Drosophila CG4572 protein and the spread of the RNAi antiviral immune signal

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UPMC SORBONNE UNIVERSITÉS

European Research Council
Established by the European Commission

THÈSE DE DOCTORAT DE L'UNIVERSITÉ PIERRE ET MARIE CURIE

Doctoral School: Life Science Complexity

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by

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Defended on September 23, 2015

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ACKNOWLEDGMENTS

First I would like to thank Carla who gave me the opportunity to join her team and perform my PhD in the best conditions. Thank you for your trust, advice and help. Thank you also for the careful correction of this manuscript.

Bertsy, thank you for accepting to supervise my work during this PhD. You taught me a lot about science and independency.

To the members of my jury, I would like to thank you for the time you spent on reading, commenting and judging my work.

To the entire lab, I would like to say thanks for the everyday help. I asked you so many questions and you have been so patient with me… thank you all very much for that.

I would like to express my deepest gratitude to Vanesa, first for her friendship in and out of the lab, then for her support, help, and advice all along this PhD.

Juan you have been very helpful and patient with me. I will miss you and your crazy animal's noise.

Lorena, thank you for your advice, for your smile and your courage that is exemplary.

For the three of you, thank you also for the introduction to the South American mate preparation! I really enjoyed sharing those moments with you.

Marine you allowed me to not be the youngest one in the lab anymore. Thank you for the support and shared time. I am with the «mamies» now.

Valerie thank you for the way you are managing the lab.

Hervé you are truly the calmest person in the world. Thank you for the help.

Lionel thank you for your precious help and comments.

To the three of you, thanks for the daily help and your support.

Brigitte, thank you for all the administrative help and for taking such good care of us.
Guys, thanks for the help, the lunches, the beers, and the discussions. After all this time that we spent together, it is hard to believe that it is almost over. I am going to miss you all very much.

I also would like to thank all the people that have been of great help during those 4 years. I would like to thanks PVP lab for their support and for being so friendly. During the ESPCI collaboration, Yann, Iman and Joelle, you made the time I spent in your lab very pleasant. Thanks to the entire Imagopole, especially Audrey, Pascal and Jean-Yves. At the Ultrapole, I would like to thank Gérard for his work and Jacomina for her precious commentaries on the electron microscopy work. I would like also to thank Helene Quach (for her cakes and help) and Nora, Mariano Dellarole for his advice, and Siham Yennek for her support and friendship along our respective PhD.
I learned a lot with you all.

Les pépites, thank you so much for all the fun, the holidays, the party, the laugh… it helped me a lot.
Mam, Dad, Zou and Bro-coli, thank you for being so supportive and present every time I needed it.
Guillaume thank you for your love, presence, your support day and night and your olympian calm.
Without all of you, this PhD would have been so much harder.

What is now proved was once only imagined.
William Blake

ABSTRACT

During a viral infection, cell survival will depend on adequately giving, receiving and processing information to establish an efficient antiviral immune response. Cell-to-cell communication is therefore essential to allow the propagation of immune signals that will confer protection to the entire organism. That is true for unicellular organisms as much as for specialized cells in multicellular organisms.

The major antiviral defense in insects is the RNA interference (RNAi) mechanism that is activated by detection of viral double-stranded RNA (dsRNA). The antiviral RNAi mechanism can be divided into cell-autonomous and non-cell autonomous. In cell-autonomous RNAi, the silencing process is limited to the cell in which the viral dsRNA is produced. In non-cell-autonomous RNAi, or systemic RNAi, the interfering effect occurs in cells different from where the viral dsRNA was produced. However, whereas cell-autonomous RNAi in insects is well described, the systemic RNAi response remains poorly characterized.

My PhD research explores the role of the *Drosophila* CG4572/DORA protein in the establishment of systemic antiviral RNAi. It also investigates the nature of the immune signals that will trigger the antiviral response in *Drosophila melanogaster*. I provide evidence for the existence of two different (but compatible) mechanisms of cell-cell communication that allow the spread of the immune signal: extracellular vesicles and tunneling nanotubes. I describe for the first time that DORA-positive extracellular vesicles in *Drosophila* carry fragments of viral RNAs that can spread and confer specific antiviral protection in flies. I also present the first characterization of tunneling nanotubes (TNTs) containing components of the RNAi machinery, DORA and dsRNA and I hypothesize on the use of TNTs in the spread of the immune signal.

Both mechanisms of cell-to-cell communication are coupled for the first time to the antiviral response in *Drosophila melanogaster*.

RÉSUMÉ

Au cours d'une infection virale, la survie des cellules dépend d'informations adéquatement distribuées, reçues et traitées, permettant l'établissement d'une réponse antivirale performante. La communication cellulaire est donc essentielle pour permettre la propagation de signaux immuns protecteurs à tout l'organisme. Cela s'avère vrai tant pour un organisme unicellulaire que pour les cellules spécialisées d'organismes multicellulaires.

Chez les insectes, la principale réponse antivirale est l'ARN interférent (ARNi), activé lors de la détection d'ARN double brin (ARNdb) d'origine virale. Le mécanisme antiviral de l'ARNi peut être divisé en deux catégories : l'ARNi cellulaire et l'ARNi systémique. Dans la première catégorie, la régulation de l'expression génique est limitée à la cellule dans laquelle l'ARNdb est produit, alors que dans la seconde, cette même régulation s'effectue dans des cellules distinctes de celles produisant l'ARNdb. Et bien que l'ARNi cellulaire chez les insectes soit maintenant bien caractérisé, l'ARNi systémique reste très peu décrit.

Mon travail de thèse explore le rôle de la protéine de drosophile CG4572, que j'ai nommé DORA, dans les mécanismes permettant l'établissement de l'ARNi systémique. J'ai également recherché la nature des signaux immuns déclencheurs de cette réponse antivirale. Dans ce manuscrit, nous démontrons l'existence de deux mécanismes différents (bien que compatibles) de communication cellulaire permettant la propagation de signaux antiviraux: des vésicules extracellulaires ainsi que des nanotubes membranaires. Nous mettons en évidence pour la première fois chez la drosophile que des vésicules contenant DORA et des fragments d'ARN viraux peuvent se propager dans les mouches en leur conférant une protection antivirale spécifique. Nous montrons également pour la première fois la présence de nanotubes membranaires qui contiennent des protéines de la machinerie ARNi ainsi que DORA et de l'ARNdb. Cette

découverte nous permet de proposer un mécanisme par lequel la propagation du signal antiviral se ferait par l'intermédiaire de ces nanotubes.

Les deux mécanismes de communication cellulaire que nous proposons sont pour la première fois associés à la réponse antivirale chez *Drosophila melanogaster*.

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CHAPTER 1

GENERAL INTRODUCTION

Adapted from:

RNAi and antiviral defense in *Drosophila*: Setting up a systemic immune response

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Developmental and Comparative Immunology 42 (2014) 85-92

The striking evidence that organelles, such as mitochondria and chloroplasts, are of bacterial origin illustrates that host-microbe interactions have existed for as long as these organisms have been in close physical contact. That such extreme case of co-evolution have occurred in nature, attest to the ongoing push and pull that hosts and microorganisms exert on one another[1,2].

The outcome of host–microorganism interactions can be either beneficial or detrimental to the microorganism, to the host (in this case the microorganism is know as pathogen), or to both the microorganism and the host[3]. Host–pathogen interactions can be pictured as an arms race between two adversaries. On one hand, the pathogen deploys virulence factors to exploit the resources of the host. On the other hand, the host fights back with immune responses to clear the pathogen or at least minimize its deleterious effects.

Over the course of their lifetime, insects interact with a wide variety of microbes. As a consequence, they have developed a fine-tuned immune system shaped over these complex host-microbe interactions. As any other organism, insects are subjected to infection by viruses. Among these viruses, some of them are solely insects viruses, some are transmitted from insect to plants, with strong impact in agronomy, for example by affecting crop production[4]. Some others, called arthropod-borne viruses (arboviruses) have the particularity of alternating between hematophagic invertebrate and vertebrate hosts. Several insects are

responsible for arbovirus transmission to human or cattle, including the mosquitoes *Aedes spp*. (e.g. Rift Valley fever virus, chikungunya virus, dengue virus, yellow fever virus), and *Culex spp*. (e.g. Rift Valley fever virus, Japanese encephalitis virus, West Nile virus). Importantly, arboviral infections are asymptomatic in insects but responsible for severe incapacitating diseases in mammalian hosts, suggesting a co-evolutionary process between insects and viruses.

One of the key factor that modulates whether an insect is competent or not to transmit a given pathogen is its immune response. Therefore, in view of the re-emerging and extending threat of arboviruses worldwide, understanding how the infection is controlled within the insect before crossover to the human host becomes essential to generate new strategies to disrupt pathogen transmission.

1. THE FRUIT FLY AS INSECT MODEL

At first due to the short generation time, the cost-effectiveness and the ease of rearing, and later due to the availability of genetic tools, the safety of use compared to hematophagous insects, and more recently, the availability of the complete genome sequence, the fruit fly *Drosophila melanogaster* has been the most extensively used insect model since the beginning of the 20th century[5-7]. As a result, *Drosophila* has become a powerful tool to work in several fields, including genetics, development, neuroscience and immunity. Impressively, two third of all human disease genes have homologues in *Drosophila*[8], which makes of the fruit fly an appropriate alternative to vertebrate models when characterizing biological processes.

The major breakthrough in immunology that can be attributed to *Drosophila* is the identification of the Toll pathway[9-11]. This pioneered the discovery of the Toll-like receptors (TLR) in mammals[12] and the subsequent understanding of the mechanisms that govern innate immunity.

Regarding insect-virus interactions, it is estimated that 40% of all fruit fly are infected with viruses[13]. Moreover, *Drosophila* can be the natural or experimental

host for a wide variety of viral pathogens (Box 1 and Box 2) and, as a consequence, a very powerful insect model to explore insect-virus interactions. Much of what is currently known about defense mechanisms in insects result from work with fruit flies.

Box 1

Models of infection: natural viral pathogens of *Drosophila*

DCV

Drosophila C Virus (DCV) belongs to the Dicistroviridae family. Its size is about 30 nm, non-enveloped, with a positive single-stranded RNA (ssRNA) genome of approximately 9,300 nucleotides (nts). The genome has two open-reading frames (ORFs) and one of the proteins encoded is a suppressor of RNAi named 1A[14]. DCV 1A binds double-stranded RNA (dsRNA) molecules preventing the action of Dicer-2 and the cleavage of dsRNA into siRNAs. When Dicistroviridae virus enters the cell, the viral genome is released into the cytoplasm, where its replication occurs in viral factories. After assembly, new virions are released from cells, potentially by lysis.

DXV

Drosophila X Virus (DXV) belongs to the Birnaviridae family with a viral particle of a size of 70 nm. It is a non-enveloped virus with a bipartite dsRNA genome. It encodes for a suppressor of RNAi, the Vp3 protein[15] that binds long and short dsRNA. Birnaviridae viruses transcribe and replicate their dsRNA genome into the viral particle in order to protect the dsRNA molecule from cellular immune sensors and from the ribonuclease Dicer-2. Once virions are produced, they are released from the cells by budding.

(source: viralzone.expasy.org)

Box 1.

2. GENERALITIES ABOUT DEFENSE MECHANISMS

The defense of higher eukaryotes against pathogens is organized into different layers. First, there is a non-specific host defense: a physical barrier, which is the skin in mammals and the cuticle for insects. The epithelium of the intestinal tract is also lined by a chitinous membrane that prevents direct contact between cells and microbes. In the gut, which constitutes the main route of

infection, the secretion of digestive enzymes, a low pH and the production of reactive oxygen species maintain a hostile environment to microbial survival[13-15].

Second, there is innate immunity, which acts coordinately at the cellular and systemic level (like antimicrobial peptides that are secreted in the hemolymph of the fly)[13,16].

The third layer is the adaptive immune response, which is present only in jawed vertebrates. Some of the most interesting characteristics of this adaptive immunity are the boosting or amplification of the immune response, as well as the immune memory, which enhances the ability of the organism to respond to future related infections[17-19]. However, insects lack an adaptive immune system and rely almost entirely on the innate immune response for defense. For instance, flies are able to trigger various defense pathways depending on the type of infecting pathogen, and most of these pathways are inter-connected.

2.1. Antimicrobial immune response in insects

Once the physical barriers are breached, the immunity relies on cellular and humoral response. The cellular immune response involves plasmatocytes (macrophages cells) that will engulf microbes by phagocytosis[20].

On the other hand, the humoral immune response relies on the production of inducible antimicrobial peptides (AMPs)[13]. This production is regulated by two NF-kB-like pathways that are differentially activated. The Toll pathway is activated upon yeast, fungal and Gram-positive infections and induces the production of specific AMPs such as Drosomycin[10,21]. The Imd pathway is activated upon Gram-negative bacteria infection and induces the production of AMPs such as Dyptericin[22,23].

Box 2

Models of infection: non-natural viral pathogens of *Drosophila*

FHV

Flock House Virus (FHV) belongs to the Nodaviridae family, with a non-enveloped viral particle which size is around 30 nm. The genome is composed of two segment of positive ssRNA of approximately 3,100- and 1,400- nts. After penetrating the host cell, nodaviridae viruses release their RNA genome into the cytoplasm. RNA1 codes for the RNA-dependent RNA polymerase, as well as a subgenomic RNA (RNA3) that codes for a viral suppressor of RNAi, the protein B2[26]. This protein binds dsRNA as well as small interfering RNAs (siRNAs)[27], and during infection can represent up to 5% of the total cellular protein content. Viral replication takes place into cytoplasmic viral factories[28]. After assembly, new viral particles are release, potentially by cell lysis.

SinV

Sindbis virus (SinV) is an arbovirus that belongs to the togaviridae family of the genus alphavirus. The viral particle is enveloped, with a size of approximately 70 nm and contains a positive ssRNA genome of 11.7 kb. The virus enters the cell through the endocytic clathrin dependent pathway[29], and when fusing with endosomes, release viral RNA in the cytoplasm. Togaviridae viruses replicate at the surface of endosomes in viral factories. After transcription and replication, new viral particles will assemble and exit the cells through the cell secretory pathway.

CrPV

Cricket Paralysis Virus (CrPV) is a Dicistroviridae virus of 27 nm with a positive ssRNA genome. This non-enveloped virus encodes for a viral suppressor of RNAi, the protein 1A[30]. CrPV 1A binds Ago-2 protein and prevents its slicing activity. As described for DCV, the replication occurs in viral factories and new virions are released by cell lysis.

VSV

Vesicular Stomatitis Virus (VSV) is an arbovirus that belongs to the Rhabdoviridae family. The viral particle is enveloped, with a size of about 70 nm and the genome is a negative ssRNA of 11.5 kb. Rhabdoviridae viruses are believed to enter cells via the endocytic clathrin dependent pathway. When the envelop of the virus fuse with the endosome membrane, the ribonucleocapsid is released in the cytoplasm. After transcription and replication, new virions will bud at the plasma membrane.

(source: viralzone.expasy.org)

Box 2.

2.2. Antiviral immune response in insects

The core antiviral response of *Drosophila* is distinct from its antimicrobial counterpart. It involves different components that can be divided into two classes depending on the mechanisms by which they are elicited: RNA interference pathway (see below, section 3) and JAK/STAT pathway. Originally studied for its involvement in development, the JAK/STAT pathway is composed of the receptor Domeless, the Janus Kinase (JAK) Hopscotch, and the STAT transcription factor, and was originally thought to be involved in antimicrobial response and response to cell damages[24-26]. However JAK/STAT deficient flies are not sensitive to fungi or bacterial infection, but are sensitive to DCV infection[25]. Together with Toll and Imd, the JAK/STAT pathway plays a role in viral infections, but their antiviral function seems to be virus-specific rather than being a general antiviral response (for examples see[25,27,28]).

Antiviral defense in insects relies mostly on another pathway of innate immunity: the RNA interference (RNAi) response[29-32].

3. RNA INTERFERENCE

RNAi is a conserved sequence-specific, gene-silencing mechanism that is induced by double-stranded RNA (dsRNA). Several RNAi-related pathways[30,33-38] have been described in many organisms and they have diverse functions, including the modulation of mRNA translation[39], establishment of chromosomal architecture[40], regulation of stem cell renewal[41] and defense against viruses and mobile genetic elements[42].

In general terms, RNAi pathways involve the production of small non-coding RNAs, and their biogenesis and function is based on two proteins: Dicer (Dcr) and Argonaute (Ago). The *Dicer* and *Ago* genes are strongly conserved in wide-ranging species including plants, invertebrates and mammals. Nevertheless, as a result of evolutionary and immune adaptation processes, there are several

paralogues of both proteins[43]. As of today, four main RNAi-related pathways have been described and they can be classified into two major groups on the basis of the origin of the small noncoding RNAs: the ‘‘endogenous’’ group, which involves small RNAs encoded within the cell and the ‘‘exogenous’’ group, which involves small RNAs not encoded by the cell.

The “endogenous” RNA group gathers (1) micro-RNAs (miRNAs) that actively regulate cellular gene expression[44]; (2) endogenous small interfering RNAs (endo-siRNAs) that regulate transposable elements[34,45]; and (3) PIWI-interacting RNAs (piRNAs) that are involved in epigenetic and post-transcriptional silencing of transposons[46-48].

The “exogenous” RNA group is only composed of small-interfering RNAs (siRNA) that are produced from virus-derived dsRNAs or non-cellular RNAs that generates dsRNA structures. The siRNA pathway was first identified as an antiviral mechanism in plants[49] and in the nematode *C. elegans*[50,51]. The link between RNAi and antiviral defense in *Drosophila* was established when Ago-2 depletion led to the accumulation of FHV[52]. Since then, several studies confirmed that the siRNA pathway was indeed the major antiviral response pathway in insects: flies deficient for Dcr-2 or Ago-2 are unable to control virus replication and as a consequence are hypersensitive to infection[29-32,53,54].

4. THE siRNA PATHWAY: THE INSECTS ANTIVIRAL DEFENSE

Long viral dsRNA molecules trigger the antiviral siRNA pathway[55]. Those viral dsRNA molecules are produced in cells that are infected with diverse types of virus (see Box 1 and Box 2): *(i)* viruses with dsRNA genomes, such as Drosophila X virus (DXV)[56]; *(ii)* viruses with DNA genomes that contain convergent transcript units, for example Invertebrate Iridescent virus (IIV6)[27,53]; and *(iii)* viruses with single-stranded RNA genomes that produce dsRNA as the result of the formation of secondary structures, such as Sindbis virus[57,58] or vesicular stomatitis virus[59]; and/or replication intermediates as for Drosophila C virus (DCV) or Semliki Forest virus[60] (Fig. 1).

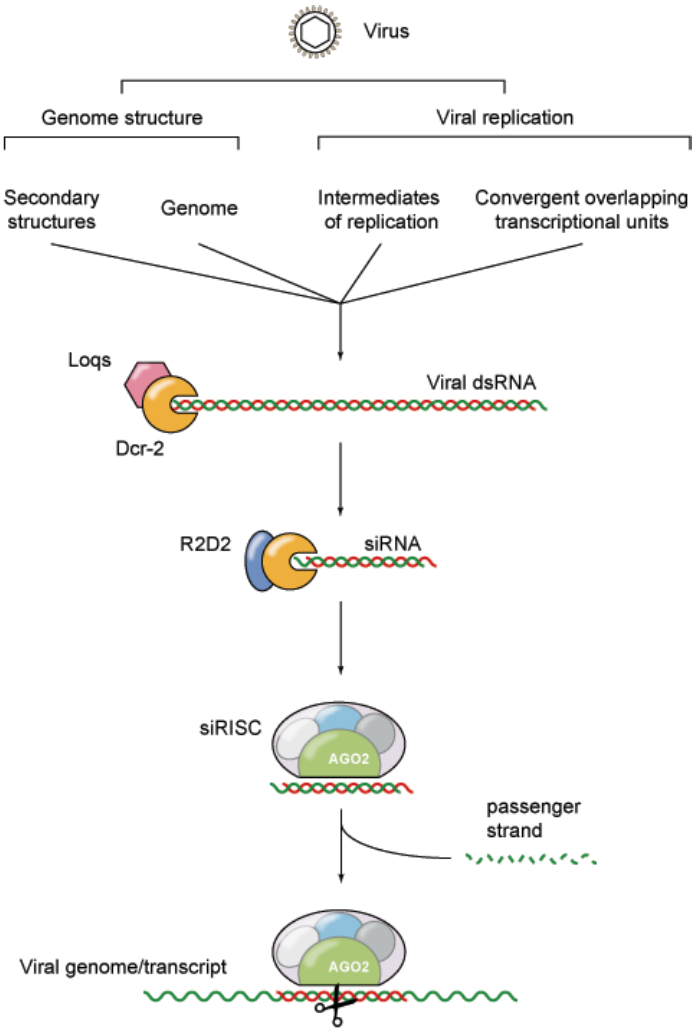

Figure 1. Cell autonomous siRNA antiviral response. Long viral dsRNA, produced during viral infection is diced by Dcr-2 with its cofactor Loqs into viral siRNAs. These siRNAs are loaded in the RISC complex where the passenger strand is removed. The guide strand in Ago-2/RISC acts as a template for RISC to recognize complementary RNA (viral genome or viral transcripts). Once found, Ago-2 cleaves the target RNA downregulating viral replication.

Viral dsRNA molecules are cleaved (or 'diced') by a ribonuclease III enzyme, Dcr-2[61] in association with its cofactor R2D2[62], into viral siRNAs of 21 nt long[63]. These viral siRNAs are loaded into the RISC (RNA-induced silencing complex) complex, where the siRNA duplex is unwound and the strand with the less stable 3'-terminus, the passenger strand, is removed. The remaining viral siRNA strand, the guide strand, is retained in Ago-2, which is the catalytic effector of RISC[64,65]. The loaded viral siRNA can bind a viral RNA (genome or transcript) by sequence complementarity leading to specific degradation of the targeted RNA mediated by

Ago-2 slicing activity[66] (Fig. 1). The complementarity of the siRNA and its target is thus the basis of the specificity of the RNAi machinery.

Recently, some reports suggested the involvement of other RNAi pathway in the control of viral infections. Indeed, piRNAs from viral origin have been detected by deep-sequencing during infections of mosquitoes with arboviruses, including dengue[67], Sindbis[68], chikungunya[69] and LaCrosse virus[70]. Hess and colleagues[67] described an *in vivo* assay using mosquitoes and dengue virus, and detected a peak in the accumulation of piRNAs at 2 days post infection. The amounts of these piRNAs then decreased during the infection, whereas siRNA production increased. This suggests that the RNAi-Dcr-2-dependent pathway is active during viral infection, but is preceded by the piRNA response. These observations lead to the notion that the piRNA pathway may initiate the antiviral process during a viral infection in mosquitoes. Schnettler and colleagues[71] provided the first functional demonstration that viral piRNAs do indeed contribute to antiviral defenses in mosquito cells infected with Semliki Forest virus, and very recently, Miesen and colleagues[72] described the production of viral piRNAs in *Aedes aegypti* mosquito cells. It is important to note that, in *Drosophila*, siRNAs accumulate during viral infection independently from piRNA production.

5. SYSTEMIC ANTIVIRAL RNAi

An important characteristic of immune systems is their ability to act both at the immediate site of infection, as well as at distal uninfected locations. Accordingly, the antiviral RNAi mechanism can be divided into cell-autonomous (at the cellular level) and non-cell autonomous (at the systemic level). In cell-autonomous RNAi, the silencing process is limited to the cell in which the dsRNA is introduced or expressed. In non-cell-autonomous RNAi, the interfering effect occurs in cells different from where the dsRNA was produced. However, whereas cell-autonomous RNAi in insects is well described, the systemic RNAi response remains poorly characterized.

5.1. Systemic RNAi in arthropods

Several lines of evidence implied the existence of a systemic component to the siRNA pathway in arthropods. Early in 2002 it was shown that dsRNA injection into the haemocoel of adult *Tribolium castaneum* (floor beetle) resulted in knockdown of zygotic genes, which was also manifested in offspring embryos, implying transfer across cell membranes[73]. In 2005, Robalino and colleagues showed that the injection of viral sequence-specific dsRNA confers potent antiviral immunity *in vivo* in the shrimp *Litopenaeus vannamei*[74]. Accordingly, endogenous shrimp genes could be silenced in a systemic fashion by the administration of cognate long dsRNA. A systemic component to antiviral RNAi was shown in mosquito cells infected with Semliki Forest virus[75]. Even if the exact mechanism is not known, the authors showed cell-to-cell spread of viral-derived siRNA, and possible long dsRNA, with concomitant inhibition of replication of the incoming viruses in cells neighboring infected cells. Also, we were able to show that in *Drosophila*, intra-thoracic injection of viral sequence-specific long dsRNA into uninfected flies conferred immunity against subsequent infection with the corresponding virus. Furthermore, infection with Sindbis virus expressing the green fluorescent protein (GFP) suppressed expression of host-encoded GFP at a distal site of infection[76]. The laboratory therefore proposed a model (Fig. 2) where cells that are lysed due to the cytopathic effect of viruses, release viral dsRNA. Through the hemolymph, the dsRNA may be distributed throughout the body and at proximal locations may be taken up into non-infected cells via specialized mechanisms. There, the dsRNA is processed into siRNAs that are loaded into RISC, programming this machinery to rapidly cleave incoming genomic viral RNA. Thus the systemic RNAi may set up a specific antiviral state that control spread of a viral infection.

In the next section I will briefly discuss general aspects of the antiviral RNAi response in organisms other than arthropods.

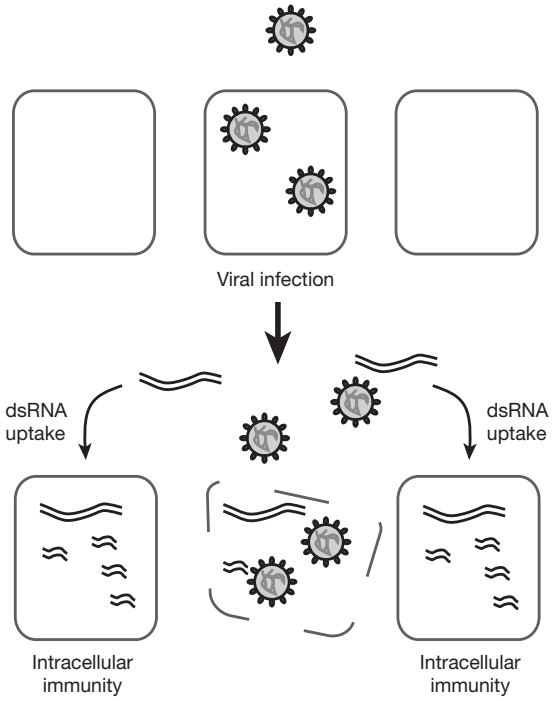

Figure 2. Model for systemic RNAi antiviral immunity in *Drosophila melanogaser*. Upon viral infection, virus-specific dsRNAs (for example, replication intermediates) are generated during the initial rounds of virus replication. After cell death or lysis, dsRNAs are taken up and processed by uninfected cells to protect them from subsequent infection, thereby preventing virus spread.
From MC Saleh *et al*. *Nature* 2009.

5.2. RNAi-mediated antiviral immunity in other organisms

5.2.1. In plants: *Arabidopsis thaliana*

In higher plants, the antiviral mechanism is based on the siRNA pathway, and involves the same factors than in *Drosophila*. Therefore, a deep look at the antiviral response in the plant model is of great help to do parallels in *Drosophila*. The *Dicer* gene family in *A. thaliana* has four members[77], the Dicer-like (DCL) proteins. DCL2-4 process long dsRNA into siRNA of 22, 24 and 21 nt respectively[78]. A functional redundancy in antiviral immunity capability of the four DCL proteins was shown as individual mutations of the proteins did not generated a change in viral susceptibility[78]. From the 10 AGO proteins that are encoded by *A. thaliana*, Ago-1 immunoprecipitates with viral siRNAs in infected tissues[79].

Beyond the difference on the number of Dicer and Ago proteins for plants and flies, plants are also able to mount a systemic antiviral response[80,81]. Indeed, most of the cells are connected through plasmodesmata[82], a structure composed of desmotubules that allow a highly regulated transport in between cells with a size exclusion limit of 1,000 Da[82,83]. Most plants viruses code for movement protein and manipulate plasmodesmata to allow large viral particle to pass between cells[84] and infect the entire plant through cell-to-cell spreading. However plasmodesmata also allow cell-to-cell spreading of the antiviral signal conferring systemic protection. For short-range movement (10-15 cells) the signal moving is siRNA produced from dsRNA in the infected cell. It involves DCL4 as well as SMD1-3 (silencing movement deficient) proteins[85]. The long-range movement is a succession of the short-range, with an additional step, the amplification of the signal.

In plants, an amplification mechanism of the immune signal (also called transitivity) occurs. This mechanism involves an enzyme called RNA-dependent RNA polymerase (RdRP)[86-88]. For the six RdRp identified in *A. thaliana*, RDR-1, 2 and 6 have confirmed roles in RNA silencing pathways. RDR6 produces *de novo* dsRNA synthesis from siRNAs, which are diced by DCL4 in secondary siRNAs. Those secondary siRNAs will therefore spread 10-15 cells around[89] limiting the infection as it spreads.

5.2.2. In worms: *C. elegans*

RNAi as an antiviral mechanism in the nematode was shown in 2005 by two papers using non-natural models of infection: the mammalian pathogen Vesicular Stomatitis virus[51] and the insect virus Flock House virus[50] (see box 1 and box 2). In both cases, virus infection was potentiated in RNAi-defective worms and inhibited in worms with an enhanced RNAi response.

A systemic component of RNAi is also present in *C. elegans*. It involves three elements: *(i)* SID proteins: SID-1, a transmembrane channel allowing dsRNA uptake[90,91] and SID-2 that allows active transport of environmental dsRNA from the intestinal lumen into cells[92]; *(ii) Fed* (feeding defective for RNAi) genes

products that are involved in dissemination of the RNA silencing signals; and *(iii)* Rsd (RNAi spreading defective) protein which mutant are defective for systemic RNAi[93,94]. Amplification (or transitivity) is also a characteristic of the RNAi response in *C. elegans*. It involves Ego-1 protein that acts as a RdRp[95,96]. It has been shown that worms were able to transmit the immune signal to their progeny, with the help of the RNAi defective 4 protein (rde-4)[97].

5.2.3. In mammals: the mouse case

In 2001, it was demonstrated that RNAi was functional in mammalian cells[63] but it was assumed that it has little or no role in vertebrate antiviral immunity[98] mostly due to the very potent interferon (IFN) response. However in 2013, two studies demonstrated the antiviral role of RNAi in mammals. Studies were performed in mice stem cells that do not produce IFN, infected with encephalomyocarditis virus (EMCV) or with Nodamura virus (NoV)[99], in hamster cells and in 7-days-old mice infected with NoV[100]. By high throughput sequencing, and in absence of the viral suppressor of RNAi B2, the authors identified viral siRNAs with a characteristic 2-nt 3' overhangs and a phasing of 22 nts, signature of Dicer processing. Furthermore, when Dicer was depleted in the stem cells, the viral small RNAs were lost. However, as none of these studies showed increased viral titers on cells deficient on RNAi or the product of virus genome cleavage by the RNAi machinery, the existence of antiviral RNAi in mammals remains highly controversial in the RNAi community[101,102].

After this brief overview of RNAi-mediated systemic antiviral immunity in other organisms, a common characteristic arose: the control of viral infections requires signaling molecules to elicit an effective response and to establish systemic immunity at the organism level. These signals must be amplified and disseminated throughout the organism to avoid pathogen propagation and establishment of the infection. Sometimes, the progeny inherits the immune signal. In *Drosophila*, although it has been postulated that there is a systemic antiviral response, neither the signal, nor the amplification mechanism or the mechanism of its dissemination, have been described.

