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Literature review of baseline information to support the risk assessment of RNAi-based GM plants

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Abstract

This report is the outcome of an EFSA procurement aiming at investigating and summarising the state of knowledge on (I) the mode-of-action of dsRNA and miRNA pathways, (II) the potential for non-target gene regulation by dsRNA-derived siRNAs or miRNAs, (III) the determination of siRNA pools in plant tissues and the importance of individual siRNAs for silencing. The report is based on a comprehensive and systematic literature search, starting with the identification and retrieval of ~190,000 publications related to the research area and further filtered down with keywords to produce focused collections used for subsequent screening of titles and abstracts. The report is comprised of an (I) Introduction to the field of small RNAs, (II) a Data and Methodologies section containing strategies used for literature search and study selection, and (III) the Results of the literature review organized according to the three main procurement tasks. The outcome of the first task reviews dsRNA and miRNA pathways in mammals (including humans), birds, fish, arthropods, annelids, molluscs, nematodes, and plants. Eight taxon-dedicated chapters are based on ~1,400 cumulative references chosen from ~10,000 inspected titles and abstracts. We review conserved and divergent aspects of small RNA pathways and dsRNA responses in animals and plants including structure and function of key proteins as well as four basic mechanisms: genome-encoded post-transcriptional regulations (miRNA), degradation of RNAs by short interfering RNA pools generated from long dsRNA (RNAi), transcriptional silencing, and sequence-independent responses to dsRNA. The outcome of the second task focuses on base pairing between small RNAs and their target RNAs and predictability of biological effects of small RNAs in animals and plants. The outcome of the last task reviews methodology, siRNA pools, and movement of small RNAs in plants. Potential transfer of small RNAs between species and circulating miRNAs in mammals is described in the final chapter.

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Summary

This report integrates a comprehensive and systematic literature analysis, starting from a collection of ~190,000 references related to the research area and filtered with specific keywords to produce a focused literature collection used for answering the specific tasks laid down in the tender specifications. The report contains (I) an introductory paragraph with a brief introduction to small RNA (II) Data and Methodologies containing strategies used for literature survey and selection, and (III) Results of the literature review are organized in three sections corresponding to the three main tasks of procurement:

I. Mode-of-action of dsRNA and miRNA pathways

This section is composed of eight chapters providing a detailed description of dsRNA and miRNA pathways in mammals (including humans), birds, fish, arthropods, annelids, molluscs, nematodes, and plants and describes functions of components of these pathways. The text is based on a selection of ~1,400 cumulative references from ~10,000 inspected titles and abstracts. We review conserved and divergent aspects of small RNA pathways and dsRNA responses in animals and plants including structure and function of key proteins. Particular attention is paid to the Dicer and Argonaute proteins. The reviewed molecular mechanisms are divided into four basic types (modules), which have distinct features. We recognize three sequence-specific modules and one heterogeneous sequence-independent module:

- **miRNA module** – post-transcriptional regulation, which is guided by individual genome-encoded small RNAs loaded on Argonaute proteins. The key factor is that there is a genome-encoded population of small RNAs with an identical defined sequence.
- **RNAi module** – elimination of RNAs by short interfering RNA pools generated from long dsRNA. The common theme is that small RNAs come as populations of RNAs with variable sequences.
- **transcriptional silencing module** – small RNAs loaded on Argonaute proteins acting in the nucleus and changing chromatin structure (DNA methylation/histone modifications). The classical example is small RNA-induced DNA methylation in plants.
- **sequence-independent responses to dsRNA** such as the interferon response and adenosine deamination. Despite the heterogeneity, the characteristic feature of the module is the coupling of a dsRNA sensor protein with sequence-independent functions, associated with signalling (e.g. innate immunity) or RNA metabolism (e.g. A-to-I editing).

These modules are interconnected as they were dynamically interacting and evolving during the course of the last 300 million years.

II. Potential for non-target (off-target) gene regulation by dsRNA-derived siRNAs or miRNAs

This part provides an overview of the potential for non-target (off-target) gene regulation by dsRNA-derived siRNAs or miRNAs. This section specifically addresses (I) sequence complementarity between target RNA and small RNA (siRNA or miRNA), (II) tolerance of mismatches between target RNA and small RNA (siRNA or miRNA), (III) parameters influencing sequence complementarity (including target recognition and repression), (IV) target recognition by small RNAs, specific targeting by miRNAs and off-targeting by siRNAs, (V) assessment of publicly available bioinformatic programs predicting target recognition, and (VI) the significance of the complementarity search for determining biological effects of small RNAs in animals and plants.

III. Determination of siRNA pools in plant tissues and importance of individual siRNAs for silencing

This part reviews (I) the current methodology used to determine the dsRNA-derived siRNA pool in plant tissues (II) dsRNA-derived siRNA pool in plant tissues and movement of small RNAs in plants, and (III) transfer of small RNAs between species and circulating miRNAs in mammals.

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1. Introduction

1.1. Background and Terms of Reference as provided by EFSA

This report is an outcome of an EFSA procurement titled "Literature review of baseline information to support the risk assessment of RNAi-based GM plants – lot 1" (reference number OC/EFSA/GMO/2015/01), awarded by the European Food Safety Authority to the Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic (IMG) in consortium with EcoMole Ltd.

1.2. Tasks as defined by EFSA

For the literature review of scientific information on RNAi to support the molecular characterisation of the RNAi-based GM plants, the following tasks were defined by EFSA.

1.2.1. Task 1: Mode-of-action of dsRNA and miRNA pathways

- Based on the available scientific literature, the selected tenderer needs to provide a detailed description of the processing of dsRNA and miRNA in plants, humans, mammals, birds, fish, arthropods, annelids, nematodes and molluscs and describe the function of all the different components of the silencing pathways in these organisms. A comparative graphical representation of these pathways should be provided.
- The selected tenderer needs to identify dsRNA and miRNA processing mechanisms and pathway components that are unique to plants, humans, mammals, birds, fish, arthropods, annelids, nematodes and molluscs and are required for normal functioning of the pathway in these specific organisms. Evidence for gene silencing by dsRNA, its derived siRNAs or miRNAs between organisms with unique processing or pathway components should be provided and discussed.

1.2.2. Task 2: Potential for non-target (off-target) gene regulation by dsRNA-derived siRNAs or miRNAs

- The selected tenderer needs to perform a comprehensive literature search on the minimal sequence complementarity needed between target RNA and small RNA (siRNA or miRNA), the parameters influencing this sequence complementarity and the extent to which mismatches between target RNA and small RNA (siRNA or miRNA) can be tolerated.
- Based on the findings of the comprehensive literature search on sequence complementarity between target RNA and small RNA, the selected tenderer is requested to assess to which extent the publicly available bioinformatic programs take into account the knowledge on sequence complementarity required to predict a silencing effect.

1.2.3. Task 3: Determination of siRNA pools in plant tissues and importance of individual siRNAs for silencing

- The selected tenderer needs to provide a comprehensive overview on the current methodology used to determine the dsRNA-derived siRNA pool in plant tissues. Possible advantages and biases of each method need to be described.
- The selected tenderer needs to perform a comprehensive literature search of the dsRNA-derived siRNA pool in different plant tissues, describe the observed variability, the parameters that influence the siRNA pool in these tissues and the movement of siRNAs, dsRNA and miRNA between different cells and tissues.

1.3. Content of the report

Section 1 summarises the content of the report and describes the terms of reference. It also includes a general introductory section (Section 1.4), providing some basic information on RNA silencing pathways. Common general principles are explained, allowing the reader to navigate through the mechanisms and functions of RNA silencing presented in the rest of the report. Section 2 describes the methodology used for the systematic search. Section 3 describes the results of the literature review of baseline information to support the risk assessment of RNAi-derived GM plants and is divided into three parts according to the tasks laid down in the procurement:

1.3.1. Part I: Mode-of-action of dsRNA and miRNA pathways

This part provides a detailed description of dsRNA and miRNA pathways in mammals (including humans), birds, fish, arthropods, annelids, molluscs, nematodes, and plants and describes functions of components of these pathways. The section starts with an introduction (section 3.1), providing some general and comparative overview on the relevant molecular components and mechanisms, which are later reviewed in a taxon-specific manner. The following section (section 3.2) is divided into eight taxon-specific chapters. Each taxon-specific chapter is organized in the same way: a taxon-dedicated introduction is followed by a review of the key protein components of the miRNA and RNAi pathways where the essential key protein factors Dicer and Argonaute are reviewed in detail. Next, the review focuses on the mechanistic aspects of the molecular mechanisms of miRNA and RNAi pathways. Finally, additional dsRNA-responding factors and pathways are discussed with particular attention to the innate immunity and RNA editing.

