RNAi AND miRNA PATHWAYS IN ARTHROPODS

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ABSTRACT

RNA silencing denotes sequence-specific repression mediated by small RNAs. In vertebrates, there are two closely related pathways, which share several protein factors: RNA interference (RNAi) and microRNA (miRNA) pathway. The miRNA pathway regulates endogenous protein-coding gene expression and has been implicated in many biological processes. RNAi generally serves as a form of innate immunity targeting viruses and mobile elements. While *Arthropoda* are an extremely large and diverse phylum, research on microRNA (miRNA) and RNA interference (RNAi) pathway in this phylum primarily used the *Drosophila melanogaster* model system and related species. Notably, both pathways are genetically separated; they utilize dedicated Dicer proteins to produce miRNAs and small interfering RNAs (siRNAs), which are sorted onto different Argonaute effector proteins. This review focuses on the miRNA pathway and pathways initiated by long dsRNA in arthropods. The first part introduces the key molecular players of RNA silencing. The second discussed biological roles of miRNA and dsRNA-induced pathways in Arthropods.

Introduction

Arthropoda are an extremely large and diverse group of animals. In fact, they are the largest animal group on Earth with a million of named species (~80% of described animal species!) and estimated tens of millions of species (Margulis and Schwartz, 1998). *Arthropoda* are typically classified into five subphyla (Fig. 1). (Regier et al., 2010). *Trilobita* are a famous extinct group of marine animals that declined in the Late Devonian extinction and completely disappeared in the Permian–Triassic extinction. *Chelicerata* include living fossil horseshoe crabs, spiders, mites, ticks, scorpions and related organisms. Their characteristic features are chelicerae appendages, which appear in scorpions and horseshoe crabs as claws while spiders use them to inject venom. *Myriapoda* have repetitive body segments carrying one or two pairs of legs and include centipedes, millipedes, and their relatives. *Crustacea*

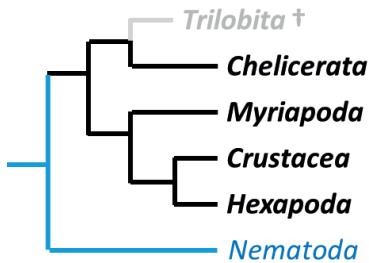

Figure 1 Simplified division of *Arthropoda* used in the text
The scheme reflects the *Mandibulata* model of arthropod phylogenetics described in (Regier et al., 2010)

are with, some exceptions (e.g. armadillo bug an relatives known as woodlice), aquatic and have differentiated segmented body and biramous appendages. They include shrimp, crayfish, lobsters, crabs, barnacles, prawns and others. *Hexapoda* comprise insects and insect-like animals with six thoracic legs.

The key model organism for arthropods is *Drosophila*, which has been a workhorse of biology for over hundred years. miRNA, RNAi and other dsRNA pathways in *Drosophila* are well understood and will serve as benchmarks for the entire phylum. *Drosophila* evolved an extensive genetic separation of miRNA and RNAi pathways where each pathway has a dedicated Dicer, dsRBP, and Argonaute protein. Given the complexity of the phylum and evolutionary time, one could question how representative of arthropods is the *Drosophila* model. However, analysis of Dicer and AGO indicates that *Drosophila* is a more-or-less acceptable model for most arthropods as the "two Dicer system" can be recognized within phylogeny of Dicer and AGOs also in *Chelicerata* (whose common ancestors with *Drosophila* branched in the most distant past), *Myriapoda*, and *Crustacea* (Palmer and Jiggins, 2015). However, it should be kept in mind that some variability could emerge during half a billion years of arthropod evolution.

Since mechanistical principles of vertebrate miRNA and RNAi pathways were introduced in the first review of this series (Svoboda, 2019) and in further detail elsewhere (Bartel, 2018), I will focus here directly on features of these pathways discovered in Arthropods. The formal structure of the report will be as in other animal taxons – upon miRNA dna RNAi molecular features of key individual components of reviewed mechanisms, I will discuss the silencing mechanisms and their biological roles. Importantly, to provide an overview of miRNA and dsRNA mechanisms in arthropods, I will focus on description of molecular mechanisms identified in *Drosophila* and will highlight and discuss significant deviations observed elsewhere in arthropods, especially in more studied organisms, such as mosquitos, flower beetle, silk moth, and shrimps.

The Microprocessor complex

Drosophila utilizes the same Microprocessor complex as the earlier discussed *Metazoa*, i.e. a complex of Drosha and DGCR8 homologs, the latter being named Pasha (partner of

Drosha) (Denli et al., 2004; Filippov et al., 2000; Landthaler et al., 2004). The complex cleaves the pri-miRNA into pre-miRNA in the nucleus. Suppression of Pasha in *Drosophila* interferes with pri-miRNA processing, leading to an accumulation of pri-miRNAs and a reduction in mature miRNAs (Denli et al., 2004; Landthaler et al., 2004). Like in other animals, Pasha is essential for processing of canonical miRNAs but is dispensable for mirtrons (Flynt et al., 2010; Martin et al., 2009; Smibert et al., 2011). *Drosophila* Pasha is possibly phosphorylated by ERK/MAPK, as suggested by phosphorylation of human DGCR8 in insect cells; the phosphorylation appears to increase protein stability without altering miRNA processing activity (Herbert et al., 2013). miRNA biogenesis in *Drosophila* also involves SmD1, a component of the *Drosophila* small nuclear ribonucleoprotein particle (snRNP), which interacts with both the microprocessor component Pasha and pri-miRNAs, and is indispensable for optimal miRNA biogenesis (Xiong et al., 2015).

Analysis of transcriptome changes upon Drosha knock-down in S2 cells identified 137 Drosha-regulated RNAs, including 11 relatively long (>10 kb) pri-miRNAs (Kadener et al., 2009). Interestingly, >100 RNAs not annotated as miRNAs could be direct targets of Drosha action (Kadener et al., 2009), which is consistent with other model systems where Drosha is having roles beyond miRNA biogenesis. Drosha-regulated RNAs contain conserved hairpins similar to those recognized by the Drosha-Pasha/DGCR8 complex in pri-miRNAs, one of such hairpins is found also in Pasha suggesting a negative feedback loop regulating miRNA-biogenesis (Kadener et al., 2009). miRNA-independent roles of the Microprocessor complex components seem to be reflected in phenotypes of some of their mutants (Luhur et al., 2014).

In terms of evolutionary diversity of the Microprocessor complex in arthropods, the miRNA pathway seemed to expand in the pea aphid (insect, *Hemiptera*), whose genome carries four expressed copies of Pasha (Jaubert-Possamai et al., 2010). At the same time, the brown planthopper (insect, *Hemiptera*), the fall armyworm (insect, *Lepidoptera*) or the desert locust (insect, *Orthoptera*) all have a single Pasha (Ghosh et al., 2014; Wynant et al., 2015; Xu et al., 2013), which appears the common case among arthropods when browsing available genome databases. Analysis of Pasha in *Litopenaeus vannamei* (shrimp) revealed high sequence conservation and nuclear localization, suggesting a well-conserved role in miRNA biogenesis (Chen et al., 2012). Conservation of miRNA pathway in shrimps is further supported by requirement for Drosha, Dicer1 and Ago1 for production of viral RNAs in infected shrimps (He and Zhang, 2012; Huang et al., 2012).