6. SETTING-UP A SYSTEMIC ANTIVIRAL RNAi IN *DROSOPHILA MELANOGASTER*

It is known that *Drosophila* cells are able to take up viral dsRNA that will trigger a specific RNA silencing response[103]. Presumably, following dsRNA uptake, an immune signal is sent by infected cells to prevent viral infection in distant non-infected cells[75,76]. Non-infected cells have to be able to ‘‘catch’’ or to ‘‘sense’’ this signal and to internalize it in order to be primed.

6.1. Uptake of the immune signal

Viral dsRNA and naked siRNA are large and charged molecules, and therefore there must be appropriate receptors if they have to enter into the cells. In *C. elegans* as already described, there are two receptors for dsRNA molecules, SID-1 and SID-2. These receptors participate in the internalization of dsRNA, which can then lead to the spread of the RNAi. Other SID proteins that participate in dsRNA transport have been described, like SID-5, which promotes the transport of the silencing signal between cells[104], or SID-3, which is needed for an efficient import of dsRNA into cells[105]. Interestingly, the extent to which RNAi spreads is coupled to the amount of dsRNA produced within cells or imported from the environment[106]. However, although dsRNA is able to enter cells in flies, it is not clear how. No receptors with a dsRNA-binding domain have been found in *Drosophila*. It appears that two Scavenger receptors, called SR-CI and Eater, are associated with dsRNA uptake[107] but further studies addressing the role of these receptors are needed.

It is also possible that dsRNA is not the signal that triggers systemic immunity, in which case, there must be another molecule. Studies in cell culture using *Drosophila* S2 cells found that free siRNAs added to the extracellular media were not taken up by the cells, and did not result in silencing of a reporter gene[107,108]. These results are consistent with the notion that the signal of

systemic immunity may be a long dsRNA, a ribonucleoprotein (RNP), or another RNA complex.

It has been shown that the endocytic clathrin dependent mechanism is involved in the uptake of dsRNA and the trigger of the antiviral RNAi response in *Drosophila*[107,108]. Below, I will briefly revisit the clathrin-mediated endocytosis pathway.

6.1.1. Clathrin-mediated endocytosis

Eukaryotic cells can take up extracellular material by a variety of different mechanisms such as phagocytosis, macropinocytosis, clathrin-dependent endocytosis, caveolin dependent endocytosis, and clathrin- and caveolin-independent pathways. Those mechanisms respond to necessary functions from maintenance of cell polarity to uptake of extracellular nutrients[109]. Endocytosis mediated by clathrin (Fig. 3) corresponds to the internalization of cargos in vesicles coated with clathrin[110]. In this pathway, when cargos have been taken up, they are sorted in early endosomes, and either sent back to the plasma membrane in recycling endosomes or targeted to more mature endosomes such as lysosomes and/or multivesicular bodies (MVBs). In this manuscript, we will further refer to MVBs as an intermediate between late endosomes and lysosomes.

The fusion of the outer membrane of MVBs with the lysosomal membrane results in delivery of the luminal MVBs vesicles to the lysosome where they are degraded together with their content[111]. The sorting of cargos into MVBs has been linked to ubiquitin[112], however not all MVBs cargos are ubiquitylated[113]. The outer membrane of MVBs can also fuse with the plasma membrane and release vesicles in the extracellular space[114,115]. Those vesicles are mainly named exosomes but also microvesicles[116]. In this manuscript I will further referred to both type of vesicles under the term “extracellular vesicles” (EVs).

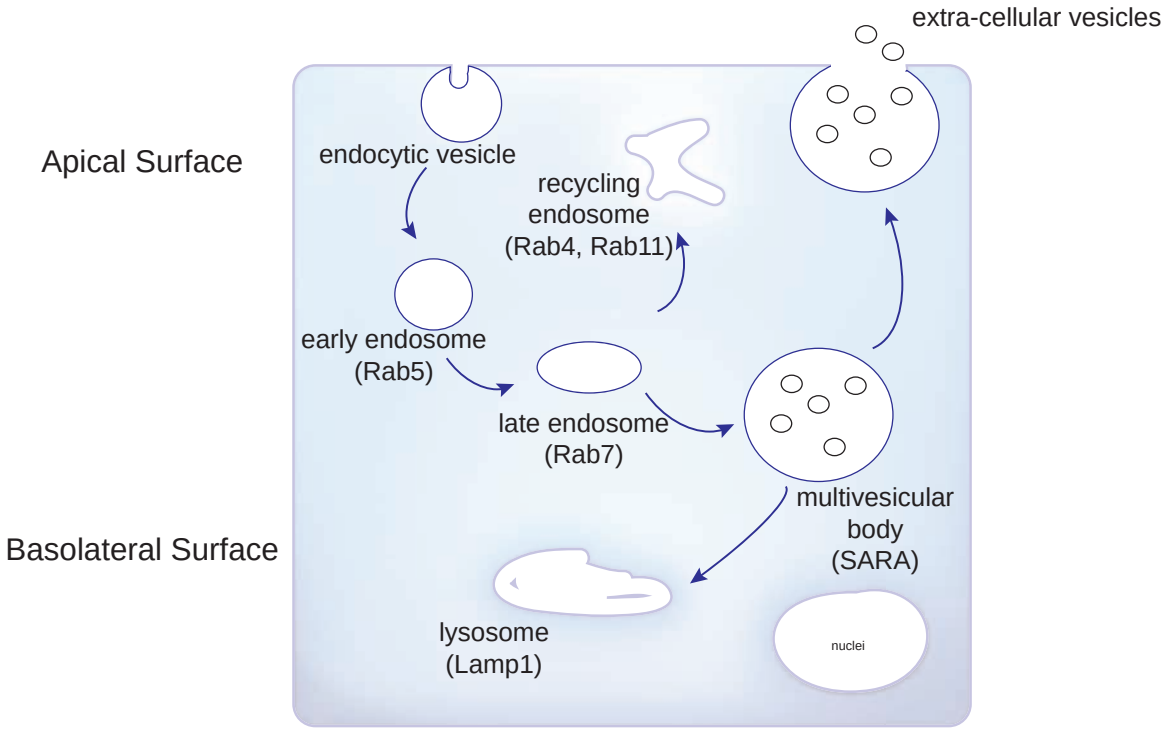

Figure 3. Clathrin mediated endocytosis. Markers for each vesicle are indicated in between brackets.

6.2. Sorting of the immune signal

Assuming the RNA nature of the signal, insights into the transport of other RNA species, for example miRNA or mRNA, may be informative about the sorting of the immune signal in flies. In murine and human cells, naked mRNA has been shown to associate with early endosomes (Rab5-positive vesicles) after endocytosis probably mediated by a Scavenger receptor. However, this entry route is not exclusive for mRNA and other negatively charged molecules, including all small RNAs and dsRNA, could potentially use it. After internalization, the mRNA was found to traffic into lysosomes where it accumulated and was then degraded by ribonucleases; however, an important proportion of the RNA could escape to the cytoplasm where they can be expressed[117] (Fig. 4).

Many RNAs are addressed to specific subcellular compartments by *(i)* a signal that they carry in their sequence or by structural motifs (cis-acting elements or localizer signals) and/or by *(ii)* associated proteins (trans-acting factors)[118]. The

cis-acting elements provide binding sites for the trans-acting factors. However, small RNAs, which may be no more than a few dozen of nucleotides long, are unlikely to encode such localizer sequences.

Therefore, the effectiveness of the silencing process not only depends on internalization of the signal; the signal must also be delivered to the appropriate site to allow the production of siRNA based sequence-specific protection.

6.3. Spread of the immune signal

During a viral infection, the immune signal needs to be shared throughout the organism to elicit a systemic antiviral response. As such, it should be possible to find dsRNA or siRNA at locations distant from the infection site. One plausible explanation for systemic spread in *Drosophila* is the lysis of the infected cells. However, this would not explain the protection observed in organisms infected with viruses that do not produce a cytopathic effect. Therefore, there must be an active process to share and alert the neighboring and distant non-infected cells to allow a specific antiviral protection mediated by RNAi.

Unlike plants, fruit flies lack plasmodesmata connecting the cells, but uses cell-to-cell communication through a plethora of structures and mechanisms. Below I will review four of them: *(i)* extracellular vesicles, *(ii)* tunneling nanotubes, *(iii)* free-protein complexes, and *(iv)* cell junctions.

6.3.1. Extracellular vesicles

As mentioned in section 6.1.1, MVBs cargos are either sorted for degradation into lysosomes or secreted as extracellular vesicles into fluids. Interestingly, MVBs have been found to be closely involved with the miRNA and the siRNA pathways[119]. In mammals, for example, Epstein-Barr virus encodes its own miRNAs that are released within exosomes with immunomodulatory properties[120].

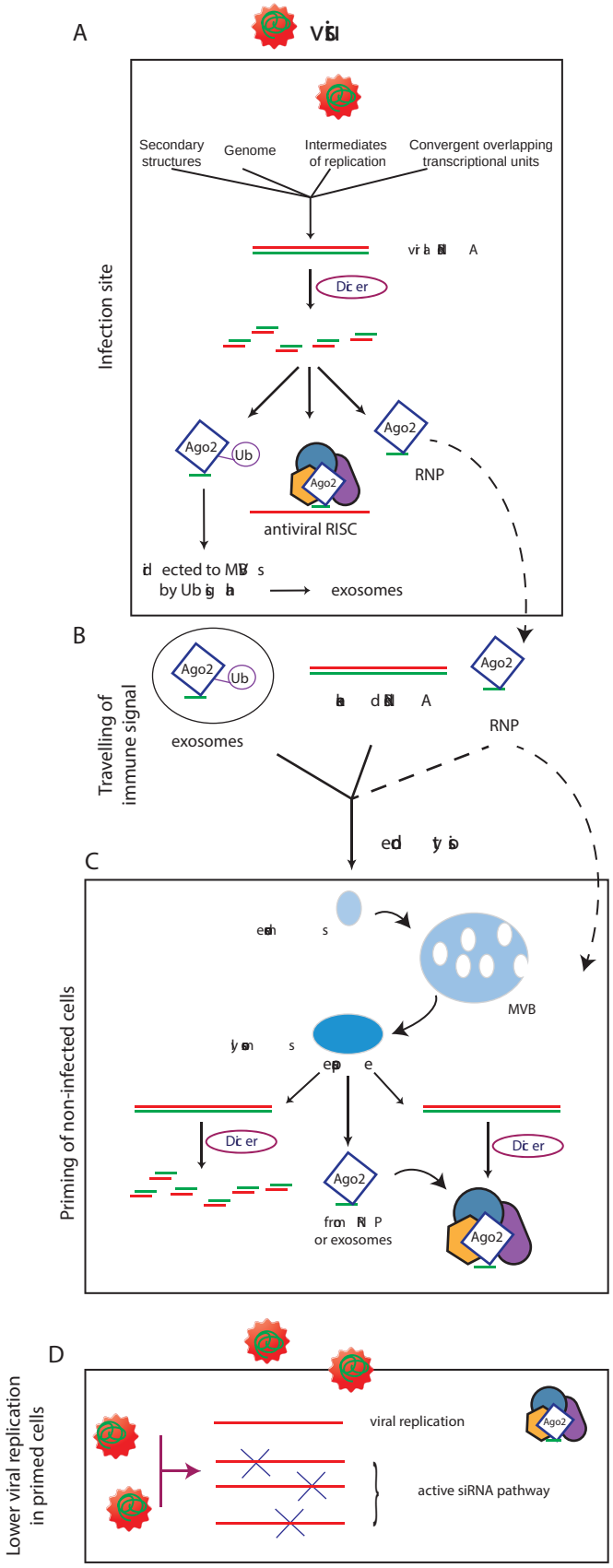

Figure 4. Hypothetical model for internalization, sorting and transmission of the RNAi-immune signal during viral infection. At the site of infection (A), the presence of dsRNA activates the antiviral response at the cellular level and sets up the immune signal for a systemic response. This signal could spread through the organism by (B) exosomes containing siRNAs or an RNP or long dsRNA from viral replication. At distant sites of infection (C) the immune signal is potentially endocytosed, followed by release from lysosomes. Long viral dsRNAs are diced and siRNAs loaded into RISC or in free RNP. Alternatively, some naked dsRNA can be diced and siRNAs directed to the RNAi machinery where active silencing control future infections (D). Adapted from Karlikow *et al.* 2013.

Several pathways for targeting proteins into MVBs have been described in flies. Monoubiquitinated proteins are sorted from the endosomes to the outer membranes of MVBs bound to the ESCRT complex (Endosomal Sorting Complex Required for Transport)[121]. It is therefore possible that dsRNA and/or siRNA could be addressed towards MVBs following their inclusion in RNPs subject to ubiquitination (Fig. 4A-B).

Exosomes can also carry mRNA and miRNA[122,123] and both endogenous and exogenous proteins, including toxins[124]. Therefore, it has been suggested that exosomes may be responsible for exchange of material between cells. Exosomes are found in many different fluids, including blood, breast milk, amniotic fluid and malignant ascites[125-127]. It is tempting to speculate that in *Drosophila* extracellular vesicles may travel through the hemolymph carrying and propagating the immune signal (Fig. 4B). There is increasing evidence that exosomes in mammals can be taken up by other cells following recognition by receptors on the plasma membrane[128,129] and that they are carriers for many and diverse cargos depending on their cell origin. The uptake of free exosomes is currently the subject of lively debate. It has been suggested that after release from a cell, exosomes are endocytosed and targeted, along the cytoskeleton, to lysosomes[130]. Other authors suggest that exosomes can be imported into cells by phagocytosis[131] or by fusion[132]. It is possible that exosomes fuse with the endocytic compartment after endocytosis and during acidification they release their content in lysosomes. However, a large part of their contents escape and these escaped contents can include proteins, such as Ago-2[133], and molecules such as siRNA and dsRNA. Silencing by small RNA is linked to endosomal trafficking[119] and it has been demonstrated that exosomes are involved in the immune system[134-138]. In mammals, for example, exosomes act as immunological mediators associated with tumor growth by exosome-mediated miRNA transfer[139,140] or by exosome-mediated antigen presentation[141]. In flies, exosome vesicles, first called argosomes, are responsible for a graded distribution of morphogens, such as Wingless[142]. Several studies show the presence of extracellular vesicles in *Drosophila* involved in development[143,144] (Fig. 4C-D).

6.3.2. Tunneling nanotubes

Tunneling nanotubes (TNTs) were first described in rat neuro-derived cells[145]. They are tubular structures, made of actin filaments that are not attached to the substratum (and therefore are not filipodia[146] or cytonemes[147]) and allow direct communication between cells *via* cytoplasmic connection. Conversely, filopodia and cytonemes are thin structures acting as bridges to connect cells (mechanism of adhesion without cytoplasmic connections) and therefore, cargos move along the structure and not inside[148].

Since their first discovery, TNTs have been found in a wide variety of mammalian cells where they act for example as route for transport of cytosolic and membrane-bound molecules, organelles, and pathogens such as HIV[149,150]; in bacteria cells where they serve for exchange of molecules[151]; and in *Drosophila* where they participate in developmental processes[147,152-155].

6.3.3. Free ribonucleoprotein-complexes

EVs and TNTs are not the only way that RNA uses to circulate. For example, small RNAs, like miRNAs have been described in body fluids as free ribonucleoprotein (RNP) complexes[156-158]. Moreover, Arroyo and colleagues[159], and Turchinovich and colleagues[160] showed that in mammals, miRNA contained in exosomes constitutes only a minority of the circulating miRNA and the bulk of miRNA is found in the plasma as RNP complexes associated with Ago protein. In addition to those finding, a very recent quantitative and stoichiometric analysis of the miRNA content of exosomes by Chevillet and colleagues[161] suggested that it was unlikely that exosomes act as vehicles for miRNA based cell-cell communication due to the very low amount of miRNA *per* exosomes.

Embedding small RNA in an RNP has several advantages as mechanism of dissemination: it may improve RNA stability and resistance to environmental damage or degradation such as extracellular RNases and it is in a ‘‘ready-state’’ to regulate gene expression in recipient cells. Additionally, proteins of the RNP could allow the entry into a specific cell. A question under consideration is whether the level of extracellular small RNAs are a mirror of their intracellular

abundances, in other words does secretion occurs in a non-selective manner, or is secretion an active process[162].

6.3.4. Cell junctions

Adherens and tight junctions were first described by Farquhar and colleague in 1963[163] and provide important adhesive contacts between cells. Tight junctions have two vital functions, the barrier function and the fence function (Fig. 5). The barrier function regulates the movement of ions and solutes in-between cells through intercellular spaces[164]. Therefore, molecules have to enter the cells by diffusion or by active transport in order to pass through the tissue. The fence function maintains cell polarity by allowing the specific functions of each cell surface in such a way that prevents molecules at one side of the cells to migrate to another side[165]. For example, the apical surface of cells is involved in receptor-mediated endocytosis and the basolateral surface is involved in exocytosis. Both surfaces have specific function, and the fence prevents the loss of this cellular polarity. Interestingly, some pathogenic bacteria and some viruses target tight junctions, leading to diseases (review in[165]). Tight junctions have also been involved in other functions, as the regulation of signal transduction[166] and the immune system[167-169].

Gap junctions are another type of junction that link cells together and allow direct communication of neighboring cells[170,171] *via* proteins channels that connect both cytoplasms (review[172]). They are reminiscent of plasmodesmata in plants. It has been described that viruses can interact with those structures to transmit toxic signals through them or to disrupt them[173,174]. Valiunas *et al*.[175] described the transfer of siRNAs through gap junctions in mammals.

Each type of cell-cell communication, besides their known physiological role, could therefore be potentially involved in the systemic antiviral immune responses, by the transfer of the immune signal and/or the pathogen.

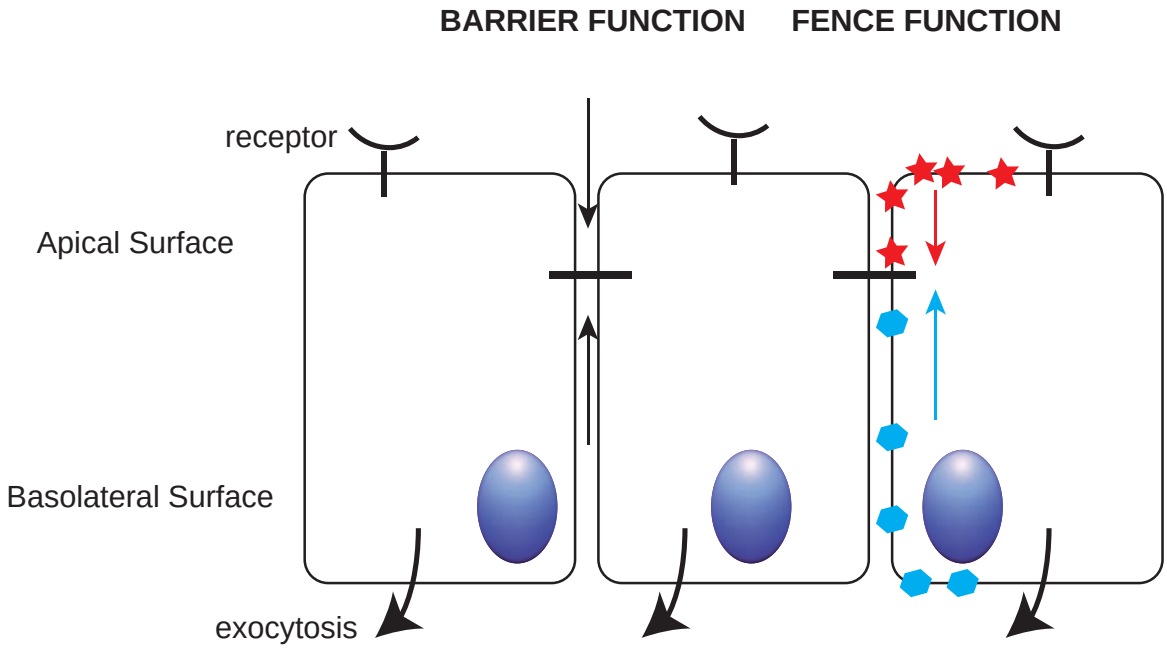

Figure 5. Tight junctions with two major roles: barrier and fence function. Cells on the left use the barrier function (black solid line between cells), which regulates the movement of ions and solutes through intercellular spaces. The cell on the right uses the fence function (black solid line between cells), to keep cellular polarity by impeding molecules (blue or red) to migrate to a different location.

Adapted from Sawada *et al*.

7. *DROSOPHILA* CG4572 PROTEIN

A genome-wide screen performed by Saleh and colleagues[108] for components of the dsRNA-transport machinery, indicated that dsRNA is taken up by an active process involving clathrin-mediated endocytosis and relies on the integrity of vesicle trafficking and protein sorting to elicit an efficient RNAi response. In this same screen, genes with unknown functions were also identified. One of these genes was CG4572.

CG4572 is a putative serine-carboxypeptidase with a catalytic triad specific to those proteins: S217, D403 and H460[176]. This domain is well evolutionary conserved and present in several proteins in species ranging from plants[177], yeast (carboxypeptidase Y), mosquitos[176], to humans (cathepsin A, also known as protective protein). Moreover, this protein carries a signal peptide (amino acids VEG-ER, where – corresponds to the cleavage site) for translocation into the

endoplasmic reticulum, where it may be subjected to several N-glycosylations. Interestingly, carboxypeptidase S in yeast is delivered from Golgi to endosomes and to MVBs[112]. In plants, several serine carboxypeptidases are predicted to be secreted[178]. In human, the vitelogenic-like protein, also a serine carboxypeptidase, localizes to exosomes[179].

Further studies from Saleh *et al*[76] demonstrated that cells depleted for CG4572 failed at silencing a reporter gene following exogenous dsRNA soaking (Fig 6). However, when the natural entry pathway (soaking) was bypassed by transfection of dsRNA, cells depleted for CG4572 were fully able to perform silencing. In addition, flies deficient for CG4572 were hypersensitive to DCV infection and died even more rapidly than Ago-2 mutant flies, suggesting a role of CG4572 in the RNAi-mediated antiviral response.

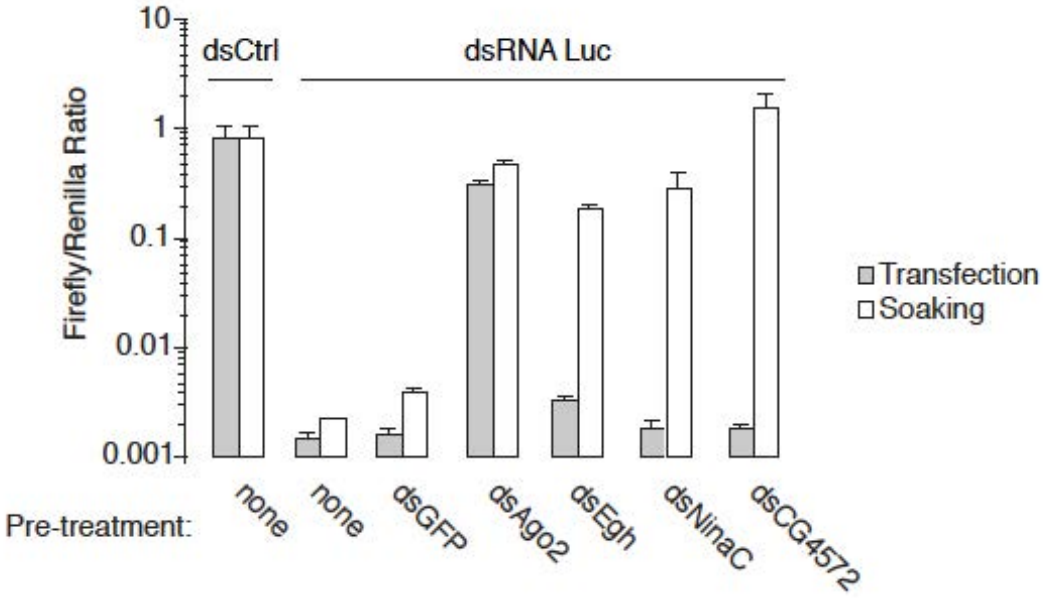

Figure 6. CG4572 is involved in RNAi transport. Silencing of luciferase expression in *Drosophila* S2 cells depleted in dsRNA uptake genes (*egh*, *NinaC* and *CG4572*) after exposure to luciferase dsRNA by either transfection (filled bars) or by soaking dsRNA in the culture supernatant (empty bars). dsGFP: dsRNA directed against GFP, used as a negative control. dsAgo2: Ago2 dsRNA control for the core RNAi machinery depletion. Ctrl (-): untreated control.
MC Saleh *et al*, *Nature* 2009.

AIM OF THE STUDY

In the context of the study of the antiviral RNAi mechanism and the establishment of a systemic RNAi response in *Drosophila melanogaster*, my PhD focused on answering a central question:

How is CG4572 protein involved in the *Drosophila* antiviral response?

Starting from the previous observation on CG4572, this protein could be involved in: *(i)* the uptake of exogenous dsRNA into the cell, *(ii)* the intracellular transport of dsRNA to the RNAi core machinery, *(iii)* the spread of the antiviral signal. As CG4572 does not present a transmembrane domain or a dsRNA binding domain, we discard a role as potential dsRNA receptor and favour a role in the transport of dsRNA or the RNAi signal.

Through a combination of cell biology, biochemistry, high-resolution imaging, mass spectrometry and high throughput sequencing approaches I tackled the characterization of this protein and its role.

Fours years into the adventure, I demonstrated that CG4572 is essential for the spread of the RNAi antiviral immune signal through extracellular vesicles. In the first part of the results, I describe how extracellular vesicles carrying CG4572 and fragments of viral RNAs can spread and confer protection against further viral infection in flies. In the second part, I dig further on the analyses of high throughput sequencing of small RNAs in extracellular vesicles. In the third and last part of the results, I identify tunneling nanotubes containing CG4572 and core components of the RNAi machinery, and hypothesized on the use of those TNT in the spread of the immune signal. Both different but compatible mechanisms of cell-cell communication are coupled, for the first time, to the establishment of systemic antiviral immunity in *Drosophila*.

CHAPTER 2

RESULTS

CHAPTER 2

PART 1

Drosophila DORA protein is essential for systemic spread of antiviral RNAi immunity through extracellular vesicles containing viral RNAs

Drosophila DORA protein is essential for systemic spread of antiviral RNAi immunity through extracellular vesicles containing viral RNAs.

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Key words

systemic immunity, RNAi spread, extracellular vesicles, viral RNA, *Drosophila*.

Abstract

An important feature of immune responses is their ability to act both at the site of infection and at distal uninfected sites. In insects, RNA interference (RNAi) mediates the antiviral immune response *via* a cell-autonomous and a systemic response. The latter remains poorly characterized. In this study we show that DORA- a *Drosophila* protein identified in a genome-wide screen for components of the dsRNA-transport machinery- interacts with core components of the RNAi machinery and is involved in systemic RNAi. Flies deficient for DORA are hypersensitive to viral infection and fail to co-suppress endogenous GFP when infecting GFP-deficient flies with a Sindbis-GFP virus. Furthermore, we demonstrate that the systemic RNAi response is mediated by extracellular vesicles carrying fragments of viral RNA, in a DORA-dependent manner. We therefore propose *Drosophila* DORA protein as the first required component for the establishment of a systemic antiviral response in invertebrates.

Introduction

Following the discovery of the RNA interference (RNAi) pathway[1] and its role in antiviral defense in plants[2] and invertebrates[3-8], it was demonstrated that the antiviral RNAi response was not only cell-autonomous, acting locally at the site of infection, but also systemic, conferring immunity at distal sites[9]. Indeed, the spread of a specific silencing signal plays a critical role in antiviral defense by initiating a systemic RNAi response in the entire organism[10]. This protects uninfected cells from infection and thus confines the deleterious effects of viral infection to a limited number of cells. In plants, systemic RNAi is dependent on the ribonuclease Dicer-like 4, and the RNA-dependent RNA polymerase RDR6 and involves the spread of 21 nt long small interfering RNAs (siRNAs) between infected and non-infected cells via plasmodesmal junctions[11-13]. In *C. elegans*, systemic RNAi is dependent on the family of the transmembrane systemic RNA interference-deficient (SID) channel protein, as well as on the presence of a RNA-dependent RNA polymerase (Ego1) for amplification of the immune signal[14-20]. In insects, very little is known about the mechanisms involved in systemic antiviral immunity[21,22] with most of the evidence coming from studies in *Drosophila melanogaster*. In flies, it is possible to confer protection against viral infection by inoculating virus-specific long dsRNA in the fly haemolymph, with a loss of protection when viral siRNAs are used due to a selective mechanism for uptake of only long dsRNA from the surroundings[22,23]. Therefore, even if is tempting to speculate that dsRNA molecules are the immune signal that spread to establish a systemic response[24], the nature of the signal and the mechanism of spread remain thus far elusive.

In the fruit fly, endosomal trafficking and multi-vesicular bodies (MVBs) are required for efficient RNAi silencing[23,25]. When MVBs fuse with the plasma membrane, they release extracellular vesicles (EVs). Those vesicles have been found in all sorts of fluids in different organisms[26-28] and EVs-containing small RNAs have been linked to immune modulation in mammals[29,30].

Here we demonstrate that *Drosophila* CG4572/DORA protein, previously identified in a genome-wide screen for RNAi transport in *Drosophila* cells[23] and

found to contribute to antiviral defense to *Drosophila* C virus infection[22], interacts with core components of the antiviral RNAi machinery and is present in EVs that contain viral RNAs. We show that EVs isolated from infected cells are able to protect wild type flies against viral infection, while EVs from DORA depleted cells, fail to immunise flies, confirming that the DORA is required to mount an RNAi-mediated systemic immune response. We propose DORA protein as the first discovered specific component for the establishment of a systemic antiviral response in invertebrates.

Results

In silico and biochemical analyses of CG4572

CG4572 is a putative serine-carboxypeptidase with a catalytic triad specific to those proteins: S217, D403 and H460[31]. This domain is well evolutionary conserved and present in several proteins in species ranging from plants[32], yeast (carboxypeptidase Y), mosquitos[31], to humans (cathepsin A, also known as protective protein). Moreover, this protein carries a signal peptide (amino acids VEG-ER, where – corresponds to the cleavage site) for translocation into the endoplasmic reticulum, where it may be subjected to several N-glycosylations.

To characterize CG4572, we raised monoclonal antibodies against the C-terminal part of the protein. After subcellular fractionation of S2 cells and western blot analyses, the protein was found in the soluble fraction (Supplementary Fig. 1a) containing the cytoplasm and small vesicles and organelles. In addition to the predicted 54 kDa protein, another unexpected band was detected at 30 kDa. To confirm the identity of the two proteins, mass-spectrometry assays were performed following CG4572 immunoprecipitation (Supplementary Fig. 1b). Both the 54 kDa and the 30 kDa proteins correspond to CG4572, with the latter corresponding to the C-terminal part of the protein. To determine if the 30 kDa protein was the product of an alternative splicing event, we analysed total RNA from S2 cells by Northern blot. Only one transcript was detected at the expected size (1800 nt), suggesting that both forms of CG4572 are the result of post-translational processing, rather than production of two distinct messenger RNAs or alternative splicing (Supplementary Fig. 1c).

CG4572 is required for an efficient antiviral response

CG4572 has been described as a component of the antiviral immune response in *Drosophila*[22,23]. To monitor its expression throughout viral infection, RT-qPCR was performed in flies infected with *Drosophila* X virus (DXV) or *Drosophila* C virus (DCV). During the course of the viral infection (Supplementary Fig. 2a and

2b), CG4572 RNA levels were stable in w^{1118} flies (wild type, wt) (Supplementary Fig. 2c and 2d), whereas they were absent in deficient flies ($CG4572^{-/-}$). The CG4572 mutation is characterized by the insertion of a PiggyBack transposon within the coding sequence of CG4572 (allele $CG4572^{c05963}$) resulting in extreme hypomorphs for the gene.