1.3.2. Part II: Potential for non-target (off-target) gene regulation by dsRNA-derived siRNAs or miRNAs

This part provides a detailed description of the minimal sequence complementarity needed between target RNA and small RNA (siRNA or miRNA) to trigger repression of gene expression. Parameters influencing the minimal sequence complementarity are also described in this part and it reviews to which extent mismatches between target RNA and small RNA (siRNA or miRNA) can be tolerated while still allowing for efficient silencing. Based on this review and based on the mechanism of target recognition by small RNAs, currently available bioinformatics programs were assessed. An overview of programs allowing prediction of target recognition is provided together with the description of the main features of these programs.

1.3.3. Part III: Determination of siRNA pools in plant tissues and importance of individual siRNAs for silencing

This part reviews the current methodology used to determine the dsRNA-derived siRNA pool in plant tissues, the composition of dsRNA-derived siRNA pools in plant tissues and movement of small RNAs in plants. Finally, this last part also describes the transfer of small RNAs between species and circulating miRNAs in mammals.

1.4. Introduction to small non-coding RNA biology

1.4.1. Mechanistic principles and roles of RNA silencing

The aim of this section is to provide a basic introduction into RNA silencing pathways before the taxon-specific systematic reviews. The idea is to bring up common general principles allowing the reader to better navigate through common and derived mechanisms and functions of RNA silencing presented in the part 3.2. While the volume of the RNA silencing and double-stranded RNA (dsRNA)-related published data is overwhelming (over 2×10^5 entries), the consensus regarding the core molecular mechanisms of RNAi and miRNA pathways is well established. Furthermore, as the field of RNA silencing evolved from parallel research in different model systems, it would benefit from a common introduction.

RNA silencing, guided by small RNA molecules (20-30 nucleotides long) includes diverse silencing mechanisms including RNA degradation, translational repression, induction of repressive chromatin, and even DNA deletions (reviewed in Ketting, 2011). Some form of RNA silencing exists in almost every eukaryote. The RNA interference (RNAi, Figure 1) and microRNA (miRNA, Figure 6) pathways are the primary focus of this report. miRNAs are genome-encoded short RNAs that regulate gene expression by translational repression and/or degradation of cognate mRNAs. The term RNAi has been originally used for sequence-specific mRNA degradation induced by long dsRNA (Fire et al., 1998). This mechanism, which employs small RNAs produced from long dsRNA, is the canonical RNAi. However, the term RNAi is also used as a common name for a broad range of RNA silencing pathways (Ketting, 2011). In this report, we use the term RNAi strictly in its original connotation.

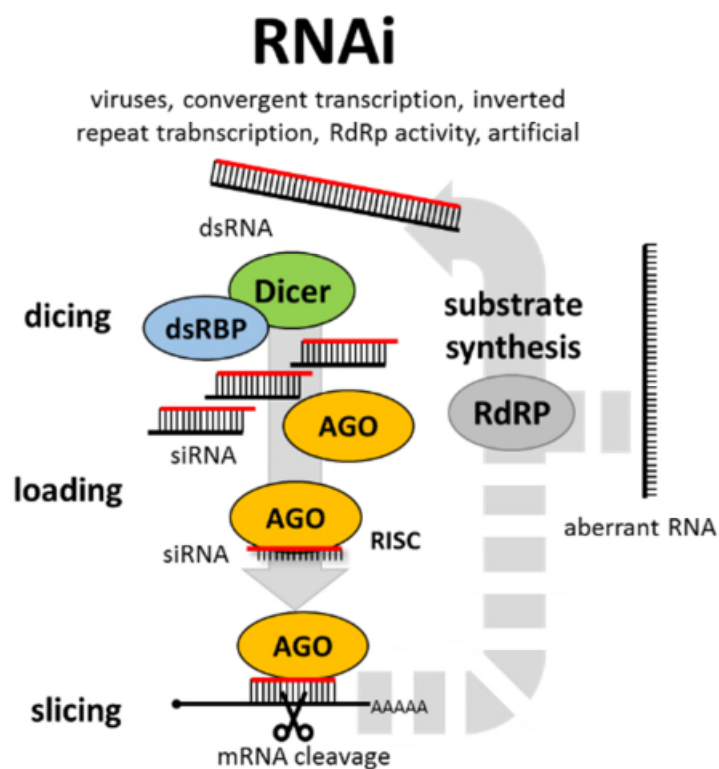


Figure 1 RNAi pathway overview

Canonical RNAi pathway is triggered by some form of long dsRNA. dsRNA can come from various sources including viruses and their replication intermediates or from base pairing of RNA molecules transcribed from the genome. Endogenous RNAs can form dsRNA either through an intramolecular duplex (hairpin dsRNA), or by base pairing RNAs transcribed in *cis* (convergent transcription) or in *trans* (from interspersed elements, pseudogenes etc.). The core mechanism of RNAi has three steps: **dicing** - cleavage of long dsRNA into siRNA duplexes by RNase III Dicer, **loading** - where one strand of siRNA duplex is selected and loaded onto an Argonaute protein forming the RNA-induced silencing complex (RISC), and **slicing** - where siRNA guides RISC to cognate RNAs. Upon making a perfect duplex with a cognate RNA, AGO proteins perform endonucleolytic cleavage of the cognate RNA in the middle of the base paired sequence. In some species, RNAi also involves an RNA-dependent RNA polymerase (RdRP), which may generate initial substrates or participate in amplification of the response by using cognate RNAs to synthesize dsRNA or small RNAs.

Historically, the first discovered RNA silencing pathway was co-suppression in plants, which appeared as sequence-specific silencing of endogenous genes induced by transgene expression (Napoli et al., 1990). The miRNA pathway was first found in 1993 in the nematode *C. elegans* (Lee et al., 1993). The idea of a conserved miRNA pathway emerged after the identification Let-7 miRNA, which was reported in 2000 and is conserved from *C. elegans* to mammals (Pasquinelli et al., 2000). RNAi was described in the meantime also in *C. elegans* (Fire et al., 1998). Around the year 2000, it became apparent that

earlier observations, which included plant co-suppression, quelling in fungi, and animal RNAi and miRNA pathways (Napoli et al., 1990; van der Krol et al., 1990; Romano and Macino, 1992; Lee et al., 1993), belong to one group of mechanisms commonly called RNA silencing.

The core principle of RNA silencing (repression mediated by a ribonucleoprotein complex guided by a small RNA, reviewed, for example, in Ketting, 2011) was deciphered during 1998-2004 using a combination of genetic and biochemical approaches. Key steps in understanding how RNA silencing works were biochemical studies in *Drosophila* embryo lysates and genetic studies in *C. elegans* and plants. The last discovery, which defined the end of an era of deciphering the key principles of RNA silencing, was the structural analysis of Argonaute 2, which revealed the last missing piece of the big puzzle: this protein carries the endonucleolytic activity executing (slicing) targeted mRNAs (discussed in detail, for example in the section 3.2.1.2).

1.4.2. RNAi and miRNA pathway components

1.4.2.1 Substrates of RNA silencing and small RNAs

RNA silencing employs different types of substrates (Figure 2). Canonical precursors of miRNAs contain short hairpin structures, which are released by the Microprocessor complex and processed by Dicer in the cytoplasm. However, additional substrates can produce non-canonical miRNAs, which can be generated in Drosha- and/or Dicer-independent manner (reviewed in Kim et al., 2009; Winter et al., 2009). Long dsRNA substrates for RNAi may come in different forms; viral replication (and RdRP activities in general) often yields blunt-end dsRNA whereas hybridization of sense and antisense RNAs yields dsRNA with single-strand overhangs. Sense and antisense transcripts can be produced in *cis* by convergent transcription or in *trans* at separate loci. Another type of dsRNA are intramolecular duplexes of dsRNA hairpins, which form upon transcription of inverted repeat. In terms of long dsRNA formation, the most efficient is RdRP synthesis followed by forming intramolecular duplexes. Sense and antisense base pairing is less likely to occur in *cis*, especially when sense and antisense RNAs are produced at distant loci. The reason is that RNAs are usually forming intramolecular secondary structures and are bound by RNA binding proteins, which may reduce probability of base pairing of complementary RNAs.

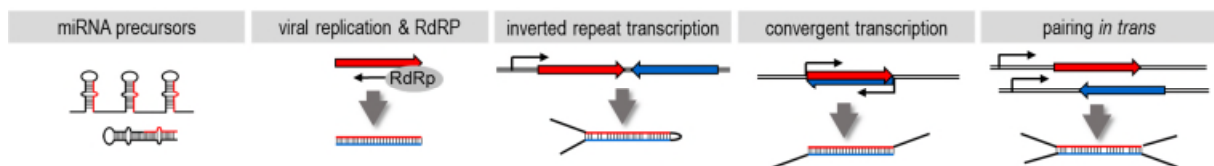


Figure 2 Substrate types in RNA silencing pathways.