Dicer

Drosophila utilizes two Dicer proteins (Fig. 2), Dicer-1 (DCR-1) and Dicer-2 (DCR-2), which are dedicated to miRNA and RNAi pathways, respectively (Lee et al., 2004). This makes *Drosophila* (and arthropods in general) unique among the reviewed metazoan model systems (Fig. 3), which employ a single Dicer protein producing multiple classes of small RNAs (miRNAs, endo-siRNAs, exo-siRNAs). Separation of miRNA and RNAi at Dicer level could have an advantage in terms of uncoupling antagonistic evolutionary forces acting on Dicer, i.e. (i) selective pressure on conservation of the miRNA pathway machinery

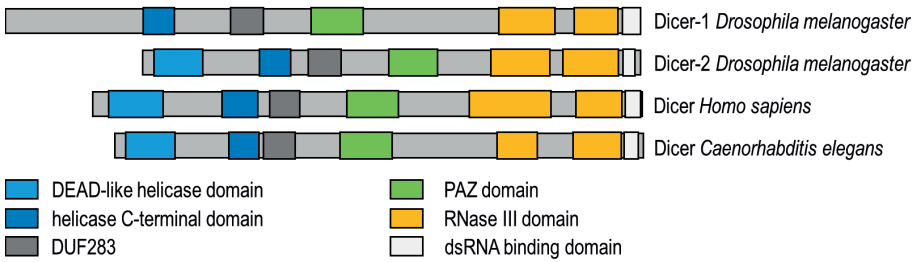

Figure 2 Comparison of *C. elegans*, human and *Drosophila* Dicer proteins
Domain composition was adopted from (Jaskiewicz and Filipowicz, 2008).

and (ii) host-pathogen arms race where Dicer evolves to avoid viral proteins interfering with its function.

The domain organization of *Drosophila* Dicer proteins is generally the same as in other metazoan Dicer proteins – they are composed of domains ordered from the N- to the C-terminus as follows: N-terminal helicase domains, a domain of unknown function DUF283, PAZ domain, RNase IIIa and RNase IIIb domains, and the C-terminal dsRBD (Fig. 2). As for other metazoan Dicers, *Drosophila* Dicer proteins have not been crystallized yet but their structure can be inferred from biochemical studies of recombinant Dicer and individual domains (Tsutsumi et al., 2011; Ye et al., 2007), the crystal structure of *Giardia intestinalis* Dicer (MacRae et al., 2007; MacRae et al., 2006), domain modelling or cryo-EM studies (Lau et al., 2012).

Dicer-1

Dicer-1 was originally identified as one of two homologs in *Drosophila*, which was able to produce siRNAs *in vitro* and participated in RNAi (Bernstein et al., 2001). Subsequent analysis of Dicer mutants showed that mutation in dicer-1 blocked processing of miRNA precursors while dicer-2 mutants were defective for processing siRNA precursors (Lee et al., 2004). However, consistent with the initial study, Dicer-1 was also implicated in RNAi (Lee et al., 2004). Biochemical analysis of Dicer-1 showed that its functional core consists of a DUF283 domain, a PAZ domain, and two RIII domains (Ye et al., 2007). With respect to the size of cleavage products, Dicer-1 apparently does not differ from other metazoan Dicers, as the typical product size is 22 nt long (Fig. 4). DCR-1 also functions in biogenesis of mirtron class of miRNAs (Okamura et al., 2007).

Dicer-1 differs from Dicer-2 in substrate specificities and ATP requirements (Jiang et al., 2005). Like human Dicer, Dicer-1 generates small RNAs in an ATP-independent manner (Jiang et al., 2005), whereas Dicer-2 or Dicer-2/R2D2 required ATP hydrolysis for efficient siRNA production (Liu et al., 2003). Dicer-1 shows a preference for pre-miRNAs (Jiang et al., 2005; Tsutsumi et al., 2011). It recognizes the single-stranded terminal loop structure of pre-miRNAs through its N-terminal helicase domain, checks the loop size and measures the distance between the 3′ overhang and the terminal loop – this allows Dicer-1 to inspect the authenticity of pre-miRNA structures (Tsutsumi et al., 2011).

In terms of evolutionary diversity of Dicer-1 in arthropods, as mentioned above, the miRNA pathway seemed to expand in pea aphid (insect, *Hemiptera*) which utilizes two active copies of Dicer 1 (Jaubert-Possamai et al., 2010; Ortiz-Rivas et al., 2012). However, this duplication is a relatively recent event while single Dicer-1 was also identified elsewhere among arthropods (Jaubert-Possamai et al., 2010; Ortiz-Rivas et al., 2012), including shrimp (Su et al., 2008), mosquito (Bernhardt et al., 2012), cockroach (Gomez-Orte and Belles, 2009) or locust (Wynant et al., 2015) species.

Dicer-2

Dicer-2 in *Drosophila* is mainly producing siRNAs from long dsRNA and functions in RNAi and antiviral defense (Galiana-Arnoux et al., 2006; Kim et al., 2006). Dicer-2 has actually a dual role in antiviral defense – apart from RNAi, it has an RNAi-independent role in promoting Toll signalling (Wang et al., 2015b), but biological aspects of Dicer-2 role will be covered later in the text.

Unlike mammalian Dicer or Dicer-1 paralog, Dicer-2 requires ATP for processive cleavage of dsRNA (Liu et al., 2003; Nykanen et al., 2001; Provost et al., 2002; Welker et al., 2011; Zhang et al., 2002). Remarkably, analysis of shapes of a mammalian Dicer and Dicer-2 by cryo-EM yielded an L-shaped reconstruction with dimensions strikingly similar to those of the human enzyme (Lau et al., 2012). Therefore, despite striking functional differences in ATP requirement and substrate preference, the overall three-dimensional architecture of Dicer is well conserved (Lau et al., 2012).

Dicer-2 contains an N-terminal helicase motif and hydrolyzes ATP; ATP hydrolysis is required for Dicer-2 to process long dsRNA, but not pre-miRNA (Cenik et al., 2011). Dicer-2 works as a dsRNA-stimulated ATPase that hydrolyzes ATP to ADP; and it was suggested that Dicer-2 helicase domain uses ATP to generate many siRNAs from a single molecule of dsRNA before dissociating from its substrate. (Cenik et al., 2011).

The helicase domain of Dicer-2 also governs substrate recognition and cleavage efficiency through discriminating among dsRNA ends. First, it was shown that the helicase domain is essential for cleaving dsRNA with blunt or 5'-overhanging termini, but not those with 3' overhangs, as in pre-miRNAs (Welker et al., 2011). Subsequently, it was found that the discrimination of termini takes place during initial binding (Sinha et al., 2015). In the absence of ATP, Dicer-2 binds 3′ overhanging (pre-miRNA-like), but not blunt termini. in the presence of ATP, Dicer-2 binds both types of termini, with highest-affinity binding observed with blunt dsRNA (Sinha et al., 2015).

An important factor in substrate discrimination and processing is inorganic phosphate, which inhibits Dicer-2 cleavage of pre-miRNAs, but not long dsRNAs (Cenik et al., 2011). It was proposed that the inorganic phosphate occupies a PAZ domain 5' phosphate binding pocket required to bind the 5' terminal phosphate of short substrates, blocking their use and restricting pre-miRNA processing in flies to Dicer-1 (Fukunaga et al., 2014). Binding of long dsRNA is not inhibited when the inorganic phosphate occupies the PAZ domain binding pocket because it also involves the helicase domain and/or the central dsRNA-binding domain, which might be combined with displacement of the inorganic phosphate from its binding pocket (Fukunaga et al., 2014)

