGCTTATCACTACGTGGACCGGTG-3'

Table 4. List of primers used for molecular cloning.

II.9. DNA analysis

II.10.1. DNA extraction

Flies were crushed in a solution of Spermidine/Spermine/Sarkosyl. The homogenate was filtered and centrifuged for 7 min at 7000 rpm, at 4°C. The liquid was removed and the pellet washed with homogenization solution. Then, the pellet was resuspended in 1.8 ml of homogenization solution, 200 µl of Sarkosyl and 10 µl of Proteinase K and incubated overnight at 55°C. Genomic DNAs were then extracted with classic phenol-chloroform procedure and precipitated with ethanol. DNAs were resuspended in TE buffer and quantified with the Nanodrop spectrophotometer.

II.10.2. Preparation for *pastrel* gene sequencing

For the sequencing of *pastrel* gene, PCR amplification was performed with Phusion® High-Fidelity DNA polymerase (NEB). 10 µl of 5x Phusion Buffer HF, 5 µl of dNTPs (2mM), 1 µl of forward and 1 µl of reverse primers, 30.5 µl of water and 0.5 µl of Phusion DNA polymerase were added to 2 µl of genomic DNA (100 ng/µl). The following program was performed on a thermocycler T3000 (Biometra): step 1: 2 min at 98°C, step 2: 10 sec at 98°C, step 3: 30 sec at 62°C, step 4: 2 min at 72°C, go to step 2 and repeat 35 times, step 5: 10 min at 72°C, step 6: pause at 16°C. PCR products were quantified with Nanodrop

spectrophotometer and control quality was performed on 1% agarose gel. Samples were then sent for sequencing with GATC Company.

II.10.3. Southern blot

Genomic DNAs and plasmid control were digested with restriction enzymes *PvuII*, *EcoRI*, *SalI* and *PstI* (Invitrogen) for 4 h at 37°C, and treated with RNase. After migration on agarose gel for 24h at 25 volts, DNAs were transferred to nylon membrane overnight in 10x SSC solution. The membrane was prehybrided 1 h at 60°C under rotation. To generate DCV probes, DCV RNAs were extracted with QIAmp Viral RNA Mini Kit (Qiagen) from viral stock, reverse transcribed with Brilliant II kit (Stratagen) and amplified with Phusion High-Fidelity DNA Polymerase (NEB). 4 probes were generated to cover the entire genome sequence and radiolabelled with RediPrime II DNA labelling system (Amersham). After prehybridation, radiolabelled probe was incubated with the membrane for 1 h at 60°C. Next, the membrane washed twice with 2x SSC 0.1% SDS solution for 15 min at 60°C and again twice with 0,1% SSC 0,1% SDS solution. Revelation was performed on BioImager FLA-5000 SERIES.

II.10.4. PCR genotyping

Single flies were crushed in 50 µl of "Squishing buffer" (10 mM of Tris-HCl, pH 8.2, 1mM of EDTA, 25mM of NaCl and 200 µg/mL of Proteinase K). The homogenate was incubated 30 min at 37°C and the Proteinase K was then inactivated at 95°C for 5 min. Then PCR was performed to genotype sensitive or resistant alleles of *pastrel*. The primers were designed with the Web-based Allele-Specific PCR assay (WASP) designing tool (Wangkumhang et al., 2007). Two sets of primers were designed: one set to amplify the sensitive allele of *pastrel* (T in position 521 on the gene) and one set to amplify the resistant allele (C in position 521 on the gene) (**Table 5**). For PCR control, we amplified the gene encoding the ribosomal protein *RpL32*. Fragments were amplified by Taq DNA polymerase (Invitrogen) with the following PCR program: Step 1: 94°C for 2 min, step 2: 94°C for 10 sec, step 3: 58°C for 15 sec, step 4: 72°C for 20 sec, go to step 2 and repeat 30 times, step 5: 72°C

for 2 min, step 6: 16°C pause. PCR products were migrated on 2% agarose gel. The expected bands are 116 bp for pastrel fragment and 500 bp for RpL32 fragment.

Primers	Sequence
RpL32 Fwd (IMU85)	5'-GTGTATTCCGACCACGTTACA-3'
RpL32 Rev (IMU86)	5'-ATACAGGCCCAAGATCGTGA-3'
Pst (521T) Fwd	5'-CAGCATGGTGTCCATGAAGTT-3'
Pst (521C) Rev	5'-CAGCATGGTGTCCATGAAGTC-3'
Pst Rev	5'-ACGTGATCAATGCTGAAAGT-3'

Table 5. List of primers used for PCR genotyping.

II.11. RNA analysis

II.11.1. RNA extraction

Total RNA were isolated from samples of 6 flies with a mixture 1:20 of TRI Reagent® RT (MRC) and phase separation reagent bromoanisole (MRC). Flies were crushed, tubes were vortexed for 30 sec and incubated at room temperature for 5 min. Tubes were next centrifuged for 15 min at 12500 rpm, at 4°C. The aqueous phase was transferred into a new tube containing 300 µl of Isopropanol. Tubes were vortexed, incubated for 10 min at room temperature and next centrifuged for 10 min at 12500 rpm at 4◦C. Supernatants were removed and the pellet washed with 70% of ethanol. After 5 min of centrifugation at 7500 rpm at 4°C, supernatants were removed and pellet were dried 10 min at 65°C. RNAs were resuspended in RNase-free water and quantified with the Nanodrop spectrophotometer.

II.11.2. cDNA synthesis

cDNAs were synthesized from total RNAs with the iScript™ cDNA Synthesis kit (Biorad). 1 µg of total RNAs was mixed with 4 µl of 5x Reaction mix, 1 µl of reverse transcriptase and nuclease-free water in a total reaction volume of 20 µl. The following program was performed on a thermocycler T3000 (Biometra): step 1: 5 min at 65°C, step 2: 5

min at 4°C, step 3: 25 min at 42°C, step 4: 15 min at 70°C, step 5: pause at 16°C. cDNAs were then diluted 20 times for qRT-PCR analysis.

II.11.3. Quantitative real time PCR

Viral RNA levels in flies were measured by qPCR from samples with 6 individuals (3 males and 3 females), using the iQTM Custom SYBR Green Supermix kit (Biorad). 2.4 µl of Supermix, 0.3 µl of forward and 0.3 µl of reverse primers, 5 µl of water were added to 20 times diluted cDNA in a total volume of 10 µl. The qPCR was performed on a CFX384 Touch™ Real-Time PCR platform (Bio-Rad), with the following program: step 1: 3 min at 95°C, step 2: 10 sec at 95°C, step 3: 30 sec at 55°C. Go to step 2 and repeat 39 times.

Viral RNA levels in cells were measured by qRT-PCR using Power SYBR Green Cell-to-CT kit (Ambion®), according to the manufacturer's instructions, on a CFX384 Touch™ Real-Time PCR platform (Bio-Rad). This kit combines all tools for RNA extraction, cDNA synthesis and qPCR.

Sequences of primers used for qPCR are indicated in **Table 6**. In all cases, gene expression was normalized to the expression of the ribosomal protein-coding gene *RpL32*.

Primers	Sequence
RpL32 Fwd	5'-GACGCTTCAAGGGACAGTATCTG-3'
RpL32 Rev	5'-AAACGCGGTTCTGCATGAG-3'
DCV Fwd	5'-TCATCGGTATGCACATTGCT-3'
DCV Rev	5'-CGCATAACCATGCTCTTCTG-3'
CrPV Fwd	5'-GCTGAAACGTTCAACGCATA-3'
CrPV Rev	5'-CCACTTGCTCCATTTGGTTT-3'
FHV Fwd	5'-TTTAGAGCACATGCGTCCAG-3'
FHV Rev	5'-CGCTCACTTTCTTCGGGTTA-3'
VSV Fwd	5'-CATGATCCTGCTCTTCGTCA-3'
VSV Rev	5'-TGCAAGCCCGGTATCTTATC-3'

SINV Fwd	5'-CAAATGTGCCACAGATACCG-3'
SINV Rev	5'-ATACCCTGCCCTTTCAACAA-3'
IIV-6 Fwd	5'-TTGTTAGGAATTGGAACTGGAA-3'
IIV-6 Rev	5'-GCCCTAGATGCTGCTTGTTC-3'
Nora Virus Fwd	5'-AACCTCGTAGCAATCCTCTCAAG-3'
Nora Virus Rev	5'-TTCTTGTCCGGTGTATCCTGTATC-3'

Table 2. List of primers used for qPCR.

II.12. Protein analysis

II.12.1. Protein extraction

Cells were homogenized in Cell Extraction Buffer (CEB, Invitrogen) mixed with 1 dose of protease inhibitor (PI) cocktail complete mini (Roche). After 30 min incubation on ice (vortexing every 10 min), cell lysate was centrifuged for 10 min at 14000 rpm and the supernatant was collected in a new tube (cell lysate, « input »). Proteins were quantified by Bradford Protein assay (Biorad). Samples were mixed with 5x Laemmli loading buffer (400 mM Tris Base, 8% LiDS, 30% Glycerol, 408 mM MAC, 0.1% bromophenol blue, final pH 6.8), incubated for 5 min at 95°C and applied to 12% SDS-PAGE gel.

II.12.2. Immnoprecipitation

50 µl of Anti-FLAG beads (Clontech) or anti-HA beads (Sigma) were mixed with 200 ul of Ab binding buffer (from Dynabeads® Protein G Immunoprecipitation Kit, invitrogen). Beads were resuspended in 200 µL Ab binding buffer and incubated for 20 min at room temperature under rotation. Then, beads were resuspended in 250 µL of CEB+PI and mixed with 250 µl of cell lysate 1h30 at 4°C for anti-FLAG beads or overnight at 4°C for anti-HA beads. After incubation, beads were washed three times with Washing buffer (from Dynabeads® Protein G Immunoprecipitation Kit, invitrogen) and resuspended in 40 µl of 5x Laemmli loading buffer. After vortexing, beads were incubated at 95°C for 3 min, centrifuged 3 min at 2000 rpm and the supernatant was collected in a new tube (« IP »).

II.12.3. Western blot

Migration of samples was performed in 1x TCG buffer (Tris/Glycine/SDS Buffer, Biorad) for 1h30 at 130 volts. Proteins were transferred to nitrocellulose membrane for 2 h at 25 volts with wet transfer system (Invitrogen). Then, the membrane was saturated for 1 h at room temperature in 3% milk (Biorad) diluted in TBST 1x (10x TBST pH 7.6 containing TrisHCl pH 7.2 100mM, NaCl 90 g/L and 0.5% Tween-20). The primary antibody was incubated overnight at 4°C in TBST 1x 0.3% milk. The membrane was washed three times for 10 min with TBST 1x and the secondary antibody was added in TBST 1x for 1 h at room temperature. After incubation, the membrane was washed three times with TBST 1x and incubated with ECL™ (Prime) Western Blotting Detection Reagent (Amersham™).

Polyclonal rabbit antiserums directed against DCV (anti-DCV-F3) and Pastrel, and the anti-actin monoclonal antibody (Millipore) were used in a dilution of 1:5000. A mouse antiserum directed against DCV (anti-DCV-1) was also used, mentioned in the figure legend. Rabbit anti-FLAG (Abcam) and mouse anti-HA (Sigma) antibodies were used in a dilution of 1:2000 and 1:2500 respectively. Secondary antibodies against mouse or rabbit (Fisher Scientific) conjugated to horseradish peroxidase were used at the dilutions of 1:5000 and 1:1000 respectively.

For biotinylation procedure, the membrane was saturated overnight at 4°C in 10% milk (Biorad) diluted in TBST 1x. After incubation, the membrane was washed three times with TBST 1x for 10 min at room temperature and 5 ml of Streptavidine-POD (ROCHE) 1:5000 diluted in TBST 1x - 2% BSA was added onto the membrane at 4°C. After 5 min of incubation, the membrane was washed three times with TBST 1x for 10 min and next two times with PBS for 10 min at room temperature. Then, the revelation was performed as described above.

II.12.4. Immunostaining

Cells were fixed for 10 min in 4% solution of paraformaldehyde (PFA). After two washes with PBS 1x – 0.1% Triton-x-100 (PBT) solution, blocking was performed by

incubation of the fixed cells with PBT supplemented with 10% Fetal Calf Serum for 30 min. Cells were then incubated for 2h at room temperature with primary antibodies. Cells were washed twice with PBT and then incubated with a secondary antibody for 1h at room temperature. For immunofluorescence assays, images were acquired using confocal LSM700 and analyzed with ImageJ. For flow cytometry analysis, cells were passed on FACS ARIA II and data were analyzed with FlowJo software.

Drosophila fat body were dissected in PBS and transferred in 4% paraformaldehyde for 30 min. Fat bodies were washed three times for 5 min with PBS-Tween 0.2% and next incubated for 1h in a solution of PBS-Tween 1%-BSA 2%. Primary antibodies were diluted in PBS-Tween 0.2%-BSA 2% and added to the tube. After overnight incubation at 4°C, fat bodies were washed three times for 5 min with PBS-Tween 0.2% and incubated with secondary antibodies diluted in PBS-Tween 0.2% for 2h at room temperature. Fat bodies were washed three times, incubated with Nile Red solution at a concentration of 1µM for 5 min at room temperature and then mounted into Vectashield containing DAPI.

Primary antibodies used for immunostaining were a mouse antiserum directed against DCV (anti-DCV-1) or CrPV (anti-CrPV-4), produced in the laboratory; a rabbit anti-serum directed against Pastrel (provided by Dr. Stéphane Noselli), a mouse anti-FHV antiserum (from Dr. Annette Schneeman), a goat anti-VSV-G-FITC antibody and an anti-beta-cop antibody (Abcam). We used secondary antibodies coupled with Alexa Fluor 488, 568 or 647 (Invitrogen).

To induce the formation of lipid droplets in *Drosophila* S2 cells and thereby facilitate observations, complete medium was supplemented with 400 µM Sodium Oleate (Sigma) / BSA 0.5%.

II.13. Statistical analysis

An unpaired two-tailed Student *t* test was used for statistical analysis of data with GraphPad Prism (GraphPad Software). The *p* values lower than 0.05 were considered statistically significant. Survival curves were plotted and analyzed by log-rank analysis (Kaplan–Meier method) using GraphPad Prism (GraphPad Software).

Part III

RESULTS

Chapter 4

Pastrel: a restriction factor for picorna-like viruses in *Drosophila*

Powerful genetic tools available in *Drosophila* combined with a large panel of model viruses able to infect fruit fly, provides a good model to identify and characterize new genes involved in antiviral defense. Viral restriction factors are pooly identified and characterized in *Drosophila*, as discussed in the Chapter 3 of the Introduction. This contrasts with the characterization of a larger number of genes restricting viral infection in mammals.

4.1. Research of the cause of susceptibility of flies to DCV infection: Identification of *pastrel* gene.

Differences of susceptibility of flies to DCV infection *in vivo* were suspected from previous studies performed in the laboratory. Strikingly, this variability in the susceptibility to DCV infection concerned not only fly lines used as control in the laboratory, but also different stocks of the same control line. In the course of the experiments carried in the laboratory to characterize the impact of mutations on resistance to DCV infection, we noticed differences in the susceptibility to infection of the lines used as wild-type controls. These correspond to different wild-type strains (*Ore-R*, *Canton-S*), or mutant flies carrying useful phenotypically visible genetic markers frequently used in our experiments (e.g., white (w^1), cinnabar (*cn*), brown (*bw*)). We therefore decided to verify this variability, and identify its cause.

4.1.a. Wide variability in the susceptibility of laboratory control flies to DCV, but not CrPV and FHV, infection.

We first confirmed that laboratory control flies showed differences in term of susceptibility to DCV infection. We collected control flies available in the laboratory and infected them by intra-thoracic injection of DCV (500 PFU). We monitored survival daily at 22°C and measured virus load after 1, 2, 3 and 4 days post-infection (**Figure 11A** and **11B**). *yw* and w^{A5001} flies were more susceptible to DCV infection compared to *Canton-S* and *DD1 cnbw* flies (**Figure 11A**). *Canton-S* and *DD1 cnbw* flies died 4 days later than *yw* and w^{A5001} flies. This difference of susceptibility to DCV infection was correlated with the DCV RNA level (**Figure 11B**). *yw* and w^{A5001} flies have higher DCV RNA levels during the course of the infection (days 2, 3 and 4) compared to *Canton-S* and *DD1 cnbw* flies (**Figure 11B**). Then we wondered whether differences in susceptibility between laboratory control flies also

concerned viruses other than DCV. We observed that susceptibility to Cricket Paralysis Virus (CrPV), a virus whose genome is closely related to DCV genome, and Flock House Virus (FHV) infection was similar among control flies, except for *DD1 cnbw* flies which displayed a small increase in sensitivity to CrPV infection, but not reflected on the virus titre (**Figure 11C** and **11E**). All flies died in 14 days after CrPV infection (5 PFU) and 12 days after FHV infection (500 PFU). The similar pattern of susceptibility between *yw*, w^{A5001}, *Canton-S* and *DD1 cnbw* flies to CrPV and FHV infection is correlated with similar viral RNA levels during the course of the infection (**Figure 11D** for CrPV and **11F** for FHV). No statistical difference was observed for CrPV RNA level at 1, 2 and 3 days post infection and FHV RNA level at 2, 4, 6 and 8 days post infection between *yw*, w^{A5001} flies (sensitive to DCV infection) and *Canton-S*, *DD1 cnbw* flies (resistant to DCV infection).

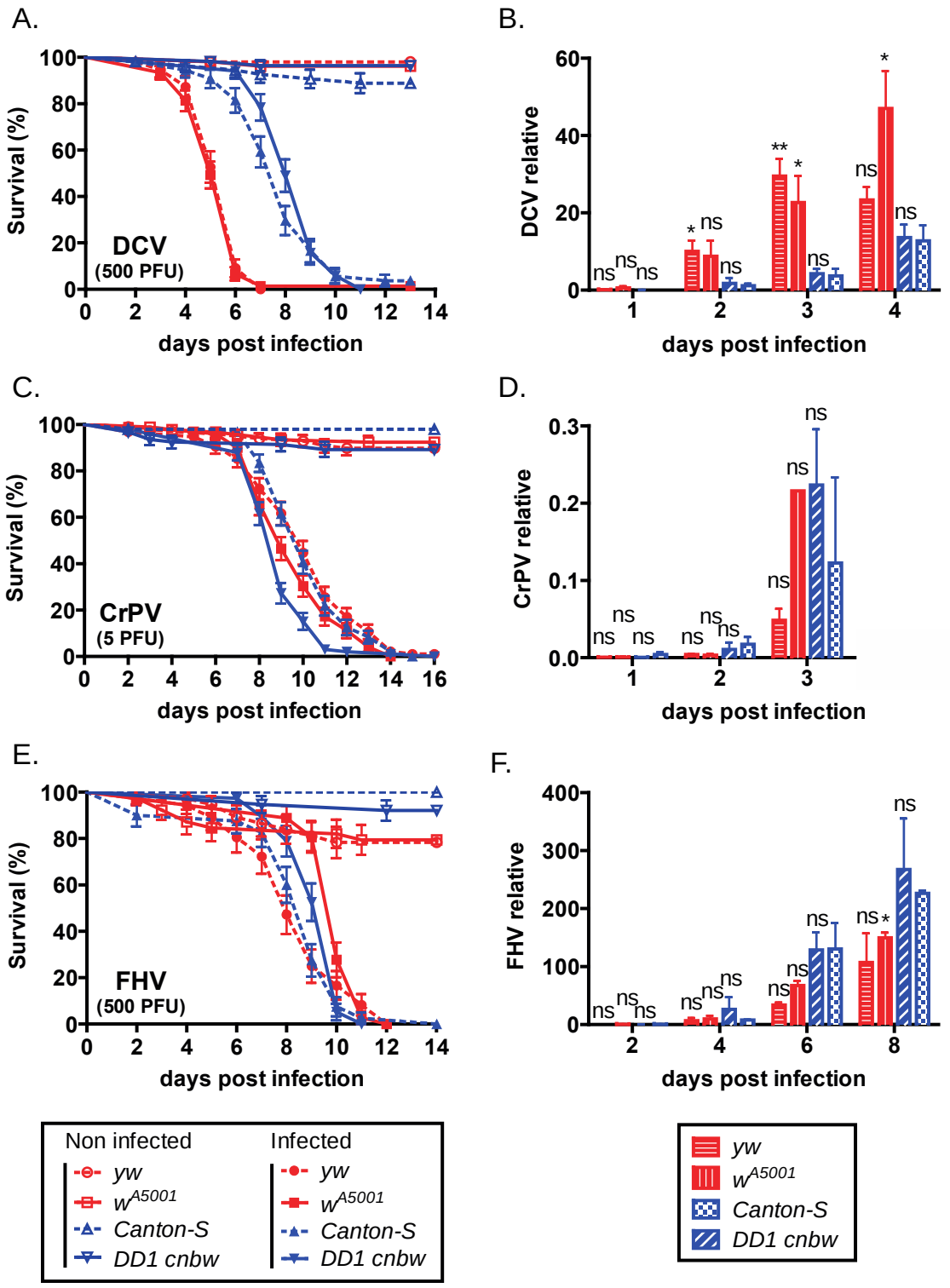


Figure 11. Wide variability in the susceptibility to DCV infection, but not to CrPV and FHV infection, between laboratory control flies. A. Flies (4-8 days) were infected with DCV (500 PFU) and survival was monitored daily at 22°C. Bar graphs represent the average standard deviation of 3 independent experiments. **B.** DCV RNA level was measured by qPCR after 1, 2, 3 and 4 days of infection. Bar graphs represent the average standard deviation of 3 independent experiments. **C.** Flies (4-8 days) were infected with CrPV (5 PFU) and survival was monitored daily at 22°C. Bar graphs represent the average standard deviation of 5 independent experiments. **D.** CrPV RNA level was measured by qPCR after 1, 2, and 3 days

of infection. Bar graphs represent the average standard deviation of 2 independent experiments. **E.** Flies (4-8 days) were infected with FHV (500 PFU) and survival was monitored daily at 22°C. Bar graphs represent the average standard deviation of 2 independent experiments. **F.** FHV RNA level was measured by qPCR after 2, 4, 6 and 8 days of infection. Bar graphs represent the average standard deviation of 2 independent experiments. For panels **B.**, **D.** and **F.**, qPCR values were normalized to the housekeeping gene *RpL32*. *Canton-S* flies were used as reference for statistical analysis.

Altogether, we observed that *yw* and w^{A5001} flies were higher sensitive to DCV infection, but not CrPV and FHV infection than *Canton-S* and *DD1 cnbw* flies. We also showed that w^{1118} flies had comparable susceptibility to DCV, CrPV and FHV infection than *yw* and w^{A5001} flies (**data not shown**).

4.1.b. Wide variability in the susceptibility of two *Ore-R* stocks to picorna-like viruses DCV and CrPV, and DNA virus IIV-6 infection, but not FHV, VSV and SINV infection.

Our data indicate that flies routinely used as controls in our experiments vary widely in their sensitivity to DCV infection, for unknown reasons. While these experiments were ongoing, one post-doctoral fellow in the laboratory noticed that the wild-type *Ore-R* strain she was using in her infections was highly susceptible to DCV infection (Dr. Stefanie Mueller, personnal communication). These *Ore-R* flies had been maintained in the laboratory of Dr. Dominique Ferrandon, and will thereafter be referred to as $Ore\text{-}R^{DF}$. We compared their resistance to DCV infection to that of an independent stock which had been maintained in our group ($Ore\text{-}R^{JLI}$). We observed a wide difference in the susceptibility of flies to DCV infection between two stocks of Oregon flies ($Ore\text{-}R^{DF}$ and $Ore\text{-}R^{JLI}$, **Figure 12A**). $Ore\text{-}R^{JLI}$ flies died 3 days later than $Ore\text{-}R^{DF}$ flies after intrathoracic injection of DCV (500 PFU) at 22°C (**Figure 12A**). The higher resistance of $Ore\text{-}R^{JLI}$ flies to DCV infection was correlated with a lower DCV RNA level (6.35-fold) at 3 days post infection (**Figure 12A**). We observed that $Ore\text{-}R^{JLI}$ flies were also more resistant to CrPV infection (5 PFU) compared to $Ore\text{-}R^{DF}$ flies and showed higher CrPV RNA levels after 3 days of infection (**Figure 12B**). Even if it is not significative in the format of our experiments, we observed a decreased CrPV RNA level in $Ore\text{-}R^{JLI}$ flies compared to $Ore\text{-}R^{DF}$ flies. In summary, $Ore\text{-}R^{JLI}$ flies are more resistant than $Ore\text{-}R^{DF}$ flies to infection by *Dicistroviridae* DCV and CrPV.

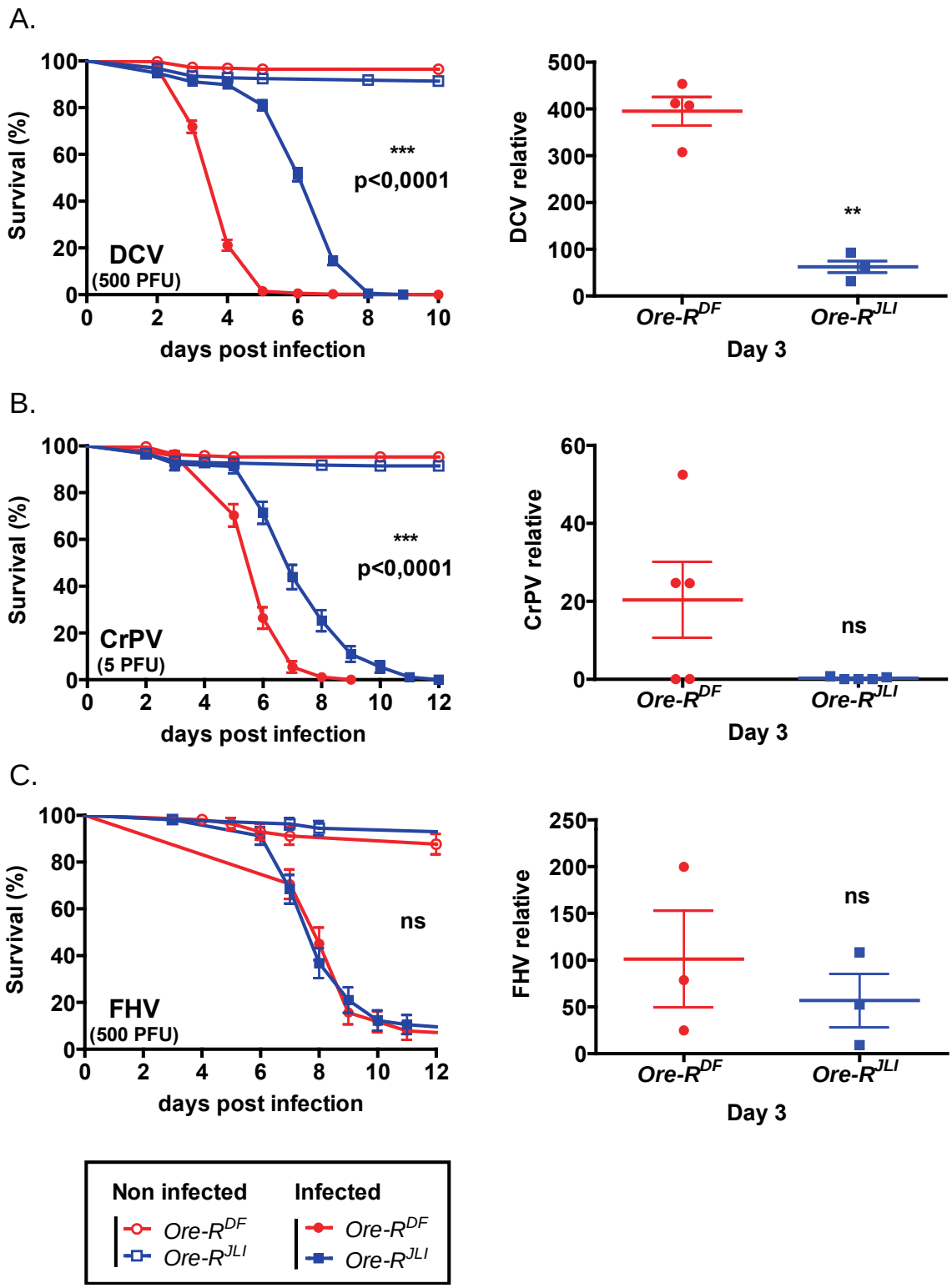


Figure 12. Wide variability in the susceptibility of two *Ore-R* stocks to DCV and CrPV infection, but not FHV infection. A. Flies (4-8 days) were infected with DCV (500 PFU) and survival was monitored daily at 22°C. Bar graphs represent the average standard deviation of 17 independent experiments. **B.** DCV RNA level was measured by qPCR after 3 days of infection. Bar graphs represent the average standard deviation of 5 independent experiments. **C.** Flies (4-8 days) were infected with CrPV (5 PFU) and survival was monitored daily at 22°C. Bar graphs represent the average standard deviation of 4

independent experiments. **D.** CrPV RNA level was measured by qPCR after 3 days of infection. Bar graphs represent the average standard deviation of 4 independent experiments. **E.** Flies (4-8 days) were infected with FHV (500 PFU) and survival was monitored daily at 22°C. Bar graphs represent the average standard deviation of 3 independent experiments. **F.** FHV RNA level was measured by qPCR after 3 days of infection. Bar graphs represent the average standard deviation of 3 independent experiments. For panels **B.**, **D.** and **F.**, qPCR values were normalized to the housekeeping gene *RpL32*.

We next tested if a difference of susceptibility was observed with other families of RNA viruses: FHV (*Nodaviridae*), VSV (*Rhabdoviridae*), SINV (*Togaviridae*) and a DNA virus: IIV-6 (*Iridoviridae*). No statistical difference was observed between survival and virus load of *Ore-R*^{JLI} and *Ore-R*^{DF} flies challenged by FHV infection (500 PFU, **Figure 12C**). We observed a difference of susceptibility of *Ore-R*^{JLI} and *Ore-R*^{DF} flies to VSV infection (5000 PFU) after 20 days of infection (**Figure 13B**). However, we believe that this difference is due to injury after injection, not to VSV infection. Indeed, we also observed a difference in survival between the two *Ore-R* stocks injected with TRIS buffer (**Figure 13A**). This is also consistent with VSV RNA level after 5 days of infection, which is similar between *Ore-R*^{JLI} and *Ore-R*^{DF} flies (**Figure 13C**). We did not observe variability in the susceptibility of *Ore-R*^{JLI} and *Ore-R*^{DF} flies to SINV infection (2500 PFU, **figure 13D**). Survival curves of SINV-infected *Ore-R*^{JLI} and *Ore-R*^{DF} flies overlap each other whereas a difference of susceptibility is observed with TRIS injections, suggesting that *Ore-R*^{DF} flies may be more resistant to SINV infection. Contradictorily, SINV RNA level was significantly higher in *Ore-R*^{DF} flies after 5 days of infection (**Figure 13E**). *Ore-R*^{DF} flies infected by the DNA virus IIV-6 showed higher sensitivity than *Ore-R*^{JLI} flies (**Figure 13F**), and a higher IIV-6 DNA level at 20 days post infection (**Figure 13G**). In summary, only IIV-6 shows differences between the two *Ore-R* stocks.

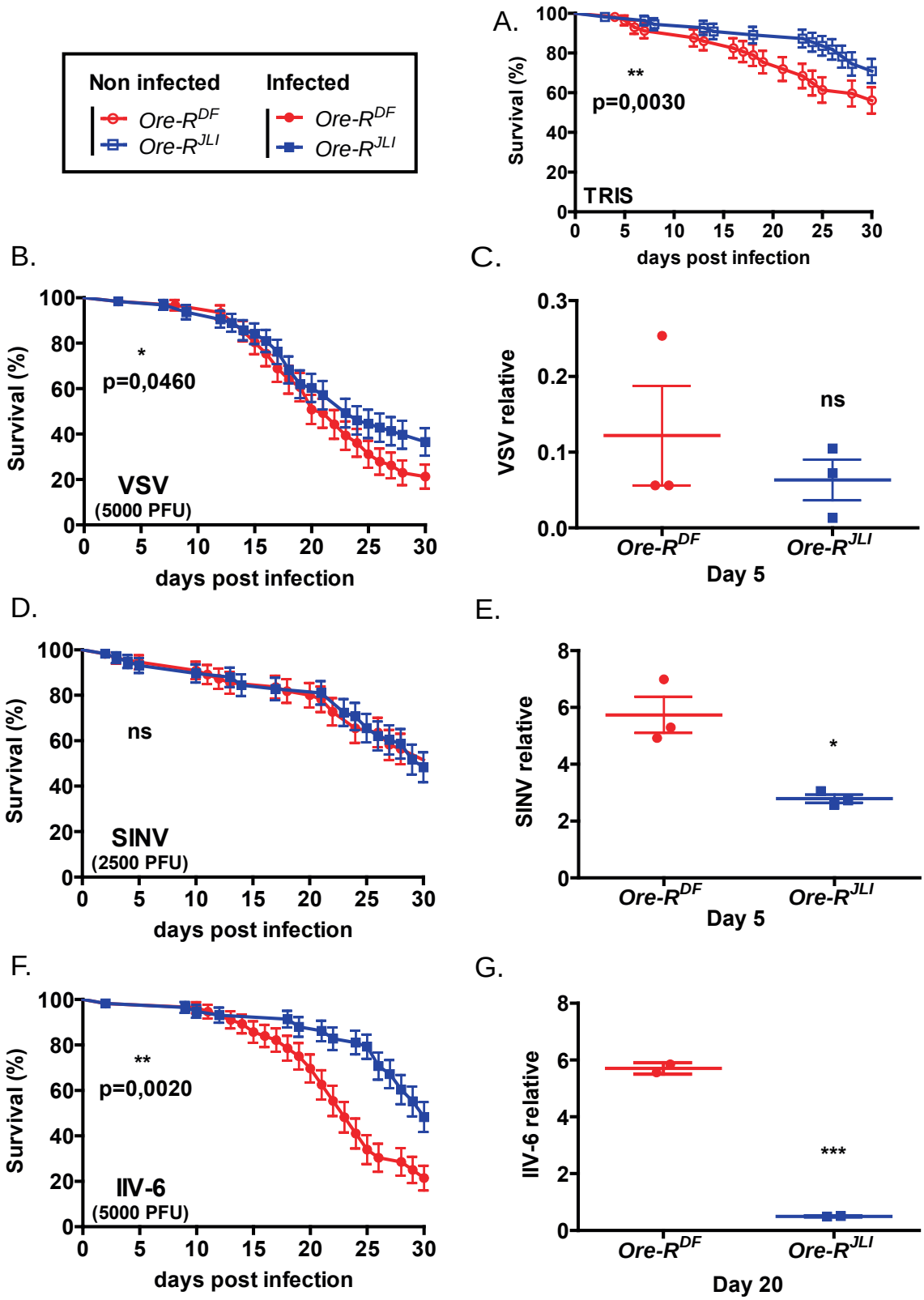


Figure 13. Variability in the susceptibility of two *Ore-R* flies to IIV-6 infection, but not VSV and SINV infections. A. Flies (4-8 days) were injected by TRIS buffer as non-infected control. **B.** Flies (4-8 days) were infected with VSV (5000 PFU) and survival was monitored daily at 22°C. Bar graphs represent the average standard deviation of 3 independent experiments. **C.** VSV RNA level was measured by qPCR after 5 days of infection. Bar graphs

represent the average standard deviation of 3 independent experiments. **D.** Flies (4-8 days) were infected with SINV (2500 PFU) and survival was monitored daily at 22°C. Bar graphs represent the average standard deviation of 3 independent experiments. **E.** SINV RNA level was measured by qPCR after 5 days of infection. Bar graphs represent the average standard deviation of 3 independent experiments. **F.** Flies (4-8 days) were infected with IIV-6 (5000 PFU) and survival was monitored daily at 22°C. Bar graphs represent the average standard deviation of 3 independent experiments. **G.** IIV-6 DNA level was measured by qPCR after 20 days of infection. For panels **C.**, **E.** and **G.**, qPCR values were normalized to the housekeeping gene *RpL32*.

We next started to investigate the cause of the variability of susceptibility between laboratory control flies. For this, we decided to focus on the viruses for which we observed the strongest effects, namely the picorna-like viruses DCV and CrPV.

4.1.c. No DCV fragments integrated in the genome of laboratory control flies.

Recently, the group of Dr. Carla Saleh revealed the presence of viral DNA forms in *Drosophila* cells infected by the RNA viruses FHV and DCV, which would play a role in the establishment of resistance to infection. The authors hypothesized that DNA forms may serve as initiators of antiviral response through the RNA interference pathway (Goic et al., 2013). We sought to verify if such DNA forms from DCV may be present in laboratory control flies and trigger antiviral resistance to DCV infection. We constructed four probes against the DCV genome and performed Southern blots to detect the eventual presence of DCV genome fragments integrated in the genome of *DD1 cnbw*, *Ore-R*^{JLI} and *w*^{A5001} flies. As a control, we designed a probe against the Toll gene (**Figure 14A**). Toll fragments were detected in *DD1 cnbw*, Ore-*R*^{JLI} and *w*^{A5001} flies. Similarly, we hybridized DCV probes (A, B, C and D) with the membrane but no DCV fragment was detected in the genome of the three *Drosophila* strains (**Figure 14C**, representative of DCV probe A hybridization). As a positive control, a plasmid containing a DCV fragment was digested by the same restriction enzymes and hybridized with DCV probe A (**Figure 14B**). It revealed DCV fragments, indicating that method is working.

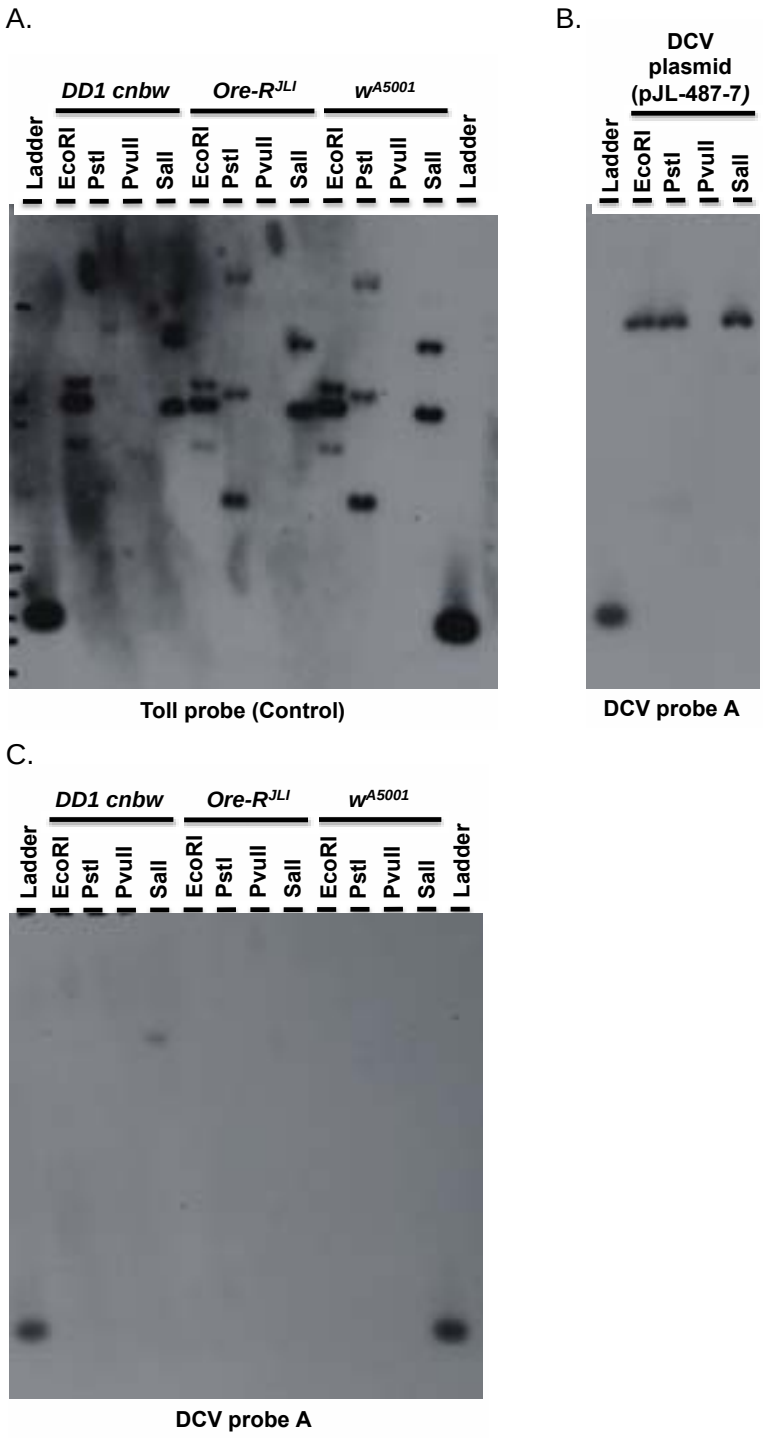


Figure 14. TOLL DNA fragments, but no DCV fragments, were detected by southern blot in *DD1 cnbw*, *Ore-R*^{JLI} and *w*^{A5001} flies. A. Southern blot hybridized with probe against TOLL gene. **B.** Plasmid with DCV fragment hybridized with probe against DCV genome. **C.** Southern blot hybridized with probe against DCV genome. For all panels, genomes of *DD1 cnbw*, Ore-*R*^{JLI} and *w*^{A5001} flies were digested by *EcoRI*, *PstI*, *PvuII* and *SalI*.

In summary, we cannot attribute the difference of susceptibility of flies to DCV infection to the presence of DCV DNA forms in the genome. However, it seemed that sensitivity or resistance to DCV infection was strain specific and not caused by a pathogen transmitted via feces (discussed after in chapter 5). At this point, we suspected that genetic determinants may confer resistance or sensitivity to DCV infection.

4.1.d. The susceptibility of flies to DCV infection is genetically transmitted

In order to identify if the cause of susceptibility of flies to DCV infection is genetic, we crossed together DCV resistant flies and DCV sensitive flies. We next infected the F1 progeny by DCV (500 PFU) to identify if these flies were sensitive or resistant to DCV infection (**Figures 15 and 16**).

The progeny resulting of the cross between *Ore-R*^{JLI} males and *Ore-R*^{DF} females was as resistant to DCV infection as the parental *Ore-R*^{JLI} flies (**Figure 15A and 15B**). These results indicate that the susceptibility to DCV infection is genetically transmitted. The progeny resulting from the reciprocal cross (*Ore-R*^{JLI} females and *Ore-R*^{DF} males) showed similar resistance to DCV infection. This indicates that the allele conferring resistance to DCV infection is autosomal-dominant. The F1 progeny recovered a lower DCV RNA level than *Ore-R*^{DF} flies, similar to *Ore-R*^{JLI} flies after 3 days of infection (**Figure 15C**).

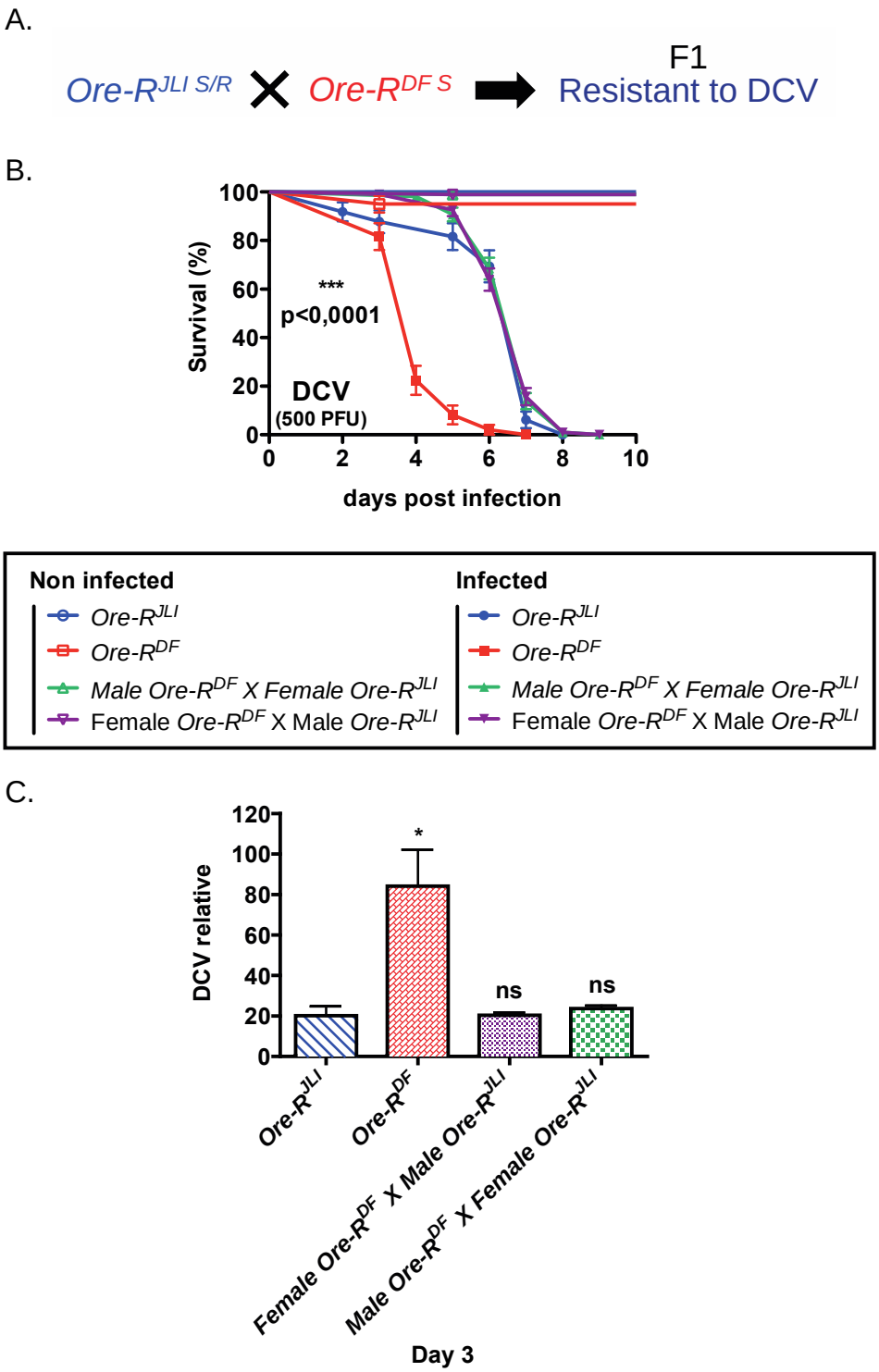


Figure 15. The susceptibility of two Ore-R stocks flies to DCV infection is genetically transmitted, and the resistant allele is autosomal-dominant. A. Representation of the cross between *Ore-R*^{JLI} and *Ore-R*^{DF} flies. **B.** Ore-R^{JLI} flies were crossed with *Ore-R*^{DF} flies at 25°C. Parent flies and F1 progeny were injected by TRIS (control, empty symbols) and DCV (500 PFU, filled symbols). Survivals were monitored daily at 22°C. Bar graphs represent the average standard deviation of 3 independent experiments. **C.** DCV RNA level was measured by qPCR after 3 days of infection. Bar graphs represent the average standard deviation of 1 experiment. qPCR values were normalized to the housekeeping gene *RpL32*.