1.4.2.2 Dicer - structure and function

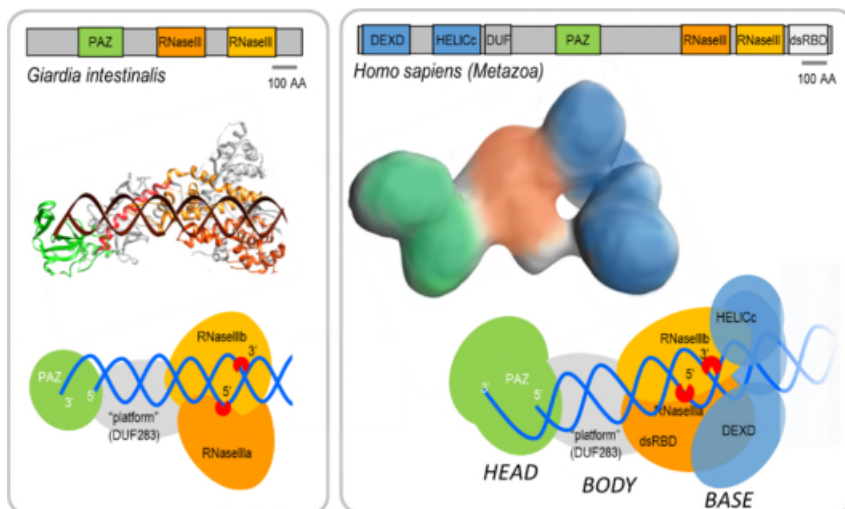
Dicer is the central enzyme for producing small RNAs in miRNA and RNAi pathways. Dicer is intensely studied (there are ~3500 references concerning Dicer). Here, we will provide information necessary to understand the basic molecular features of Dicer; properties of Dicer in different species will be discussed in taxon-specific parts of section 3.2, on *Processing of dsRNA and miRNA in plants, humans, mammals, birds, fish, arthropods, annelids, nematodes and molluscs*.

Dicer, first identified in 2001 as the enzyme processing long dsRNA into siRNAs (Bernstein et al., 2001), generates small RNAs in RNAi and many other (but not all) RNA silencing pathways (reviewed for example in Jaskiewicz and Filipowicz, 2008). Dicer is a large (~200 kDa), multi-domain RNase III endonuclease cleaving various forms of duplex dsRNA. Dicer carries two RNase III domains and several other domains that are typically found in Dicer-like proteins in multicellular eukaryotes (Figure 3). These include an N-terminal helicase domain, piwi/Argonaute/zwille (PAZ) domain, domain of

unknown function DUF283, and a C-terminal dsRNA binding domain (dsRBD, duplicated in some plant Dicers).

Figure 3 Dicer structure

Understanding of Dicer structure and function comes largely from the crystal structure of the shorter Dicer of *Giardia intestinalis*, which was successfully crystallized and provided the first direct structural insight into its function (left panel). Mammalian (human) Dicer was not crystallized yet. The model shown in the right panel comes from cryo-electron microscopy analysis. Dicer cleaves dsRNA from the termini, which are bound by the PAZ domain. Each of the RNase III domains cleaves one strand at a defined distance from the PAZ domain.



The ribonuclease activity of Dicer requires magnesium ions. Dicer preferentially cleaves dsRNA at the termini but it can also cleave internally with low efficiency (Provost et al., 2002; Zhang et al., 2002). Cleavage produces small (21-27 nt long) RNA duplexes with two nucleotide 3' overhangs and 5' monophosphate and 3' hydroxyl groups at RNA termini. Dicer functions as a molecular ruler, measuring the length of the substrate from the PAZ domain to RNase III domains where it is cleaved. The PAZ domain binds the end of dsRNA, with high affinity to 3' protruding overhangs (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003; Ma et al., 2004); these termini are typical for canonical miRNA precursors and processive cleavage of long dsRNA. RNase IIIa and IIIb domains form a single processing center containing two catalytic „half-sites“, each cleaving one strand of the duplex and producing short dsRNA with 2 nt 3' overhang. RNase IIIa domain is processing the protruding 3'-OH-bearing strand and RNase IIIb cuts the opposite 5'-phosphate-containing strand (Zhang et al., 2004).

The crystal structure of the full length Dicer from *Giardia intestinalis*, which represents a minimal Dicer model (MacRae et al., 2006; MacRae et al., 2007), showed that the RNase III domains form a catalytic center connected with the PAZ domain by a long α -helix („connector“ helix), which is implicated in determining the product length. The connector helix is supported by a platform-like structure containing the DUF283 domain, which has a dsRBD-like fold (Dlagic, 2006) and perhaps mediates protein-protein interaction (Qin et al., 2010). Full-length Dicer proteins from animals and plants were not crystallized. However, recent advances in cryo-electron microscopy (cryo-EM) provide additional insights into Dicer structure and function in other models, which utilize more complex Dicer proteins. These results will be summarized in corresponding taxon-specific sections.

Some organisms, like mammals, *Caenorhabditis*, or *Trypanosoma*, utilize a single Dicer protein to produce both siRNAs and miRNAs. In contrast, *Drosophila* utilizes two Dicer paralogs, DCR-1 to produce miRNAs and DCR-2 to produce siRNAs. Some species utilize even more paralogs with distinct functions and different cleavage product lengths (e.g. four Dicer paralogs in *Arabidopsis thaliana* (reviewed in Meins et al., 2005). Animal Dicer proteins are typically found in the cytoplasm. Plants employ multiple Dicer isoforms, some of which have a dedicated nuclear localization.

1.4.2.3 dsRNA binding proteins (dsRBPs)

Dicer interacts with many proteins of which two protein types stand out: (I) Argonaute proteins (discussed next), which receive small RNAs produced by Dicer, and (II) dsRNA-binding proteins (dsRBPs) with tandemly arrayed dsRBDs. dsRBPs facilitate substrate recognition, cleavage fidelity, and Argonaute loading. However, despite a similar domain organization, these proteins evolved distinct roles in small RNA biogenesis by Dicer and sorting onto Argonautes in different model organisms. These roles are discussed for each taxon separately in the section 3.2.

1.4.2.4 Argonaute proteins – structure and function

Argonaute proteins have a molecular weight of ~100 kDa and carry four distinct domains: the central PAZ domain, the C-terminal PIWI (P-element induced wimpy testis), the N-terminal domain, and the MID domain between PAZ and PIWI domains (Figure 4). The PAZ domain binds the 3' end of a short RNA in a sequence-independent manner (Lingel et al., 2003; Song et al., 2003; Lingel et al., 2004; Ma et al., 2004). Structural studies of archaeal Argonaute homologs showed that the PIWI domain has an RNase H-like fold (Parker et al., 2004; Song et al., 2004; Ma et al., 2005; Yuan et al., 2005). A small RNA is anchored with its 3' end in the PAZ domain. The 5' phosphate of the small RNA is buried in a pocket at the interface between the MID domain and the PIWI domain (reviewed in Jinek and Doudna, 2009). The 5' end of the base pairing cognate RNA enters between the N-terminal and PAZ domains and its 3' end exits between the PAZ and MID domains.

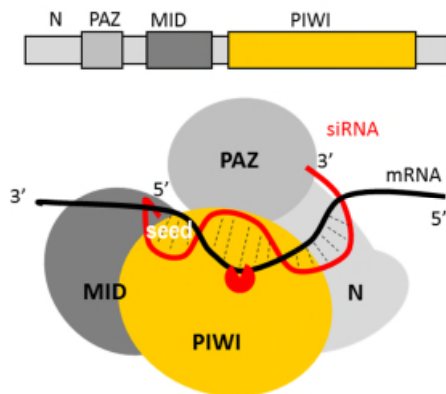


Figure 4 Argonaute protein structure

The order of domains in an Argonaute protein. The scheme shows how Argonaute cleaves a perfectly complementary RNA, which becomes accessible by the catalytic center in the PIWI domain upon base pairing with a small RNA. Nucleotides 2-8 of the small RNA initiate the interaction with the cognate RNA and form the so-called "seed", which has a highly predictive value for miRNA binding sites and siRNA off-targeting.

Argonaute proteins can be divided into three distinct groups (reviewed in Faehnle and Joshua-Tor, 2007): (1) AGO proteins, found in all kingdoms, (2) PIWI proteins found in animals, and (3) WAGO proteins found only in nematodes. The WAGO subfamily was described last, so it is not recognized in the older literature, which typically divides Argonaute proteins into AGO and PIWI subgroups (e.g. Carmell et al., 2002). Metazoan Argonaute proteins functioning in the RNAi pathway include RDE-1 (exogenous RNAi) and ERGO-1 (endogenous RNAi) in *C. elegans*, AGO-2 in *Drosophila*, and AGO2 in mammals. Other Argonautes act in the miRNA and other pathways employing small RNAs.