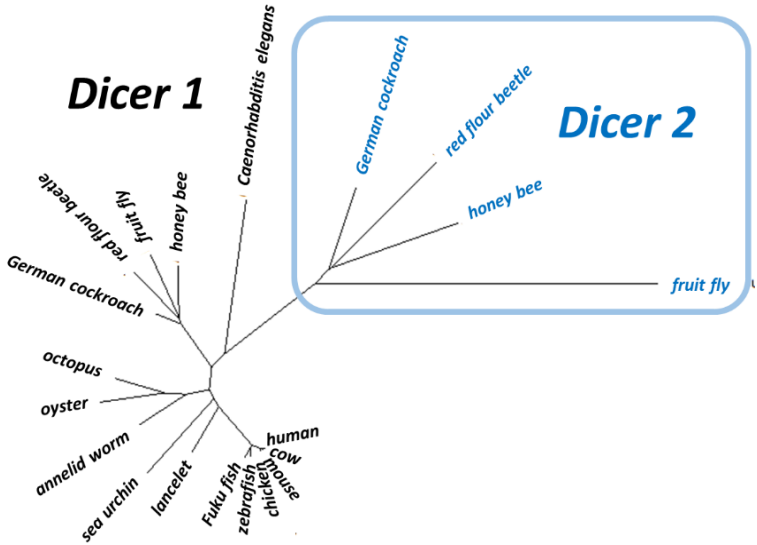

Figure 3 Metazoan Dicer phylogeny
The unrooted tree shows phylogenetic relationships of Dicer proteins in Metazoa. The blue frame depicts Dicer2 homologs in arthropods. As the length of each branch indicates evolutionary distance (or sequence divergence), it is apparent that arthropod's Dicer 2 proteins acting in RNAi are evolving at much faster pace than Dicer 1 protein, which function in the miRNA pathway. This is consistent with the above-mentioned notion of antagonistic evolutionary forces acting on Dicer where the miRNA pathway functionality is being conserved while the RNAi functionality is evolving during the host-pathogen arms race where Dicer evolves to avoid viral proteins interfering with its function.

In terms of evolutionary diversity of Dicer-2 in arthropods, most species seem to use only one Dicer-2 but some underwent duplication, such as Daphnia (*Crustacea*, two Dicer-2 paralogs) or *Metaseiulus* (*Chelicerata*, five Dicer-5 paralogs) (Palmer and Jiggins, 2015). Among the experimentally approached species, one Dicer-2 was reported in experimental results from silk moth (Kolliopoulou and Swevers, 2013), mosquito (Leger et al., 2013), cockroach (Lozano et al., 2012), Hessian fly (Kolliopoulou and Swevers, 2013), planthopper (Zhang et al., 2013), emerald ash borer (Zhao et al., 2015), mite (Hoy et al., 2016), bumble bee (Niu et al., 2016), or shrimp (Niu et al., 2016).

dsRBPs in arthropods – R2D2 and LOQS homologs

Drosophila also utilizes Dicer partner dsRBPs with tandemly arranged dsRBDs – Loquacious (LOQS) and R2D2. The first Dicer partner dsRBP in *Drosophila* is Loquacious, which was found to associate with Dicer-1, suggesting that the miRNA pathway in *Drosophila* employs a distinct dsRBP in substrate routing (Forstemann et al., 2005; Saito et al., 2005). However, it was also found that Dicer-2-generated siRNAs in the endogenous RNAi pathway depend preferentially on Loquacious and not on R2D2, the canonical Dicer-2 partner

(Czech et al., 2008). it turned out that Loquacious gene actually produces three protein isoforms , which associate with Dicer-1 and miRNA pathway (LOQS-PA and LOQS-PB isoform) and Dicer-2 and RNAi (LOQS-PD isoform) (Fukunaga et al., 2012; Hartig et al., 2009; Miyoshi et al., 2010a; Zhou et al., 2009).

LOQS-PB uses the second dsRNA-binding domain to bind pre-miRNA and the third dsRNA-binding domain to interact with Dcr-1. Both domains of LOQS-PB are required for efficient miRNA production by enhancing the affinity of Dcr-1 for pre-miRNA (Ye et al., 2007).

LOQS-PD and R2D2 function sequentially and non-redundantly in the endogenous RNAi pathway. LOQS-PD stimulates DCR-2-mediated processing of dsRNA whereas R2D2 acts downstream during RISC loading (Hartig and Forstemann, 2011; Marques et al., 2010; Miyoshi et al., 2010a). Taken together, LOQS and R2D2 contribute to the profound mechanistic separation of miRNA and RNAi pathways, which evolved in *Drosophila* (and presumably in arthropods in general).

R2D2 associates with Dicer-2 and acts in RNAi; it was co-purified with Dicer-2 during purifying siRNA-generating activity from *Drosophila* S2 cell lysates (Liu et al., 2003). Although R2D2 bears 33% similarity to RDE-4 (see the section *Nematoda*) its role is different. R2D2 does not influence DCR-2 enzymatic activity (Liu et al., 2003) but restricts Dicer-2 function to processing of long dsRNAs (Cenik et al., 2011; Fukunaga et al., 2014). It also facilitates passing the cleavage product to AGO2 excluding miRNA-like duplexes with imperfect base pairing (Tomari et al., 2004a). R2D2 has two roles – it is sensing siRNA thermodynamic asymmetry for strand selection and it is a licensing factor for entry of authentic siRNAs into the RNAi pathway (Nishida et al., 2013; Tomari et al., 2004b).

Unlike the Microprocessor complex, Dicer or Argonautes, dsRBPs seem undergo various functional adaptations between different taxons (compare, for example TARBP2, RDE-4, R2D2 or LOQS). This possibly also happens among arthropods. An example is the lack of the RNAi-associated LOQS-PD isoform outside *Drosophila* (Haac et al., 2015). Analysis of dsRBPs in the mosquito *Aedes aegypti* revealed absence of LOQS-PD isoform, conserved roles of R2D2 and LOQS-PB, and LOQS-PA role in biogenesis of both, miRNAs and endo-siRNAs (Haac et al., 2015).

Argonaute proteins

AGO proteins of arthropods are conserved, i.e. their domain composition is the same as that of mammalian proteins, which was discussed in detail (Peters and Meister, 2007). Arthropods have varying number of Argonaute proteins of the AGO and PIWI clade but it seems that their archetypal state is two AGO proteins, each being associated with one Dicer and one type of small RNA (Palmer and Jiggins, 2015).

AGO1 and its loading with miRNAs

AGO1 RISC loading is similar to that of human RISC assembly described earlier (Yoda et al., 2010). Dicer-1/LOQS-PB heterodimer functions in assembling AGO1 RISC, which

is preferentially loaded with miRNA/miRNA* duplexes while siRNAs are being excluded from the assembly (Tomari et al., 2007).

AGO1 requires ATP for miRISC loading, presumably to trigger the dynamic conformational opening of AGO proteins so that they can accept small-RNA duplexes (Kawamata et al., 2009). Unwinding of miRNA-miRNA* duplexes is a passive process that does not require ATP or slicer activity of Ago1 (Kawamata et al., 2009).

Two distinct AGO complexes were identified (Miyoshi et al., 2009): (i) AGO1-Dicer-1 complex with pre-miRNA processing activity where the resultant mature RNA was loaded onto AGO1 within the complex – this complex corresponds to miRLC (miRISC loading complex) (ii) the AGO1-GW182 complex with excluded DCR-1, containing mature miRNA no pre-miRNA processing activity – this complex corresponds to miRISC. AGO1 loading also involves R3D1-L, a dsRBP that functions as a cofactor interacting with Dicer-1 and AGO1 (Jiang et al., 2005).

AGO1 might also have miRNA-independent role in translational repression where AGO1 is recruited to mRNA via an RNA-binding protein SMAUG and not through miRNA:mRNA interaction (Pinder and Smibert, 2013).