We then tested the ability of $CG4572^{-/-}$ flies to cope with different viruses. Flies were inoculated in the thorax with DCV (Fig. 1a), Flock House virus (FHV) (Fig. 1b) or Cricket Paralysis virus (CrPV) (Fig. 1c) and survival rates were monitored daily. $CG4572^{-/-}$ flies were hypersensitive to these viruses, showing a lower lifespan compared with wt flies or even Dicer-2 mutant flies ($Dcr\text{-}2^{-/-}$). However, no difference in survival between wt and $CG4572^{-/-}$ flies were observed during infection with Sindbis virus (SinV) (Supplementary Fig. 3a), Vesicular stomatitis virus (VSV) (Supplementary Fig. 3b) and *Drosophila* X virus (DXV) (Supplementary Fig. 3c).

As CG4572 protein displayed a major effect on the antiviral response against non-enveloped, positive stranded RNA viruses, we explored the possibility that CG4572 acts as a viral restriction factor. To test this hypothesis, *Drosophila* S2 cells were transfected with different concentrations of expression plasmids to drive CG4572, and then infected with DCV and DXV. If CG4572 acts as a restriction factor, its overexpression should hinder viral replication. However, no differences in viral load over time were observed, precluding a role of CG4572 as a viral restriction factor (data not shown).

Finally, to exclude the possibility that the CG4572 protein could be involved in a broad-spectrum immune response, flies were infected with Gram-positive bacteria, *Miccrococus luteus* (data not shown) or with Gram-negative bacteria, *Listeria monocytogenens*. As observed in Fig. 1d, $CG4572^{-/-}$ flies were as sensitive as wt flies to bacterial infection, precluding a role of CG4572 as a broad immunity component. Taken together, the results unveil an essential and specific role of CG4572 in the fly antiviral response.

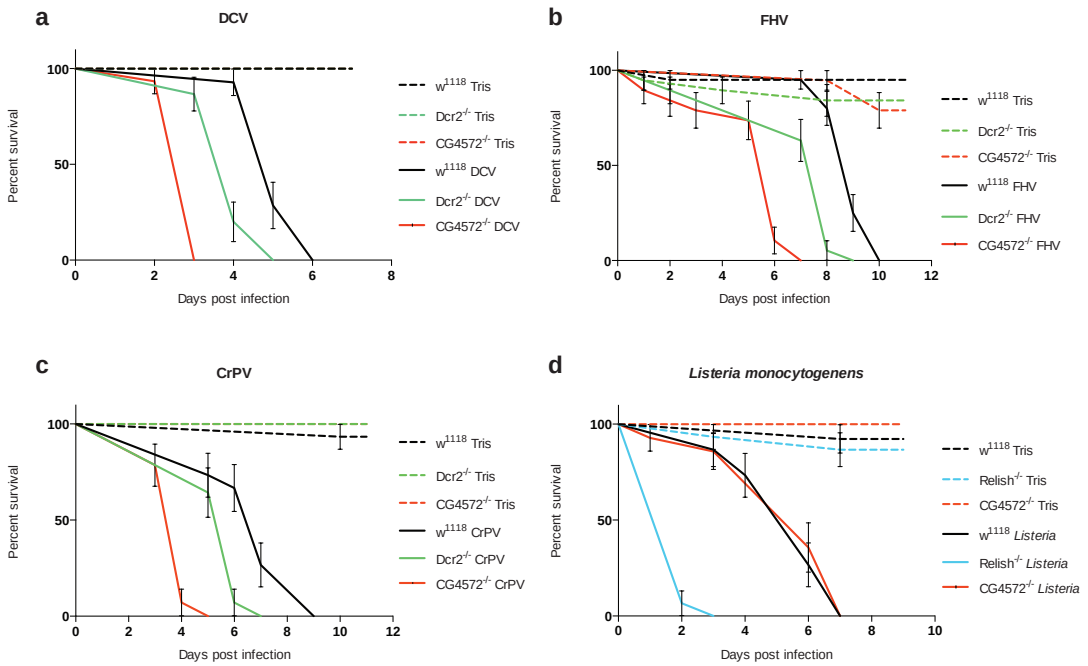

Figure 1
Karlikow *et al*.

Figure 1. Increased viral susceptibility in CG4572 deficient flies. Five-days old flies were injected in the thorax with (**a**) Drosophila C virus (DCV) at 100 $TCID_{50}$/fly, (**b**) Flock house virus (FHV) at 500 $TCID_{50}$/fly, (**c**) Cricket paralysis virus (CrPV) at 100 $TCID_{50}$/fly or (**d**) *Listeria monocytogenens* at 1,000 bacteria/fly. Mortality was monitored daily. For each assay w^{1118} flies, in black, were used as wild type (wt) control; Dicer-2 deficient flies ($Dcr\text{-}2^{-/-}$), in green, were used as a control of defective antiviral RNAi pathway. *Listeria monocytogenens* was used to determine if CG4572 has a role in the response to bacterial infection. Relish deficient flies ($Relish^{-/-}$, in blue) were used as a positive control of a deficient anti-bacterial response. In each experiment, Tris injection was used to control the impact of the injection *per se* (black dashed line). Data from one experiment representative of three. The values represent the mean and SD of three independent groups of 15 flies each by experiment.

CG4572 interacts with core components of the RNAi machinery

Using the monoclonal antibody against CG4572, immunoprecipitation of S2 cell lysates were performed and co-purifying factors were analysed by lc-ms/ms mass-spectrometry. In three independent experiments, 11 putative partners of CG4572 were identified. In addition, 33 other possible interacting proteins were found in two out of three experiments (Fig. 2a and Table 1). We detected proteins related to: 1- cellular transport, such as twinstar (*Drosophila* homologue of cofilin), Map205 (CG1483) and Shrub (an ESCORT-III component)[33,34]; 2- immune and

stress responses, such as Hopscotch and HSP83. This category also contained proteins known to interact with RNAi components, such as Vig and its homologue Vig-2, dFMR1 (Wan, 2000, Mol Cell Biol) (*Drosophila* homologue of the human FMR1 protein)[35,36], Ago-2, the main effector of the antiviral RNAi pathway[37], and Dmp68[36] (Table 1). As these data suggested an association of CG4572 with core components of the RNAi machinery and as the RNAi pathway is essential for the antiviral response, we further validated these interactions. Co-immunoprecipitations of CG4572, Ago-2 and dFMR1 proteins were performed (Fig. 2b), confirming that CG4572 interacts with both Ago-2 and dFMR1 in a specific manner. In addition, confocal microscopy shows co-localization of CG4572 with Ago-2 (Fig. 2c) in small punctuate structures reminiscent of vesicles, reinforcing that CG4572 specifically interacts with the RNAi effector complex RISC.

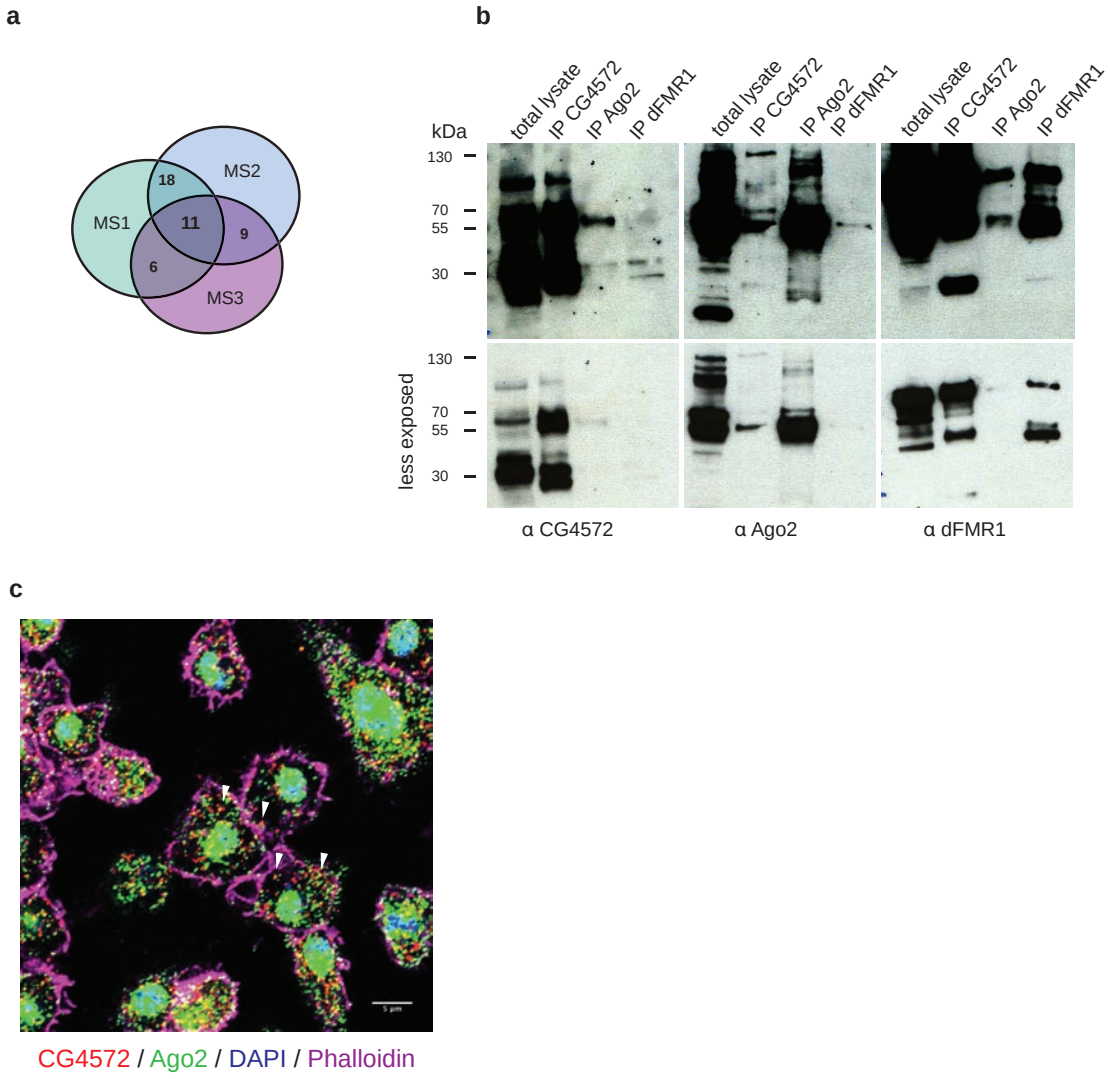

Figure 2
Karlikow *et al.*

Figure 2. CG4572 interacts with RNAi factors. (**a**) Using the soluble fraction of S2 cells, three independent immunoprecipitation assays were performed with a monoclonal antibody that recognizes CG4572. Co-immunoprecipitating factors were identified by mass-spectrometry (Table 1). Eleven proteins were identified in three independent mass spectrometry and 33 other proteins were found twice. (**b**) Immunoprecipitation of CG4572 or Ago-2 or dFMR1 was performed on the soluble fraction of S2 cells lysates to confirm mass-spectrometry results. WB using the respective antibody assessed CG4572 interactions with Ago-2 or dFMR1. (**c**) CG4572 (red) and Ago-2 (green) proteins localize together in punctuate structures as shown by confocal microscopy. Arrowheads show examples of localization of CG4572 and Ago-2 proteins in the same structure.

Table 1. CG4572 interacting candidates. List of proteins interacting with CG4572, detected by mass spectrometry. Grouping was performed following Gene Ontology terms. Hits indicate the number of times proteins were detected on three independent mass spectrometry assays.

Gene Ontology Term	ID	Name	Hits
RNA binding	CG5836	Splicing Factor 1	2
	CG5442	Sc35	2
	CG10851	B52	3
	CG5654	Ypsilon Schachtel	3
	CG9273	Replication Protein A2	2
	CG9983	Hrb98DE	2
	CG3800	CHCC-type zinc finger protein	2
Immune and stress response	CG6203	dFMR1	2
	CG1594	Hopscotch	2
	CG6988	Pdi	2
	CG1242	Hsp83	3
	CG4170	Vig	2
	CG11844	Vig-2	2
	CG10279	Dmp68	1
	CG7439	Ago-2	1
Transport	CG12051	Actin 42A	2
	CG1913	Tubulin 1α	2
	CG9277	Tubulin 1β	3
	CG6948	Tubulin 1β	2
	CG8055	Shrub (ESCORT-III complex)	2
	CG9412	Rasputin	2
	CG4254	Cofilin / Twinstar	3
	CG1483	205 kDa microtubules associated protein	2
	CG14996	Transgelin (Chd64)	2
	CG4800	Translationally-controled tumor protein homolog	2
	CG8280	Elongation factor 1α	3
	CG6341	Probable Elongation factor 1β	3
	CC4572	Serine carboxypeptidase	2
		Ribosomal protein S3, S3a, S5a, S18	3
		Ribosomal protein S2, S9, S14a, S26, S30, L14, L22, LP0, LP2	2
	CG11901	Elongation factor 1γ	2
	CG8759	Nascent polypeptide associated complex protein alpha subunit	2
	CG11999	glycosyltransferase	2
		Histone 4	2
	CG4337	mtSSB	2
Unclassified	CG30412		2

Table 1
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CG4572 is involved in systemic antiviral RNAi

Because CG4572 interacts with components of the RNAi machinery and flies deficient for CG4572 cannot mount an effective antiviral response, we next analysed whether the core RNAi machinery was impaired in CG4572 deficient flies. To this end, we first analysed viral siRNA production following viral infection. CG4572$^{-/-}$ flies were able to generate viral siRNAs during DCV infection (Supplementary Fig. 4a and 4b respectively) as well as after injection of exogenous dsRNA (Supplementary Fig. 4c and 4d respectively). Second, we analysed Ago-2 catalytic activity (slicing) in wild type and CG4572$^{-/-}$ embryo extracts. Both wild type and in CG4572$^{-/-}$ flies were competent in Ago-2 mediated slicing, although slicing seems somewhat less efficient in the latter (Supplementary Fig. 4e). Therefore the absence of CG4572 does not impair the cell-autonomous RNAi response and does not seem to be responsible for the extreme sensitivity of CG4572$^{-/-}$ flies to viral infection.

Given that CG4572$^{-/-}$ flies were not protected from a viral challenge after sequence specific dsRNA inoculation ([22] and data not shown) and that the cell-autonomous siRNA pathway was functional in these flies, we analyzed whether CG4572 is involved in systemic antiviral RNAi. Using a previously described approach[22], we examined whether infection with SinV carrying a GFP reporter gene could silence an ubiquitous endogenous GFP signal at a distal site. To do so, wt-GFP or CG4572$^{-/-}$-GFP flies were infected with SinV expressing GFP (SinV-GFP) or firefly luciferase (SinV-Luc). SinV was detected in fly heads and bodies at 2 days post infection (dpi) (Fig. 3, lane 3-5; 8-10 for wt flies, and 13-15; 18-20 for CG4572$^{-/-}$ flies). Yet at 1 dpi, co-suppression of endogenous GFP was already detectable in heads of wt flies (Fig. 3 lane 2), but not in CG4572$^{-/-}$ flies (Fig. 3 lane 12). Co-suppression of GFP was not detected following infection with SinV-Luciferase virus (Fig. 3 lane 1-4 for wt flies, and 11-14 for CG4572$^{-/-}$ flies), showing the sequence specificity of the silencing. Taken together, our results suggest that CG4572$^{-/-}$ flies are unable to effectively spread the immune silencing signal and therefore are impaired in systemic RNAi. As the spread of an immune signal is affected by the absence of CG4572, we proposed to name CG4572 after

Dora the explorer. DORA is essential to allow the immune signal to spread (to explore) to uninfected tissues.

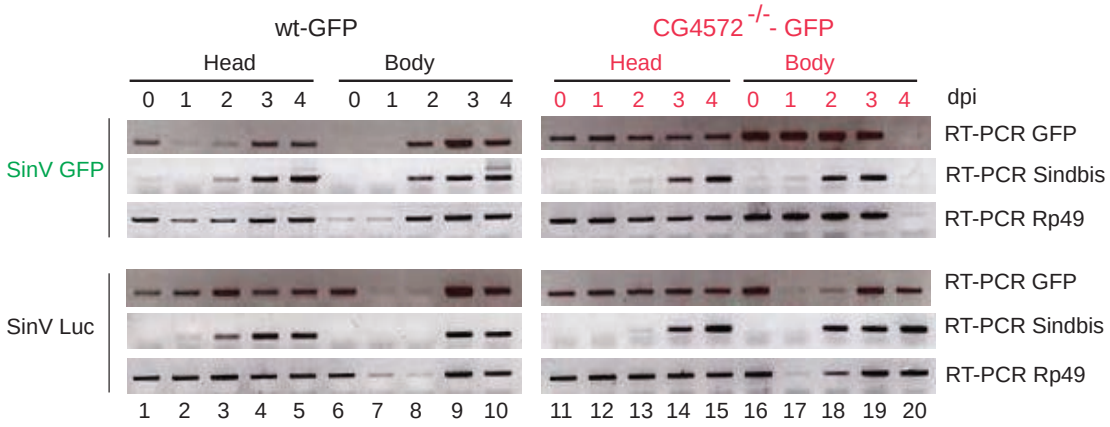

Figure 3
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Figure 3. CG4572 deficient flies are unable to mount a systemic antiviral response. Five-days old wild type w^{1118} flies (wt) and CG4572 deficient flies ($CG4572^{-/-}$) expressing eGFP (under the control of a tubulin promoter) were injected with 5000 pfu Sindbis-luciferase or Sindbis-GFP virus. Pools of 5 flies were collected daily up to day 4 post-infection. Sindbis viral replication was monitored by RT-PCR in heads and bodies as well as the expression of GFP. Rp49 was used as endogenous control for gene expression.

DORA is found in late endocytic compartments

Immunofluorescence staining with DORA monoclonal antibodies shows localization in cytoplasmic vesicles (Supplementary Fig. 5a). We further analysed subcellular localization of DORA in S2 cells by confocal microscopy. Signal was detected in late endocytic vesicles positive for SARA (marker of multivesicular bodies, MVBs)[38] (Fig. 4a, numerical zoom in Fig. 4b) and in Rab7 positive vesicles, a marker of late endosomes[39] (Fig. 4c, numerical zoom in Fig. 4d). The same localization for DORA was found *ex vivo*, in wing disc of *drosophila* larvae that expressed Rab7-GFP or SARA-GFP (Supplementary Fig. 5b and 5c).

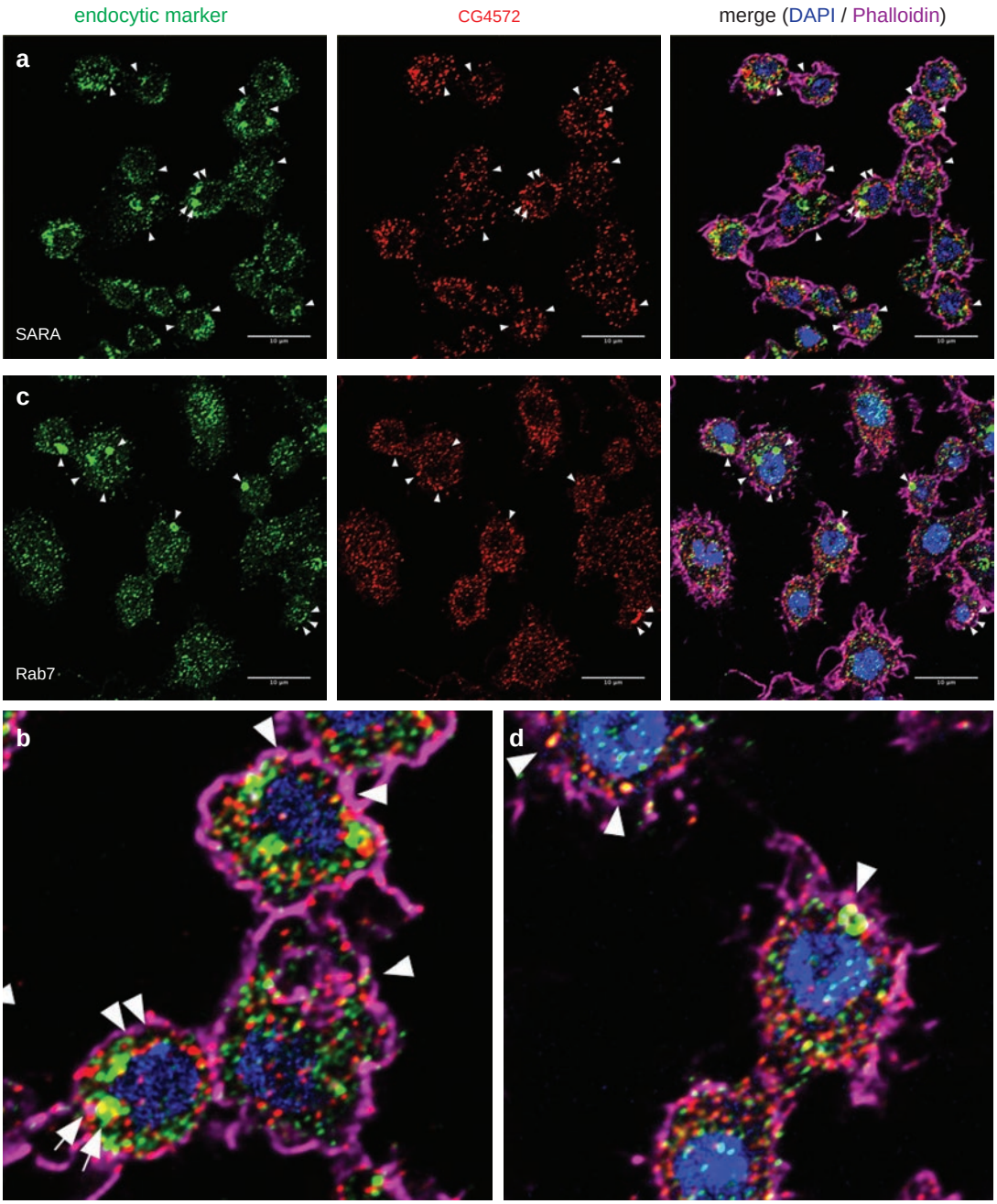

Figure 4
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Figure 4. CG4572 protein localizes in late endosomal compartments. Cellular localization of CG4572 was assessed by immunofluorescence followed by confocal microscopy. Endocytic markers were labeled in green and CG4572 in red. Confocal microscopy using (**a**) CG4572 antibody (red) and SARA (green) or (**c**) CG4572 antibody (red) and Rab7 (green). (**b**) and (**d**) show a numerical zoom of the merged image of panel (**a**) and (**c**). Arrows indicate some colocalization of CG4572 and endocytic markers. DAPI, in blue, labels the nuclei and Phalloidin, in magenta, labels the F-actin and is used to define the cell edge.

DORA is present in extracellular vesicles containing fragments of viral RNAs as well as viral siRNAs

When MVBs fuse with the plasma membrane, their inner vesicles are released outside the cells as extracellular vesicles (EVs). Given that DORA was specifically found in late endosomal compartments and that EVs play a potential role in intercellular communication, we analysed whether DORA was present in EVs. Due to their capacity to efficiently uptake dsRNA from media, S2R+ cells (persistently infected with DAV, DXV, DCV and FHV viruses) were used, as well as S2R+ cells in which a short dsRNA hairpin was expressed allowing the stable depletion of DORA (S2R+_shCG4572, Supplementary Fig. 6a,). Both cell lines were incubated with dsRNA against SinV, dsRNA against GFP (as a control) or infected with SinV (Fig. 5a). Three days later, EVs were purified following the protocol described by Thery ([40] with modifications) and the criteria published by Lotvall *et al*.[41] were applied to assess the quality and purity of EVs. To rule out the possibility that collected EVs were the product of cell damage/death, we checked cell viability (Supplementary Fig. 6b). EVs from both cell lines were purified by Optiprep discontinuous gradient to separate EVs from viruses. The quality of the separation was verified by western blot and DORA was detected in the 10% Optiprep-fraction of the gradient (Supplementary Fig. 6c), clearly separated from the virus fraction (10-35% fraction). The same samples were analysed by electron microscopy and EVs were detected (Supplementary Fig. 6d). Once the quality of the samples was checked, high throughput sequencing of small RNAs was performed on RNA purified from EVs.

As shown in Figure 5b, EVs from cells soaked on dsRNA against SinV contained viral siRNAs (vsiRNAs) corresponding to the dsRNA. EVs from cells infected with SinV contained fragments of viral RNA (Fig. 5c), corresponding to the entire genome, but no vsiRNAs. However, in both cases, EV content was specifically enriched in viral RNAs, as shown by the low proportion (less than 1%) of detectable small RNAs against endogenous transposons or miRNA (Supplementary Fig. 6e).

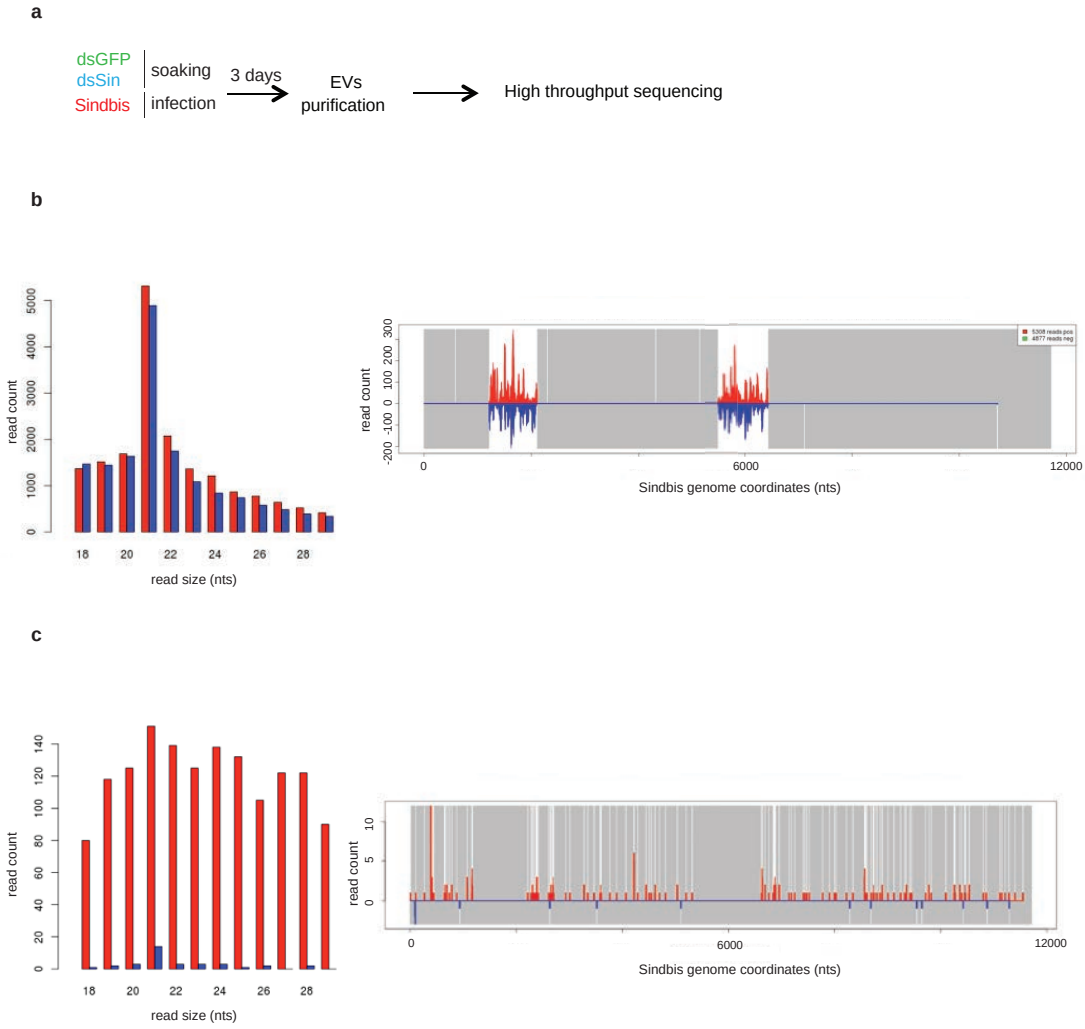

Figure 5
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Figure 5. CG4572-positive EVs are loaded with viral small RNAs as well as fragments of viral RNA. Extracellular vesicles (EVs) were purified by Optiprep gradient. (**a**) Experimental design of high throughput sequencing on EVs. (**b**-**c**) Left panel: Size distribution of viral reads corresponding to (**b**) EVs from cells that soaked dsSin or (**c**) EVs from cells infected with Sindbis virus. Right panel: Alignment of 21 nts siRNAs on Sindbis genome. Positive strand small RNAs are presented in red and negative strand in blue. The gray bars represent nucleotides position without coverage. For (**c**) left and right panel, the regions corresponding to dsSin were extracted during analysis in order to avoid risk of cross-contamination.

DORA-positive extracellular vesicles containing fragments of viral RNA protect against viral infection

Given that DORA depletion inhibited systemic RNAi and considering its localization in EVs, we tested whether DORA-positive EVs containing vsiRNAs and other viral RNAs could have a role in establishing systemic immunity. In order to mimic this situation, we developed an immunization protocol based on inoculating flies with EVs (Figure 6a). The same EVs that were purified for deep sequencing were injected in wild type flies. After two days, flies were challenged with SinV-Luciferase virus. Viral replication was assessed by Luciferase activity 48 hours after infection. Flies inoculated with EVs containing siRNAs (from cells that soaked dsRNA against SinV) were not protected against SinV-Luc infection (Fig. 6b, green versus blue). In contrast, flies inoculated with EVs containing fragments of viral RNAs (from cells that were infected with SinV virus) were efficiently protected against SinV-Luc infection (Fig. 6c green versus red). Moreover, the protection was lost in flies inoculated with EVs from cells depleted for DORA. Together, our results indicate that EVs secreted from cells during acute viral infection are specifically loaded with fragments of viral RNAs and not vsiRNAs. These viral RNAs are capable of immunizing wild type flies against a virus challenge in a DORA-dependent manner.

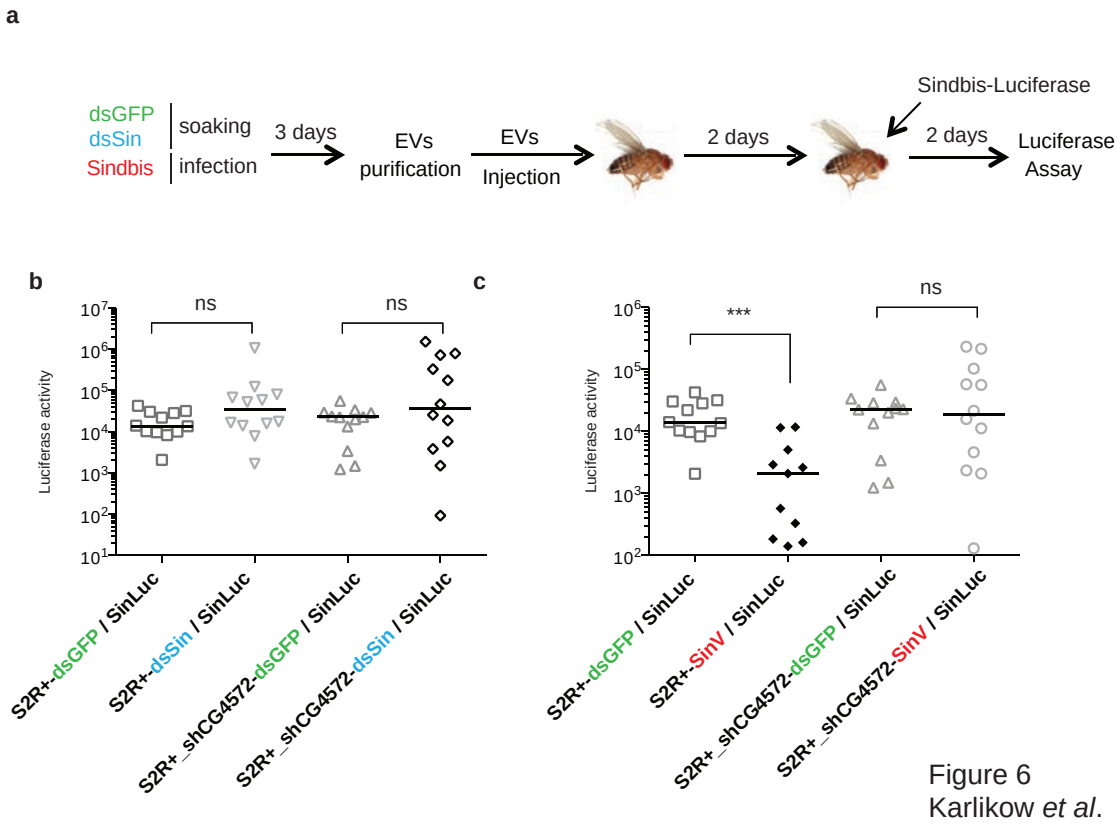

Figure 6
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Figure 6. EVs containing fragments of viral RNA protect flies. (**a**) Experimental design of the EVs based immunization. (**b**, **c**) Five days old wt flies were inoculated with 100 µL of EVs preparation. 2 days later, flies were infected with 3000 pfu of Sindbis-Renilla and renilla activity in single flies was measured 2 days later. (**b**) Immunization was performed with EVs from S2R+ cells that either soaked dsGFP (as control) or dsSin (blue versus green). (**c**) Immunization was performed with EVs from S2R+ cells that either soaked dsGFP (as control) or that were infected with Sindbis (red versus green). ***p=0.0007. In both cases, immunization was performed with EVs coming from S2R+ cells or S2R+ cells depleted for CG4572. Statistical analysis was performed with Prism 6 software using a non-parametric Mann-Whitney test (n=12 for each condition).

