Bugs Are Not to Be Silenced: Small RNA Pathways and Antiviral Responses in Insects

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Annu. Rev. Virol. 2016. 3:573–89

The *Annual Review of Virology* is online at virology.annualreviews.org

This article's doi: 10.1146/annurev-virology-110615-042447

Keywords

insects, viruses, antiviral immunity, small RNAs, siRNAs, viral suppressors

Abstract

Like every other organism on Earth, insects are infected with viruses, and they rely on RNA interference (RNAi) mechanisms to circumvent viral infections. A remarkable characteristic of RNAi is that it is both broadly acting, because it is triggered by double-stranded RNA molecules derived from virtually any virus, and extremely specific, because it targets only the particular viral sequence that initiated the process. Reviews covering the different facets of the RNAi antiviral immune response in insects have been published elsewhere. In this review, we build a framework to guide future investigation. We focus on the remaining questions and avenues of research that need to be addressed to move the field forward, including issues such as the activity of viral suppressors of RNAi, comparative genomics, the development of detailed maps of the subcellular localization of viral replication complexes with the RNAi machinery, and the regulation of the antiviral RNAi response.

INTRODUCTION

Insects: a class of arthropods with a three-segment body, three pairs of legs, compound eyes, and one pair of antennae

RNA interference (RNAi): biological mechanisms guided by small RNA molecules (21–30 nt) enabling the sequence-specific recognition of cognate nucleic acid target sequences and their degradation, translational arrest, or transcriptional regulation

Antiviral RNAi: innate immune response based on RNAi mechanisms triggered by the presence of dsRNA of viral origin

Small interfering RNAs (siRNAs): small RNA molecules (21–23 nt) of cell-endogenous (transposons) or cell-exogenous (virus) origin

Dipteran: a member of *Diptera*, an order of insects characterized by the presence of one pair of membranous wings and one pair of hind wings

Insects were among the first animals to colonize Earth's ecosystems approximately 500 million years ago (1, 2) and now constitute the most diverse class of living organisms in terms of described species (**Figure 1**) (3). Insects act as key participants in most terrestrial ecosystems by cycling nutrients, maintaining soil structure and fertility, pollinating plants and dispersing their seeds, controlling populations of other organisms, and serving as a food source for other organisms (4).

Insects have captured human attention since early times. Indeed, the earliest objects made by humans to symbolize insects date from the Paleolithic period (10,000 years ago); these objects depict beetles (5). For centuries, insects such as the silkworm (*Bombyx mori*) and the honeybee (*Apis mellifera*) have been protected and cherished because of their silk and honey production, respectively, but others, such as the locust (*Locusta* spp.), have been battled because of their devastating effects on crops. Once their capacity to act as vectors for human, livestock, and plant pathogens was established, insects became a primary research interest. In recent years, and particularly during the writing of this review, mosquitoes transmitting arboviruses (such as the dengue, West Nile, and Zika viruses) and insects transmitting agricultural diseases have become the focus of study, leaving other insect species aside. Today, few scientists study the behavior, ecology, biology, physiology, and immunity of other insects. Considering that insects constitute the most abundant taxa on Earth, returning to broader research about basic insect biology will positively influence research on all orders of life.

Like every other organism on Earth, insects interact with diverse pathogens. In contrast to vertebrates, insects lack an adaptive immune system and rely only on innate immune responses to cope with these pathogens. In a very general manner, the innate immune system, which is highly conserved in all metazoans, is based on the recognition of conserved pathogen-derived molecular motifs, called pathogen-associated molecular patterns (PAMPs), by host-encoded pattern-recognition receptors (PRRs) (6, 7). In insects, PAMP recognition by PRRs induces the rapid activation of the Toll, Imd, and Jak/Stat signal-transduction pathways, which lead to both humoral (e.g., secretion of antimicrobial peptides, lysozymes, or other microbe-targeting substances) and cellular (e.g., programmed cell death and autophagy) defense responses (6). These pathways were first described in studies of insect host defense against bacteria and fungi and were later shown to function in antiviral defense (6, 8, 9). Another evolutionarily conserved defense mechanism against viral infection is active in insects, the RNA interference (RNAi) mechanism, which is the subject of this review (10–12).

RNAi-based responses mediate robust antiviral activity in plants, nematodes, and insects (10, 13, 14). Antiviral RNAi is triggered in host cells following the sensing and subsequent cleavage of virus-derived double-stranded RNA (dsRNA) into small interfering RNAs (siRNAs) that guide the sequence-specific recognition of complementary viral RNA molecules, leading to their degradation and the blockade of viral replication. A remarkable characteristic of this mechanism relies on the fact that it is both broadly active, because it is triggered by dsRNA molecules derived from virtually any virus, and extremely specific, because it targets only the particular viral sequence that initiated the process.

Most of our knowledge on the innate immune system in insects comes from studies of the fruit fly (*Drosophila melanogaster*) and the mosquito (6, 8). *D. melanogaster* has been the insect model of choice since the early twentieth century (15), and it is followed in popularity by the mosquito, the various species of which constitute the main vectors for numerous human viral pathogens throughout the world (16). Consequently, research on insect immunity is heavily focused on these two dipterans, and the RNAi field is no exception. Reviews covering different facets of the RNAi antiviral immune response in the fruit fly and the mosquito have been published elsewhere