AGO2 and its loading with siRNAs

The model of RNAi RISC loading in *Drosophila* suggests that RISC assembly occurs in several steps, which involve a several complexes (Tomari and Zamore, 2005). The first complex is formed by siRNA, R2D2 and DCR-2, also known as R1 or R2/D2/DCR-2 initiator (RDI) complex (Kim et al., 2007; Pham et al., 2004), which develops into a mature form of the RISC loading complex RLC (Tomari and Zamore, 2005). The RLC determines strand selection and recruits AGO2 (and other proteins) to form pre-RISC (Kim et al., 2007), which contains duplex siRNA. Finally, the release of the passenger strand from the duplex produces holo-RISC, which can base pair with complementary mRNA substrates. The loading is assisted by Hsc70/Hsp90 chaperones (Iwasaki et al., 2015; Miyoshi et al., 2010b).

The coupling of dsRNA cleavage and RISC assembly is a matter of debate. It was suggested that, after cleavage, small-RNA duplexes need to dissociate from Dicer and then rebind to a sensor of the thermodynamic asymmetry of the duplex, because the guide strand of an siRNA will be at random orientation (Tomari et al., 2004b).

AGO2 requires ATP for RISC loading (Kawamata et al., 2009; Pham et al., 2004; Tomari et al., 2004a). ATP is presumably used to trigger the dynamic conformational opening of AGO proteins so that they can accept small-RNA duplexes (Kawamata et al., 2009).

Strand selection in fly RLC is controlled by R2D2. Analysis of the interaction of DCR-2/R2D2 complex with siRNA duplexes showed that R2D2 orients the complex according to thermodynamic stabilities of siRNA strands and binds the 5’ phosphate of the passenger strand at the thermodynamically more stable end (Tomari et al., 2004b). Thus, R2D2 functions as a licensing factor for routing siRNAs into the RNAi pathway. Interestingly, a thorough analysis of AGO2 complexes revealed that, unlike mature miRNAs, which are loaded on AGO1, complementary strands of mature miRNAs (miRNA*) are efficiently loaded on AGO2 in DCR2/R2D2-dependent manner (Ghildiyal et al., 2010; Okamura et al.,

2011). Thus, the role of R2D2 in sorting small RNAs is wider and extends into the miRNA pathway.

The final step in assembly of an active RISC is the release of the passenger strand from the siRNA duplex. *Drosophila* is Armitage helicase is a candidate for a mechanism separating the two siRNA strands while the guide remains bound to AGO2 (Tomari et al., 2004a). However, experimental data support a simple solution where passenger strand cleavage by AGO2 slicer activity liberates the single-stranded guide siRNA strand from the pre-RISC complex (Kim et al., 2007; Matranga et al., 2005; Miyoshi et al., 2005). Removal of siRNA passenger strand cleavage products is assisted by C3PO endoribonuclease, which was identified as a RISC-enhancing factor that promotes RISC activation (Liu et al., 2009). The cleavage-assisted mechanism is typical for AGO2-loaded fly and human siRNAs in the RNAi pathway while passenger strand cleavage is not important for loading miRNAs (Matranga et al., 2005).

Small RNA sorting and mRNA targeting by AGO1 and AGO2

Drosophila sorts Dicer-produced small RNAs onto functionally distinct AGO proteins where AGO1 is dedicated to the miRNA pathway while AGO2 served for RNAi. Small RNA sorting is initiated by substrate recognition and continues through sorted loading onto the AGO proteins. Small-RNA duplexes are actively sorted into AGO-containing complexes according to their intrinsic structures (Forstemann et al., 2007; Tomari et al., 2007). Importantly, separation of miRNA and RNAi at the level of small RNA sorting onto AGO1 and AGO2 is not completely pre-determined by small RNA origins (Tomari et al., 2007). It was found that miRNA*s are often loaded as functional species into AGO2 (Czech et al., 2009; Ghildiyal et al., 2010; Okamura et al., 2009). Furthermore, miRNAs produced by Dicer-1 and LOQS can be loaded by Dicer-2 and R2D2 into an AGO2 RISC (Forstemann et al., 2007). Finally, siRNAs derived from long hairpin RNA genes (hpRNA) also show a hybrid biogenesis combining RNAi factors DIcer-2 and AGO2 and Loquacious isoform (Okamura et al., 2008c).

Subsequently, AGO2-RISC mediates RNAi while only AGO1 is able to repress mRNAs with central mismatches in miRNA-binding sites (Forstemann et al., 2007). At the same time, AGO1 cannot mediate RNAi, because it is an inefficient nuclease with a catalytic rate limited by the dissociation of reaction products (Forstemann et al., 2007). AGO1 and AGO2 RISCs also differ in mechanisms of translational repression – AGO1-RISC represses translation primarily by ATP-dependent deadenylation while Ago2-RISC competitively blocks the interaction of eIF4E with eIF4G and inhibits the cap function (Fukaya et al., 2014; Iwasaki et al., 2009). AGO1-mediated translational repression involves GW182 in the same manner as in mammals (GW182 is separately described further below). miRNA-mediated silencing involves recruitment of PABP, CCR4-NOT deadenylase and decapping complex to RISC (Behm-Ansmant et al., 2006; Chekulaeva et al., 2011; Eulalio et al., 2008; Fukaya and Tomari, 2011; Huntzinger et al., 2010; Huntzinger et al., 2013; Moretti et al., 2012; Rehwinkel et al., 2005). miRNA-mediated repression occurs on ribosome complexes but is independent of ribosomal scanning(Antic et al., 2015; Kuzuoglu-Ozturk et al., 2016).

Evolutionary perspective

As mentioned above, the archetypal state arthropod state is two AGO proteins, AGO1 and AGO2. Apart from *Drosophila*, such a situation is found in *Daphnia* (*Crustacea*) and *Metaseiulus* (mite, *Chelicerata*) (Palmer and Jiggins, 2015). However, upon detailed inspection, one frequently finds variability in the number of AGO proteins across the phylum or even across smaller taxonomic units. The miRNA pathway seemed to expand in pea aphid (insect, *Hemiptera*), whose genome two expressed copies of ago1, one of which (ago1b) shows signs of positive selection (Jaubert-Possamai et al., 2010). At the same time, a single AGO1 but duplications of AGO2 were found *Ixodes* (tick, *Chelicerata*, three AGO2 paralogs), *Strigamia* (centipede, *Myriapoda*, two AGO2 paralogs), *Mesobuthus* (scorpion, *Chelicerata*, six AGO2 paralogs) or *Tetranychus* (spider mite, *Chelicerata*, six AGO2 paralogs) (Palmer and Jiggins, 2015). *Penaeus monodon* (black tiger shrimp) has four functionally diversified AGO paralogs (Dechklar et al., 2008; Leebonoi et al., 2015; Phetrungnapha et al., 2013; Yang et al., 2014b). Analysis of Argonaute genes across 86 Dipteran species showed that variation in copy number can occur rapidly, and that there is constant flux in some RNAi mechanisms; this suggests that Argonautes undergo frequent evolutionary expansions that facilitate functional divergence (Lewis et al., 2016).

Additional miRNA and RNAi factors

There is a large number of accessory factors beyond those described above. For example, a systematic screen of 40% of the genome for genes acting in the miRNA pathway yielded 45 mutations in 24 genes and an estimate of ~100 genes are required to execute the miRNA program (Pressman et al., 2012). Here, we will describe several additional factors, which have been associated with miRNA or RNAi pathways.

Nibbler – Nibbler is a 3'-5; exoribonuclease involved in trimming 3' ends of miRNAs and piRNAs (Feltzin et al., 2015; Han et al., 2011; Liu et al., 2011; Wang et al., 2016; Yang et al., 2014a). In the miRNA pathway, Nibbler shortens distinct longer miRNAs during RISC assembly, yielding miRNA isoforms that are compatible with the preferred length of AGO1-bound small RNAs (Han et al., 2011; Liu et al., 2011). It has been estimated that about a quarter of miRNAs undergoes such a trimming (Han et al., 2011).