We also crossed resistant *Canton-S* and *DD1 cnbw* flies with sensitive w^{A5001} and *yw* flies respectively. We observed that the progeny resulting of these crosses, and also reciprocal crosses, was resistant to DCV infection, as the resistant parental flies (**Figure 16A and 16B**).

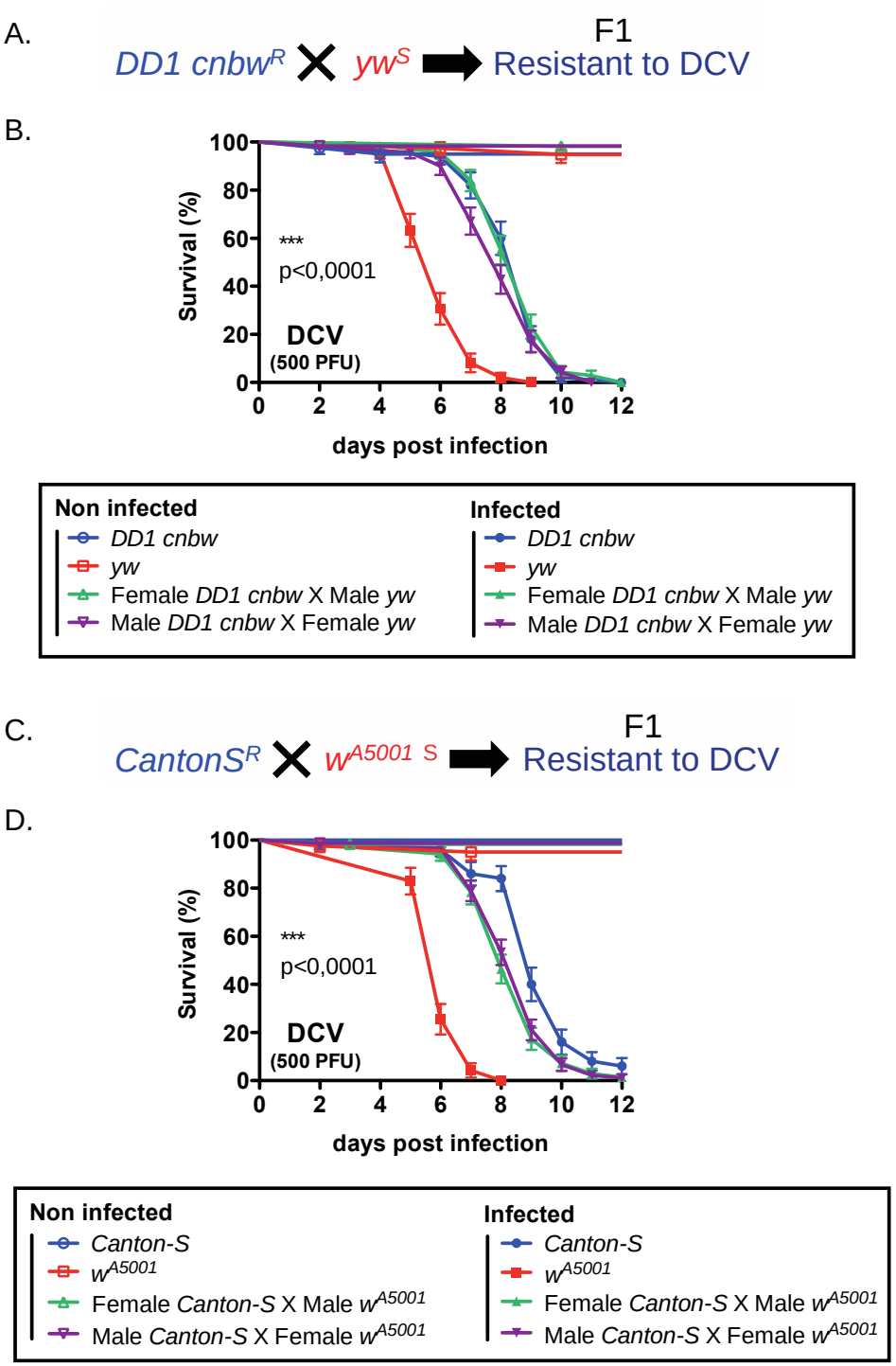


Figure 16. The susceptibility of flies to DCV infection is genetically transmitted, and the resistant allele is autosomal-dominant. A. Representation of the cross between *DD1 cnbw* and *yw* flies. **B.** *DD1 cnbw* and *yw* flies were crossed at 25°C. Parent flies and F1 progeny were injected by TRIS (control, empty symbols) and DCV (500 PFU, filled symbols). Survivals were monitored daily at 22°C. Bar graphs represent the average standard deviation

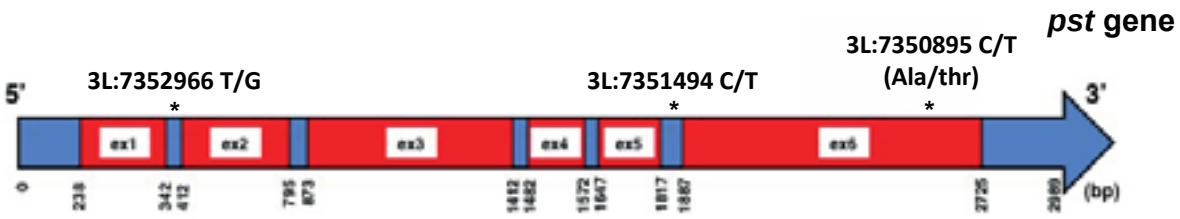
of 3 independent experiments. **C.** Representation of the cross between *Canton-S* and w^{A5001} flies. **D.** *Canton-S* and w^{A5001} flies were crossed at 25°C. Parent flies and F1 progeny were injected by TRIS (control, empty symbols) and DCV (500 PFU, filled symbols). Survivals were monitored daily at 22°C. Bar graphs represent the average standard deviation of 3 independent experiments. For all panels, sensitive flies are indicated in red and resistant flies are indicated in blue.

Altogether, these results indicate that the susceptibility to DCV infection between two stocks of *Ore-R* flies or between different laboratory control flies is caused by genetic determinants on the second and/or third chromosome. Interestingly, it was reported in the literature that differences of susceptibility to DCV infection between *Charolles* and *Nagasaki* wild-type flies may be caused by genetic determinants on the third chromosome (**Thomas-Orillard et al, 1995**). At that time, we heard from Prof. Francis Jiggins in Cambridge that a gene on the third chromosome, named *pastrel*, was determining the susceptibility of flies to DCV infection (personal communication with Prof. Jean-Luc Imler). We then looked for polymorphisms in the *pastrel* gene which could correlate with the sensitivity or resistance of our *Drosophila* strains to DCV infection.

4.1.e. Correlation between the polymorphism in *pst* gene and DCV susceptibility

The complete sequencing of the *pastrel* gene from our resistant (*Canton-S* and *DD1 cnbw*) and sensitive (*yw* and w^{A5001}) flies allowed the identification of three single nucleotides polymorphisms (SNPs) correlating with their degree of susceptibility to DCV infection (**Figure 17A and 17B)**. Two of them are present in introns 1 and 5 (3L:7352966 T/G and 3L:7351494 C/T respectively). The last SNP is present in exon 6 of *pastrel* gene and induces an amino acid substitution (3L:7350895 C/T (Ala/Thr)).

A.



B.

Strains	Phenotype with DCV	*pst* allele
yw	Sensitive	Sensitive [1]
W^{A5001}	Sensitive	Sensitive [1]
CantonS	Resistant	Resistant [2]
DD1 cnbw	Resistant	Resistant [2]

[1] *pst* sensitive allele : 3L:7352966 G – 3L:7351494 T – **3L:7350895 T**
[2] *pst* resistant allele : 3L:7352966 T – 3L:7351494 C – **3L:7350895 C**

Figure 17. Susceptibility of flies to DCV infection is correlated with polymorphisms in *pastrel* gene. A. Representation of *pastrel* gene with exons indicated in red squares. Three SNPs identified as correlated with DCV susceptibility are indicated in bold. Two SNPs are present in introns. The third SNP is present in exon 6 and induces an amino acid substitution. **B.** Fly strains sensitive or resistant to DCV infection display sensitive or resistant alleles of *pastrel*, respectively.

In addition to SNPs, we also noticed after amplification of the *pastrel* gene from flies for sequencing, that duplications with deleted version of *pastrel* gene were present in some of our fly stocks and *Drosophila* cell lines. The amplification of the *pastrel* gene from laboratory control flies with primers designed at each of its extremity produced a single band of 2,989 kb, the expected length of *pastrel* gene (**Figure 18A**). Unexpectidely, additional bands of *pastrel* were amplified from a deficiency line, a mutant for the transcription factor Dif, and some *Drosophila* cell lines (S2, S2R+ and KC167 cell lines) (**Figure 18B**). We also amplified the *pastrel* gene from two lines used in genome wide association study (GWAS) by Prof. Jiggins (lines 45 and 101). Flies from line 45 are resistant to DCV infection whereas flies from line 101 are sensitive (**data not shown**). Unexpectidely, we amplified additional bands of *pastrel* from the line 101, but a single band from line 45, with the expected size of *pastrel* gene (**Figure 18C**). The sequencing of the lower band revealed that a *pastrel* truncated version of 1755 kb length was present in the genome of these flies and cells (**Figure 18D**). The deletion is extended from position 1072 in exon 6 to position 2306 in exon 2, with two adenosines at the junction. The other bands were characterized by the group of Prof Jiggins.

They further analysed whether the presence of these duplications is associated with the susceptibility of flies to DCV infection but they did not find a significative association (personnal communication, Prof. Jiggins).

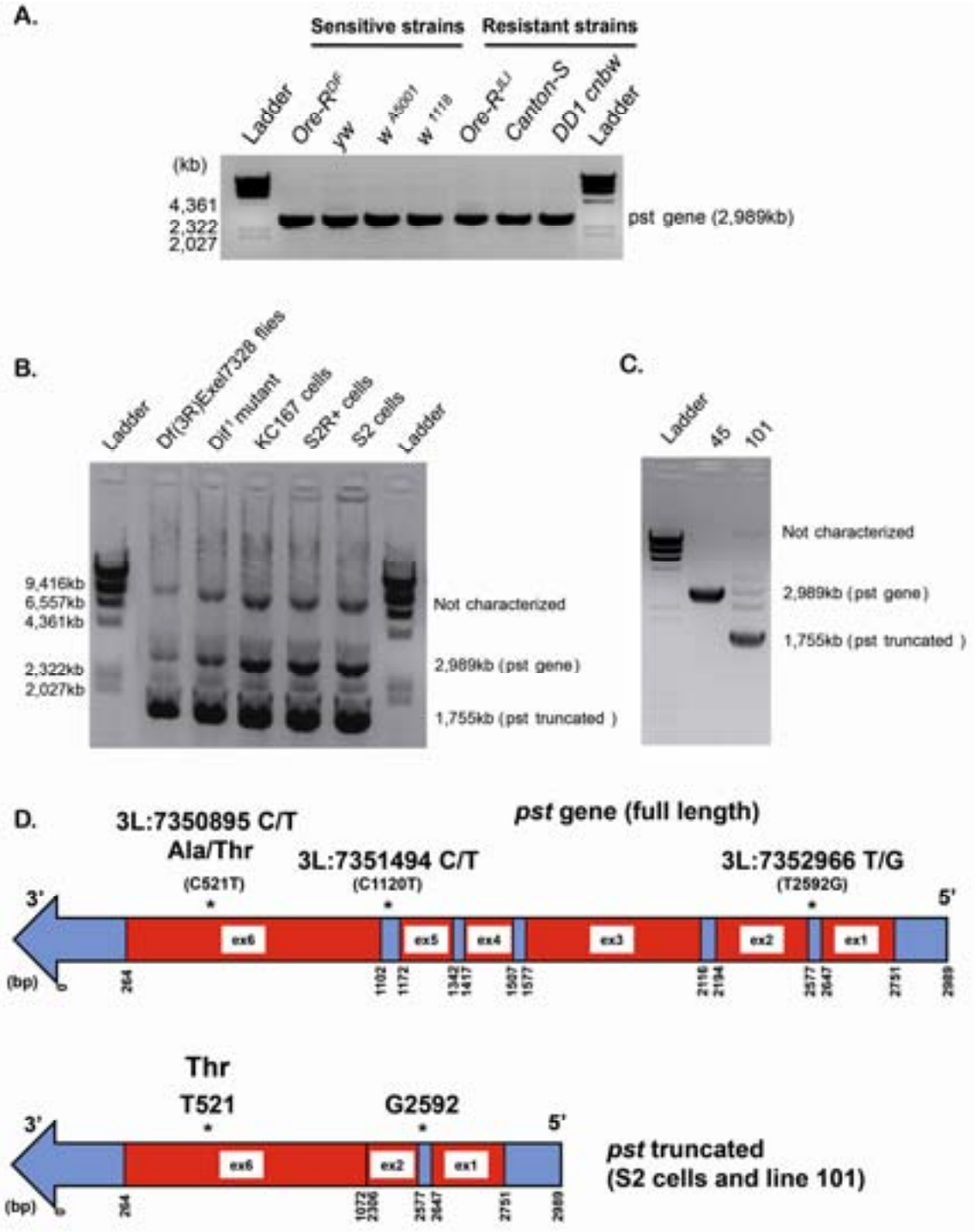


Figure 18. Identification of a deleted version of pastrel gene present in the genome of some flies and cells. A. to **C.** PCR amplification on genomic DNA from flies or cells with

specific primers for each extremity of *pastrel* gene, followed by gel electophoresis. **D.** Representation of the truncated version of *pastrel*, sequenced from S2 cells and fly line 101.

Surprisingly, the sequencing of *pastrel* gene from *Ore-R*^{JLI} and *Ore-R*^{DF} flies revealed new polymorphisms not found in the other laboratory control flies (**Figure 19**). Interestingly, the T521C SNP in exon 6 of *pastrel* gene was also correlated with the susceptibility of flies to DCV infection. Moreover, we observed previously that *Ore-R*^{DF} flies die in 5 days and *yw* and *w*^{A5001} flies die in 7 days. *Ore-R*^{DF} flies die also earlier after CrPV infection than other control flies. The high sensitivity of *Ore-R*^{DF} flies to DCV and CrPV infection may correlate with the new polymorphisms found in this strain. We also observed that wild-type *Charolles* flies were highly sensitive to DCV infection, comparable to *Ore-R*^{DF} (**data not shown**). The polymorphisms in the *pastrel* gene of *Charolles* flies remain to be characterized, to verify whether unusual polymorphisms of *Ore-R*^{DF} flies are present in *Charolles* flies, and thereby may correlate with the high sensitivity of both strains to DCV infection. At least, we confirmed that they display the SNP 3L:7350895 Thr (T at position 521 in the gene (T521)), present in all DCV sensitive flies. Additionaly, the susceptibility of *Charolles* flies to CrPV infection remains to be investigated.

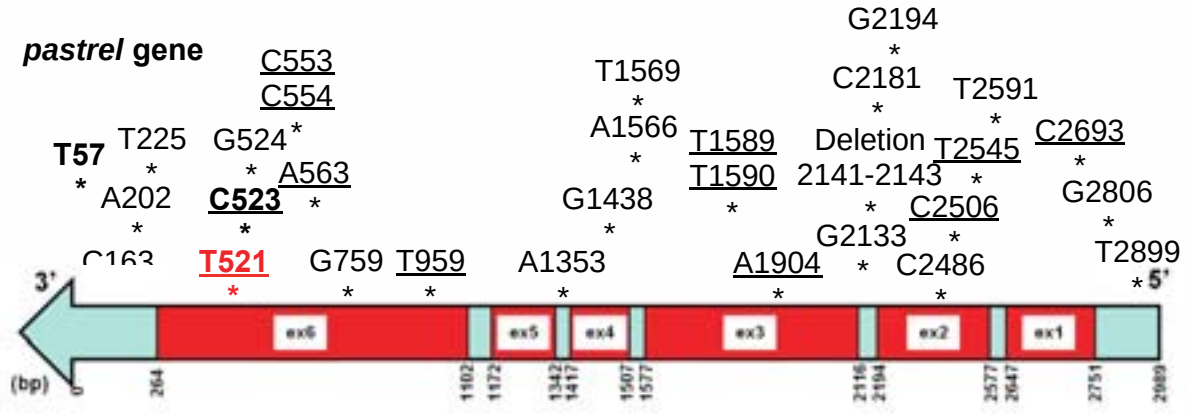


Figure 19. SNPs found exclusively in *pastrel* gene of *Ore-R*^{DF} flies may correlate with higher sensitivity to DCV and CrPV infection. SNPs were found in *pst* gene of *Ore-R*^{DF} but not in *Ore-R*^{JLI}, *w*^{A5001}, *DD1 cnbw* or *Canton-S*. SNPs found only by comparing *pst* gene of *Ore-R*^{DF} and *Ore-R*^{JLI} are indicated in bold. SNPs resulting in amino acid substitution are underlined. T521 (in red) is the SNP determined as sensitive allele.

The group of Pr. Jiggins classified the SNP in exon 6 (3L:7350895 Ala/Thr) as mainly responsible of differences of susceptibility to DCV infection, even if they do not exclude the participation of other SNPs in the phenotype (Magwire et al., 2012). On our side, we also observed that this SNP always correlated with sensitivity or resistance to DCV infection. We therefore used this SNP to diagnose the presence of sensitive or resistant alleles of *pastrel*. In this way, we developed allele specific PCR assay for fast genotyping of the fly strains used in the laboratory (**Figure 20**).

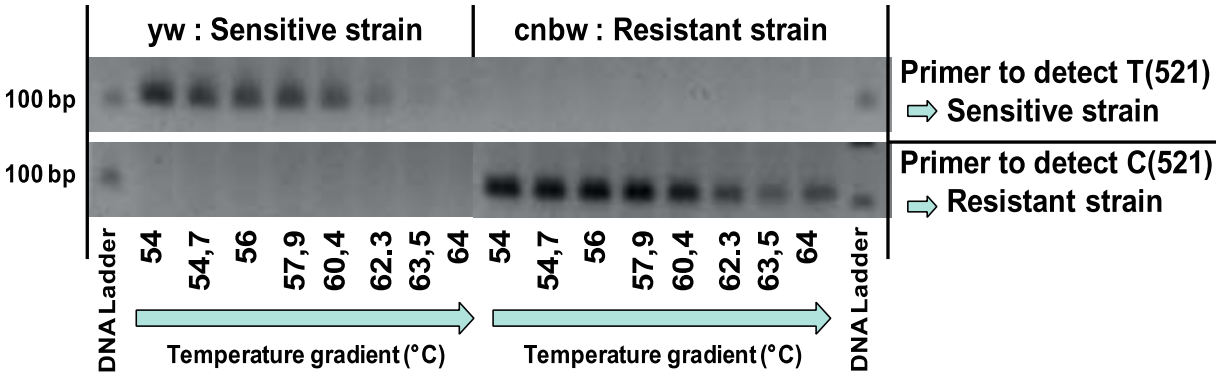


Figure 20. Allele specific PCR assay for fast genotyping of the sensitive and resistant alleles of *pastrel* gene. Primers to detect SNP 3L:7350895 Ala/Thr (or C521T) were designed using Web-based Allele-Specific PCR assay designing tool (Wangkumhang et al., 2007). PCR was performed with temperature gradient from 54°C to 64°C.

In order to clarify if polymorphisms present in the *pastrel* gene could impact on the susceptibility of flies to DCV infection, we decided to check whether the *pastrel* gene is required to resist to DCV infection.

4.2. Knockdown of *pst* gene increases the susceptibility of flies to DCV and CrPV infection

We obtained flies mutants for *pastrel* gene (Pst^1 flies) from Dubnau et al, 2003. Unfortunately, we did not find any *p* element inserted in the *pastrel* gene of these flies (**data not shown**). Moreover, normal *pst* RNA expression was detected by qPCR (**data not shown**). Even if we observed a high sensitivity of Pst^1 flies to DCV infection (**data not shown**), we cannot attribute it to the absence of *pastrel* gene expression but rather to sensitive *pst* allele

(*pst* genotyping). Without any *pastrel* mutant fly available, we therefore investigated the effect of knocking down *pastrel* gene expression by RNA interference on DCV infection in flies. We used flies from the VDRC collection, which express inverted repeats under the control of the yeast UAS-Gal4 promoter. Before starting the crosses, we genotyped *pastrel* allele of flies to be in the same genetic background for *pastrel* gene. All flies had *pastrel* sensitive allele (**data not shown**).

We first knocked down *pastrel* gene with the strong driver Actin-Gal4 but we were not able to obtain flies with the right genotype. This suggests that *pastrel* gene expression may be involved in fly development, in addition to its putative antiviral function. To resolve this problem, we used an Actin-Gal4-Tubulin-Gal80^{ts} system in which Gal4 expression is repressed by Gal80 at the restrictive temperature of 18°C. After the emergence of progeny, we transferred flies 0-4 days old at 29°C during 4 days to inactivate Gal80 and allow *pastrel* gene knockdown. Flies injected by TRIS buffer were perfectly fine (**Figure 21A**). We infected 4-8 days old flies with DCV (500 PFU) and transferred them to 22°C. We observed a higher DCV sensitivity for *pastrel*-silenced flies compared to control flies (**Figure 21B**). Death occurred at a median of 3.5 days for *pastrel* knocked down flies and 5.5 days for control flies. The DCV RNA level was also increased in *pastrel*-depleted flies compared to control flies during the course of the infection.

As previously mentionned, *Canton-S*, *DD1 cnbw*, *yw* and w^{A5001} flies did not show a wide variability in their susceptibility to CrPV infection, indicating that polymorphisms in *pastrel* gene did not affect CrPV susceptibility (**Figure 11**). However, there was a difference of 3 days in the susceptibility to CrPV infection between *Ore-R*^{DF} and *Ore-R*^{JLI} flies (**Figure 12**) and *Ore-R*^{DF} showed unique polymorphisms (**Figure 19**). Thus, we also verified if pastrel controls CrPV infection. Interestingly, we observed that *pastrel*-silenced flies died faster than control flies after CrPV infection (**Figure 21C**). This higher susceptibility was also correlated to a higher CrPV RNA level at 2, 3 and 4 days post infection.

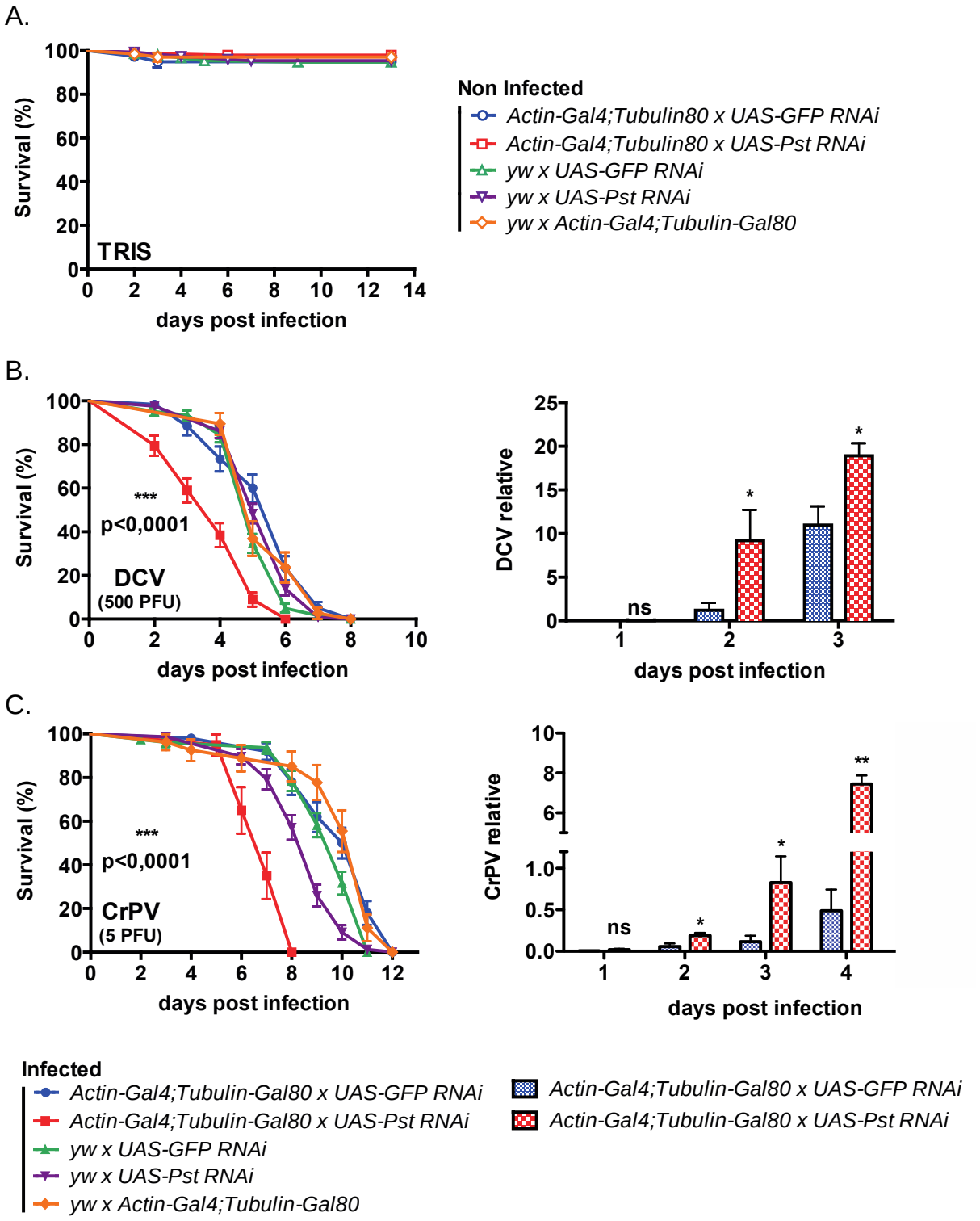


Figure 21. Knockdown of *pastrel* gene in whole flies increases their susceptibility to DCV and CrPV infection. A. Flies were injected by TRIS buffer as control (empty symbols). **B.** Flies were infected with DCV (500 PFU). DCV RNA level was measured by qPCR after 1, 2 and 3 days of infection. Bar graphs represent the average standard deviation of 2 independent experiments. **C.** Flies were infected with CrPV (5 PFU). CrPV RNA level was measured by qPCR after 1, 2, 3, and 4 days of infection. Bar graphs represent the average standard deviation of 2 independent experiments. For all panels, flies were crossed at 18°C and progeny (0-4 days) was collected at 29°C for 4 days. Flies (4-8 days) were infected with DCV or CrPV and survivals were monitored at 22°C.

To conclude, these results indicate that *pastrel* restricts infection by DCV and CrPV *in vivo*.

We looked at microarrays data from FlyAtlas to know which tissues harbor a high level of *pastrel* expression. Interestingly, we found that *pastrel* gene is highly expressed in the fat body (**Figure 22**).

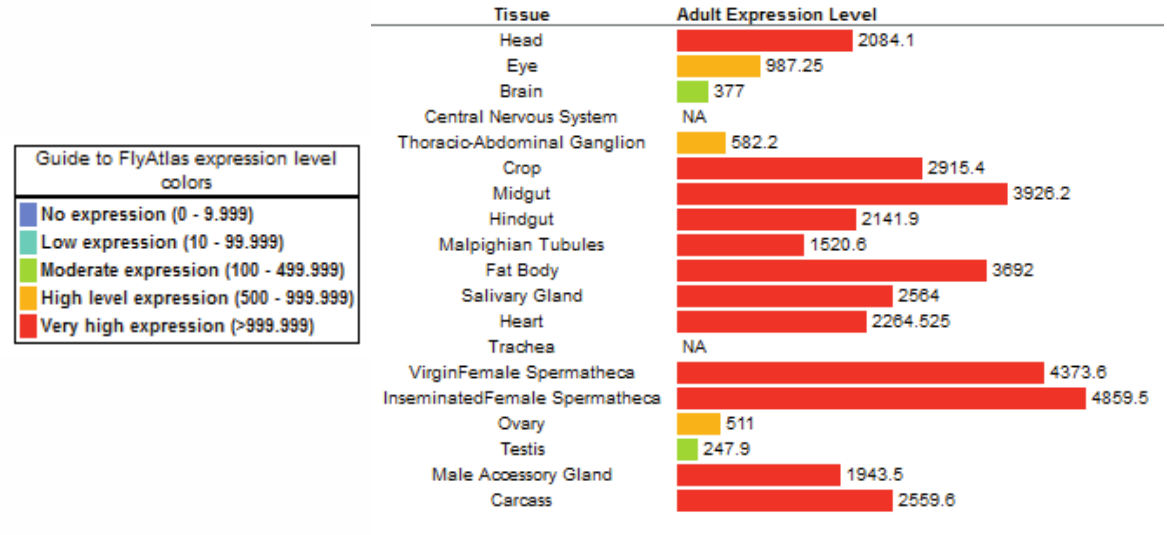


Figure 22. *pastrel* gene expression in organs and tissues from adult *Canton-S* flies. These data were adapted from FlyAtlas Anatomy Microarray.

The fat body is a major tissue targeted by DCV for replication (discussed in Chapter 1). We observed that silencing of *pastrel* in this tissue also increases viral titer and susceptibility to infection (**Figure 23B** and **23C**). As a control, flies were insensitive to TRIS buffer injection (**Figure 23A**). The silencing of *pastrel* also affects the resistance of flies to CrPV infection (**Figure 23D**). This higher susceptibility to CrPV infection is correlated with a higher viral titer (**Figure 23E**).

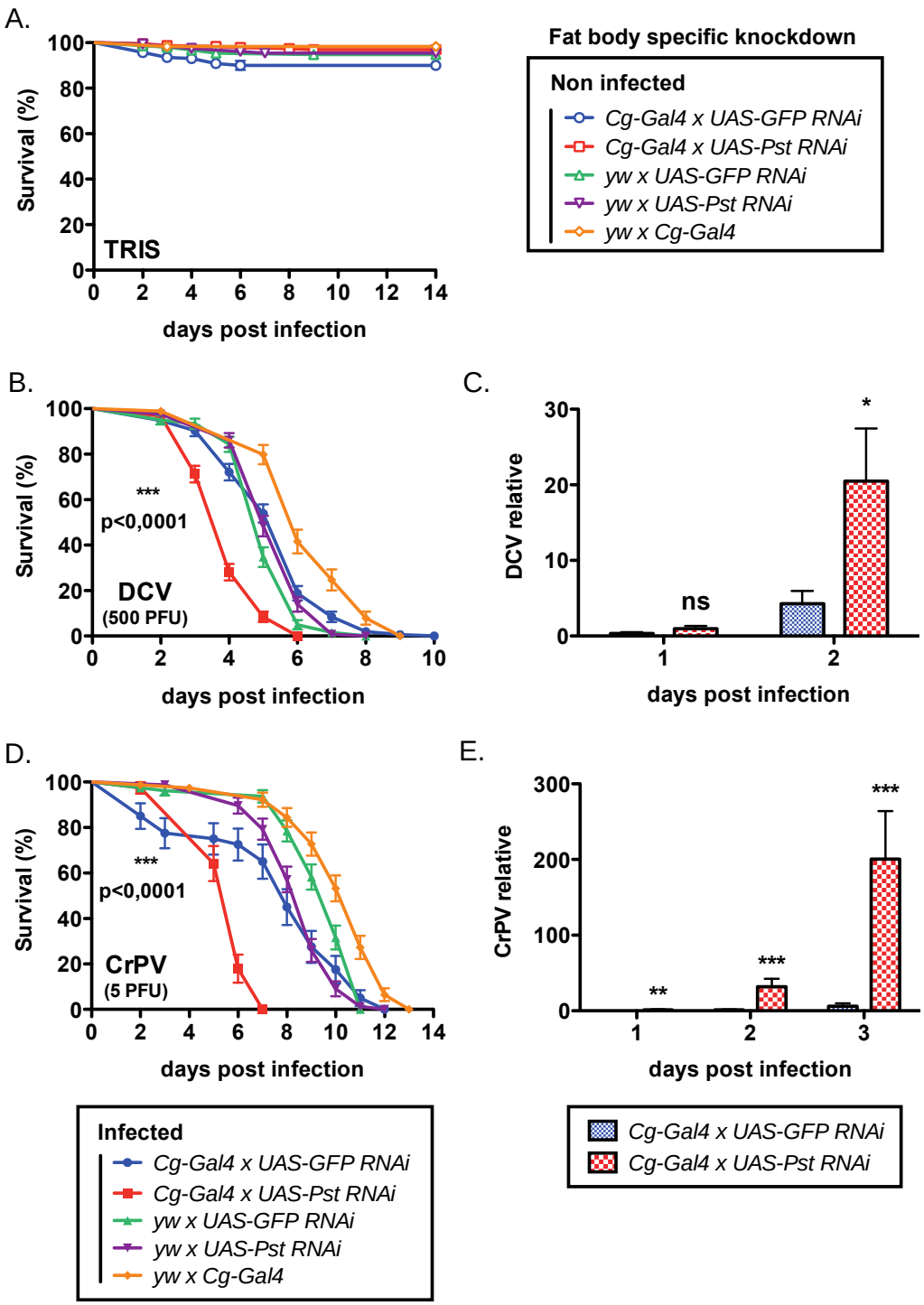


Figure 23. Specific knockdown of *pastrel* gene in the fat body increases the susceptibility of flies to DCV and CrPV infection. A. Flies were injected by TRIS buffer as control (empty symbols). **B.** Flies were infected with DCV (500 PFU). **C.** DCV RNA level was measured by qPCR after 1 and 2 days of infection. Bar graphs represent the average standard deviation of 3 independent experiments. **D.** Flies were infected with CrPV (5 PFU). **E.** CrPV RNA level was measured by qPCR after 1, 2 and 3 days of infection. Bar graphs represent the average standard deviation of 1 experiment. For panels **A**, **B**, **C**, and **D**. Flies were crossed at 25°C, fly progeny (4-8 days) was infected and survivals or virus load were monitored at 22°C (except panel **E.**, 25°C). For panels **A**, **B**, and **D**, bar graphs represent the average standard deviation of at least 2 independent experiments.

By contrast, silencing *pastrel* expression in midgut epithelial cells, where we did not detect DCV infection, had no effect on fly susceptibility (**Figure 24A** and **24B**), nor on viral titer (**Figure 24C**).

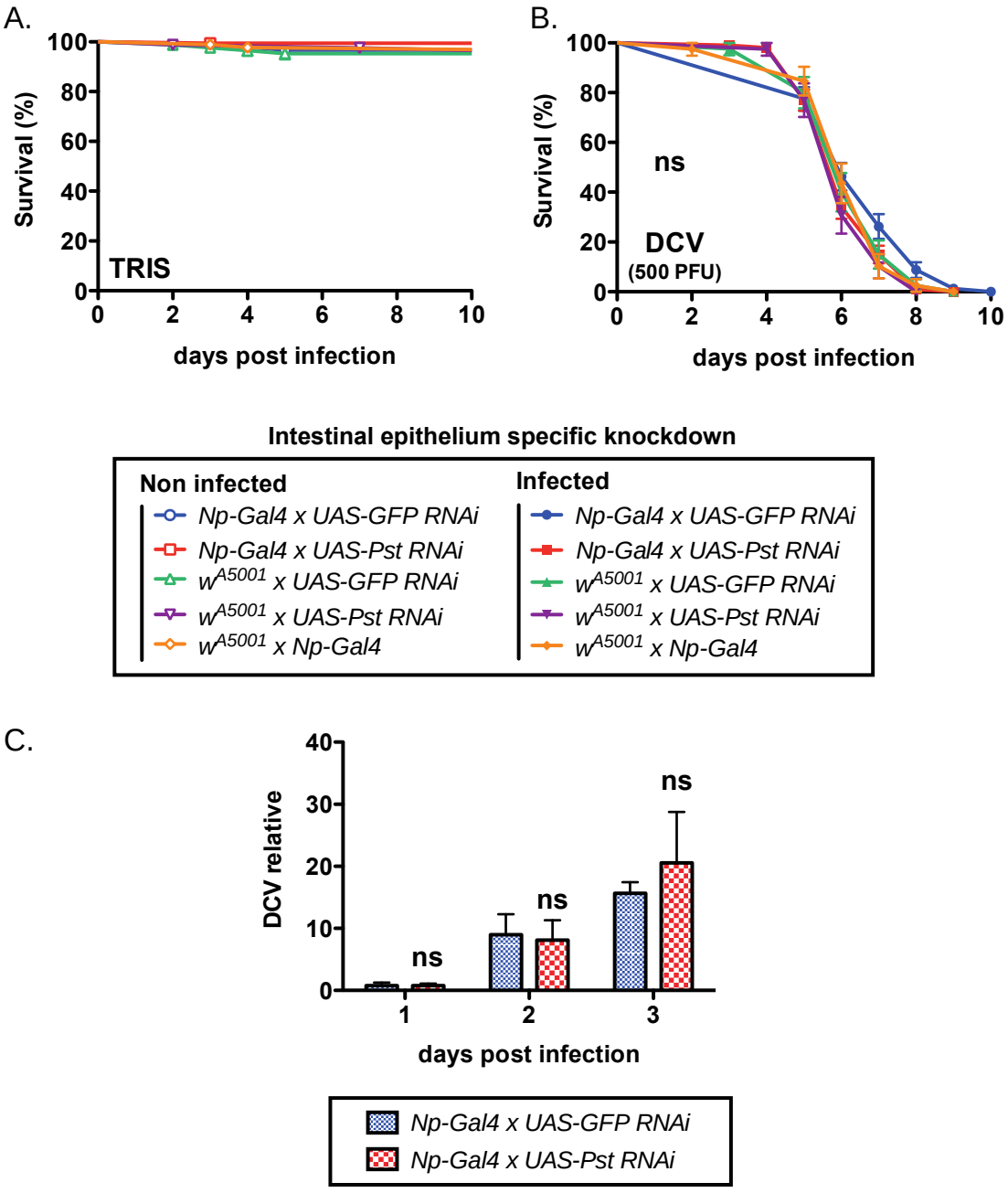


Figure 24. Specific knockdown of *pastrel* gene in the intestinal epithelium of flies does not affect the resistance to DCV infection. A. Flies were injected by TRIS buffer as control (empty symbols). **B.** Flies were infected with DCV (500 PFU). **C.** DCV RNA level was measured by qPCR after 1, 2 and 3 days of infection. For all panels, flies were crossed at 25°C, the progeny (4-8 days) was infected and survivals or virus load were monitored at 22°C. Bar graphs represent the average standard deviation of at least 2 independent experiments.

Altogether, these data confirm that *pastrel* controls the susceptibility of flies to DCV and CrPV infection. Interestingly, the control of both DCV and CrPV viral infections relies on the expression of *pastrel* in the fat body. Additionaly, our data indicate that *pastrel* gene expression in the epithelial midgut does not contribute to the control of DCV infection. Our group further investigated the localization of DCV in the gut. We were able to find DCV particles in the smooth muscles around the gut but not in the epithelial midgut (**data not shown**). This may explain the absence of increased susceptibility to DCV infection after *pastrel* knockdown in the epithelial midgut.

4.3. Polymorphisms in *pastrel* gene do not affect protein stability

We showed that knockdown of *pastrel* gene expression increases sensitivity of flies to DCV and CrPV infection. We wondered if sensitivity to DCV infection correlated with the expression level of Pastrel protein. This may explain the differences of susceptibility of flies to DCV infection. To address this point, we monitored Pastrel protein expression by western blot in *yw*, w^{A5001} (DCV sensitive flies) and *Canton-S*, *DD1 cnbw* (DCV resistant flies), using an antibody raised against the Pastrel protein in the group of Dr. Stéphane Noselli (**Figure 25**). The Pastrel protein level was similar between DCV sensitive and resistant flies. This result indicates that the polymorphisms in the *pastrel* gene have no effect on expression or stability of the Pastrel protein.

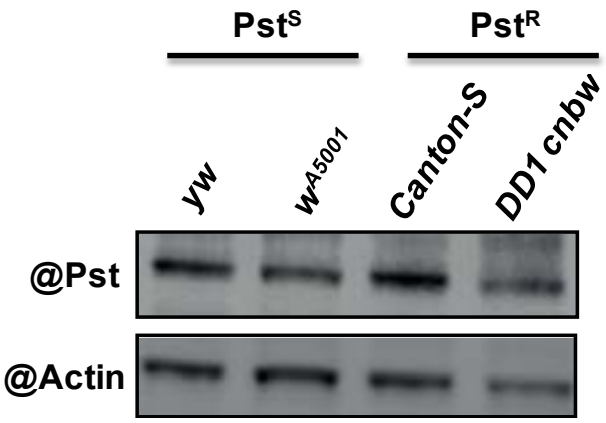


Figure 25. Flies expressing Pst^S and Pst^R forms show similar levels of Pst protein expression. Pst protein level from Pst sensitive flies (*yw* and w^{A5001}) and Pst resistant flies (*Canton-S* and *DD1 cnbw*) was revealed by western blot with an antibody against Pst. Antibody against Actin protein was used as loading control.

4.4. Does Pastrel overexpression restrict DCV infection in flies ?

Loss of function experiments showed that *pastrel* gene expression is necessary to control DCV infection. We wondered if overexpression of Pastrel protein in flies may control DCV infection. To address this question, we constructed vectors expressing, under the control of HSP-promoter, sensitive and resistant versions of Pastrel, tagged by the FLAG epitope in N-terminal. Plasmid constructions were sent to Pr. Jiggins for making transgenic flies and testing effect of Pastrel overexpression on DCV infection. This work is in progress.

In parallel, we obtained from Dr. Stéphane Noselli transgenic flies expressing a Pst-GFP fusion protein under the control of the UAS promoter. We crossed UAS-Pst-GFP flies with yw;Actin-Gal4 flies at 25°C to obtain flies expressing Pst-GFP under actin promoter. *Pastrel* allele of these flies was genotyped: all flies had the *pastrel* sensitive allele, but the *pastrel* transgene was the resistant allele. So we overexpressed the resistant version of the *pastrel* gene in flies with sensitive background for pastrel. We checked that these flies expressed Pst-GFP by looking for GFP under fluorescence microscope (**Figure 26A**). Then, flies were challenged with DCV and FHV (500 PFU) and survival was monitored at 22°C. Overexpression of Pst-GFP in whole flies did not increase their resistance to DCV infection, nor to FHV infection (**Figure 26B** and **26C** respectively). These results indicate that overexpression of Pastrel is not sufficient to restore better survival of flies challenged by DCV. We are now investigating if Pastrel overexpression reduces DCV titer in flies.

