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

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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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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

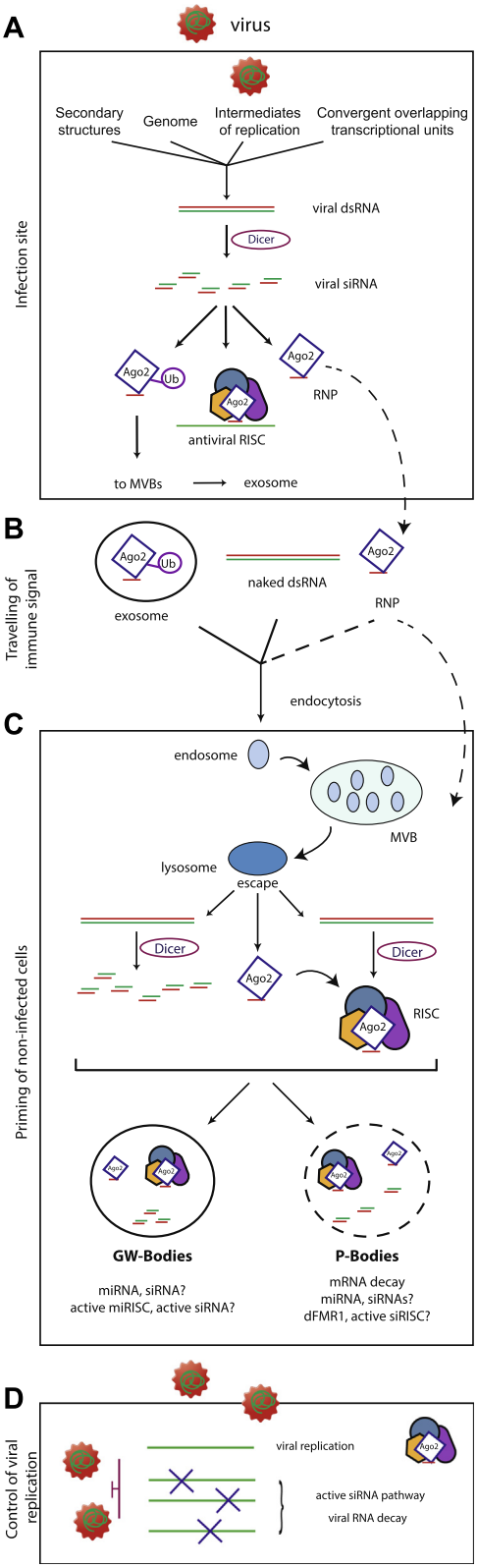

Fig. 1. Hypothetical model for internalization, sorting and transmission of the RNAi-immune signal during virus infection. At the site of infection (A), the presence of dsRNA activates the antiviral response at the cellular level and sets up the immune signal for a systemic response. This signal could 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

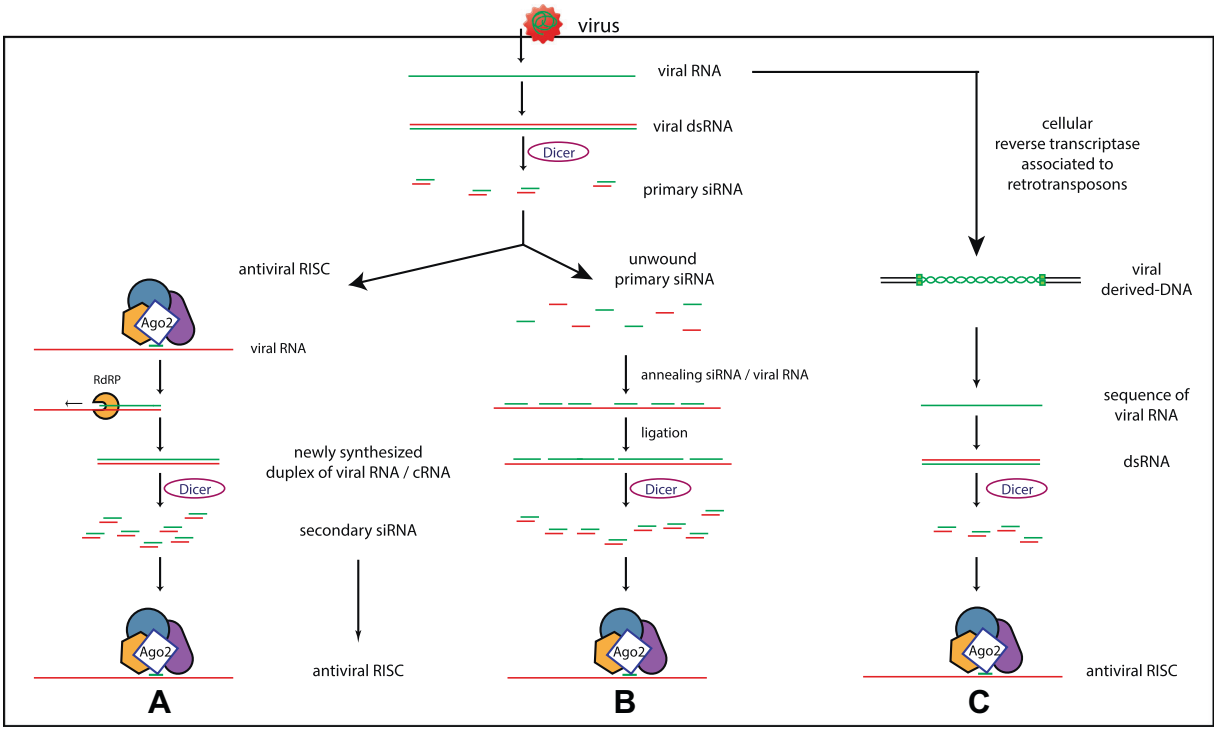

Fig. 2. Proposed mechanisms for amplification of the antiviral RNAi response. (A and B) During virus infection, genomic dsRNA, secondary structures, convergent overlapping transcriptional units or replication intermediates forming dsRNA are diced into primary siRNAs. The siRNA can be loaded in RISC complex 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.

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