HEN1 – HEN1 (Pimet, Dmhen1)is an enzyme catalyzing addition of a 2'-O-methyl group at the 3' end of small RNAs (Horwich et al., 2007; Saito et al., 2007). While this modification is predominantly found on piRNAs in *Drosophila*, it was also found on siRNAs and miRNAs (Abe et al., 2014; Horwich et al., 2007; Yang et al., 2014a). Functionally, 2'-O-methylation of siRNAs loaded on AGO2 prevents tailing and trimming of siRNAs (Ameres et al., 2010). Generally HEN1 and Nibbler thus have antagonistic activities at the 3' end of small RNAs where Nibbler promotes small RNA trimming while Hen1 prevents it (Ameres et al., 2010; Yang et al., 2014a). 2'-O-methylation is also found on select miRNA isoforms and appeared to increase with age while its reduction was associated with neurodegeneration and shorter life span (Abe et al., 2014).

nucleotidyltransferases – tailing of short RNAs is mediated by terminal nucleotidyltransferases, which produce 3' uridylation or adenylation. **PAPD4** has been identified as a primary miRNA adenylating enzyme in *Drosophila*, adenylation did not appear to affect miRNA stability on a genome-wide scale (Burroughs et al., 2010). Another non-canonical adenylase is **Wispy**, which is responsible for adenylation of miRNAs and biologically it may facilitate clearance of maternal miRNAs in the embryo (Lee et al., 2014). Uridylation is mediated by **Tailor**, which is a uridylyltransferase that is required for the majority of 3' end modifications of microRNAs in *Drosophila* and predominantly targets mirtron hairpins (Reimao-Pinto et al., 2015; Westholm et al., 2012).

GW182 – GW182 is the key co-factor of AGO1 in miRISC. Its role has been described in detail in the mammalian section, he we will briefly note its key features with respect to arthropods. GW182 and its interaction with AGO1 were found to be required for miRNA-mediated repression in *Drosophila* cells (Behm-Ansmant et al., 2006; Eulalio et al., 2008; Rehwinkel et al., 2005). miRNA-mediated repression also required the decapping complex DCP1:DCP2 and CCR4-NOT deadenylase (Behm-Ansmant et al., 2006; Rehwinkel et al., 2005). Multiple domains of GW182 contribute to miRNA-mediated repression (Chekulaeva et al., 2009; Chekulaeva et al., 2011; Chekulaeva et al., 2010; Eulalio et al., 2009). Similarly to mammals, *Drosophila* GW182 directly interacts with PABP and CCR4-NOT (Chekulaeva et al., 2011; Fukaya and Tomari, 2011; Huntzinger et al., 2010; Huntzinger et al., 2013; Moretti et al., 2012).

Armitage – RNA helicase, which was identified as a maternal effect gene required for RNAi (Tomari et al., 2004a). Armitage is probably not required for RISC activity. Instead, it was proposed to facilitate removal of the passenger strand during RISC formation (Tomari et al., 2004a). Armitage was also implicated in piRNA biogenesis (Huang et al., 2014; Murota et al., 2014; Nagao et al., 2010; Qi et al., 2011; Saito et al., 2010).

dFMR1 – *Drosophila* ortholog of human fragile X mental retardation protein (FMRP) was identified as a RISC component (Caudy et al., 2002; Ishizuka et al., 2002; Pham et al., 2004). dFMR1 is associated with ribosomes through interaction with ribosomal proteins L5 and L1 and with complexes containing miRNAs (Ishizuka et al., 2002). dFMR1 is not a conserved RISC component involved in RNAi as depletion of dFMR1 reduces RNAi efficiency in *Drosophila* S2 cells but not in mammals (Caudy et al., 2002). dFMR has been also implicated in the piRNA pathway (Bozzetti et al., 2015; Jiang et al., 2016).

VIG – Vasa Intronic Gene (Caudy et al., 2002; Pham et al., 2004). VIG is a conserved protein, which encodes a putative RNA binding protein, whose depletion reduces RNAi efficiency (Caudy et al., 2002). *Vig* mutants are more susceptible to viral infections in *Drosophila* (Zambon et al., 2006). Whether this role of VIG is coupled with its presence in the RISC complex is not known. There is no evidence that SERBP1, the closest mammalian VIG homolog, would be associated with RISC. VIG was also implicated in heterochromatin formation (Gracheva et al., 2009).

Tudor-SN – Tudor Staphylococcal Nuclease is a protein containing five staphylococcal/micrococcal nuclease domains and a Tudor domain. It is a component of the RISC in *C. elegans*, *Drosophila* and mammals (Caudy et al., 2003; Pham et al., 2004). The role of Tudor-SN in RISC RNAi remains enigmatic. TSN is not the „slicer" (Schwarz et al., 2004) and its knock-down in silk moth cells had not effect on RNAi efficiency (Zhu et al.,

2012). In *Drosophila*, Tudor-SN has also been linked to piRNA pathway regulation (Ku et al., 2016).

DMP68 (RM62) – this conserved helicase was co-purified with AGO1 and dFMR1 (Ishizuka et al., 2002). This helicase seems to be required for RNAi in S2 cells where depletion of DMP68 results in inhibition of RNAi (Ishizuka et al., 2002). Whether DMP68 is needed for RISC formation or for RISC activity/stability is not known. Later publications on RM62 linked it to antiviral response (Zambon et al., 2006) and to other mechanisms than RNAi.

CRIF1 – the *Drosophila* homolog of the mammalian CR6-interacting factor 1 (CRIF1), was identified as a potential new regulator of the RNAi pathway during a screen for genetic mutations in *Drosophila* that alter the efficiency of RNAi. CRIF1 loss-of-function mutant flies are deficient in exo-RNAi, in siRNA biogenesis and in antiviral immunity. (Lim et al., 2014)

RdRPs in arthropods

One of the less understood areas of RNA silencing in arthropods is utilization of RdRPs. A phylogenetic analysis suggests that RdRPs in RNA silencing pathways have a monophyletic origin, i.e. evolved from a single ancestral RdRP (Cerutti and Casas-Mollano, 2006; Murphy et al., 2008). A simple TBLASTN search with C. elegans RRF-1 proteins sequence reveals RdRPs in species across *Metazoa*, including *Cnidaria* (hydra), *Nematoda* (RdRPs in *C. elegans* will be discussed later), *Mollusca* (oyster), *Hemichordata* (acorn worm), or *Urochordata* (sea squirt). At the same time, RdRPs seem to be absent in other groups including *Platyhelminthes*, *Annelida*, and vertebrates. What this implies for arthropods is that the common ancestors of protostomes and deuterostomes still had RdRPs and that RdRPs were repeatedly lost in different taxons. We performed a diagnostic TBLASTN search with C. elegans RRF-1 proteins sequence also for the major groups of arthropods and we have identified RdRP homologs in diverse representatives of the subphylum *Chelicerata* (spiders, horseshoe crab, ticks, mites) but none in the remaining subphyla – *Myriapoda*, *Crustacea*, and *Hexapoda*. This would suggest that RdRP was lost early in the arthropod evolution, being retained only in *Chelicerata*. Thus, one could assume that except of *Chelicerata*, RNA silencing does not employ RdRP-generated secondary siRNAs like those found *in C. elegans*.