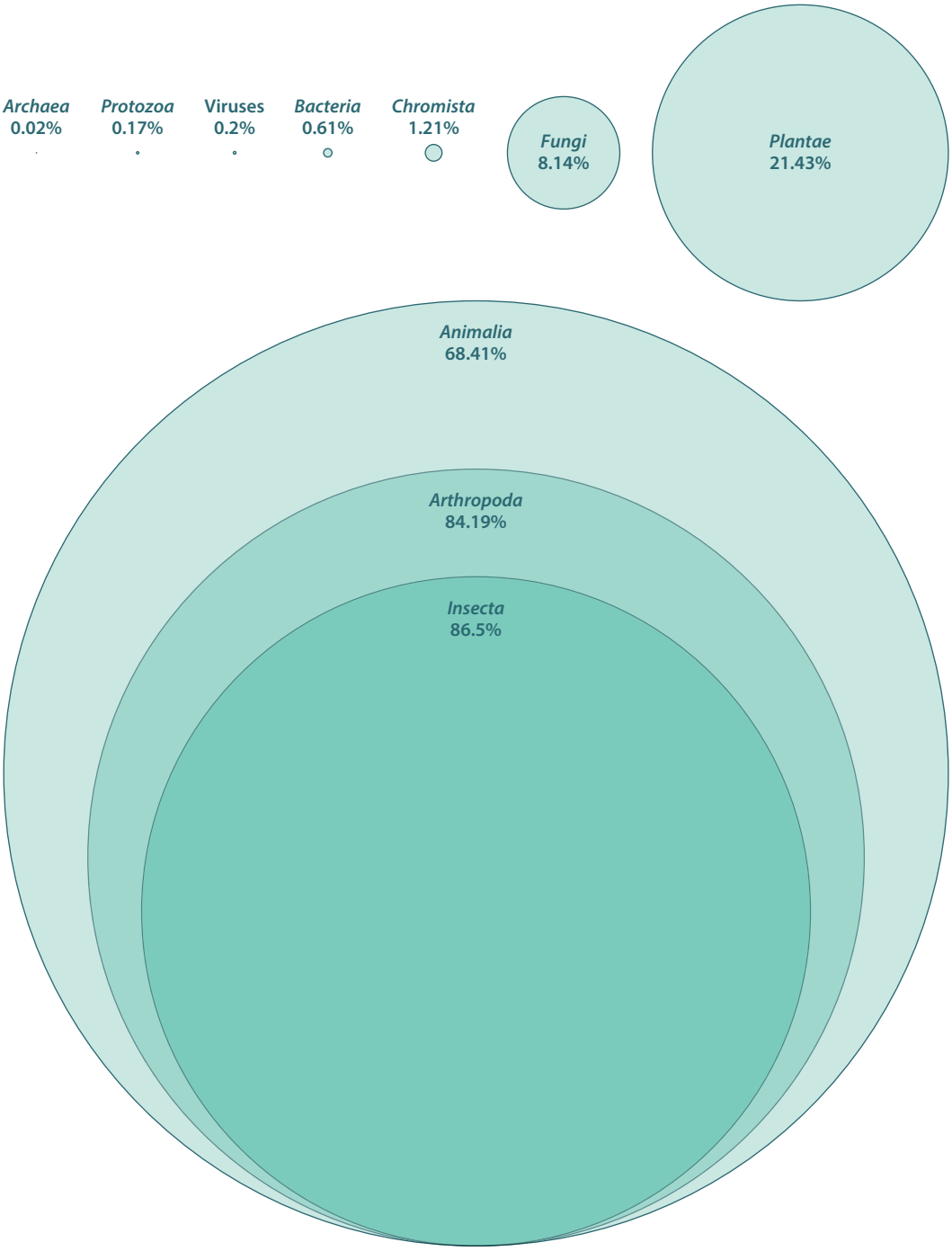

Figure 1

Insects rule. The *Insecta* class represents 86.5% of all species described in the *Animalia* kingdom. Indeed, insects are among the most extensive and diverse groups of life. The size of each circle is proportional to the number of species in each kingdom, including viruses.

(8, 10, 17). In this review, we build a framework for the discussions that are needed to advance future investigation. For several years, research on antiviral RNAi in insects has been centered on established conceptual frameworks. We use this review to briefly discuss what is known and to focus on the remaining questions and avenues of research that need to be addressed to move the field forward, including investigation of the activity of viral suppressors of RNAi, the use of comparative genomics, the development of detailed maps of subcellular localization of viral replication complexes with the RNAi machinery, and the regulation of the antiviral RNAi response.

MicroRNAs (miRNAs): small RNA molecules (21–23 nt) derived from host-encoded miRNA loci

PIWI-interacting RNAs (piRNAs): small RNA molecules (26–30 nt) derived from host-encoded piRNA loci or transposable elements

ARGONAUTE (AGO) proteins: endoribonucleases from the ARGONAUTE protein family characterized by PAZ (PIWI-AGO-Zwille) and PIWI domains

DICER (DCR) proteins: endoribonuclease proteins from the RNase III family of nucleases; able to recognize and cleave dsRNA molecules into small dsRNA molecules

RNA-induced silencing complex (RISC): enzymatic complex containing small RNA molecules and AGO proteins as the main active components for guiding and catalyzing sequence-specific recognition of target sequences

SMALL RNA PATHWAYS, THEIR BIOLOGICAL FUNCTIONS, AND THEIR INVOLVEMENT IN ANTIVIRAL DEFENSE

Three main small RNA–based silencing pathways are known in insects: the microRNA (miRNA), siRNA, and PIWI-interacting RNA (piRNA) pathways. Although they all use small RNAs (from 21 to 30 nt) to guide the sequence-specific recognition of target sequences by an ARGONAUTE (AGO) protein family member, these small RNAs differ in their origin, their biogenesis, the nature and fate of their targets after recognition, and their biological function. We use research on *D. melanogaster* to briefly describe the basis of each pathway (**Figure 2**) (for a detailed description, see 18–20).