In RNAi, one strand of a siRNA produced by Dicer serves as a sequence-specific guide in the RNA-induced silencing complex (RISC), which is the effector complex of RNAi. The key component of RISC is an Argonaute family protein (AGO), which binds the selected siRNA strand and uses it as a sequence-specific guide recognizing mRNAs that will be degraded. Argonaute is the „slicer“ (Liu et al., 2004; Meister et al., 2004; Song et al., 2004), i.e. the enzyme catalyzing the cleavage of the cognate mRNA in the canonical RNAi pathway (Figure 1). The active site in the PIWI domain is positioned such that it cleaves the mRNA opposite the middle of the siRNA guide (Song et al., 2004). However, only some Argonautes function as slicers. In other cases, silencing is mediated by additional proteins forming a complex with an Argonaute. There is a long list of Argonaute-interacting protein factors; they will be described separately in the taxon-dedicated sections.

While the minimal active RISC contains only the „slicing“ Argonaute protein and the guide strand of siRNA, RISC activity was found in different models and cell types to reside in ~200 kDa, ~ 500 kDa, or 80S complexes (Nykanen et al., 2001; Martinez et al., 2002; Mourelatos et al., 2002; Pham et al., 2004). Multiple proteins either contribute to RISC formation or might regulate RISC activity, stability, target selection, mode of repression or otherwise contribute to RISC function

1.4.2.5 RNA-dependent RNA polymerase (RdRPs) proteins

RdRPs can contribute to RNA silencing in two ways – either by converting single-stranded RNA to dsRNA or by synthesizing short RNAs, which could function as guides. RdRP is an ancestral component of RNA silencing since RdRP orthologs were identified in RNA silencing pathways in plants,

fungi and some animals: QDE-1 in *Neurospora crassa* (Cogoni and Macino, 1999), EGO-1 and RRF-1 in *C. elegans* (Sardon et al., 2000; Grishok et al., 2001), SDE1/SGS2 in *Arabidopsis* (Mourrain et al., 2000; Dalmay et al., 2001), and Rdp1 in *Schizosaccharomyces pombe* (Hall et al., 2002; Volpe et al., 2002). Homologs of these RdRPs exist in numerous metazoan taxons, including *Nematoda* (e.g. *C. elegans*), *Cnidaria* (hydra), *Chelicerata* (tick), *Hemichordata* (acorn worm), *Urochordata* (sea squirt) but appear absent in others, including *Platyhelminthes* (planaria), *Hexapoda* (*Drosophila*), or *Craniata* (vertebrates). 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). The fact that RdRP orthologs are found in other protostomes and deuterostomes but not in *Drosophila* or mammals suggests a repeated loss of the ancestral RdRP component of RNA silencing. Whether RdRP activity completely disappeared from RNAi in *Drosophila* and mammals is unclear but transitive RNAi generating secondary sequences upstream of the region targeted by siRNAs was not observed in *Drosophila* or mouse (Schwarz et al., 2002; Roignant et al., 2003; Stein et al., 2003).

1.4.3. Diversity of RNAi and miRNA pathway functions

1.4.3.1 RNAi pathway

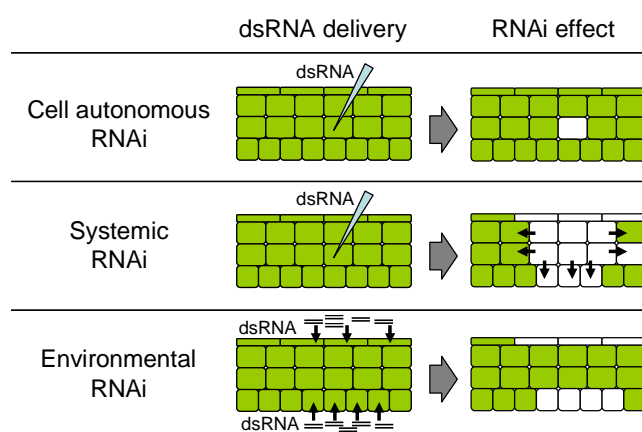
The RNAi pathway (Figure 1) has three main steps: (1) the cleavage of long dsRNA by Dicer into siRNAs, (2) loading of small RNAs on the RISC, and (3) recognition and cleavage of cognate RNAs by the RISC. In addition to this core pathway, two extensions of the pathway, which are restricted to some animal species, should be mentioned: (1) an amplification step, in which RdRPs generate secondary siRNAs and (2) systemic RNAi where an RNAi response can spread across cellular boundaries.

Because dsRNA often originates from viruses, the role of RNAi has been viewed as a form of native immunity. While this role is experimentally supported in some models, RNAi may also have other roles in maintaining genome integrity, and control of gene expression.

RNA viruses generate dsRNA during their replication cycle in host cells. DNA viruses often produce complementary sense and antisense transcripts, which can form dsRNA upon annealing. Thus, dsRNA is a common marker of viral infection and it is recognized by different mechanisms mediating an innate immune response. The idea that RNA silencing may function as a form of innate immunity is supported by several lines of evidence, which were first found in plants and later also in invertebrates (reviewed in (Xie and Guo, 2006; Marques and Carthew, 2007): 1) siRNAs derived from viral sequences were found in infected organism (Hamilton and Baulcombe, 1999), 2) inhibition of RNA silencing resulted in increased viral replication (Mourrain et al., 2000), and 3) some viruses produce suppressors of RNA silencing (Voinnet et al., 1999).

Figure 5 Cell autonomous, systemic and environmental RNAi

RNAi can either act in a cell autonomous manner, i.e. affecting only cells directly exposed to dsRNA (e.g. by injection). Cell non-autonomous RNAi includes systemic RNAi, where the RNAi effects propagates across cellular boundaries (e.g. mediated by transport of small RNAs) or dsRNA can be picked up from the environment (the bottom scheme). Note that systemic and environmental RNAi can actually coexist, such as in *C. elegans*.



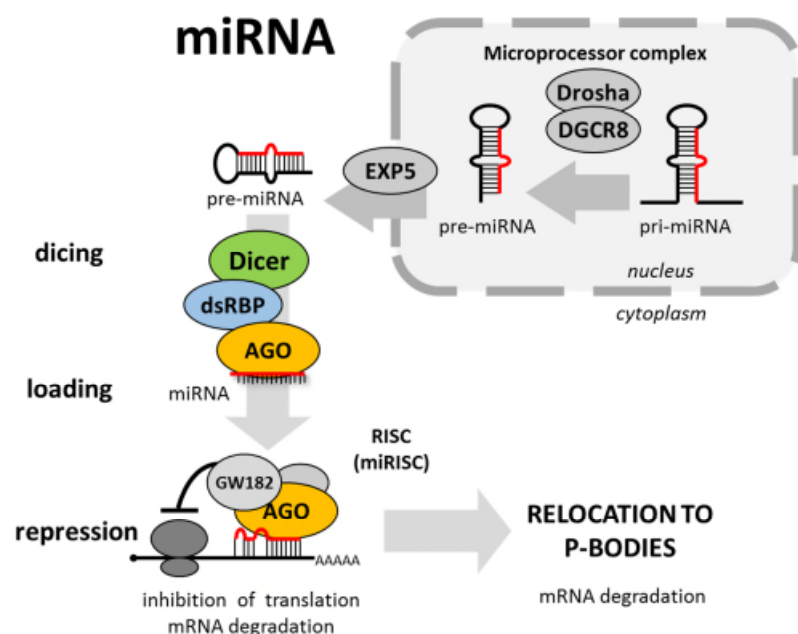
Systemic and environmental RNAi

RNAi can either act in a cell autonomous manner, i.e. affecting only cells directly exposed to dsRNA, or can propagate across cell boundaries (Figure 5). Two modes of non-cell autonomous RNAi are recognized: (1) environmental RNAi involves processes where dsRNA is taken up by a cell from the environment. (2) systemic RNAi includes processes where a silencing signal spreads from a cell across cellular boundaries into other cells. Both modes can be combined and systemic RNAi can follow environmental RNAi. At least two pathways for dsRNA uptake were described: (1) a specific transmembrane channel-mediated uptake and (2) an alternative endocytosis-mediated uptake (reviewed in Whangbo and Hunter, 2008; Huvenne and Smagghe, 2010).

The non-cell autonomous RNAi was observed already during the first RNAi experiments in *C. elegans* (Fire et al., 1998). When animals were microinjected with dsRNA into head, tail, intestine or gonad arm, or even just soaked in dsRNA solution or fed by bacteria expressing dsRNA, these treatments induced a specific null phenotype in the whole animal and even in its progeny, demonstrating a surprising ability of dsRNA to cross cellular boundaries (Fire et al., 1998; Tabara et al., 1998; Timmons and Fire, 1998). Non-cell autonomous RNAi has been discovered also in parasitic nematodes (Geldhof et al., 2007), hydra (Chera et al., 2006), planaria (Newmark et al., 2003; Orii et al., 2003), insects (Tomoyasu et al., 2008; Xu and Han, 2008), or plants (Himber et al., 2003).