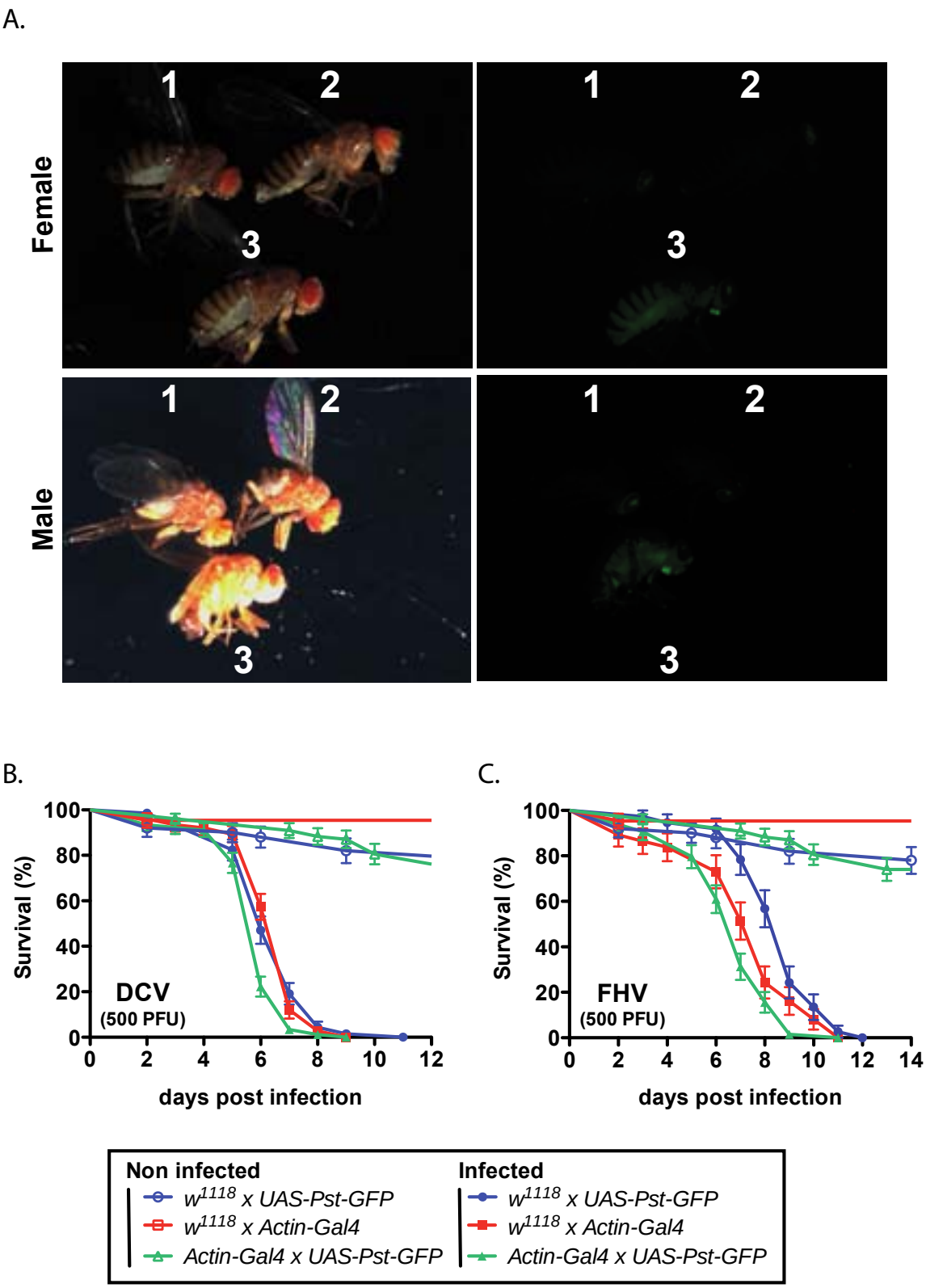


Figure 26. Overexpression of Pst-GFP in flies does not increase resistance to DCV infection. A. GFP fluorescence was observed in flies overexpressing Pst-GFP (3) and not in genetic controls (1 and 2). **B.** and **C.** Flies were crossed at 25°C and progeny (4-8 days) was injected by TRIS buffer as control (empty symbols), DCV (500 PFU, panel **B**) and FHV (500 PFU, panel **C**). Survivals were monitored at 22°C. Bar graphs represent the average standard deviation of 3 independent experiments.

4.5. Study of *pastrel* gene *in vitro*: *Drosophila* S2 cells

In order to further characterize the function of Pastrel, we decided to switch to an *ex vivo* system of infection, which is more amenable to experimental manipulation. We have shown *in vivo* that *pastrel* plays a role in the sensitivity or resistance of flies to DCV and CrPV infection. Then, we decided to further characterize the effect of *pastrel* gene expression on the DCV and CrPV infectious cycle by using *Drosophila* S2 cells. We had first to confirm that S2 cells system is suitable to study the effect of *pastrel* on DCV infection.

4.5.a. Knockdown of *pastrel* gene increases DCV susceptibility in *Drosophila* cells

We infected S2 cells with DCV (MOI 1) and followed the viral RNA level at different times after infection (**Figure 27A**). We observed that DCV replicates in S2 cells. We also noticed that *pastrel* gene expression is constantly increased by 25% during the course of the infection, compared to non infected S2 cells (except after 24h of infection). The induction of *pastrel* gene expression is virus-dependant, but not time-dependant (**Figure 27B**).

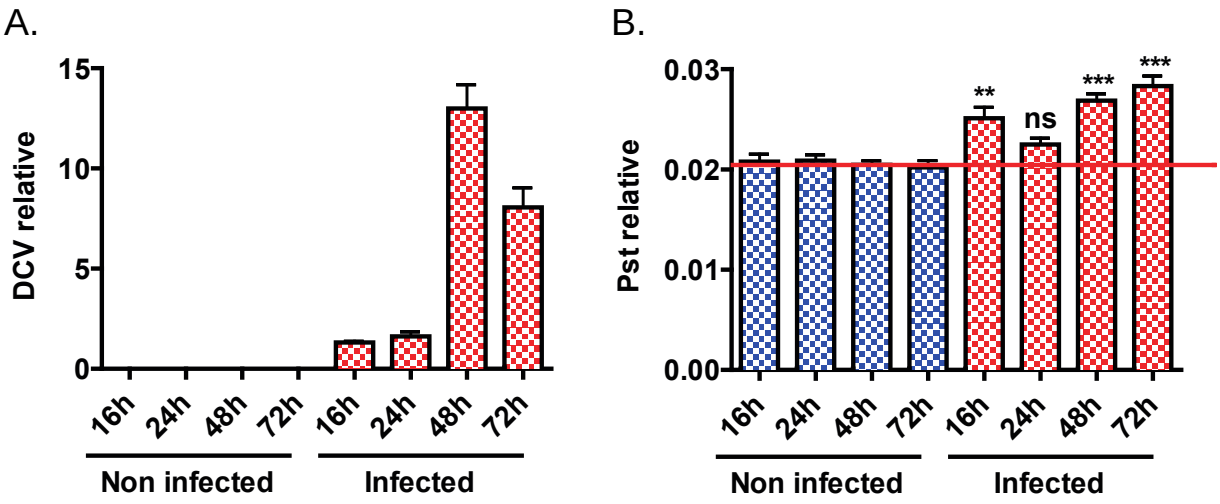


Figure 27. DCV replicates in *Drosophila* S2 cells and *pastrel* gene expression is increased after infection. A. and B. S2 cells were infected, or not, by DCV (MOI 1). DCV and Pst RNA levels were measured by qPCR after 16h, 24h, 48h and 72h of infection (panels **A.** and **B.** respectively). qPCR values were normalized to the housekeeping gene *RpL32*. Bar graphs represent the average standard deviation of 1 experiment (12 wells per condition).

We first confirmed that the increased DCV RNA level observed *in vivo* after *pastrel* gene knockdown is also observed *ex vivo* in *Drosophila* S2 cells. *Drosophila* S2 cells were treated for 5 days with two sets of double stranded RNAs (dsRNAs, **Figure 28A**), recognizing different regions of the *pastrel* gene in order to rule out off-target effects. Cells were then infected with DCV (MOI 1) for 16h. *Drosophila* S2 cells treated with dsRNAs against *pastrel* gene for 5 days showed a 50% reduction of *pastrel* gene expression (**Figure 28B**). This knockdown of *pastrel* gene expression led to a 2-fold increase in DCV RNA level after 16h of infection (**Figure 28C**).

We also observed by western blot that knockdown of *pastrel* gene expression increases DCV capsid protein level in cells and in the supernatant after 48h of infection (**Figure 28D**). We titered DCV infectious particles liberated in the supernatant after 16h of infection. The treatment of *Drosophila* S2 cells with dsRNAs against *pastrel* gene increased by almost 1 log DCV particles liberated in the supernatant (**Figure 28E**).

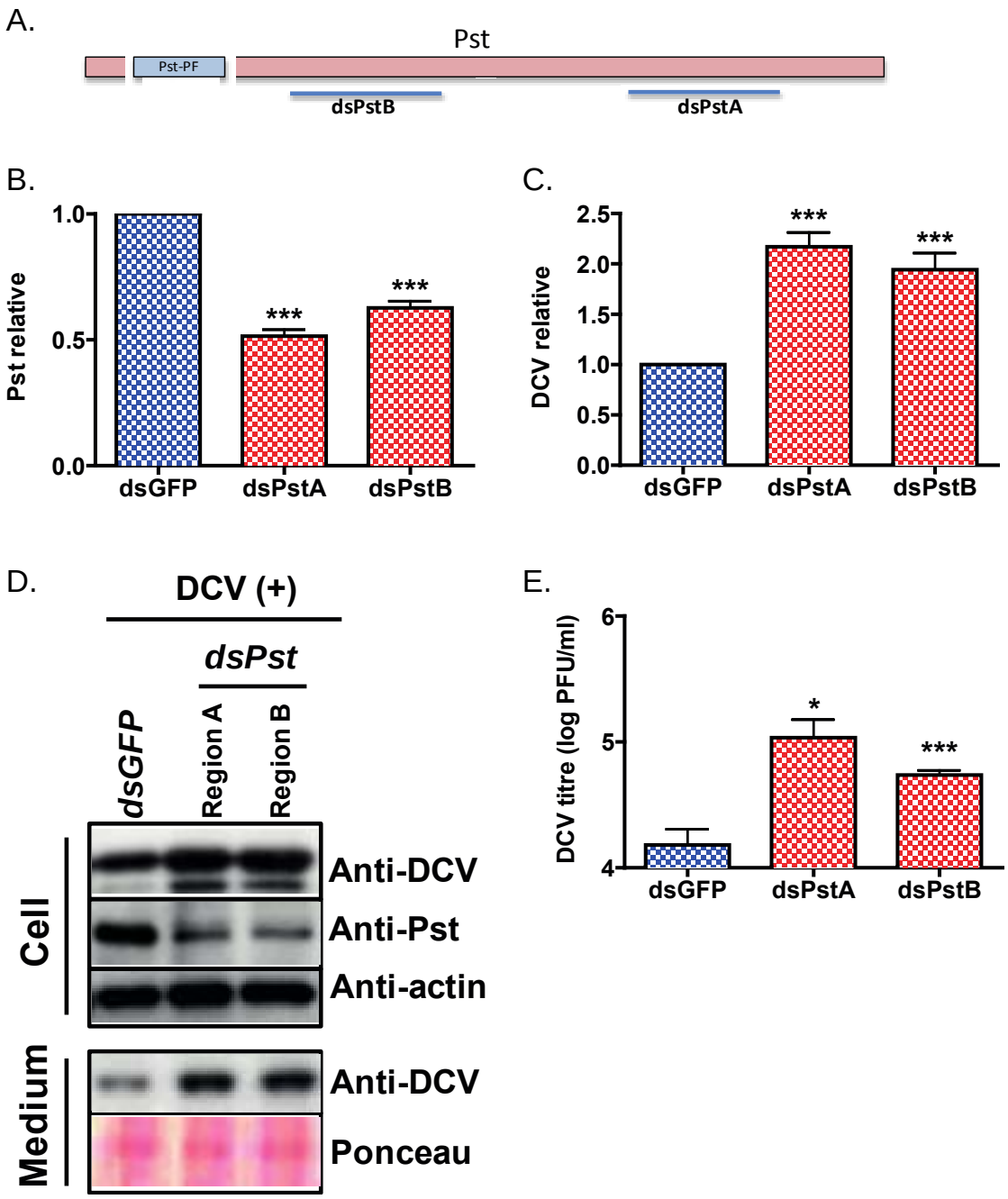


Figure 28. Knockdown of *pastrel* gene in S2 cells increases DCV infection. A. Representation of pastrel RNA with dsRNAs designed against two different regions of pastrel (region A and B). **B.** Pst RNA level was measured by qPCR after 16h of infection by DCV (MOI 1) in S2 cells treated during 5 days by dsRNAs against GFP and Pst (region A and B). **C.** DCV RNA level was measured by qPCR after 16h of infection in S2 cells treated during 5 days by dsRNAs against GFP and Pst (region A and B). **D.** DCV protein level from S2 cell lysate and supernatant, revealed by western blot. Cells were treated for 5 days by dsRNA against GFP and Pst (region A and B), and infected during 48h by DCV (MOI 1). **E.** DCV titration after 16h of infection in S2 cells treated by dsRNAs against GFP or Pst (region A and B). For panels **B**, **C**, and **E**, qPCR values were normalized to the housekeeping gene *RpL32*. Bar graphs represent the average standard deviation of 4 independent experiments for panels **B** and **C**, and 3 independent experiments for panel **E**. Western blot from panel **D** is representative of 3 independent experiments.

We achieved a greater knocked down of *pastrel* gene expression in *Drosophila* S2 cells by performing two dsRNAs treatments (2 x 5days) instead of one as in **Figure 28B**. These two successives treatments of S2 cells with dsRNAs against *pastrel* gene (region A and B) reduced of 3.2-fold the expression of *pastrel* gene, consequently increasing of 5-fold DCV RNA level (**Figure 29A** and **29B**). Compared to single dsRNAs treatment (**Figure 28B**), we observed higher DCV RNA level in cells treated twice with dsRNAs against *pastrel*, probably because Pst RNA level was lower after two dsRNAs treatments than one. This suggests that *pastrel* may control DCV infection in a dose-dependent manner.

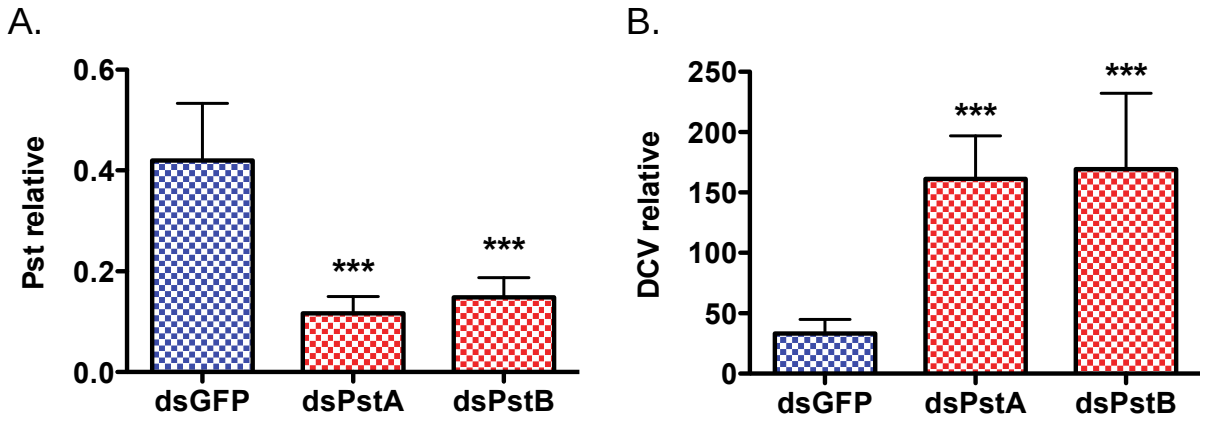


Figure 29. Knockdown of *pastrel* gene in S2 cells increases DCV infection. A. Pst RNA level was measured by qPCR after 16h of infection by DCV (MOI 1) in S2 cells treated twice during 10 days by dsRNAs against GFP and Pst (region A and B). **B.** DCV RNA level was measured by qPCR after 16h of infection in S2 cells treated twice during 10 days by dsRNAs against GFP and Pst (region A and B). For both panels, qPCR values were normalized to the housekeeping gene *RpL32*. Bar graphs represent the average standard deviation of 1 experiment.

We have confirmed that knockdown of *pastrel* gene expression, as in flies, increases DCV infection in *Drosophila* S2 cells. We next checked if CrPV infection in *Drosophila* S2 cells was affected after knockdown of *pastrel* gene. CrPV RNA level was increased after 10 days of treatment by dsRNAs against *pastrel* gene followed by 16h of infection in *Drosophila* S2 cells (**Figure 30**). *Pastrel* gene expression was 4.5-fold decreased after dsRNA treatment (**Figure 30A**) and CrPV RNA level was 4.9-fold increased (**Figure 30B**).

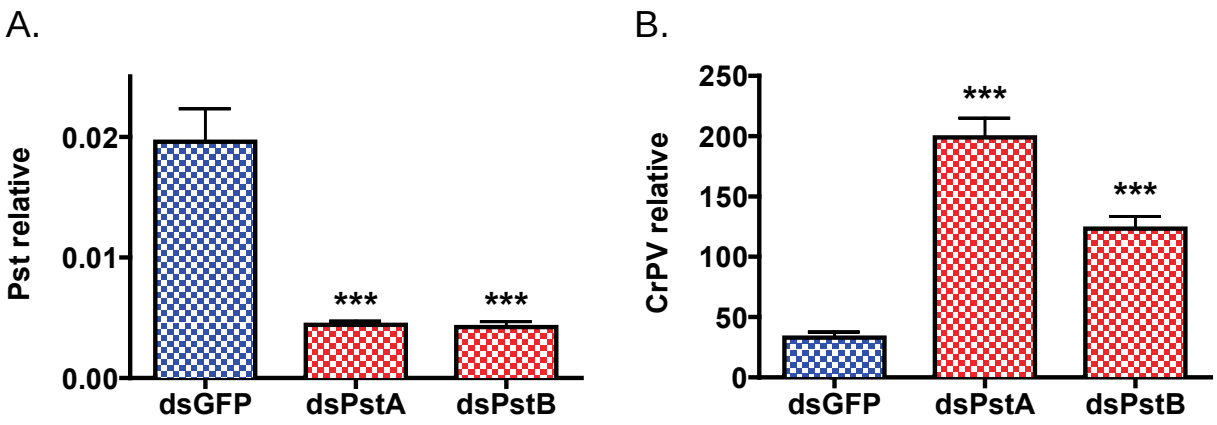


Figure 30. Knockdown of *pastrel* gene in S2 cells increases CrPV infection. A. Pst RNA level was measured by qPCR. **B.** CrPV RNA level was measured by qPCR. *Drosophila* S2 cells were treated for 10 days by dsRNAs against GFP and Pst (region A and B). Cells were then infected with CrPV (MOI 0,01) for 16h. Bar graphs represent the average standard deviation of 2 (out of 4) independent experiments (n=2x6 wells). qPCR values were normalized to the housekeeping gene *RpL32*.

To conclude, the knockdown of *pastrel* gene in *Drosophila* S2 cells increases DCV and CrPV infection. Finally, *pastrel* gene expression controls DCV and CrPV infections *in vivo* and *in vitro*. We wondered if the overexpression of *pastrel* gene in *Drosophila* S2 cells may control DCV and CrPV infections.

4.5.b. Overexpression of Pastrel protein restricts DCV and CrPV infection

We constructed plasmids to express the sensitive and resistant versions of Pastrel protein in *Drosophila* S2 cells under the control of an actin promoter. We added a Red Fluorescent Protein (RFP) tag in N or C-terminal of Pastrel protein for further intracellular localization studies (discussed in **section 4.6**). As a control, we used the empty vector expressing RFP only. We overexpressed these constructions in *Drosophila* S2 cells by transient transfection (**Figure 31A**), and then we infected these cells with DCV. The DCV RNA level was significantly reduced in *Drosophila* S2 cells overexpressing Pastrel protein fusions (**Figure 31B**). However, the effect was smaller than expected.

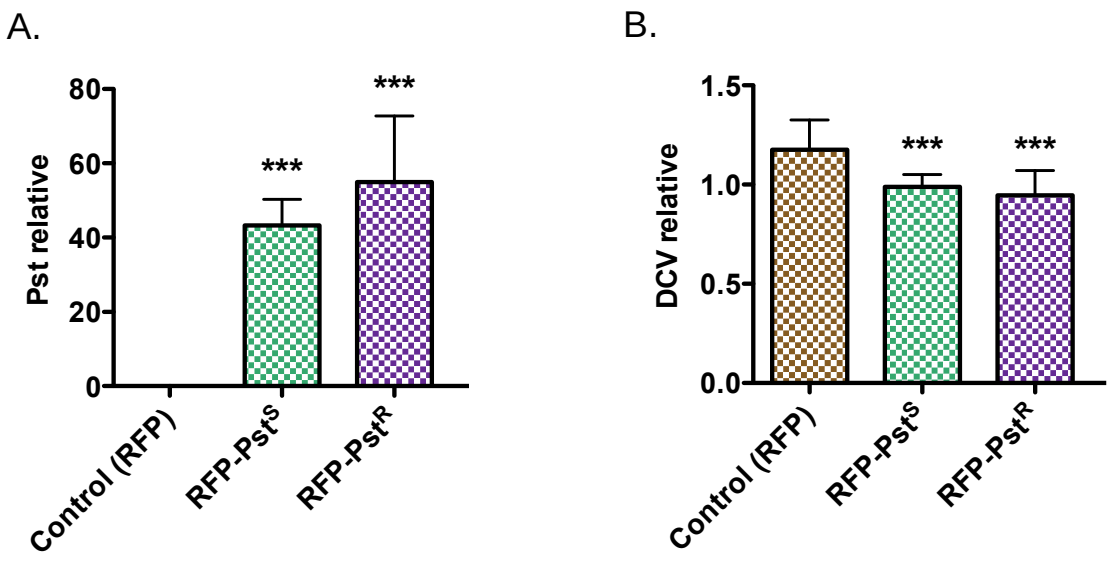


Figure 31. Transient overexpression of Pastrel full lentgh slightly reduces DCV infection in *Drosophila* S2 cells. A. Pst RNA level was measured by qPCR. **B.** DCV RNA level was measured by qPCR. *Drosophila* S2 cells were transfected by vectors expressing full-length sensitive and resistant versions of Pastrel. Cells were then infected with DCV (MOI 1) for 16h. Bar graphs represent the average standard deviation of 1 experiment (n=12 wells). qPCR values were normalized to the housekeeping gene *RpL32*. Control cells expressing RFP were used as reference for statistical analysis.

Then, we wanted to check, thanks to RFP tag added on Pastrel protein, if the overexpression was homogeneous in the population of cells transfected by the vectors. Only few cells showed a high level of Pastrel expression. Interestingly, we noticed by immunofluorescence assay that cells overexpressing the sensitive or resistant versions of Pastrel were never infected by DCV (**data not shown**). We also noticed that DCV does not infect each S2 cells in cell culture, even if the multiplicity of infection (MOI) is equal or greater than one. We hypothesized that the moderate effect of Pastrel expression on DCV may result from the low transfection efficiency of Pastrel fusion proteins, coupled to the low efficiency of infection, reducing the probability of DCV to meet a cell overexpressing Pastrel protein. This could mask the effect of Pastrel overexpression on DCV infection. Thus, it appeared necessary to establish stable cell lines overexpressing Pastrel protein fusions to by-pass this problem. Stable cell lines overexpressing sensitive and resistant versions of Pastrel tagged in N or C-terminal by RFP were screened by Fluorescence-Activated Cell Sorting (FACS) analysis (**Figure 32A**). The clones that had a cell population with an homogenous expression of Pastrel fusions, or RFP only, were selected and infected with DCV (MOI 1). The overexpression of Pastrel constructs was also confirmed by qPCR (**Figure 32B** and **32C**).

Stable cell lines overexpressing Pastrel fusions showed a remarkable decrease of DCV RNA level after 16h and 48h of infection, compared to cells stably expressing RFP as a control (**Figure 32B** and **32C**). We used two independent clones for each Pastrel constructions to avoid positional effect after random integration of our constructs in the genome of *Drosophila* S2 cells. Independent clones showed similar restriction of DCV infection after 16h and 48h of infection. Surprisingly, both sensitive and resistant Pastrel constructs were able to restrict DCV infection. We did not observe a higher resistance to DCV infection in clones overexpressing the resistant version of Pastrel protein compared to clones overexpressing the sensitive version of Pastrel protein. Thus, the high level of expression of the tagged Pastrel protein appears to abolish the difference between the two versions of the Pastrel protein.

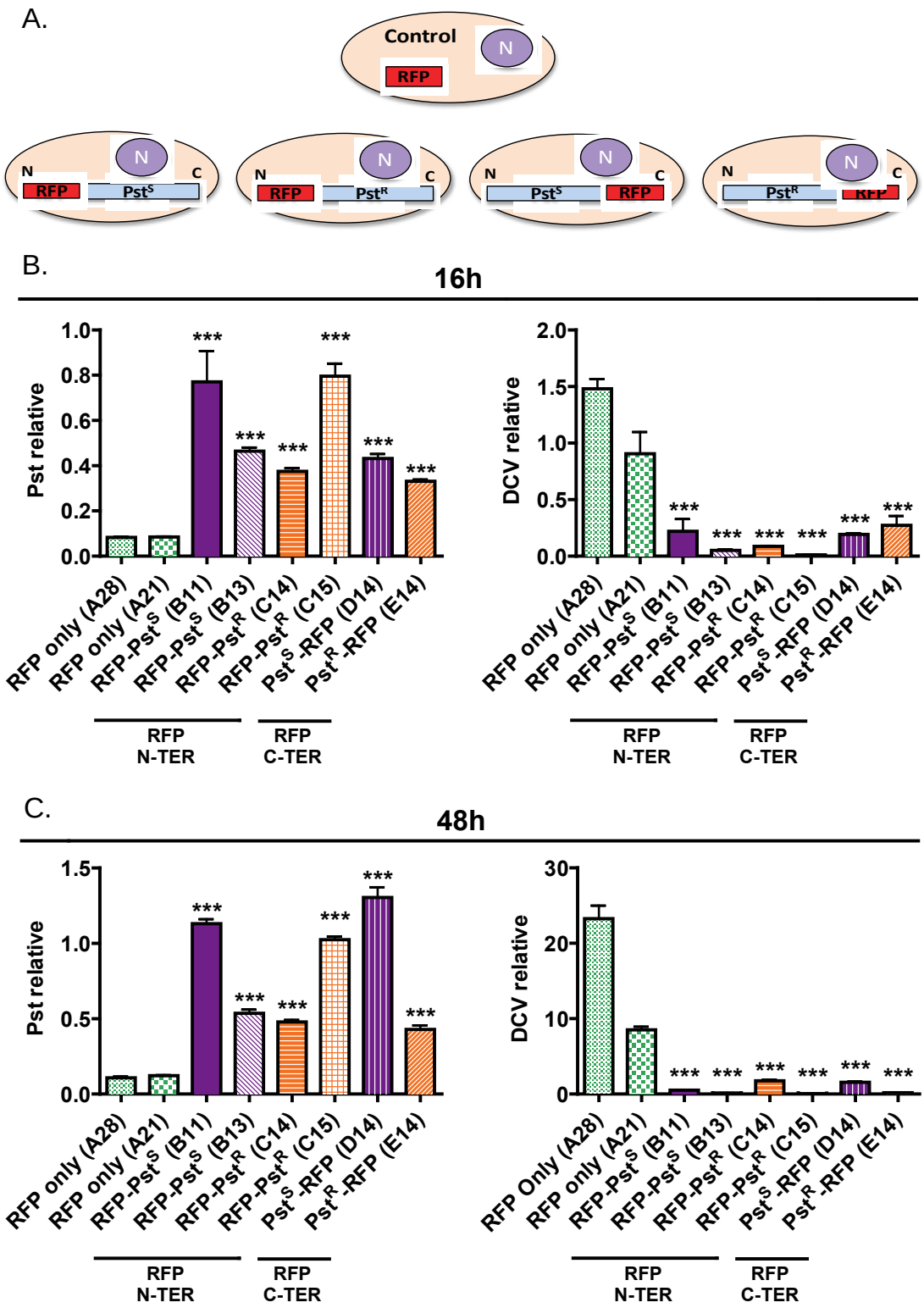


Figure 32. *Pst* overexpression decreases DCV RNA level after 16h and 48h of infection. **A.** Stable cell lines were established to overexpress RFP (control) or Pst sensitive and resistant forms tagged by RFP in N or C-terminal. **B. and C.** DCV and Pst RNA levels were measured by qPCR after 16h (**B.**) or 48h (**C.**) of infection in cells overexpressing RFP and Pst fusions. Bar graphs represent the average standard deviation of 3 independent experiments for panel **B** and 2 independent experiments for panel **C**. qPCR values were normalized to the housekeeping gene *RpL32*. Clones identifications are indicated in brackets.

We have observed a decrease in DCV RNA levels in *Drosophila* S2 cells overexpressing Pastrel fusions. We further analyzed the effect of Pastrel overexpression by looking at the DCV capsid protein level. By FACS, we were able to discriminate cells infected by DCV from cells non infected by using an antibody against DCV capsid protein. A large subset of cells overexpressing RFP was stained by DCV capsid antibody after 48h of infection (**Figure 33A**). By contrast, cells overexpressing Pastrel protein fused with RFP were mostly not stained by DCV capsid antibody after 48h of infection (**Figure 33B to 33E**). The DCV capsid staining was even comparable to the non infected condition, which is antibody background staining. Overexpresion of sensitive and resistant versions of Pastrel showed comparable levels of restriction of DCV infection (**Figure 33**, panels **B**, **D** and **C**, **E** respectively). We noticed a better restriction of DCV infection in clones overexpressing Pastrel with RFP tag in N-terminal (panels **B** and **C**) compared to ones with RFP tag in C-terminal (panels **D** and **E**). A small population of DCV infected cells is detected in clones overexpressing Pastrel-RFP fusions, as if RFP tag in C-terminal of Pastrel would destabilize its antiviral function. This may indicate that the C-terminal region of Pastrel participates in the restriction of DCV infection.

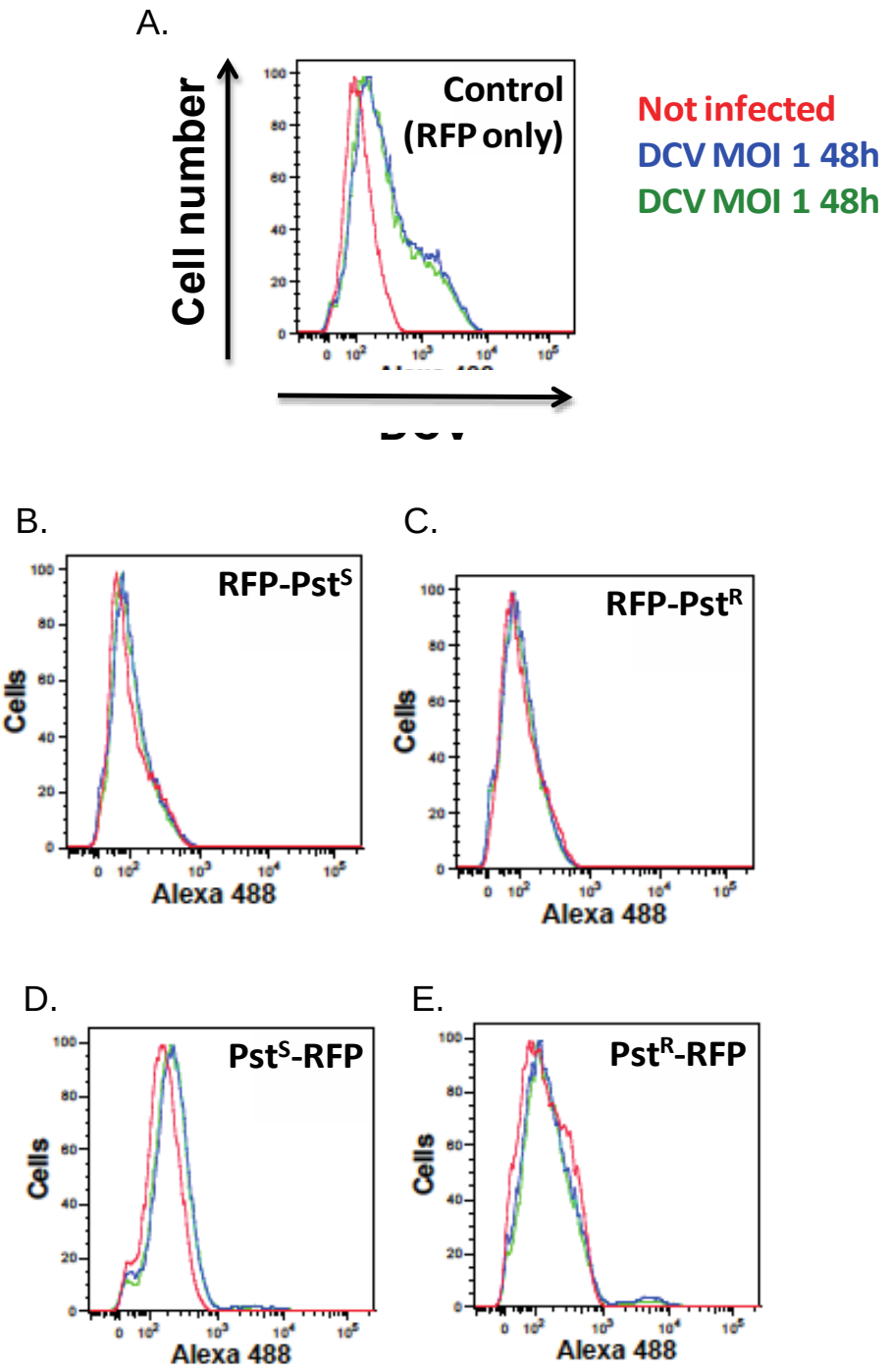


Figure 33. Pst^S and Pst^R overexpression restricts DCV infection. Stable cell lines expressing RFP only (control, panel **A**), Pst sensitive and resistant versions tagged by RFP in N-terminal (panels **B** and **C**) or C-terminal (panels **D** and **E**), were infected, or not, by DCV (MOI 1) for 48h. Cells were stained with an antibody against DCV capsid and analyzed by FACS ARIA II. Panels are representative of 3 independent experiments. These data were confirmed by 3 other independent experiments with independent clones.

The overexpression of sensitive and resistant versions of Pastrel in *Drosophila* S2 cells restricts DCV infection. We wondered if the better resistance to DCV infection acquired by S2 cells overexpressing Pastrel could be transmitted to *Drosophila* S2 cells which express endogenous sensitive version of Pastrel (determined by *pastrel* genotyping). To answer this question, *Drosophila* S2 cells and cells overexpressing Pastrel (sensitive and resistant versions) or RFP (control) were mixed together with a ratio of 1:1. Mixed population of cells were infected with DCV (MOI 1) for 48h, stained by DCV capsid antibody and analyzed by FACS (**Figure 34**). As a control, both mixed populations of *Drosophila* S2 cells and cells overexpressing RFP were infected by DCV at 48h post infection (**Figure 34A**). By contrast, only *Drosophila* S2 cells were infected by DCV after 48h of infection when mixed with cells overexpressing sensitive or resistant versions of Pastrel (**Figure 34B** and **34C** respectively). A lower amount of *Drosophila* S2 cells was infected by DCV after mixing with cells overexpressing Pastrel, compared to S2 cells mixed with cells overexpressing RFP. This can be explained by a lower quantity of viral particles liberated in the supernatant when S2 cells are mixed with cells overexpressing pastrel, as the total number of DCV infected cells is lower compared to the control. We conclude that the restriction of DCV infection by Pastrel is cell-autonomous. Cells overexpressing sensitive and resistant versions of Pastrel are better protected from DCV infection than normal S2 cells, and do not transmit this higher protection to *Drosophila* S2 cells.

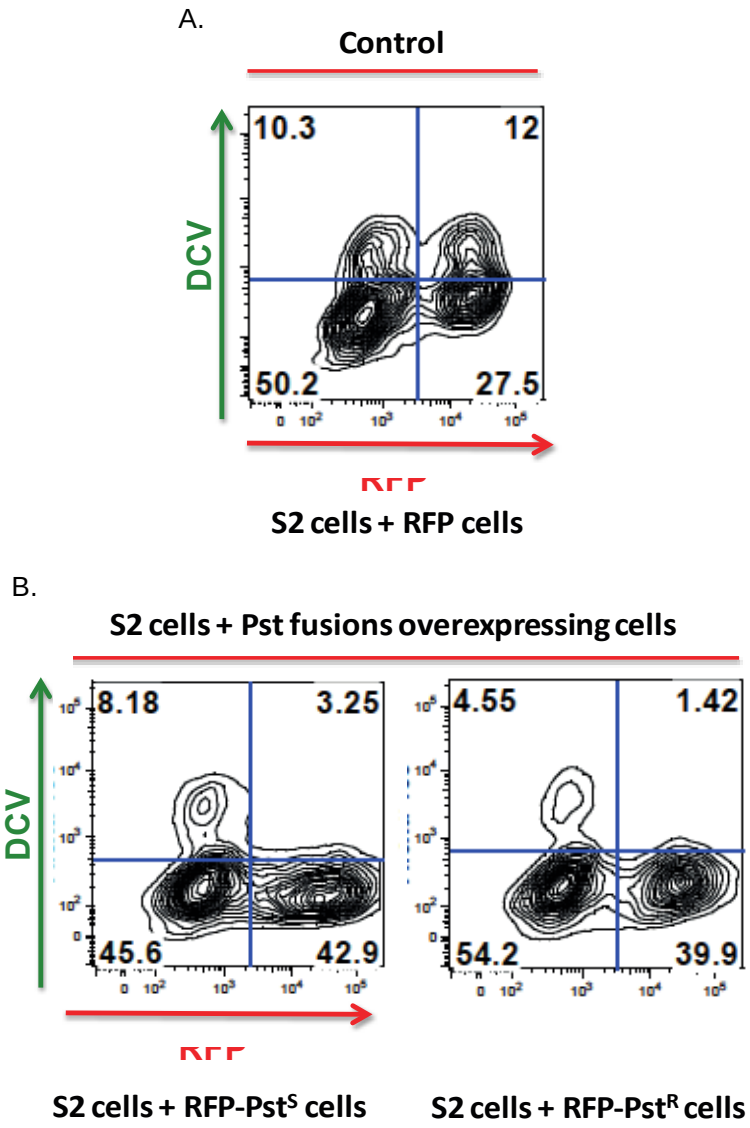


Figure 34. Cells overexpressing Pst are better protected from DCV infection compared to cells with endogenous Pst expression. A. *Drosophila* S2 cells were mixed with stable cell lines overexpressing RFP. **B.** *Drosophila* S2 cells were mixed with stable cell lines overexpressing sensitive and resistant versions of Pastrel tagged by RFP in N-terminal. S2 cells and stable cell lines were mixed in a 1:1 ratio and infected with DCV (MOI 1) for 48h. Cells were then stained with antibody against DCV capsid and analysed by FACS ARIA II. Panels are representative of 1 experiment. These data were confirmed by 1 experiment with independent clones.

We investigated if overexpression of Pastrel, which restricts DCV infection, also protects cells from CrPV infection. We selected clones overexpressing sensitive and resistant version of Pastrel which showed highest protection to DCV infection and measured CrPV RNA level after 16h and 48h of infection. Cells overexpressing Pastrel (**Figure 35A** and **35C**)

showed reduced CrPV RNA level at 16h and 48h post infection compared to cells overexpressing RFP (**Figure 35B** and **35D** respectively).

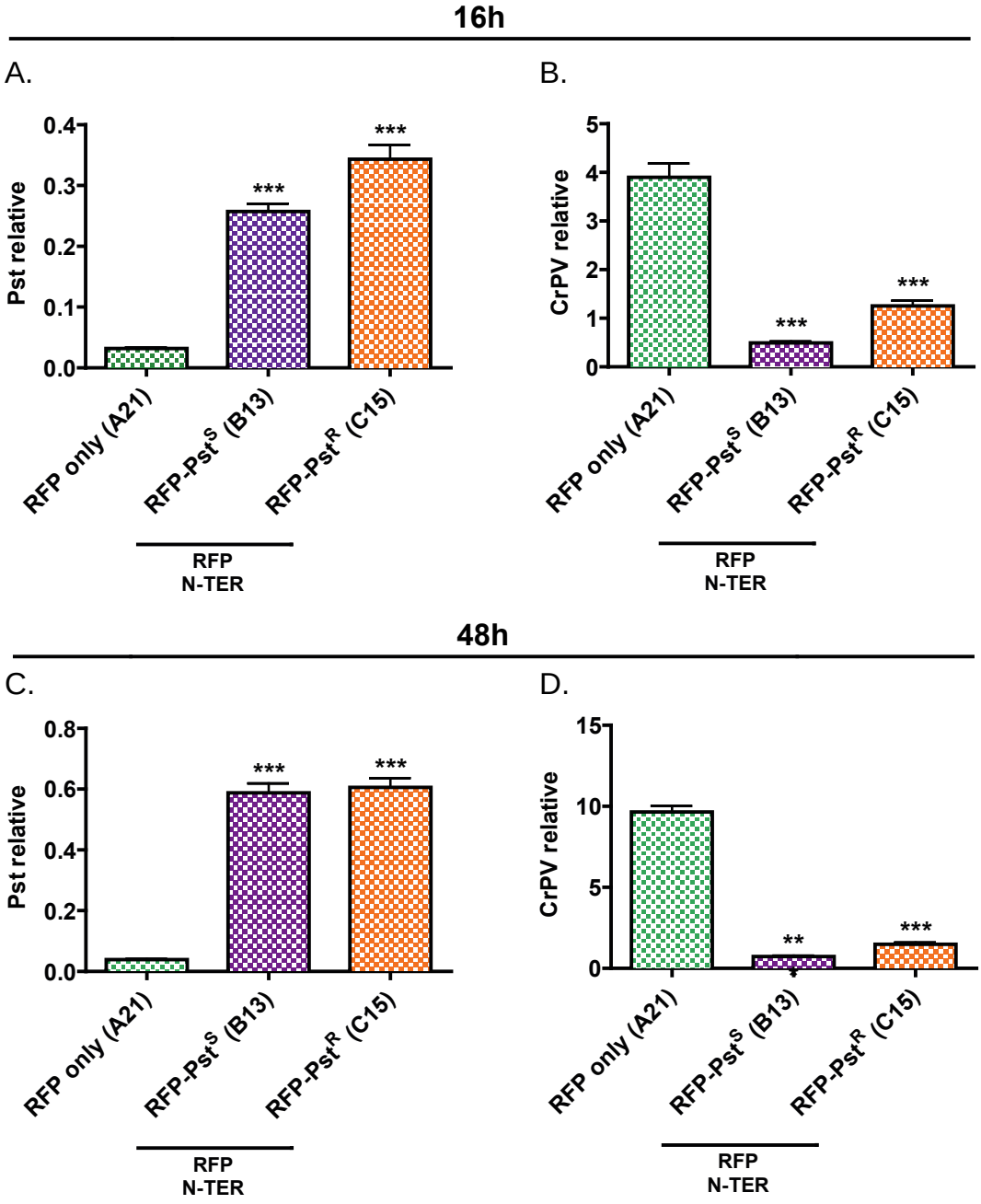


Figure 35. Pst^S and Pst^R overexpression decreases CrPV RNA level after 16h and 48h of infection. A. and C. Pst RNA level was measured by qPCR after 16h (**A.**) and 48h (**C.**) of infection (MOI 0.1). **B.** and **D.** CrPV RNA level was measured by qPCR after 16h (**B.**) and 48h (**D.**) of infection (MOI 0.1). For all panels, bar graphs represent the average standard deviation of 2 independent experiments. qPCR values were normalized to the housekeeping gene *RpL32*. Clones identifications are indicated in brackets.

The overexpression of Pastrel in *Drosophila* S2 cells restricts both DCV and CrPV infection. For further confirmation, immunofluorescence assays were performed with antibodies against DCV and CrPV capsid. After 48h of infection, stable cell lines overexpressing sensitive (**Figure 36A**, panel **B**) and resistant (**Figure 36A**, panel **C**) versions of Pastrel fusions showed reduced number of cells infected by DCV, compared to stable cell lines overexpressing RFP (**Figures 36A**, panel **A**). For statistical analysis, we counted the number of cells overexpressing sensitive and resistant versions of Pastrel, or RFP, that were infected by DCV (**Figure 36B**). Around 30% of cells overexpressing RFP were infected by DCV. By contrast, only 1.8% of cells overexpressing sensitive or resistant versions of Pastrel were infected by DCV. Western blot analysis with DCV capsid antibody revealed that the level of DCV particles was almost not detectable in cells overexpressing Pastrel protein, compared to cells overexpressing RFP (**Figure 36C**).