The miRNA pathway is initiated by the expression of genome-encoded miRNA gene transcripts. These primary miRNAs are capable of folding back on themselves to form one or more dsRNA stem-loop structures that trigger the pathway. The primary miRNAs are processed in the cell nucleus by a protein complex formed by DROSHA and PASHA to produce the precursor miRNA, which is exported to the cytoplasm (21, 22). Precursor miRNAs are then further processed into 21- to 23-nt small dsRNA (miRNA) duplexes by another enzymatic complex formed by the DICER1 (DCR1) protein and LOQUACIOUS (LOQS)-PA or LOQS-PB (23, 24). The miRNA duplex produced in this reaction is loaded into the ARGONAUTE1 (AGO1)-containing RNA-induced silencing complex (RISC). One strand of the duplex, the miRNA*, is released from the complex and quickly degraded, forming a mature RISC that contains only one small RNA strand (25). RISCs harboring miRNAs primarily target protein-coding mRNAs, producing either translational inhibition or mRNA degradation. Target recognition by miRNA does not require perfect homology. The miRNA pathway is active in both somatic and germ-line tissues.

The siRNA pathway can be triggered in cells by either endogenous or exogenous dsRNA molecules. Endogenous dsRNA molecules are produced from long genomic transcripts capable of forming extensive fold-back structures or double-stranded regions generated by intermolecular hybridization of overlapped transcripts (26, 27). Exogenous dsRNA molecules can be derived from any environmental source, such as viral dsRNA molecules. In the siRNA pathway, dsRNA is recognized and processed in the cytoplasm by DICER2 (DCR2) into 21-nt siRNA duplexes harboring 2-nt 3′ overhangs (22). After being diced, siRNA duplexes are loaded into the ARGONAUTE2 (AGO2)-containing RISC. The biogenesis and loading of siRNA duplexes into the RISC requires the activity of LOQS and R2D2 as DCR2 cofactors. The LOQS-PD isoform and R2D2 are required for the production of siRNAs derived from endogenous dsRNA triggers, and R2D2 is primarily recruited in the production of virus-derived siRNAs (vsiRNAs) (28, 29). Once loaded into the RISC, one strand of the siRNA duplex, termed the passenger strand, is eliminated from the RISC. The single-stranded siRNA that remains in the RISC, termed the guide strand, is then 2′-*O*-methylated at its 3′-terminal nucleotide by the RNA methyltransferase DmHEN1 (30, 31), resulting in a mature, active RISC. Sequence-specific recognition mediated by the retained siRNA guide strand, which requires complete complementarity, then induces target RNA cleavage via the slicing activity of AGO2. Although endogenous siRNA targets are mostly