Discussion

To establish systemic immunity, the spread of an immune signal from infected cells and its sensing and internalization by non-infected cells are crucial steps. Although systemic spread of a specific antiviral RNAi activity in *Drosophila* was previously shown[22], the precise nature of the mobile signal and the mechanism of its systemic spread remain to be defined. Here we demonstrated that *Drosophila* DORA protein is an essential component of the immune response elicited by virus infection in flies. DORA is the first critical component described for the establishment of systemic antiviral immunity in insects.

Our most significant finding is the requirement of DORA-positive extracellular vesicles for the immunization of non-infected flies against viral infection. DORA could affect four major steps on EVs function: the loading, the sorting from the donor cell, the docking, or their processing in the target cell. The absence of DORA therefore could: *(i)* impair the function of MVBs (EVs loading and sorting), which could explain the reduced slicer activity of Ago-2 (as described by Lee and colleague[25]) and the concomitant retention of the RNAi immune signal inside cells; *(ii)* impair the docking of EVs in distant target cells, therefore a dysfunction in the sensing of the signal; *(iii)* impair the internalization and processing of EVs and their contents, resulting in dysfunction in the RNAi signal internalization at the distal site of infection.

A striking result is the nature of the immune signal travelling within EVs. The fact that EVs containing viral siRNAs failed to immunize flies, strongly suggested that vsiRNAs are not the RNAi immune signal, contrary to the situation observed in plants. However, by the immunization protocol, the sorting and the docking of EVs are bypassed and it could be possible that the processing of EVs is dysfunctional in such a way that vsiRNAs cannot get released from the EVs. Surprisingly, EVs purified from infected cells, were enriched in fragments of viral RNAs ranging in size of 19-51 nts (upper length-limit of the deep sequence protocol used), which were distributed through all of the Sindbis genome. It is tempting to propose that systemic spread of antiviral RNAi is mediated by large viral fragments with intramolecular base-pairing structures released from infected cells in EVs. These viral fragments contained in EVs would enable *(i)* the spread of the immune signal to distant locations, and *(ii)* the presence of the immune signal in non-infected tissues without the need of an RdRp-mediated amplification process. Therefore, fragments of viral RNA with dsRNA structures (degradation products, abortive transcripts, incomplete viral genomes, etc) would be the RNAi-immune signal, and the vsiRNAs produced from the viral fragments, would be the immune effectors that confer a pre-existing immunity at the cellular level to uninfected cells.

Another interesting consideration is the specificity of the antiviral function of DORA only with non-enveloped positive strand RNA viruses. As our results

indicate that DORA is not acting as a viral restriction factor, it is enticing to hypothesize that DORA is involved in the sorting of fragments of viral RNA corresponding to viruses that would localize their replication and assembly in endocytic compartments, in close proximity to DORA localization. For the viruses that localize in different cell compartments, alternative modes to spread the RNAi signal could be envisioned, as the recently described tubular nanotube structures that carry RNAi machinery and dsRNA (Karlikow et al. Submitted to Scientific Reports).

To our knowledge, this is the first demonstration of a cellular protein in arthropods that contributes to the spread of an antiviral signal. Whether the role of DORA in systemic immunity is evolutionarily conserved deserves further exploration.

Methods

Fly stocks. Virus- and *Wolbachia*- cleared flies were reared on standard medium at 25°C.

The following genotypes were used:

Wild-type (w^{1118}): w^{1118}; ; were obtained from the Bloomington Drosophila Stock Center.

Dcr-2 mutant: y^{d2} w^{1118}, P{$ry^{+t7.2}$=ey-FLP.N}2; Dcr-$2^{L811fsX}$;

CG4572 mutant (CG4572$^{-/-}$): w^{1118}; ; PBac{w^{+mC}=PB}CG4572^{c05963}

CG4572$^{-/-}$-GFP: w^{1118}; ; PBac{w^{+mC}=PB}CG4572^{c05963}, P(w^{+mC}=tub-GFP}/TM6, Sb^1 Tb^1

wt-GFP: w* ; ; P{w^{+mC}=Tub84B-EGFP.NLS}3

Relish: w^{1118}; ; $Rel^{E20}e^{s}$

UAS flies were kindly provided by Pr. Gonzalez-Gaitan:

UAS-SARA-GFP: w*; P{w^{+mC}=UAS-GFP::Sara}/CyO ;

UAS-Rab7-GFP: w*; P{w^{+mC}=UAS-GFP::Rab7}/CyO ;

EnGal4: w; P{w[+mW.hs]=en2.4-GAL4}e16E (kindly provided by Dr. Franck Coumailleau).

Fly crossing, dissection and immunofluorescence of wing discs. GFP larvae were generated by crossing [w; UAS-Rab-7-GFP/CyO] or [w; UAS-SARA-GFP/CyO] flies with [EnGal4] flies. The transcription factor Gal4 activated the UAS promoter, generating Rab-7-GFP or SARA-GFP larvae. Wing discs from larvae were dissected in PFA 4% on ice, incubated in PFA 4% for 20 min at RT and washed twice in PBS for 5 min. Wing discs were permeabilized in PBS-Triton X-100 0,1% for three times 5 min and immunofluorescence was performed as described below.

Cell culture: *Drosophila* S2 cells (Schneider, 1972, Life Technologies) were cultured at 25°C in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% heat inactivated fetal bovine serum (FBS, Life Technologies), 2 mM glutamine (Life Technologies), 100 $U.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ streptomycin (Life Technologies).
Stable cell lines were generated by co-transfecting $1x10^7$ S2 cells with pCoBlast plasmid (Life Technologies) as selection vector and a pValium plasmid containing a short hairpin against CG4572 (TRiP, Harvard Medical School) as expression vector at a 1:19 ratio. Transfections were performed using Effectene transfection reagent (Quiagen) according to the manufacturer's instructions. The next day, cells were washed and kept at 25°C. Three days later, medium was replaced and the selective agent added (blasticidin at 25 µg/mL). Medium was then changed every 4-5 days with the selective agent, and selection occurred in 2-3 weeks.

Immunofluorescence. *Drosophila* cells were cultured overnight (o/n) on coverslips at a concentration of $1x10^6$ cells/mL in complete Schneider's *Drosophila* medium. Cells were then fixed in paraformaldehyde diluted in PBS to 4% (PFA, Alfa Aesar) during 15 minutes (min) and washed two times in PBS for 5 min. Following the fixation step, permeabilization was done in PBS 0.1% Triton X-100 (PBS-Triton) for 5 min three times. Cells were then incubated with primary antibodies in PBS-Triton supplemented with 3% FBS for at least 1 hour (h) at RT

(mouse αCG4572 1/500 is a home-made antibody, rabbit anti-SARA kindly provided by Pr. Gonzales-Gaitan, rabbit αAgo2 1/500 (Abcam) and rabbit αRab7 1/2,000 kindly provided by Pr. Nakamura). After three washes in PBS-Triton, cells were incubated with secondary antibody (Alexa-Fluor 1/1,000, Life Technologies), DAPI (1/10,000, Life Technologies) and Phalloidin-647 (Alexa-Fluor 1/200, Life Technologies) diluted in PBS-Triton with 3% FBS for 1 h at RT. Cells were washed two times in PBS-Triton for 5 min, once in PBS and finally mounted on glass slide with Fluoromount G (eBioscience) and imaged with a confocal microscope LSM 700 inverted (Zeiss) at a 63X magnification oil immersion lens. Confocal stacks were reconstructed with Huygens Professional software (SVI).

Fly infection. 5-days old females flies were injected in the thorax with 50 nL of the appropriate virus dilution in 10 mM Tris using a Drummond nanoject injector. Mortality was monitored daily.

For bacterial infection, *Listeria monocytogenens* was pre-cultured in BHI medium supplemented with chloramphenicol. *Micrococcus luteus* was pre-cultured in LB at 37°C. Bacteria were washed twice in PBS, resuspended in PBS at $2x10^7$ per mL and this solution was used to inject flies with 50 nL (1,000 cell / fly) as previously described.

Western Blot. Protein extracts were incubated at 95°C for 5 min in 1X Laemmli Buffer containing 5% β-mercaptoethanol and separated on 4-15% polyacrylamide gels (Mini-PROTEAN TGX Precast Gels, BioRad). Proteins were then transferred with a semi-dry system (TransBlot Turbo Transfer System). Nitrocellulose membranes were incubated in blocking buffer (5% fat free milk in PBS supplemented with 0,1% of Tween-20, PBS-T, Euromedex) for 45 min. Membranes were then incubated with primary antibody (mouse αCG4572 1/1,000 home-made, rabbit αAgo-2 1/1,000 Abcam, mouse αFMR1 1/2,000 Abcam, mouse αTubulin 1/5,000 Sigma, mouse αSyntaxin 1/300 DSHB, rabbit αFHV 1/10,000 home-made) diluted in fat free milk PBS-T for two hours, washed three times in PBS-T for 5 min each, and incubated for one hour in secondary antibody (ECL anti-mouse or ECL anti-rabbit, GE Healthcare) diluted 1/5,000 in fat free

milk PBS-T. Finally membranes where washed three times in PBS-T, incubated 5 min with SuperSignal West Pico Chemiluminescent Substrate, or with Femto (Thermo Scientific) and visualized on Amersham Hyperfilm ECL (GE Healthcare).

Mass Spectometry. 50 or 100 µg of immunoprecipitated CG4572 were loaded onto a linear immobilized pH gradient (3-10) for charge separation in the first dimension. The second separation was carried on 15% SDS-PAGE. Selected spots were cut and analyzed by tandem mass spectrometry (MS/MS).

CG4572 interactome. Three sets of experiments were performed, adapted from[42].

- Protein digestion and labelling: immunoprecipitated CG4572 and binding proteins solubilised in PBS (pH 8.0) were reduced and alkylated with first DTT at 10 mM final for 2h, at 37°C; iodoacetamide was added for the last 30 min at 50 mM final, in dark. After 3 washes in PBS on microcolumn (Millipore, cut off 10 kDa), primary amines were labelled with 1 mM N-hydroxysuccinimidobiotin (NHS) -biotin (Thermo scientific) for 2h at RT according to manufacturer's instructions. Then, NHS was saturated using 200 mM hydroxyalamine, and the proteins were washed 3 times with ammonium bicarbonate (50 mM) on microcolumn. Proteins were fractionated in two parts, one for the proteolysis using trypsin (10 ng modified sequencing grade trypsin; Roche; 37°C, overnight) and the other for proteolysis with Lys-C (10 ng, Roche; 37°C, overnight). Resulting peptides were purified using streptavidin beads (Thermo scientific) according to manufacturer's instructions (150 µL of resins incubated 1h at RT with the samples). After washing with PBS, bound peptides were eluted with DTT (5 mM final, 30 min, RT), acidified with 10 µL of 10% aqueous formic acid and desalted using ziptip C18 micro column (Millipore).

- Identification of peptides by capillary Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS): Peptides (6 µL) were purified on a capillary reversed-phase column (nano C18 Acclaim PepMap100 Å, 75 µm i.d., 15-cm length; Dionex) at a constant flow rate of 220 nL/min, with a gradient of 2% to 40% buffer B in buffer A in 45 min; buffer A: H2O/acetonitrile(ACN)/ FA 98:2:0.1 (vol/vol/v);

buffer B water/ACN/FA 10:90:0.1 (vol/vol/v). The MS analysis was performed on a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (LTQ-FT Ultra; ThermoFisher Scientific) with the top-seven acquisition method: MS resolution 60,000, mass range 470–2,000 Da, followed by seven MS/MS (LTQ) on the seven most intense peaks, with a dynamic exclusion for 90 s.
Raw data were processed using Xcalibur 2.0.7 software. The database search was done using Mascot search engine (Matrix Science Mascot 2.2.04) on the 17 *D.melanogaster* protein databank. Proteome Discoverer 1.3 (ThermoFisher Scientific) and Mascot were used to search the data and filter the results. The following parameters were applied: MS tolerance 10 ppm; MS/MS tolerance 0.5 Da; semi-tryptic or semi-AspN peptides or semi V8-DE; two miss cleavages allowed; partial modifications carbamidomethylation (C), oxidation (M), acetyl (K, N-ter), thiopropionation (K, N-ter). Proteins identified by at least two peptides with a high confidence were validated.

RT-PCR. Total RNA was isolated with TRIzol (Life Technologies) according to manufacturer's instructions, followed by RNase-free DNase I treatment (Roche Diagnostics). RT was performed with 1 µg of total RNA, using SuperScript II reverse-transcriptase and random hexamer primers (Life Technologies). PCR was performed using DreamTaq DNA polymerase (ThermoFisher Scientific). Oligonucleotide primers were as follow:
GFP-F, CACATGAAGCAGCACGACTT
GFP-R, TGCTCAGGTAGTGGTTGTCG
Sin-F, AAGGATCTCCGGACCGTACT
Sin-R, TTCCGGGAGCGTTGATATAC
Rp49-F, AAGGGTATCGACAACAGAGTGC
Rp49-R, ACAAATGTGTATTCCGACCACG

Quantitative real-time RT-PCR. Total RNA was isolated with TRIzol (Life Technologies) according to manufacturer's instructions, followed by RNase-free DNase I treatment (Roche Diagnostics). RT was performed with 1 µg of total RNA

using iSCript reverse-transcriptase (BioRad) and qPCR with FastStart SYBR Green Master (Roche).

Oligonucleotide primers were as follow:

CG4572-F, TGGCCCCGTGGATAGCCGAA

CG4572-R, CTTCGCGGGGAGCCACCTTG

DXV-F, GACGTAAAGAGAGAACCCCA

DXV-R, CGAGACTGGGTTTCGATGTTC

DCV-F, CCGGAAGCGCATTGTATTGG

DCV-R, GGAGTTGGGTGCTGAACTGA

Rp49-F, CGGATCGATATGCTAAGCTGT

Rp49-R, GCGCTTGTTCGATCCGTA

Northern blot. Total RNA from *Drosophila* S2 cells was isolated with TRIzol (Life Technologies). RNA (24 µg) was separated by electrophoresis through 1.5% denaturing agarose gels, then transferred to a Nytran SuperCharge membrane with the Turbo Blotter system (Whatman). RNA was crosslinked to membranes by ultraviolet irradiation (Stratalinker) and was prehybridized for 2 h at 42°C in ULTRAhyb Ultrasensitive Hybridization Buffer (life technologies). DNA probes corresponding to the 5' region (nts 2-310) and the 3' region (nts 1011-1297) of CG4572 CDS were labeled with ^{32}P using Ready-to-Go kit (GE Healthcare) then added to the hybridization buffer, followed by incubation overnight at 42°C. Membranes were washed 2 x 5 min in 2X SSC, 0.1% SDS at 42°C, and 1 x in 0.1X SSC, 0.1% SDS at 42°C and then exposed to a PhosphoImager screen. Probes were stripped by boiling of the membrane twice in 0.1% SDS for a second round hybridization with Rp49 as a 'housekeeping' control.

dsRNA synthesis. dsRNAs were generated by *in vitro* transcription using T7 RNA polymerase. Overnight incubation in T7 buffer (80 mM HEPES-KOH pH7.5, 24 mM $MgCl_2$, 2 mM Spermidine, 40 mM DTT) was followed by RNAase-free DNase I treatment (Roche Diagnostics), acid-phenol/chloroform (Life Technologies) purification and dilution at 3 µg/µL in H_2O. Annealing was

performed at 62°C for 15 min and dsRNAs were allowed to cool-down slowly at room temperature.

Oligonucleotide primers containing the T7 sequence in their 5'-end were designed as follows (F, forward; R, reverse):

dsGFP-F,TAATACGACTCACTATAGGGAGAAGTGGAGAGGGTGAAGGTGA

dsGFP-R, TAATACGACTCACTATAGGGAGAAAAGGGCAGATTGTGTGGAC

dsSin1-F, TAATACGACTCACTATAGGGAGATCTGCCGATCATAGCACAAG

dsSin1-R, TAATACGACTCACTATAGGGAGACTTCTTAACGCAACGCTTC

dsSin2-F, TAATACGACTCACTATAGGGAGAGGATCAATTTTCGACGGAGA

dsSin2-R, TAATACGACTCACTATAGGGAGATTGAATGTCGCTGAGTCCAG

Small RNA libraries from EVs: Total RNA from EVs was isolated with TRIzol (Life Techonlogies). 19-150 nts length small RNAs were purified from a 15% acrylamide/bisacrylamide (37.5:1), 7 M urea gel as described in[43]. Purified RNAs were used for library preparation using the kit NEBNext Multiplex Small RNA Library Prep for Illumina (E7300 L) with the 3' adapter from IDT (linker 1) and in-house designed indexed primers. Libraries were diluted to 4 nM and sequenced (51 single 'reads') using NextSeq® 500 High Output Kit v2 (75 cycles) on a NextSeq 500 (Illumina). 'Reads' were analyzed with in-house Perl scripts.

Bioinformatics analysis of small RNA libraries

The quality of fastq files was assessed using graphs generated by "FastQC"[44]. Using "cutadapt"[45], quality and adaptors were trimmed from each read. For small libraries, only the reads with a detectable adaptor sequence were kept (fragments < 30 bases). For other libraries, adaptor sequence was removed when it's possible. Only reads with acceptable quality were retained. A second set of graphs were generated by "FastQC" on the fastq files create by "cutadapt". Mapping was produced by "bowtie1" [46] with the "-v 1" option (one mismatch between the read and its target). miRNA and endogenous piRNA were search by mapping against specific miRNA and transposons databases of Flybase. "bowtie1" generates results in "sam" format. All "sam" files were analysed by different tools of the package "samtools"[47] to produce "bam" indexed files. To

analysed these "bam" files, we generated different kind of graphs using home made R scripts[48] with several Bioconductor libraries[49] as "Rsamtools" or "Shortreads".

Slicing assay. The slicing experiment was performed on *Drosophila* embryos as described in[50] with minor modifications.

EVs purification. Cells were grown three days in conditioned media (media centrifuged o/n at 100,000 g, 4°C in a Beckman SW32Ti rotor to remove any microvesicles that could be contained in the FBS; and filtered). Media was then recovered and centrifuged for 5 min at 300 g to remove living cells. The supernatant was recovered and spun down 5 min at 2,500g. The pellet contained dead cells and big cell debris. The supernatant was collected and spun down 30 min at 10,000 g at 4°C. The new supernatant was spun down o/n at 4°C at 100.000g in a Beckman SW45Ti. The pellet containing EVs and contaminating proteins was resuspended in 1 mL PBS and loaded on an Optiprep discontinuous gradient (described below) and ran for 4 h at 100,000g at 4°C in a Beckman SW41Ti rotor. All the fractions were recovered and Western Blot was performed to assess the separation of EVs from viral particles. Finally all fractions were resuspended in PBS an ultracentrifuged in a SW32Ti rotor, at 100,000g for 4 h at 4°C to remove as much Optiprep as possible. Pellets were resuspended in 150 µL Tris/NaCl 150 mM and subjected to total RNA extraction or directly inoculated into flies.

Optiprep gradient preparation. Briefly, the working solution at 50% Optiprep was prepared by diluting 5 volumes of Optiprep 60% with 1 volume of Homogenization Buffer 6X (250 mM sucrose, 3 mM EGTA, 120 mM HEPES-KOH pH 7.4). A discontinuous iodixanol gradient was prepared by diluting the working solution with Homogenization Buffer 1X (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH pH 7.4) to prepare the 10–35 and 50% Optiprep fractions.

Electron microscopy for EVs. 4 µl of each sample were spotted on glowdischarged carbon coated grids (EMS, USA) and negatively stained with NanoW (Nanoprobes, USA). Samples were then observed at 120 kV with a Tecnai G2. Images were recorded using an FEI 4K Eagle camera and TIA software (FEI, USA).

Fly immunization experiment. 100 nL of EVs preparation were injected in 5-days old wt flies. 48 hours later, flies were challenged with 100nL of SinV-Renilla (1500 pfu / 50 nL). 48 hours later, flies were collected and luciferase activity was measured as described below.

***Renilla* Luciferase Assay.** *Renilla* Luciferase was measured using the Renilla luciferase assay system (Promega). Individual flies were collected in 200 µL of Renilla Luciferase assay lysis buffer diluted to 1X in H_2O, and homogenized using pellet pestles (Sigma-Aldrich). 20 µL of each sample were transferred to a white 96-well plate. 20 µL of *Renilla* luciferase assay reagent were added in each well containing samples. Luminescence was immediately measured on a Glomax 96 microplate luminometer (Promega).

Statistical analyses. Statistical analyses were performed with Prism 6 software using a non-parametric Mann-Whitney test.

Acknowledgments

We thank members of the Saleh lab and specially V. Mongelli for fruitful discussions and R. van Rij for critical reading of the manuscript; J. Santos and L. Tomé for advice on Optiprep gradients; I. Haddad for advice on mass-spec analyses; F. Coumailleau for supervision of fly crossings; M. Gonzalez-Gaitan (University of Geneva, Switzerland) for UAS mutant flies; Pr A. Nakamura (RIKEN Center for Developmental Biology, Japan) for antibody to Rab7; TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947) for providing the shCG4572 plasmid vector used in this study.

This work was supported by the French Agence Nationale de la Recherche (ANR-09-JCJC-0045-01), the European Research Council (FP7/2007-2013 ERC StG 242703 and FP7/2013-2019 ERC CoG 615220) and the French Government's Investissement d'Avenir program, Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases" (grant n°ANR-10-LABX-62-IBEID) to M.C.S.

Author contributions

Conceived and designed the experiments: MK, BG and MCS. Performed most of the experiments: MK. Mass Spectrometry: CL, PL, YV and JV. Co-suppression in mutant flies, CG4572 antibodies, Northern blot: BG. Slicing activity: KWRvC and RvR. Preparation of small RNAs libraries: HB. Bioinformatics analyses: LF. Electron microscopy of EVs: GPA. Data analysis and interpretation of results: MK, BG, and MCS. Wrote the paper: MK and MCS.

Competing financial interests

The authors declare no competing financial interests.

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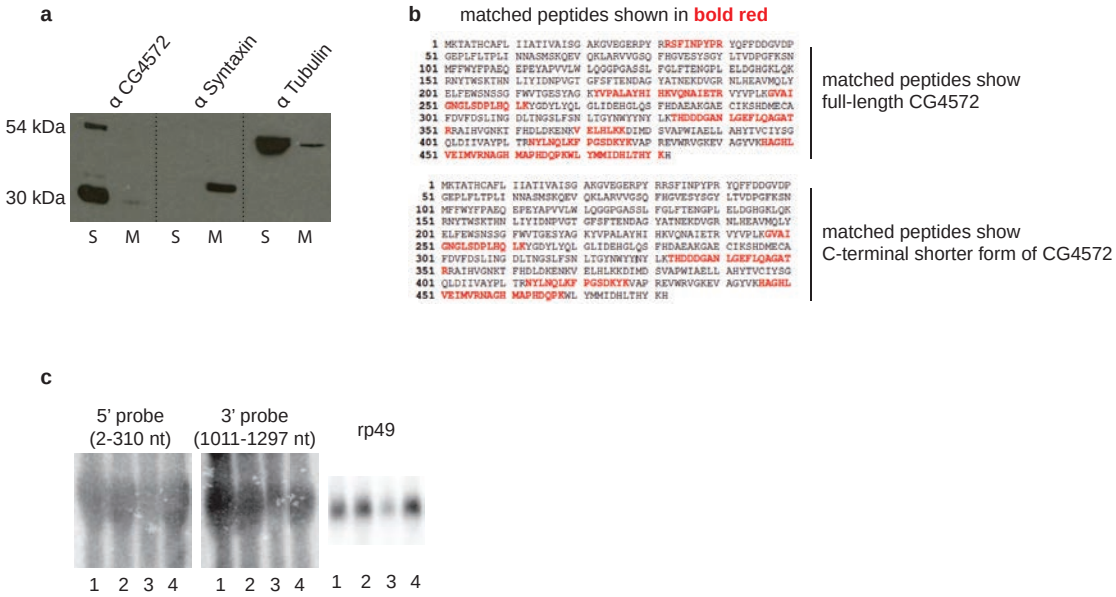

Supplementary Figure 1
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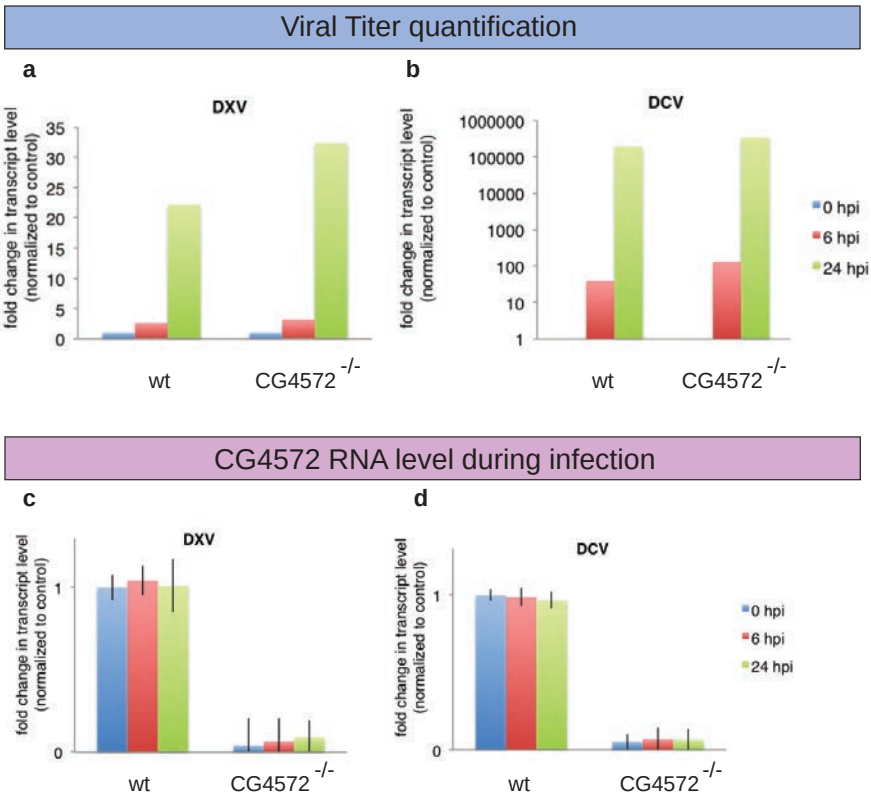
Supplementary Figure 1. CG4572 protein has two forms and localize in the soluble fraction of *Drosophila* cells. (**a**) S2 cells were fractionated and the subcellular localization of CG4572 was established by Western blot using a monoclonal antibody for CG4572, anti-Syntaxin antibody as marker of membrane fraction (M) and an anti-Tubulin antibody as marker of the soluble fraction (S). (**b**) Mass-spectrometry was used to define the two bands detected by the anti-CG4572 antibody by Western blot (the expected size for CG4572 at 54 kDa and a small protein at ~30 kDa). Peptides highlighted in bold red are identified by Mass-spectrometry and confirm that both bands are products of the CG4572 gene. (**c**) Northern blot analyses was performed to detect CG4572 RNA in non-infected S2 cells (lane 1), in S2 cells infected with Flock House virus (FHV) at 24 hours post infection (lane 2), in S2 cells infected with FHV at 4 days post infection (lane 3) and in S2 cells persistently infected with FHV (lane 4). One probe corresponded to the 5' of CG4572 (position 2 to 310 over CG4572 CDS), and the other one to the 3' (position 1011 to 1297 over CG4572 CDS). Rp49 probe was used as an endogenous control.



Supplementary Figure 2
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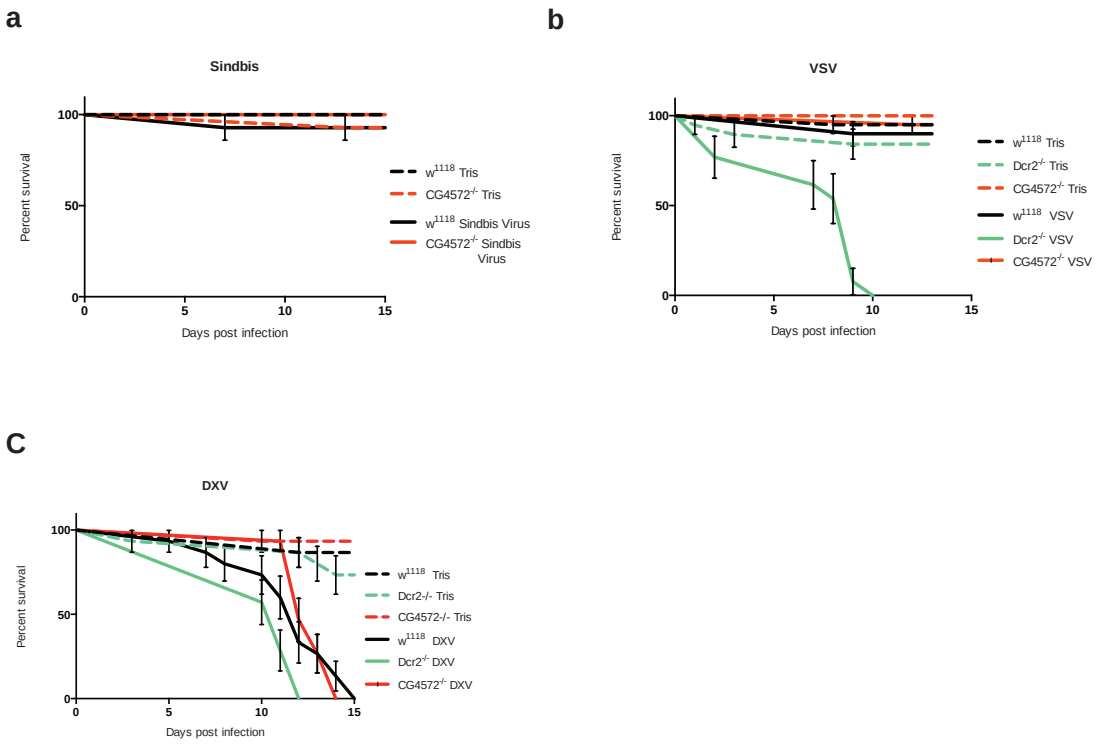
Supplementary Figure 2. Stable RNA expression of CG4572 during viral infection. Viral replication was assessed by RT-qPCR in wt and CG4572$^{-/-}$ flies during the course of a viral infection at 0, 6 and 24 hrs post infection (hpi). (**a**) DXV infection (inoculated at 100 $TCID_{50}$/fly). (**b**) DCV infection (inoculated at 100 $TCID_{50}$/fly). CG4572 RNA expression level was monitored during the infections for (**c**) DXV or (**d**) DCV. (**a-d**) is one representative experiment of three independent ones. For each time points, 3 technical replicates of 5 flies each were analyzed in triplicate. Histogram bars represent the mean of 9 identical samples, with error bars corresponding to standard deviation. All samples are normalized to rp49, as endogenous control.