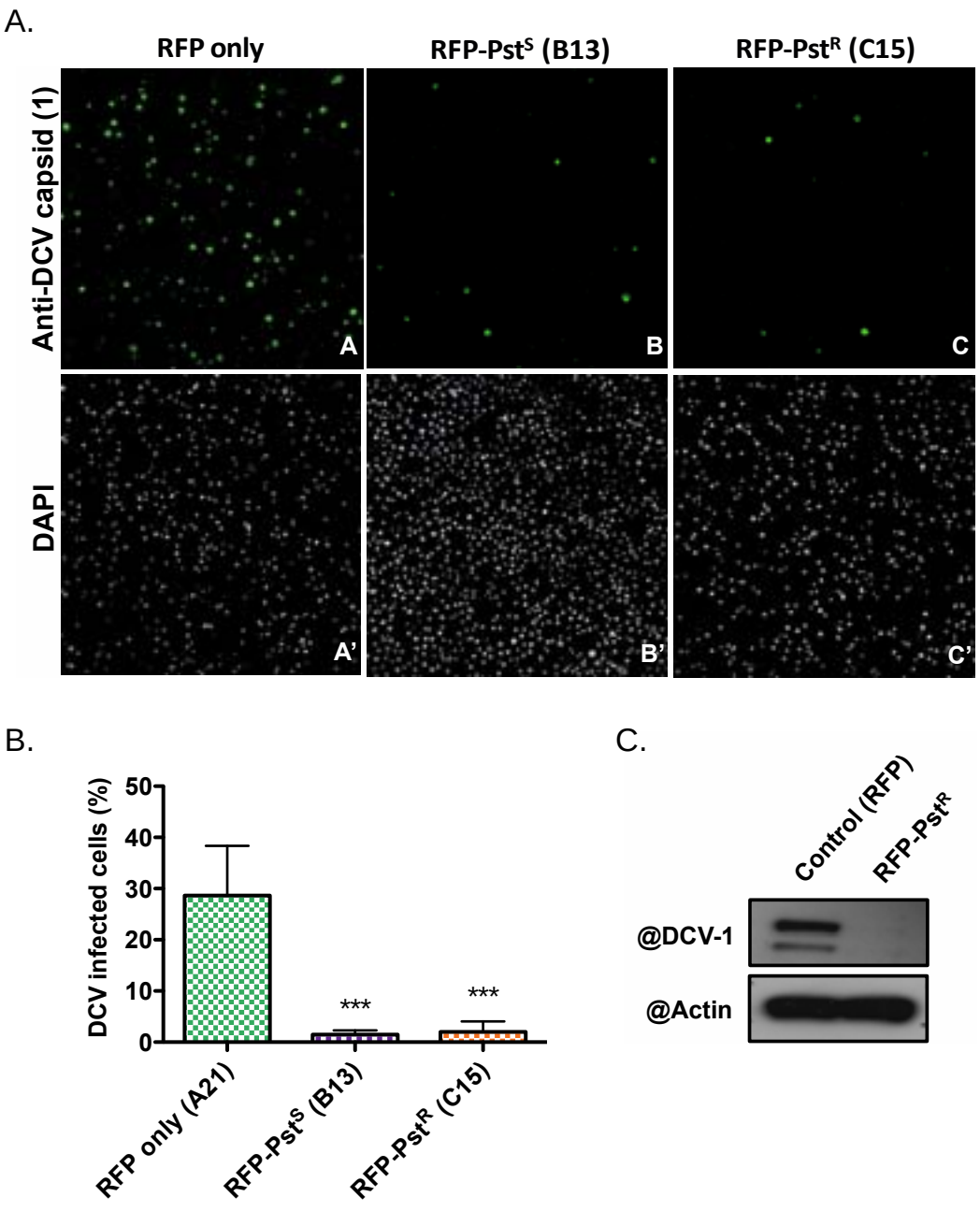


Figure 36. Overexpression of Pst^S and Pst^R decreases the number of cells infected by DCV. A. Stable cell lines overexpressing RFP only (control, panels A and A'), Pst sensitive (panels B and B') and resistant (panels C and C') versions tagged by RFP in N-terminal, were infected with DCV (MOI 1) for 48h. Cells were stained with an antibody against DCV capsid (anti-DCV-1, panels A, B and C). DAPI staining is represented in panels A', B' and C'. Pictures were taken by confocal LSM700. **B.** DCV infected cells were quantified using ImageJ. Bar graphs represent the average standard deviation of 9 pictures. Panels are representative of 2 independent experiments. **C.** Stable cell lines overexpressing RFP and Pst resistant version tagged by RFP in N-terminal were infected with DCV (MOI 1) for 24h. DCV protein level was revealed by western blot with antibody against DCV (@DCV-1). Antibody against Actin protein was used as loading control. Clones identifications are indicated in brackets.

Similarly, cells overexpressing sensitive (**Figure 37A**, panel **B**) and resistant (**Figure 37A**, panel **C**) versions of Pastrel were less infected by CrPV than cells overexpressing RFP (**Figure 37A**, panel **A**). 9% of cells overexpressing RFP were stained with antibody against CrPV capsid. Strikingly, only 0.5% of cells overexpressing sensitive and resistant versions of Pastrel were stained by CrPV antibody after 24h of infection (**Figure 37B**).

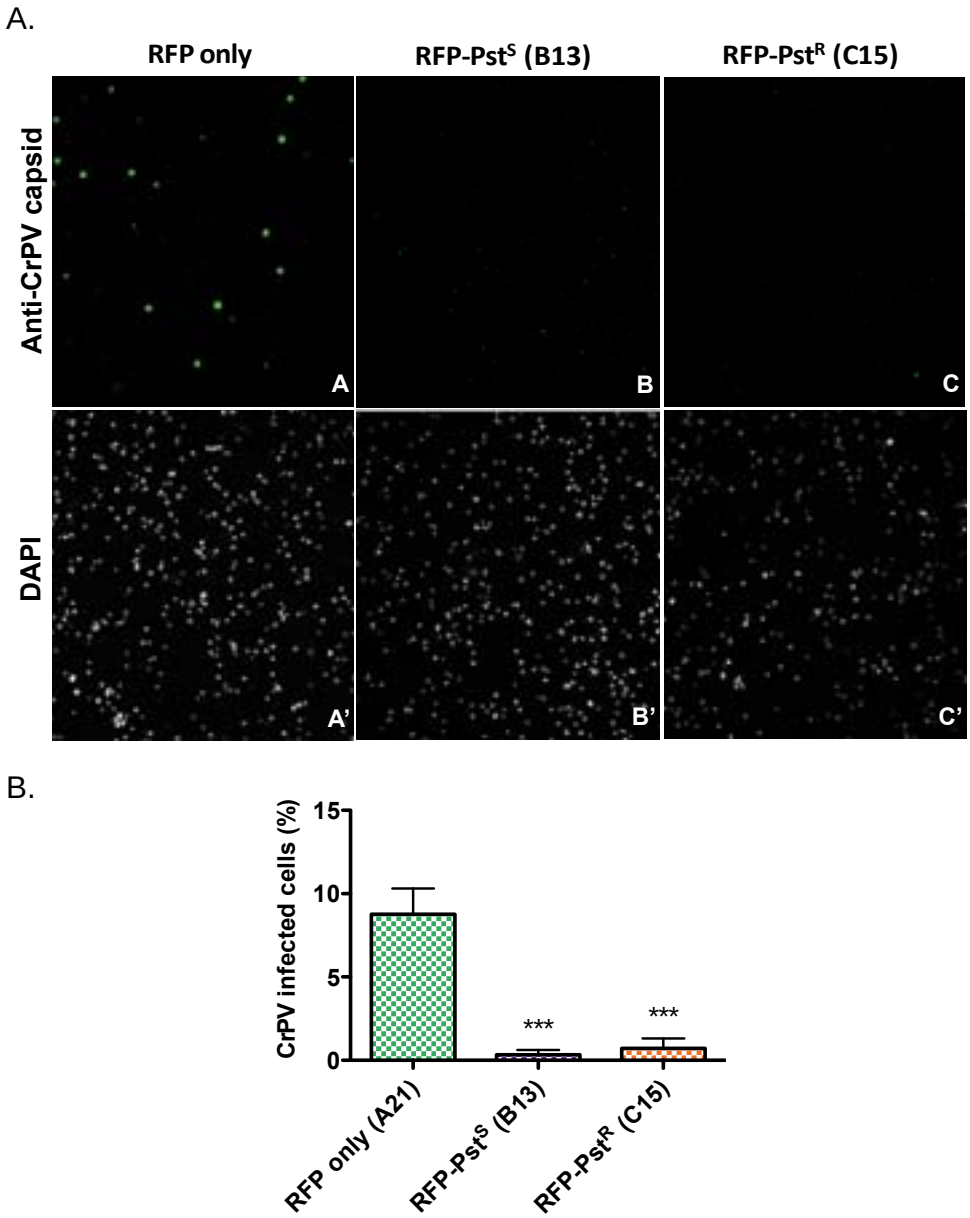


Figure 37. Overexpression of Pst^S and Pst^R decreases the number of cells infected by CrPV. A. Stable cell lines overexpressing RFP only (control, panels A and A'), Pst sensitive (panels B and B') and resistant (panels C and C') versions tagged by RFP in N-terminal, were infected with CrPV (MOI 0.01) for 24h. Cells were stained with an antibody against CrPV capsid (anti-CrPV-4, panels A, B and C). DAPI staining is represented in panels A', B' and C'. Pictures were taken by confocal LSM700. **B.** CrPV infected cells were quantified using ImageJ. Bar graphs represent the average standard deviation of 9 pictures. Panels are representative of 1 experiment. Clones identifications are indicated in brackets.

By contrast, the overexpression of sensitive (**Figure 38**, panel **B**) and resistant (**Figure 38**, panel **C**) versions of Pastrel did not protect cells from FHV infection (**Figure 38**). No statistical difference was observed between the number of cells overexpressing sensitive and resistant versions of Pastrel, plus RFP, and stained by antibody against FHV capsid (**Figure 38B**).

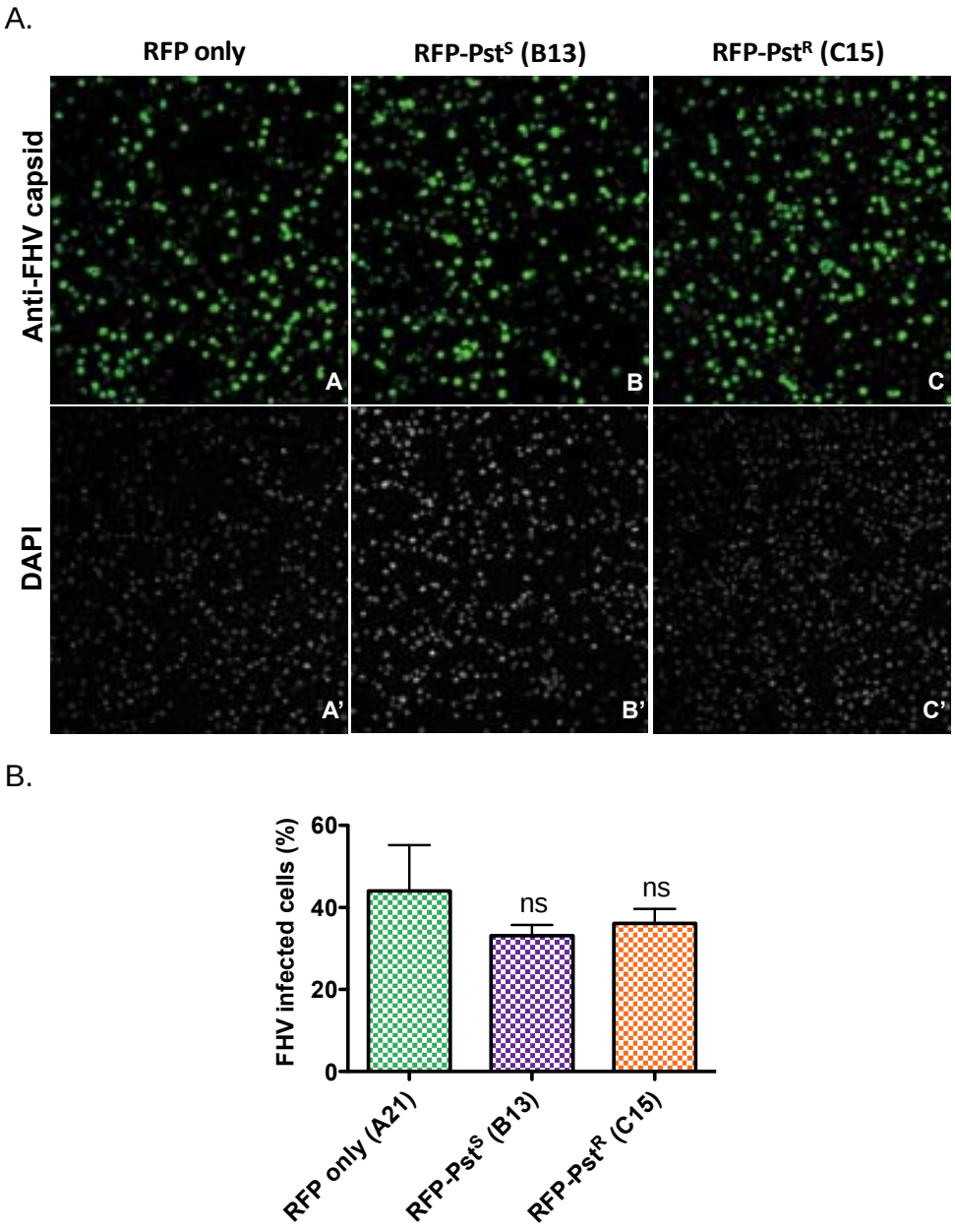


Figure 38. Overexpression of Pst^S and Pst^R does not affect the number of cells infected by FHV. A. Stable cell lines overexpressing RFP only (control, panels A and A'), Pst sensitive (panels B and B') and resistant (panels C and C') versions tagged by RFP in N-terminal were infected with FHV (MOI 1) for 24h. Cells were stained with an antibody against FHV capsid (anti-FHV-5588, panels A, B and C). DAPI staining is represented in panels A', B' and C'. Pictures were taken by confocal LSM700. **B.** FHV infected cells were quantified using ImageJ. Bar graphs represent the average standard deviation of 4 pictures. Panels are representative of 1 experiment. Clones identifications are indicated in brackets.

Overexpression of sensitive (**Figure 39**, panel **B**) and resistant (**Figure 39**, panel **C**) versions of Pastrel did not affect the number of cells stained by VSV antibody after 48h of infection, compared to cells overexpressing RFP (**Figure 39**, panel **A**). The number of cells infected by VSV was similar between clones overexpressing RFP, sensitive and resistant versions of Pastrel (**Figure 39B**).

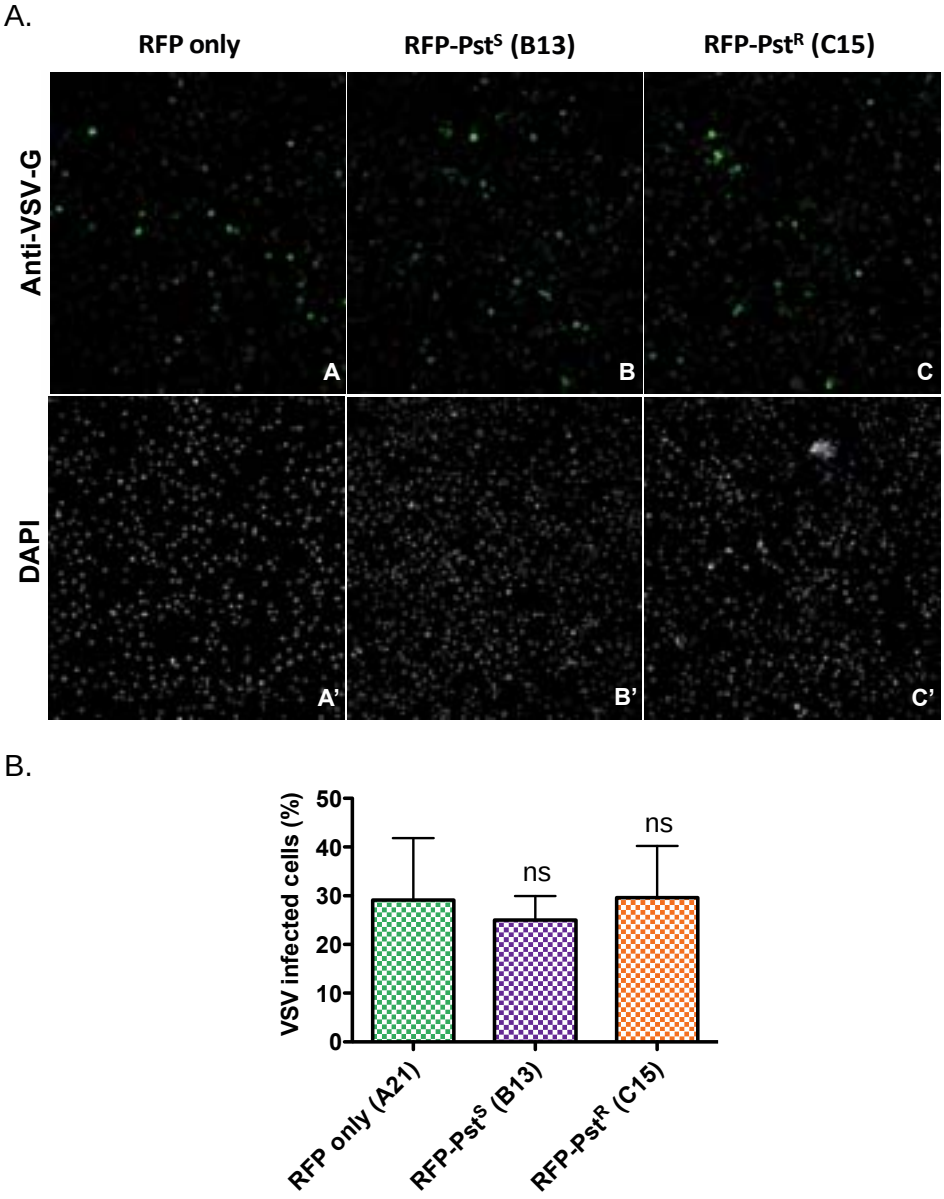


Figure 39. Overexpression of Pst^S and Pst^R does not affect the number of cells infected by VSV. A. Stable cell lines overexpressing RFP only (control, panels A and A'), Pst sensitive (panels B and B') and resistant (panels C and C') versions tagged by RFP in N-terminal were infected with VSV (MOI 25) for 48h. Cells were stained with an antibody against glycoprotein (G) of VSV (anti-VSV-G-FITC, panels A, B and C). DAPI staining is represented in panels A', B' and C'. Pictures were taken by confocal LSM700. **B.** VSV infected cells were quantified using ImageJ. Bar graphs represent the average standard deviation of 9 pictures. Panels are representative of 1 experiment. Clones identifications are indicated in brackets.

Altogether, these results indicate that Pastrel restricts infection by *Dicistroviridae* DCV and CrPV in *Drosophila* S2 cells. However, from our immunofluorescence assay, Pastrel does not affect the infection by *Nodaviridae* FHV and *Rhabdoviridae* VSV. This is in agreement with similar susceptibility to FHV and VSV infection of flies expressing sensitive or resistant versions of Pastrel (see sections **4.1.a** and **4.1.b**). We then looked for the step of *Dicistroviridae* infectious cycle which may be impaired by the antiviral function of Pastrel.

4.5.c. Pst antiviral function acts at early steps of DCV infectious cycle

We first investigated if the overexpression of Pastrel affects the binding of DCV particles on the surface of *Drosophila* S2 cells. We performed binding assay by infecting cells with DCV (MOI 20) and incubating them at 4°C for 2h. At this temperature, endocytosis is blocked. So the virus can bind his receptor but cannot enter in the cells. After washing cells, DCV RNA level was measured by qPCR and no statistical difference was observed between cells overexpressing sensitive and resistant versions of Pastrel and RFP (**Figure 40**). As a control, cells were infected for 2h at 25°C. In this condition, we began to see a difference of DCV RNA level between cells overexpressing sensitive and resistant versions of Pastrel and cells overexpressing RFP only. Even if this difference was not significative, it may indicate a early antiviral effect of Pastrel on DCV infectious cycle.

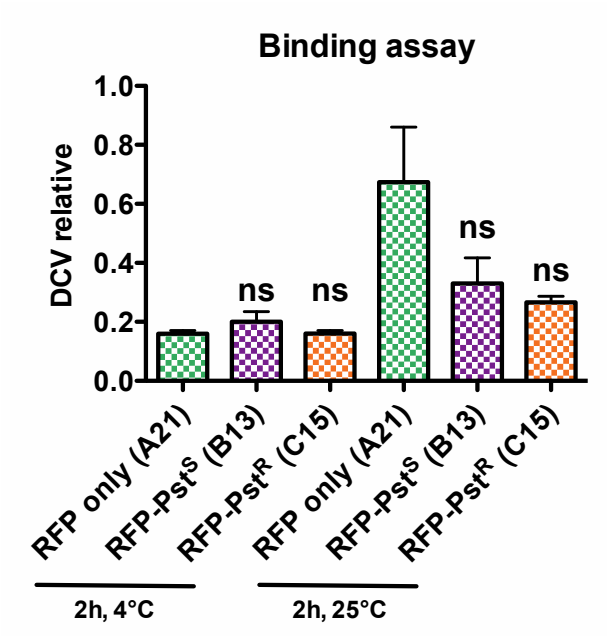


Figure 40. Pst^S and Pst^R overexpression does not affect binding of DCV on cells. Stable cell lines overexpressing RFP only (control), sensitive and resistant versions of Pst tagged by RFP in N-terminal, were infected with DCV (MOI 20) for 2h at 4°c to allow DCV binding or 25°C to allow DCV binding and entry. DCV RNA level was measured by qPCR and normalized to *RpL32*. The average standard deviations are representative of 1 out of 5 independent experiments. Clones identifications are indicated in brackets.

Pastrel does not affect the binding of DCV particles on *Drosophila* S2 cells. We wondered if the following step, the entry of the virus, is impaired by the antiviral action of Pastrel. To answer this question, we performed a viral entry assay. Cells were infected with DCV (MOI 20), incubated for 2h at 4°C, washed with PBS and switched to 25°C for 3h to allow virus entry. Pastrel overexpression in cells was confirmed by qPCR (**Figure 41A**). By qPCR, we observed a significant decrease of DCV RNA level in cells overexpressing either the sensitive or resistant version of Pastrel compared to cells overexpressing RFP, after 3h of infection (**Figure 41B**).

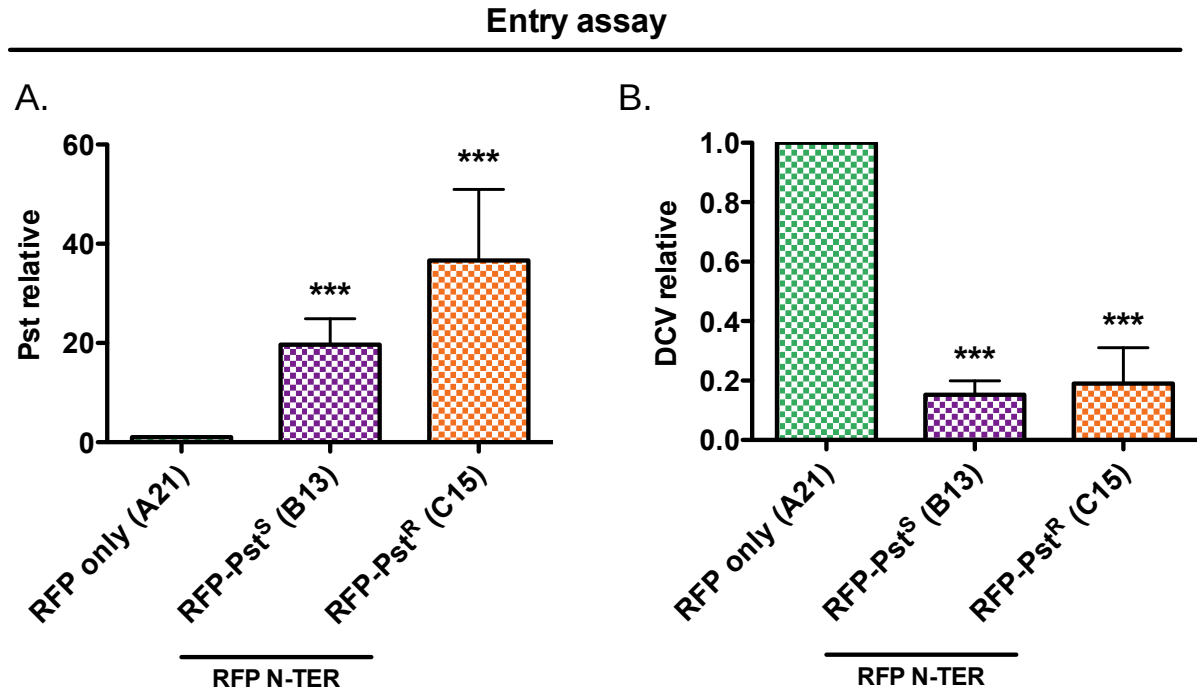


Figure 41. Pst^S and Pst^R overexpression affects entry of DCV in cells. Stable cell lines overexpressing RFP only (control), sensitive and resistant versions of Pst tagged by RFP in N-terminal, were infected with DCV (MOI 20) for 2h at 4°c to allow DCV binding and switched at 25°C for 3h to allow DCV entry. **A.** Pst RNA level was measured by qPCR. **B.** DCV RNA level was measured by qPCR. qPCR values were normalized to the housekeeping gene *RpL32* and control RFP only was used as reference for analysis. Bar graphs represent the average standard deviation of 4 independent experiments. Clones identifications are indicated in brackets.

To summary, Pastrel impairs DCV infection after 3h of infection, without affecting DCV binding. To precisely decipher when does Pastrel begin to exerss its antiviral function, DCV RNA level was measured in cells overexpressing sensitive and resistant versions of Pastrel, cells overexpressing RFP as control, after 2h incubation at 4°C followed by 0, 1, 2, 3,

24h incubation at 25°C (**Figure 42**). DCV RNA level started to be strongly reduced from 1h after DCV entry in cells overexpressing sensitive and resistant versions of Pastrel, compared to cells overexpressing RFP (**Figure 42A**). Interestingly, this experiment revealed a progression of DCV infection in cells overexpressing Pastrel. However, this progression of DCV infection was remarkably lower compared to cells overexpressing RFP. DCV RNA level moderately increased between early time points (1, 2 and 3h of infection) and later time point (24h of infection) in cells overexpressing Pastrel, compared to cells overexpressing RFP. The same experiment was performed with CrPV. Surprisingly, the kinetic of CrPV infection at early time points was different to the one of DCV (**Figure 42B**). A peak of CrPV infection was observed in cells overexpressing RFP but not in cells overexpressing sensitive and resistant versions of Pastrel at 1h after CrPV infection. A second peak of CrPV replication probably appeared after 3h of infection because the CrPV RNA level in S2 cells and cells overexpressing RFP was strikingly increased at 24h post infection. CrPV RNA level was also increased in cells overexpressing sensitive and resistant versions of Pastrel after 24h of infection compared to early time points (1, 2, 3h of infection) but lower than in control cells (**Figure 42B**).

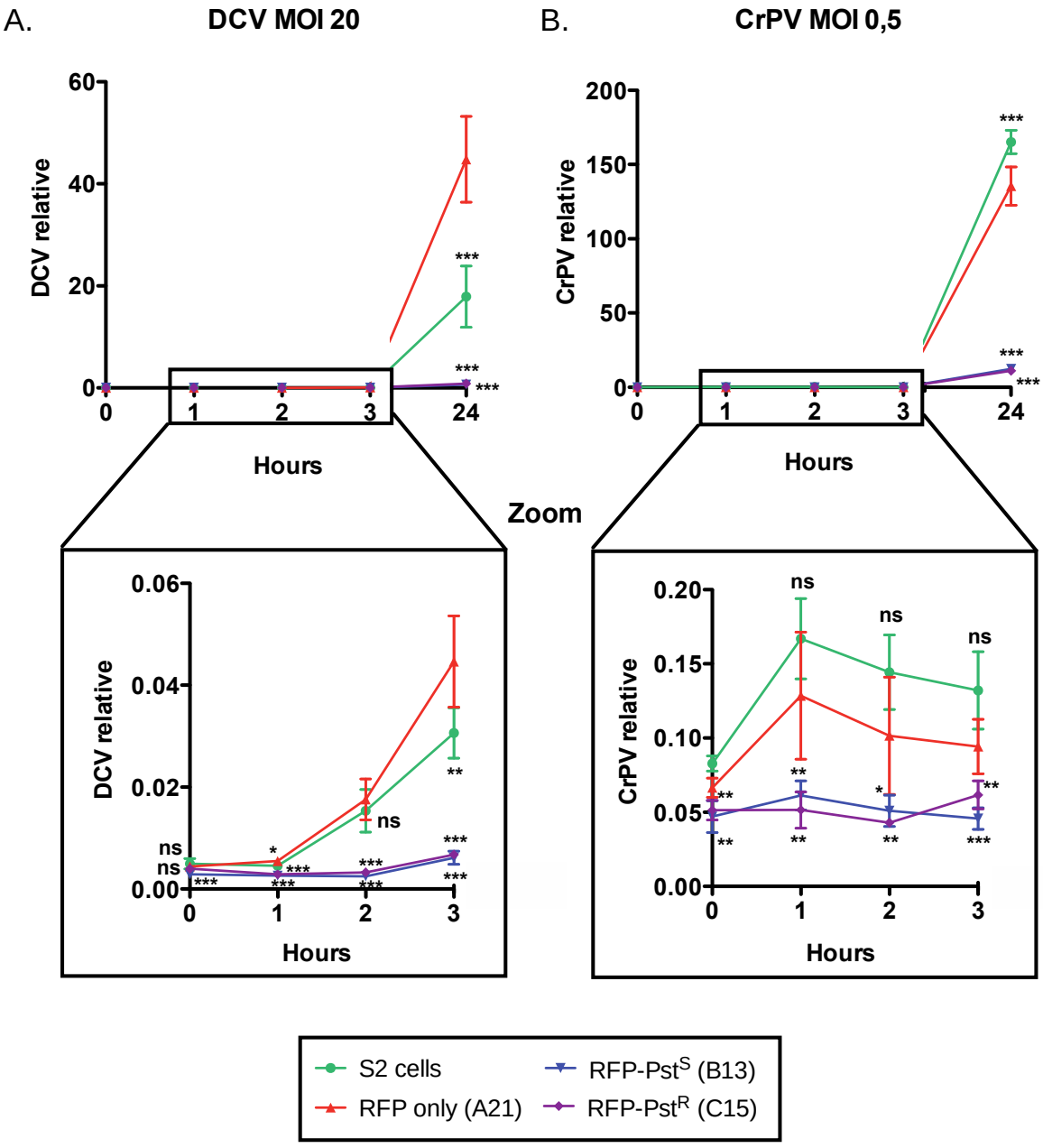


Figure 42. Pst^S and Pst^R overexpression affects DCV and CrPV early after infection. *Drosophila* S2 cells and **s**table cell lines overexpressing RFP only (control), sensitive and resistant versions of Pst tagged by RFP in N-terminal, were infected with DCV (MOI 20) or CrPV (MOI 0.5) for 2h at 4°c to allow virus binding on cell surface and then transfered at 25°C for 0, 1, 2, 3 and 24h. **A.** DCV RNA level was measured by qPCR. **B.** CrPV RNA level was measured by qPCR. qPCR values were normalized to the housekeeping gene *RpL32* and control RFP only was used as reference for analysis. Bar graphs represent the average standard deviation of 1 experiment. Clones identifications are indicated in brackets.

4.5.d. Pst does not affect CrPV IRES translation

Unlike the other viruses not affected by Pastrel, *Dicistroviridae* rely on Internal Ribosome Entry Site (IRES) for the translation of structural and non structural proteins. To test if the overexpression of Pastrel affects the IRES driven translation in *Drosophila* cells, we co-transfected vectors expressing Renilla luciferase under CrPV 5' IRES translation and Firefly luciferase under cap-dependant translation. Dual luciferase assay was performed 48h after transfection. Cells overexpressing Pastrel (sensitive and resistant versions) and RFP showed similar levels of Renilla luciférase, indicating that overexpression of Pastrel does not affect CrPV 5' IRES translation (**Figure 43A**). We also showed that intergenic region (IGR) IRES of CrPV (CrPV IGR IRES) mediated translation was not affected by the overexpression of Pastrel (**Figure 43B**). Surprisingly, a statistically significant increase of CrPV IGR IRES translation was observed after overexpression of the resistant version of Pastrel. However, this effect was minor and the significance of this observation is not clear at present.

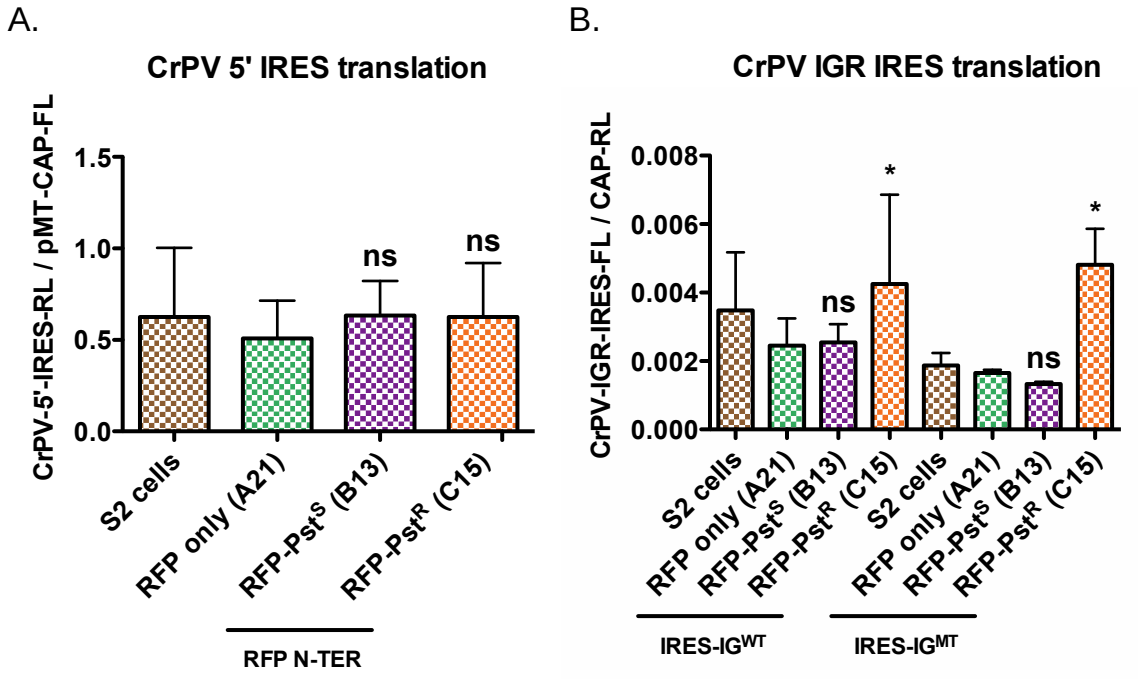


Figure 43. Pst^S and Pst^R overexpression does not affect CrPV 5' IRES and CrPV IGR IRES translation. A. Cells were co-transfected by vectors expressing Renilla luciferase under CrPV-5'-IRES translation and Firefly luciferase under cap-dependant translation. **B.** Cells were transfected by a vector expressing Firefly luciferase under CrPV-IGR-IRES translation and Renilla luciferase under cap-dependant translation. For both panels, 500 µM $CuSO_4$ were added the day after transfection and luminescence was read 72h after transfection. Bar graphs represent the average standard deviation of 3 independent experiments for panel **A** and 2 independent experiments for panel **B**. Clones identifications are indicated in brackets.

4.5.e. Pst is not involved in protein secretion

In 2006, the team of Pr. Malhotra performed a screen to identify genes involved in protein secretion in *Drosophila* S2 cells (Bard et al., 2006). Pastrel was found to play a role in protein secretion. This function may affect DCV infectious cycle if Pastrel perturbated secretion of DCV proteins during the infectious cycle. We first confirmed the role of Pastrel in protein secretion. The vector expressing horseradish peroxidase (HRP) fused with secretion signal peptide (ss-HRP) was kindly sent by Pr. Malhotra. After induction of ss-HRP production by copper, peroxydase activity was measured from an aliquot of medium by chemiluminescence. Luminescence was comparable between cells treated with dsRNAs against *pastrel* gene (region A + B) and GFP (negative control), but significantly reduced for cells treated with dsRNAs against beta-COP and Syntaxin5 (**Figure 44A**). These two proteins are known to be involved in protein secretion and were used as positive controls. The knockdown of *pastrel* gene did not affect protein secretion.

The vector expressing ss-HRP was also transfected in cells overexpressing sensitive and resistant versions of Pastrel and cells overexpressing RFP, as a control. The luminescence measured from medium of cells overexpressing Pastrel and RFP was comparable, but higher than one from normal *Drosophila* S2 cells (**Figure 44B**), suggesting that either transfection or overexpression of exogenous protein stimulates the secretory pathway.

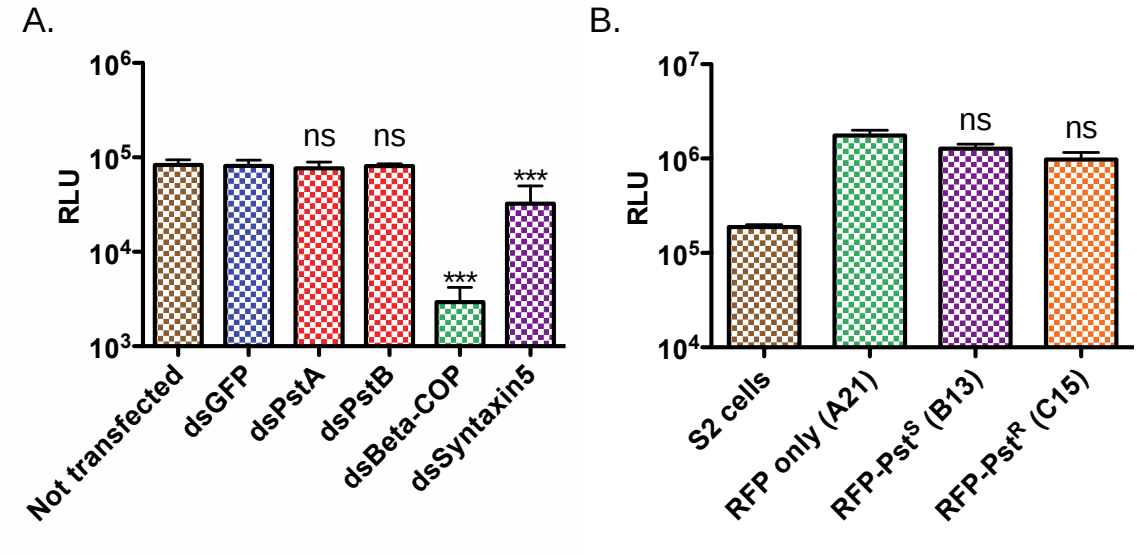


Figure 44. Pst is not involved in protein secretion. A. S2 cells were treated by dsRNA against GFP, Pst (region A + B), beta-COP and Syntaxin5 during 5 days. Cells were next transfected with vector expressing ss-HRP under methallothionein promoter (pMT-ss-HRP). The day after, ss-HRP expression was stimulated by addition of 500 µM $CuSO_4$ and cells

were incubated for 16h. Then, luminescence was read by luminometer after addition of ECL reagent to supernatant. **B.** S2 cells and stable cell lines were transfected by pMT-ss-HRP vector, treated by 500 µM $CuSO_4$ for 16h, and luminescence was read by luminometer to quantify secretion. Bar graphs represent the average standard deviation of 2 independent experiments.

Overall, the knockdown and overexpression of Pastrel does not affect protein secretion and the effect reported by Malhotra and colleagues may have reflected an off-target effect.

4.5.f. Study of Pst topology by a Biotin-Streptavidin revelation system

Transmembrane (TM) prediction softwares were used to determine putative TM domains of Pastrel protein. However, we noticed that different softwares predict a different number of TM domains for Pastrel protein. This was particularly disturbing for our analysis because some programs predicted an odd or even number of TM domains, which completely changes the topology of the protein. We computed the analysis from different programs to define a consensus of 6 putative TM domains predicted for Pastrel protein (**Figure 45**).

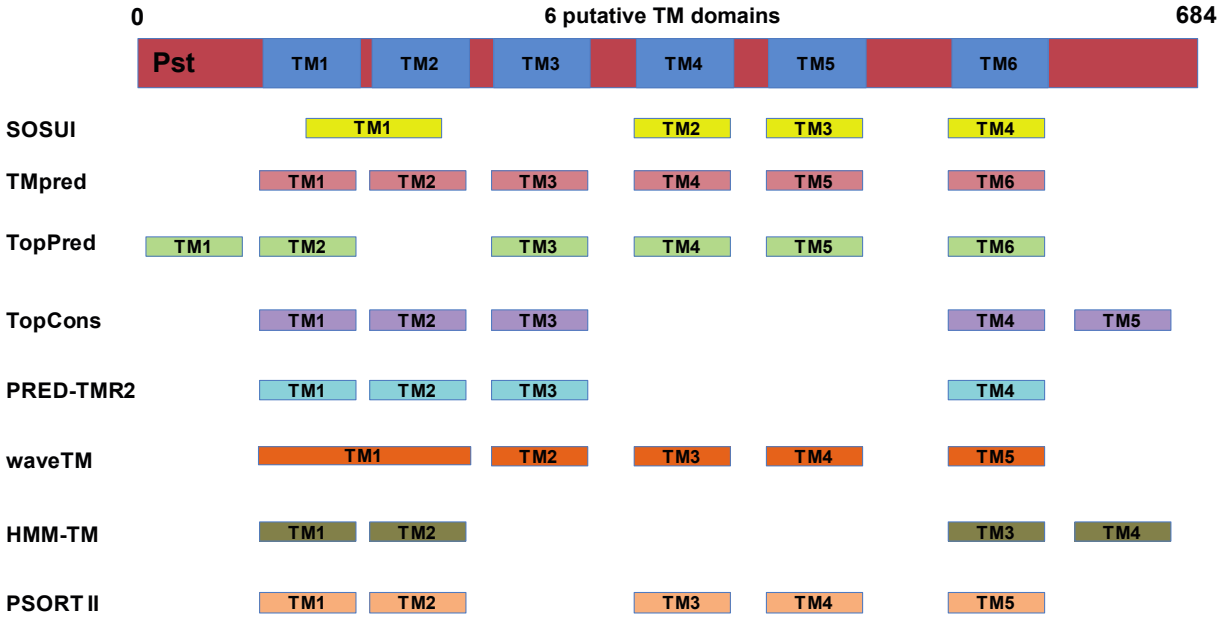


Figure 45. Prediction of 6 putative TM domains in Pastrel protein. Programs for TM prediction were used to identify the number of putative TM domains in Pastrel protein.

Prediction software gave also an ambigous answer whether the N- and C-terminal extremities of Pastrel protein face the cytosol or the lumen (**data not shown**). The SNP 3L:7350895 Ala/Thr, associated with the sensitivity or resistance of flies to DCV infection, is present in the C-terminal region. We wondered whether this region faces the cytosol or the lumen. Thus, we developed a new technique based on in situ biotinylation to determine if the N and C-terminal regions of Pastrel protein face the cytosol. A peptide of 15 amino acids, called Biotin Acceptor Peptide (BAP), was fused to Pastrel protein in N or C-terminal. As a control, BAP was fused to N and C-terminal extremities of the single-pass type I transmembrane receptor TOLL. These constructs were transfected in cells expressing the bacterial biotin-protein ligase BirA in the cytosol. BirA specifically recognizes and attaches a biotin to the single lysine residue of the BAP sequence. Therefore, if the BAP sequence is exposed in the cytosol, accessible to BirA, it will be biotinylated (**Figure 46A**). It was then easy and fast to reveal biotinylation after immunoprecipitation of the constructs and western blot using streptavidine-HRP. The C-terminal extremity of TOLL faces the cytosol and was biotinylated whereas the N-terminal extremity of TOLL, which faces the lumen of the secretory apparatus and then the exterior of the cell, was not (**Figure 46B**). Using the same procedure, we observed that both Pastrel constructs with BAP in N or C-terminal were biotinylated (**Figure 46C**). This indicates that N and C-terminal regions of Pastrel are exposed in the cytosol. This observation suggests that the C-terminal region of Pastrel, where the polymorphism is located, is potentially exposed to viral proteins or particles present in the cytosol.