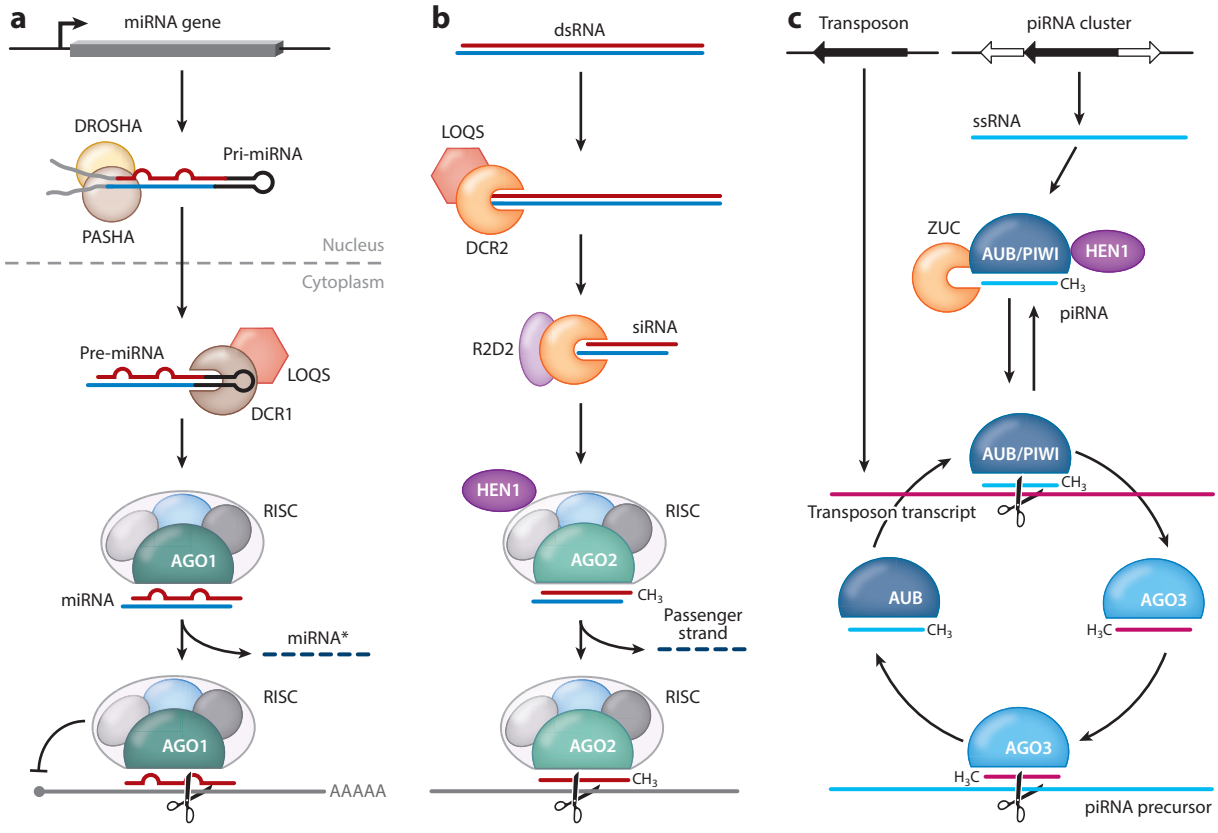

Figure 2

The elements of RNA interference (RNAi) in *Drosophila melanogaster*. (*a*) The microRNA (miRNA) pathway is initiated by the transcription of miRNA genes. Primary miRNA (pri-miRNA) transcripts are first processed by the DROSHA-PASHA complex in the nucleus and then exported to the cytoplasm as precursor miRNAs (pre-miRNAs). There, DICER1 (DCR1) and LOQUACIOUS (LOQS) complete the processing and deliver the mature miRNA to the ARGONAUTE1 (AGO1)-containing RNA-induced silencing complex (RISC). The miRNA* is eliminated, and the guide miRNA directs translational repression or cleavage of the cognate mRNA. (*b*) The small interfering RNA (siRNA) pathway is initiated by double-stranded RNA (dsRNA) of viral or genomic origin that is recognized and cleaved by DICER2 (DCR2) with the help of LOQS. The resulting double-stranded siRNAs are delivered to the ARGONAUTE2 (AGO2)-containing RISC by DCR2 and R2D2. The passenger strand is eliminated, and the guide siRNA, methylated by HEN1, directs the degradation of the target RNA via AGO2 catalytic activity. (*c*) The PIWI-interacting RNA (piRNA) pathway is initiated when primary piRNAs are processed from single-stranded RNA (ssRNA) precursors derived from transposable element sequences, named the piRNA clusters. ZUCCHINI endonuclease (ZUC) cleaves the primary piRNA precursors and generates the 5′ end of the mature piRNAs. The precursors are then loaded into PIWI or AUBERGINE (AUB) proteins, trimmed to reach their final lengths, and methylated by HEN1 to become mature piRNAs. Cleavage of the complementary active transposon transcript by primary piRNAs loaded into AUB proteins initiates the second biogenesis round and leads to the production of secondary piRNAs that are loaded into ARGONAUTE3 (AGO3). This amplification cycle is named ping-pong. Figure adapted with permission from Reference 103.

transposons and protein-coding mRNAs, vsiRNAs recognize virus-derived sequences. As with the miRNA pathway, the siRNA pathway is ubiquitously expressed.

Molecules initiating the piRNA pathway are single-stranded RNA (ssRNA) precursors transcribed from chromosomal loci that mostly consist of remnants of transposable element sequences, called piRNA clusters (32). Biogenesis of piRNAs involves two steps, primary processing and secondary amplification. Production of piRNAs is DCR independent and mainly relies on the

activity of PIWI proteins, a subclass of the AGO protein family (33). Primary piRNAs are processed from ssRNA transcripts derived from piRNA clusters. ZUCCHINI endonuclease (ZUC) cleaves primary piRNA precursors and generates the 5′ end of mature piRNAs (34–36). The cleaved precursor is loaded into PIWI or AUBERGINE (AUB) proteins and then trimmed by an unknown nuclease to reach its final length. After trimming, piRNAs undergo a final 3′-end 2′-*O*-methyl nucleotide modification catalyzed by DmHEN1 (30, 31) to yield mature piRNAs. Primary piRNAs harbor a 5′ uridine bias (U1) (37). Cleavage of the complementary active transposon RNA by primary piRNAs loaded into AUB proteins initiates the second round of biogenesis, which leads to the production of secondary piRNAs that are loaded into ARGONAUTE3 (AGO3). During this ping-pong, or amplification, cycle, AUB and AGO3 proteins loaded with secondary piRNAs mediate the cleavage of complementary RNA, generating new secondary piRNAs that are identical in sequence to the piRNA that initiated the cycle. The complementary secondary piRNAs usually have a 10-nt overlap and contain an adenine at position 10 (A10) (38). Most data indicate that the piRNA pathway is mainly active in germ-line tissues, where it acts as a genome guardian by cleaving transposon RNA or transcriptionally silencing transposable elements.

Until recently, only the siRNA pathway was known to contribute to the antiviral response through the production of vsiRNAs. However, virus-derived small RNAs with piRNA features have been found by using next-generation sequencing in *Aedes* mosquitoes and mosquito cell lines infected by arboviruses (39–44). These descriptive studies were followed by direct functional analyses demonstrating that (*a*) depletion of PIWI4 protein enhances replication of Semliki Forest virus (positive-sense ssRNA, *Togaviridae*) without interfering with virus-derived piRNA production in Aag2 cells (45) and (*b*) both PIWI5 and AGO3 are required for piRNA biogenesis derived from Sindbis virus (positive-sense ssRNA, *Togaviridae*) in the same cell line (46). Nevertheless, functional in vivo data remain scarce, and more work needs to be done to fully understand to what extent the piRNA pathway contributes to antiviral defenses as a general mechanism, not only in mosquitoes during arboviral infections but also in the context of other insect-virus interactions. Interestingly, studies from our laboratory have shown that virus-derived piRNAs are not produced by *D. melanogaster*, and the piRNA pathway thus does not appear to be required for antiviral defense in this organism (47).