Whether RdRP activity completely disappeared from RNAi in *Drosophila* (and those arthropods lacking an RdRP ortholog) is not completely understood but available data suggest that it is could the case. One should consider also the option that a missing RdRP ortholog in RNA silencing could be replaced by another RdRP, for example by horizontal transfer of some viral RdRP. In fact, there is a report of RdRP activity in *Drosophila* (Lipardi et al., 2001) but, was contradicted by experiments demonstrating the absence of transitive RNAi generating secondary sequences upstream of the region targeted by siRNAs (Roignant et al., 2003; Schwarz et al., 2002), so the issue remained unresolved. Later, dELP1, a non-canonical RdRP conserved in all eukaryotes, was suggested to associate with Dicer-2 and function as an RdRP (Lipardi and Paterson, 2009). However, the article was later retracted because the measured biochemical activity did not seem to be an authentic

RdRP (Lipardi and Paterson, 2011). Taken together, as of now, there is no evidence for RdRP activity yielding secondary siRNAs and transitive RNAi in arthropod species lacking orthologs of ancestral RNA silencing-related RdRPs.

miRNA-mediated control of gene expression

miRNAs play physiologically important roles in arthropods, as suggested by phenotypes of mutants of miRNA pathway. The loss of Dicer-1 or Ago1 causes embryonic lethality (Lee et al., 2004; Pressman et al., 2012). Dicer-1 is also needed to maintain ovarian stem cells in *Drosophila* (Jin and Xie, 2007). Similarly, Ago1 is essential for oocyte formation and maintenance of germline stem cells in *Drosophila* (Azzam et al., 2012; Yang et al., 2007) and for locust oogenesis (Song et al., 2013). The miRNA pathway can also participate in response to physiological cues (Dekanty et al., 2010) or in regulation of immunity in arthropods, as shown for the Plasmodium infection mosquito (Winter et al., 2007). The miRNA pathway apparently expanded in pea aphid (insect, Hemiptera), whose genome contains four pasha, two dcr-1 and two ago2 paralogs, all of which are expressed and where one of the ago1 paralogs shows signs of positive selection (Jaubert-Possamai et al., 2010). Notably, these expansions occurred concomitantly within a brief evolutionary period. it has been speculated that the miRNA pathway diversity could contribute to adapted phenotypes, which the pea aphid is able to produce from a single genotype (Jaubert-Possamai et al., 2010).

Drosophila's miRNA annotation in mirBase (Kozomara and Griffiths-Jones, 2014) is likely the most thoroughly done; it includes meta-analysis of $> 10^9$ raw reads from 187 RNA-seq libraries comprising diverse developmental stages, specific tissue- and cell-types, mutant conditions, and/or Argonaute immunoprecipitations yielded a thorough annotation of miRNA loci, including definition of multiple phased by-products of cropping and dicing, abundant alternative 5' termini of certain miRNAs, frequent 3' untemplated additions, and potential editing events (Berezikov et al., 2011). Considering incomplete miRNA annotations in other species, miRBase numbers indicate that arthropods might have about one order of magnitude less miRNAs than mammals suggesting somewhat less expanded miRNA-mediated control of gene expression. In addition, there are only a few miRNAs conserved between *Drosophila* and mammals (the best known is Let-7 (Pasquinelli et al., 2000)).

Arthropods also employ non-canonical miRNAs. These include mirtrons that bypass Drosha processing (Martin et al., 2009; Okamura et al., 2007; Ruby et al., 2007). In addition, specific miRNA loops may accumulate as non-canonical miRNAs on AGO1 and mediate miRNA-type repression (Okamura et al., 2013). Another non-canonical miRNA was identified in rDNA arrays. Its processing requires Dicer-1 but not the Microprocessor complex and it is conserved among *Diptera* (Chak et al., 2015).

RNAi pathway in arthropods

Although *Drosophila* Dcr-2 or Ago-2 are nonessential and mutants can develop to adults with no strong phenotypes under standard laboratory conditions (Deshpande et al., 2005;

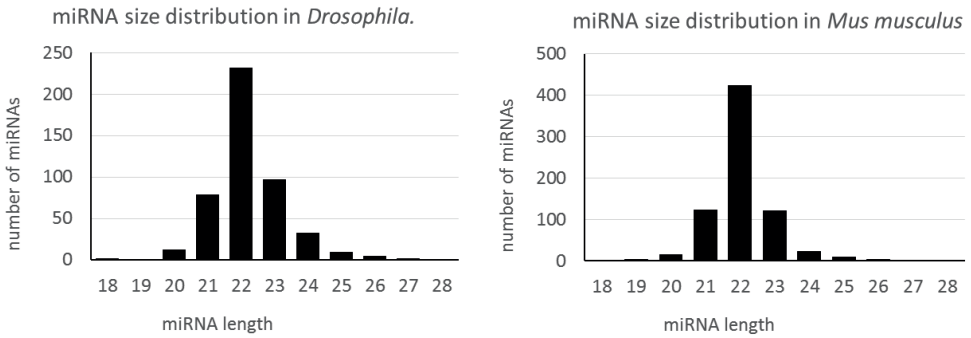

Figure 4 *D. melanogaster* miRNA lengths
The left graph depicts size distribution of all 466 miRNAs of a *Drosophila melanogaster* deposited in the miRBase (version 21). For comparison, the right graph shows size distribution of 721 high-confidence murine miRNAs.

Lee et al., 2004; Li et al., 2013; Xu et al., 2004), some minor phenotypes appear and severe defects in embryonic development have been noted in these mutants upon exposure to temperature perturbations (Deshpande et al., 2005; Li et al., 2013; Lucchetta et al., 2009).

RNAi is functional across other arthropods subphyla, including *Chelicerata* (ticks and mites (Hoy et al., 2016; Kurscheid et al., 2009; Schnettler et al., 2014)) and *Crustacea* (shrimps (Chen et al., 2011; Hoy et al., 2016; Huang and Zhang, 2013; Jariyapong et al., 2015; Maralit et al., 2015; Sabin and Cherry, 2013; Yang et al., 2014b). We did not find published functional RNAi data for *Myriapoda* but genomic analyses show that they have the necessary machinery (Palmer and Jiggins, 2015). In this section, we will discuss the role of antiviral RNAi and endo-siRNAs in *Drosophila* and arthropods in general.

Antiviral RNAi

RNAi plays a key role in innate immunity in arthropods and a large volume of the reviewed literature across the taxon dealt with antiviral role of RNAi. As it could be expected, most of the mechanistic data came from the *Drosophila* model.

The first evidence for antiviral RNAi in arthropods emerged in 2002 from a study that used flock house virus (FHV) is inducing RNAi (and is being targeted by RNAi) *Drosophila* host cells (Li et al., 2002). Infection of 14 different *Drosophila* RNA silencing mutants with a dsRNA X virus (DXV) showed that all but three lines were significantly more susceptible to viral infection (reduced survival and elevated viral titers) than normal flies. Moreover, replication of DXV was sequence-specifically inhibited (but not absolutely blocked) by „immunizing“ *Drosophila* S2 cells with dsRNA from the coding region of DXV before infection (Zambon et al., 2006). Remarkably, increased susceptibility was observed not only for mutants of the RNAi pathway, such as *r2d2*, *armi*, or *ago2*, but also for mutants of the piRNA pathway (*aubergine* and *piwi*), suggesting that RNAi is not the only RNA silencing pathway in *Drosophila* that can respond to a viral infection (Morazzani et al., 2012). The engagement of the piRNA pathway also extends to mosquitos (Leger et al.,

2013; Schnettler et al., 2013a; Vodovar et al., 2012). A number of studies provided ample mechanistic evidence that RNAi plays an essential role in antiviral response in *Drosophila* (Galiana-Arnoux et al., 2006; Nayak et al., 2010; van Rij et al., 2006; Wang et al., 2006).

The antiviral role of RNAi is conserved across the entire phylum. here, we will just list a few representative examples of taxons across arthropods with documented antiviral role of RNAi.