Supplementary Figure 3
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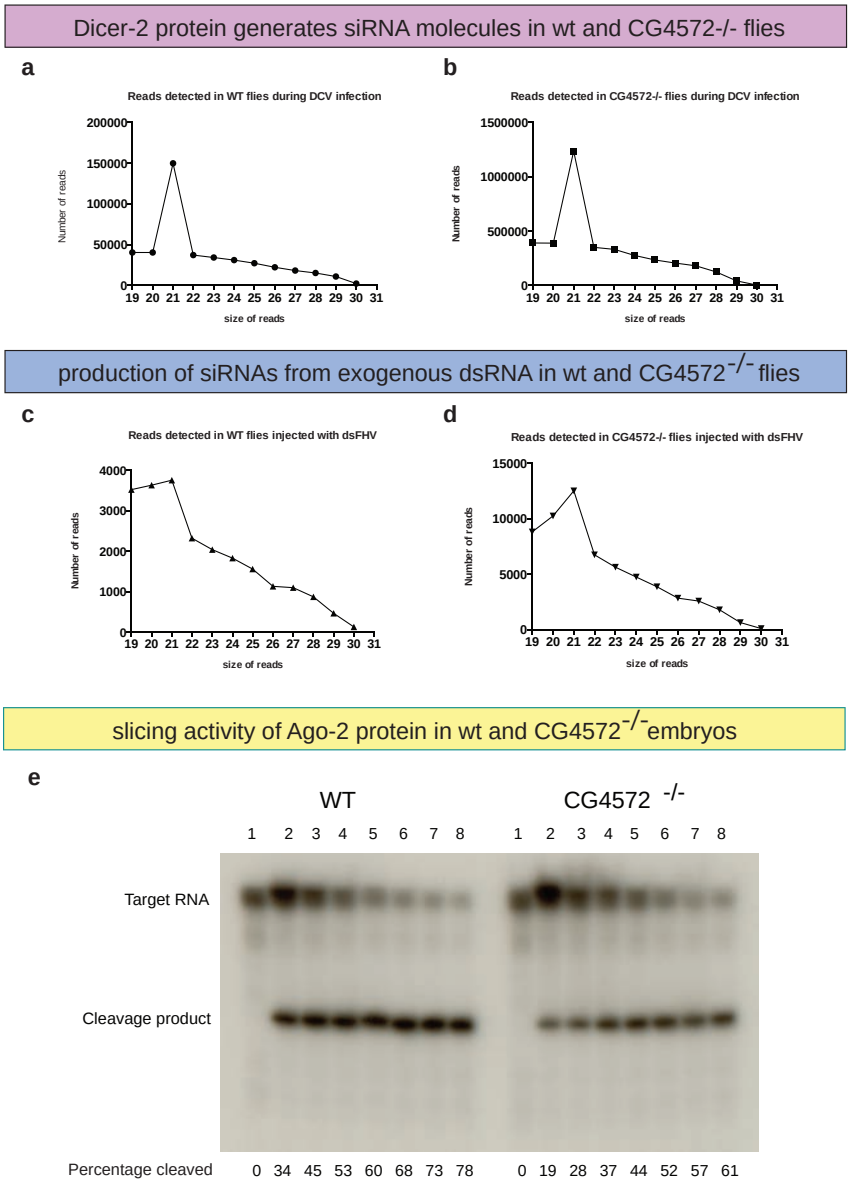
Supplementary Figure 3. Survival curves following virus infection. Five-days old flies were injected with (**a**) Sindbis virus, an enveloped positive stranded RNA virus (500 pfu/fly), (**b**) VSV, a non-enveloped negative stranded RNA virus (5000 pfu/fly) and (**c**) DXV, a non-enveloped double stranded RNA (dsRNA) (100 $TCiD_{50}$/fly). Wild type, $Dcr2^{-/-}$ and $CG4572^{-/-}$ flies were used. As in Figure 1, Tris injection was used to control the impact of the injection *per se*. Data from one experiment representative of three. The values represent the mean and SD of three independent groups of 15 flies each by experiment.



Supplementary Figure 4
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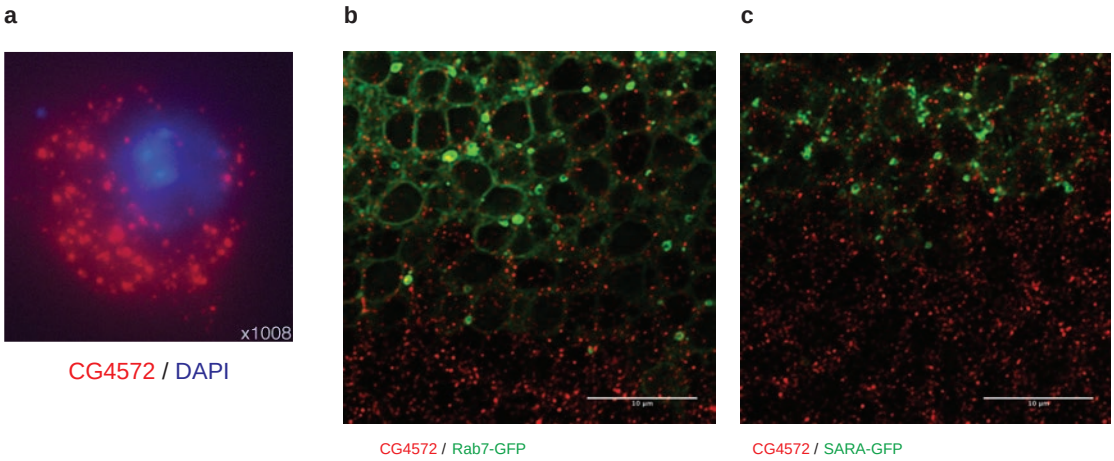
Supplementary Figure 4. CG4572 does not interfere with cell autonomous RNAi. 3 days old wild type or CG4572$^{-/-}$ flies were inoculated in the thorax with DCV (100 $TCID_{50}$/fly) or with 50 nL of dsRNA against Flock house virus (3 µg/µL). Two days after inoculation, total RNA was extracted and a deep-sequencing analysis was performed. (**a**-**b**) Length distribution of viral reads for DCV infection in wt and CG4572$^{-/-}$ flies. (**c**-**d**) Length distribution of reads for dsRNA against FHV (dsFHV) in wt and CG4572$^{-/-}$ flies. (**e**) Slicing assay lysates of wt (left panel) and CG4572$^{-/-}$ (right panel) embryos. Target RNA: Cap-labeled target RNA was incubated with control siRNA (lane 1, 180 min incubation) or specific siRNA (lane 2-8) and RNA was purified, separated on a polyacrylamide gel, and exposed to a film. Lane 2, 15 min incubation; lane 3, 30 min; lane 4, 45 min; lane 5, 60 min; lane 6, 90 min; lane 7, 120 min; lane 8, 180 min). No cleavage product was observed for the nonspecific control siRNA, whereas a specific cleavage product was observed for the specific siRNA (lane 2-8). Percentage cleavage is indicated at the bottom of the figure. The figure is representative for at least three experiments using independent embryo lysates.



Supplementary Figure 5
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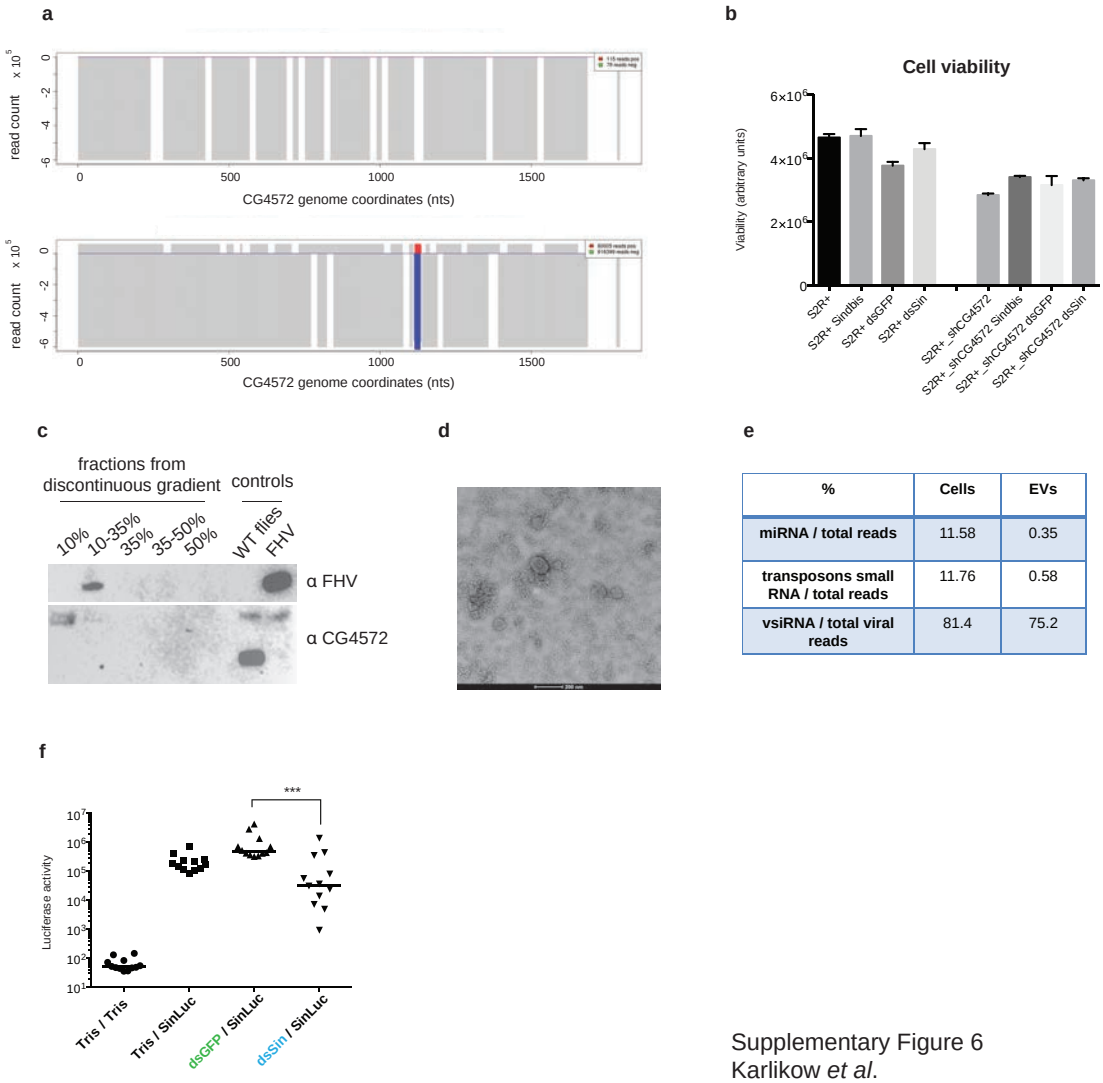
Supplementary Figure 5. CG4572 localizes *in vivo* in late endocytic compartments. (**a**) Immunofluorescence followed by confocal microscopy was performed on S2 *drosophila* cells. (**b**) Wing discs of L3 larvae expressing Rab-7-GFP (marker of late endosomes) or (**c**) SARA-GFP (marker of multivesicular bodies) were dissected, stained for CG4572 (in red) and imaged on a confocal microscope. Size bar represent 10 µm.



%	Cells	EVs
miRNA / total reads	11.58	0.35
transposons small RNA / total reads	11.76	0.58
vsiRNA / total viral reads	81.4	75.2

Supplementary Figure 6
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Supplementary Figure 6. Validation steps on EVs collection procedure. (**a**) S2R+ (top panel) or S2R+ stable cell line expressing an endogenous hairpin against CG4572 (bottom panel) were analyzed by deep sequencing. Alignment of small RNAs reads on CG4572 CDS region. A miRNA specific for CG4572 were produced only in cells expressing the short hairpin against CG4572, as visualized by positive (red) and negative (blue) strands of the miRNA. The gray bars represent nucleotides without coverage. (**b**) Cell viability for cells used to purify EVs. (**c**) Purification by Optiprep discontinuous gradient of the vesicular fraction containing CG4572 protein. Different density fractions were analyzed by WB for the presence of CG4572 and FHV, which persistently infects S2R+ cells. (**d**) Electron microscopy on the Optiprep fraction that contains CG4572. (**e**) Percentage of reads for miRNA, transposons and viral siRNAs of 21 nts that were found in EVs, compared to the percentage present in S2R+ cells. (**f**) wt flies were injected with Tris, dsGFP (as unrelated control, green) or dsSin (blue) to verify that the immunization protocol was functional. *** p=0.0009. Statistical analysis was performed with Prism 6 software using a non-parametric Mann-Whitney test (n=12 for each condition).

CHAPTER 2

PART 2

Extracellular vesicles and their RNA content: High throughput sequencing analysis

Here, I present in detail the various information we obtained from analyses of the high throughput sequencing performed on the RNA content of extracellular vesicles. The results of the immunization experiment correlated to these analyses, provide clues on the nature of the immune signal during systemic antiviral RNAi.

8. SMALL RNA CONTENT OF EVs

8.1. EVs purified from cells that soaked on dsSin

EVs purified from S2R+ cells that soaked dsSin, were not able to immunize flies against SinV infection (Chapter 2, part 1). However, these EVs contained siRNAs produced from the dsSindbis fragments (Fig. 7a). These siRNAs mapped on the positive (red) and the negative (blue) strand of SinV, in the region of the genome corresponding to the dsRNA fragments used (Fig. 7b).

As mentioned previously, S2R+ cells are persistently infected with different viruses (DCV, DAV, ANV, DXV, CrPV, DBV, FHV). Interestingly, viral siRNAs corresponding to all these viruses were found inside EVs. As an example, I present in Fig 7c-d the size distribution of viral reads for DCV and their location on DCV genome.

Therefore, EVs are loaded with siRNAs (from soaked dsRNA) and with vsiRNAs (from persistent virus infection) and their incapacity to immunize flies is not due to an absence of siRNAs.

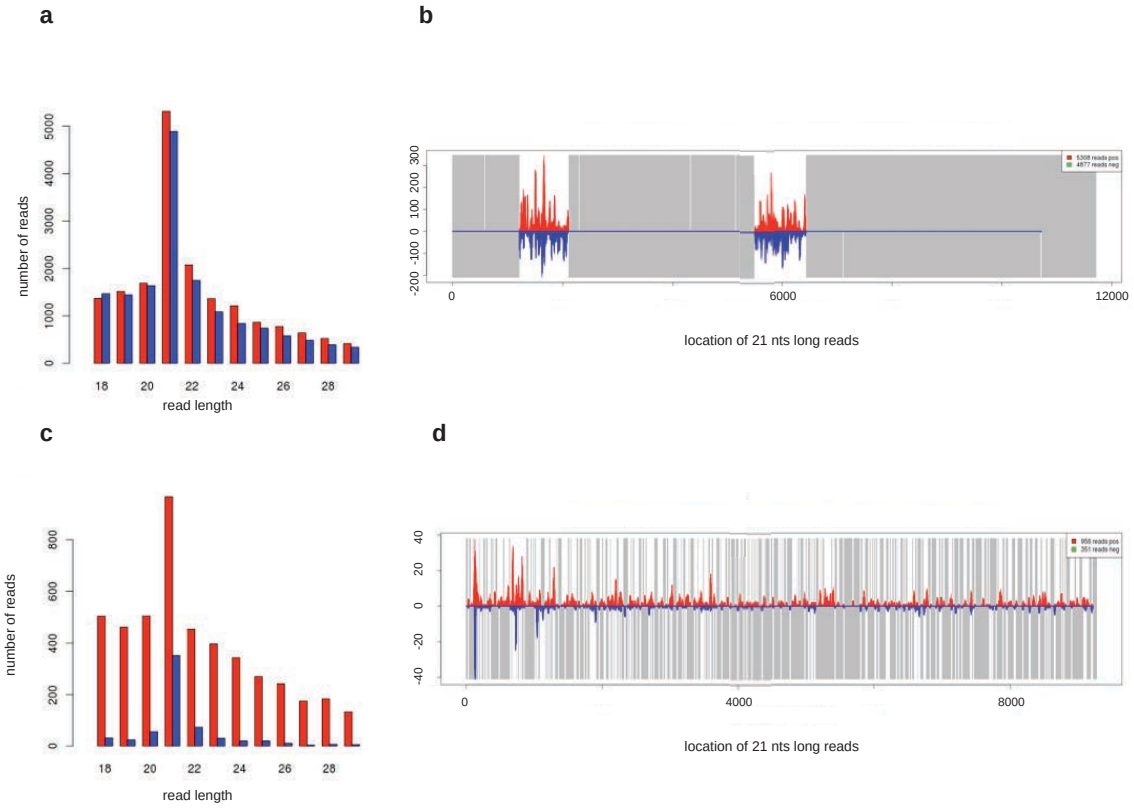

Figure 7. Deep-sequencing analysis of EVs purified after soaking of dsRNA into S2R+ cells. (**a**) Length and number of Sindbis RNA reads. (**b**) Alignment of 21 nts long Sindbis reads along Sindbis virus genome. (**c**) Length and number of DCV RNA reads. (**d**) Alignment of 21 nts long DCV reads along DCV virus genome. Positive strand small RNAs are presented in red and negative strand in blue. The gray bars represent nucleotides position without coverage.

8.2. EVs purified from cells infected with Sindbis virus

EVs purified from cells infected with SinV were able to immunize non-infected wt flies against further Sindbis infection. Surprisingly, these EVs did not contained vsiRNAs against Sindbis (Fig. 8a) as no clear peak at 21 nts was detected. A very low number of reads at 21 nts were mapped along Sindbis genome (Fig. 8b). However, those EVs contained vsiRNAs against all the other viruses persistently infecting S2R+ cells (i.e., DCV Fig. 8c-d).

The absence of SinV vsiRNAs, prompted us to search into other RNAs detected during deep sequencing of EVs.

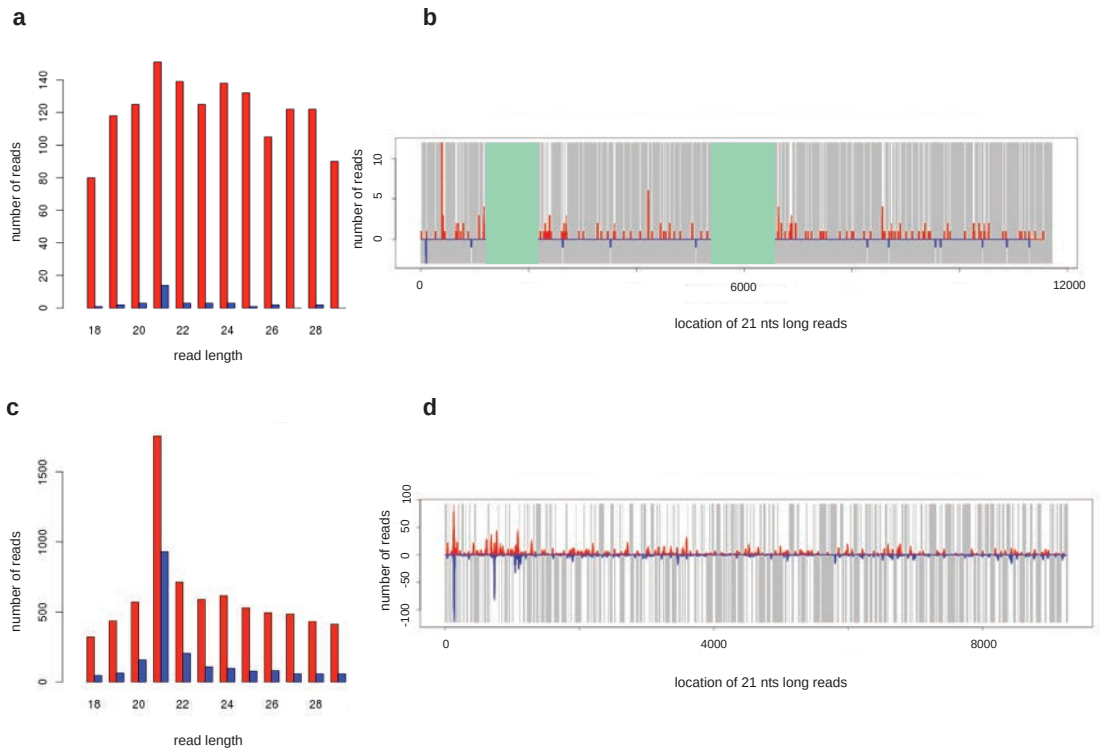

Figure 8. Deep sequencing analysis of EVs purified after acute infection with Sindbis. (**a**) Length and number of Sindbis RNA reads. (**b**) Alignment of Sindbis 21 nts long reads along Sindbis virus genome. Regions corresponding to dsSin were extracted during analysis in order to avoid risk of cross-contamination (green rectangle). (**c**) Length and number of DCV RNA reads. (**d**) Alignment of DCV 21 nts long RNA reads along DCV virus genome. Positive strand small RNAs are presented in red and negative strand in blue. The gray bars represent nucleotides position without coverage.

9. LONG RNA CONTENT OF EVs

The small RNA deep sequencing protocol that we used, allows the sequencing of small RNAs from 18 up to 51 nts. Therefore, all RNAs of- or longer than- 51 nts would be detected as reads of 51 nts.

9.1. EVs purified from cells that soaked on dsSin

As seen in Figure 9a, two types of RNA molecules were detected in EVs that failed at immunize wt flies: siRNAs of 21 nts (see above and Fig 7a-b) and longer RNAs (peak at 51 nts) that were also perfectly double stranded (Fig. 9b). Those fragments of 51 nts localize at the same position than the dsSin the cells were soaked with (Fig. 9b) and are therefore fragments of dsSin. The same size distribution of viral reads was observed for DCV (Fig. 9c-d) with a peak at 21 nts and another at 51 nts, with reads mapping along DCV genome. The 51 nts long RNA fragments were almost entirely corresponding to the positive strand of DCV, and we assumed they represented products of viral degradation or abortive viral genomes.

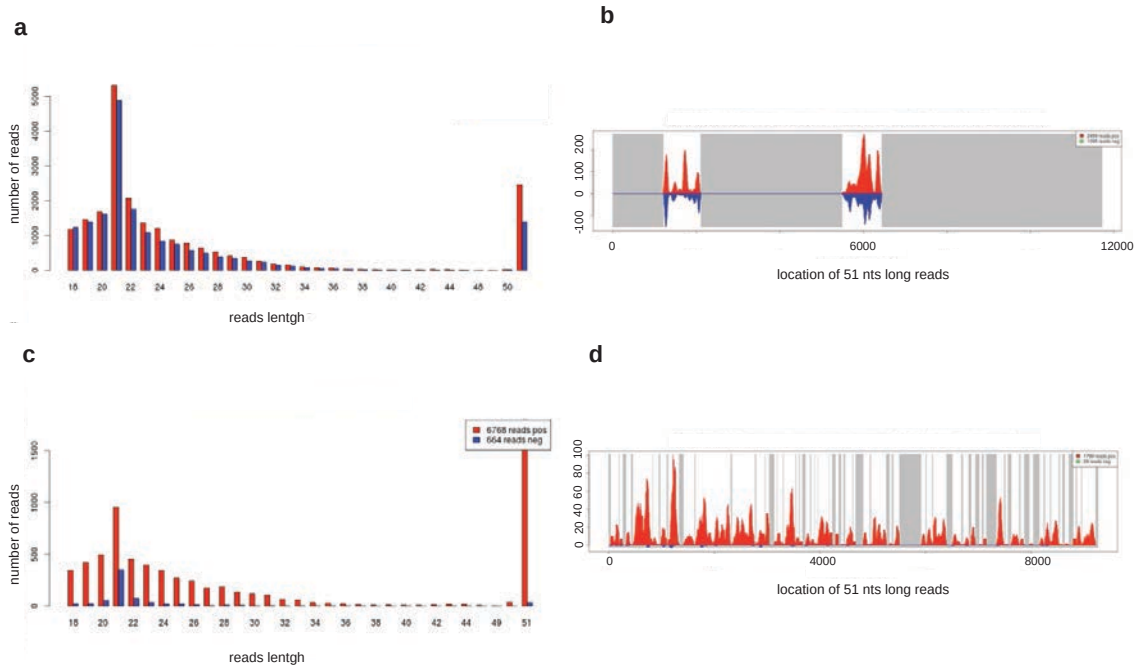

Figure 9. EVs purified from cells that soaked on dsSindbis contain fragments of viral RNA. (**a**) Length and number of Sindbis RNA reads. (**b**) Alignment of Sindbis 51 nts long reads along Sindbis virus genome. (**c**) Length and number of DCV RNA reads. (**d**) Alignment of DCV 51 nts long RNA reads along DCV virus genome. Positive strand small RNAs are presented in red and negative strand in blue. The gray bars represent nucleotides position without coverage.

9.2. EVs purified from cells infected with Sindbis virus

As shown in Figure 8a-b, no vsiRNAs against Sindbis were detected during Sindbis infection of S2R+ cells, however, 51 nts fragments of viral RNA were detected and these reads aligned all over Sindbis genome (Fig. 10a-b). These reads corresponded only to the positive strand of SinV. As for DCV, the same results as for the precedent section were obtained: in addition of the vsiRNAs, a peak at 51 nts RNAs mostly corresponding to the positive strand of the virus was detected (Fig. 10c-d).

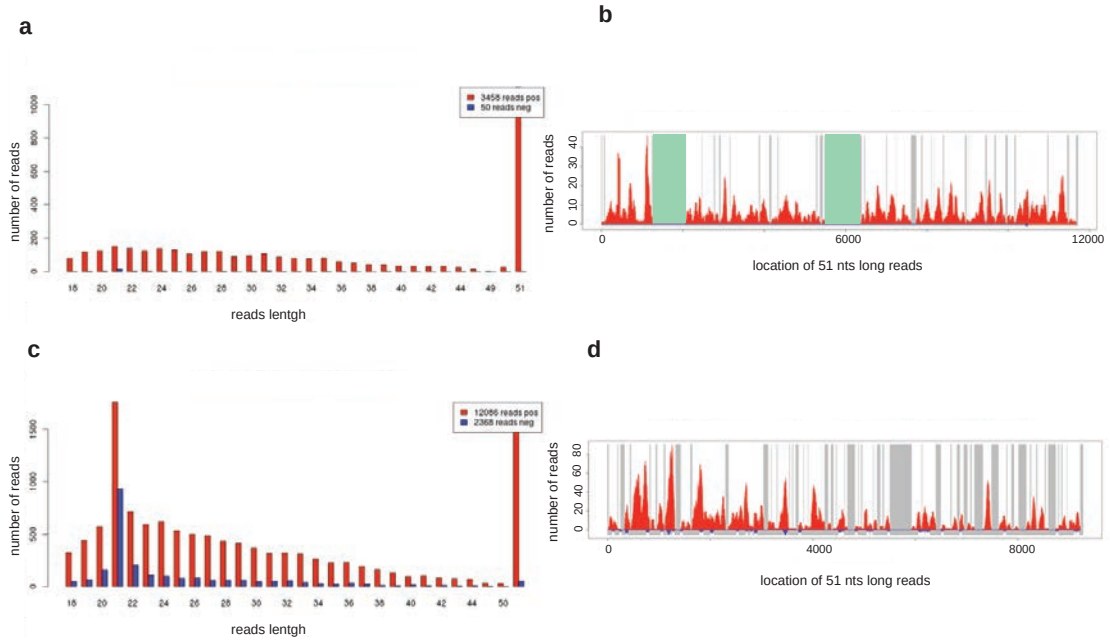

Figure 10. EVs purified after Sindbis acute infection contain fragment of viral RNA. (**a**) Length and number of Sindbis RNA reads. (**b**) Alignment of Sindbis 51 nts long reads along Sindbis virus genome. Regions corresponding to dsSin were extracted during analysis in order to avoid risk of cross-contamination (green rectangle). (**c**) Length and number of DCV RNA reads. (**d**) Alignment of DCV 51 nts long RNA reads along DCV virus genome. Positive strand small RNAs are presented in red and negative strand in blue. The gray bars represent nucleotides position without coverage.

The following points summarize the first part of the bioinformatics analysis:

- Only EVs purified from cells infected with SinV were able to immunize flies.
    - These EVs were depleted on vsiRNAs corresponding to SinV, but not in vsiRNAs corresponding to the viruses persistently infecting S2R+ cells.
    - These EVs were rich in viral fragments of at least 51 nts matching the genome of SinV (positive strand) or the genome of the persistent viruses.

- EVs purified from cells soaked with dsSin but not infected with SinV, failed to immunize flies.
    - These EVs were loaded with siRNAs from the dsRNA fragment that cells soaked, as well as vsiRNAs from the persistently infecting viruses.
    - These EVs were also loaded with viral fragments of at least 51 nts corresponding to the dsRNA fragment of the soaked dsRNA, or to the genome of the persistent viruses (positive strand).

This first part of the analysis strongly suggests that vsiRNAS are not the immune signal that is spreading but it appears that long RNA fragments are.
We decided therefore to analyze the composition of long viral RNA fragments detected in the EVs.

10. QUANTIFICATION OF THE RNA CONTENT OF EVs

The immunization experiment developed in Chapter 2 part 1, used three types of EVs: *(i)* EVs purified from S2R+ cells soaked with dsGFP (Fig. 11, in green); *(ii)* EVs purified from S2R+ cells soaked with dsSin (Fig. 11, in blue); and *(iii)* EVs purified from S2R+ cells infected with SinV (Fig. 11, in pink).

We calculated for these three types of EVs, the percentage of different types of RNAs relative to the total number of reads of RNA that were sequenced and we plotted them (Figure 11). The RNA content for different RNA types was the same for the three different EV populations.

However, we knew that EVs content was not the reflection of the total cellular RNA. Indeed, EVs contained less than 1% of miRNA and transposons small RNAs, compared to their origin cells (that contained more than 10%). This observation, not only confirm the accuracy of our EVs purification protocol but also indicated that EVs were specifically enriched for viral small RNAs.

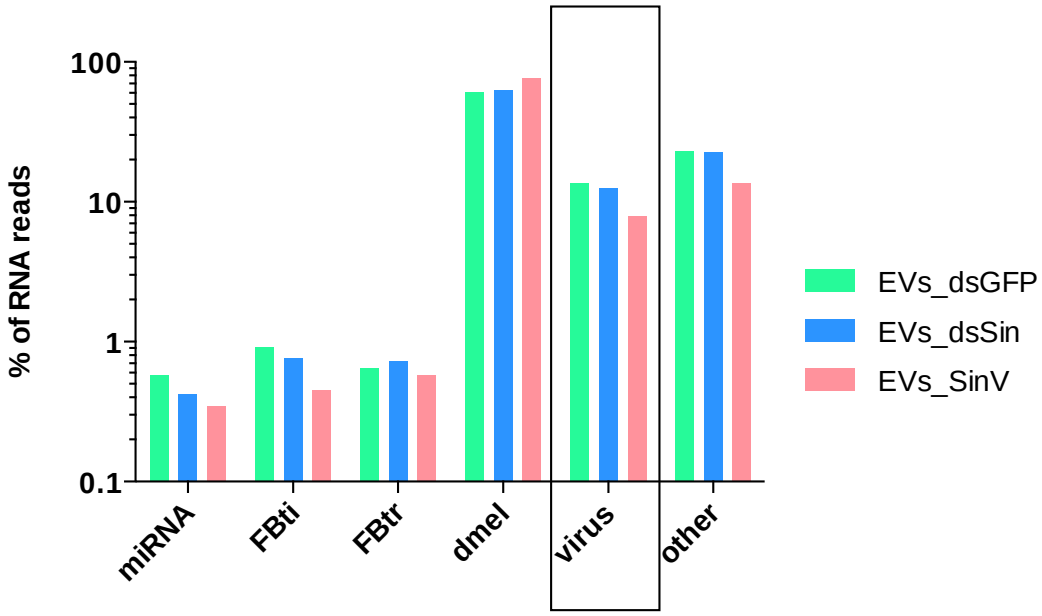

Figure 11. RNA composition of extracellular vesicles. Reads detected from three different EVs preparation were analyzed for the presence of miRNA reads, transposons small RNAs (FBti) reads, RNA transcripts (FBtr) reads, *Drosophila melanogaster* transcripts reads, viral RNA or other RNA. The amount of RNA is expressed as the log of the percentage of the total RNA per EVs.