Figure 6 Canonical animal miRNA pathway overview

miRNAs are genome-encoded. Their synthesis typically starts with Pol II-mediated transcription of long primary miRNA transcripts (pri-miRNAs), which carry one or more short hairpins. These are released as precursor miRNAs (pre-miRNAs) by the nuclear "Microprocessor complex". Pre-miRNAs are transported into the cytoplasm via Exportin 5. In the cytoplasm, Dicer cleaves the pre-miRNA and one strand of the duplex is loaded onto an AGO protein, which forms the core of the effector complex (RISC or miRISC). The effector complex contains additional proteins, which mediate translational repression or RNA degradation. The key bridge between AGO and proteins mediating deadenylation and decapping is GW182 protein. Targeted mRNAs usually localize to P-bodies, which are cytoplasmic foci associated with RNA metabolism.



1.4.3.2 miRNA pathway

miRNAs are genome-encoded short RNAs that regulate gene expression by mediating translational repression and/or degradation of cognate mRNAs. miRNAs play important roles in many processes and are one of the most common small RNAs found in animal and plant cells. Animal miRNA biogenesis starts with long primary transcripts (pri-miRNAs), which are processed by the nuclear "Microprocessor" complex, into short hairpin intermediates (pre-miRNAs). Pre-miRNAs are transported to the cytoplasm where they are further processed by Dicer into a small RNA duplex, from which is one RNA strand loaded onto an Argonaute protein where it guides recognition and repression of cognate mRNAs (Figure 6).

The AGO-containing effector complex has been given different names; here we will refer to it as miRNA-Induced Silencing Complex (miRISC). The mechanism of action of an AGO-containing effector complex varies and may include either translational repression and/or RNAi-like endonucleolytic cleavage. Functional base pairing of animal miRNAs with their mRNA targets appears to involve little

beyond the “seed” region comprising nucleotides 2 to 8 of the miRNA (Brennecke et al., 2005; Sontheimer, 2005). Pairing between miRNAs and mRNAs in plants is typically much more extensive and results in direct endonucleolytic cleavage.

Imperfect miRNA:mRNA base pairing in animals generally results in translational repression (Hutvagner and Zamore, 2002; Doench et al., 2003), which is coupled with mRNA degradation (Bagga et al., 2005; Lim et al., 2005). The molecular mechanism of mRNA degradation induced by imperfect base pairing differs from the RNA-like cleavage described above (Schmitter et al., 2006) and involves mRNA deadenylation and decapping activities (Djuranovic et al., 2012; Nishihara et al., 2013; Chen et al., 2014; Rouya et al., 2014). RNA degradation might actually be the dominant component of cognate gene repression (Eichhorn et al., 2014).

Repressed mRNAs, miRNAs, and AGO proteins localize to cytoplasmic foci known as P-bodies (Liu et al., 2005; Pillai et al., 2005), which contain mRNA degrading enzymes such as the decapping complex, deadenylases, and the exonuclease XRN1 (reviewed in Decker and Parker, 2012).

Thousands of miRNAs have been annotated in different species. The central miRNA database miRBase (<http://www.mirbase.org>, Kozomara and Griffiths-Jones, 2014) includes 2588 human, 1915 murine, 466 *Drosophila melanogaster*, 434 *Caenorhabditis elegans*, and 427 *Arabidopsis* mature miRNAs (release 21). miRNAs have been implicated in countless cellular and developmental processes; in some cases are changes in their expression linked to pathological conditions. Bioinformatics estimates suggest that miRNAs might directly target over 60% of mammalian genes (Friedman et al., 2009); miRNA-dependent regulation in invertebrates and plants are less extensive.

There are only a few miRNAs conserved between *Drosophila* and mammals and it is not clear if there are any conserved miRNA genes between plants and animals. Animal miRNAs seem to emerge from random formation of Drosha/Dicer substrates (discussed in detail in Svoboda and Cara, 2006). Newly evolving miRNAs likely form a considerable portion of annotated miRNAs, especially in species where miRNAs were intensely studied by next generation sequencing (NGS), which can identify low-abundance miRNAs. The newly emerging miRNAs either acquire significant repressive functions and become retained during evolution or they become lost. Furthermore, target repertoire of individual miRNAs can evolve fast since a single point mutation can weaken an existing regulation or create a new one. Other relevant pathways in *Metazoa*

1.4.4. Adenosine deamination

A-to-I editing is mediated by Adenosine Deaminases Acting on RNA (ADARs), enzymes that carry dsRBD and recognize both inter- and intramolecular dsRNAs longer than 20-30 bp (Nishikura et al., 1991). ADARs are found in animals (including earliest branching groups) but not plants, yeasts or protozoa (Nishikura, 2010; Grice and Degnan, 2015). ADARs convert adenosines to inosines, which are interpreted as guanosines during translation. It was predicted that more than 85% of pre-mRNAs may be edited, predominantly in the non-coding regions (Athanasiadis et al., 2004). Many long perfect dsRNAs (>100 bp) undergo extensive editing with a conversion of approximately 50% of adenosines to inosines (Nishikura et al., 1991; Polson and Bass, 1994). On the other hand, short RNAs (~20-30 bp) or imperfect long dsRNAs are edited selectively; usually only a few adenines at specific sites are deaminated (Lehmann and Bass, 1999).

RNA editing can negatively influence RNAi in several ways. First, ADARs can compete with RNAi for dsRNA substrates including siRNAs. A change of a single base in a sequence may result either in destabilization of dsRNA structure (inosine-uridine pair) or in its stabilization (inosine-cytidine pair) (Nishikura, 2010). This transition in the local and global stability of dsRNA structure can influence further processing of dsRNA, such as the selection of the effective miRNA strand (Bartel, 2004; Meister and Tuschl, 2004; Du and Zamore, 2005). While moderate deamination (one I-U pair per siRNA) does not prevent Dicer processing to siRNAs (Zamore et al., 2000), hyperediting (~50 % of deaminated adenosines) can make dsRNA resistant to Dicer processing (Scadden and Smith, 2001). Hyperedited dsRNA is also degraded by Tudor-SN (TSN) nuclease (Scadden, 2005).

Moreover, editing affects target recognition; a mismatch between siRNA and target mRNA can reduce RNAi efficacy (Scadden and Smith, 2001) or modify target specificity (Kawahara et al., 2007b). This is well documented for miRNAs. Several pri-miRNAs (e.g. miR-142) are known to undergo editing, which inhibits Drosha cleavage or causes even degradation of pri-miRNA by TSN (Scadden, 2005; Yang et al., 2006; Nishikura, 2010). In other cases, pri-miRNA editing does not influence Drosha activity but inhibits processing of pre-miRNA by Dicer (e.g. miR-151) (Kawahara et al., 2007a). Last but not least, RNA editing might also inhibit export of miRNAs from the nucleus (Nishikura, 2010)

An important connection between A-to-I editing and RNAi was found in *C. elegans*. In contrast to mice, where *Adar1* and *Adar2* are essential for normal development (Higuchi et al., 2000; Wang et al., 2000), *Adr* null phenotype manifests only as behavioral defects in *Drosophila* and *C. elegans* (Palladino et al., 2000; Tonkin et al., 2002). Specifically, *Adr-1* or *adr-2* mutant worms exhibit a defective chemotaxis but this phenotype can be rescued when worms lacking *Adar* are crossed with RNAi-defective strains (Tonkin and Bass, 2003).

1.4.5. Interferon pathway

Mammalian somatic cells can respond to dsRNA in a sequence-independent manner. A pioneering work by Hunter et al. showed that different types of dsRNA can block translation in reticulocyte lysates (Hunter et al., 1975). Analysis of the phenomenon identified protein kinase R (PKR) that is activated upon binding to dsRNA and blocks translation by phosphorylating the alpha subunit of eukaryotic initiation factor 2 (eIF2 α) (Meurs et al., 1990). Activation of PKR represents a part of a complex response to foreign molecules known as the interferon response (reviewed in Sadler and Williams, 2007), which includes activation of the NF κ B transcription factor and a large number of interferon-stimulated genes (ISGs) (Geiss et al., 2001). In addition to PKR, several other proteins recognizing dsRNA induce the interferon response, including helicases RIG-I and MDA5, which sense cytoplasmic dsRNA and activate interferon expression, and the 2',5'-oligoadenylate synthetase (OAS), which produces 2',5'-linked oligoadenylates that induce general degradation of RNAs by activating latent RNase L, and specific Toll-like receptors (TLRs) (reviewed in Gantier and Williams, 2007; Sadler and Williams, 2007).

There is an evolutionary connection between RNAi and the interferon response. Mammalian RNA helicases *Ddx58*, *Dhx58* and *Ifih1*, which are involved in immune response, are the closest homologs of helicases involved in processing of long dsRNA during RNAi in *C. elegans*. Notably, DDX58, also known as RIG-I, is an established component of the interferon response to long dsRNA (Yoneyama et al., 2004). This suggests that the interferon response, which has a common trigger and evolved after the RNAi pathway, adopted several components from the latter pathway. It remains to be determined whether these and other components of RNAi lost during evolution their function in RNAi entirely or mediate some form of a crosstalk between RNAi and interferon response.