RNAi is an antiviral system in mosquitos, where RNAi also comes into contact also with viruses that infect humans, such as Dengue Virus Type 2 (Sanchez-Vargas et al., 2009), O'nyong-nyong virus (Keene et al., 2004), Sindbis virus (Adelman et al., 2012; Campbell et al., 2008), West Nile virus (Paradkar et al., 2012), Rift Valley Fever virus (Leger et al., 2013), Arbovirus (Schnettler et al., 2013b; van Cleef et al., 2014), Mosinovirus (Schuster et al., 2014), Culex Y virus (van Cleef et al., 2014). Importantly, viruses facing RNAi-based innate immunity evolve different RNAi inhibitors, proteins, which interfere with various stages of siRNA biogenesis. Such proteins have been identified in most of the aforementioned viruses. Needless to say that antiviral RNAi was reported also from other hexapods, such as silk moth (Liu et al., 2015; Zografidis et al., 2015). Antiviral RNAi was also reported for *Chelicerata* (ticks and mites (Hoy et al., 2016; Schnettler et al., 2014)) or *Crustacea* (shrimps (Hoy et al., 2016; Huang and Zhang, 2013; Jariyapong et al., 2015; Maralit et al., 2015; Sabin and Cherry, 2013; Yang et al., 2014b).

Endogenous RNAi in the germline and soma

Severe defects in embryonic development have been noted in *Drosophila* mutants lacking *Dcr-2* or *Ago2*, exposed to temperature perturbations while these mutants otherwise develop under standard laboratory conditions to normal adults with no specific phenotype (Lucchetta et al., 2009). This indicated that one of the functions of endo-siRNA pathway is to stabilize embryonic development under environmental stress (Lucchetta et al., 2009). Subsequent analysis of the distinct phenotypes in RNAi-defective mutants (Deshpande et al., 2005; Li et al., 2013) and RNA-seq data lead to recognition of diversity of the endogenous RNAi pathway and various types of endo-siRNAs.

A substantial source of *Drosophila* endo-siRNAs comes from mobile elements (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008a). Endo-siRNAs in somatic tissues and cultured cells thus complement piRNAs that are responsible for genome surveillance predominantly in the germline. Importantly, as endo-siRNAs and piRNAs were found that originate from the same loci, it is possible that piRNA and endo-siRNA pathways might be interdependent in repression of mobile elements in *Drosophila* (Ghildiyal et al., 2008). The notion of interdependence in mobile element repression also resonates with above-mentioned piRNA contribution to antiviral defense.

Other endo-siRNAs map to protein-coding genes and potentially contribute to control of gene expression. Among them a significant portion maps to protein-coding regions (Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008a; Okamura et al., 2008b). However, only endo-siRNAs derived from a small number of loci are produced in sufficient amount to reduce target mRNA levels, as exemplified by the *esi-2* locus-derived endo-siRNAs targeting DNA damage-response gene *Mus-308* (Czech et al., 2008).

Table 1 Numbers of annotated miRNAs in selected arthropods in miRBase 22.1. Note: only species with >100 annotated miRNA precursors are shown for Hexapoda

subphylum	species	miRNA precursors	mature miRNA
Chelicerata	*Ixodes scapularis*	49	49
	Rhipicephalus microplus	24	24
	Tetranychus urticae	52	92
Myriapoda	*Strigamia maritima*	3	4
Crustacea	*Daphnia pulex*	44	45
	Triops canciformis	148	160
Hexapoda	*Aedes aegypti*	122	164
	Apis mellifera	254	262
	Acyrthosiphon pisum	123	97
	Bombyx mori	487	563
	Drosophila melanogaster	258	469
	Drosophila simulans	148	213
	Drosophila virilis	180	328
	Plutella xylostella	133	127
	Tribolium castaneum	342	590

Another type of *Drosophila* endo-siRNAs arises from overlapping antisense transcripts observed in hundreds of protein-coding loci (Okamura et al., 2008a). Abundance of such endo-siRNAs is generally low. Potential mRNA targets of such endo-siRNAs are not upregulated in *Ago2*-deficient flies, suggesting that these endo-siRNAs are not involved in post-transcriptional control of mRNA levels under physiological conditions (Czech et al., 2008). Interestingly, a dsRNA/endo-siRNA-binding protein Blanks, which associates with DCR-2 and forms an alternative Argonaute-independent functional RISC complex, has a role in spermatogenesis (Gerbasi et al., 2011). As Blanks deletion does not affect transposon activity, this finding suggests a role for endo-siRNAs in regulation of protein-coding mRNAs in *Drosophila* sperm development.

Yet another distinct type of endo-siRNAs are those derived from hairpin RNAs, whose biogenesis involves HEN1, canonical RNAi factors Dicer-2 and AGO2 plus miRNA factor Loquacious (Okamura et al., 2008c), specifically the LOQS-PD isoform(Zhou et al., 2009). One of the roles of hairpin RNA-derived endo-siRNAs is regulation of gene expression (Wen et al., 2015).

Systemic RNAi

Some insects, such as red flour beetle *Tribalism*, have efficient systemic RNAi where injection of adults causes RNAi effects in the progeny (Bucher et al., 2002; Tomoyasu et al., 2008). One of the well-known systemic RNAi factors is SID-1, a transmembrane protein transporting dsRNA across cell boundaries, which was first identified in *C. elegans*

(Feinberg and Hunter, 2003). Ectopic expression of SID-1 is sufficient for permitting a systemic RNAi through dsRNA soaking in insect cells (Feinberg and Hunter, 2003; Mon et al., 2013; Shih and Hunter, 2011).

Non-cell autonomous RNAi exists across arthropods where different taxa have different numbers of sid-1 homologs. Importantly, some taxons lack systemic RNAi, which is not necessarily accompanied with the absence of sid-1 homologs; systemic RNAi can also be developmentally restricted or simply inefficient despite the expression of sid-1 homologs (Tomoyasu et al., 2008). Importantly, SID-1 is not the only system of systemic RNAi as was demonstrated in the locust model (Luo et al., 2012) or in Tribalism (Bucher et al., 2002; Tomoyasu et al., 2008).

There is a number of insect species, in which was found systemic RNAi or at least sid-1 homologs, include the aforementioned red flour beetle, Colorado potato beetle (Cappelle et al., 2016), juvenile grasshopper (Dong and Friedrich, 2005), brown planthopper (Xu et al., 2013), oriental leaf worm moth (Gong et al., 2015), diamondback moth (Wang et al., 2014), silk moth (Tomoyasu et al., 2008), honeybee (Honeybee Genome Sequencing, 2006), soybean aphid (Bansal and Michel, 2013), cotton/melon aphid (Xu and Han, 2008), grain aphid (Xu and Han, 2008). Beyond *Hexapoda*, there was also one report of sid-1 homolog in *Crustacea* (shrimp) (Labreuche et al., 2010; Maralit et al., 2015).

Sid-like genes were not found in *Drosophila* (Roignant et al., 2003), Hessian fly (Shreve et al., 2013), or mosquito *Anopheles gambiae* (Blandin et al., 2002). However, it should be kept in mind that this is not a conclusive evidence for absence of non-cell autonomous RNAi. For example, non-cell autonomous RNAi could be experimentally achieved also in *Drosophila* (Dzitoyeva et al., 2003) and it has been proposed that it would have a natural role in conjunction with antiviral RNAi in adult flies (Saleh et al., 2009).

Dicer-dependent nuclear silencing

Small RNAs in plants and fungi can mediate transcriptional silencing via chromatin remodelling/DNA methylation. A nuclear role and chromatin remodelling has been also attributed to the PIWI-loaded piRNA class of small RNAs protecting genome integrity in the germline. The role of Dicer-dependent small RNAs in transcriptional silencing in *Metazoa* is poorly understood and, in some cases, controversial. In any case, studies in *Drosophila* yielded some, albeit heterogeneous, evidence connecting Dicer-dependent small RNAs with transcriptional silencing and chromatin changes.