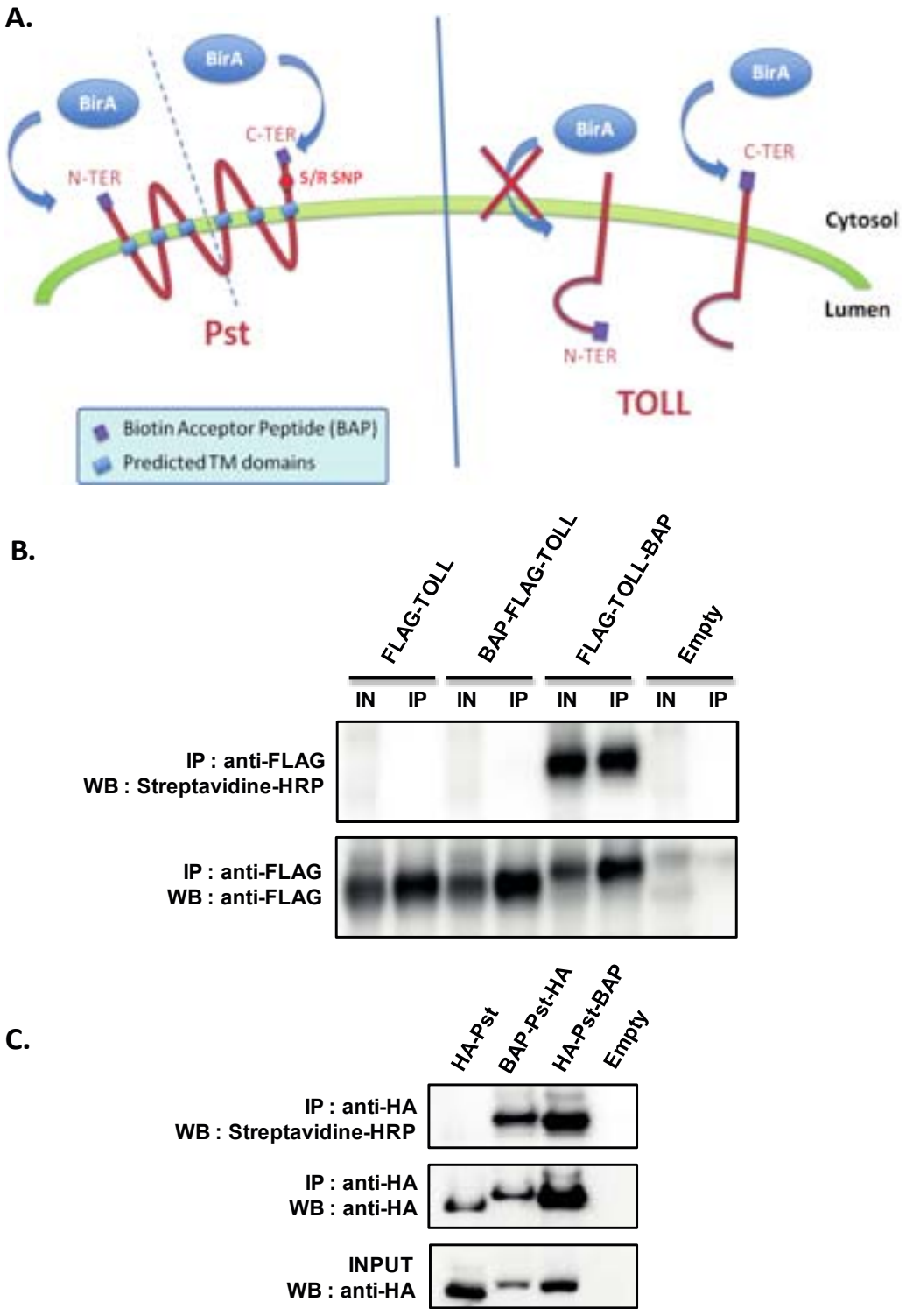


Figure 46. Topology of N and C-ter regions of Toll and Pst. A. Principle of the method: A 15 amino acid long Biotin Acceptor Peptide (BAP) was fused to Pst and TOLL proteins in N or C terminal. These constructs were then transfected in cells expressing the bacterial biotin-protein ligase BirA, which specifically recognizes and attaches a biotin to the single lysine residue of the BAP sequence. If the BAP sequence is exposed in the cytosol, accessible to BirA, this sequence is biotinylated. **B.** and **C.** Transfection of Pst or TOLL constructs in cells expressing BirA protein for 72h. Cells were then lysed, immunoprecipitated with anti-FLAG (**B.**) or anti-HA (**C.**) beads overnight at 4°C and biotinylation was revealed by addition of Streptavidin-HRP for 5 min at 4°C.

4.5.g. The C-terminal region of Pst is required for its antiviral function

We showed that Pastrel restricts DCV and CrPV infection in *Drosophila*. To decipher which region of Pastrel protein confers its antiviral function, successive deletions were performed in full length Pastrel protein (1-682) from the end of the last predicted transmembrane domain. Indeed, the SNP (3L:7350895 Ala/Thr), responsible of the sensitivity or resistance of flies to DCV infection, is present in the C-terminal region of Pastrel. Then, we hypothesized that the C-terminal region of Pastrel carries antiviral activity. Stable cell lines overexpressing a truncated version of Pastrel (1-562), tagged by RFP in N-terminal, were firstly established (**Figure 47A**). The overexpression of the full length Pastrel protein and Pastrel truncated in C-terminal (1-562) was confirmed by qPCR. The overexpression of Pastrel (1-562) did not restrict DCV infection after 48h, by contrast to overexpression of Pastrel full length (**Figure 47B**). DCV RNA level in cells overexpressing Pastrel (1-562) (clone E9) was similar to control cells, or even greater (clone E12).

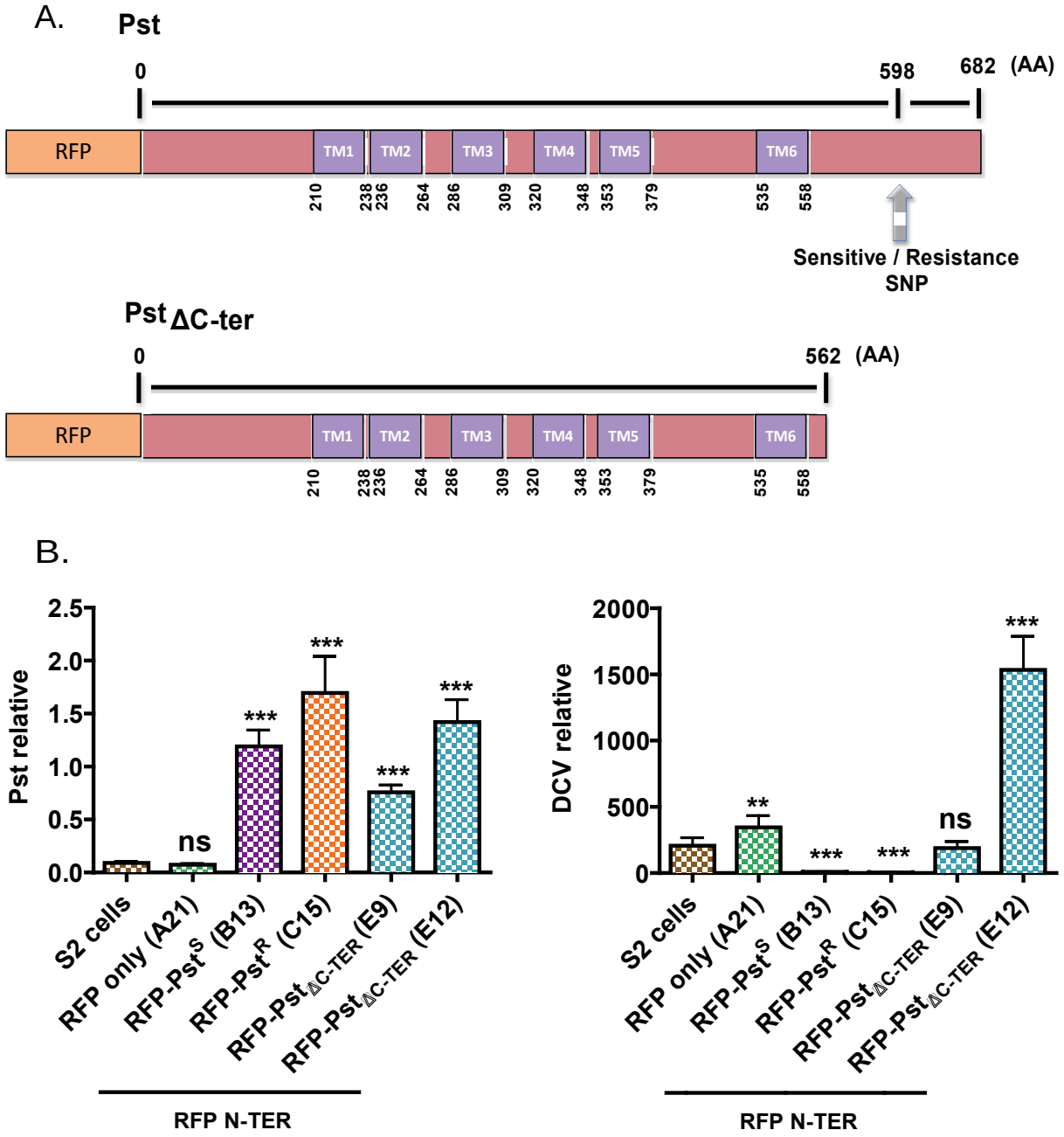


Figure 47. Overexpression of Pst deleted in C-ter does not affect DCV RNA level. A. Schema of Pst full lentgh and C-ter deleted version tagged in N-ter by RFP. Representation of 6 putative transmembrane domains (TM) predicted by different softwares. **B.** S2 cells, stable cell lines overexpressing RFP only (control), sensitive and resistant versions of Pst full length and truncated tagged by RFP in N-terminal, were infected with DCV (MOI 1) for 48h. Pst RNA level (left panel) and DCV RNA level (right panel) were measured by qPCR. qPCR values were normalized to the housekeeping gene *RpL32*. Bar graphs represent the average standard deviation of 1 experiment. Clones identifications are indicated in brackets.

For further confirmation, immunofluorescence assays were performed with antibodies against DCV capsid after 48h of infection. The number of cells overexpressing sensitive and

resistant versions of Pastrel which were stained by DCV antibody after 48h of infection (**Figure 48**, panels **C** and **D**) was dramatically decrased compared to cells overexpressing RFP (**Figure 48**, panels **A** and **B**) and cells overexpressing Pastrel truncated in C-terminal (**Figure 48**, panels **E** and **F**). DAPI staining is showed as control (**Figure 48**, panels **A'** to **F'**). The antiviral effect of Pastrel overexpression was significative compared to cells overexpressing RFP and Pastrel truncated in C-terminal (clone E9) (**Figure 48B**). Another clone expressing Pastrel truncated in C-terminal (clone E12) did not display resistance to DCV infection, but rather an increased number of DCV infected cells.

Overall, these results uncover the role of the C-terminal region of Pastrel protein in antiviral function.

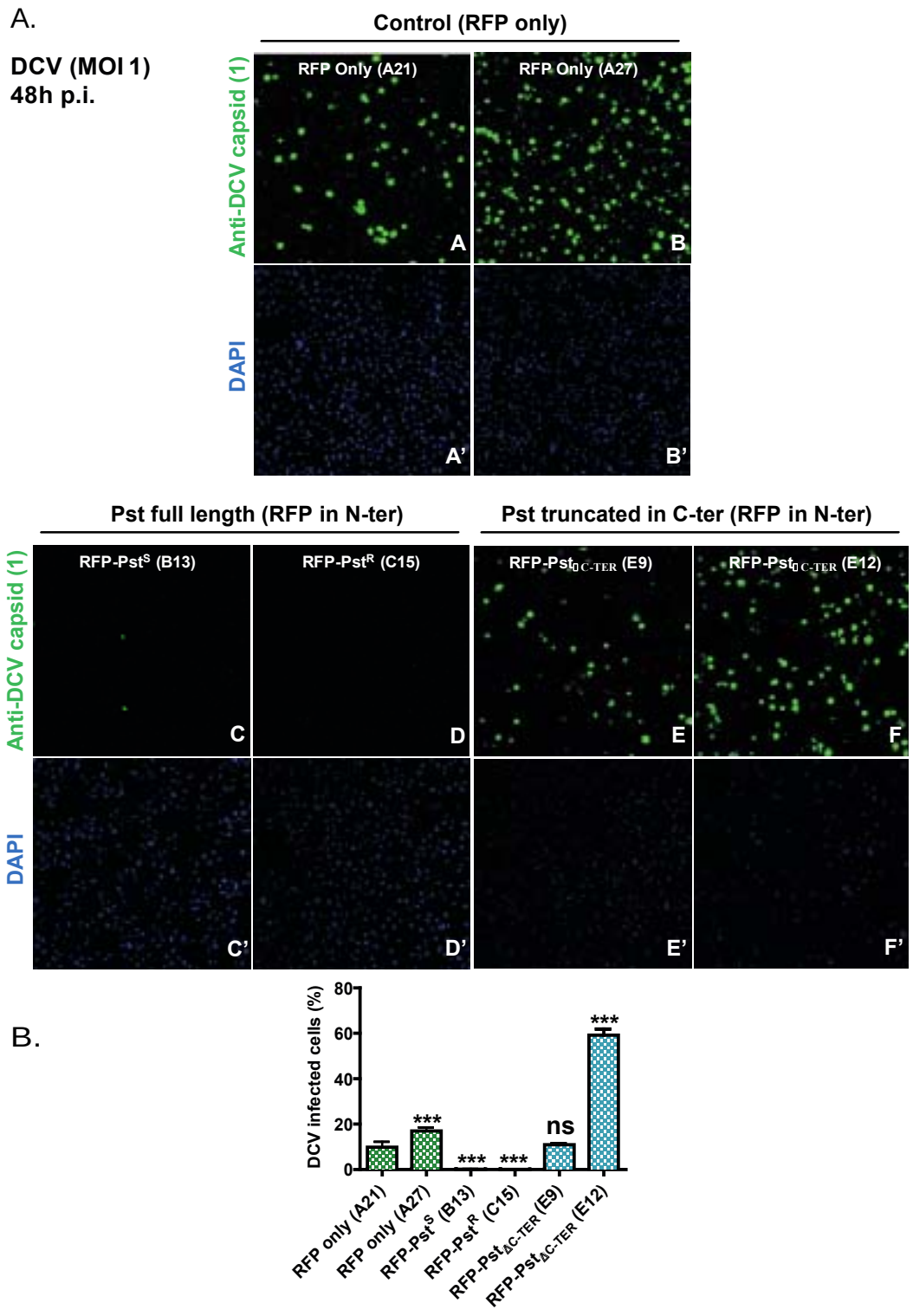


Figure 48. Overexpression of Pst deleted in C-ter does not restrict DCV infection. A. Stable cell lines overexpressing RFP only (control, panel A, A' and B, B'), Pst sensitive (panel C and C') and resistant (panel D and D') versions of Pst full length or truncated in C-terminal (panels E, E' and F, F') tagged by RFP in N-terminal, were infected with DCV (MOI 1) for 48h. Cells were stained with an antibody against DCV capsid (anti-DCV-1, panels A, B, C, D, E and F). DAPI staining is represented in panels A', B', C', D', E' and F'. Pictures were taken by confocal LSM700. **B.** DCV infected cells were quantified using ImageJ. Bar graphs represent the average standard deviation of 9 pictures. Panels are representative of 1 experiment. Clones identifications are indicated in brackets.

4.6. Study of Pst localization in cells

We carried out immunofluorescence assays to decipher the localization of endogenous Pastrel protein in *Drosophila* S2 cells and in stable cell lines overexpressing sensitive and resistant versions of Pastrel fused with RFP. The endogenous Pastrel protein (**Figure 49A**) and RFP-Pst fusion protein (**Figure 49B**) form cytoplasmic aggregates with a dot-like structure.

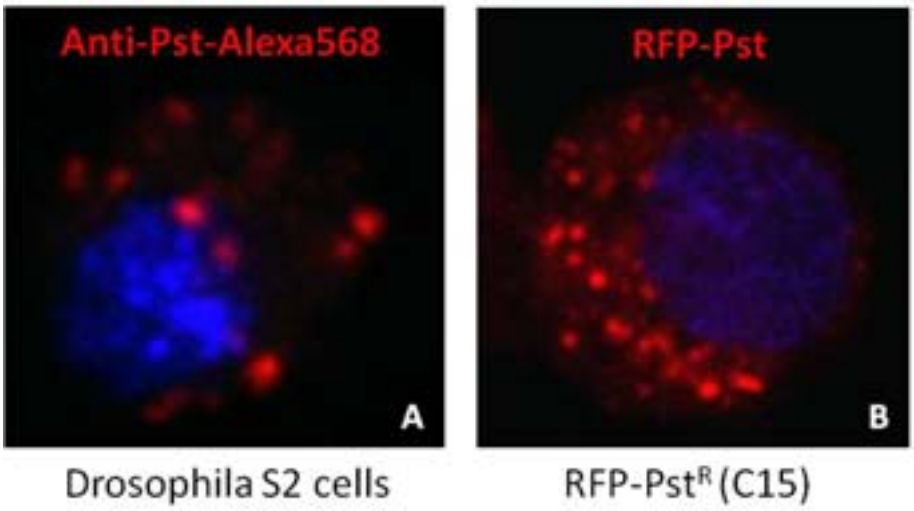


Figure 49. Endogenous Pst and Pst fusion proteins exhibit a vesicular pattern in the cytoplasm of *Drosophila* cells. A. *Drosophila* S2 cells were stained with antibody against Pst and DAPI. **B.** Cell expressing RFP-Pst fusion. Pictures were taken by confocal LSM700.

We showed above that overexpression of Pastrel truncated in C-terminal does not restrict DCV infection. We then assessed if this loss of antiviral function may be related to a change in cellular protein localization. Pastrel protein truncated in C-terminal did not form cytoplasmic aggregates (**Figure 50**, panels **E** and **F**), as overexpression of single RFP (**Figure 50**, panels **A** and **B**). This contrasts with overexpression of sensitive and resistant full length versions of Pastrel protein which form cytoplasmic aggregates (**Figure 50**, panels **C** and **D**). The truncated Pst form is diffused in the cells, comparable to the overexpression of RFP only (**Figure 50**, panels **A** and **B**).

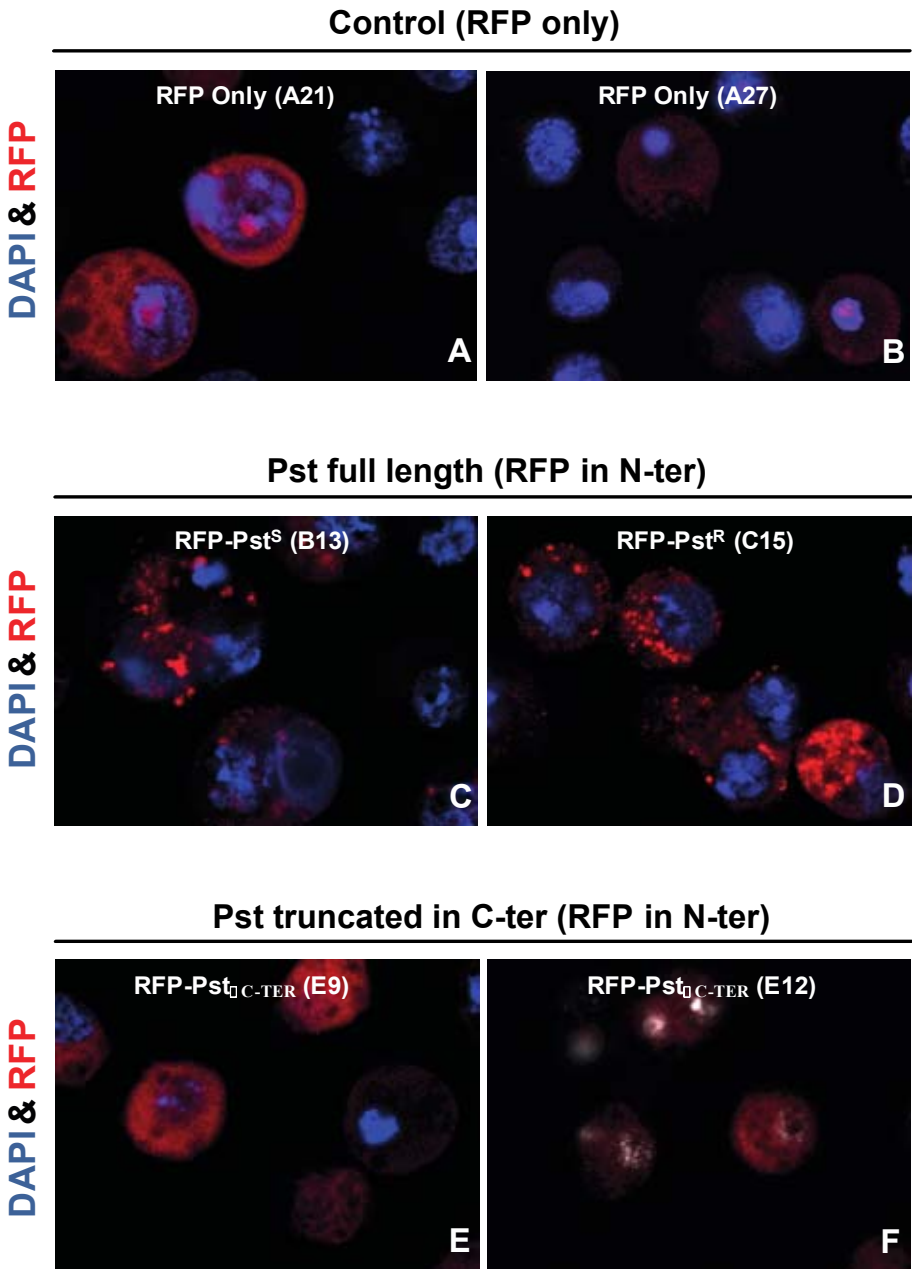


Figure 50. Deletion of C-terminal region of Pst modifies its intracellular localization. Stable cell lines overexpressing RFP only (control, panel A and B), Pst sensitive (panel C) and resistant (panel D) versions of Pst full length or truncated in C-terminal (panels E and F) tagged by RFP in N-terminal were stained with DAPI and observed for RFP by confocal LSM700. Panels are representative of 1 experiment. Clones identifications are indicated in brackets.

In addition to its antiviral function, these results uncover a role of the C-terminal region of Pastrel protein in protein localization. These data suggest that the localization of Pastrel in the cells may also be important for its antiviral function. Then, we investigated the localization of Pastrel protein in the cells.

4.6.a. Pst co-localizes with lipid droplets in non-infected cells

We used a large number of markers for cellular compartments (e.g. ER, Golgi, mitochondria, early endosomes) to decipher the localization of Pastrel in *Drosophila* S2 cells. None of these markers colocalized with either endogenous Pastrel protein or RFP-Pastrel fusions (**data not shown**).

The team of Dr. Kuhnlein performed a proteomic analysis of lipid droplets from *Drosophila* larvae fat body. They found that Pastrel is associated with lipid droplets specifically in adp^{60} mutants which are flies genetically predisposed for obesity (Beller et al., 2006). Therefore, we carried out co-staining of *Drosophila* S2 cells with antibody against Pastrel and Nile Red, a marker of lipid droplets (Greenspan et al., 1985). We observed that some aggregates of Pastrel protein colocalize with lipid droplets (**Figure 51**).

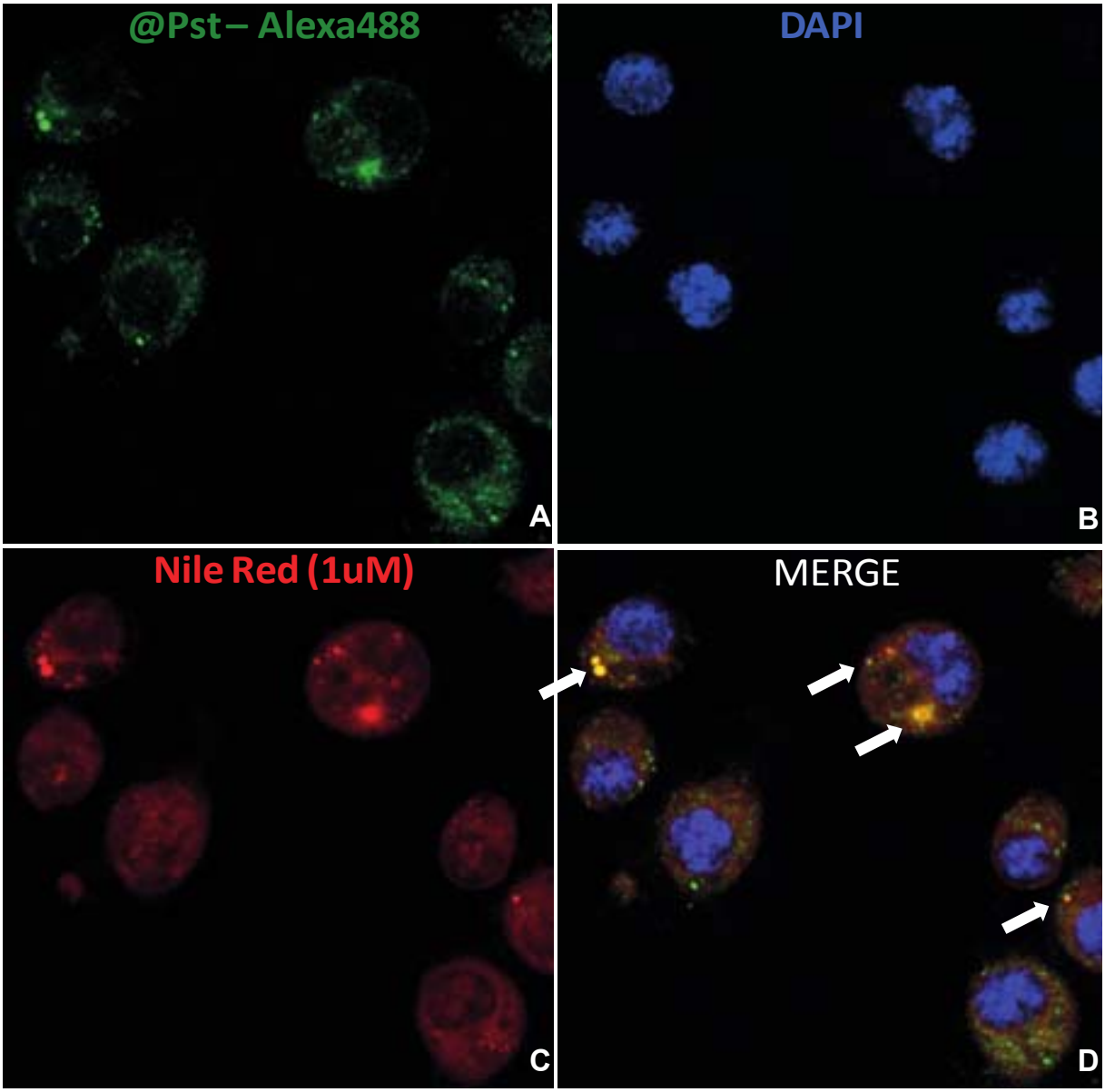


Figure 51. Pst colocalizes with lipid droplets stained by Nile Red. *Drosophila* S2 cells were stained with antibody against Pst (panel A) and DAPI (panel B). Cells were next incubated for 5 min with Nile Red 1µM to stain lipid droplets (panel C). Merge picture is represented in panel D. Pictures were taken by confocal LSM700. Panels are representative of 2 independent experiments.

4.6.b. Enrichement of COP-I vesicles staining in the areas where Pastrel and DCV localize

It is reported that COP-I vesicles are required for DCV replication (Cherry et al., 2006). The knockdown of COP-I components dramatically reduces DCV replication in

Drosophila cells. We wondered if Pastrel overexpression may affect the number and distribution of COP-I vesicles. We stained cells overexpressing Pastrel fused with RFP with an antibody against beta-COP, a component of COP-I vesicles. We observed an enrichment of COP-I vesicles in the areas where Pastrel aggregates localize in the *Drosophila* cells (**Figure 52**). Interestingly, the laboratory of Dr. Kuhnlein showed that COP-I complexes regulate lipid homeostasis, modifying protein composition at the surface of lipid droplets (Beller et al., 2008). This may link Pastrel with COP-I vesicles and lipid droplets.

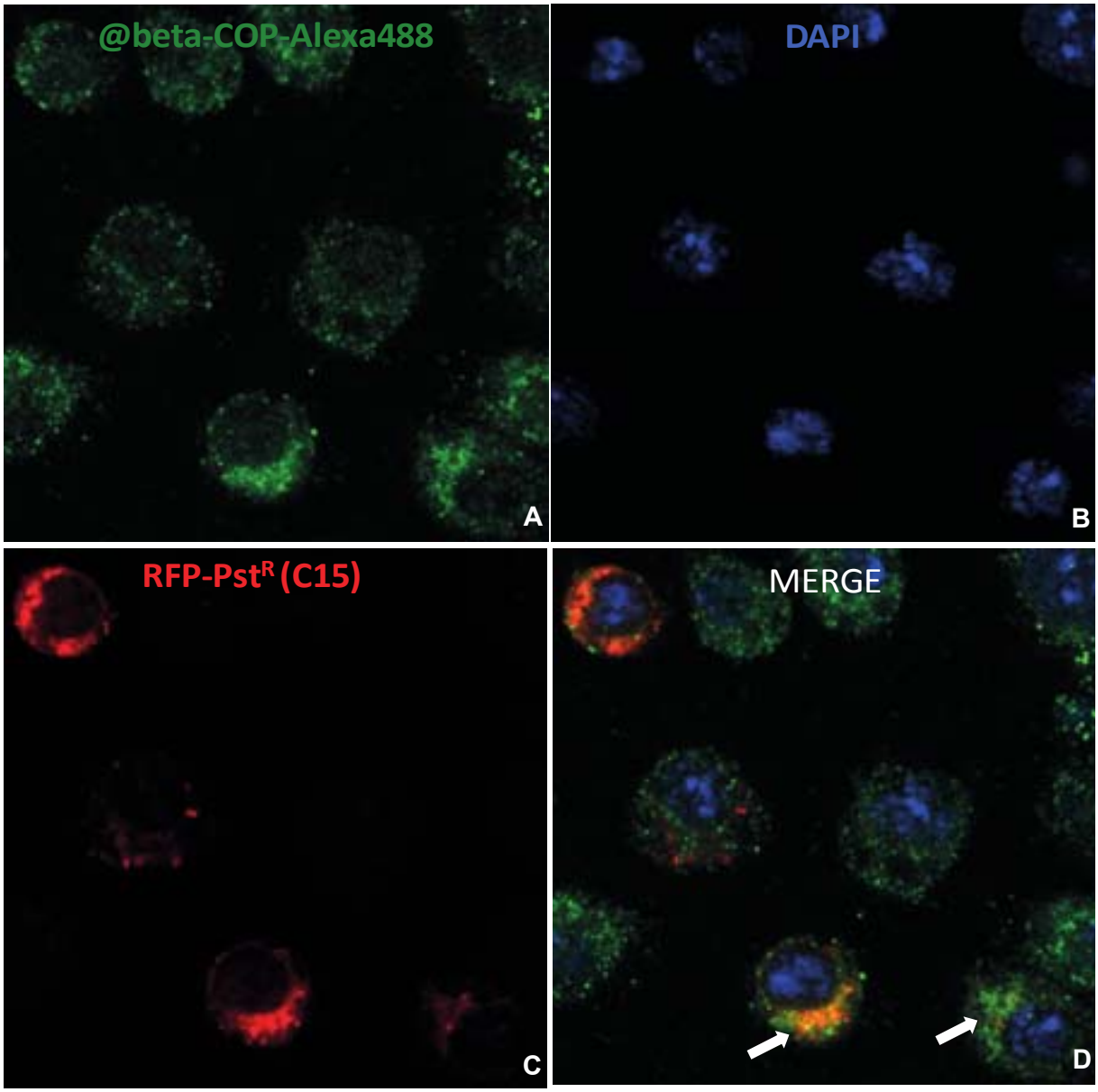


Figure 52. beta-COP staining is enriched in areas of Pst fusion aggregates. Stable cell line overexpressing resistant version of Pst was stained with antibody against beta-COP (panel A) and DAPI (panel B). Pst was observed thanks to RFP tag in N-terminal (panel C). Merge picture is represented in panel D. Pictures were taken by confocal LSM700. Panels are representative of 1 experiment.

Furthermore, *Drosophila* S2 cells were infected with DCV (MOI 1) for 48h and co-stained with antibodies against DCV capsid and beta-COP. The localization of beta-COP in *Drosophila* S2 cells was enriched in the areas where DCV staining was strong (**Figure 53**, panel **D**). Unfortunately, it was not possible to triple label cells with antibodies against DCV, Pastrel endogenous and beta-COP.

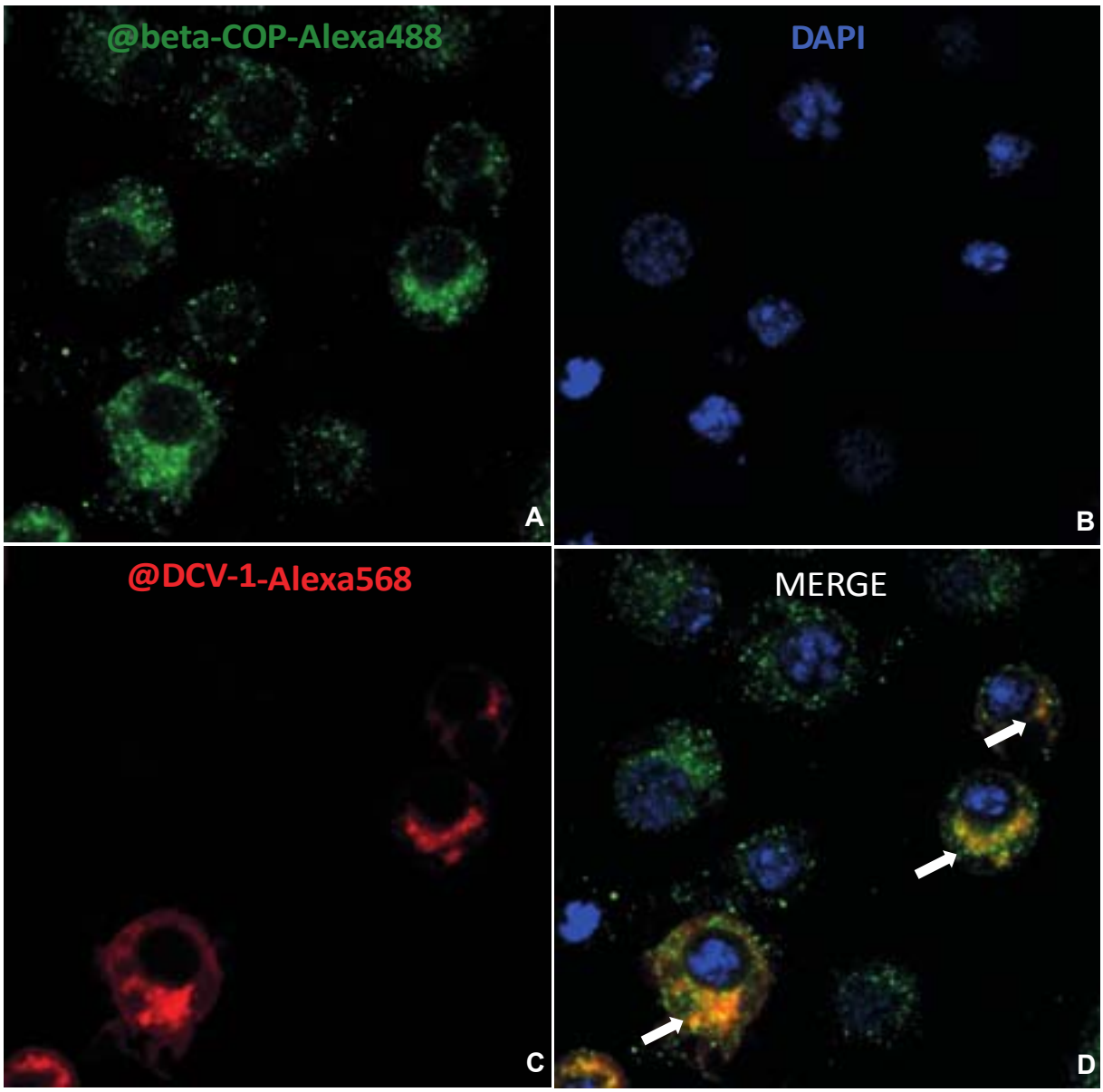


Figure 53. Colocalization between DCV capsid and beta-COP. *Drosophila* S2 cells were infected with DCV (MOI 1) for 48h. Cells were then stained with antibody against beta-COP (panel A), DAPI (panel B) and DCV capsid (anti-DCV-1, panel C). Merge picture is represented in panel D. Pictures were taken by confocal LSM700. Panels are representative of 1 experiment.

4.6.c. Pst co-localizes with DCV and CrPV capsid proteins during infection

Drosophila S2 cells were infected with DCV (MOI 1) for 48h and co-stained with Pastrel and DCV capsid antibodies (**Figure 54**, panel **A** and **B**). Pastrel aggregates were colocalized with DCV capsid staining (**Figure 54**, panel **C**). The colocalization of Pastrel protein with DCV capsid was also observed *in vivo* in the fat body of infected flies (**Figure 54**, panel **F**).

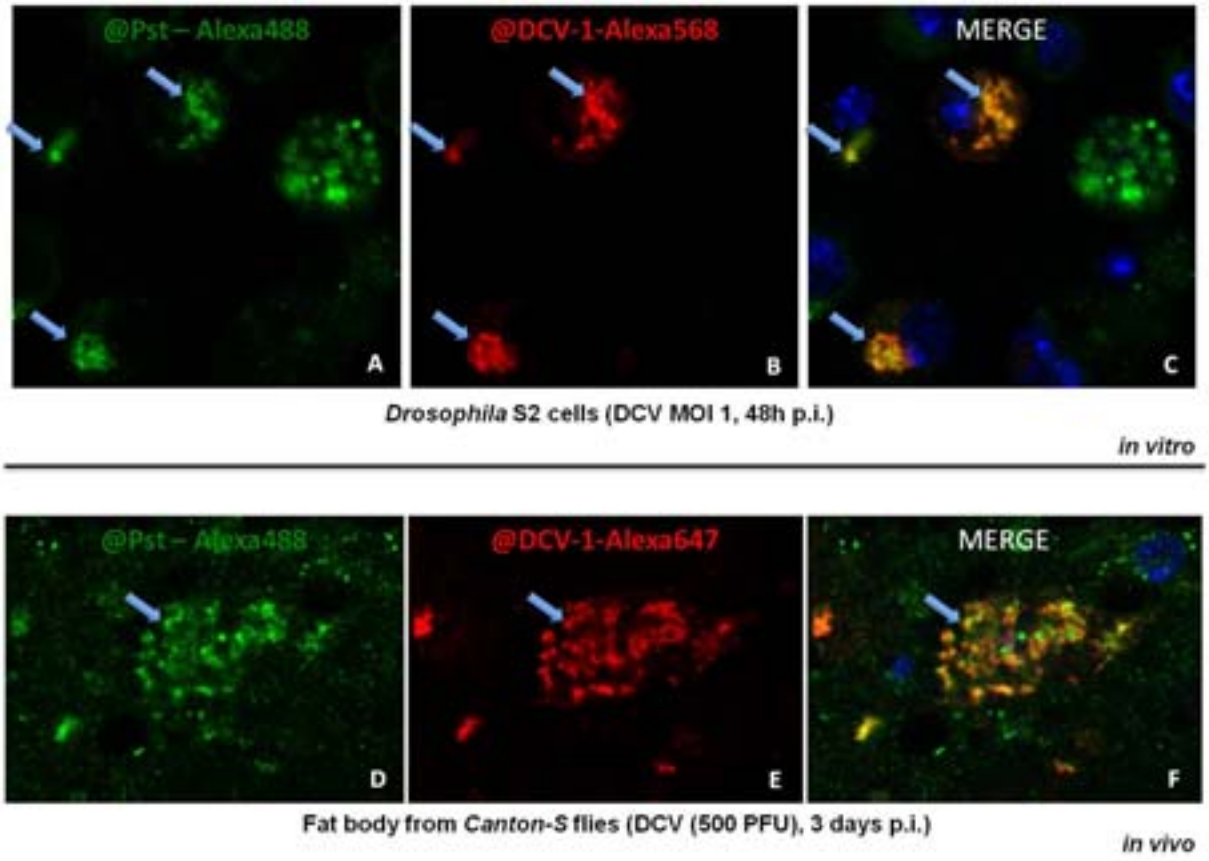


Figure 54. Pst colocalizes with DCV capsid staining in *Drosophila* S2 cells and in the fat body of infected flies. *Drosophila* S2 cells were infected with DCV (MOI 1) for 48h. *Canton-S* flies were infected with DCV (500 PFU) for 3 days at 25°C and the fat body was dissected for immunostaining. Drosophila cells and fat body were stained with antibody against Pastrel (panel A and D) and DCV capsid (anti-DCV-1, panel B and E). Nuclei were stained with DAPI solution. Merge picture is represented in panel C and F. Pictures were taken by confocal LSM700. Panels are representative of 2 independent experiments.

Similarly, CrPV capsid staining colocalized with Pastrel aggregates after 24h of infection by CrPV (MOI 0.01) in *Drosophila* S2 cells (**Figure 55**, panel **C**) and also in the fat body of infected flies (**Figure 55**, panel **F**).

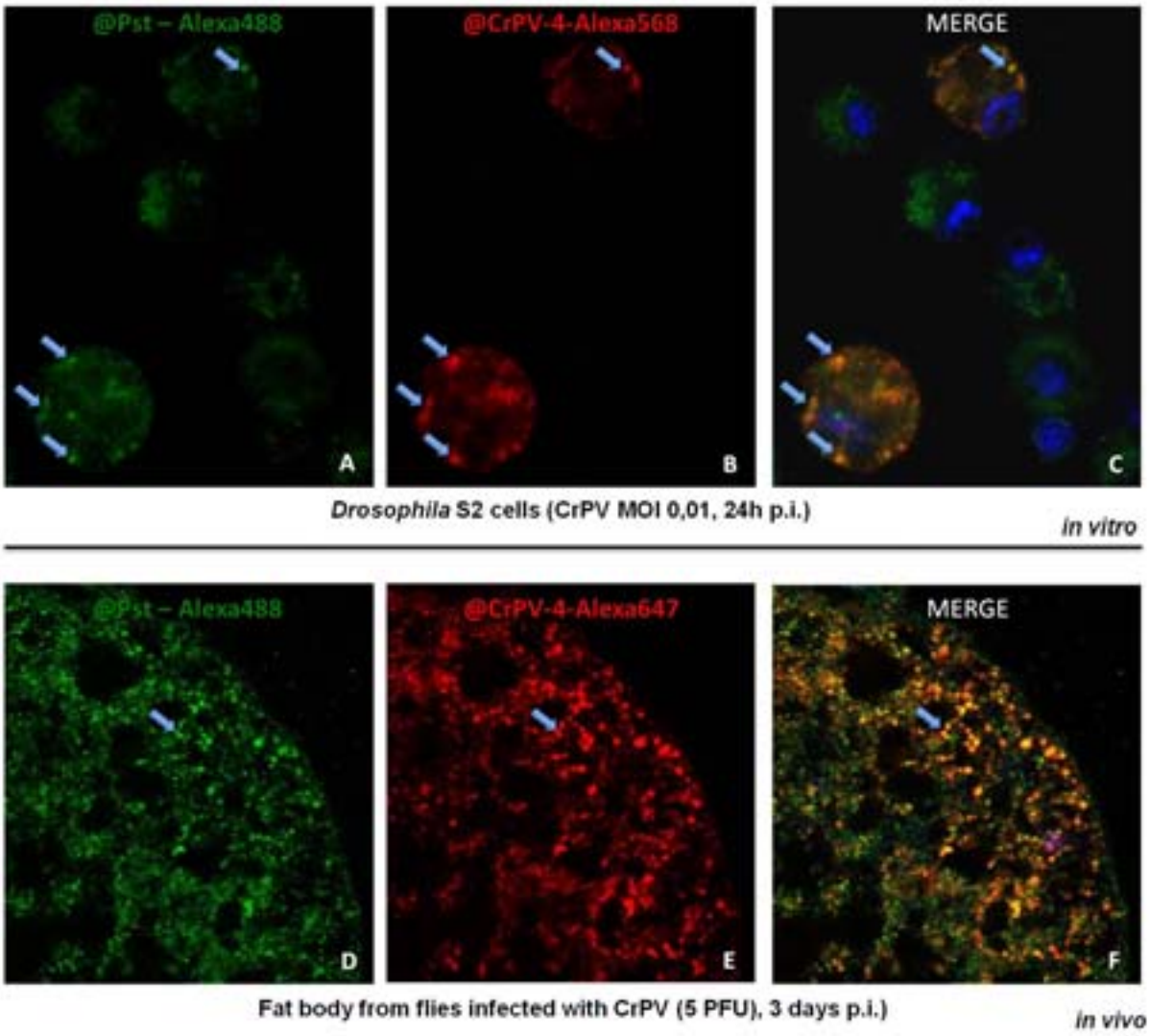


Figure 55. Pst colocalizes with CrPV capsid staining in S2 cells and in *Drosophila* fat body. *Drosophila* S2 cells were infected with CrPV (MOI 0.01) for 24h. Flies were infected with CrPV (5 PFU) for 3 days at 25°C and the fat body was dissected for imunostaining. *Drosophila* cells and fat body were stained with antibody against Pst (panels A and D) and CrPV capsid (anti-CrPV-4, panels B and E). Nuclei were stained with DAPI. Merge picture is represented in panel C and F. Pictures were taken by confocal LSM700. Panels are representative of 1 experiment.

4.6.d. Does DCV colocalize with lipid droplets ?

It is known that *Flavivirus* Hepatitis C Virus (HCV) requires lipid droplets for its infectious cycle in human hepatocytes. To determine if DCV capsid protein is present at the surface of lipid droplets, *Drosophila* S2 cells were treated with oleic acid (400 µM) and infected with DCV (MOI 1) for 48h. The oleic acid treatment allows to induce the formation of lipid droplets in cells (Beller et al., 2008). Indeed, we noticed that lipid droplets are small and not detected in every cell in normal conditions (see **Figure 51**, panel **D**), probably because *Drosophila* S2 cells are hemocyte-like cells and thereby not specialized for lipid storage like hepatocyte cells in Humans. Cells were then co-stained with antibody against DCV capsid protein (**Figure 56**, panel **A**) and Nile Red (**Figure 56**, panel **B**). No staining of DCV capsid was observed at the surface of lipid droplets (**Figure 56**, panel **C**).