Insect miRNAs are involved in the regulation of diverse biological processes, including metabolism, development, differentiation, apoptosis, and innate immune responses (48). Viral infections can alter the regulation of host miRNAs that may either promote or interfere with viral infection by influencing the expression of host immune response genes or viral genes involved in the establishment of infection (49–53). Viral genomes can encode their own miRNAs, which function to regulate viral gene expression or manipulate the host immune response. Among the few insect viral miRNAs identified, most come from viruses with double-stranded DNA (dsDNA) genomes, such as *Heliothis virescens ascovirus* (*Ascoviridae*), *Bombyx mori* nucleopolyhedrosis virus, and *Autographa californica* nucleopolyhedrovirus (51, 52, 54, 55). Nevertheless it has been suggested that miRNA-like molecules are encoded by two flaviviruses (positive-sense ssRNA): West Nile virus and dengue virus (56, 57). The West Nile virus miRNA-like molecule is thought to facilitate viral replication by targeting cellular mRNA, and the dengue virus miRNA-like molecule is thought to regulate viral replication by targeting a viral nonstructural protein sequence (56, 57). The role of viral and cellular miRNAs during the antiviral response remains an enigma, but it seems to constitute a key feature of an intricate host-pathogen struggle, in which both virus and host miRNAs and proteins aim to control virus and host gene expression (58–60). The elucidation of such interactions requires complex and refined research that fully addresses the biological implications and consequences beyond providing mere descriptions and miRNA annotations.

VIRAL SUPPRESSORS OF RNAi AND VIRAL COUNTER-DEFENSE MECHANISMS

The existence of viral proteins with the capacity to interfere with different components of the RNAi machinery, the viral suppressors of RNAi (VSRs), highlights the relevance of the RNAi antiviral defense as the center of the host-pathogen encounter. Several viral suppressors have been identified in insect viruses with positive-sense ssRNA genomes (*Nodaviridae*, *Dicistroviridae*, *Flaviviridae*, Nora viruses with unassigned family), dsRNA genomes (*Birnaviridae*), and DNA genomes (*Ascoviridae*, *Iridoviridae*) (61). Curiously, viral suppressors for negative-sense ssRNA viruses have not been reported. These VSRs exert their action by four main mechanisms: (*a*) binding long dsRNA to prevent DCR2 cleavage, (*b*) binding siRNAs to prevent their loading into the RISC, (*c*) directly interacting with DCR2 or AGO2 proteins to prevent their action, and (*d*) degrading siRNAs. In some cases, one VSR protein may combine more than one of these modes of action (for reviews of insect VSRs, see 10, 61).

Most VSRs have been characterized using ex vivo systems and in tissue culture using RNAi reporter systems or recombinant viruses (62, 63) without consideration of the natural context of an infection and the role of the VSR in pathogenesis and transmission. Despite the utility of these RNAi and VSR reporter systems, they have important limitations. Viral proteins usually display more than one function, and their ectopic overexpression in a system designed to detect suppression might produce false-positive results. In these types of experiments, it is difficult to prove whether a viral protein with a dsRNA-binding domain is or is not a VSR. For example, ectopically expressed dsRNA-binding proteins act as VSRs in reporter assays in plants (64). Indeed, *Escherichia coli* RNase III and mammalian orthoreovirus (MRV) σ3 protein expressed in plants both display VSR activity. Strikingly, *E. coli* RNase III is a global gene expression regulator in bacteria, influencing posttranscriptional control of mRNA stability or mRNA translational efficiency (65). MRV σ3 is a multifunctional protein that serves as a virion outer capsid protein and mediates several activities throughout the viral life cycle (66). Whether MRV σ3 can be considered to be a VSR in its natural biological context remains unknown. Another limitation of studying VSRs is the host specificity of VSR activity. For example, VP1 proteins encoded by *D. melanogaster* Nora virus (DmelNV) and *Drosophila immigrans* Nora-like virus (DimmNV) act as VSRs by interacting with AGO2 and blocking target cleavage. However, DimmNV VP1 cannot act as a VSR in a *D. melanogaster* S2 cell reporter system. DimmNV VP1 can interact only with *D. immigrans* AGO2 (67). Of all known insect VSRs, the B2 protein of Flock House virus (FHV) has been most extensively studied. Its absence during infection in adult flies causes a defect in FHV replication and the accumulation of FHV-derived siRNAs (68). The study that found this was conducted using *D. melanogaster*, which is not the natural host of FHV. However, this kind of direct experimental evidence about the role of VSRs in the viral life cycle and during infection of the host is lacking for VSRs from other insect viruses.

An interesting facet of VSRs is their potential capacity to interfere with the normal activity of small RNA pathways other than the vsiRNA pathway, due to their mode of action. Although the ectopic expression of VSRs in plants was found to interfere with the miRNA pathway and lead to pleiotropic developmental defects (69, 70), analogous experiments performed using *D. melanogaster* have demonstrated that VSRs do not affect global miRNA biogenesis and function (71). These results can be reconciled by the fact that plant siRNA and miRNA pathways share biogenesis factors, and both vsiRNAs and miRNAs can be loaded into AGO1 (72), whereas in flies, the main factors of these pathways are not commonly shared. Additionally, ectopic expression of some VSRs suppresses RNAi induced by exogenous dsRNA in embryos and by endogenous siRNAs in adult flies. These results suggest that, although VSRs might not have a conspicuous

effect on the miRNA pathway, they appear to block the endogenous siRNA pathway, a result that is further supported by other work from the same group (73). Determining whether VSRs interfere with small RNA pathways in a general manner requires more research on *D. melanogaster*, as well as on other insect species.

Finally, because the piRNA pathway is involved in the antiviral response, should we expect to identify VSRs of the piRNA pathway? If so, what would be the counter-defense mechanism of viruses against antiviral piRNAs?