The best known small RNA-dependent transcriptional silencing mechanism is the piRNA pathway, which controls transcriptional silencing of retrotransposons (reviewed in Fu and Wang, 2014; Haase, 2016; Han and Zamore, 2014; Sato and Siomi, 2013). We do not cover the piRNA pathway as it is neither miRNA nor RNAi; piRNAs are not produced from a dsRNA but from complementary single-stranded transcripts through a concerted action of multiple factors. In any case, the piRNA pathway occasionally intersects with RNAi and miRNA pathways. For instance, it was shown that a functional miRNA pathway is required for the piRNA-mediated transcriptional silencing of mobile elements (Mugat et al., 2015). The mechanistic link seems to be provided by two specific miRNAs, miR-14 and miR-34

(Mugat et al., 2015). This highlights the issue of discerning miRNA-mediated effects on chromatin and direct chromatin regulation by small RNAs.

A possible existence of transcriptional silencing mediated by Dicer-dependent small RNAs emerged from several analyses. First, it was found that AGO1 is found in the nucleus and cytosol in early embryos and that repeat induced silencing and transcriptional co-suppression were disrupted by Ago-1 mutation (Pushpavalli et al., 2012). The effect was accompanied by reduced H3K9me2 and H3K27me3 histone modifications (Pushpavalli et al., 2012). However, it is not clear whether this phenomenon is caused by a direct endo-siRNA-mediated heterochromatin induction or an indirect effect of miRNAs (similarly to the situation mentioned in the previous paragraph). Another possible bridge between the miRNA pathway and transcriptional regulation is Glioma amplified sequence41 (Gas41), a chromatin remodeler, implicated in repeat-induced transgene silencing, which also interacts with Dicer-1 (Gandhi et al., 2015). Others proposed that AGO1 (and Dicer-2) interacts with RNA pol II and contribute to heterochromatin formation (Kavi and Birchler, 2009).

AGO2 has been implicated in alternative splicing and transcriptional silencing, which included Polycomb group complex (associated with H3K27 methylation) (Taliaferro et al., 2013). Again, it is not clear how direct and indirect mechanism is responsible for the observed effects. RNAi machinery is not certainly an essential component of polycomb-mediated silencing as it was demonstrated that RNAi pathway is dispensable for the polycomb-mediated silencing of the homeotic Bithorax Complex (Cernilogar et al., 2013). AGO2 was also implicated in chromatin insulator function that would be independent of RNAi (Moshkovich et al., 2011). AGO2 was localized by chromatin immunoprecipitation to euchromatin but not heterochromatin and co-localized and physically interacted with CTCF/CP190 chromatin insulators (Moshkovich et al., 2011). AGO2, together with Dicer-2 and R2D2 was implicated in H3K9 methylation, suggesting that endo-siRNAs might regulate heterochromatin (Fagegaltier et al., 2009). A possible bridge between RNA silencing and chromatin could be VIG, the aforementioned RISC component (Gracheva et al., 2009). Chromatin-related factors also emerged from screens for RNAi and miRNA genes (Ghosh et al., 2014; Pressman et al., 2012).

Taken together, despite a relatively large volume of evidence, there is still not a consensus and a validated model explaining how would miRNAs or endo-siRNAs guide chromatin remodelling in *Drosophila* (or in arthropods in general).

Other dsRNA response pathways in arthropods

While RNAi is an important antiviral innate immunity mechanism in arthropods, it should be pointed out that it is not the only one and that the innate immunity of arthropods is much more complex. A study of five chelicerates, a myriapod, and a crustacean revealed traces of an ancient origin of innate immunity, with some arthropods having Toll-like receptors and C3-complement factors that are more closely related in sequence or structure to vertebrates than other arthropods (Palmer and Jiggins, 2015). Thus, apart from a robust and sequence-specific RNAi, arthropods also have a largely unexplored potential

to mount a sequence-independent response to dsRNA (reviewed in Wang et al., 2015a). Such sequence-independent response would, for example, explain increased expression of apoptosis-related genes 24 hours upon exposing shrimps to encapsulated dsRNA (Jariyapong et al., 2015).In fact, shrimp has several interferon system-related genes such as dsRNA-dependent protein kinase PKR and Toll-like receptor 3, which are induced upon dsRNA exposure (Wang et al., 2013). Furthermore, *Crustacea* have many genes homologous to genes of the vertebrate interferon response suggesting that they might combine sequence-specific and sequence-independent innate immunity response to nucleic acids (Wang et al., 2013).

ADAR

Similarly to all previously discussed model systems, *Drosophila* (and presumably all arthropods) have A-to-I editing system. *Drosophila* has a single ADAR related to vertebrate ADAR2 (Barraud et al., 2012). ADAR is developmentally regulated and essential gene (Palladino et al., 2000). *Drosophila* ADAR edits convergent transcripts (Peters et al., 2003), antisense read-through transcripts of KP elements (Peters et al., 2003) as well as miRNAs (Chawla and Sokol, 2014). *Drosophila* ADAR edit primary microRNA (pri-miRNA) transcripts to alter the structural conformation of these precursors resulting in positive or negative modulation of miRNA expression or its activity (Chawla and Sokol, 2014; Cui et al., 2015). Despite its biochemical activity would imply it, there is no good evidence to show that ADAR antagonizes RNAi in *Drosophila* (Paro et al., 2012).

Summary

Taken together, arthropods are an extremely large and diverse taxon, characterized by an extended genetic separation of miRNA and RNAi pathways (Fig. 5). The separation is not complete and structure of small RNAs appearing in the system strongly influences their sorting onto AGO proteins. The main arthropod model system – Drosophila – lost the RdRP component of RNA silencing, which seems to be also the case for *Hexapoda*, *Crustacea* and *Myriapoda* but not *Chelicerata*.

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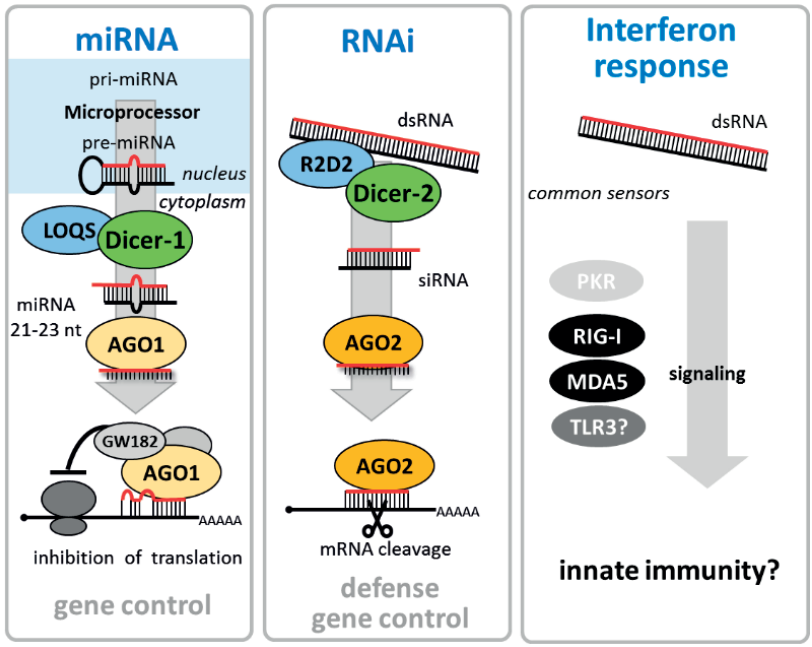

Figure 5 Overview of discussed arthropod pathways
dsRNA and miRNA pathways in *Arthropoda* show separation of miRNA and RNAi pathways, each utilizing a more-or-less dedicated set of proteins for small RNA biogenesis and effector complex.

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