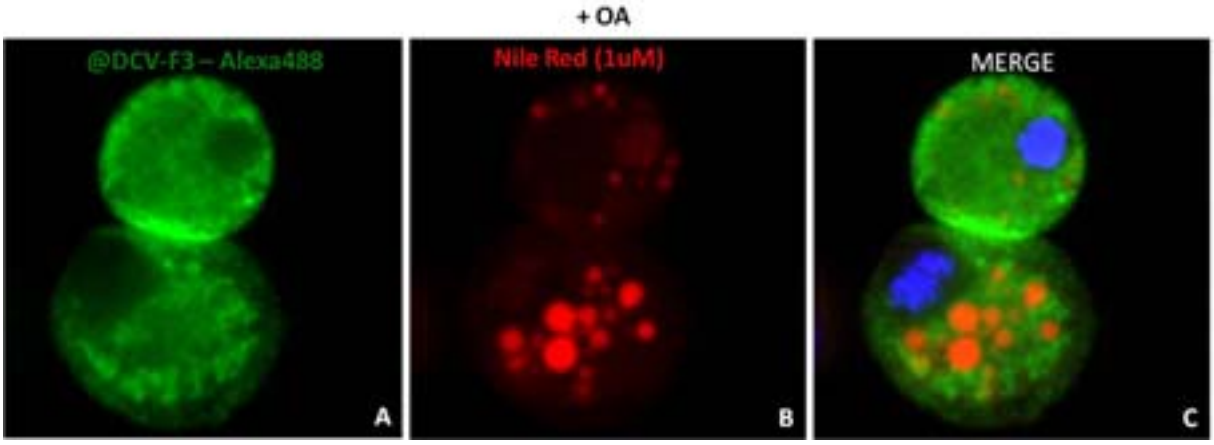


Figure 56. DCV capsid staining does not colocalize with the surface of lipid droplets in *Drosophila* S2 cells. *Drosophila* S2 cells were treated by oleic acid (400 µM) and infected with DCV (MOI 1) for 48h. Cells were next stained with antibody against DCV capsid (anti-DCV-F3, panel A) and then incubated 5 min with Nile Red (1 µM, panel B). Nuclei were stained with DAPI. Merge picture is represented in panel C. Pictures were taken by confocal LSM700. Panels are representative of 1 experiment.

We showed above that DCV infects the fat body of flies which is thought to be homologous to the human liver. The fat body is a lipid storage organ, thus it is more relevant to see whether DCV, and also Pastrel protein, colocalize with lipid droplets than in *Drosophila* S2 cells. We performed a triple labeling of Pastrel protein (**Figure 57**, panel **A**), DCV capsid (**Figure 57**, panel **B**) and lipid droplets (**Figure 57**, panel **C**). In the fat body, we observed that DCV capsid and Pastrel protein colocalize each other and also with small dots of Nile Red staining, but not with large lipid droplets.

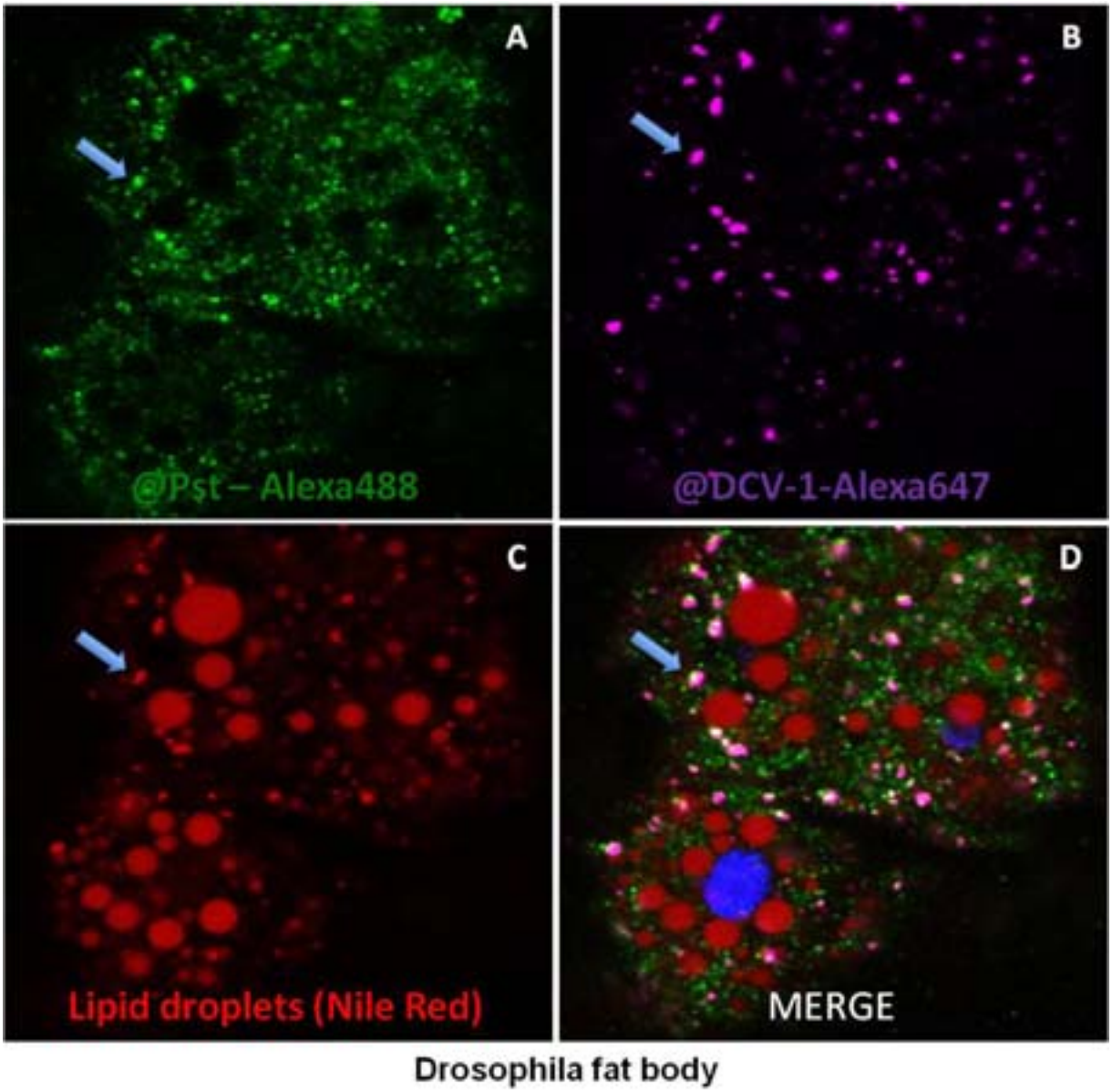


Figure 57. DCV capsid and Pastrel protein colocalize with some dots of Nile Red staining, but not with large lipid droplets in the fat body of DCV-infected flies. w^{A5001} flies were infected with DCV (500 PFU) for 3 days at 25°C. The fat body from w^{A5001} flies was next stained with antibody against Pastrel protein (anti-Pst, panel A), DCV capsid (anti-DCV-1, panel B), DAPI and then incubated 5 min with Nile Red (1 µM, panel C). Merge picture is represented in panel D. Pictures were taken by confocal LSM700. Panels are representative of 1 experiment.

We also observed that DCV infection affects the number and the size of lipid droplets in the regions of the fat body which are highly infected by the virus (**Figure 58**, panel **D**). The lipid droplets are smaller in size compared to regions of the fat body that are not stained with DCV antibody. Unexpectidely, it also seems that Pastrel staining is weaker in regions highly infected with DCV.

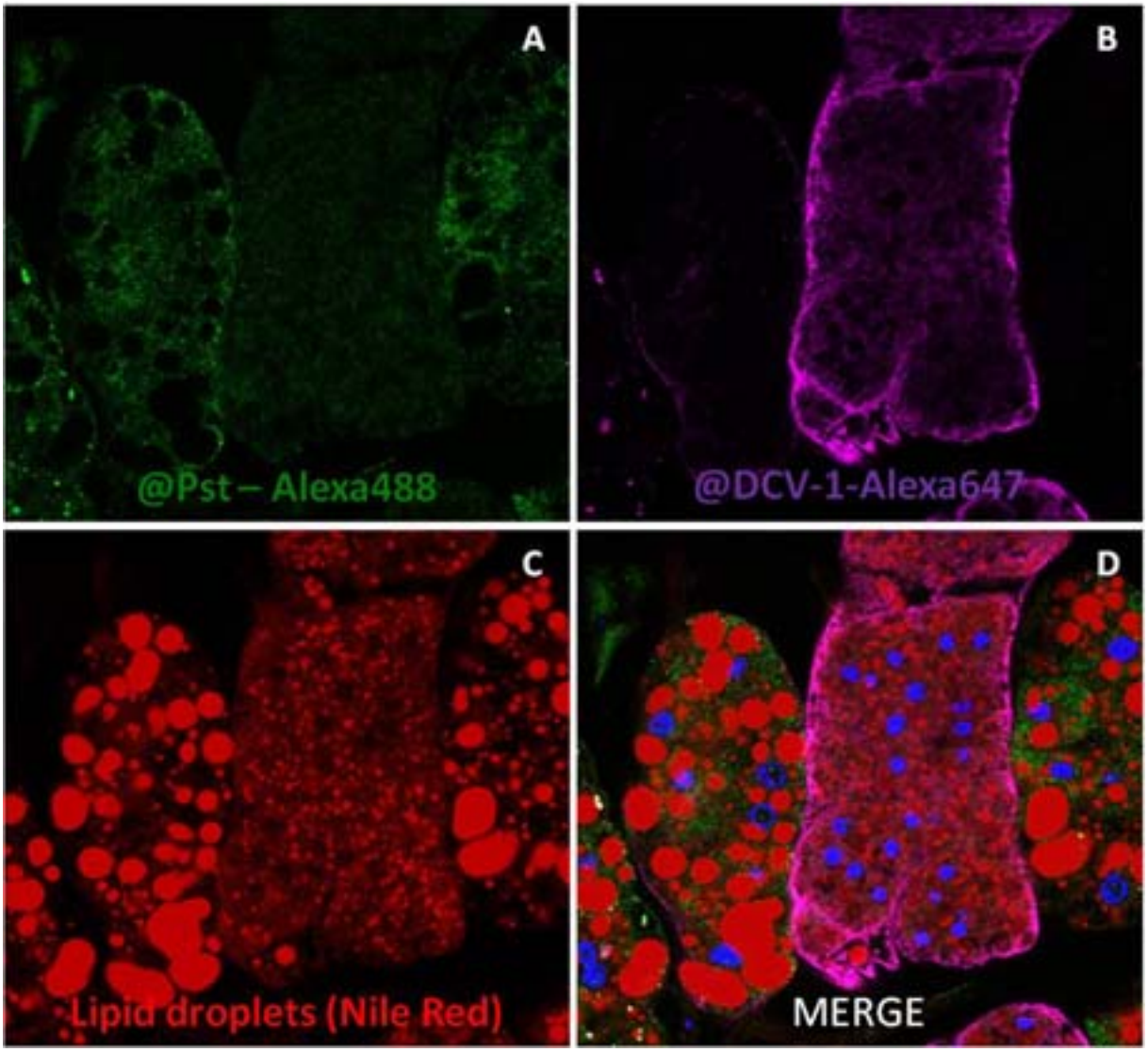


Figure 58. DCV infection affects lipid droplets morphology in the fat body of DCV-infected flies. w^{A5001} flies were infected with DCV (500 PFU) for 3 days at 25°C. The fat body from w^{A5001} flies was next stained with antibody against Pastrel protein (anti-Pst, panel A), DCV capsid (anti-DCV-1, panel B), DAPI and then incubated 5 min with Nile Red (1 µM, panel C). Merge picture is represented in panel D. Pictures were taken by confocal LSM700. Panels are representative of 1 experiment.

It may be possible that DCV infection uses lipids stored in lipid droplets, thereby degrading lipid droplets as illustrated by **Figure 59**. Whether DCV infection induces lipolysis remains to be determined.

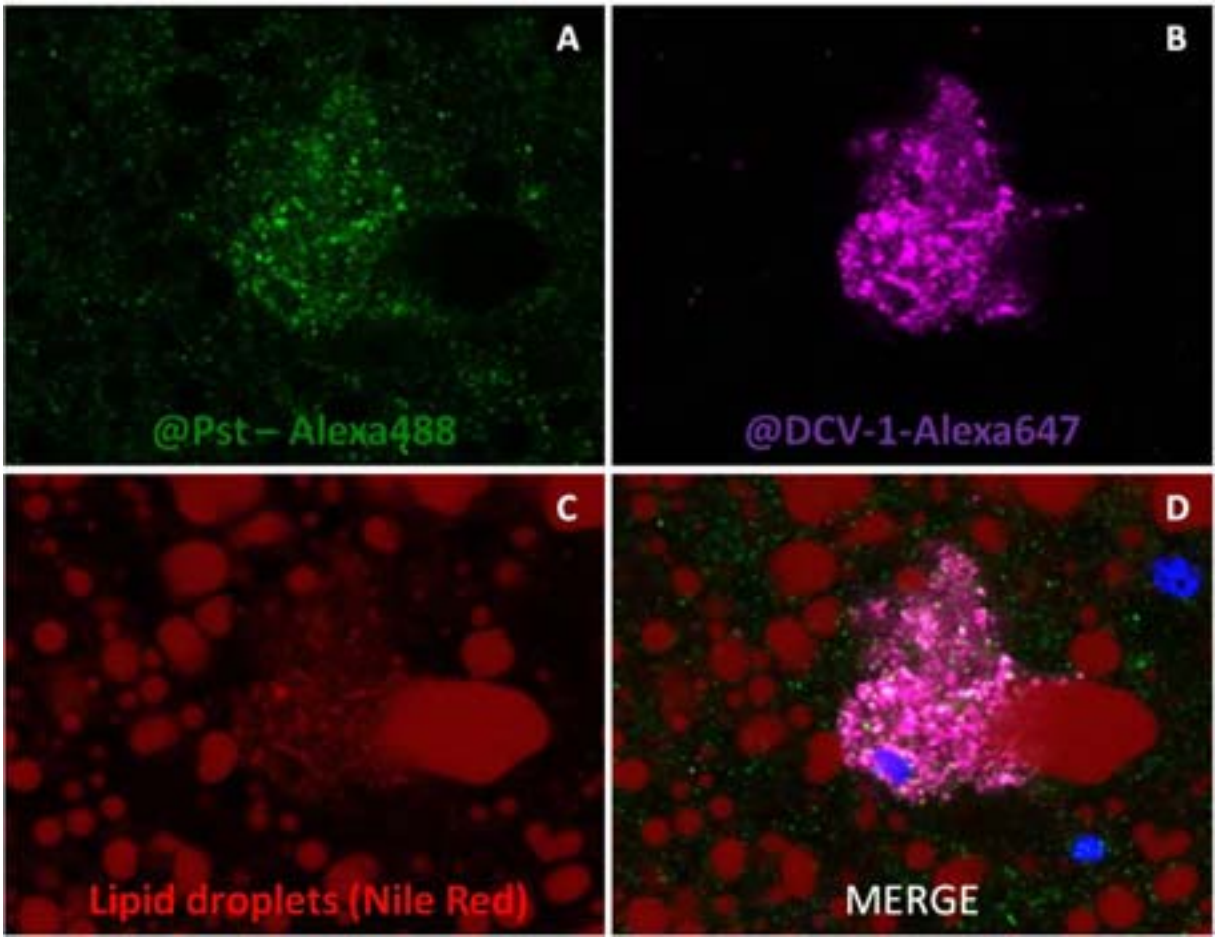


Figure 59. DCV seems to induce the degradation of large lipid droplets in the fat body of DCV-infected flies. w^{A5001} flies were infected with DCV (500 PFU) for 3 days at 25°C. The fat body from w^{A5001} flies was next stained with antibody against Pastrel protein (anti-Pst, panel A), DCV capsid (anti-DCV-1, panel B), DAPI and then incubated 5 min with Nile Red (1 µM, panel C). Merge picture is represented in panel D. Pictures were taken by confocal LSM700. Panels are representative of 1 experiment.

4.6.e. DCV infection was not affected by the knockdown of genes involved in lipid metabolism

Studies conducted on human hepatocytes implicated genes involved in lipid metabolism as important for the life cycle of HCV. For example, Diacylglycerol acyltransferase-1 (Dgat1), which generates triglycerides, was reported to direct HCV core protein and NS5A to lipid droplets (Camus et al., 2013a; Herker et al., 2010). The tail interacting protein of 47 kDa (TIP47) was shown to interact with NS5A and facilitate HCV replication (Ploen et al., 2013; Vogt et al., 2013). TIP47 is a member of the PAT (Perilipin, ADRP and TIP47) protein family that coat lipid droplets and regulate their biogenesis (Bickel

et al., 2009; Bulankina et al., 2009). Both Dgat1 and Tip47 are conserved in *Drosophila*. Thus, we wanted to verify if genes involved in lipid metabolism and identified to be important for HCV infection in Human, could affect DCV infection in *Drosophila* S2 cells. The *Drosophila* lipase Brummer (Bmm, homolog of Atgl in mammals) and the triglyceride synthase Midway (mdy, homolog of Dgat1 in mammals) are involved in lipolysis and lipogenesis, respectively (Beller et al., 2008). The knockown of these genes did not affect DCV RNA level after 16h of infection in *Drosophila* S2 cells (**Figure 60**). The knockdown of Lsd-1 and Lsd-2 (homologs of PAT-domain proteins Adrp and Tip47 respectively) also did not change DCV RNA level compared to cells treated with dsRNAs against GFP (negative control) (**Figure 60**). We observed a significative increase of DCV RNA level when both Lsd-1 and Lsd-2 were silenced, but the effect was minor compared to the depletion of *pastrel*. As mentioned above, this negative result may be related to the fact that S2 cells are hemocytes, and do not store large quantities of lipids.

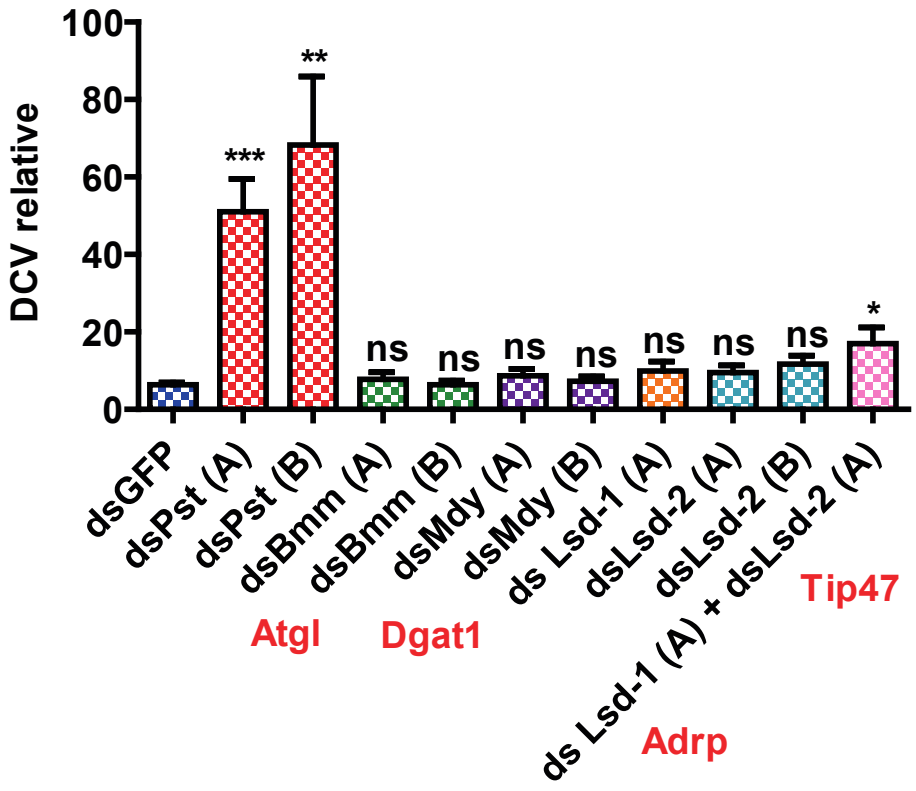


Figure 60. Knockdown of genes involved in lipid metabolism has no effect on DCV infection in *Drosophila* S2 cells. *Drosophila* S2 cells were treated by dsRNAs for 5 days and next infected with DCV (MOI 1) for 16h. DCV RNA level was measured by qPCR and normalized to the housekeeping gene *RpL32*. Bar graphs represent the average standard deviation of 1 experiment.

Chapter 5

Effect of Nora virus on the susceptibility of *Drosophila* to pathogens

5.1. Correlation between the presence of Nora Virus and the susceptibility of flies to DCV infection

The analysis of small RNA libraries previously conducted in the laboratory allowed to identify the presence of Nora Virus in our stocks of *yw* flies (**Figure 61**).

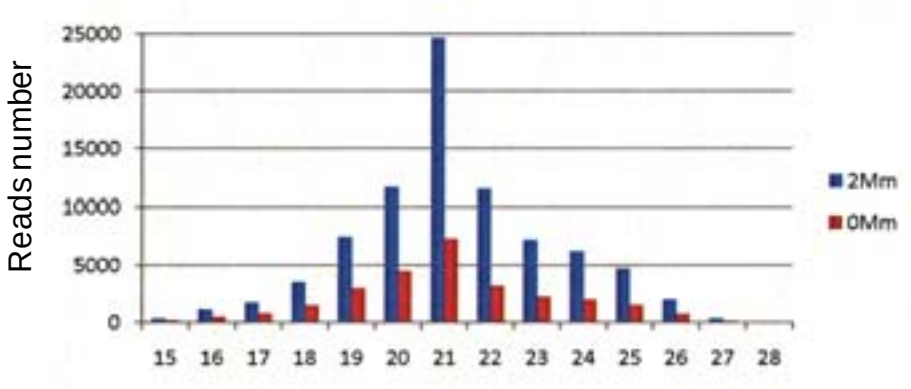


Figure 61. Small RNAs matching with Nora Virus genome sequence were detected in *yw* flies. The profile of Nora Virus small RNAs with a peak at 21 nucleotides indicates that siRNAs are produced in *yw* flies against Nora Virus genome. Blue and red bars indicate reads against Nora Virus genome respectively with 2 and 0 mismatches.

Therefore, we wondered whether this virus was widely spread in flies of our laboratory. We decided to diagnose the presence of Nora virus in our control flies (**Table 7**). Interestingly, we noticed that flies with higher sensitivity to DCV infection (*Ore-R*^{DF}, *yw*, *w*^{A5001} and *w*^{1118}) were persistently infected by Nora Virus. By contrast, we did not detect Nora Virus in the *Ore-R*^{JLI} and *Canton-S* flies which are resistant to DCV infection. The only exception was *DD1 cnbw* which was positive for Nora virus even though it is resistant to DCV infection. We therefore hypothesized that the presence of Nora Virus in flies could increase their sensitivity to DCV infection.

Strains	Phenotype with DCV	Nora virus detection (by qPCR)
Ore-R^{DF}	Sensitive	Yes
Ore-R^{JLI}	Resistant	No
yw	Sensitive	Yes
w^{1118}	Sensitive	Yes
w^{A5001}	Sensitive	Yes
Canton-S	Resistant	No
DD1 cnbw	Resistant	Yes

Table 7. The presence of Nora Virus in flies correlates with the susceptibility to DCV infection (except for *DD1 cnbw* flies (in red) which are resistant to DCV infection but persistently infected by Nora Virus). Nora Virus RNA level was detected by qPCR from whole flies.

5.2. Does Nora Virus persistent infection contribute to the susceptibility of flies to DCV infection ?

To test this hypothesis, we cured *Ore-R^{DF}* flies from Nora Virus infection. Eggs from Nora Virus infected *Ore-R^{DF}* flies were dechorionated with bleaching solution as previously described (Habayeb et al., 2009b). As a control, Nora Virus RNA genome was measured in whole flies, gut and rest of the body of emerged flies (**Figure 62**). Nora Virus was described as an enteric virus, which is highly present in the gut (Habayeb et al., 2009b). We expected that qPCR from gut samples would be more sensitive. Nora Virus RNA level was detected in *Ore-R^{DF}* flies, but not *Ore-R^{DF}* bleached flies and *Ore-R^{JLI}* flies (**Figure 62**). Nora Virus RNA level was enriched in gut samples from *Ore-R^{DF}* flies, but not detected in gut samples from *Ore-R^{DF}* bleached flies.

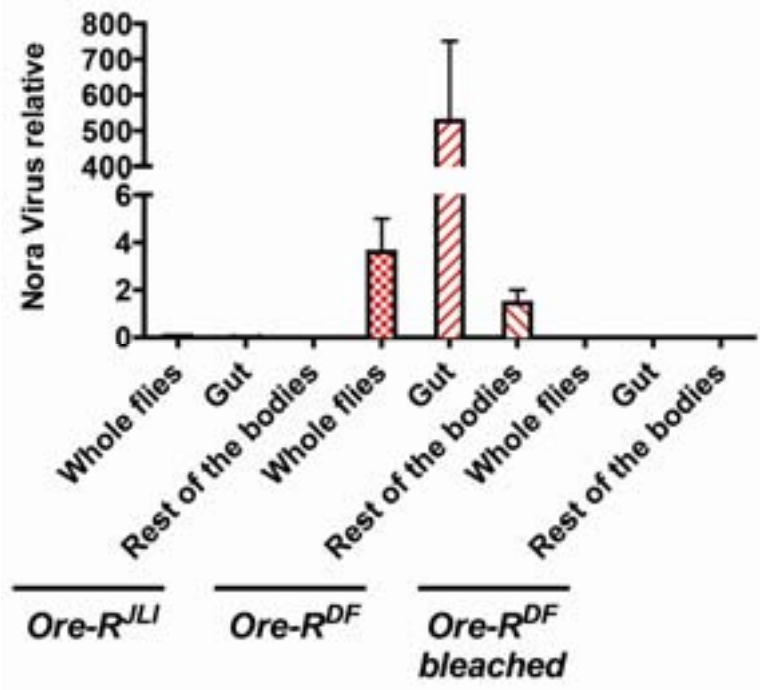


Figure 62. Nora Virus RNA genome was detected in *Ore-R*^{DF} samples but not in *Ore-R*^{JLI} and *Ore-R*^{DF} bleached samples. Nora Virus RNA genome was detected by qPCR from whole flies, gut and rest of the bodies from *Ore-R*^{JLI}, *Ore-R*^{DF} and *Ore-R*^{DF} bleached flies. qPCR values were normalized to the housekeeping gene *RpL32*. Bar graphs represent the average standard deviation of 1 experiment with duplicates of 10 flies.

Since we showed that bleaching was efficiently clearing Nora Virus infection from *Ore-R*^{DF} flies, these flies were challenged by DCV infection (500 PFU) to test if absence of Nora Virus persistent infection impacted their susceptibility to DCV infection (**Figure 63**). *Ore-R*^{DF} bleached flies, cured from Nora Virus infection, showed comparable susceptibility to DCV infection than *Ore-R*^{DF} flies, persistently infected by Nora Virus (**Figure 63A** and **63B**). Susceptibility to DCV infection did not change in next generations after eggs bleaching (**data not shown**). To be sure that bleaching of eggs itself did not increase sensitivity of flies to DCV infection, eggs from *Ore-R*^{JLI} flies were bleached and emerging flies were also challenged by DCV. *Ore-R*^{JLI} bleached flies did not show an increased sensitivity to DCV infection (**Figure 63A**). Besides, DCV RNA level was measured in flies by qPCR after 3 days of infection. *Ore-R*^{JLI} flies and *Ore-R*^{JLI} bleached flies had comparable levels of DCV RNA genome, and it was also the case between *Ore-R*^{DF} flies and *Ore-R*^{DF} bleached flies (**Figure 63C**). DCV RNA level was increased in *Ore-R*^{DF} flies and *Ore-R*^{DF} bleached flies compared to *Ore-R*^{JLI} flies and *Ore-R*^{JLI} bleached flies. Finally, DCV RNA level in flies correlates with their susceptibility to DCV infection.

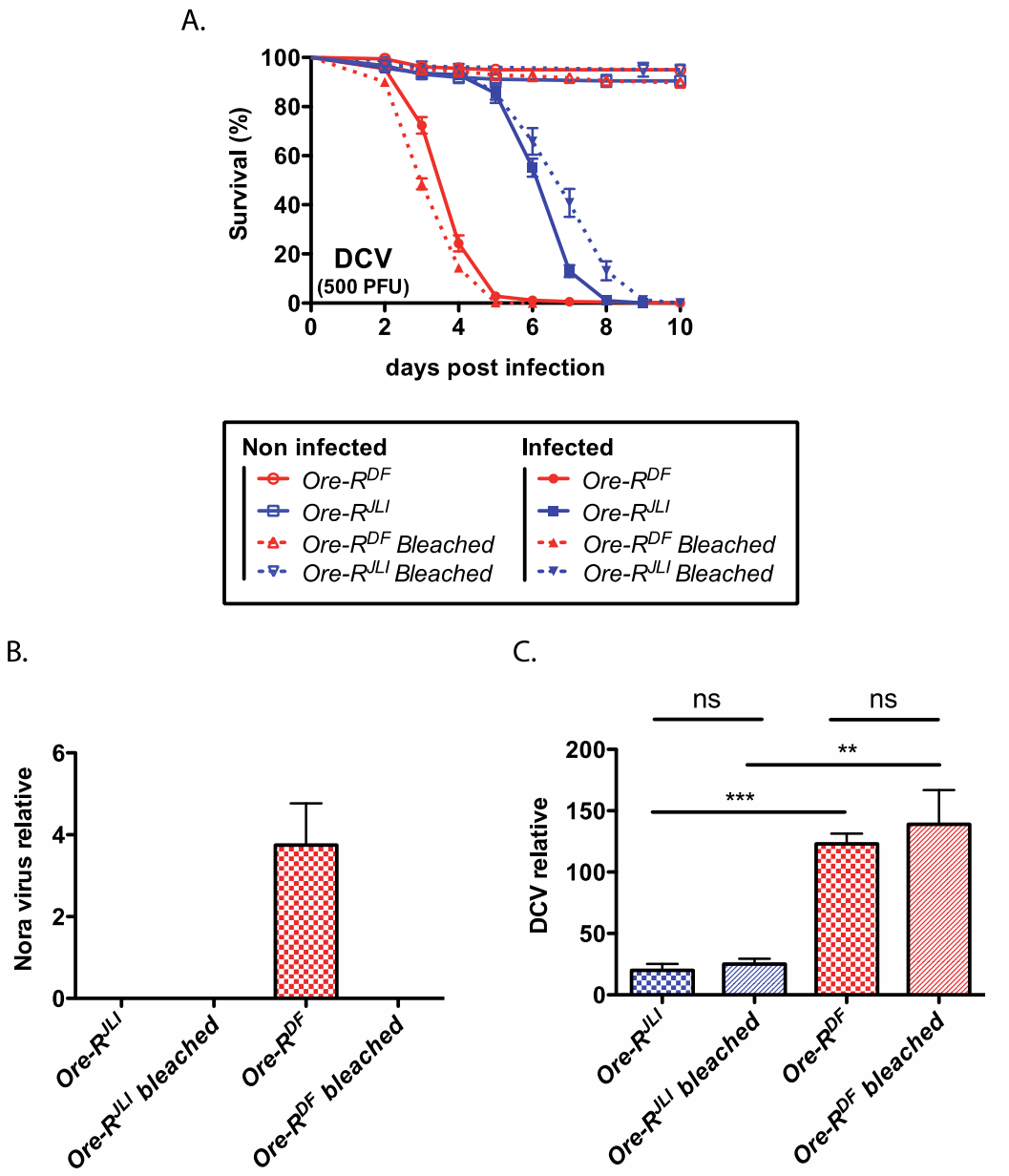


Figure 63. *Ore-R*^{DF} bleached flies, cured from Nora Virus infection, are still highly sensitive to DCV infection, comparable to Nora Virus persistently infected *Ore-R*^{DF} flies. **A.** *Ore-R*^{DF}, *Ore-R*^{JLI} and respective bleached flies were injected by TRIS (control, empty symbols) and DCV (500 PFU, filled symbols). Survivals were monitored daily at 22°C. Bar graphs represent the average standard deviation of at least 3 independent experiments. **B.** Nora Virus RNA level was measured by qPCR after 3 days of infection by DCV (500 PFU). **C.** DCV RNA level was measured by qPCR after 3 days of infection. For panels **B** and **C**, qPCR values were normalized to the housekeeping gene *RpL32*. Bar graphs represent the average standard deviation of 2 independent experiments.

As a further confirmation, reciprocal experiment was performed. *Ore-R*^{JLI} flies, not infected by Nora Virus, were contaminated with feces from Nora Virus infected *Ore-R*^{DF} flies. *Ore-R*^{JLI} flies contaminated by Nora Virus did not present an increased sensitivity to DCV infection, unlike Nora Virus infected *Ore-R*^{DF} flies (**Figure 64**). Nora-contaminated

Ore-R^{JLI} flies were even slightly more resistant to DCV infection than naïve *Ore-R*^{JLI} flies. This may be explained by a *Drosophila* antiviral defense triggered by Nora Virus, which may impact susceptibility to DCV.

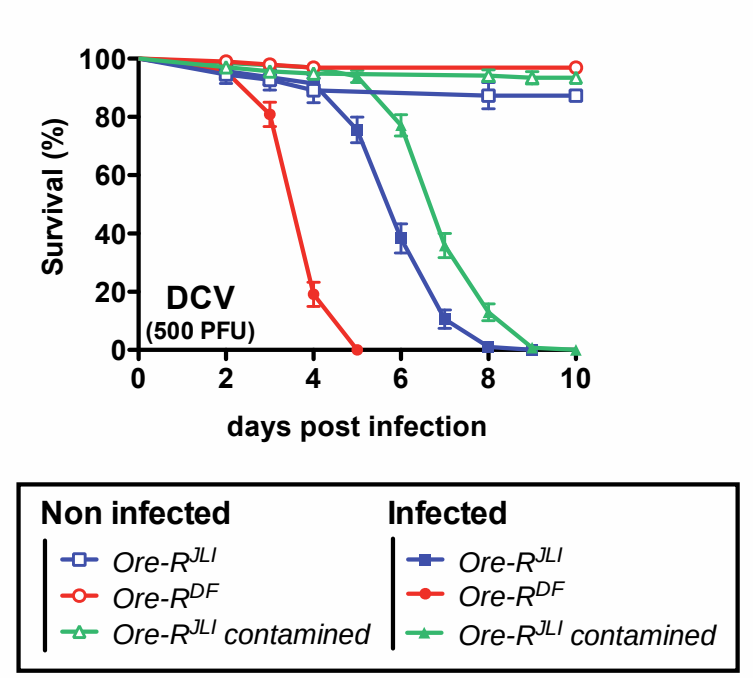


Figure 64. *Ore-R*^{JLI} flies, contaminated by Nora Virus, are still resistant to DCV infection, comparable to *Ore-R*^{DF} flies. *Ore-R*^{DF}, *Ore-R*^{JLI} and *Ore-R*^{JLI} contaminated flies were injected by TRIS (control, empty symbols) and DCV (500 PFU, filled symbols). Survivals were monitored daily at 22°C. Bar graphs represent the average standard deviation of at least 3 independent experiments.

5.3. Does *pastrel* affect Nora Virus persistent infection ?

Surprisingly, it seemed difficult to maintain a stable Nora Virus persistent infection in next generations of *Ore-R*^{JLI} flies after Nora Virus contamination. It appeared that the first generation of *Ore-R*^{JLI} flies was infected by Nora virus, although high variation in virus titer was observed between samples, as described by (Habayeb et al., 2009b), but then Nora Virus infection tended to be cleared in the next generations (**data not shown**). Thus, we decided to decipher if *Ore-R*^{DF} flies, which carry sensitive alleles of *pastrel*, may allow a better proliferation of Nora Virus than *Ore-R*^{JLI} flies, which carry a resistant allele of *pastrel*. We therefore contaminated *Ore-R*^{DF} bleached flies and *Ore-R*^{JLI} flies with Nora Virus and we compared the level of Nora Virus in both contaminated-*Ore-R* flies (named parents) and in the first generation after contamination (F1). Interestingly, we observed that Nora Virus RNA level in *Ore-R*^{JLI} flies was lower than in *Ore-R*^{DF} flies after one generation (**Figure 65**). In

addition to DCV and CrPV, *Ore-R*^{DF} flies appear more sensitive to Nora Virus infection compared to *Ore-R*^{JLI} flies.

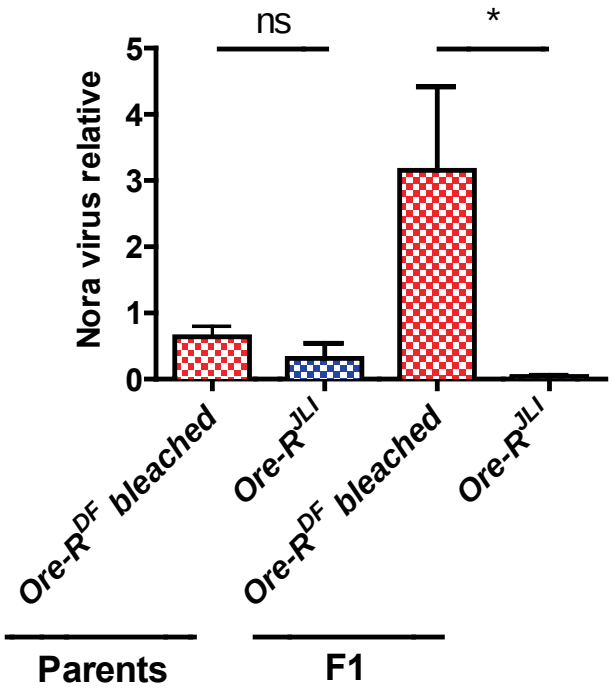


Figure 65. *Ore-R*^{DF} flies are more sensitive to Nora virus infection compared to *Ore-R*^{JLI} flies. *Ore-R*^{DF} bleached flies and *Ore-R*^{JLI} flies were contaminated with feces from *Ore-R*^{DF} flies at 25°C for 5 days. Then, Nora virus RNA level was measured by qPCR in these flies and also in 4-8days flies from the first generation. Bar graphs represent the average standard deviation of 1 experiment.

Overall, our data indicate that Nora Virus is not responsible for the differences of susceptibility to DCV infection between *Drosophila* strains. However, we uncovered that *Ore-R*^{DF} flies, which carry sensitive alleles of *pastrel*, are more sensitive to Nora Virus infection, suggesting that pastrel may also control Nora Virus infection.

The group of Dr. Dominique Ferrandon got interested in this work as they study the effect of intestinal pathogens on the gut. As we hypothesized for DCV infection, we started a collaboration to test if the presence of Nora Virus may impact intestinal infections by pathogenic gram-negative bacteria. Interestingly, it came out that Nora Virus increases the sensitivity of flies to *Pseudomonas aeruginosa* (PA14) and *Serratia marcescens*. They also observed an increased renewal of intestinal epithelium in Nora Virus persistently infected flies.

Chapter 6

RNAi pathway controls infection by a DNA virus in *Drosophila*

6.1. Broad RNA interference-mediated antiviral immunity in Drosophila (Kemp et al, 2013)

In the beginning of my thesis, the siRNA pathway was reported by many groups including ours to be involved in the control of infections by RNA viruses. However, it was unknown whether the siRNA pathway can control a DNA virus infection. This question had been adressed by a post-doctoral fellow in the laboratory and I contributed to this project during my PhD thesis. I observed that *Dcr-2* mutant flies (*Dcr-2*^{R416X}) infected with the DNA virus IIV-6 display an increased susceptibility and a higher viral titer compared to control flies. Accordingly, flies mutant for another null allele of Dcr-2 (*Dcr-2*^{L811fsx}) were highly sensitive to IIV-6 infection and the sensitivity was rescued in mutants carrying a transgene of the genomic region of wild-type Dcr-2. Flies mutants for other components of the siRNA pathway, *R2D2* and *AGO2*, displayed an increased sensitivity to IIV-6 infection. In collaboration with the group of Dr. Sébastien Pfeffer, I constructed a small RNA library from flies and cells infected with IIV-6, allowing the identification of IIV-6-derived siRNAs. V-siRNAs were predominantly of 21 nts, indicating that they are generated by Dcr-2. Indeed, they were not detected in *Dcr-2* mutant flies. Surprisingly, we noticed that v-siRNAs were not uniformly distributed along the viral genome, but rather hot spots of v-siRNAs were observed along the viral genome. Dr. Simona Paro showed that these hot spots are correlated with convergent transcription occuring in these regions. Overall, these data indicate that the siRNA pathway is a broad antiviral defense mechanism, controlling infections by RNA viruses, but also a DNA virus. These results were recently published in the paper hereafter, in the Journal of Immunology.

The Journal of Immunology

Broad RNA Interference–Mediated Antiviral Immunity and Virus-Specific Inducible Responses in *Drosophila*

Cordula Kemp,*,[1] Stefanie Mueller,*,[1,2] Akira Goto,* Vincent Barbier,* Simona Paro,* François Bonnay,* Catherine Dostert,* Laurent Troxler,* Charles Hetru,* Carine Meignin,* Sébastien Pfeffer,[†] Jules A. Hoffmann,* and Jean-Luc Imler*,[‡]

The fruit fly *Drosophila melanogaster* is a good model to unravel the molecular mechanisms of innate immunity and has led to some important discoveries about the sensing and signaling of microbial infections. The response of *Drosophila* to virus infections remains poorly characterized and appears to involve two facets. On the one hand, RNA interference involves the recognition and processing of dsRNA into small interfering RNAs by the host RNase Dicer-2 (Dcr-2), whereas, on the other hand, an inducible response controlled by the evolutionarily conserved JAK-STAT pathway contributes to the antiviral host defense. To clarify the contribution of the small interfering RNA and JAK-STAT pathways to the control of viral infections, we have compared the resistance of flies wild-type and mutant for Dcr-2 or the JAK kinase Hopscotch to infections by seven RNA or DNA viruses belonging to different families. Our results reveal a unique susceptibility of *hop* mutant flies to infection by *Drosophila* C virus and cricket paralysis virus, two members of the *Dicistroviridae* family, which contrasts with the susceptibility of *Dcr-2* mutant flies to many viruses, including the DNA virus invertebrate iridescent virus 6. Genome-wide microarray analysis confirmed that different sets of genes were induced following infection by *Drosophila* C virus or by two unrelated RNA viruses, Flock House virus and Sindbis virus. Overall, our data reveal that RNA interference is an efficient antiviral mechanism, operating against a large range of viruses, including a DNA virus. By contrast, the antiviral contribution of the JAK-STAT pathway appears to be virus specific. ***The Journal of Immunology*****, 2013, 190: 650–658.**

Viruses represent an important class of pathogens, causing serious concern for human health, as well as important economic losses in crops and animals. Because they replicate inside cells, and rely for the most part on host cell molecular machineries for their replication, viruses pose specific challenges to the immune system. Two major strategies of antiviral resistance have been described. In mammals, viral infection is first detected by pattern recognition receptors of the Toll- and RIG-I–like families that sense the viral nucleic acid and trigger the induction of IFNs and other cytokines (1). These factors activate the production of antiviral molecules, such as protein kinase R or oligo-2′, 5′-adenylate synthetase, that contain the infection and contribute to the activation of the adaptive immune response (2). In plants, viral nucleic acids are recognized by enzymes of the Dicer family, which produce small interfering RNAs (siRNAs) of 21–24 nucleotides. These siRNAs are then loaded onto molecules of the Argonaute (AGO) family and will guide them toward RNAs with complementary sequences; targeted RNAs are then either sliced by AGO, or their translation is inhibited. This RNA interference (RNAi) mechanism provides efficient and sequence-specific protection against viral infections (3).

RNAi also plays an important role in the control of viral infections in insects, as shown by the production of virus-derived siRNAs in infected flies, and the increased susceptibility to viral infection of *Drosophila* mutants for the genes *Dcr-2* and *AGO2* (3–6). In addition, several reports indicate that an inducible response also contributes to the control of viral infections (7–15). We previously showed that infection with *Drosophila* C virus (DCV), a member of the *Dicistroviridae* family, leads to induction of some 130 genes (11). Analysis of the regulation of one of these genes, *vir-1*, revealed the presence of functionally important binding sites for the transcription factor STAT in its promoter. The induction of *vir-1*, as well as several other DCV-induced genes, was found to be dependent on the gene *hopscotch* (*hop*), which encodes the only JAK kinase in *Drosophila*. Furthermore, *hop* mutant flies succumb more rapidly than do wild-type controls, with a higher viral load, to DCV infection (11). The Toll and immune deficiency (Imd) pathways, initially characterized for their role in the control of bacterial and fungal infections, were

*CNRS-UPR9022, Institut de Biologie Moléculaire et Cellulaire, 67084 Strasbourg Cedex, France; [†]CNRS-UPR9002, Institut de Biologie Moléculaire et Cellulaire; 67084 Strasbourg Cedex, France; and [‡]Faculté des Sciences de la Vie, Université de Strasbourg; 67083 Strasbourg Cedex, France

[1]C.K. and S.M. contributed equally to this work.