As the RNA composition of EVs purified after dsGFP soaking was the same as the two others, we continued the analysis only with EVs purified after dsSin soaking (blue) and after Sindbis infection (pink). It is not clear why one type was able to immunize non-infected flies and the other type was not. To address this question, we analyzed the viral RNA composition of both EVs in more detail.

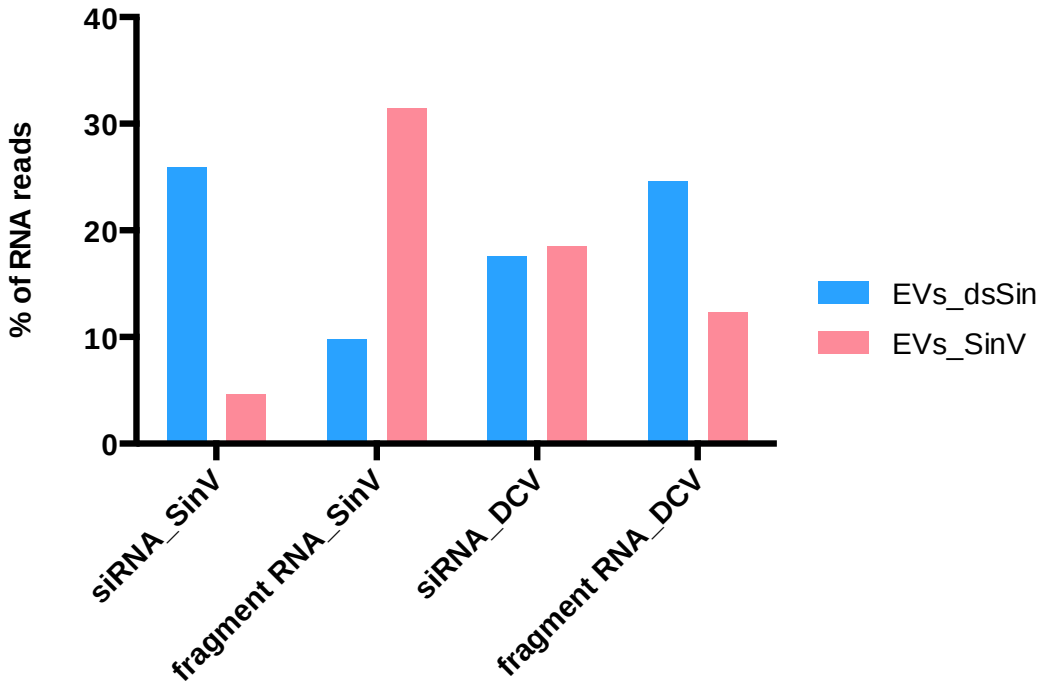

Figure 12. Viral RNA composition of extracellular vesicles. Viral RNA reads were separated as siRNA and other fragments of viral RNA (51 nts reads) matching for Sindbis or DCV virus. The amount of RNA is expressed as the percentage of the viral RNA per EVs.

Figure 12 shows that EVs from cells that soaked on dsSindbis (dsSin, blue) contained 5 times more vsiRNAs (26%) than EVs from cells infected with SinV (SinV, pink) (only 4.5%).

Inversely, EVs from cells that soaked on dsSindbis contained only 10% of 51 nts viral RNA fragments, in comparison to the 32% of viral RNA fragments found in EVs from cells infected with SinV.

We applied the same analysis to DCV reads, present in both EVs preparations. We detected a very similar composition in term of viral siRNAs and a difference, although much less drastic, in long viral RNAs of 51 nts length.

This analysis strongly suggests that siRNAs are not the immune signal that spread through EVs in a systemic manner during viral infection. It also indicates a non-dsRNA nature of the systemic immune signal in *Drosophila*. Taken together, the bioinformatics analyses point to long viral RNA fragments as the putative immune signal.

CHAPTER 2

PART 3

Drosophila cells use tunneling nanotubes to transport dsRNA and RNAi machinery between cells

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In revision in *Scientific Reports*

Brief introduction to TNTs section

While studying CG4572 and Ago-2 interactions by confocal microscopy, I noticed a stream of positive dots for Ago-2 between cells (Fig 13). These dots did not attach to the substratum and therefore were not filopodia, and were positive for F-actin staining. As their aspect reminds to membranous tubular connections between cells, I decided to investigate these structures.

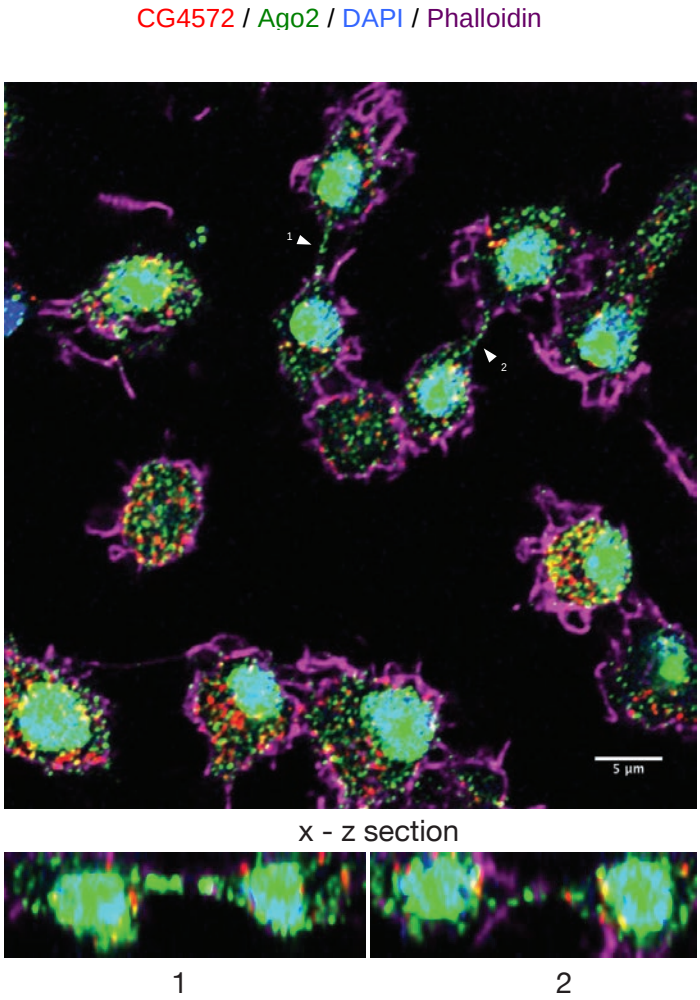

Figure 13. Stream of Ago-2 dots between cells. The intriguing structures are marked by arrows numbered 1 and 2. The (x-z) sections are shown below. Size bar is 5 µm.

***Drosophila* cells use tunneling nanotubes to transport dsRNA and RNAi machinery between cells**

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Summary

Tunneling nanotubes (TNTs) function as highways for the transport of organelles, cytosolic and membrane-bound molecules, and pathogens between cells. During viral infection in the model organism *Drosophila melanogaster*, a systemic RNAi antiviral response is established presumably through the transport of a silencing signal from one cell to another *via* an unknown mechanism. Because of their role in cell-cell communication, we investigated whether TNTs could be a mediator of the silencing signal. Here, we describe for the first time the presence of TNTs in different *Drosophila* cell types. TNTs associated with components of the RNAi machinery including Argonaute 2, dsRNA, and CG4572. Moreover, TNTs were more abundant during viral, but not bacterial, infection. Our results suggest that TNTs are one of the mechanisms by which the primed antiviral RNAi machinery is transported between infected and non-infected cells to trigger systemic antiviral immunity in *Drosophila*.

Highlights

- Tunneling nanotubes, a form of cell-cell communication, are present in *Drosophila* cells
- The RNAi machinery, dsRNA and viral proteins localize within tunneling nanotubes (TNTs)
- TNTs are more abundant during viral infection
- TNTs may facilitate cell-cell communication to establish a systemic antiviral response

Introduction

To establish systemic immunity and protect both the site of initial infection and the entire organism, all multicellular species have developed sophisticated ways to communicate immune signals. These signals must be disseminated between cells locally and throughout the organism to avoid pathogen propagation and establishment of the infection. Not long ago, it was proposed that mammalian immune cells (such as dendritic cells and macrophages) transmit signals to distant cells through a network of physically connected tunneling nanotubes (TNT)[1-4]. TNTs were first described in rat neuro-derived cells[5], and since then in a wide variety of mammalian cells where they act as a route for transport of cytosolic and membrane-bound molecules, organelles, and pathogens such as HIV[6-10]. Similar structures are widely observed. In higher plants, plasmodesmata (a structure composed of desmotubules)[11] are continuously lined by the plasma membrane allowing the transport of molecules such as nutrients, hormones, regulatory proteins, and RNA from one cell to another[12-14]. In bacteria, nanotubes bridge neighboring cells for exchange of molecules within and between species[15]. Filamentous connections that resemble nanotubes link gametes during malarial parasite reproduction in the mosquito midgut[16]. In *Drosophila*, cytonemes in the wing imaginal disc are a type of filopodia in which morphogen signaling proteins move between producing and target cells[17-19].

Insects are well-known vectors of a variety of pathogens including viruses, bacteria, protozoa and nematodes[20]. Although insect-borne viral diseases have been a threat to humans since recorded history, insect-virus interactions and mechanisms of insect antiviral immunity remain poorly characterized[21]. The discovery of RNA interference (RNAi) as the major antiviral immune mechanism in invertebrates[22-25] has opened new avenues to understand insect immunity. RNAi refers to sequence-specific RNA-dependent silencing mechanisms[26,27] that regulate various processes such as gene expression[28], epigenetic modifications[29] and defense against pathogens[30]. Antiviral RNAi is naturally triggered by virus-derived double-stranded RNA (dsRNA) molecules. These long viral dsRNA molecules prompt the small-interfering RNA (siRNA) pathway[26], silencing both

viral dsRNA replicative intermediates as well as viral genomes[31-33].

The RNAi mechanism is described as either cell-autonomous or non-cell-autonomous[26,34]. In cell-autonomous RNAi, the silencing process is limited to the cell in which the dsRNA is introduced or expressed. In non-cell-autonomous RNAi, the interfering effect occurs in cells distinct from those where the dsRNA was produced. Non-cell-autonomous RNAi presumes that a silencing signal is transported from one cell to another via an unknown mechanism to establish antiviral systemic immunity[35,36].

Because of their role in cell-cell communication, we investigated whether membrane-tunneling nanotubes could be one of the mediators that connect *Drosophila* cells in order to establish a systemic RNAi-mediated antiviral immune response. We describe for the first time the presence of TNTs in different *Drosophila* cell types. The nanotubes were open-ended and were associated with components of the RNAi system including Argonaute 2, dsRNA, and CG4572. We postulate that the spread of the silencing signal in insects relies, among other cellular mechanisms, on TNT intercellular connections.

Results

Drosophila cells are connected to neighboring cells by membrane tunneling nanotubes

To test for the presence of membranous connections or nanotubes between cells, we established two stable *Drosophila* S2 cell lines: one expressing dsRed and the other eGFP, each under the control of an actin promoter. This allowed us to distinguish cell-cell connectors from remnants of incomplete cytokinesis events. Cells were mixed 1:1, adhered overnight on glass coverslips, fixed and analyzed by confocal microscopy. Membrane projections connecting cells were readily observed (Figure 1a-c). These projections could be half red/half green (Figure 1a, arrow), entirely red towards a green cell (Figure 1b, arrow) or

entirely green towards a red cell (Figure 1c, arrow). The membrane projections contained F-actin, as evidenced by positive staining with fluorophore-conjugated phalloidin (Figure 1d). However, they were not attached to the substratum, which would be characteristic of filopodia. Together, these features are indicative of membrane tunneling nanotubes (TNT)[37]. Similar membrane projections were identified in another *Drosophila* cell line, Kc167 (Supplementary Figure 1), suggesting that membrane bridges and TNTs may be a general feature in *Drosophila*.

To investigate the structure of these tubes, and to further confirm the confocal results, we performed scanning electron microscopy (SEM) and correlative microscopy on S2 cells. SEM revealed the presence of projections connecting neighboring cells (Figure 1e-f). Correlative microscopy indicated that these connections had the same features of TNT observed by confocal microscopy, including non-adherence and the presence of F-actin (Supplementary Figure 2)[19]. The average diameter of the TNTs was 250 nm (n=12), in agreement with the diameter already published[2,5]. Neighboring cells were often connected by a single nanotube (Figure 1e); but multiple nanotube connections were also observed (Figure 1f). When cell contacts were examined at higher magnification, (Figure 1g and h), open connections to the cell surface were visible. Thus, while there remains a debate as to whether TNTs have open or closed ends[5,10,37-39], our data suggest that they could be open ended.

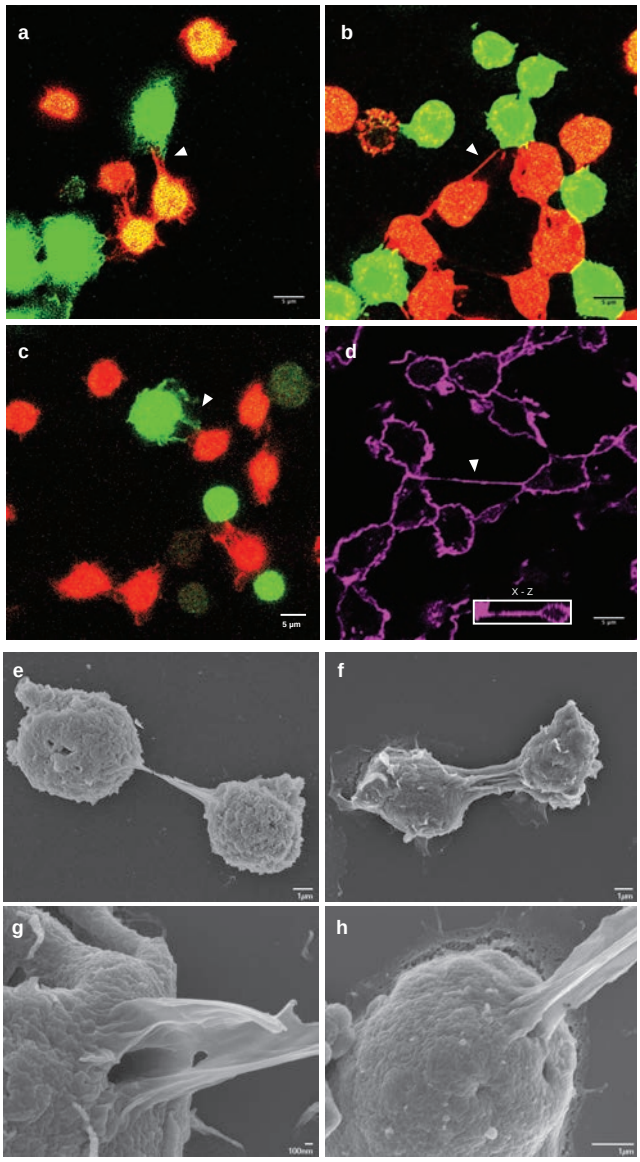

Figure 1: Tunneling-nanotubes are present in *Drosophila* cells. Stable cell lines expressing eGFP or dsRed under the control of an actin promoter were mixed at a 1:1 ratio, grown overnight and examined by confocal microscopy (**a**, **b** and **c**). Cells were stained for F-actin using Phalloidin 647 Alexa-Fluor (**d**). Arrows indicate projections between cells and bars represent 5 µm. The inset in (d) depicts the corresponding (*x-z*) section through the marked TNT (arrow). Scanning electron microscopy of S2 cells showing projections between cells (**e** and **f**) as well as possible open-ended termini of TNTs (**g** and **h**).

Figure 1
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The RNAi machinery localizes within TNTs

To determine if the TNTs participate in RNAi signaling, we looked for the presence of RNAi markers in association with TNTs. We also looked for late endosomal vesicles described as essential for an effective RNAi response[40-43]. Rab7 (Figure 2a-c), a marker for late endosomes, and Ago2, the main actor of the antiviral RNAi response[44], were each detected in TNTs and could be observed localizing within the same tubule (Figure 2b, arrow and 2c). Following infection

with Flock house virus (FHV), Ago2, dsRNA (Figure 2d-f) and CG4572 (Figure 2g-i), a *Drosophila* protein that has been involved in the spread of the RNAi signal *in vivo*[36], localize within TNTs. These data support the hypothesis that TNTs play a role in cell-cell communication during the RNAi response.

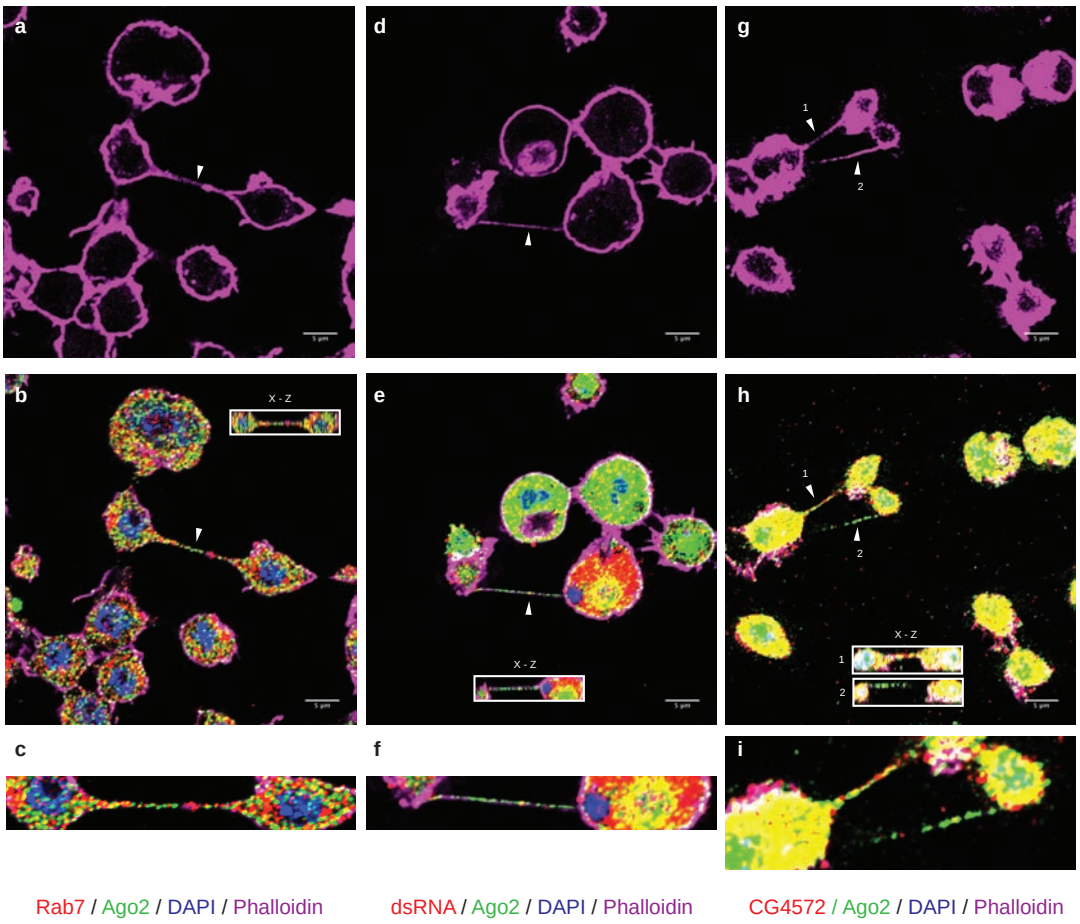

Figure 2
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Figure 2: The RNAi machinery localizes in TNTs. Immunofluorescence and confocal microscopy in S2 cells. Cells were stained for F-actin using Phalloidin 647 Alexa-Fluor to show membrane continuity between connected cells (**a**, **d**, **g**). Rab7 and Ago-2 (**b**, **c**), dsRNA and Ago-2 (**e**, **f**) and CG4572 protein and Ago-2 (**h**, **i**) were detected in TNTs. DAPI is used to mark nuclei. The insets in (**b**, **e**, **h**) depict the corresponding (*x-z*) section through the marked TNT (arrow). Higher magnification images of TNTs (arrow) and RNAi proteins are shown in (**c**, **f**, **i**).

Virus infected cells show more abundant TNTs

To explore a possible role for TNTs in antiviral immunity, we then tested whether the abundance of TNTs changed in relation to the infection status of the cell. Non-infected S2 cells (S2n) or cells persistently infected with FHV (S2p) were adhered on coverslips for 12 hours and at least 1300 cells were examined by confocal microscopy as above. There was a significant increase in the number of TNT connections in virus-infected cells, with connections observed in 10.75% of S2p cells but in only 3.75% S2n cells (Figure 3a). Connections were found in 9.35% of S2R+ cells, another *Drosophila* cell line persistently infected with DAV, FHV and DXV (Figure 3a). TNT formation could be a consequence of stress due to infection rather than a means of cell-cell communication during viral infection. Therefore, we counted TNTs during infection of S2 cells with a bacterium *Erwinia carotovora*. As shown in Figure 3a, after 8 hours of bacterial infection, bacteria-infected cells display as many TNTs as non-infected cells (4.08% and 4.57% respectively, n>600). We then checked for the presence of viral proteins associated with the TNTs of infected cells using polyclonal antibodies generated against viral particles. Readily detectable levels of DCV (Figure 3b-c) and FHV (Figure 3d-e) capsid protein were present in TNTs together with CG4572, raising the possibility that TNTs are a mechanism for cell-cell spread of virus infection.

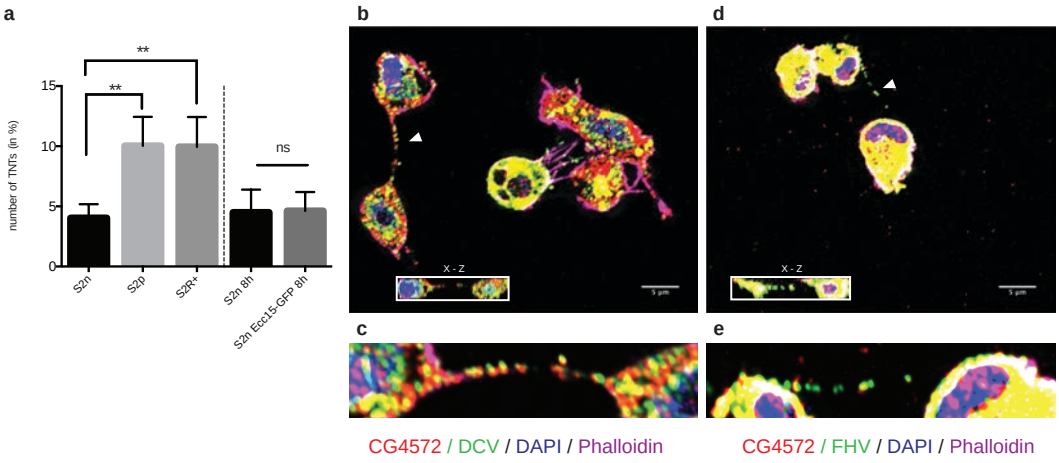

Figure 3
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Figure 3: TNTs are more abundant during viral infection. (**a**) Non-infected S2 cells (S2n), S2 cells persistently infected with either FHV (S2p), or with DAV, DXV and FHV (S2R+)), and S2 cells infected with bacteria (S2n Ecc15-GFP) were plated on glass coverslips overnight as described in Materials and Methods. Cells were stained for DAPI and Phalloidin, and TNTs were counted in at least 1000 cells per treatment group. Error bars indicate standard deviation. **p<0.005, ns: non-significant (non-parametric Mann-Whitney test). (**b** - **d**) Immunofluorescence during acute infection of S2 cells with DCV (**b**, **c**) or FHV (**d**, **e**). Cells were stained for CG4572 and viral capsid. DAPI and Phalloidin were used to mark nuclei and F-actin, respectively. The insets in (**b**, **d**) depict the corresponding (*x-z*) section through the marked TNT (arrow). Higher magnification images of TNTs (arrow), viral proteins and CG4572 are shown in (**c**, **e**).

Discussion

Intercellular communication must be highly selective and tightly regulated as it is essential for the survival of any organism. In recent years, cell-cell connections between animal cells including tunneling nanotubes and filopodia were discovered and proposed to allow the trafficking of cytoplasmic material[5], the transmission of calcium signals[45], or pathogens[10,46]. Here we identify TNTs in *Drosophila*, a model organism to study innate immunity, and we provide evidence that they also function in cell-cell communication in response to virus infection. *Drosophila* cells show TNTs that are positive for F-actin staining, are non-adherent, and are seemingly open-ended. The TNTs were found to contain components of the RNAi system, including Ago2, dsRNA, Rab7, and CG4572[42].

In addition, they increased in abundance during virus infection and contained viral capsid proteins, some of which localized with components of the RNAi machinery. Together, this suggests a role for TNTs in establishing systemic RNAi anti-viral immunity.

Both insects and derived cell lines can survive in the presence of several viruses without any major fitness cost or cytotoxic response, the so-called persistent viral infection. The systemic RNAi response may facilitate the persistent state by limiting virus replication and cytotoxicity associated with spread of an acute virus infection[47]. If so, cell-cell signaling of the antiviral response via TNTs may also play a role establishing viral persistence.

In 2011, Lopez-Montero et al.[48] observed that mosquito cells infected with an arbovirus (Bunyamwera virus) developed a complex network of filopodial-like bridges and proposed they could serve for virus propagation but most likely to spread protective signals between cells. Our results suggest that TNTs are one of the mechanisms by which the primed antiviral RNAi machinery is transported from a donor to an acceptor cell to trigger intracellular antiviral immunity in the latter. The presence of viral capsid protein associated with the TNTs raise the possibility that TNTs may also be a means of cell-to-cell spread of virus, similar to HIV[10].

The observation that TNTs are present in *Drosophila* S2 cells constitutes a powerful model to study biogenesis of TNTs and mechanisms of cell-cell communication. For example, while TNTs are widely thought to play a role in cell-cell communication, it remains controversial if they are open or closed-ended[37]. We found evidence for open-ended tubes, yet it is not known if the RNAi components travel within the tubules, or on the surface of the tubules, and more studies are needed to resolve this question. Additionally, as *Drosophila* is a genetically tractable small animal susceptible to a number of natural virus infections, approaches could be developed to explore the relevance of TNTs for antiviral immunity *in vivo*, a question still pending in biology.

Experimental procedures

Cell culture:

Drosophila S2 cells (Schneider, 1972, invitrogen) were cultured at 25 °C in Schneider's *Drosophila* medium (invitrogen) supplemented with 10 % heat inactivated fetal bovine serum (FBS, invitrogen), 2 mM glutamine (invitrogen), 100 $U.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ streptomycin (invitrogen).

Transfection and stable cell line establishment:

$1x10^7$ cells were cotransfected with pAc5.1B-eGFP (plasmid #21181 from AddGene) or with pAc-dsRed (kindly provided by Dr F. Coumailleau) and pCoBlast vector (invitrogen) in a 19:1 ratio using Effectene reagent (Qiagen). Five days later, cells were selected by replacing Schneider's complete *Drosophila* media with fresh one supplemented with Blasticidin (25 $\mu g.mL^{-1}$, Euromedex). By 14 days later, 98% of cells expressed eGFP or dsRed protein.

Immunofluorescence:

Drosophila cells were cultured overnight (o/n) on coverslips at a concentration of 10^6 cells/mL in complete Schneider's *Drosophila* medium, as described above. Cells were then fixed in paraformaldehyde diluted in PBS to 4% (PFA, Alfa Aesar) during 15 minutes (min) and washed two times in PBS for 5 min. Following the fixation step, permeabilization was done in PBS 0.1% Triton X-100 for 5 min three times. Cells were then incubated with primary antibodies in PBS-Triton supplemented with 3% FBS for at least 1 hour (hr) at RT (rabbit αFHV 1/500, rabbit αDCV 1/500, and mouse αCG4572 1/500 are three home-made antibodies, mouse anti-dsRNA αK1 1/1000 Scicons, rabbit αAgo2 1/500 Abcam or mouse αAgo2 1/100 kindly provided by Pr. Siomi and rabbit αRab7 1/2000 kindly provided by Pr. Nakamura). After three washes in PBS-Triton, cells were incubated with secondary antibody (Alexa-Fluor 1/1000, invitrogen), DAPI (1/10.000, life technologies) and Phalloidin-647 (Alexa-Fluor 1/200, invitrogen) diluted in PBS-Triton with 3% FBS for 1 hr at RT. Cells were washed two times in PBS-Triton for 5 min, once in PBS and finally mounted on glass slide with Fluoromount G (eBioscience) and imaged with a confocal microscope LSM 700

inverted (Zeiss) at a 63X magnification oil immersion lens. Brightness and color balance in some images have been changed only in order to increase visibility of TNTs. Confocal stacks were reconstructed with Huygens Professional software (SVI).

Virus and bacteria infections:

Drosophila S2 cells were cultured on glass coverslip as previously described, during an o/n acute infection for DCV (1 M.O.I.) or FHV (1 M.O.I.). The next day, cells where harvested, washed, fixed, and immunofluorescence was performed. For persistently infected cells with DCV or FHV, cells were grown as described and IF was performed. For bacteria infections, *Drosophila* S2 cells were washed twice (5 min at 500 rpm) with Schneider media without antibiotics and plated on glass-coverslips at $5x10^5$ per well o/n at 25°C. Bacterium *Erwinia carotovora carotovora 15* (Ecc15-GFP) (kindly provided by B. Lemaitre, [49]) was grown o/n at 29°C in LB supplemented with Rifampicin. The next day, bacteria were washed twice in PBS (5 min at 1000 rpm) and incubated on the glass-coverslip plated S2 cells at 250.000 bacteria/well (OD_{600}=0,3=$1x10^8$ cell/mL) for 45 min at 25°C. When adding bacteria on cells, a short spin (1 min at 3000 rpm) was performed for bacteria to sit on the cells. After 45 min of incubation, the bacteria-containing media was removed and replace with fresh Schneider media without antibiotics. *Drosophila* S2 cells infected with Ecc15-GFP were harvested at 8 hours post infection, fixed in PFA 4%, stained for DAPI and Phalloidin, and TNTs were counted as described above.

Correlative microscopy: CLEM-SEM

Cells were first imaged by fluorescence microscopy on an alphanumeric coded, grid-patterned glass (MatTek dishes). Samples were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) o/n at 4 °C, then washed in 0.2 M cacodylate buffer (pH 7.2), postfixed for 1 hr in 1 % osmium and rinsed with distilled water. Cells were dehydrated through a graded ethanol series followed by critical point drying with CO_2. Dried specimens were gold/palladium sputter-coated with a gun ionic evaporator PEC 682. The samples are imaged in a JEOL

JSM 6700F field emission scanning electron microscope operating at 5 kV.

Author contributions: Conceived and designed the experiments: M.K, V.M., B.G., C.Z. and M.C.S. Performed the experiments: M.K., C.S. and V.M. Analyzed the data: M.K, C.S., V.M., I.B., C.Z., B.G. and M.C.S. Wrote the paper: M.K., S.C. and M.C.S.

Acknowledgements:

We thank members of the Saleh lab and M. Vignuzzi for critical reading of the manuscript; S. Abounit for advice on TNTs; Pr A. Nakamura (RIKEN Center for Developmental Biology, Japan) for antibody to Rab7; Pr H. Siomi (Keio University School of Medicine, Japan) for Ago-2 antibody and F. Coumailleau for dsRed plasmid. This work was supported by the French Agence Nationale de la Recherche (ANR-09-JCJC-0045-01), the European Research Council (FP7/2007-2013 ERC StG 242703 and FP7/2013-2019 ERC CoG 615220) and the LabEx IBEID (Integrative Biology of Emerging Infectious Diseases) program to M.C.S.