1.4.6. References

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2. Data and Methodologies

2.1. Data

To search for different types of publications and unpublished work that would provide information on the review questions, multidisciplinary citation databases and databases of grey literature listed in the following table were used.

Table 1 Information sources used in the carried out extensive literature searches.

Information source	Link
Web of Science™ Core Collection	http://webofknowledge.com/WOS
Scopus	http://www.scopus.com/
PubMed	http://www.ncbi.nlm.nih.gov/pubmed
ProQuest Dissertations & Theses Full Text: The Sciences and Engineering Collection	http://search.proquest.com/

The following sections provide a brief description about the searched databases, while Table 2 lists the parameters applied for the searches in individual databases.

PubMed

PubMed comprises over 25 million citations for biomedical literature from MEDLINE, life science journals, and online books. PubMed citations and abstracts include the fields of biomedicine and health, covering portions of the life sciences, behavioural sciences, chemical sciences, and bioengineering. PubMed also provides access to additional relevant web sites and links to the other NCBI molecular biology resources.¹

Web of Science™ Core Collection

Web of Science™ Core Collection provides multidisciplinary content covering over 12,000 of the highest impact journals worldwide (including open access journals) and over 160,000 conference proceedings. The collection features current and retrospective coverage in the sciences, social sciences, arts, and humanities, with coverage dating back to 1900.²

Scopus

More than 60 million records are indexed in Scopus, including over 21,500 peer-reviewed journals, of which more than 4,200 are full open access. Scopus indexes also articles-in-press (i.e., articles that have been accepted for publication) from more than 5,000 international publishers.³

ProQuest Dissertations & Theses Full Text: The Sciences and Engineering Collection

ProQuest Dissertations and Theses - Full text is the world's most comprehensive collection of dissertations and theses. The database includes millions of searchable citations to dissertation and theses from around the world from 1861 to the present day together with over a million full text dissertations and offers full text for most of the dissertations added since 1997 and strong retrospective full text coverage for older graduate works.⁴

¹ Source: http://www.ncbi.nlm.nih.gov/books/NBK3827/#pubmedhelp.PubMed_Quick_Start

² Source: http://wokinfo.com/products_tools/multidisciplinary/webofscience/

³ Source: <https://www.elsevier.com/solutions/scopus/content>

⁴ Source: <http://proquest.libguides.com/pqdt>

Table 2 Example of parameters applied for the searches in individual databases with miRN as keyword.

Information source	Search limited to	Example of a search query for articles with "mirna" keyword
PubMed	no limitations	"mirna"[All Fields]
Web of Science	<ul style="list-style-type: none"> • Topic (TS) • Web of Science™ Core Collection 	TS=("mirna") Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC Timespan=All years
Scopus	<ul style="list-style-type: none"> • Title • Abstract • Keywords • Document Type: Article 	TITLE-ABS-KEY ("mirna") AND DOCTYPE (ar)
ProQuest Dissertations & Theses Full Text: The Sciences and Engineering Collection	<ul style="list-style-type: none"> • Title (TI) • Abstract (AB) • Key Fields (KF) 	TI,AB,KF("mirna") ^(a)

^(a)Equal to ti("mirna") OR ab("mirna") OR kf("mirna")

In addition to the above listed databases used for study retrieval, miRBase⁵ was used to identify keywords related to individual taxons of interest. The miRBase database is a searchable database of published miRNA sequences and annotation. Based on miRBase, all taxons with published information on miRNA were identified and in each taxon-specific search, references from miRBase related to particular species of interest were added to the set of references for screening of titles and abstracts.

2.2. Methodologies

The methodology designed for the systematic literature search described in this report followed three key principles: 1. Methodological rigour and coherence in the retrieval and selection of studies; 2. Reproducibility; 3. Transparency.

To ensure that these principles are implemented in the followed methodology, the designed search methodology is based on the methods and techniques described in the EFSA guidance on application of systematic review methodology to food and feed safety assessments to support decision-making.⁶

Section 2.2.1 summarises in a generic way the key steps of the designed literature search methodology, which is further described in the following sections. Description of the methodology presented here is further complemented with the search protocol for compilation of a comprehensive set of scientific and grey literature in the field attached to this report as Appendix A.

⁵ Available at <http://www.mirbase.org/>

⁶ Available at http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/1637.pdf

2.2.1. Compilation of a comprehensive set of scientific and grey literature

Step 1: Searches in bibliographic databases

To compile a comprehensive set of scientific and grey literature related to RNA interference and dsRNA and miRNA pathways, keywords and phrases listed in Table 3 were used as search queries in the databases described in section 2.1.

Table 3 Keywords and phrases used in search queries to compile a comprehensive set of scientific and grey literature related to RNA interference, dsRNA and miRNA pathways.

21u rna	double strand* rna	rna interference	rnase l	ago1
ago2	argonau*	Dicer	dsrna	mirna or microrna
oligoadenylate	Piwi	Pkr	ptgs	r2d2
Rasirna	Rnai	sirna	tarbp2	rde-1 or rde1
trbp2	wago	gene silenc*		

The set of keywords and phrases used in searches aiming at compiling a comprehensive set of scientific and grey literature related to RNA interference, dsRNA and miRNA pathways (Table 3) was derived using word frequency analysis of titles and abstracts of 647 publications the project team had at its disposal before this literature search was carried out and which are highly relevant for the studied topic. The most frequently used terms and keywords relating to the topic were included in the set of keywords for the systematic literature search. The search was piloted with the set of keywords retrieved from the initial set of publications and the search results were screened to identify further relevant keywords which were added to the set of keywords for the systematic literature search. Since the initial set of 647 publications used for identification of relevant keywords covers the whole period from the discovery of RNAi to present, it reflects the evolution of the terminology in the field, minimising the risk that a keyword important for the search was missed.

Step 2: Citation pearl growing using publications known to be landmark publications in the field

To ensure that publications likely to be highly relevant to answer the Extensive Literature Search (ELS) questions are included in the compiled comprehensive set of scientific literature, 47 publications known to be landmark in the field were used as pearls in citation pearl growing search strategy complementing keyword searches in bibliographic databases. Using the citation pearl growing search strategy, publications citing these 47 publications (listed in Appendix C) as well as publications cited by these 47 pearls were retrieved through Web of Science.