COMPARATIVE GENOMICS: NATURAL VARIATIONS OF RNAi COMPONENTS AMONG INSECTS

As mentioned above, most of what we know about the antiviral role of small RNA–based pathways comes from studies of *D. melanogaster* and mosquitoes. Nevertheless, other insects of economic interest, such as honeybees (*A. mellifera*), silkworms (*B. mori*), red flour beetles (*Tribolium castaneum*), and plant virus-transmitting hemipteroids (aphids, whiteflies, and thrips), are being extensively studied (74–77). The existence of RNAi in different types of insects has been shown by (*a*) the presence of small RNA species detected by next-generation sequencing and (*b*) the use of RNAi as a tool for understanding the role of endogenous genes or for affecting the insect life cycle as a method of pest control. However, the increasing number of genome sequencing projects, with 138 insect genome sequences now available (78), allows for detailed comparative genomic and phylogenetic analyses of the RNAi pathway components present in different insect species. **Table 1** shows the number of gene copies for the key components of the small RNA pathways, the DCR and AGO protein family members, in some insect orders and species. This list is not exhaustive but illustrates that small RNA–encoding genes differ in copy number among insect species. Phylogenetic analyses of small RNA pathway components have indicated that loci encoding these molecules expanded or contracted during the course of evolution (79–81). For example, miRNA gene expansions are found in the pea aphid (*Acyrthosiphon pisum*), the Russian wheat aphid (*Diuraphis noxia*), and the housefly (*Musca domestica*). Gene expansions of siRNA are found in the locust (*Locusta migratoria*), the Russian wheat aphid, the red flour beetle (*T. castaneum*), the parasitoid wasp (*Nasonia vitripennis*), diverse species of *Glossina* (tsetse flies), the fruit fly, and the housefly (82; also see figure 2 in 79). The piRNA pathway genes expanded in both pea and Russian wheat aphids; the parasitoid wasp; two types of ants (*Camponotus floridanus* and *Harpegnathos saltator*); and mosquitoes from the *Aedes*, *Anopheles*, and *Culex* genera. Duplication and diversification of small RNA pathways are widely present among insects, indicating that these changes are not rare and have occurred even at the species level.

Although gene duplications are a frequent source of novelty in host genome defense repertoires, the evolutionary fate of specific duplicates can vary. In most cases, one of the duplicated copies will be eliminated; alternatively, both copies may be retained but may acquire differentiated profiles of expression or different functions (83, 84). The frequent expansion of genes in the RNAi pathway is often accompanied by changes in their evolutionary rates. Some of these changes might be explained by the involvement of these genes in host-pathogen interactions. For example, in *D. melanogaster*, genes involved in pathogen defense evolve much more quickly than the rest of the genome. AGO2 is a clear example of this observation, and PIWI and AUB also evolve extremely quickly (85), which is thought to be attributable to adaptation to the ever-changing landscape of transposition activity in the fly. Another interesting example arises when studying the involvement of the piRNA pathway in antiviral responses in two dipterans, the fruit fly and the mosquito. Although virus-derived piRNAs are present in mosquitoes, recent work from our lab (47) has indicated that they are not produced during viral infections in *D. melanogaster*. Although flies encode

Table 1 Number of gene copies for key components of the small RNA pathways in some insect orders and species

Order	Species	Common name	MicroRNA (miRNA)			Small interfering RNA (siRNA)		PIWI-interacting RNA (piRNA)		
			DROSHA	DCR1	AGO1	DCR2	AGO2	PIWI, PIWI/AUB	AUB	AGO3
Orthoptera 	*Locusta migratoria*	Locust	1	1	1	2	2	—	—	—
Hemiptera 	*Acyrthosiphon pisum*	Pea aphid	1	2	2	1	1	8	0	2
	Diuraphis noxia	Russian wheat aphid	1	1	2	1	2	5	0	1
	Nilaparvata lugens	Brown planthopper	1	1	1	1	1	1	1	1
Coleoptera 	*Tribolium castaneum*	Red flour beetle	1	1	1	1	2	1	0	1
Hymenoptera 	*Apis mellifera*	Honeybee	1	1	1	1	1	1	1	1
	Nasonia vitripennis	Parasitoid wasp	1	1	1	1	2	0	3	1
	Camponotus floridanus	Bull ant	1	1	1	1	1	1	2	1
	Harpegnathos saltator	Indian jumping ant	1	1	1	1	1	1	2	1
Lepidoptera 	*Bombyx mori*	Silkworm	1	1	1	1	1	1	1	1
	Manduca sexta	Tobacco hornworm	1	1	1	1	1	1	—	1
	Danaus plexippus	Monarch butterfly	1	1	1	1	1	1	1	1
	Heliconius melpomene	Postman butterfly	1	1	1	1	1	1	1	1
Diptera 	*Drosophila melanogaster*	Fruit fly	1	1	1	1	1	1	1	1
	Musca domestica	Housefly	1	2	1	—	2	1	1	1
	Glossina morsitans	Tsetse fly	—	—	1	—	3	1	1	3
	Aedes aegypti	Yellow fever mosquito	1	1	1	1	1	7	0	1
	Anopheles gambiae	Malaria mosquito	1	1	1	1	1	2	0	1
	Culex pipiens	House mosquito	1	1	1	1	2	6	0	1

Dash indicates that the number is not known; 0 indicates that copies were not found.

three PIWI proteins (PIWI, AUB, and AGO3), the PIWI family is expanded to eight members (PIWI1–7 and AGO3) in the mosquito *Aedes aegypti* and to seven in *Culex pipiens* (86). It is possible that during speciation and diversification of piRNA pathway proteins, the piRNA pathway gained functions beyond the repression of transposon activity in mosquitoes while remaining exclusively focused on the control of transposons in the fly. Alternatively, the piRNA pathway may have lost an ancestral antiviral function since the last common ancestor of flies and mosquitoes. With the advent of new sequencing technology and increasing access to metagenomic data, the field of comparative genomics and evolution should continue to expand knowledge about the origin, role, and diversity of RNAi genes in insects. These studies will establish a foundation for comprehensive designs of wet-lab approaches to different insect-virus combinations to unlock the secrets of antiviral RNAi in insects.