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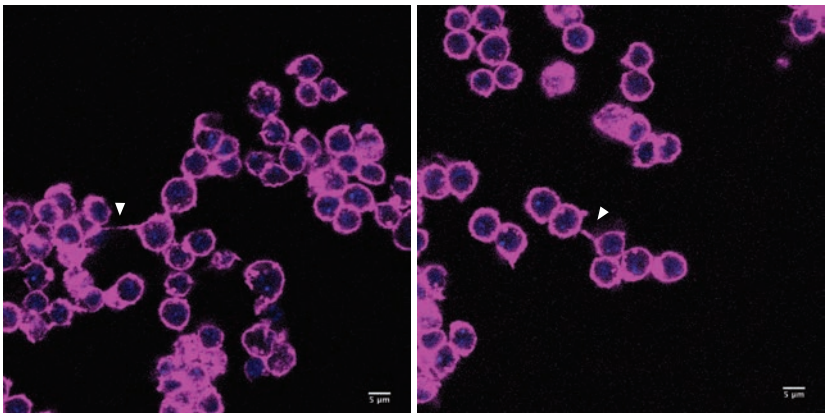
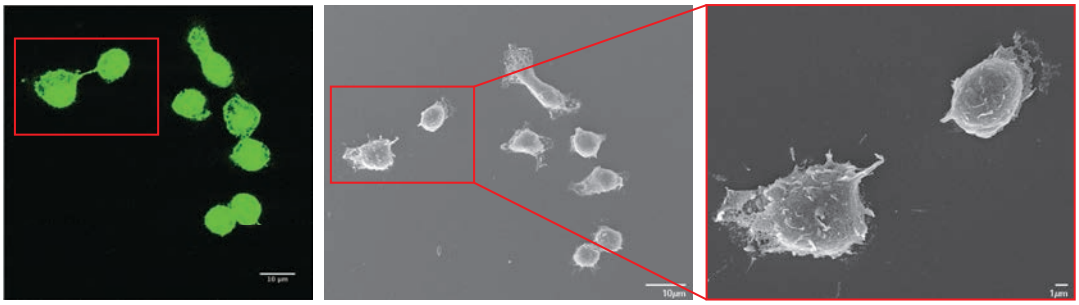


DAPI / Phalloidin

Supplementary Figure S1:

Tunneling-nanotubes are present in Drosophila Kc167 cells. Immunofluorescence and confocal microscopy. Cells were stained for F-actin using Phalloidin 647 Alexa-Fluor to show membrane continuity between connected cells. DAPI is used to mark nuclei.

Supplementary Figure S1
Karlikow *et al*.



Supplementary Figure S2:

Correlative microscopy of TNTs. S2 cells were grown overnight on an alphanumeric coded, grid-patterned glass and imaged for Ago2-containing TNTs in confocal microscopy (red square). The alphanumeric code allowed localization, and scanning electron microscopy was performed on the exact same cells.

Supplementary Figure S2
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CHAPTER 3

DISCUSSION & PERSPECTIVES

General Discussion

The model proposed by Saleh and colleagues in 2009[76], satisfactorily explained how the immune signal could travel in the insect to confer protection against a lytic virus infection: when cells are lysed, viral dsRNAs will be released in the extracellular compartments. From there, viral dsRNA will spread and be taken up at distal sites of the infection[108]. However, this model fell short to explain how systemic immunity could be reached in the case of non-lytic viruses.

My work shed light on this complex mechanism by highlighting two non-exclusive mechanisms of spread:

1- the role of DORA-positive EVs in the communication of the immune signal during infection. Indeed, the immunization experiment with DORA-positive EVs loaded with viral fragments showed an efficient protection of wild type flies against viral infection. It seems then that EVs containing the immune signal would be secreted from infected cells and internalized by non-infected cells to set-up the antiviral response in a DORA-dependent manner.

2- the discovery that TNTs in *Drosophila* cells contain Ago-2, dsRNA and DORA. TNTs were more abundant during viral infection, suggesting that those structures may be involved in the transmission of the immune signal from a donor-infected cell to a target non-infected cell.

The results obtained during my PhD emphasize the notion that systemic antiviral RNAi rely on several mechanisms, depending on virus infection type (lytic *vs.* non-lytic), viral replication localization, presence or absence of host proteins, etc. Systemic antiviral RNAi could then be set by free dsRNA as well as by EVs loaded with the immune signal and/or TNTs that transport the same or a different signal.

However, several questions remained unanswered and I will discuss them below.

11. CG4572 / DORA PROTEIN

During my PhD I showed that *Drosophila* DORA protein interacts with core components of the RNAi machinery (Ago-2 and dFMR1 protein), localizes in late endocytic compartments and is involved in immunization process through extracellular vesicles containing fragments of viral RNAs. Additional experiments could shed light on other aspects of the protein characterization, and its general function in the cell.

11.1. Concerning DORA protein characterization

DORA protein is produced from a single mRNA that, when translated, code for a two-form protein with a serine carboxypeptidase domain characterized by a catalytic triad S217, D403 and H460. It would be interesting to study if the serine carboxypeptidase domain of DORA is involved in its antiviral function. Using site-directed mutagenesis, we produced plasmids were the catalytic triad was mutated at a single, two, or at the three positions of the catalytic triad. By lack of time, I could not perform experiments with these plasmids. However, the transfection of these plasmids in cells depleted for DORA could answer two questions: *(i)* How are the two forms of DORA produced? By an endo-proteolytic cleavage involving the catalytic triad? In this case, mutating the triad would impair the formation of both forms. Or by the action of another protease? In this case, mutations of the catalytic triad would not impair the formation of both forms of the protein. *(ii)* Which form of DORA is involved in the antiviral function of the protein? Is it the full-length protein of 54 kDa or the short form of 30 kDa that is active in this process? To answer this question I developed, in an ongoing collaboration with ESPCI ParisTech (Ecole Supérieure de Physique et Chimie Industrielle de la ville de Paris), a protocol based on N-terminal peptide labeling, in order to find the precise position of the first amino acid of the 30 kDa form. The identity of the N-terminus of the 30 kDa form, will allow us to clone both forms of DORA and to perform rescue experiment in cells depleted for DORA. Even more interesting,

rescue experiments could be performed by introducing the short, or the long form that cannot be cleaved into deficient flies for DORA. Viral titers on flies expressing the long or the short form of DORA will be measured following viral infection. In this way, the involvement of the long form, the short form, or both forms of DORA on the antiviral response can be addressed.

The localization of DORA has been assessed with the help of *in vitro* and *in vivo* techniques. I did test not only markers for late endosomes (Rab7) and MVBs (SARA), but also for early endosomes (Rab5); for recycling endosomes (Rab4 and Rab11); for P-Bodies (DmMe31B); and for lysosomes (Lamp-1). None of them, except for SARA and Rab7, showed clear localization with DORA. However, endosomal compartments are highly changing vesicles, and I believe that a determination of a strict localization of DORA with a unique marker of the endocytic pathway could be a task difficult to accomplish. However, performing immunogold labeling electron microscopy with DORA could help to further confirm the subcellular localization observed for this protein.

11.2. Concerning DORA role in the antiviral response

11.2.1. DORA virus-specificity

The siRNA pathway exerts broad antiviral activity and affects both RNA and DNA viruses. Thus, we were quite surprised by the hypersensitivity of DORA deficient flies only to non-enveloped positive-stranded RNA viruses (Chapter 2, part 1).

Therefore we sought of DORA as a viral restriction factor, like Pastrel[180] or Ars2[181], as their action is virus dependent. However, overexpression of DORA in S2 cells followed by virus infection did not produce a detectable reduction on viral loads in the supernatant, ruling out this hypothesis.

A common characteristic of the viruses that DORA deficient flies are hypersensitive to (CrPV, DCV and FHV), is that each of them has a viral suppressor of RNAi (VSR) (described in Box 1 and 2 of the introduction). I hypothesized then, that DORA was interacting with VSRs, but it was quite unlikely

that a unique protein could prevent the function of these three VSRs that act very differently on RNAi. This hypothesis was also tampered with the experiment of overexpression of DORA. Moreover, DXV also encode for a VSR, the Vp3 protein that binds long and short viral dsRNAs, and the presence or the absence of DORA did not seem to affect the antiviral response against this virus.

Another common point I was able to find, only for CrPV, DCV and FHV, was that their replication occurs in viral factories. Viral factories are inclusions of membranes in the cytoplasm of infected cells, where viruses replicated while being shield from the host defense. One could hypothesize that if viral factories are included in endosomal membranes, as DORA localizes in these compartments, the physical proximity between viruses and DORA would explain the virus specificity observed. Whether DORA is involved in the destabilization of viral factories allowing the RNAi machinery to access viral dsRNA deserves further exploration.

11.2.2. DORA cellular function

My research showed that DORA was found in EVs carrying an antiviral immune signal. Preliminary data strongly suggest that DORA presence is mandatory to confer a significant immunization to non-infected flies. But what the main function of DORA is, remains a relevant question. Several scenarios can be proposed:

- DORA acts at the secretion level. If that is the case, in the absence of DORA the signal could be retained into the cells. Alternatively, the immune signal could not be loaded in EVs when DORA is not expressed.
- DORA is involved in the internalization of secreted EVs. If that is the case, in the absence of DORA the signal cannot be internalized at distal sites of infection and/or cannot reach the RNAi core machinery to be processed.

An interesting observation that can help us to understand the role of DORA was made during purification of EVs. When EVs were purified from cells depleted for DORA, fewer EVs were detectable by comparison with EVs from DORA-

expressing S2 cells. Dynamic light scattering (DLS) measurements of DORA-depleted EV samples, consistently showed different plots, suggesting a sample poor in components. This observation, together with the reduced Ago-2 slicing activity observed in DORA deficient flies (Chapter 2, part 1) are interesting leads into DORA function. As Ago-2 and DORA localize in late endocytic compartments, I propose that the absence of DORA impairs the normal turnover of MVBs. In this way, MVBs would secret less EVs that would not be sufficient to efficiently immunize flies. It is also possible that the loading of EVs with fragments of viral RNAs is impaired in cells depleted for DORA. In this situation, even if EVs are correctly secreted from cells, they are not carrying the immune signal to distal sites (Fig. 14, left panel). RNA high throughput sequencing of EVs purified from cells depleted for DORA would much probably help to answer this question.

Another important consideration when seeking for DORA function, is the nature of the RNA molecules loaded in DORA-positive EVs. The bibliography in organisms that use RNAi as antiviral response, such as plants, worms, and mosquitos, places vsiRNAs at the foundation of the specific antiviral response. And even if I was able to detect siRNAs in EVs from cells that soaked dsSin, these EVs were unable to immunize wt flies. DORA-positive EVs that contained fragments of viral RNA, immunized flies against a further related infection. As there is increasing evidence that exosomes in mammals can be taken up by other cells types[128,129,182], it is tempting to speculate that these fragments of viral RNAs inside EVs, are able to enter distal cells and reach the RNAi core machinery. Because the immunization was lost in the absence of DORA, the mechanism could rely on DORA, as the protein being responsible for the uptake of EVs at distal site (Fig. 14, right panel). However, as EVs containing siRNAs against SinV were unable to immunize flies, it is more likely that DORA is involved at the infection site impairing the release of EVs, the loading of their cargoes or both, rather than on the docking of EVs in uninfected cells. It is worth mentioning that from an experimental point of view, some improvements could be done to obtain purified EVs (see footnote[1]) that will be used to confirm all the previous results.

[1] As S2R+ cells are persistently infected with a wide array of viruses, to optimize the separation of viruses from EVs, I pelleted the supernatant over an Optiprep gradient.

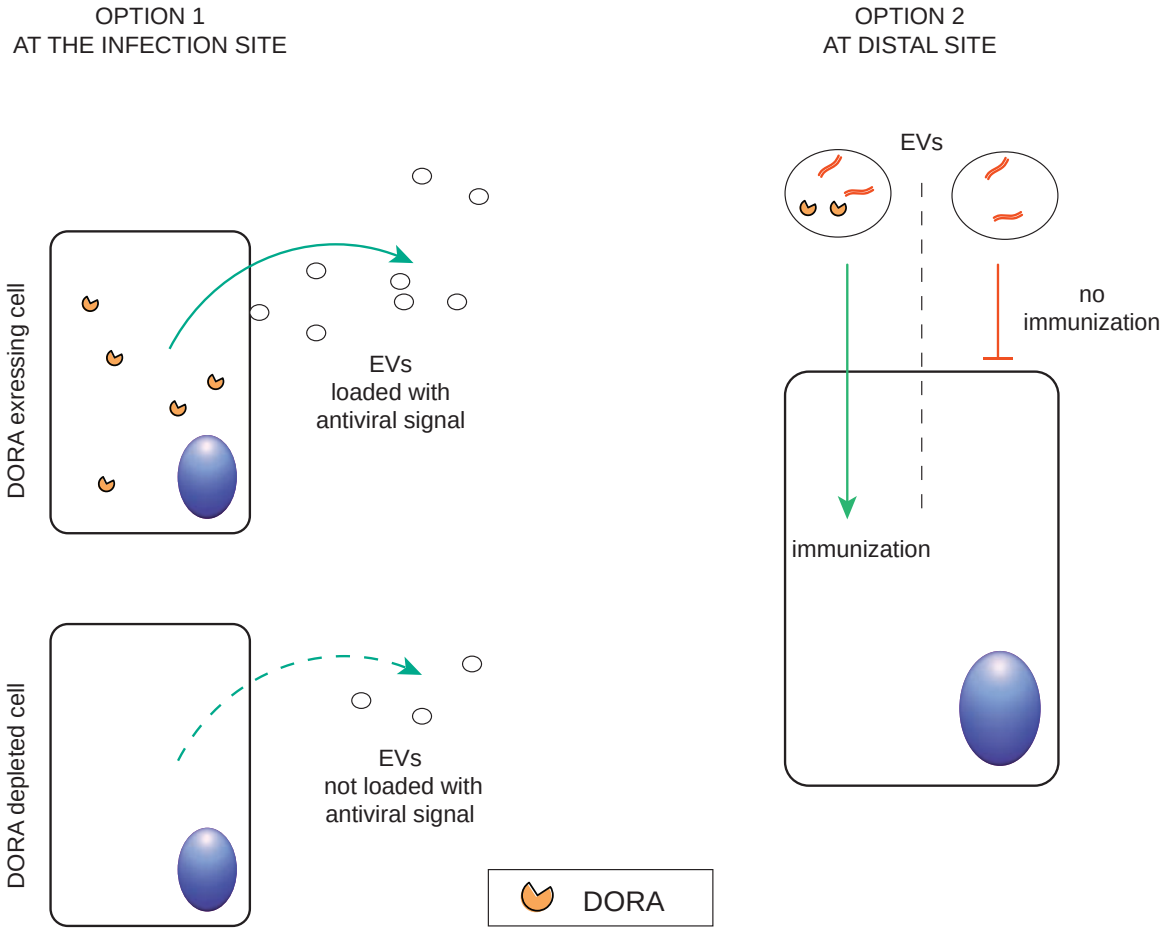

Figure 14. Different alternatives for the role of DORA. Option 1 considers DORA at the infection site. EVs secreted from cells expressing DORA protein (upper cell) are loaded with the immune signal and are able to immunize. Cells depleted for DORA (bottom cell) could still release EVs but to a lesser extent, and those EVs failed in loading with the immune signal. Option 2 considers DORA at a distal site of infection. DORA-positive EVs loaded with the immune signal are able to dock on the target cell, allowing immunization. In the absence of DORA, EVs would not be internalized into the target cell, hindering the immunization process.

However, as DORA belongs to EVs, a flotation experiment where EVs go to the top of the gradient (less density) and not to the bottom should be performed. In this way EVs would be readily separate from non-enveloped viruses (at the bottom of the gradient). The only set back for this new approach, is that Sindbis virus is an enveloped virus and although its density should not exactly correspond to EVs density, there is a risk of contamination of the EVs fraction with Sindbis virus. Therefore, in order to confirm my results, I will purify EVs using floatation and a non-enveloped virus such as DCV. This approach will be further developed in the second part of the discussion.

As for the name I chose for this protein, I hesitated between Spondophorin and DORA. The first one comes from antic Greek where Spondophores were travelling all over the country to announce the holy truce during the Olympic game. I wanted to do a parallel with CG4572 that is travelling at distal site of the infection to alert cells and help them to mount an antiviral response. Finally I chose DORA, as she is a very well known explorer (Dora the Explorer).

11.3. Concerning DORA and TNTs

During my PhD I discovered another new route for the RNAi machinery to spread and potentially transmit an immune signal: tunneling nanotubes (TNTs). Since their discovery[145] TNTs were well characterized for the dissemination of molecules, organelles and also viruses[149,150,183-185]. Here we showed that the nanotubes were associated with components of the RNAi machinery including Ago-2, dsRNA, and DORA. We postulated that the spread of the silencing signal in insects relies, among other cellular mechanisms, on TNT intercellular connections. Two major interrogations however remain: whether transfer occurs in these TNTs and how to address the exact role of TNTs during viral infection.

For the transfer issue, live-microscopy experiments would be the best way to answer this question. Alternatively, we could address the transport of color dyes (such as DiI and DiO stains) between cell lines expressing GFP and dsRed. If the color dye is found in TNTs structures between red and green cells, transfer could be assumed.

As for the function of TNTs during viral infection, the depletion of these structures could help us. If we imagine this experiment, the TNTs detected in infected *Drosophila* cells could be serving three purposes: 1- allowing viral transmission, as is the case for HIV-1[150]. In this case, the disruption of TNTs during infection should result in a lower viral titer in cell culture; 2- allowing the components of the RNAi machinery and its related vesicles (Ago-2, dsRNA, DORA and Rab7) to be transported in between cells in order to generate an intracellular immunity in uninfected cells. In this case, the disruption of TNTs should result in higher viral titers; 3- allowing virus transmission as well as the

transport of the RNAi machinery to the receptor cell that will set up an infection and an antiviral response. In this case, disruption of TNTs should not modify viral titers.

But at present, it remains unfeasible to investigate into these hypotheses that would require the disruption of TNTs during viral infection. The only method described to disrupt TNTs is to inhibit actin polymerization by latrunculins A and B. However, the disruption of actin polymerization strongly affects intra- and intercellular movement of viruses such as entry and budding[186] therefore precluding its utilization and leaving us, for the moment being, at the speculation level.

12. NATURE OF THE IMMUNE SIGNAL

The different results obtained while searching for DORA function, give us hints on the nature of the immune signal during systemic RNAi in *Drosophila*. Most of the information comes from the small RNA sequencing of EVs (Chapter 2, part 2) and from the immunization protocol I developed using EVs (Chapter 2, part 1).

Interestingly, canonical viral siRNAs corresponding to all the viruses that persistently infect S2R+ cells were present in EVs. One could then hypothesize that the loading of EVs is non-specific. However, cellular small RNAs, such as miRNAs and transposons small RNAs, were almost totally excluded from EVs, suggesting that loading of small RNAs into EVs is a specific process for viral RNAs.

During the immunization experiment, EVs containing siRNAs from the processing of the dsSin, were not able to immunize against an ulterior SinV infection. Perfect dsRNA fragments (>51 nts) from the same precursor (dsSin) were also detected inside EVs (Fig. 7 and Fig. 9) but also failed to confer protection. Even though it is possible that the amount of siRNAs contained in EVs is just not enough to trigger a systemic immunization, these results strongly suggest that dsRNA molecules are not the primary antiviral signal that spread

during infection. Confirming this observation, when S2R+ cells were acutely infected with SinV, EVs were loaded specifically with SinV RNA fragments (positive strand only, Fig 8 and Fig. 10) but not with SinV siRNAs or dsRNAs. These EVs were capable of conferring protection to non-infected flies.

What is then the process by which EVs loaded with viral RNA fragments can transfer antiviral immunity to target cells or tissues?

The most evident way to answer this question is the presence of secondary structures (intramolecular base-pairing structures) inherent to any long RNA molecules. When EVs reach the target cell and the cargo is released, the fragments of RNA presenting dsRNA-like secondary structures could be recognized by Dicer-2 and processed into viral siRNAs of 21 nts long, triggering the antiviral response. In strong support of this idea, in the laboratory was observed that flies inoculated with the genomic RNA of Sindbis virus mutated in the polymerase (unable to replicate), were immunized against SinV infection (Juan A. Mondotte, personal communication). This suggests that the secondary structures on the genomic RNA of SinV constitute the immune signal that will trigger the antiviral RNAi response.

Nevertheless, it is important to consider what would happen if the purification of EVs that I performed was not selective enough to separate SinV from the EVs fraction. Would it be possible that the remaining virus is enough to trigger the protection when EVs are transferred to non-infected cells? Is this protection observed independently of the content of EVs? As mentioned above, a new protocol for purification of EVs will be applied and I will be able to answer this question. However, it has been published[187] that inoculation of wild type flies with a small dose of DCV, was not able to immunize the same flies against a second infection. Therefore, we could assume that the traces of SinV (if any) present in EVs preparation would not be enough to trigger the EVs-mediated protection we observed.

To further discard (or confirm) a possible role of vsiRNAs on the spread of the immune signal, it would be interesting to perform the EVs immunization protocol on Dcr-$2^{-/-}$ flies. In this case, if the signal transported in EVs is vsiRNAs, Dcr-$2^{-/-}$ flies should be protected. On the contrary, if the signal is dsRNA, or fragments of

viral RNAs containing secondary structures, vsiRNAs from these precursors could not be produced due to the lack of Dicer-2 and immunization would fail.

Finally, I find important to understand if the protection mediated by EVs occurs only during acute- or also during persistent- infections. To answer this question, I will develop a stable cell line in non-infected *drosophila* S2 cells that are depleted for DORA protein. Then I will compare EVs secreted from DORA-positive S2 cells and DORA-depleted cells in three conditions: *(i)* after soaking of dsDCV, *(ii)* after acute infection with DCV, *(iii)* during persistent infection with the same virus. EVs immunization protocol associated with RNA high throughput sequencing on EV content will help answer if EVs participate in protection during persistent and/or acute infection.

Taken together all my results and the different considerations discussed, I propose the following model for the systemic spread of an antiviral signal during infection (Fig 15). When a cell is infected with a virus, viral dsRNA molecules trigger the cell-autonomous antiviral response and, as a consequence, viral siRNAs (21 nts long) are produced. These cells, completely overwhelmed by the burden of viral replication, produce high amounts of viral RNAs but also fragments of viral RNA as result of degradation products, abortive transcripts, incomplete viral genomes, etc. Through EVs and/or TNTs, viral RNAs fragments (> 51 nts) with secondary structures and/or dsRNA molecules are sent to distal sites, where they are recognized and processed by the RNAi machinery setting-up an antiviral systemic response.

Model for the systemic spread of an antiviral immune signal

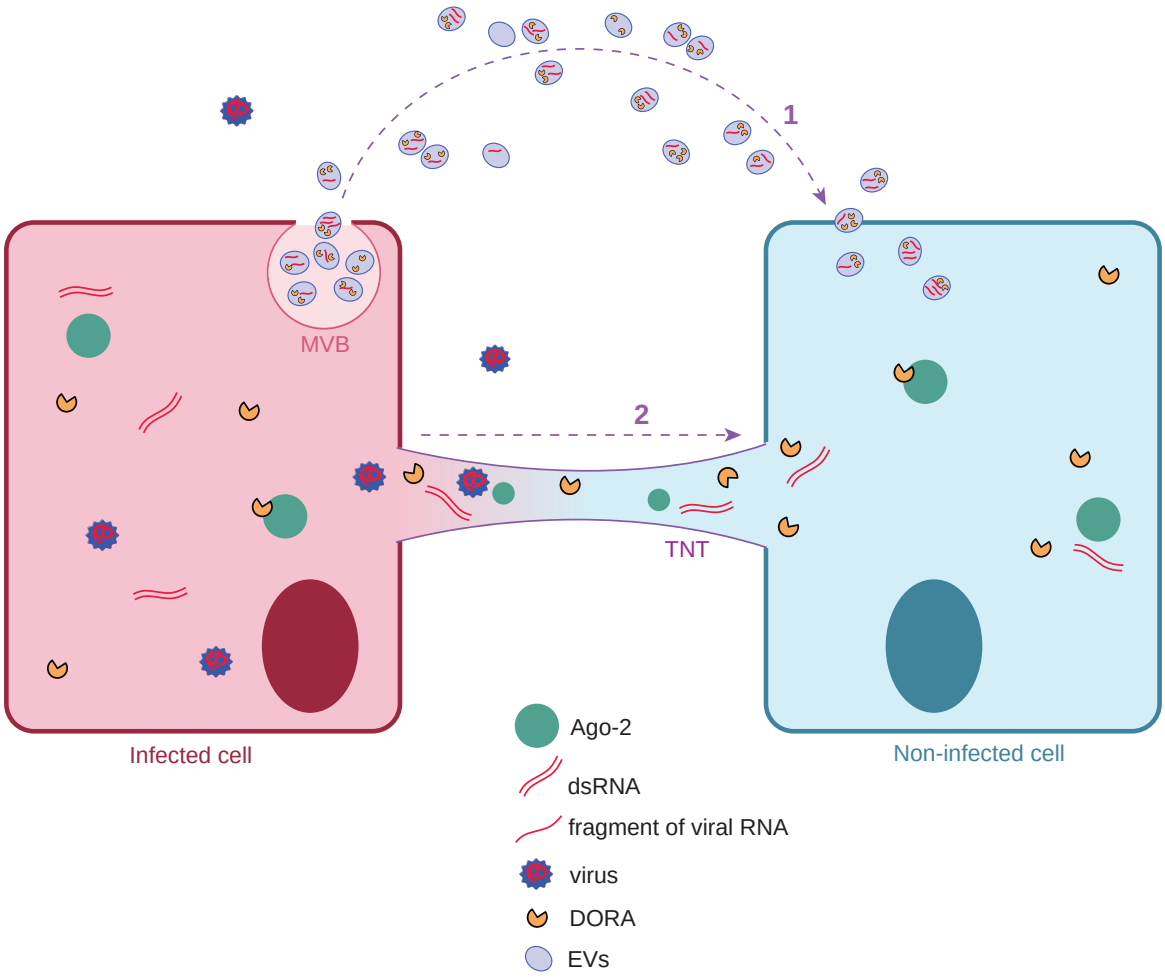

Figure 15. Model for the systemic spread of the antiviral immune signal. From the infected cell (**1**) EVs containing fragments of viral RNA and DORA could be released out of the cells and take up by non-infected cell. (**2**) During viral infection TNTs will increase in number and allow molecules such as Ago-2, DORA and dsRNA to spread to non-infected cells. By both mechanisms non-infected cell will be immune-primed and will efficiently control the incoming viral infection.

Perspectives

I believe that the research I performed on DORA protein and TNTs unveil important information on the nature of the antiviral signal, and on the mechanisms by which this immune signal propagates in insects. This information will have great impact to manipulate and enhance the control of viral replication in invertebrates, and as a consequence, the control of viral transmission from insects to mammals, to plants…

In recent years, different alternatives have been employed to eradicate vector mosquitoes, like pesticide vaporization and genetically modified mosquitoes. But each of them have sequentially failed due to negative ecological impact, development of pesticides resistance by mosquito or a quickly outcompetition of introduced genetic modified mosquitoes by the natural population. It would be then interesting to develop new approaches to help mosquitoes to better fight, and therefore less transmit viruses.

My work revealed that DORA-positive extracellular vesicles loaded on antiviral immune signals were efficiently protecting *Drosophila*. Could we imagine the development of a prophylactic treatment using stable and long lasting nanoparticles charged with antiviral signals against viruses such as Dengue? These nanoparticles could spread on aqueous surface where mosquito larvae develop and adults drink. Larvae would feed on this water, and ingest the nanoparticles and their content. The presence of nanoparticles charged with antiviral signals, would help non-infected mosquitoes to develop a pre-immunization against a specific virus before they get infected.

By helping insects such as mosquitos to fight viral infection, we would be fighting at the same time viral transmission to, for example, humans, and development of epidemics. We need to learn how to live together with insect vectors instead of only trying to get ride of them.

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ANNEX

Developmental and Comparative Immunology 42 (2014) 85–92

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

RNAi and antiviral defense in *Drosophila*: Setting up a systemic immune response ☆

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ARTICLE INFO

Article history:
Available online 14 May 2013

Keywords:
Innate immunity
Insects
Drosophila
RNAi
Small RNAs
Arboviruses

ABSTRACT

RNA interference (RNAi) controls gene expression in eukaryotic cells and thus, cellular homeostasis. In addition, in plants, nematodes and arthropods it is a central antiviral effector mechanism. Antiviral RNAi has been well described as a cell autonomous response, which is triggered by double-stranded RNA (dsRNA) molecules. This dsRNA is the precursor for the silencing of viral RNA in a sequence-specific manner. In plants, systemic antiviral immunity has been demonstrated, however much less is known in animals. Recently, some evidence for a systemic antiviral response in arthropods has come to light. Cell autonomous RNAi may not be sufficient to reach an efficient antiviral response, and the organism might rely on the spread and uptake of an RNAi signal of unknown origin. In this review, we offer a perspective on how RNAi-mediated antiviral immunity could confer systemic protection in insects and we propose directions for future research to understand the mechanism of RNAi-immune signal sorting, spreading and amplification.

1. Introduction

Arthropods are of enormous importance to ecology, economy and health. Some of them, such as sand flies, mosquitoes and ticks, are vectors for numerous pathogens, including viruses. Among them, arboviruses (arthropod-borne viruses) are transmitted by insects upon biting vertebrates. Several arboviruses are responsible for worldwide epidemics and high mortality or morbidity rates in humans, such as dengue and chikungunya virus. The insect vectors of arboviruses have to control these viral infections to maximize their survival and minimize the associated fitness cost. Thus, the insect antiviral response is an important factor for viral transmission and dissemination.

Since the beginning of the 20th century, the fruit fly *Drosophila melanogaster* has been the most widely used insect model. As a result, *Drosophila* has become a powerful tool to work in several fields, including genetics, development, neuroscience and immunity. This is due to the availability of genetic tools, the short generation time, the safety of use compared to hematophagous insects, and more recently, the availability of the complete genome sequence. Consequently, much of what is currently known about defense mechanisms in insects results from work with fruit flies. This review therefore focuses on research performed using *Drosophila*, although some examples and works in other models, such as in *Caenorhabditis elegans*, are addressed.

The defense of higher eukaryotes against pathogens is organized into different layers. First, there is a non-specific host defense: a physical barrier, which is the skin in mammals and the cuticle for insects. The gut epithelia can also be considered as an anatomical barrier as it protects against infections during feeding (Buchon et al., 2010; Davis and Engstrom, 2012). Second, there is innate immunity, which acts coordinately at the cellular and systemic level. The third layer is the adaptive immune response, which is present only in jawed vertebrates. Some of the most interesting characteristics of this adaptive immunity are the boosting or amplification of the immune response, as well as the immune memory, which enhances the ability of the organism to respond to future related infections. However, insects lack an adaptive immune system and thus, the immune defense relies almost entirely on the innate immune response. For instance, flies are able to trigger various defense pathways depending on the type of infecting pathogen, and most of these pathways are inter-connected. For fungal or bacterial infections, the Toll, Imd and Jak/STAT pathways have been implicated (Agaisse et al., 2003; De Gregorio et al., 2002). Although these pathways also play a role in viral infections, their antiviral function seems to be virus-specific rather than being a general antiviral response (see for example Dostert et al., 2005; Zambon et al., 2005; Kemp et al., 2013). A comprehensive description and discussion of these antiviral mechanisms are presented in this special issue by Sara Cherry and colleagues. Antiviral defense

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http://dx.doi.org/10.1016/j.dci.2013.05.004

in insects relies also on another pathway of innate immunity: the RNA interference (RNAi) response (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006).