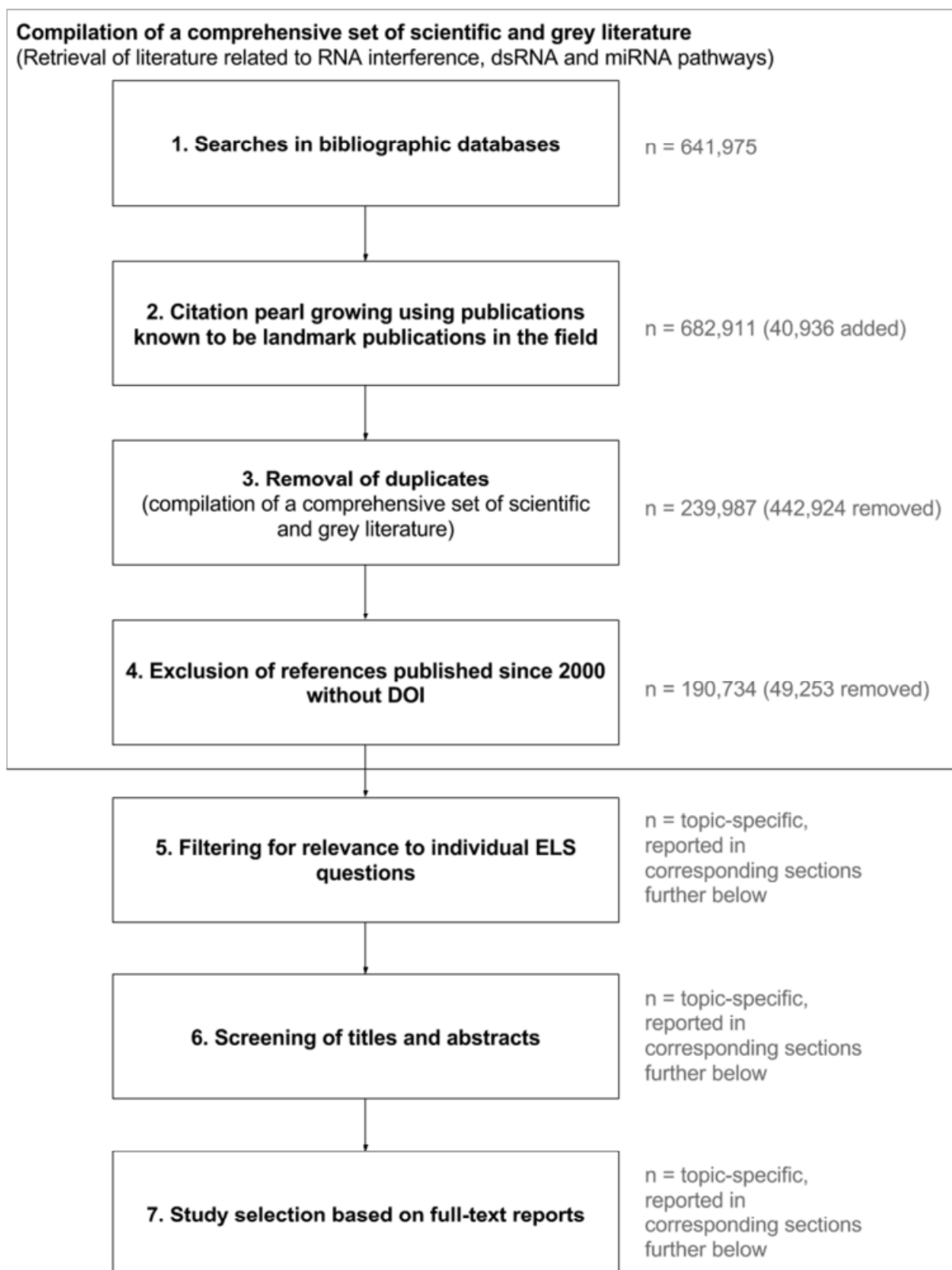
Step 3: Removal of duplicates

Due to the fact that the searches were performed in several different databases and with many keywords yielding similar results, the initial set of retrieved references included many duplicates. These were removed comparing DOIs and citation data of retrieved references.

Step 4: Exclusion of references published since 2000 without DOI

Since 2000, publishers use digital object identifiers (DOIs) to identify electronically published materials⁷. All references published since 2000 lacking a DOI were therefore excluded. References published before 2000 were passed on to filtering for relevance and screening of titles and abstracts regardless of presence or absence of DOI.

⁷ https://www.doi.org/overview/DOI_article_ELIS3.pdf

Figure 7 Schematic overview of the key steps of the designed literature search methodology.

2.2.2. Filtering for relevance to individual ELS questions

The comprehensive set of scientific and grey literature compiled according to the methodology described in section 2.2.1. contains over 190,000 references. To reduce the quantity of references to a manageable number suitable for screening of titles and abstracts by experts of the project team, a filtering using keywords specific to the ELS questions for the individual review topics was carried out. The ELS questions and the review topics were designed to address the questions posed by EFSA in the tender specifications.

2.2.3. Part I: Mode-of-action of dsRNA and miRNA pathways

2.2.3.1 *Mammals*

ELS questions

How is dsRNA and miRNA processed in mammals?

What is the function of all the different components of the silencing pathways in mammals?

Which dsRNA and miRNA processing mechanisms and pathway components are unique to mammals?

Which pathway components are required for the normal functioning of a specific pathway in mammals?

Keywords / phrases used for filtering the comprehensive set of scientific and grey literature related to RNA interference and dsRNA and miRNA pathways:

To select relevant studies concerning dsRNA and miRNA pathways in mammals, the following combinations of keywords were used to select mammals-related studies from the comprehensive set of literature compiled earlier:

mammals OR mammal OR mouse OR mice OR human OR "homo sapiens" OR "mus musculus"

Justification of the selection of taxon-representing keywords: While there are many more studied mammalian species, two mammalian species stand out by a large margin as the most explored: *Mus musculus* and *Homo sapiens*. The search with the above keywords yielded > 100 000 entries, suggesting that it should contain enough in-depth information for the purpose of the report, i.e. describing the molecular mechanism of dsRNA and miRNA pathways. It is very unlikely that inclusion of any other mammalian species would yield any important information concerning the molecular mechanism of these studied pathways that would not be already present in human and mouse data. There are two lines of evidence supporting and justifying this decision; 1) the studied molecular mechanisms are well conserved across vertebrates and mouse and human model systems were historically benchmarking the studied molecular mechanism. Thus, if the purpose is to describe the molecular mechanism in mammals in depth, this work must be primarily based on human and mouse data. 2) since a literature search analysis for birds, more distantly related than any other mammal, provided essentially a similar outcome as the mammalian search, expanding the analysis by including more closely related mammalian species would not have added additional information.

Results

The initial filtering of the comprehensive set of scientific and grey literature using the above listed keywords yielded 98,263 entries. Studies passed on to the screening of titles and abstracts were further limited to studies that included at least one of the following keywords in the title or abstract: miRNA, microRNA, RNAi, siRNA, Dicer, Argonaute or dsRNA. This filtering lead to 63,358 entries of which 4,398 were subsequently screened for relevance (title and abstract). Studies were selected for screening for relevance to the ELS questions based on the keywords listed in the following Table 4 showing the statistics of included and excluded studies – if a keyword listed in the table was present in the title or abstract, the paper was selected for screening for relevance.

Table 4 Breakdown of studies screened (titles and abstracts) for relevance to ELS questions.

	included	excluded		total screened
		no description of dsRNA or miRNA processing	not relevant for mammals	
TOTAL	2561	1327	510	4398
DGCR8	139	21	12	172
Drosha	265	49	20	334
TRBP	91	25	10	126
TARBP2	17	3	0	20
TRBP2	8	7	3	18
dsRBP	9	0	3	12
dsRBD	40	20	15	75
PACT	42	14	3	59
Dicer	1030	410	158	1598
Argonaut*	570	326	127	1023
AGO1	105	33	24	162
AGO2	361	119	51	531
AGO3	31	2	1	34
AGO4	29	2	3	34
PAZ	39	26	8	73
PIWI	103	34	28	165
GW182	55	8	9	72
TNRC6	39	19	2	60
LIN28+uridylation	11	0	1	12
RdRP	12	5	34	51
miRNA	1621	519	169	2309
siRNA	942	700	294	1936
dsRNA	486	241	200	927
siRNA+exosome	35	2	40	77
miRNA+exosome	87	4	31	122
miRNA+extracellular	87	7	27	121
ADAR	74	11	36	121
ADAR+structure	35	3	13	51
ADAR+RNAi	9	0	3	12
ADAR+siRNA	16	0	14	30
ADAR+miRNA	28	4	10	42
PKR	145	46	134	325
PKR+structure	41	2	23	66
PKR+RNAi	19	6	12	37
PKR+siRNA	46	26	49	121
PKR+miRNA	13	10	0	23
RIG-I	80	20	45	145
RIG-I+structure	20	5	8	33
RIG-I+RNAi	13	1	9	23
RIG-I+siRNA	32	18	28	78
RIG-I+miRNA	18	0	3	21
MDA5	33	5	17	55
MDA5+RNAi	3	0	1	4
MDA5+siRNA	12	4	12	28
MDA5+miRNA	1	0	0	1
MDA5+structure	6	0	3	9
OAS	42	42	64	148
OAS+dsRNA	35	37	60	132

Upon screening of titles and abstracts and manual annotation, 2,561 entries were retained as relevant for the ELS questions. Irrelevant (and therefore excluded) studies were 1) articles concerning mammalian biology but unrelated to the studied mechanisms, and 2) articles related to the studied mechanisms, which did not bring any relevant information, e.g. studies, which just used RNAi to knock-down a gene of interest.

Complete set of inclusion/exclusion criteria applied in the screening of titles and abstracts is documented in the following Table 5.

Table 5 Inclusion/exclusion criteria applied in the screening of titles and abstracts.

Inclusion criteria	Exclusion criteria
Population	
Studies describing the processing of dsRNA and/or miRNA in mammals (including humans)	Studies describing the processing of dsRNA and/or miRNA in other organisms
Outcome	
Processing mechanisms of dsRNA and/or miRNA are described	
Study design	
All study designs are eligible	
Geography	
Studies from all countries are eligible	
Recency	
All studies are eligible regardless of the publication date	
Language	
No languages are excluded	
Publication type	
Peer-reviewed original research articles	Reviews, news, letters, brief communications, communications arising, technical reports, project reports, non-scientific media publications (newspapers, popular magazines, etc.), patents

Due to complexity of the performed search and broadness of the review topic, the results of the study selection process for specific fields are further commented in the following sections.