WHERE AND WHEN? SUBCELLULAR LOCALIZATION OF THE RNAi-VIRUS INTERACTION

Several independent studies have indicated that the antiviral RNAi machinery is triggered by the presence of viral dsRNAs in the cell. In most cases, the source of these viral dsRNAs is viral replication intermediates (see table 1 in 10). Additionally, RNA viruses can be retrotranscribed into a viral DNA form by cellular reverse transcriptases. The transcription of these viral DNA forms is involved in the dsRNA biogenesis that triggers the RNAi response (see figure 1 in 87). However, a question surrounds this evidence: When and where in the cell does the RNAi machinery encounter the viral replication apparatus?

Viral replication is a complex and regulated process that may occur in the nucleus (dsDNA viruses and some negative-sense ssRNA viruses) or the cytoplasm (positive-sense ssRNA viruses, dsRNA viruses, and some negative-sense ssRNA viruses) (**Figure 3**). Despite biological differences, replication of most positive-sense ssRNA viruses is associated with, and localized to, host-derived membranes. Positive-sense ssRNA viruses form vesicular invaginations to use as an optimized environment to support viral replication and assembly. The viral dsRNA intermediate is mainly detected inside these membranous structures (88), which are connected to other membranes and open to the cytoplasm through pore-like structures (89). These membranous structures, or spherules (88), are derived from mitochondria during infection with members of the family *Nodaviridae*, from endosomes for *Togaviridae*, from the endoplasmic reticulum and Golgi for *Flaviviridae*, from the Golgi for *Bunyaviridae*, and from autophagosomes for *Picornaviridae*. Viruses from other families, such as *Iridoviridae*, *Poxviridae*, and *Reoviridae*, form electron-dense cytoplasmic inclusions called viroplasms that house viral replication. In the case of dsRNA reoviruses, replication of the genome takes place inside viral particles. Viral dsRNA produced during viral replication is mainly localized inside viral membranous or protein structures, which may be a mechanism to protect or delay recognition of PAMPs in vertebrates (90, 91). The same rationale can be applied to insect viruses and antiviral RNAi.

The subcellular localization of RNAi is not precisely understood, although it has been proposed to occur in the cytosol. In plants and mammals, RNAi proteins are associated with endomembrane systems in the cell (92). For insects, information is scarce. R2D2 and DCR2 have been proposed to localize in discrete cytoplasmic bodies in *Drosophila* cells, termed D2 bodies (93), and the AGO1-containing RISC localizes to mRNA decay centers, suggesting that this is the final step of the RNAi pathway, when target RNA is degraded (94).

In *Drosophila* somatic cells from germ tissue, the primary piRNA biogenesis machinery (PIWI protein and others) localizes in perinuclear cytoplasmic structures called Yb bodies (95). In germline cells, both primary and secondary piRNA biogenesis machineries localize in other perinuclear,