[2]Current address: Bernhard-Nocht-Institut for Tropical Medicine, Molecular Entomology, Hamburg, Germany

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The sequences presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE31542 and to the National Center for Biotechnology Information Small Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number GSE41007.

Address correspondence and reprint requests to Prof. Jean-Luc Imler, IBMC-CNRS/Université de Strasbourg, Rue Rene Descartes, Strasbourg, Alsace 67084, France. E-mail address: JL.Imler@unistra.fr

The online version of this article contains supplemental material.

Abbreviations used in this article: AGO, Argonaute; CrPV, cricket paralysis virus; Dcr-2, Dicer-2; DCV, *Drosophila* C virus; dpi, day postinfection; DXV, *Drosophila* X virus; FHV, Flock House virus; IIV-6, invertebrate iridescent virus type 6; Imd, immune deficiency; MEKK1, MEK kinase 1; RNAi, RNA interference; SINV, Sindbis virus; siRNA, small interfering RNA; *TotM*, *Turandot M*; Upd, unpaired; VSV, vesicular stomatitis virus.

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also thought to play a part in the control of viral infections. Whereas the Toll pathway was associated with resistance to the *Drosophila* X virus (DXV) (15), the Imd pathway was implicated in the control of Sindbis virus (SINV) (7) and cricket paralysis virus (CrPV) (9).

Altogether, the data in the present literature point to the involvement of both RNAi and an inducible expression of effector molecules to counter viral infections in insects (5, 16). However, whereas RNAi was shown to contribute to resistance to several RNA viruses (with either single-stranded genomes of both polarities or double-stranded genomes), most studies on the inducible response have so far focused on a single virus. As a result, the global significance of the inducible response for the control of viral infections remains poorly understood. In particular, it is unclear at present if the JAK-STAT pathway is involved in a general antiviral response, providing broad antiviral immunity, or if it acts specifically on a critical step in the replication cycle of a specific virus or virus family. To address this important question, we have compared the resistance of a mutant for the JAK-STAT pathway to infection by seven RNA or DNA viruses. We find that *hop* mutant flies are more susceptible than wild-type controls to infections by the *Dicistroviridae* DCV and CrPV, but exhibit either no or a weak phenotype for other viruses, suggesting that the JAK-STAT pathway–dependent inducible response is virus specific. Genome-wide transcript profiling shows that infection by two other RNA viruses, Flock House virus (FHV; *Nodaviridae*) and SINV (*Alphaviridae*), leads to upregulation of ≥400 genes, which only partially overlap with those induced by DCV. Overall, our data indicate that the siRNA pathway exerts broad antiviral activity and affects both RNA and DNA viruses, with virus-specific inducible responses contributing to the control of viral infections in *Drosophila*.

Materials and Methods

Fly strain culture and infection

Oregon-R (*OR*) and *yw* were used as wild-type control flies. The $hop^{M38/msv1}$, $Dcr\text{-}2^{L811fsX}$, and $Dcr\text{-}2^{R416X}$ mutant flies were previously described (17–19). A genomic rescue of the *Dcr-2* gene was established with the Fosmid FlyFos017074 (transgeneome.mpi-cbg.de) inserted at the landing site attP40 (2L), and the transgenic chromosome was recombined with the deficiency Df(2R)BSC45, which uncovers the *Dcr-2* locus. For the rescue experiments, *Dcr-2* mutants were crossed with the deficiency Df(2R) BSC45 or the Df(2R)BSC45–*Dcr-2* rescue line. Flies were fed on standard cornmeal–agar medium at 25°C. All fly lines were tested for *Wolbachia* infection and cured whenever necessary. Viral stocks were prepared in 10 mM Tris-HCl, pH 7.5, with the exception of vesicular stomatitis virus (VSV), which was used directly from Vero cell culture supernatant [VSV 4×10^9 PFU/ml; DCV 5×10^{10} PFU/ml; CrPV 1×10^9 PFU/ml; FHV 5.5×10^9 PFU/ml; DXV 4.4×10^7 PFU/ml, invertebrate iridescent virus type 6 (IIV-6) 4.4×10^{11} PFU/ml; and SINV 5×10^8 PFU/ml]. Infections were performed with 4- to 6-d-old adult flies by intrathoracic injection (Nanoject II apparatus; Drummond Scientific) with viral particles, indicated in the figure legends. Injection of the same volume (4.6 nL) of 10 mM Tris-HCl, pH 7.5, was used as a control. For bacterial infection, flies were pricked with a thin needle previously dipped in a concentrated overnight culture of *Escherichia coli* and *Micrococcus luteus* in Luria–Bertani medium. Infected flies were then incubated at room temperature, or at 29°C in the case of $hop^{M38/msv1}$ and the corresponding control flies, and monitored daily for survival, or frozen for RNA isolation and virus titration at the indicated time points.

Cell culture and virus titration

Vero R cells were grown in DMEM (Invitrogen) supplemented with 10% FCS (Biowest), penicillin/streptomycin (Invitrogen), nonessential amino acid mix (Invitrogen), 10 mM pyruvate (Life Technologies), and 200 mM L-glutamine (Invitrogen). Kc167 and S2 cells were grown in Schneider's medium (Biowest) supplemented with 10% FCS, GlutaMAX (Invitrogen), and penicillin/streptomycin (100× mix, 10 mg/ml/10,000 U; Invitrogen). VSV and SINV were titrated from infected flies by plaque assay on Vero R cells. DCV, CrPV, FHV, and IIV-6 were titrated on Kc167 (DCV, CrPV, and FHV) or S2 (IIV-6) cells by the Reed–Muench method to calculate 50% tissue culture–infective dose and converted to PFU with a conversion factor of 0.7.

RNA analysis

Total RNA from infected flies was isolated using TRI Reagent RT bromoanisole solution (MRC), according to the manufacturer's instructions. Total RNA, 1 μg, was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad). The reverse transcription was run in the T3000 Thermocycler (Biometra), with the following PCR program: step 1: 65°C for 5 min, step 2: 4°C for 5 min, step 3: 25°C for 10 min, step 4: 42°C for 60 min, and step 5: 70°C for 15 min. A total of 100 ng cDNA was used for quantitative real-time PCR, using the iQ Custom SYBR Green Supermix Kit (Bio-Rad). The PCR was performed using the CFX384 Real-Time System (Bio-Rad) with the following program: step 1: 95°C for 3 min, step 2: 95°C for 10 s, step 3: 55°C for 30 s, repeated 39 times from step 2. Primers used for qPCR were as follows: *RpL32* (forward 5′-GACGCTTCAAGGGACAGTATCTG-3′; reverse 5′-AAACGCGGTTCTGCATGAG-3′), *vir-1* (forward 5′-GATCCCAATTTTCCCATCAA-3′; reverse 5′-GATTACAGCTGGGTGCACAA-3′), *drosomycin* (forward 5′-CGTGAGAACCTTTTCCAATATGATG-3′; reverse 5′-TCCCAGGACCACCAGCAT-3′), and *diptericin* (forward 5′-GCTGCGCAATCGCTTCTACT-3′; reverse 5′-TGGTGGAGTGGGCTTCATG-3′). *Turandot M* (*TotM*), *upd*, *upd2*, and *upd3* expression levels were quantified using the Brilliant II QRT-PCR Core Reagent Kit, 1-step (Stratagene). The reaction took place in a total volume of 20 μl, using the Taqman Gene Expression Assay [*TotM* (Dm02362087 s1), *upd* (*os*) (Dm01843792_g1), *upd2* (Dm01844134 g1), *upd3* (custom-designed upd3exon2-ANY), and RpL32 (Dm02151827 g1), all from Applied Biosystems]. We used the 7500 Fast Real-Time PCR System (Applied Biosystems) with following PCR program: step 1: 45°C for 30 min, step 2: 95°C for 10 min, step 3: 95°C for 15 s, step 4: 60°C for 1 min, repeated 39 times from step 3. In all cases, gene expression was normalized to the ribosomal protein gene *RpL32*.

For IIV-6, the expression of the annotated genes *206R*, *224L*, *244L*, and *261R* was assessed by strand-specific RT-PCR. We used SuperScript III Reverse Transcriptase specifically adapted for gene-specific priming and followed the manufacturer's protocol (Invitrogen). Briefly, primer pairs were designed to amplify regions of the IIV-6 genome exhibiting or not exhibiting a high density of small RNA reads. Total RNA,1 μg, extracted from infected S2 cells was reverse transcribed with 2 pmol of either forward (F) or reverse (R) primer and 200 U of SuperScript III Reverse Transcriptase. The reaction was then incubated for 1 h at 55°C. Then 1 μl of the resulting cDNA was used to perform 25 cycles of PCR, using Taq DNA polymerase (Invitrogen) and both F and R primers. The primer pairs were as follows: 206R (forward: 5′-AAGGAAAGTGGCGAGTACGA-3′, reverse 5′-AACAAACCCGTTTTCTTCCA-3′); 224L (forward: 5′-CCACCATCACATTGACCTTG-3′, reverse: 5′-ATAAGCGAACCCGAAATCA-3′); 244L (forward: 5′-TGGAAAAGAGTGGTCCCATTT-3′, reverse: 5′-TGTACCTCCCGGAAGATTT-3′); 261R (forward: 5′-CAGCCCCATCCGAATTACTA-3′, reverse: 5′-CTGCAACTGCAGAAATTTGA-3′). The PCR bands were sequenced to verify their viral origin.

Statistical analysis

An unpaired two-tailed Student *t* test was used for statistical analysis of data with GraphPad Prism (GraphPad Software). The p values < 0.05 were considered statistically significant. Survival curves were plotted and analyzed by log-rank analysis (Kaplan–Meier method) using GraphPad Prism (GraphPad Software).

DNA microarray analysis

For each sample, Tris-injected, DCV-infected (11), and FHV- and SINV-infected, three biologically independent samples comprising 45 male Oregon-R flies were used. RNA extraction, biotinylation, and hybridization to Affymetrix *Drosophila* GeneChip microarrays (Affymetrix) were performed as described (20). The Affymetrix Microarray Suite 5.0 (Affymetrix) or Excel (Microsoft) with a combination of built-in functions and custom formulae was used for data analysis. Raw data were sorted with the "absent-marginal-present flags" generated by the Microarray Suite functions. Although an absent flag might indicate that no mRNA of a particular type was present in a sample, marginal flags and absent flags may indicate problems with the hybridization; therefore, only data points marked as present in at least one replicate were retained. The remaining data mass for each microarray was then normalized to itself, making 1 the median of all the measurements. A gene was considered induced if present in at least one replicate, with a virus/Tris ratio higher than 2 for at least one of the time points. Classification of gene functions was analyzed by David

Bioinformatics Resources 6.7 (21). The data set for FHV and SINV was submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE31542.

Assembly, sequencing, and analysis of small RNA libraries

The small RNA library of S2 cells and whole flies was constructed as described (22) and sequenced by the Illumina 2G Analyzer. Reads were then aligned to a reference consisting of the IIV-6 genome from the National Center for Biotechnology Information (accession code NC_003038) using the Bowtie program with standard parameters in genome assembly. Reads aligning to the IIV-6 genome with a maximum of one mismatch were retained and analyzed using in-house Perl scripts and Excel. Sequences were submitted to the National Center for Biotechnology Information Small Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?) under the accession number GSE41007.

Results

RNAi provides broad antiviral protection in Drosophila

Several independent studies, including our own, have established that RNAi, and more precisely the siRNA pathway, serves as an efficient host defense against RNA viruses. These include viruses with a single-stranded genome of both (+) and (−) polarity and dsRNA viruses (23–30), and we confirmed that flies mutant for *Dcr-2* died more rapidly than wild-type controls when they were infected with DCV, CrPV, FHV, SINV, VSV (*Rhabdoviridae*), and DXV (*Birnaviridae*) (data not shown). Next, we addressed the question whether the siRNA pathway also participated in the control of a DNA virus infection, and infected wild-type and RNAi mutant flies with IIV-6 (*Iridoviridae*). Infection of *Dcr-2* mutant flies led to a more rapid and intense appearance of blue color, which is characteristic of the accumulation of iridescent viral particles, than in wild-type controls (Fig. 1A). *Dcr-2*$^{-/-}$ flies were significantly more susceptible to IIV-6 infection than were the corresponding wild-type (Fig. 1B). A fraction of *Dcr-2*$^{-/-}$ flies injected with buffer also died in the course of the experiment, confirming the increased sensitivity to stress associated with mutations of the siRNA pathway (31). The decreased survival time correlated with a 20-fold increased viral load in *Dcr-2* mutant flies at 10 d postinfection (dpi) (Fig. 1C). Similar results were obtained when a different null allele of *Dcr-2* was used, and the IIV-6 susceptibility phenotype was rescued by a wild-type genomic *Dcr-2* transgene (Fig. 1D). The *r2d2*$^{-/-}$ and *AGO2*$^{-/-}$ null mutant flies also exhibited increased sensitivity to IIV-6 (Fig. 1E). *AGO2*$^{-/-}$ flies contained more viral DNA than did wild-type controls, confirming that this gene participates in the control of infection (Fig. 1F).

We next sequenced small RNA libraries prepared from IIV-6–infected S2 cells or adult flies. We observed several hundreds of thousands of reads matching the IIV-6 genome in both infected S2 cells and wild-type flies, but not in control noninfected S2 cells (Supplemental Table I). The large majority of these reads had a size of 21 nucleotides, which is characteristic for processing by the RNase Dicer-2 (Dcr-2). This peak was absent from the library prepared from infected *Dcr-2*$^{-/-}$ mutant flies (Fig. 2A). These data indicate that Dcr-2 generates 21-nucleotide IIV-6–derived siRNAs in infected flies, and raise the question of the nature of the substrate used by Dcr-2 in the context of this infection. As previously reported for RNA viruses, the number of reads matching

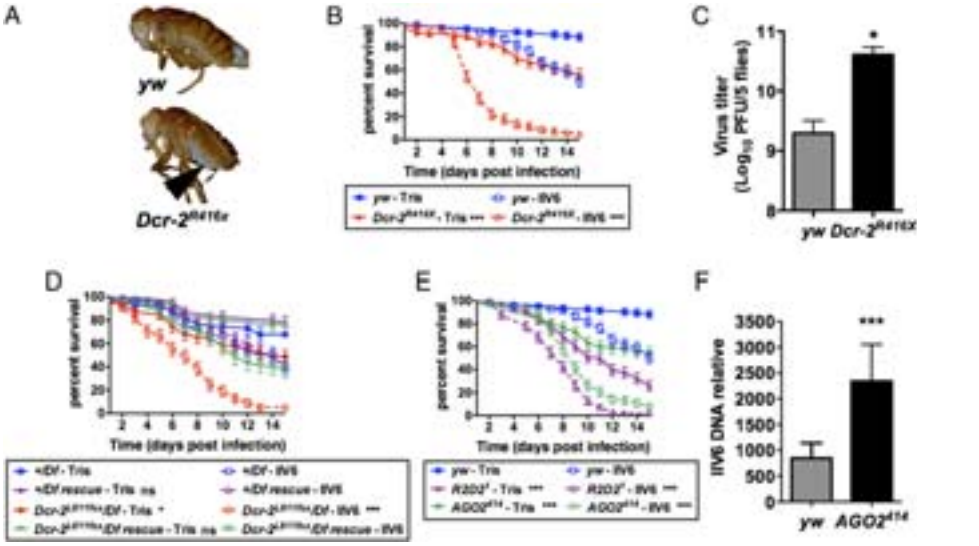

FIGURE 1. Dcr-2 is involved in host defense against the DNA virus IIV-6. (**A**) Upon injection of IIV-6 (5000 PFU) in wild-type (*yw*) and *Dcr-2*^{R416X} mutant flies, typical blue paracrystalline structures appeared earlier in the abdomen (arrowhead) of the mutant flies. Representative individuals 10 dpi are shown. (**B**) Groups of 20 wild-type (*yw*) or *Dcr-2*^{R416X} mutant flies were injected with IIV-6 or Tris, and survival was monitored daily. The difference between the wild-type and *Dcr-2* mutant flies is statistically significant. (**C**) Viral titer in groups of five wild-type (*yw*) or *Dcr-2*^{R416X} mutant flies was monitored 10 dpi. (**D**) Rescue of the hemizygous *Dcr-2*^{L811fsX} for the IIV-6 susceptibility phenotype by a transposon expressing a wild-type *Dcr-2* transgene. *Dcr-2*^{L811fsX} hemizygous flies (*Dcr-2*^{L811fsX}/Df) are significantly more susceptible than *Dcr-2*^{L811fsX} hemizygous flies complemented by a wild-type *Dcr-2* transgene (*Dcr-2*^{L811fsX}/Df rescue). Df is Df(2R)BSC45, a deficiency that fully uncovers the *Dcr-2* locus. All control and genomic rescued flies are in *CantonS* background. (**E**) Survival rate of wild-type (*yw*), *R2D2*^{1}, and *AGO2*^{414} mutant flies upon IIV-6 or Tris injection. (**F**) IIV-6 DNA load was determined by quantitative PCR in four groups of six flies of the indicated genotype at 10 dpi. For all panels, the data represent the mean and SD of at least three independent experiments, and the difference between controls and mutant flies is statistically significant. $*p < 0.05$, $***p < 0.001$. All experiments are performed at 22°C (A, C, F) or 25°C (B, D, E).

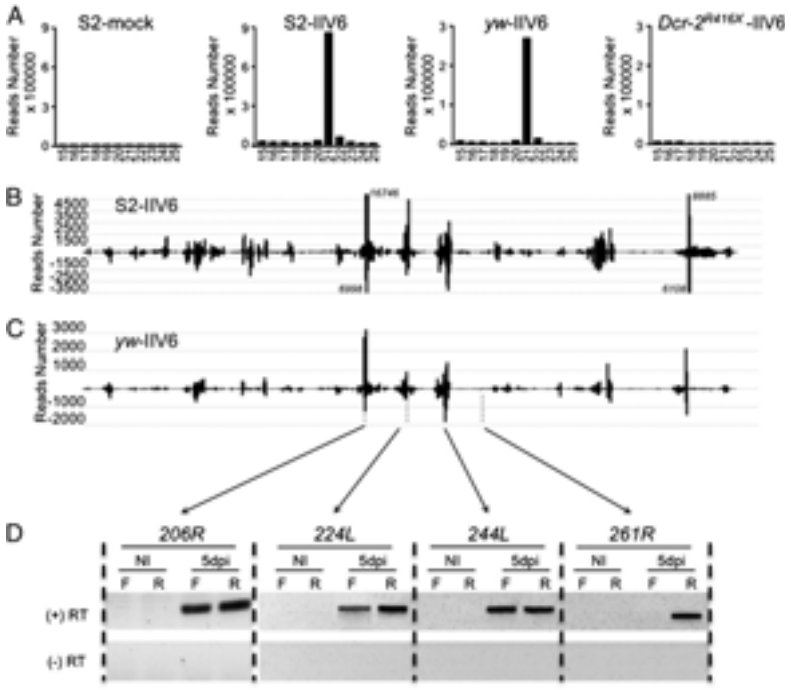

FIGURE 2. Virus-derived siRNAs in S2 cells and *Drosophila* adult flies infected by the DNA virus IIV-6. RNA was extracted 5 dpi from S2 cells infected by IIV-6 (MOI 0.01) and adult wild-type (*yw*) or mutant ($Dcr\text{-}2^{R416X}$) flies injected with IIV-6 (5000 PFU per fly). (**A**) Size distribution of the small RNAs matching the viral genome in S2 cells and adult flies of the indicated genotype. (**B** and **C**) Distribution of the 21-nucleotide siRNAs from the S2 cell (B) and *yw* adult fly (C) libraries along the IIV-6 genome. Each IIV-6–derived small RNA is represented by the position of its first nucleotide. The IIV-6–derived small RNAs matching the upper and lower strand of the DNA genome are respectively shown above (positive reads number) and below (negative reads number) the horizontal axis, which represents the 212482bp genome. In (B), the number of reads for four peaks going off-scale is indicated next to them, in italics. (**D**) Strand-specific RT-PCR with primers corresponding to the annotated viral genes *206R*, *224L*, *244L*, and *261R*. The experiment was performed in the presence (+) or absence (−) of RT. NI, Noninfected; F and R, forward and reverse strand primer used for reverse transcription.

each strand of the viral genome was very similar (Supplemental Table I). However, unlike RNA viruses, the virus-derived siRNAs were not uniformly distributed along the viral genome. Rather, several hotspots were observed, revealing that specific regions of the viral genome generate the siRNAs (Fig. 2B, 2C). These peaks do not correlate with the intensity of transcription of the viral genome, and some highly transcribed regions are located in areas not generating significant levels of siRNAs (32). The strong symmetry of the peaks observed in S2 cells and wild-type flies suggests that these regions are transcribed on both strands and generate dsRNA. Indeed, we could detect bidirectional transcription in the areas of the viral genome covered by the peaks (Fig. 2D). By contrast, transcription of only one strand of the DNA genome was detected for the locus 261R, which is located in a region that does not produce significant amounts of siRNAs. Overall, these results indicate that the siRNA pathway in *Drosophila* can also protect against a DNA virus infection.

The JAK kinase Hopscotch does not confer broad antiviral immunity

To test the contribution of the JAK-STAT pathway in antiviral immunity in *Drosophila*, we injected loss-of-function mutants of the JAK kinase Hopscotch ($hop^{M38/msv1}$) with different ssRNA, dsRNA, and DNA viruses. As previously described, $hop^{M38/msv1}$ mutant flies die more rapidly than do wild-type controls following DCV infection, and contain ~10-fold more virus (Fig. 3A). By contrast, we did not observe significant differences in survival between wild-type and $hop^{M38/msv1}$ mutant flies upon infection with the alphavirus SINV (Fig. 4A), and the viral titers 2 dpi were not significantly different in wild-type and $hop^{M38/msv1}$ mutant flies (data not shown), indicating that the JAK-STAT pathway does not contribute to resistance to this virus. The $hop^{M38/msv1}$ mutant flies, as well as wild-type flies, also resisted infections by the rhabdovirus VSV and by the nodavirus FHV (Fig. 4B, 4C). A slight reduction in survival was observed in the case of the dsRNA virus DXV (*Birnaviridae*) and the DNA virus IIV-6 (Fig. 4D, 4E). However, the difference between wild-type and $hop^{M38/msv1}$ mutant flies was only statistically significant in the case of DXV infection. Furthermore, we did not observe statistically significant differences in the DXV and IIV-6 viral titers in wild-type and $hop^{M38/msv1}$ mutant flies in the format of our assays (data not shown).

Overall, our data indicate that the JAK-STAT pathway is critical for host defense against DCV, but plays a minor role for DXV and IIV-6 and is essentially dispensable in the case of FHV, SINV, and VSV. We therefore tested CrPV, another member of the *Dicistroviridae* family known to infect *Drosophila*. We observed a decrease in survival and a significant increase in viral titers in CrPV-infected $hop^{M38/msv1}$ mutant flies compared with wild-type flies (Fig. 3B). In conclusion,

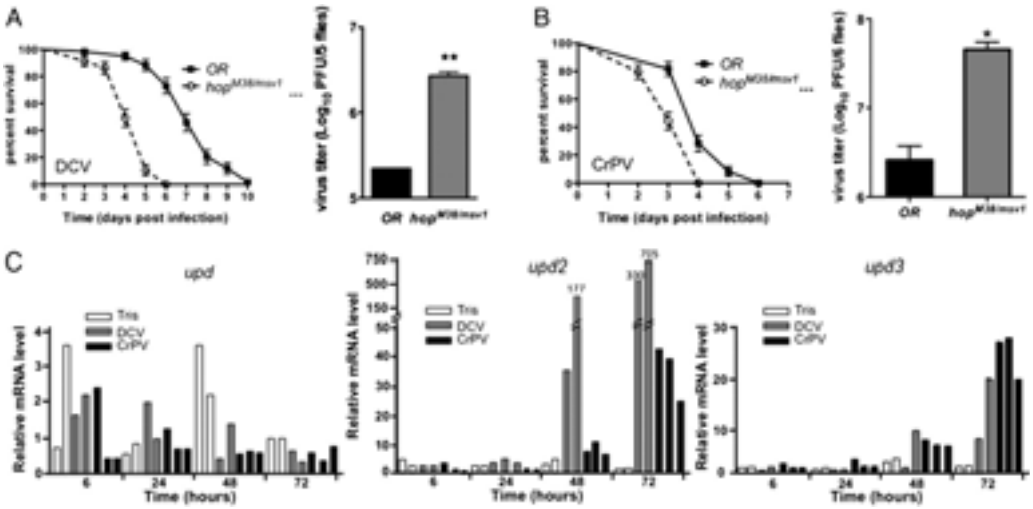

FIGURE 3. The JAK kinase Hopscotch is involved in host defense against DCV and CrPV. (**A** and **B**) Groups of 20 wild-type (OR) or *hopscotch* ($hop^{M38/msv1}$) mutant flies were injected with DCV (500 PFU) (A) or CrPV (5 PFU) (B), and survival was monitored daily. The experiment was repeated three times, and data represent the mean and SD. In the *right panels*, viral titer was determined in groups of five flies 2 dpi for DCV (A) and 1 dpi for CrPV (B). The data represent the mean and SD of three independent experiments, and the difference between wild-type and *hop* mutant flies is statistically significant. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (**C**) DCV and CrPV infection triggers induction of the genes *upd2* and *upd3*, which encode cytokines activating the JAK/STAT pathway. Flies were infected with DCV or CrPV, and expression of *upd*, *upd2*, and *upd3* was monitored in groups of 10 flies at the indicated time points by Taqman quantitative PCR. The results of at least two independent experiments are shown.

our data indicate that the JAK-STAT pathway in *Drosophila* confers protection against some viruses—in particular, the *Dicistroviridae*—but does not provide broad antiviral immunity.

Inducible gene expression in FHV- and SINV-infected flies

The above results raised the question of whether an inducible response contributes to host defense against viruses other than DCV and CrPV. We therefore conducted a genome-wide microarray analysis using Affymetrix DNA microarrays to monitor gene expression in flies infected by FHV (2 and 3 dpi) or SINV (4 and 8 dpi), and compared the data with those obtained for DCV infection (1 and 2 dpi). The time points for this analysis were chosen to take into account the different kinetics of replication and colonization of *Drosophila* by the different viruses (11, 24). For each virus, we observed a large overlap between the genes induced at the first and second time points. We then pursued our analysis, focusing on the genes induced either at the first or at the second time point. The microarray data revealed that 487 and 201 genes were induced or

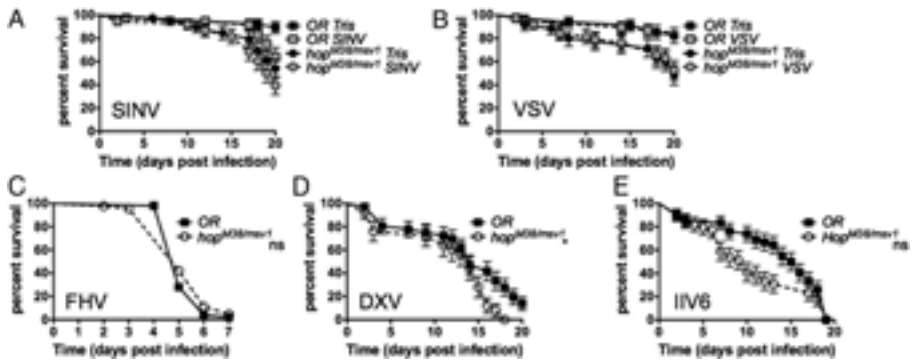

FIGURE 4. Susceptibility of flies mutant for the JAK kinase Hopscotch to infection by SINV, VSV, FHV, DXV, and IIV-6. Groups of 20 wild-type (OR) or *hop* mutant flies were injected with SINV (**A**), VSV (**B**), FHV (**C**), DXV (**D**), or IIV-6 (**E**), and survival was monitored. For VSV and SINV, the Tris buffer control injection is also shown, because *hop* mutant flies exhibited decreased survival at 29°C after day 16 upon both buffer and virus injection. Kaplan–Meier analysis of the results of at least two independent experiments reveal a statistically significant difference in survival between wild-type and *hop* mutant flies only in the case of DXV. $*p < 0.05$.

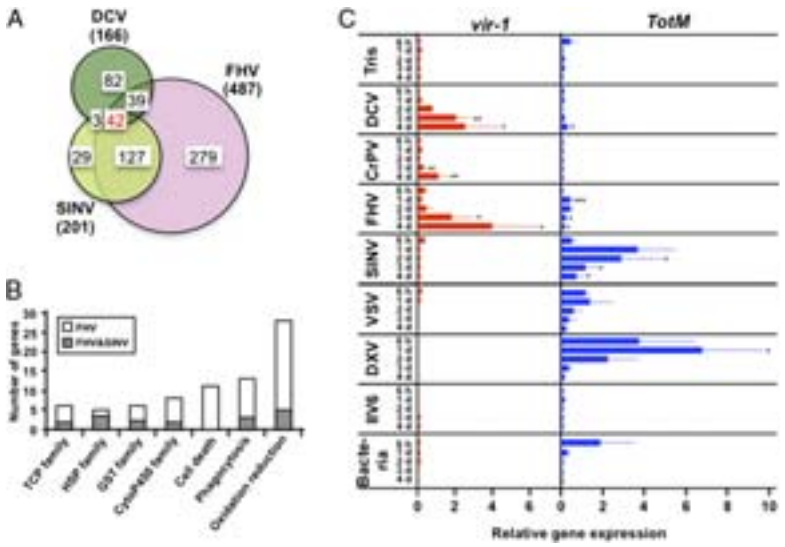

FIGURE 5. Microarray analysis of gene induction following infection by DCV, FHV, or SINV. (**A**) Venn diagram showing the number of upregulated genes (by a factor of at least 2) following infection by the three viruses. The total number of genes regulated by each virus is indicated in parentheses. (**B**) FHV and SINV induce members of the same gene families, but FHV triggers a stronger response. The numbers of genes belonging to seven gene ontology functional categories induced by both FHV and SINV or by FHV only are shown. (**C**) Expression of *vir-1* and *TotM* by quantitative PCR normalized for the expression of the housekeeping gene *RpL32*. Groups of 10 wild-type (OR) flies were injected with Tris buffer or the viruses DCV, CrPV, FHV, SINV, VSV, DXV, or IIV-6 or pricked with a needle dipped in a concentrated pellet of the Gram-positive bacterium *M. luteus* and the Gram-negative bacterium *E. coli*. RNA was extracted at 6 h, 1 d, 2 d, 3 d, and 4 d after challenge. The data represent the mean and SEs of at least two independent experiments. The p values were calculated for each time point individually versus the Tris-injected control. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

upregulated by a factor of at least 2 upon infection by FHV and SINV, respectively. When analyzed with the same criteria, 166 genes were induced by DCV (Fig. 5A, Supplemental Table II).

The data of this transcriptomic analysis call for two comments. First, we note that 42 genes were induced by all three viruses (Fig. 5A). We compared this set of genes with microarray studies performed on flies infected by fungi and bacteria (both extra- and intracellular) to identify a potential signature specific for viral infections (Supplemental Table III). We observed that a number of genes, such as *Frost*, are upregulated similarly by all types of infections, suggesting that they are induced by the stress of the infection, rather than by recognition of specific characteristics of the infecting microorganism. Of interest, other genes, such as *Vago*, *Obp99b*, *Mal-B1*, *Nmda1*, *CG8147*, *CG1572*, *l(2)gd1*, *CG14906*, *CG10911*, and *Tsp42EI*, appear to be induced only in response to viral infections, and may represent the core of an inducible antiviral gene expression program. The case of *Obp99b* is particularly striking, as this gene is strongly upregulated by FHV, SINV, and DCV, but inhibited following other types of infection. Clearly, the regulation and function of this molecule deserves further investigation. The genes *CG4680*, *Eip75B*, *Sp7*, and *CG10916* are induced both by the viruses and by the intracellular bacterium *Listeria* (33), suggesting that they may participate in the defense against intracellular intruders (Supplemental Table III).

A second comment is that the majority of upregulated genes are induced by only one or two of the viruses, revealing virus-specific responses. Of interest, 84% of the genes upregulated by SINV are also induced by FHV, pointing to a strong similarity between the responses to the two viruses. FHV induced a higher number of genes than did Sindbis virus, and only 34% of the genes induced by FHV are also induced by SINV (Fig. 5A). It is intriguing, though, that many of the genes induced solely by FHV, but not by SINV, are members of the same gene families as the genes coinduced by both FHV and SINV. This peculiarity underlines the basic similarities between the transcriptional response to the two viruses. In addition, several genes associated with cell death are induced by FHV, but not SINV, which may reflect the higher virulence of FHV (Fig. 5B, Supplemental Tables II, III). Only 22% and 16% of the genes induced by SINV and FHV, respectively, are also induced by DCV, indicating that DCV, on one hand, and FHV and SINV, on the other hand, trigger different inducible responses (Fig. 5A). We did not detect in our microarrays expression of the genes encoding the unpaired (Upd) cytokines, which activate the JAK-STAT pathway in *Drosophila*. However, quantitative RT-PCR analysis revealed that *upd2* and *upd3*, but not *upd*, are induced or upregulated following DCV and CrPV infection (Fig. 3C).

Virus-specific pattern of gene induction

To further characterize the transcriptional response triggered by different viruses, wild-type flies were injected with DCV, CrPV, FHV, SINV, VSV, DXV, and IIV-6, and gene induction was measured at 6 h postinfection and 1, 2, 3, and 4 dpi. Gene expression was monitored by quantitative RT-PCR, which provides a more accurate quantification of gene expression than does hybridization to short oligonucleotide probes on microarrays (34). We monitored expression of the DCV-induced gene *vir-1* (11) and of *TotM*, which, according to the microarrays, is induced by FHV and SINV infection. We confirmed the induction of *vir-1* by DCV and FHV (11) and detected a milder but significant induction of this gene by CrPV infection. By contrast, no induction of *vir-1* by SINV, VSV, DXV, and IIV-6 was observed (Fig. 5C). For *TotM*,

we confirmed the induction by FHV at different time points. In addition, we observed that *TotM* expression was significantly induced by DCV at late time points of infection (4 dpi). We note that induction of *TotM* by SINV, VSV, and DXV was 10–20 times stronger than the induction by FHV (Fig. 5C). The DNA virus IIV-6 did not induce *TotM* at any measured time point. Interestingly, we observed different profiles for *vir-1* and *TotM* induction after viral challenge. Overall, the viruses that kill wild-type flies rapidly (within 10 d), such as DCV, CrPV, and FHV, were potent inducers of *vir-1*, whereas less pathogenic viruses, such as SINV, VSV, and DXV, did not induce *vir-1*. The opposite trend was observed for *TotM*, which was most potently induced by SINV, VSV, and DXV. The different pattern of induction of *vir-1* and *TotM* suggests that the two genes may be regulated differently, even though both were previously shown to be regulated by the JAK-STAT pathway (11, 17). Indeed, the MAP3K MEK kinase 1 (MEKK1) and the Imd pathways are also known to contribute to the induction of *TotM* induction in some contexts (17, 35).

Some antimicrobial peptide genes were also upregulated according to the microarrays, suggesting an overlap between antiviral immunity and antibacterial–antifungal defenses. We observed an enrichment for genes regulated by the Toll pathway [e.g., the cytokine Spaetzle (Spz) and the antifungal peptides Drosomycine (Drs) and Metchnikowine (Mtk)] in the DCV-specific set of genes (Supplemental Table II). We also noted an enrichment of Imd pathway–regulated genes, such as the antibacterial peptides Attacin-A and -C, Diptericin-B, and the transcription factor Relish, in the genes upregulated by both DCV and FHV. However, when expression of *diptericin* and *drosomycin*—two markers of activation of the Imd and Toll pathways, respectively—was monitored by quantitative RT-PCR, none of the viruses triggered an induction comparable to that of bacterial and fungal infections, although the wounding associated with the injection procedure clearly led to some expression of the genes (Supplemental Fig. 1).

Discussion

We have investigated the involvement of RNAi and the evolutionarily conserved JAK-STAT signaling pathway in the resistance to a panel of seven viruses representing several important families, including the arboviruses SINV and VSV. Our data provide a contrasting picture: on the one hand, a broad antiviral immunity based on RNAi contributing to the defense against both RNA and DNA viruses, and on the other hand, a virus-specific transcriptional response involving the JAK-STAT pathway but playing a critical role only in the case of *Dicistroviridae* infection.

RNAi protects against a DNA virus infection

The present study extends work from several groups, including our own, showing that flies mutant for the siRNA pathway are more sensitive than wild-type flies to a large panel of RNA viruses, and reveals that Dcr-2 is also required for the control of the DNA virus IIV-6. We note, however, that the increase of viral titer in siRNA pathway–mutant flies is not as strong as in the case of some RNA viruses [e.g., VSV (25)]. This finding could reflect either the expression of a viral suppressor of RNAi by IIV-6 or the fact that only a portion of the viral genome is targeted by siRNAs. Indeed, this virus encodes an RNaseIII enzyme, which could cleave siRNA duplexes, as previously reported in plants infected by the sweet potato chlorotic stunt virus (36). The involvement of Dcr-mediated immune responses against DNA virus infections was previously noted in plants, in which secondary structures in the transcribed viral RNAs, or dsRNAs formed from overlapping bidirectional transcripts, can be processed into siRNAs (37, 38). Production of dsRNA from DNA viruses also occurs in animal cells, as demonstrated by the critical role played by the dsRNA receptor TLR3 in the sensing of herpesvirus infection in mammals (39, 40). Our data are consistent with a model whereby dsRNA generated from convergent transcription of the IIV-6 genome is processed by Dcr-2 and triggers RNAi. Thus, we conclude that RNAi provides an efficient and highly specific RNA-based defense against many types of viruses in *Drosophila* and probably other insects. This conclusion parallels the situation described in plants. The vertebrates, which largely rely on the induction of IFNs to counter viral infections, appear to be the exception among multicellular organisms (1). Of interest, however, the DExD/H box helicase domains found in Dcr enzymes and RIG-I–like receptors, which sense the presence of viral RNAs in cells infected by RNA and DNA viruses, are phylogenetically related (10). This finding suggests that an essential domain of a core molecule from the ancestral antiviral response, RNA silencing, was at some point recruited to sense viral RNAs in vertebrates and to subsequently activate a signaling pathway leading to production of IFNs.

Virus-specific induced gene expression in Drosophila

Microarrays are powerful tools to monitor the global transcriptome of infected cells and compare the response to different infections. Despite its limitations for accurate measurements of the magnitude of expression changes, this technology provides useful information on changes in gene expression (34). In this article, using whole-genome Affymetrix microarrays to analyze the transcriptome of flies infected by DCV, FHV, or SINV, we report the existence of virus-specific responses to infection. These results are in keeping with a previous study pointing to autophagy as an antiviral defense mechanism against VSV, but not DCV, infection (14). The three viruses we used belong to different families and present different characteristics that make them valuable for the current study. For example, 1) DCV and FHV replicate rapidly and kill *Drosophila* upon injection, whereas SINV does not at the dose used (11, 24); 2) DCV is a natural pathogen of *Drosophila*, whereas FHV and SINV have not been found in wild *Drosophila* populations (41); 3) FHV and DCV possess, respectively, a strong and moderate viral suppressor of RNAi, whereas SINV presumably does not (28, 42, 43). The three viruses also have different tissue tropism and may be associated with tissue-specific modifications in the physiology of the infected host. For example, FHV was recently shown to be a cardiotropic virus, affected by potassium channels regulating heart function (44), whereas DCV infection causes intestinal obstruction (S. Chtarbanova and J.-L. Imler, manuscript in preparation).

Comparison of the transcriptomes of the flies infected by the three viruses revealed more similarities between FHV and SINV than between each of these and DCV. This may reflect the co-evolution of DCV with its host, and the fact that this virus may have learned to ward off the antiviral arsenal of its host. Indeed, DCV induces fewer genes than does FHV, even though the two viruses replicate with similar kinetics and lead to the rapid death of the flies. The genes induced by FHV and SINV encode chaperonins (Tcp or Hsp), glutathione transferases, cytochrome P450s, stress markers (Tot family), thioester-containing proteins, and cytoskeletal regulators, suggesting an involvement of oxidative stress and phagocytosis in the response to these viruses. The two viruses also upregulate the gene *egghead* (*egh*), which encodes a molecule involved in the uptake of dsRNA and antiviral immunity (27). Despite the large overlap between the genes upregulated by FHV and SINV, the former induce a more intense transcriptional response than the latter. This observation may reflect the more aggressive replication of FHV in *Drosophila*. Indeed, the genes specifically induced by FHV include not only additional members of the families mentioned above (Hsp, Tcp, Gst, cytP450, thioester-

containing proteins), supporting the idea of a more intense response, but also genes associated with cell death. In addition, FHV upregulates several molecules previously connected to innate immunity in *Drosophila*, such as Hel89B (45), POSH (46), or MEKK1 (35), or molecules that may downmodulate the strong response to virus infection (e.g., the genes *CG9311* and *Pez*, encoding tyrosine phosphatases). Finally, we note that FHV induced eight genes encoding factors with RNA binding domains, including four DExD/H box helicases, which may participate in the sensing and neutralization of viral nucleic acids. This specificity may reflect a response of the host to counter the effect of the strong suppressor of RNAi B2, a dsRNA-binding protein (47).

An intriguing aspect of the transcriptome of virus-infected flies is the upregulation of genes regulated by the Toll and Imd pathways. We observed an enrichment of Toll pathway target genes induced in flies infected by DCV, but not FHV or SINV, suggesting that DCV infection triggers this pathway. Among the genes induced by DCV, but not by the two other viruses, we also note the presence of *Ect4*, which encodes a TIR domain cytoplasmic molecule. The mammalian ortholog of this gene, SARM, was proposed to participate as a negative regulator of TLR signaling in some antiviral defenses (48). Two other genes regulated by DCV and possibly establishing a connection between RNA silencing and the inducible response are worth mentioning: *headcase* was identified in a screen as a regulator of the siRNA pathway (49), whereas *CG9925* encodes a protein with a Tudor domain, a characteristic of several components of the Piwi-interacting RNA pathway (50).

Unlike the Toll-regulated genes, several genes regulated by Imd were induced in flies infected by DCV or FHV, although not by SINV. The Toll and Imd pathways play a well-characterized role in the regulation of bacterial and fungal infections, through the regulation of genes encoding antimicrobial peptides. These genes are also upregulated by viral infection, although not significantly, compared with buffer injection. This low level of induction most likely explains our inability to detect antimicrobial peptides in the hemolymph of DCV-infected flies (51). Although not formally establishing that the Toll and Imd pathways participate in the antiviral response, these results certainly do not rule out such a role (7, 9, 15). Alternatively, induction of the antimicrobial genes may involve the transcription factor FOXO, a known regulator of stress resistance, and may occur independently of the Toll and Imd pathways (52). Whatever the mechanism of induction, the biological significance of this weak induction of molecules normally active in the micromolar range is unclear. One possibility is that the *Drosophila* antimicrobial peptides carry additional functions that do not require high-level expression. For example, some mammalian β-defensins play a dual role in innate immunity and, in addition to their antibacterial properties, interact with chemokine receptors with affinities in the nanomolar range, thus mediating chemoattraction of phagocytic cells (53).

Dicistroviridae-*specific contribution of the JAK-STAT pathway to antiviral immunity*

An unexpected finding reported in this article is that *hop* mutant flies have a clear phenotype for DCV and CrPV, but not for the other viruses tested. This observation indicates that the JAK-STAT pathway, in addition to RNAi, participates in host defense against members of the *Dicistroviridae* family. DCV infection leads to induction of the genes encoding the cytokines Upd2 and Upd3, which may subsequently activate the JAK-STAT pathway in noninfected cells, triggering an antiviral program of gene expression. Altogether, our results highlight that the contribution of the inducible response to the control of DCV is similar to that of RNAi, as flies mutant for either RNAi or the inducible JAK-STAT pathway succumb to infection 2–3 d before the controls, with an ~10-fold increase in viral titer.

Interestingly, even though *hop* mutant flies appear to be specifically sensitive to *Dicistroviridae*, other viruses activate the JAK-STAT pathway. Indeed, we observed a slight increase in the lethality of *hop* mutant flies postinfection with DXV and IIV-6. In *Aedes* mosquitoes, the JAK/STAT pathway was also shown to activate a defense against Dengue, a member of the *Flaviviridae* family (54). We also note that the JAK-STAT pathway–regulated gene *vir-1* (11) is induced by DCV and CrPV, but also FHV, even though *hop* mutant flies resist FHV infection much as do wild-type flies. One hypothesis to explain this apparent paradox is that some genes may be induced in a JAK-STAT–independent manner in the context of viral infections. For example, the gene *TotM*, which is induced by several viruses normally resisted by *hop* mutant flies, can be induced by the MEKK1 pathway, in addition to the JAK-STAT pathway (35). Indeed, we observed that *TotM* remains fully induced by FHV and SINV in *hop* mutant flies (C. Dostert and J.-L. Imler, unpublished observations). However, this hypothesis cannot account for the induction of *vir-1* by FHV, because it is strongly reduced in *hop* mutant flies (C. Dostert and J.-L. Imler, unpublished observations). This finding suggests that some aspects of the JAK-STAT–induced response may be redundant of other defenses for FHV, but not for DCV. The fact that FHV triggers a stronger transcriptional response than does DCV (Fig. 5) is consistent with this hypothesis.