RNAi is a conserved sequence-specific, gene-silencing mechanism that is induced by double-stranded RNA (dsRNA). Several RNAi-related pathways (Aravin et al., 2006; Czech et al., 2008; Girard et al., 2006; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; van Rij et al., 2006) have been described in many organisms and they have diverse functions, including the modulation of mRNA translation (Valencia-Sanchez et al., 2006), establishment of chromosomal architecture (Hall et al., 2002), regulation of stem cell renewal (Carmell et al., 2002) and defense against viruses and mobile genetic elements (Chung et al., 2008). In general terms, RNAi pathways involve the production of small non-coding RNAs, and their biogenesis and function is based on two proteins: Dicer (Dcr) and Argonaute (Ago). The *Dicer* and *Ago* genes are strongly conserved in wide-ranging species including plants, invertebrates and mammals. Nevertheless, as a result of evolutionary and immune adaptation processes, there are several paralogues of both proteins. Consequently, a number of functions have been described for various Ago and Dcr paralogues, including their involvement in the antiviral responses. As of today, four main RNAi-related pathways have been described and they can be classified into two major groups on the basis of the origin of the small non-coding RNAs: the "endogenous" group, which involves small RNAs encoded within the cell and the "exogenous" group, which involves small RNAs not encoded by the cell.

"Endogenous" RNAs:

(i) Micro-RNAs (miRNA) are mostly encoded by intergenic regions in the nuclear DNA. In *Drosophila*, their biogenesis is dependent on Drosha and Pasha in the nucleus, that process the primary transcript (pri-miRNA) into a pre-miRNA (Denli et al., 2004); then the pre-miRNA is exported from the nucleus to the cytoplasm, where it is processed by Dcr-1 together with its cofactor Loquacious (a dsRNA binding protein) to generate the mature miRNA. In association with Ago1 (Okamura et al., 2004) miRNAs actively regulate cellular gene expression by several mechanisms ranging from cleavage of cellular transcripts to translational inhibition. They may also act at the transcriptional level through, for example, chromatin reorganization (Pushpavalli et al., 2012).

(ii) Endogenous small interfering RNAs (endo-siRNA), are encoded by transposable elements or other genomic regions that produce transcripts capable of forming dsRNA structures. They regulate genes and transposable elements. In *Drosophila*, this pathway is dependent on Dcr-2, a variant of Loquacious (loqs-PD) (Zhou et al., 2009) and Ago2 (Kawamura et al., 2008).

(iii) PIWI-interacting RNAs (piRNA) are encoded by clusters of genes throughout the genome. They are mostly known for their roles in epigenetic and post-transcriptional gene silencing of transposons and other genetic elements in the germ line. Besides, there is evidence implicating piRNAs in the antiviral response in mosquitoes (Morazzani et al., 2012; Vodovar et al., 2012). The biogenesis of piRNAs in *Drosophila* is dependent on PIWI, Aubergine (Aub) and Ago3 proteins. This RNAi pathway is Dcr independent (Olivieri et al., 2010).

"Exogenous" RNAs:

(i) Small interfering RNAs (siRNA) are produced from virus-derived dsRNAs or non-cellular RNAs that generate dsRNA structures. The siRNA pathway works as an antiviral response in invertebrates and plants, targeting both viral dsRNA replicative intermediates as well as viral genomes. The biogenesis of siRNA is dependent on Dcr-2, R2D2 and Ago2.

To understand how insects combat and control viral infections, we will first consider the RNAi pathway at a cellular level during a viral infection. We will then address how cells may establish intracellular immunity at sites distant from the infected cells, and finally discuss how antiviral RNAi generates a systemic response.

2. RNAi as an antiviral defense

Viral dsRNA molecules are produced in cells that are infected with diverse types of virus: (i) viruses with dsRNA genomes, such as *Drosophila* X virus (DXV) (Dobos et al., 1979); (ii) viruses with DNA genomes that contain convergent transcript units, for example Invertebrate Iridescent virus (IIV6) (Bronkhorst et al., 2012; Kemp et al., 2013); and (iii) viruses with single-stranded RNA genomes produce dsRNA as the result of the formation of secondary structures, such as Sindbis virus (Myles et al., 2008; Fragkoudis et al., 2009) or vesicular stomatitis virus (Sabin et al., 2013); and/or replication intermediates as for *Drosophila* C virus (DCV) or Semliki Forest virus (Siu et al., 2011).

Those long viral dsRNA molecules trigger the antiviral siRNA pathway (Fire et al., 1998). They are cleaved (or 'diced') by a ribonuclease III enzyme, Dcr-2 (Bernstein et al., 2001) in association with its cofactor R2D2 (Liu et al., 2003), into viral small interfering RNAs (siRNA) of 21 nt long (Elbashir et al., 2001). These viral siRNAs are loaded into a pre-RISC complex, where the siRNA duplex is unwound and the strand with the less stable 3′-terminus, the passenger strand, is removed. The remaining viral siRNA strand, the guide strand, is retained in Ago2/holo-RISC, which is the catalytic effector of the RISC complex (Okamura et al., 2004; Rand et al., 2004). The loaded viral siRNA can bind a viral RNA (genome or transcript) by sequence complementarity leading to specific degradation of the targeted RNA mediated by Ago2. The complementarity of the siRNA and its target is thus the basis of the specificity of the RNAi machinery.

The siRNA pathway appears to be the main antiviral response in insects: flies deficient for Dcr-2 or Ago2 are unable to control virus replication and as a consequence are hypersensitive to infection (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). Recently, some reports suggest the involvement of other RNAi pathways in the control of viral infections. Indeed, piRNAs from viral origin have been detected by deep-sequencing during infections of mosquitoes with arboviruses, including dengue (Hess et al., 2011), Sindbis (Vodovar et al., 2012), chikungunya (Morazzani et al., 2012) and LaCrosse virus (Brackney et al., 2010). Hess and colleagues (Hess et al., 2011) described an *in vivo* assay using mosquitoes and dengue virus, and detected a peak in the accumulation of piRNAs at 2 days post infection. The amounts of these piRNAs then decreased during the infection, whereas siRNA production increased. This suggests that the RNAi-Dcr-2-dependent pathway is active during viral infection, but that it is preceded by the piRNA response. These observations lead to the notion that the piRNA pathway may initiate the antiviral process during a viral infection in mosquitoes. It is important to note that, in *Drosophila*, siRNAs accumulate during viral infection independently from piRNA production. It has been suggested that piRNAs may serve as epigenetic and genomic "security guards". Recently Schnettler and colleagues (Schnettler et al., 2013) provided the first functional demonstration that viral piRNAs do indeed contribute to antiviral defenses in mosquito cells infected with Semliki Forest virus.

Interestingly, viruses like Epstein-Barr virus encode miRNAs, which can interfere with the mammalian immune response. These

viral miRNAs are predicted to target cellular regulators of cell proliferation, apoptosis (Riley et al., 2012) and components of signal transduction pathway among others (Marquitz and Raab-Traub, 2012). Klase and colleagues proposed that HIV-1 TAR element is processed by Dicer to produce a viral miRNA that is detectable in infected cells, which contribute to viral latency (Klase et al., 2007). Arthropod viruses have been predicted to encode miRNA, but there has been scarce biological or experimental demonstration that they are indeed produced. In 2012, Hussain and colleagues (Hussain et al., 2012) showed that West Nile virus encodes a miRNA in its 3' untranslated region. This miRNA is only detected in mosquito cells but not in mammalian cells infected with this virus. Regulation by this miRNA, named KUN-miR-1, increases the cellular GATA4 mRNA, which leads to a higher viral replication.

Several lines of evidence imply the existence of a systemic component to the siRNA pathway in arthropods: (i) the *in vivo* uptake of exogenous dsRNA; (ii) an increased sensitivity of dsRNA uptake mutants to viral infection; and (iii) the trans-silencing effects on endogenous genes following Sindbis virus infection in *Drosophila*. Early in 2002 it was shown that dsRNA injection into the haemocoel of adult *Tribolium castaneum* (floor beetle) resulted in knockdown of zygotic genes, which was also manifested in offspring embryos, implying transfer across cell boundaries (Bucher et al., 2002). In 2005, Robalino and colleagues showed that the injection of viral sequence-specific dsRNA confers potent antiviral immunity *in vivo* in the shrimp *Litopenaeus vannamei* (Robalino et al., 2005). Accordingly, endogenous shrimp genes could be silenced in a systemic fashion by the administration of cognate long dsRNA. A systemic component to antiviral RNAi was shown in mosquito cells infected with Semliki Forest virus (Attarzadeh-Yazdi et al., 2009). Even if the exact mechanism is not known, the authors showed cell-to-cell spread of viral-derived siRNA, and possible long dsRNA, with concomitant inhibition of replication of the incoming viruses in cells neighboring infected cells. Also, we were able to show that in *Drosophila*, intra-thoracic injection of viral sequence-specific long dsRNA into uninfected flies conferred immunity against subsequent infection with the corresponding virus. Furthermore, infection with Sindbis virus expressing the green fluorescent protein (GFP) suppressed expression of host-encoded GFP at a distal site of infection (Saleh et al., 2009).

Therefore, systemic RNA silencing pathways seem to exist in at least some arthropods. The link between systemic RNAi and antiviral defense awaits further mechanistic confirmation. Through the rest of this review we offer a perspective on how the RNAi-mediated antiviral immunity could confer systemic protection and we propose directions for further study to understand the mechanism of RNAi-immune signal sorting, spreading and amplification.

3. Setting up antiviral immunity at systemic level

The control of viral infections in mammals requires signaling molecules to elicit an effective response and to establish systemic immunity at the organismal level. These signals must be amplified and disseminated throughout the organism to avoid pathogen propagation and establishment of the infection. In *Drosophila*, although it has been postulated that there is a systemic antiviral response, neither the signal, nor the amplification mechanism, nor the mechanism of its dissemination, has been described. Nevertheless, relevant data are starting to emerge.

3.1. Uptake and sorting of the immune signal

Drosophila cells can take up viral dsRNA that triggers a specific RNA silencing response (Caplen et al., 2000). Presumably, following dsRNA uptake, an immune signal is sent by infected cells to prevent viral infection in distant non-infected cells (Saleh et al., 2009; Attarzadeh-Yazdi et al., 2009). Non-infected cells have to be able to "catch" or to "sense" this signal and to internalize it in order to be primed. Here, we will address the possible triggers, the signals and their sorting.

For the uptake of viral dsRNA or other viral infection signals by non-infected cells, the first barrier to be crossed is the plasma membrane, a bilayer of phospholipids with associated proteins. The plasma membrane is selectively permeable to small and uncharged molecules; however, most molecules are unable to freely diffuse through it. Various membrane proteins act as receptors and/or transporters allowing adequate and selective entry of molecules into the cell. It has been shown that the endocytic clathrin-dependent mechanism is involved in the uptake of dsRNA and this triggers an antiviral RNAi pathway in *Drosophila* (Saleh et al., 2006; Ulvila et al., 2006). Indeed, the specific uptake of dsRNA by cells is abolished by treatment with Bafilomycin-A1, an inhibitor of V-H-ATPase (a component of the endosome-lysosomal acidification process). Viral dsRNA and naked siRNA are large and charged molecules, and therefore there must be appropriate receptors if they have to enter into the cells. In *C. elegans* there are two receptors for dsRNA: SID-1 allows passive inter-cellular transport (Feinberg and Hunter, 2003; van Roessel and Brand, 2004; Winston et al., 2002), whereas SID-2 allows active transport of environmental dsRNA from the intestinal lumen into cells (McEwan et al., 2012). These receptors participate in the internalization of dsRNA, which can then lead to the spread of the RNAi. Other SID proteins that participate in dsRNA transport have been described, like SID-5, which promotes the transport of the silencing signal between cells in *C. elegans* (Hinas et al., 2012), or SID-3, which is needed for an efficient import of dsRNA into cells (Jose et al., 2012). Interestingly, the extent to which RNAi spreads is coupled to the amount of dsRNA produced within cells or imported from the environment (Jose et al., 2011). However, although dsRNA is able to enter cells in flies, it is not clear how. No receptors with a dsRNA-binding domain have been found in *Drosophila*. It appears that two Scavenger receptors, called SR-CI and Eater, are associated with dsRNA uptake (Ulvila et al., 2006) but further studies addressing the role of these receptors are needed.

It is also possible that dsRNA is not the signal that triggers systemic immunity, in which case, there must be another molecule. However, studies in cell culture using *Drosophila* S2 cells found that free siRNAs are not taken up by the cells, and/or do not result in silencing of a reporter gene when freely added to the extracellular media (Saleh et al., 2006; Ulvila et al., 2006). These results are consistent with the notion that the signal of systemic immunity may be a long dsRNA, a (RNP), or another RNA complex.

Assuming an RNA nature of the signal, insights into the transport of other RNA species, for example miRNA or mRNA, may be informative about the uptake of the immune signal in flies. In murine and human cells, naked mRNA has been shown to associate with early endosomes (Rab5-positive vesicles) after endocytosis probably mediated by a Scavenger receptor. However, this entry route is not exclusive for mRNA and other negatively charged molecules, including all small RNAs and dsRNA, could potentially use it. After internalization, the mRNA was found to traffic into lysosomes where it accumulates and is then degraded by ribonucleases; however, an important proportion of the RNA escapes to the cytoplasm where it can be expressed (Lorenz et al., 2011) (Fig. 1B and C).

Many RNAs are addressed to specific subcellular compartments by (i) a signal that they carry in their sequence or by structural motifs (*cis*-acting elements or localizer signals) and/or by (ii) associated proteins (*trans*-acting factors) (Bashirullah et al., 1998). The *cis*-acting elements provide binding sites for the *trans*-acting

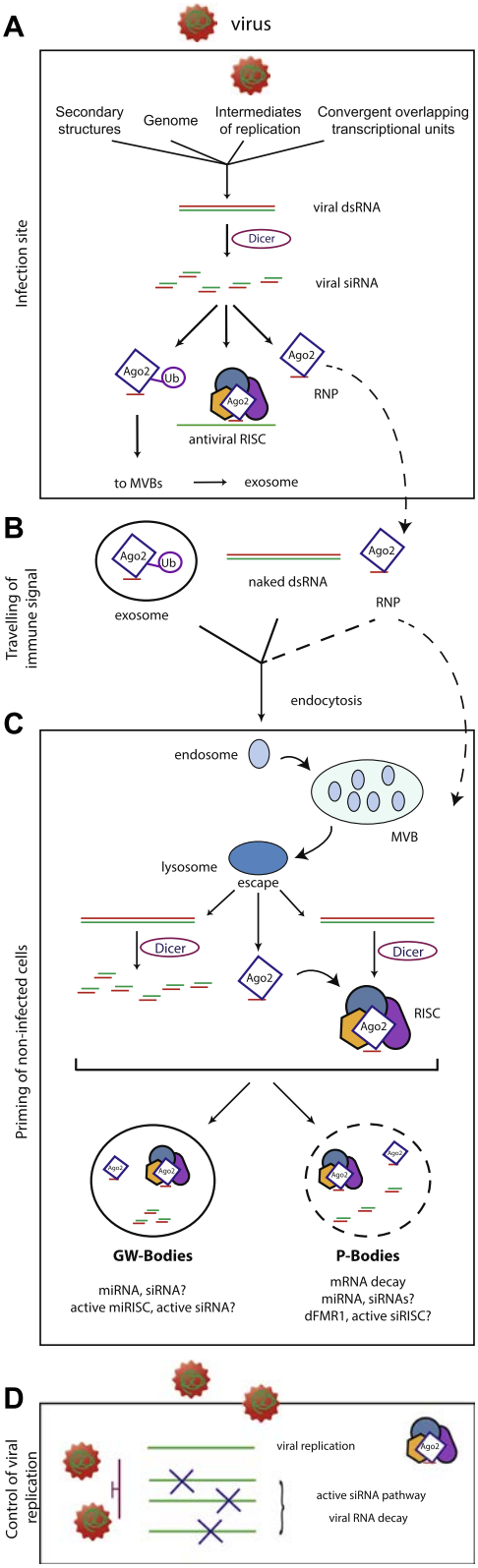

Fig. 1. Hypothetical model for internalization, sorting and transmission of the RNAi-immune signal during virus infection. At the site of infection (A), the presence of dsRNA activates the antiviral response at the cellular level and sets up the immune signal for a systemic response. This signal could spread through the organism by (B) exosomes containing siRNAs or an RNP or long dsRNA from viral replication. At distant sites of infection (C) the immune signal is potentially endocytosed, followed by release from lysosomes. Long viral dsRNA is diced and siRNAs loaded into RISC or in free RNP. Alternatively, some naked dsRNA can be diced and siRNAs direct to GW-bodies or P-bodies where active silencing and viral mRNA decay control future infections (D).

factors. However, small RNAs, which may be no more than a few dozen of nucleotides long, are unlikely to encode such localizer sequences.

Therefore, the effectiveness of the silencing process not only depends on internalization of the signal; the signal must also be delivered to the appropriate site to allow the production of siRNA-based sequence-specific protection. The internalization of transmembrane proteins and cargo can provide a clue on this process. Endocytic vesicles with transmembrane proteins and ligands are internalized and they deliver their cargo to the early endosomes where the cargo is sorted to (i) be sent back to cell surface through recycling endosomes or (ii) remain in the early endosome, which develop into multi-vesicular bodies (MVBs) by invagination of their membranes (Felder et al., 1990; Hurley and Emr, 2006; Matsuo et al., 2004). If the acidification continues, the number of internal vesicles will increase; late endosomes (multi-vesicular endosomes) fuse with lysosomes and their contents are then destroyed. MVBs are either sorted for degradation into lysosomes, although a proportion of internalized mRNAs can escape to be expressed in the cytoplasm, or secreted as exosomes into extracellular fluids. Interestingly, MVBs have been found to be closely involved with the miRNA and the siRNA pathways (Lee et al., 2009). In mammals, for example, Epstein-Barr virus encodes its own miRNAs that are released within exosomes with immunomodulatory properties (Pegtel et al., 2010). Several pathways for targeting proteins into MVBs have been described in flies. Mono-ubiquitinated proteins are sorted from the endosomes to the outer membranes of MVBs bound to the ESCRT complex (Endosomal Sorting Complex Required for Transport) (Katzmann et al., 2001). It is therefore possible that dsRNA and/or siRNA could be addressed towards MVB following their inclusion in RNP subject to ubiquitination (Fig. 1C). Although the role of MVBs in the traffic and sorting of the immune signal has thus far not been addressed, their key roles in vesicular trafficking make them good candidates to start exploring the mechanism of dsRNA transport.

Another interesting candidate for the accumulation and processing of dsRNA and/or siRNA, as part of the intracellular immunity system, are GW-bodies (Fig. 1C). In mammals, these cytoplasmic foci are physically associated with MVBs, which are involved in the post-transcriptional regulation of eukaryotic gene expression (Anderson and Kedersha, 2009). They are enriched in mRNAs, small RNAs (miRNA and siRNA) and RNA-binding proteins associated with the RNAi pathway, such as Ago2 (which has also an mRNA degradation function in mammals) and GW182 (Jakymiw et al., 2005; Liu et al., 2005; Sen and Blau, 2005) of which there is an homologue in *Drosophila* (Rehwinkel et al., 2005). In mammalian cells, GW-bodies are essential for the miRNA pathway (active miRISC is recruited into GW-bodies (Gibbings et al., 2009; Lee et al., 2009)), and the disruption of this structure impairs the silencing of endogenous genes. Transfected siRNA are also found in these GW-bodies (Jakymiw et al., 2005).

Foci very similar to GW-bodies are also found in *Drosophila*, *C. elegans* and mammalian cells and are called P-bodies (Fig. 1C) (Sheth and Parker, 2003; Jain and Parker, 2013). One of the main differences between GW-bodies and P-bodies is that P-bodies possess decapping proteins involved in mRNA decay. One of the functions associated with AIN-1 protein (the GW182 homologue in *C. elegans* (Ding et al., 2005)) is the translocation of miRNAs to P-bodies. The recruitment of miRNA into P-bodies appears to allow the decay of target mRNA, and there may be a similar mechanism for viral siRNA and viral RNA in *Drosophila*. There are two lines of evidence supporting this possibility: (i) various components of the RISC complex have been detected in P-bodies (*i.e*, presence of dFMR1 (Caudy et al., 2002; Ishizuka et al., 2002)) and (ii) active silencing pathways are necessary for P-bodies to form in *Drosophila* although P-bodies are not required for silencing (Eulalio et al.,

2007). Once their formation is initiated, decapping enzymes are recruited; these enzymes are involved in mRNA decay, which may allow the degradation of viral mRNA (deadenylation and digestion by exonucleases) (Fig. 1D). This mechanism is a possible second route for promoting intracellular immunity.

3.2. Spread of the immune signal

During a viral infection, the immune signal, whatever its nature, needs to be shared throughout the organism if a systemic antiviral response is to develop. As such, it would be possible to find dsRNA or siRNA at locations distant from the infection site. One plausible explanation for systemic spread is the lysis of the infected cells. However, this would not explain the protection observed in organisms infected with viruses that do not display a cytopathic effect. Therefore, there must be an active process to share and alert the neighboring and distant non-infected cells to allow a specific antiviral protection mediated by RNAi.

Exosomes are tiny vesicles generated from MVBs when they fuse to the plasma membrane (Fig. 1A and B), and they can carry mRNA and miRNA (Valadi et al., 2007; Huan et al., 2013) and both endogenous and exogenous proteins, including toxins (Zhang et al., 2009). Therefore, it has been suggested that exosomes may be responsible for exchange of material between cells. Exosomes are found in many different fluids, including blood, breast milk, amniotic fluid and malignant ascites (Denzer et al., 2000; Lasser et al., 2011; Runz et al., 2007). Then, it is tempting to speculate that in *Drosophila* they may travel through the hemolymph carrying and propagating the immune signal. There is increasing evidence that exosomes in mammals can be taken up by other cells following recognition by receptors on the plasma membrane (Miyanishi et al., 2007; Nolte-'t Hoen et al., 2009) and that they are carriers for many and diverse cargos depending on their cell origin. The uptake of free exosomes is currently the subject of lively debate. It has been suggested that after release from a cell, exosomes are endocytosed and targeted, along the cytoskeleton, to lysosomes (Tian et al., 2012). Other authors suggest that exosomes can be imported into cells by phagocytosis (Feng et al., 2010) or by fusion (Parolini et al., 2009). It is possible that exosomes fuse with the endocytic compartment after endocytosis and during acidification, they release their content in lysosomes. However, a large part of their contents escape and these escaped contents can include proteins, such as Ago2 and GW182 (Gibbings et al., 2009), and molecules such as siRNA and dsRNA. Silencing by small RNA is linked to endosomal trafficking (Lee et al., 2009) and it has been demonstrated that exosomes are involved in the immune system (Admyre et al., 2007). In mammals, for example, exosomes act as immunological mediators associated with tumor growth by exosome-mediated miRNA transfer (Kogure et al., 2011; Liu et al., 2006). In flies, exosome-like vesicles, called argosomes, are responsible for a graded distribution of morphogens, such as Wingless (Greco et al., 2001). This newly described route for intracellular communication has become a topic under intense study and we expect that it will soon become clear whether exosomes have an antiviral role during viral infection in insects.

Exosomes are not the only way that small RNAs use to circulate. Arroyo and colleagues (Arroyo et al., 2011) showed that in mammals, miRNA contained in exosomes constitutes only a minority of the circulating miRNA and the bulk of miRNA is found in the plasma as ribonucleoprotein complexes associated with Ago protein. Embedding small RNA in an RNP has several advantages as a mechanism of dissemination: it may improve RNA stability and

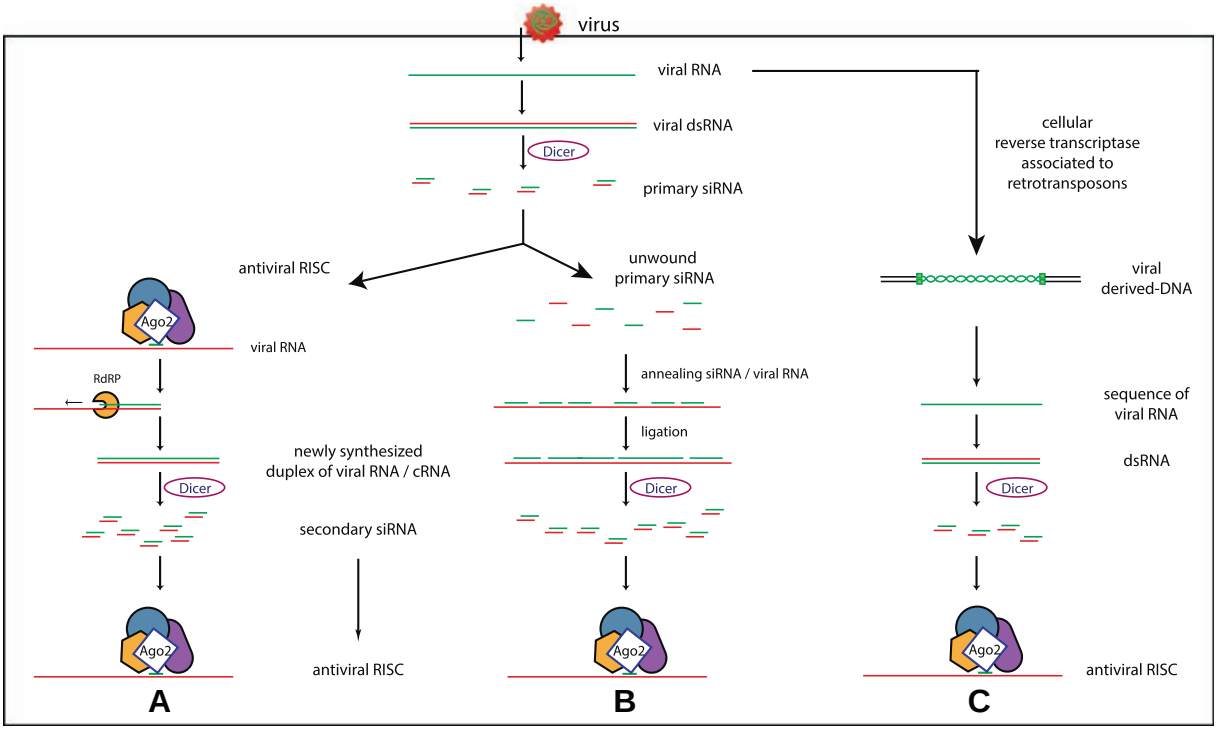

Fig. 2. Proposed mechanisms for amplification of the antiviral RNAi response. (A and B) During virus infection, genomic dsRNA, secondary structures, convergent overlapping transcriptional units or replication intermediates forming dsRNA are diced into primary siRNAs. The siRNA can be loaded in RISC complex to direct the cleavage of viral RNA. Additionally, the siRNA now in duplex with the viral RNA, can be used as template for a cellular RNA-dependent RNA-polymerase (RdRP). cRNA: complementary RNA (A). A new dsRNA is generated and diced, producing secondary siRNAs, also loaded in antiviral RISC. Alternatively, by an unknown mechanism, the primary siRNA duplex can be unwound. The single-stranded primary siRNA anneals with viral RNA (B). By ligation, new dsRNA is produced, diced into secondary siRNAs that are loaded into RISC, thereby amplifying the initial immune signal. (C) Cellular reverse transcriptase associated to retrotransposons produce a viral derived-DNA form from the viral RNA. This viral derived-DNA form is transcribed and produces dsRNA, which will in turn be diced, generating siRNAs. These DNA-derived siRNAs are loaded in antiviral RISC amplifying the canonical RNAi antiviral response.

resistance to environmental damage or degradation such that it is in a "ready-state" to regulate gene expression in recipient cells.

A graphic representation of the concepts developed in Sections 3.1 and 3.2 regarding internalization, sorting and transmission of the RNAi-immune signal is presented in Fig. 1.

3.3. Amplification of the immune signal

In a number of organisms, including plants and *C. elegans*, gene inactivation by silencing persists through cell division, can be spread to other tissues, and is heritable (Wianny and Zernicka-Goetz, 2000; Vaistij et al., 2002; Chuang and Meyerowitz, 2000). Therefore, even with very few inducer molecules of dsRNA, there must be a mechanism for the self-sustaining nature of RNAi. When *C. elegans* is fed or transfected with dsRNA, the dsRNA is diced into siRNA duplexes that are called primary siRNA. Primary siRNA triggers the specific silencing of the target RNA. A thorough study of the population of small RNAs recovered after dicing of the dsRNA revealed the presence of primary siRNAs as well as siRNAs with sequence characteristics (*i.e.* 5′-triphosphate) that excludes them from being digestion products of the dsRNA. These siRNAs are named secondary siRNA (Sijen et al., 2001). Several mechanisms for the biogenesis of secondary siRNA have been proposed (Fig. 2): (i) single-stranded (ss) siRNA may anneal with its target RNA, and could serve as primer for producing a complementary strand of the template RNA (cRNA); the resulting cRNA/RNA duplex would then constitute a dsRNA that could be degraded by Dcr, and again, loaded into RISC complexes. This mechanism allows the amplification of the initial signal, producing secondary siRNAs, which were not part of the initial dsRNA. This mechanism relies on an RNA-dependent RNA-polymerase (RdRP) (Tijsterman et al., 2002) (Fig. 2A). (ii) ss siRNAs may anneal to the RNA target by complementarity covering its entire length. A kinase may ligate siRNAs to form a new cRNA and consequently a cRNA/RNA duplex (a dsRNA molecule) (Nishikura, 2001). The dicing of this new dsRNA would produce secondary siRNAs, allowing the amplification of the signal, and the silencing of the target RNA (Fig. 2B).

Despite years of intense research, there have been no conclusive evidence for secondary siRNAs or RdRP activity in *Drosophila*. Interestingly, we recently described that during persistent viral infections non-retroviral RNA viruses can exploit cellular reverse transcriptases to produce a DNA form of viral origin early during the infection (Goic et al., 2013). This DNA of viral origin produces transcripts that can generate dsRNA, which in turn boosts siRNA-mediated immunity. The notion that DNA of viral origin can have immune functions had already been suggested for Israeli acute paralysis virus in *Apis mellifera* (honeybees): insects carrying a DNA insertion with substantial sequence identity with the RNA virus were resistant to the virus (Maori et al., 2007). It is then tempting to speculate that in the absence of a canonical RdRP that accounts for the production of secondary small RNAs in insects, the mechanism of transforming viral RNA > DNA > RNA > small RNA, could be amplifying the antiviral immune response throughout the insect's life (Fig. 2C). By the mechanism proposed, the RNAi-immune response is triggered by viral dsRNA replication intermediates, and amplified and boosted through newly generated viral DNA-derived dsRNA molecules. Future studies should shed light on the role of endogenized viral sequences in the amplification of the immune signal.

4. Closing remarks

There have been great advances over the last decade in our knowledge about immunity in non-mammalian models including plants and arthropods. Among them, the discovery of RNAi as an immune response revolutionized our way of conceiving host–virus interactions. This helped to improve the control of agricultural pests (Huang et al., 2006) and also contributed to our understanding of mammalian immunology.

Nowadays, the antiviral RNAi in *Drosophila* is well characterized as a cellular response based on the nuclease activities of Dcr-2 and Ago2. This response has also been found in almost every insect model tested. However, less is known about the role of the RNAi response as an immune system. The inducer signal for a systemic response remains still unknown. Nevertheless, evidence points to a signal of RNA nature (dsRNA, small RNA or RNP). The way in which these molecules are released from the infected site to be sensed and internalized by non-infected cells are also open questions. New findings and applications from the cell biology field will allow in-depth understanding of the role of endocytosis and trafficking vesicles in the RNAi systemic immunity in invertebrates.

Acknowledgements

We thank V. Mongelli, M. Vignuzzi and R.P. van Rij for critical reading of the manuscript. Supported by the French Agence Nationale de la Recherche (ANR-09-JCJC-0045-01) and the European Research Council (FP7/2007–2013 ERC 242703) to M.-C.S.

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