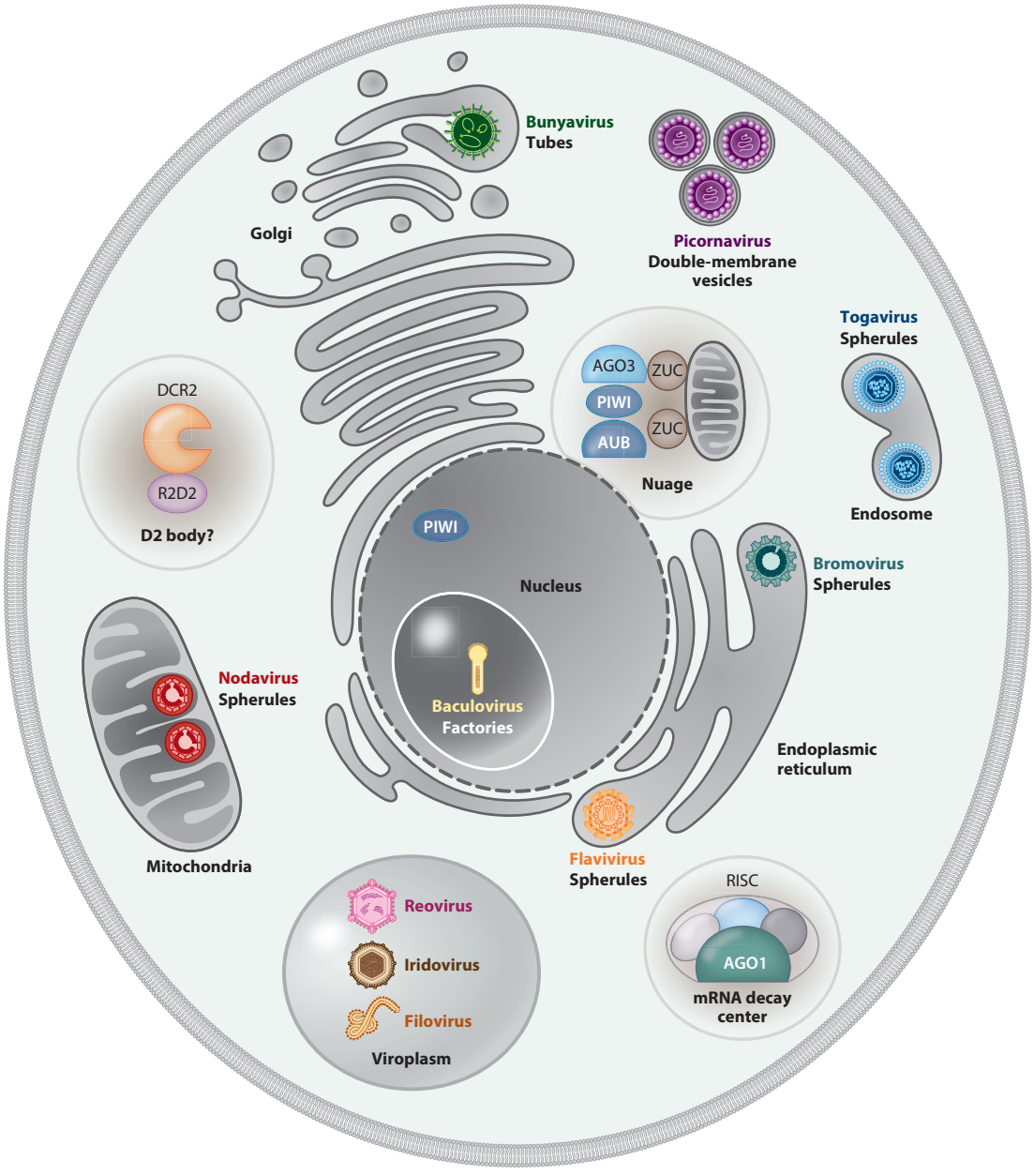

Figure 3

RNAi, where are you? Subcellular localization of viral replication sites and RNAi machinery. Viral replication occurs in specialized membranous environments within host cells. The site at which the RNAi machinery encounters viral replication intermediates is unknown. Abbreviations: AGO, ARGONAUTE; DCR, DICER; RNAi, RNA interference; ZUC, ZUCCHINI endonuclease.

electron-dense cytoplasmic structures known as nuage (**Figure 3**) (20). ZUC localizes to the outer membrane of mitochondria (36).

Important questions remain in considering the steps of antiviral RNAi. For example, where do DCR proteins and viral dsRNA meet? Is DCR2 (an ~255-kDa protein) trapped inside the spherules or the viroplasm during viral replication? Are there any signals that bring DCR2 to the viral replication site? If DCR2 is cutting viral dsRNA inside these membranous structures, how are siRNAs released to reach AGO2? Is it possible to find AGO2 and the RISC (~80S) in viral replication sites? Can the entire RNAi process occur inside viral replication sites and in parallel with viral replication? Once AGO2 cuts the target viral RNA, where does RNA degradation occur? Does the sliced viral RNA leave the replication site and enter an RNA decay center? Do pore-like openings serve as entry or exit portals for the RNAi machinery? Optimizing cell biology techniques—such as cell fractionation, membrane flotation, immune precipitation, electron microscopy, and electron tomography together with live-cell imaging—in insect cells could be a starting point for answering these questions.

REGULATION OF THE ANTIVIRAL RNAi PATHWAY

A general and widespread consequence of PAMP recognition by PRRs during the innate immune response in insects is the transcriptional induction of antimicrobial effector molecules (6, 96). Viral dsRNA molecules act as PAMPs in insects, triggering the expression of innate immune response genes (97–100). However, no such induction has been observed in insects for antiviral RNAi response–related genes following viral infection. Of the numerous studies of antiviral RNAi in the fruit fly and the mosquito, the subject of whether there is a basal and constant level of RNAi protein expression or whether expression is induced by viral infection has not been sufficiently addressed. In our lab, infection of *Drosophila* S2 cells with *Drosophila* C virus (DCV) or FHV did not change expression levels of DCR and AGO proteins. Injecting or feeding adult *D. melanogaster* with DCV does not affect the expression level of AGO2. However, there is some evidence that RNAi-related genes are induced at a transcriptional level in some experimental systems. AGO1–3 and PIWI mRNA expression in *B. mori* is induced 6 h after infection with *B. mori* nucleopolyhedrosis virus and is lost by 12 h after infection (101). A study using *D. melanogaster* suggested that the FOXO transcription factor regulates the expression of core components of the small RNA pathways in response to stress. FOXO binds to AGO1, AGO2, and DCR2 promoters and might therefore play a role in regulating their expression (102).

In *D. melanogaster*, DCR2 and AGO2 proteins are shared by both endogenous and exogenous siRNA pathways. Because the endogenous siRNA pathway is involved in the control of transposon expression in somatic tissues, that relationship could explain the constitutive expression of this pathway. However, the situation in the context of a viral infection is unclear. For example, would both endogenous and exogenous siRNA pathways share one fixed amount of RNAi effector proteins? Would the regulation rely on changes in rates of protein turnover? Could the virus be affecting a cellular function linked to the endogenous siRNA pathway that we have not yet identified? If DCR and AGO proteins are constitutively expressed and not activated by the presence of the pathogen, is it possible that their antiviral role is secondary?

CONCLUSIONS

New research is starting to shed light on the diversity of mechanisms used in insects for antiviral defense. These studies point to the uniqueness of each natural pathosystem (i.e., the natural insect-virus system) that is essential to understanding the contribution of antiviral RNAi, and they show

that in some cases the use of model systems may not have yielded generalizable concepts. Although time-consuming to determine, the contribution of the RNAi pathway to the antiviral response, as well as the interactions of RNAi with other immune pathways, should be established for each individual pathogen-insect combination. Understanding these contributions and interactions will illuminate fundamental concepts in biology; clarify age-old unexplained observations; and open the door to new views, concepts, and theories about pathogen-host relationships.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank members of the Saleh lab for fruitful discussions and Marco Vignuzzi and Ronald van Rij for critical review of the manuscript. This work was supported by the European Research Council (FP7/2013–2019 ERC CoG 615220) and the French government's Investissement d'Avenir program (Integrative Biology of Emerging Infectious Diseases Laboratory of Excellence; grant number ANR-10-LABX-62-IBEID) to Maria-Carla Saleh.

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