A key question pertains to the nature of the receptor detecting *Dicistroviridae* infection and triggering the JAK-STAT–dependent inducible response. Our data point to the induction of a specific subset of genes, including the JAK-STAT–regulated gene *vir-1* (11), by fast-killing viruses such as DCV and CrPV, but also FHV, which replicate rapidly to high titers upon injection in flies. Of note, *vir-1* induction is not affected in flies expressing the dsRNA-binding protein B2, or in *Dcr-2* mutant flies, indicating that this gene is not induced following sensing of dsRNA (10). This finding suggests that sensing tissue damage and/or cell death could contribute to this inducible response, a hypothesis corroborated by the association of the JAK-STAT pathway with the cellular response to a variety of stresses (17, 55–57).

In conclusion, our data confirm that, beyond RNAi, an inducible response contributes to the control of some viral infections in *Drosophila*. However, this response is complex, and great care should be exercised before generalizing the results obtained with one single virus species. This unexpected complexity probably reflects the intricate association of viruses with their host cells in different tissues, their different strategies of replication or protein expression, or their acquisition of suppressors of host defense.

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Disclosures

The authors have no financial conflicts of interest.

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Part IV

DISCUSSION

IV.1. Pastrel, a new gene restricting infection by picorna-like viruses in *Drosophila*

IV.1.a. Polymorphisms in pastrel gene are correlated with the susceptibility to DCV infection

It was striking to observe such a wide variability in the susceptibility of flies used as control in the laboratory to DCV infection. In 1995, Thomas-Orillard and colleagues already reported a difference of susceptibility of wild-type flies to DCV infection (Thomas-Orillard et al., 1995). They compared the susceptibility of 15 strains of *Drosophila melanogaster* isolated from different geographic areas to DCV infection. Whereas 14 strains displayed 100% lethality after 3 to 6 days post-infection, around 50% of flies from Nagasaki strain were still alive 10 days after infection with the same dose of virus. Interestingly, the lethality of strains was not correlated with their geographic origin. They observed that the progeny resulting of the direct and reciprocal cross between a sensitive strain (Charolles) and a resistant strain (Nagasaki) is resistant to DCV infection, comparable to the Nagasaki strain. It indicates that the phenotype of DCV resistance is dominant and independent of sex chromosomes. They performed crosses between a stock with chromosomes balancers and the Nagasaki flies, revealing that the resistant phenotype is associated with the third chromosome. Accordingly, our data indicate that the susceptibility of laboratory control flies to DCV infection is genetically transmitted in an autosomal-dominant manner. Indeed, the direct and reciprocal cross between a sensitive line and a resistant line result in a progeny that is resistant to DCV infection.It is important to mention that all flies used in our study are free of Wolbachia infection. Wolbachia are Gram-negative bacteria widely spread in laboratory stocks of *Drosophila* (Clark et al., 2005). It was recently reported that Wolbachia mediates protection against RNA viruses (Hedges et al., 2008; Teixeira et al., 2008). Whether the presence of Wolbachia was not diagnosed in their study, Thomas-Orillard and colleagues did not observe any maternal effect, which allows us to exclude a role of Wolbachia in the resistance of Nagasaki flies to DCV infection. However, other studies observed variations in the susceptibility of flies to DCV infection, but associated with a maternal effect (Plus and Golubovsky, 1980), pointing out a possible contribution of Wolbachia in the resistance to

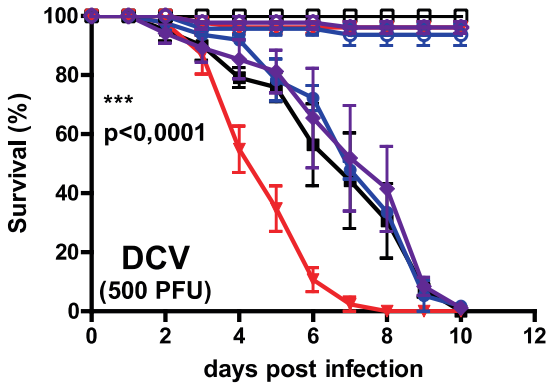
DCV infection. This highlights the importance to test the presence of Wolbachia in flies aimed at being used for experiments with viruses. Our investigation of the gene involved in the susceptibility of flies to DCV infection jumped forward with the genome wide association study (GWAS) performed by Pr. Francis Jiggins, and recently published (Magwire et al., 2012). They identified 6 SNPs in the *pastrel* gene, present on the third chromosome, that are associated with the resistance to DCV infection. Interestingly, as Thomas-Orillard and colleagues observed that the resistance to DCV infection is conferred by the third chromosome, it remains to be determined whether polymorphisms in *pastrel* gene may be involved in the resistance of Nagasaki flies to DCV infection. Our sequencing analyses of the *pastrel* gene in our laboratory control flies allowed us to identify 3 SNPs that were correlated with the sensitivity or resistance to DCV infection. Accordingly, these 3 SNPs were also identified by Pr. Jiggins: SNPs 3L:7352966 T/G and 3L:7351494 C/T present in introns 1 and 5 respectively; SNP 3L:7350895 Ala/Thr present in exon 6. The three other SNPs identified by Pr. Jiggins (SNPs 3L:7350452 T/G and 3L:7350453 A/G present in the 3'UTR region and SNP 3L:7352880 Glu/Gly present in exon 2 were either not found, or not systematically correlated with the susceptibility to DCV infection in our sample of laboratory control flies. We used prediction software to test if the two SNPs present in intron may affect the splicing of *pastrel* gene but splicing was predicted to be similar between sensitive and resistant versions of *pastrel* gene. Surprisingly, most of the flies from our laboratory that were genotyped for *pastrel* gene by allele specific PCR displayed a sensitive allele of *pastrel*. In agreement with our observations, the study of Thomas-Orillard and colleagues highlights that the sensitive phenotype is more abundant in wild-type strains of *Drosophila melanogaster* than the resistant phenotype (14 sensitive strains out of 15 strains tested). Accordingly, a previous study by Jousset and colleagues revealed that 39 strains of *Drosophila melanogaster* captured in the wild from different geographic areas were all sensitive to DCV infection ((Jousset, 1976), mentioned in Thomas-Orillard et al., 1995). They did not find resistant flies. We can speculate that the sensitive allele of *pastrel* may confer an advantage for the host compared to the resistant allele. Surprisingly, we found an increased number of SNPs in the *pastrel* gene of *Ore-R*^{DF} flies compared to other flies sequenced for*pastrel*gene (*Ore-R*^{JLI}, *w*^{A5001}, *DD1 cnbw* or *Canton-S*). These polymorphisms may correlate with the highest sensitivity of *Ore-R*^{DF} flies to DCV infection and also CrPV infection. Whereas *yw*, *w*^{A5001}, *DD1 Cnbw*, *Canton-S* and *Ore-R*^{JLI} flies display 100% lethality after 12 days of CrPV infection, *Ore-R*^{DF} flies are remarkably more sensitive to CrPV infection, displaying 100%

lethality after 8 days of infection. This discrepancy may be correlated with the polymorphisms of *pastrel* gene that are exclusive to *Ore-R*^{DF} strain (discussed below). It would also suggest that polymorphisms other than the ones identified for DCV may affect CrPV susceptibility. All fly lines tested in our study displayed a similar susceptibility and equivalent viral titre after FHV infection. This observation is in agreement with the GWAS from Prof. Jiggins, which did not find a significant association between the polymorphisms in *pastrel* gene and the susceptibility to FHV infection (Magwire et al., 2012). In our study, we went further in the analysis of the susceptibility of flies to viral infections by using two arboviruses (VSV and SINV) and IIV-6, a DNA virus. Whereas we did not observe a relevant difference in the susceptibility of *Ore-R*^{DF} and *Ore-R*^{JLI} flies to VSV and SINV infections, *Ore-R*^{DF} flies displayed an increased sensitivity to IIV-6 infection and a higher viral titre after 20 days of infection, compared to *Ore-R*^{JLI} flies. Further experiments will be required to decipher if *pastrel* controls IIV-6 infection. Importantly, we excluded that DCV integrated DNA forms may contribute to the resistance of our flies to DCV infection by southern blot with DCV DNA probes, revealing the absence of such DCV fragments in the genome of *w*^{A5001}, *DD1 Cnbw* and *Ore-R*^{JLI} flies. It is reported that mutations can affect the stability of proteins (Tokuriki and Tawfik, 2009). To rule out that polymorphisms may affect the stability of Pastrel protein and therefore be responsible of the difference of susceptibility of flies to DCV infection, we showed that sensitive and resistant flies display similar levels of Pastrel protein.

IV.1.b. Pastrel genotyping is required before conducting experiments with DCV

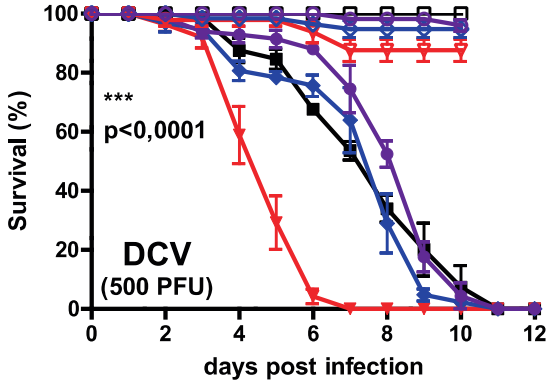
The wide difference in the susceptibility of laboratory control flies to DCV infection was a major concern because these control flies are used as reference in experiments aimed at testing the effect of a *Drosophila* mutant on viral infection. For example, flies mutant for components of the IMD pathway, *imd*^{sdk} and *Tab2*^{glr3}, were highly sensitive to DCV infection but the sensitivity was restored after crossing with lines carrying deficiencies in the chromosomal regions containing these genes, similar to control flies (**Figure 66A** and **66B**, data from Dr. Akira Goto). My study of Pastrel allowed to highlight that the sensitivity of both IMD mutant lines was conferred by sensitive alleles of *pastrel*. Of note, most deficiency lines tested from Bloomington harbored the resistant allele of *pastrel*, enabling complementation of the sensitive allele.

A.



Non infected	Infected	*pastrel* alleles
DD1 cnbw	*DD1 cnbw*	Resistant
Imd^{sdk}	*Imd*^{sdk}	Sensitive
Imd^{sdk}*/Df(2R)PC4*	*Imd*^{sdk}*/Df(2R)PC4*	Sensitive/Resistant
DD1 cnbw/Df(2R)PC4	*DD1 cnbw/Df(2R)PC4*	Resistant

B.



Non infected	Infected	*pastrel* alleles
DD1 cnbw	*DD1 cnbw*	Resistant
Tab2^{glr3}	*Tab2*^{glr3}	Sensitive
Tab2^{glr3}*/Df(2R)Exel6069*	*Tab2*^{glr3}*/Df(2R)Exel6069*	Sensitive/Resistant
DD1 cnbw/Df(2R)Exel6069	*DD1 cnbw/Df(2R)Exel6069*	Resistant

Figure 66. *Pastrel* is responsible of the sensitivity of *imd*^{sdk} and *Tab2*^{glr3} mutant flies to DCV infection. A. and **B.** Flies were crossed at 25°C and experiments were performed at 22°C. Pastrel alleles were genotyped by PCR. Bar graphs represent the average standard deviation of 3 independent experiments (data from Dr. Akira Goto).

Mutant flies for the transcription factor Dif (Dif^{1}), operating in the Toll pathway, are highly sensitive to fungus *Beauveriabassiana* (**Figure 67A**). Additionally, we also observed that Dif^{1} mutant flies display an increased sensitivity to DCV infection and a higher viral titer (**Figure 67A** and **67B**). The sensitivity of Dif^{1} mutant flies, containing sensitive alleles of

pastrel, was partially restored after crossing with deficiency lines harboring resistant alleles of *pastrel*. This indicates that beside the effect of *pastrel*, mutations in Dif affect the resistance of flies to DCV infection. Our observations suggest that Toll pathway may be involved in the control of DCV infection.

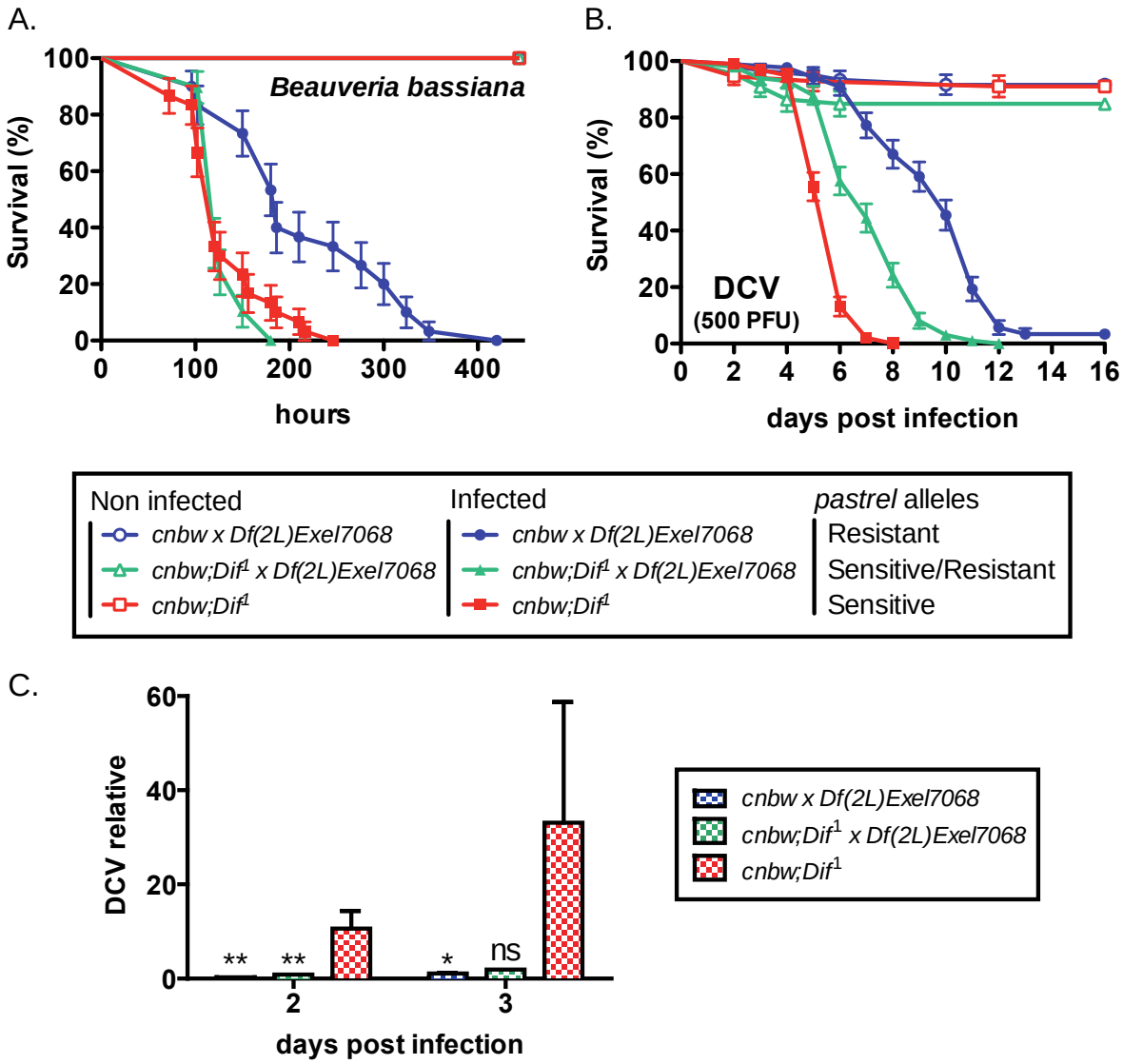


Figure 67. The transcription factor Dif, operating in the Toll pathway, is involved in the control of DCV infection. A. Flies were infected with *Beauveria bassiana* by contact with an overnight culture of spores. **B.** Flies were infected with DCV (500 PFU) and survivals were monitored at 22°C. **C.** DCV RNA levels were measured by qPCR after 2 and 3 days of infection at 22°C. Bar graphs represent the average standard deviation of 2 independent experiments.

Furthermore, we also noticed a strong variability in the susceptibility to DCV and CrPV infection between two *Ore-R* stocks. In an experiment with these viruses, this could be a big issue for the interpretation of the results, which would depend on the *Ore-R* line picked

up. Therefore, it is primordial to genotype the allele of *pastrel* before conducting experiments with DCV to avoid misinterpretations of DCV phenotypes.

IV.1.c. Pastrel gene: duplications and deletions

The amplification of the *pastrel* gene from laboratory control flies with primers for both extremities resulted in a PCR product of 2989 kb, the expected length of *pastrel* gene. However, additional bands of *pastrel* were amplified from *Drosophila* cell lines, Dif mutant flies and a deficiencyline, but also from one line sent by Prof. Jiggins and used in GWAS study. We sequenced the lower band and characterized a *pastrel* truncated version of 1755 kb length. The deletion is extended from position 1072 in exon 6 to position 2306 in exon 2. The upper band was further characterized by Chuan Cao, a PhD student in the group of Prof. Jiggins. They found two other alleles: one with a duplication of full length *pastrel* gene flanked at one extremity by parts of Sec63 and CTCF genes; one with a duplication of *pastrel* gene flanked by a truncated copy of *pastrel* gene. They did not find a significant association between these duplications and the resistance to DCV. However, it is intriguing that such duplications occurred for *pastrel* gene in *Drosophila*. Interestingly, many immune related genes are under positive selection driven by the interactions between hosts and pathogens, and particularly molecules that directly face pathogens such as PRRs (Jiggins and Kim, 2006; Sackton et al., 2007). For example, *Drosophila* has 12 PGRPs genes (Werner et al., 2000), 4 TEPs genes (Lagueux et al., 2000). Gene duplication creates genetic diversity and innovation, leading to specialization of the immune genes. The APOBEC3 family of viral restriction factors is one archetype of an antiviral gene locus that evolved by duplication and specialization to face viruses (Münk et al., 2012). Whether the duplications of *pastrel* gene may result of adaptive evolution in response to viruses remains to be investigated.

IV.1.d. Pastrel gene controls *Dicistroviridae* infection

Loss-of-function experiments

The knockdown of *pastrel* gene in whole flies carrying sensitive alleles increases their sensitivity to DCV and CrPV infection and these flies display a higher viral load compared to control flies. These observations were reproduced in cell culture, where the depletion of

pastrel facilitates DCV and CrPV infection. These data clearly indicate that *pastrel* gene expression controls the infection by picorna-like viruses DCV and CrPV, and that the sensitive allele retains some antiviral activity. Our findings validate the observations of Prof. Jiggins (Magwire et al., 2012) and extend the analysis to another member of *Dicistroviridae* family, CrPV. We pushed further the analysis by knocking down *pastrel* gene expression in aspecific tissue targeted by DCV, the fat body, by using Cg-Gal4 driver. We observed an increased sensitivity of flies to DCV and CrPV infection, correlated with a higher viral titre. These data reveal that the expression of *pastrel* in the fat body controls DCV and CrPV infection. This organ is a key player in the humoral immune response against bacterial and fungal infections, by secreting AMPs into the hemolymph (Lemaitre and Hoffmann, 2007). Moreover, Dcr-2 was reported to drive an inducible antiviral response in the fat body with the expression of Vago that may control DCV infection (Deddouche et al., 2008). However, at present, we cannot rule out that this control of DCV and CrPV infection also relies on hemocytes because Cg-Gal4 driver also affects gene expression in these macrophage-like cells. Accordingly, it has been proposed that hemocytes play a role in the defense against CrPV infection (Costa et al., 2009). Moreover, we have reported in the paper attached to this manuscript that the Jak-STAT pathway specifically contributes to the defense against *Dicistroviridae* (Kemp et al., 2013). We have demonstrated that DCV and CrPV infection triggers the expression of Upd-3, an hemocyte-released cytokine that binds the fat body Domeless receptor to activate the Jak-STAT pathway (Agaisse et al., 2003). Unpaired-3 ligand establishes a connection between hemocytes and the fat body in the antiviral response against *Dicistroviridae*. Additionally, our loss-of-function experiments in *Drosophila* S2 cells, which are hemocyte-derived cells, indicate that the expression of *pastrel* controls the infection by DCV and CrPV. To address this hypothesis, we may perform RNAi experiments with hemolectin-Gal4 driver to assess if the expression of *pastrel* in hemocytes contributes to the defense against *Dicistroviridae in vivo*. The depletion of *pastrel* in the epithelium midgut, a tissue not targeted by DCV, does not affect the susceptibility of flies to DCV infection. This suggests that the restriction is mediated in the cells where DCV can replicate, like the fat body, suggesting a mechanism of cell-autonomous restriction. In agreement with this hypothesis, the presence of cells overexpressing Pastrel mixed with normal S2 cells was not sufficient to protect these cells from DCV infection. Accordingly, prediction software did not find a peptide signal in Pastrel protein and the protein is not detected in the supernatant of *Drosophila* S2 cells (data not shown).

Gain-of-function experiments

Our data reveal that the overexpression of Pastrel fused with RFP in *Drosophila* S2 cells confers protection against DCV and CrPV infection, but not FHV and VSV infection. These findings are in agreement with loss-of-function experiments, and clearly demonstrate that Pastrel protein restricts infection by two picorna-like viruses. Surprisingly, we did not observe differences in the restriction of DCV infection between sensitive and resistant versions of Pastrel protein. This discrepancy with the difference of susceptibility to DCV infection observed *in vivo* between flies expressing sensitive and resistant versions of Pastrel may have different explanations: the overexpression of Pastrel fusions are under the control of Actin promoter, a strong promoter that may mask the difference of antiviral activity between both alleles. Another hypothesis is that the single SNP 3L:7350895Ala/Thr is not sufficient to confer sensitivity or resistance to DCV infection, but one or others SNPs may interfere. In agreement with this hypothesis, the statistical analysis performed to measure the significance of the SNP 3L:7350895 Ala/Thr in the resistance to DCV infection indicates that this SNP is responsible of 47% of the heritability (Magwire et al., 2012). This suggests that other parameters may play a role in the phenotype and remain to be determined. The overexpression of Pst-GFP in flies does not affect their susceptibility to DCV, indicating that the overexpression of Pastrel is not sufficient to reduce DCV-induced fly death. The quantification of viral particles after overexpression of Pastrel remains to be determined but we expect to observe a decrease of the viral titre, as we showed *in vitro*. Indeed, we have preliminary data, not illustrated in this manuscript, revealing that the overexpression of Pastrel in the highly infected pericardial cells restricts DCV infection in these cells. As pericardial cells play a role in the filtration of hemolymph (Das et al., 2008), it remains possible that DCV particles circulating in the hemolymph accumulate rather than replicate in pericardial cells. For this reason, we are now verifying that the overexpression of Pastrel in these cells does not affect their function of filtration. Additionally, we have established, in collaboration with Pr. Jiggins, transgenic flies expressing sensitive and resistant alleles of *pastrel* under the control of HSP promoter. We are now comparing their susceptibility to DCV and CrPV infection.

Loss- and gain-of-function experiments clearly demonstrate that Pastrel restricts infection by picorna-like viruses DCV and CrPV, which reflects the exact feature of a viral

restriction factor. Whereas most viral restriction factors are upregulated after viral infection by interferon in mammals, pastrel seems constitutively expressed, therefore establishing an antiviral state prior infection.Indeed, our data indicate that the expression of *pastrel* is around 1.25 fold upregulated in DCV-infected cells.

IV.1.e. Towards the characterization of Pastrel antiviral activity

Which step of the virus cell cycle is affected by Pastrel?

Our data reveal that the overexpression of Pastrel decreases DCV RNA level in *Drosophila* S2 cells, which subsequently decreases the quantity of viral particles produced and liberated in the supernatant, thereby affecting the progression of the infection. We have dissected steps prior or concomitant to viral replication. We show that Pastrel does not affect IRES-mediated translation, suggesting that DCV replication is not affected by depletion through ineffective translation of viral proteins involved in replication. However, we cannot exclude that Pastrel targets viral proteins for degradation, which is a strategy widely used by viral restriction factors in mammals (see chapter 3 of introduction). Binding assays indicate that Pastrel does not affect the fixation of viral particles onto the surface of *Drosophila* S2 cells, but we observe that Pastrel display an antiviral effect after 2 hours of infection. Our concern is now to decipher if Pastrel affects a step after viral binding but prior replication, such as viral entry and uncoating. A critical point is to know if Pastrel has an antiviral effect prior viral replication or not. To address this question, we measured the apparition of the anti-genome by strand-specific PCR and showed that this hallmark of virus replication appears 2 hours after infection (data from Dr. Laurent Daeffler). Thus, the apparition of the anti-genome correlates with the occurrence of Pastrel-mediated restriction, suggesting that Pastrel may affect viral replication. Further investigations remain to be done to clearly demonstrate that Pastrel affects viral replication, but not viral entry. A key experiment would be to use DCV infectious clone or DCV replicon system to bypass the entry process and investigate the effect of Pastrel on virus replication. A co-staining of Pastrel protein and dsRNAs or immunoelectron microscopy with anti-Pastrel antibody staining in DCV-infected cells may be also informative, to know if Pastrel is present in viral factories.

The antiviral activity of Pastrel is conferred by its C-ter region, exposed in the cytosol

Our data demonstrate that the overexpression of Pastrel truncated in C-terminal (1-562) does not restrict DCV infection, by contrast to the overexpression of the full length protein. In agreement with this observation, the SNP 3L:7350895Ala/Thr, responsible of the sensitivity or resistance of flies to DCV infection, is present in this region (amino acid 598). Our data reveal that the region from amino acid 563 to 682 displays the antiviral activity of Pastrel. We are now investigating if this region 563-682 of Pastrel is sufficient to confer its antiviral activity. Additionally, we want to further dissect this region by looking if other truncated versions of Pastrel (1-595; 1-601; 1-625; 1-652) restrict DCV infection. Additionally, we have developed a new approach to study the topology of a protein and we uncover that the N- and C-terminal extremities of Pastrel protein are exposed in the cytosol, suggesting that the antiviral domain of Pastrel may be accessible to viral particles in the cytoplasm.

Localization of Pastrel protein

Unexpectedly, we observe that Pastrel loses its localization in cytoplasmic aggregates when the region 563-682 is deleted. This region may direct its localization in cells, in addition to confer its antiviral function, suggesting that localization of Pastrel may be also important to exert its antiviral activity. We have observed that some cytoplasmic aggregates of Pastrel colocalize with lipid droplets stained by Nile Red. Accordingly, Pastrel was reported to be present in lipid droplets from *Drosophila* larvae fat body of mutant flies with obesity phenotype (Beller et al., 2006). However, some discrepancies exist: another lipid droplet marker, BODYPI$^{493/503}$ does not colocalize with Pastrel aggregates, but colocalizes with lipid droplets stained by Nile Red. Moreover, we do not observe a clear colocalization of DCV capsid with the surface of lipid droplets whereas DCV clearly colocalizes with Pastrel. It remains possible that Pastrel aggregates that colocalize with lipid droplets are different from the ones that colocalize with DCV. We also observe an enrichment of COP-I vesicles in the areas where Pastrel aggregates, but also DCV capsids, localize. This may be interesting because COP-I complexes are required for the generation of viral factories and thereby DCV replication (Cherry et al., 2006). Unfortunately, we do not have the tools to perform a triple labeling on S2 cells because both anti-beta-COP and anti-Pastrel antibodies where produced in rabbit but we plan to stain cells overexpressing RFP-Pst fusion. We have observed that Pastrel protein clearly colocalizes with capsids from DCV and CrPV, *in vitro* in *Drosophila* S2 cells and *in vivo* in *Drosophila* fat body. Interestingly, we noticed a marked decrease in

size of lipid droplets in highly DCV-infected portions of the fat body, as if lipid droplets were used during DCV infection. Overall, our staining experiments establish a connection between Pastrel protein, COP-I vesicles, lipid droplets and DCV infection but the interplay between these four actors remains to be characterized.

Connection between COP-I and viruses

COP-I complexes are required for DCV replication (Cherry et al., 2006). Additionally, many reports indicate that the GBF1-Arf1-COPI pathway is involved in the replication of poliovirus, coxsackievirus B3, enterovirus 71 (*Picornaviridae*) (Belov et al., 2008; Lanke et al., 2009; Wang et al., 2012a), mouse hepatitis coronavirus (*Coronaviridae*) (Verheije et al., 2008), VSV (*Rhabdoviridae*) (Cureton et al., 2012), and HCV (Goueslain et al., 2010; Tai et al., 2009). COP-I vesicles are also important for IAV infection (Sun et al., 2013a).

Connection between COP-I and lipid droplets

COP-I complexes regulate lipid homeostasis by modifying the protein composition at the surface of lipid droplets (Beller et al., 2008). COP-I promotes the targeting of adipose triglyceride lipase (ATGL) onto lipid droplets in *Drosophila* S2 cells (Beller et al., 2008) and Hela cells (Soni et al., 2009). This enzyme is involved in the first step of triacylglycerols lipolysis. A recent study suggests in an artificial system model that proteins delivery to lipid droplets is mediated by the direct interaction of COP-I with lipid droplets (Thiam et al., 2013).

Connection between lipid droplets metabolism and viruses

Many viruses require lipid synthesis for their efficient replication (reviewed in Chukkapalli et al., 2012). The usurpation of lipid metabolism probably influences the composition of membranes, changing their fluidity and curvatures to allow the formation of replication complexes or viral factories. It is reported that some viruses use lipid droplets for their replication (reviewed in (Camus et al., 2013b; Herker and Ott, 2012)). Dengue virus capsid is targeted onto lipid droplet and allows efficient viral particle formation and Dengue virus infection increases the number of lipid droplets per cell (Samsa et al., 2009). The inhibition of lipid droplet formation with C75, an inhibitor of the fatty acid synthase (FASN), inhibits DENV replication. Another study reveals a depletion of lipid droplets by autophagy process in DENV infected cells, releasing fatty acids used to produce ATP by β-oxydation,

promoting DENV replication (Heaton and Randall, 2010). Lipid droplets are also involved in viroplasms formation and replication of rotaviruses (Cheung et al., 2010), and assembly of infectious HCV particles (Miyanari et al., 2007). Interestingly, a recent paper connects a component of the innate immune response, IKK-α, to the accumulation of lipids and HCV assembly (Li et al., 2013b). The helicase DDX3X recognizes the 3'UTR region of HCV and activates the inhibitor of nuclear factor-κB kinase α (IKK-α) which is translocated in the nucleus and through CBP/p300 factor, induces the transcription of SREBP. This non-canonical pathway is independent of NF-κB. SREBP is a transcriptional factor that upregulates lipogenic genes expression (Horton et al., 2002), thereby enhancing lipid droplet formation and facilitating HCV assembly. The depletion of IKK-α increases lipid storage and inhibits HCV assembly whereas its overexpression increases the number of lipid droplets and HCV infection. (For review, (Camus and Ott, 2013; Felmlee and Baumert, 2013)). Interestingly, the viral restriction factor Viperin localizes on lipid droplets (Hinson and Cresswell, 2009a) and affects the replication of HCV (Wang et al., 2012b). Viperin is also proposed to regulate lipid metabolism during HCMV infection (Seo and Cresswell, 2013). In *Drosophila*, it is reported that the knockdown of HLH106, the ortholog of mammalian SREBP, leads to a 38.2-fold decrease of DCV replication (Cherry et al., 2006). Accordingly, the knockdown of CG3523, the ortholog of mammalian FASN, decreases DCV replication of a factor of 10.6-fold, indicating that fatty acid synthesis is required for DCV replication.

Overall, it is tempting to speculate that some viruses may require COP-I for their replication because of its function in lipid droplets metabolism. COP-I-mediated lipolysis may provide a source of lipids for the formation of viral factories. Whether Pastrel may affect COP-I-mediated lipolysis by its presence on lipid droplets in *Drosophila* S2 cells or wheter Pastrel could inhibit COP-I activity is one of our hypothesis for its antiviral function (**Figure 68**).

The analysis of Pastrel protein (CG8588) is not informative to find its antiviral function

The gene CG8588 was reported to be involved in memory (Dubnau et al., 2003) and it was named Pastrel in reference to Pavlov's dogs. Pavlov performed behavioral experiments with dogs to understand learning and memory processes. We obtained flies mutant for *pastrel* gene used by Dubnau and colleagues but we did not confirm that *pastrel* gene was inactivated. Thus, the involvement of *pastrel* in memory remains questionable. Pastrel was

reported to be involved in protein secretion but we did not reproduce these observations (Bard et al., 2006). Whereas the antiviral activity of many viral restriction factors was guessed by looking at their domain homology with other proteins, the alignment of *pastrel* gene with sequences databases is not informative. Pastrel protein sequence has homologies with proteins CG7730 and CG32243 but their function is unknown. Pastrel has orthologs in insects like other *Drosophila* species, and with less homology, in mosquitoes (*Anopheles* species, *Aedesaegypti* and *Culex quinquefasciatus*), in a pea aphid. A related gene seems present in nematode *C. elegans* but *pastrel* is not conserved in vertebrates. Pastrel protein has no conserved domains or motifs. A unique protein, CG8368, was found to interact with Pastrel in a yeast two-hybrid system (Giot et al., 2003). CG8368 also interacts with Arf79F, the ortholog of mammalian Arf1, involved in the formation of COP-I vesicles (Spang, 2002). CG8368 protein displays a motif of 3'-5' exonuclease activity and a nucleic acid binding motif. Interestingly, the preliminary analysis of small RNA libraries from DCV-infected flies reveals that many v-siRNAs are not 21-nts length, indicating that they do not result from Dicer-2 processing (communication from Dr. Simona PARO). Whether Pastrel may recruit CG8368 to degrade DCV RNAs is another hypothesis that we want to address **(Figure 68)**. The small RNA profiling of sensitive and resistant flies infected by DCV is under progress. If our hypothesis is correct, we expect that sensitive flies display less degradation of DCV RNAs than resistant flies. Nora virus small RNAs of other size classes than 21-nts were observed by Van Mierlo and colleagues and they predominantly derive from the genome (van Mierlo et al., 2012). Their origin remains unclear and they propose that they result from non-specific degradation. To fit with our hypothesis, Pastrel should therefore affect Nora virus infection.

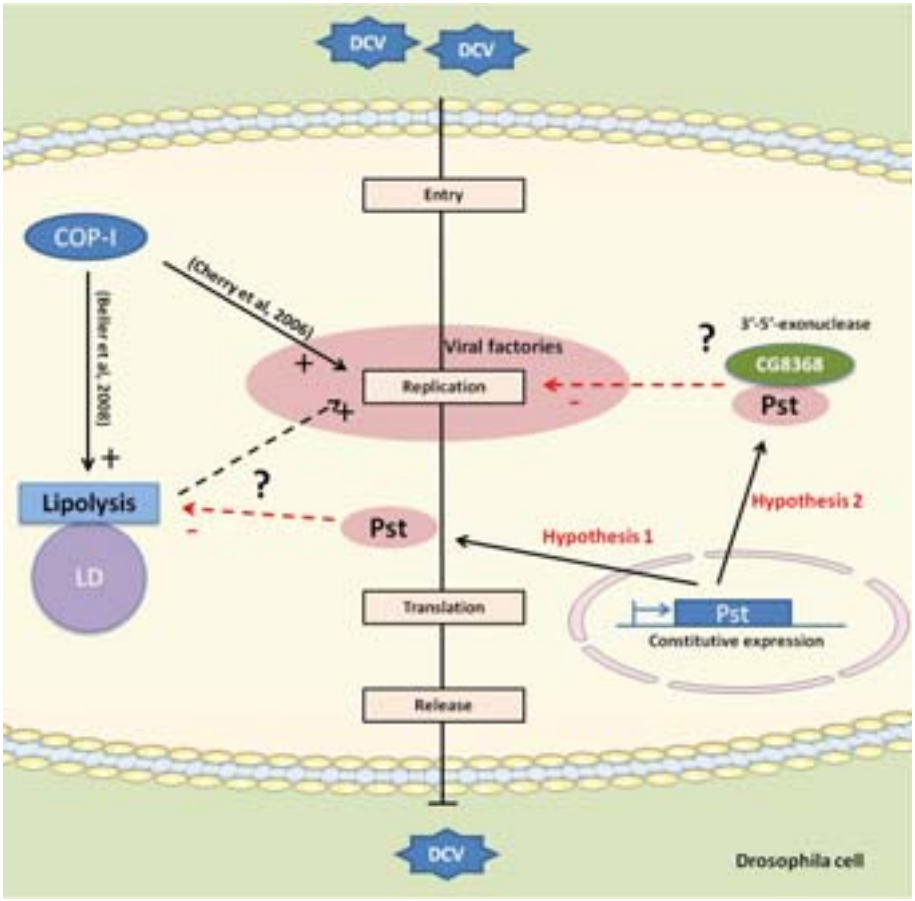


Figure 68. Speculative model for the antiviral action of Pst on DCV replication. Hypothesis 1: Our data indicate that DCV affects the size of lipid droplets in the fat body, suggesting that lipolysis is activated during infection. Additionnaly, COP-I was shown to modulate lipolysis and may be required for the formation of viral factories for DCV replication. As we found that Pst colocalized with Nile Red staining in Drosophila S2 cells and was also enriched in regions stained with anti-beta-COP antibody, we hypothesize that Pastrel may target and inhibit COP-I-mediated lipolysis. **Hypothesis 2:** Pst was shown to interact with a putative 3'-5'-exonuclease (CG8368) by yeast-two-hybrid, and may recruit this enzyme to direct the degradation of viral RNAs.

IV.2. Does Pastrel affect Nora virus infection?

We have observed a correlation between the sensitivity of flies to DCV infection and the presence of persistent Nora virus (except for *DD1 Cnbw*flies). Our results clearly demonstrate that Nora virus does not contribute to the increased sensitivity of flies to DCV infection. Indeed, *Ore-R*^{DF} flies cured from Nora virus infection remain highly sensitive to DCV infection whereas *Ore-R*^{JLI} flies contaminated by Nora virus are still resistant to DCV infection. By contrast, we got promising data for an effect of Nora virus on the pathogenicity of bacterial infections in collaboration with the team of Dr. Dominique Ferrandon. Once we

observed that *pastrel* alleles mainly account for the susceptibility of flies to DCV infection, we wondered if *pastrel* may affect Nora virus persistent infection. The mechanisms controlling a persistent virus infection are a topic of high investigations. It is still unclear how *Drosophila* controls Nora virus persistent infection. The group of Dr. Hultmak claimed that RNAi, Toll and Jak-Stat pathways do not control Nora virus infection (Habayeb et al., 2009a) whereas the group of Dr. Van Rij observed Nora virus siRNAs and defined VP1 as a VSR (van Mierlo et al., 2012). Our data reveal that *Ore-R*^{JLI} flies display lower Nora virus RNA level than *Ore-R*^{DF} flies after contamination by feces, indicating that flies with sensitive allele of *pastrel* are easier infected by Nora virus. In addition to DCV and CrPV, our observations strongly suggest that Pastrel may control the infection by another picorna-like virus, Nora virus. At present, working with Nora virus presents some limitations because the infection is asymptomatic, highly variable between individuals and we do not have a cell culture model *in vitro*. For example, the commonly used *Drosophila* S2 cells are restrictive to Nora virus infection. However, the identification of Nora virus-derived siRNAs in OSS cells suggests that these cells can be infected by Nora virus (Wu et al., 2010). In agreement with our hypothesis, the expression of *pastrel* is 5.9-fold lower in OSS cells than S2 cells (data from modENCODE project). We plan to investigate if these cells could replicate Nora virus. We aim to test if the overexpression of *pastrel* controls Nora virus infection.

IV.3. The siRNA pathway controls IIV-6 infection

Our data demonstrate that the RNAi pathway is a broad antiviral defense against RNA viruses but also a DNA virus. Dicer-2 null mutant flies display an increased sensitivity to IIV-6 infection compared to control flies and the susceptibility is restored with a Dicer-2 genomic rescue. Accordingly, R2D2 and Ago-2 mutant flies are more sensitive to IIV-6 infection. The deep sequencing of small RNAs from *Drosophila* S2 cells and flies infected by IIV-6 revealed the presence of v-siRNAs matching with the sequence of IIV-6 genome. V-siRNAs are predominantly 21-nts length, indicating that they result from Dicer-2 processing. Interestingly, v-siRNAs are not uniformly distributed along the viral genome, as observed for RNA viruses (Mueller et al., 2010), but they are produced from specific regions of the viral genome. These regions match with regions of convergent transcription which may lead to the formation of dsRNAs, substrates of Dicer-2. In agreement with our findings, the group of Dr. Van Rij made similar observations (Bronkhorst et al., 2012).

Concluding remarks

This work identified and characterized new aspects of the antiviral immunity in *Drosophila*. The role of the siRNA pathway in the defense against RNA viruses was extended to a DNA virus, emphasizing its broad contribution to the antiviral defense of *Drosophila* (Kemp et al., 2013). Additionally, the work presented in this thesis reveals the role of a new antiviral protein, Pastrel, as an important actor for the control of picorna-like viruses in *Drosophila*. We have also promising expectations for a role of Pastrel in the control of persistent Nora virus infection. Loss- and gain-of-function experiments support the classification of Pastrel protein as a viral restriction factor, establishing an antiviral state in prevention of viral infection. This new viral restriction factor identified in *Drosophila* may shed light towards the understanding of intrinsic antiviral immunity in insects.We believe that new viral restriction factors will be identified in insects. Our demonstration of the importance to genotype the allele of *pastrel* before conducting *in vivo* experiments will surely impact past and future studies with DCV. The mechanism of Pastrel-mediated restriction is under investigation in our laboratory. As DCV and CrPV share similarities with *Picornaviridae*, we plan to investigate if the expression of Pastrel in mammalian cells may affect picornaviruses infection. To address this question, we have already constructed vectors expressing sensitive and resistant versions of Pastrel under the control of CMV promoter.

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Vincent BARBIER

Pastrel, a restriction factor for picorna-like viruses in *Drosophila melanogaster*

Résumé

La drosophile est un excellent modèle pour l'étude des mécanismes moléculaires de l'immunité innée, y compris les virus. Elle a permis la caractérisation de mécanismes de défense immunitaire conservés au cours de l'évolution, tel que les voies Toll et IMD qui régulent l'expression des peptides antimicrobiens induits en réponse aux infections fongiques et bactériennes. Deux types de réponse sont impliqués dans le contrôle des infections virales chez la drosophile : une réponse inductible et l'ARN interférence. Nous avons montré que l'ARN interférence est un mécanisme global de défense antivirale puisqu'il contrôle l'infection par un virus à ADN, en plus des virus à ARN tel que le virus C de la drosophile (DCV). Le virus DCV, apparenté aux *Picornaviridae*, est un pathogène naturel de la drosophile. Nous avons également observé que la résistance de mouches contrôles à l'infection par le virus DCV est dépendante du fond génétique. Elle est d'ailleurs corrélée à des polymorphismes présents dans un gène porté par le chromosome III : le gène *pastrel*. Nos expériences de perte et gain de fonction indiquent que ce gène code pour un facteur de restriction viral, bloquant l'infection par le virus DCV mais aussi par le virus de la paralysie du cricket (CrPV). Cette restriction apparait dans les premières heures après infection. La région C-terminale de la protéine Pastrel est nécessaire à son activité antivirale ainsi qu'à sa localisation dans les cellules. La protéine Pastrel co-localise avec le Rouge de Nil, un marqueur des gouttelettes lipidiques. Ainsi, nos résultats suggèrent un lien entre le métabolisme lipidique et le blocage d'une infection virale chez la drosophile.

Mots-clés : *Drosophila melanogaster*, défense antivirale, immunité innée, immunité intrinsèque, facteur de restriction, virus C de la drosophile, virus de la paralysie du cricket, virus Nora.

Abstract

Since the discovery of the evolutionarily conserved TOLL and IMD pathways, involved in antifungal and anti-bacterial immune responses, the fruit fly *Drosophila melanogaster* is used as a model to study the molecular mechanisms of innate immunity. To defend against viral pathogens, *Drosophila* relies on two main facets: the RNA interference (RNAi) pathway and virus specific inducible responses. We show that the RNAi pathway plays a role in the control of a DNA virus, in addition to RNA viruses. We also observe that the fly genetic background can modulate the resistance to infection by *Drosophila* C virus (DCV), a natural pathogen of *Drosophila*. This resistance to DCV infection is correlated with polymorphisms in a gene named *pastrel*, localized on the left arm of the third chromosome. Our loss- and gain-of-function experiments indicate that *pastrel* encodes a molecule opposing infection by picorna-like viruses DCV and also Cricket Paralysis virus (CrPV), raising the question of the mechanism involved. This restriction appears early after infection. The C-terminal region of Pastrel protein is important for its antiviral activity and its localization in vesicular structures co-localizing with Nile Red staining, a marker for lipid droplets. Altogether, our data suggest a connection between lipid droplets and restriction of viral infection in *Drosophila*, as already described in mammals between the restriction factor Viperin, present on lipid droplets, and the replication of the human pathogen Hepatitis C Virus.

Keywords: *Drosophila melanogaster*, antiviral defense, innate immunity, intrinsic immunity, restriction factor, *Drosophila* C virus, Cricket Paralysis virus, Nora virus.