Microprocessor

The systematic search yielded 265 and 139 relevant articles containing the keyword Drosha or DGCR8 respectively. The text in section 3.1 summarizes data from literature concerning the molecular mechanism of the initial step of miRNA biogenesis. Literature that passed through the systematic search but is not explicitly discussed, largely deals with experimental manipulation of the Microprocessor complex in order to perturb the miRNA pathway. This includes 31 reports dealing with phenotypes appearing upon the genetic loss of the Microprocessor complex components (and the consequent loss of miRNAs) in various tissues or cell types (Wang et al., 2007; Babiarz et al., 2008; Chong et al., 2008; Sugatani and Hruska, 2009; Yi et al., 2009; Bezman et al., 2010; Melton et al., 2010; Suh et al., 2010; Babiarz et al., 2011; Fenelon et al., 2011; Zhdanova et al., 2011; Chen et al., 2012; Hsu et al., 2012; Knuckles et al., 2012; Teta et al., 2012; Wu et al., 2012; Fan et al., 2013; Gomez-Cabello et al., 2013; Jeker et al., 2013; Busskamp et al., 2014; Kim et al., 2014a; Sugatani et al., 2014; Zimmermann et al., 2014; Bartram et al., 2015; Belair et al., 2015; Kobayashi et al., 2015; Modzelewski et al., 2015; Ohana et al., 2015; Bartram et al., 2016; Kim et al., 2016; Rowley et al., 2016). These reports represent the best available models for analyzing requirements for the miRNA

pathway in different mammalian tissues and cell types. The remaining literature that was considered relevant but was not cited in this review, includes redundancies (studies providing the same kind of information as those explicitly cited in this report) or information that is not essential for understanding the molecular mechanism of miRNA processing in mammals. Nonetheless, these reports are attached to this report in the dataset with references to the included studies.

Dicer

Literature search with the keyword Dicer yielded 1598 hits, which were manually screened (title and abstract). Thousand references were found relevant for the covered topic. The 410 references excluded as irrelevant were mainly individual supplementary items with own DOIs, which were annotated as a separate reference (e.g. 10.1371/journal.pone.0027438.s001), duplicated references, retracted references (e.g. 10.1371/journal.pgen.1003791), annotated database entries (e.g. 10.2210/pdb3x1o/pdb), patents not containing any unique information for reviewing the molecular mechanism, and several articles clearly unrelated to the covered topic (e.g. [10.1016/j.str.2007.10.018]). ~200 references were annotated as irrelevant for mammals: they mentioned mammals, humans or mice but data came from a non-mammalian system (a typical example: 10.1016/j.molcel.2015.05.033 or 10.1038/NPLANTS.2014.27), or they were not bringing any relevant mechanistic information (e.g. 10.5037/jomr.2013.4101). Still, the included 1030 Dicer-related references could not be all systematically assembled into a concise report on Dicer. The reason is that Dicer is the key player in small RNA biogenesis in RNAi and miRNA pathways; hence, it is intensely studied and appears in countless contexts in the literature. For example, there were over 200 references reporting phenotypes of Dicer knock-out in the mouse model. Thus, we organized the text into a focused section, for which distinct reference selections from the pool of Dicer references could be made or where the recent references provided an accurate up-to-date picture of the molecular mechanism without the need for citing all related literature from the last decade. In any case, the complete list of included references is attached to this report, so that the accuracy of the text can be assessed whenever needed. This will be a recurring theme of all sections where the systematic literature search yielded a high number of more or less relevant references, which could not be all included and cited for the sake of text comprehensibility.

RdRP

The literature search for mammalian RdRPs related to RNA silencing yielded 12 original research papers related to mammalian RdRPs that could be put in some context of RNA silencing. Of these, four articles actually dealt with viral RdRPs and silencing of viruses by exogenous siRNAs and not with an RdRP possibly involved in RNAi (Lee et al., 2002; Meng et al., 2006; Nygardas et al., 2009; Moon et al., 2016).

A search for Aquarius related data (28 papers citing the original one and text search by gene name) suggested that Aquarius is an RNA/DNA helicase involved in R-loop processing (Sollier et al., 2014).

AGO

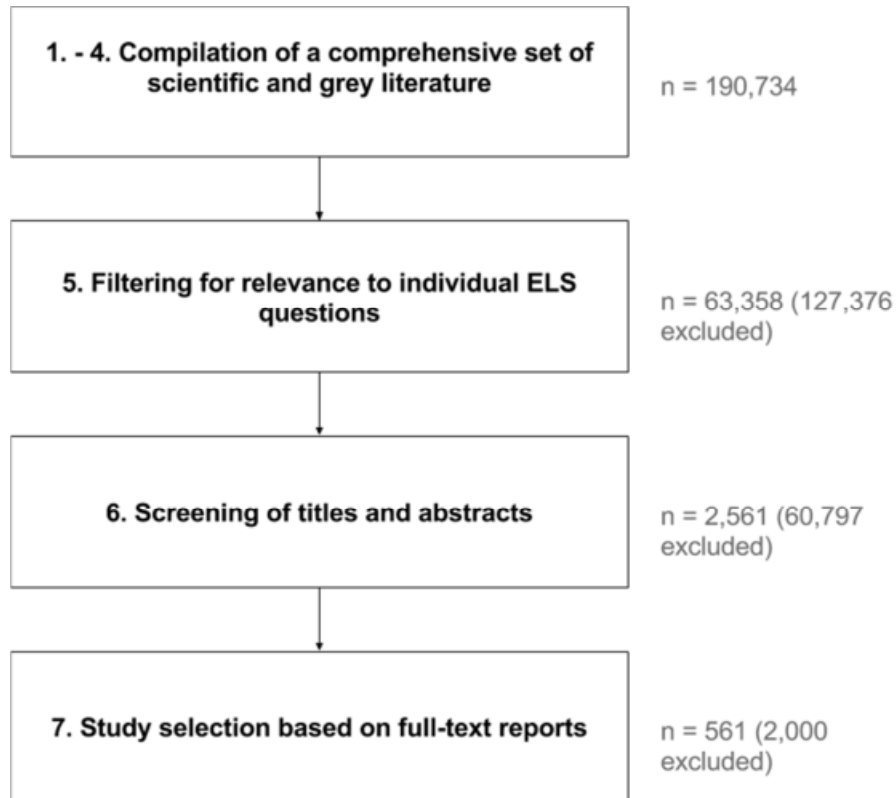
The literature search yielded over 600 references for Argonaute proteins in mammals (keyword search Argonaut* or AGO1 or AGO2 or AGO3 or AGO4). Similarly, to the section on the mammalian Dicer, not all AGO-related manually-selected references could be systematically assembled in the text. The text was therefore organised into a focused section, for which distinct reference selections from the pool of references could be made or where the recent references provided an accurate up-to-date picture of the molecular mechanism without the need for citing all related literature from the last decade. The complete list of included references is attached to this report.

Exosomal RNAs

There is a large volume of literature concerning circulating or exosomal or extracellular miRNAs. Most of the literature is associated with the field of biomarkers. Since our aim was to provide an overview, we focused our search using keywords (miRNA AND extracellular) OR (miRNA AND exosome). This yielded 93 references of which 62 were found relevant.

The following Figure 8 provides statistics of included and excluded studies in each step of the study selection process.

Figure 8 Statistics of included and excluded studies concerning dsRNA and miRNA processing in mammals.



2.2.3.2 *Birds*

ELS questions

How is dsRNA and miRNA processed in birds?

What is the function of all the different components of the silencing pathways in birds?

Which dsRNA and miRNA processing mechanisms and pathway components are unique to birds?

Which pathway components are required for the normal functioning of a specific pathway in birds?

Keywords / phrases used for filtering the comprehensive set of scientific and grey literature related to RNA interference and dsRNA and miRNA pathways:

To select relevant studies concerning dsRNA and miRNA pathways in birds, the following combinations of keywords were used to select bird-related studies from the comprehensive set of literature compiled earlier:

[birds OR bird OR chicken OR finch OR gallus OR taenopygia](#)

Justification of the selection of taxon-representing keywords: Among the bird species, two truly stand out as the main experimental model systems for birds: *Gallus gallus* (chicken) and *Taenopygia guttata* (Zebra Finch). Chicken is an old common model system for embryology and virology, Zebra Finch is a common model for studying biology of vocal communication. Both species have sequenced genomes that are available through the UCSC Genome Browser and are the only birds species with annotated miRNAs in MirBase.

Results

The initial filtering of the comprehensive set of scientific and grey literature using the above listed keywords yielded 1406 entries. Studies passed on to the screening of titles and abstracts were further limited to studies which include at least one of the following keywords in the title or abstract: miRNA, microRNA, RNAi, siRNA, Dicer, Argonaute or dsRNA. This filtering lead to 793 entries which were screened for relevance (title and abstract).

Upon screening of titles and abstracts and manual annotation, 278 entries were retained as directly relevant for the review questions. We selected studies, which contained i) any information concerning key molecular components of pathways of interest (such as Dicer, Argonaute, PKR, OAS), ii) relevant information concerning any miRNAs feature (expression, annotation, function), and iii) relevant information concerning RNA interference, including its use. Of note is that articles in the categories ii) and iii) were collected comprehensively despite almost all of them had no relevance to the review questions. However, omitting any of such publications would not have an impact on the review of molecular mechanisms of RNA silencing in birds because these entries all show that the molecular mechanism of RNAi is present and can be induced by commonly used RNAi inducers – long dsRNA, siRNA, and shRNA.

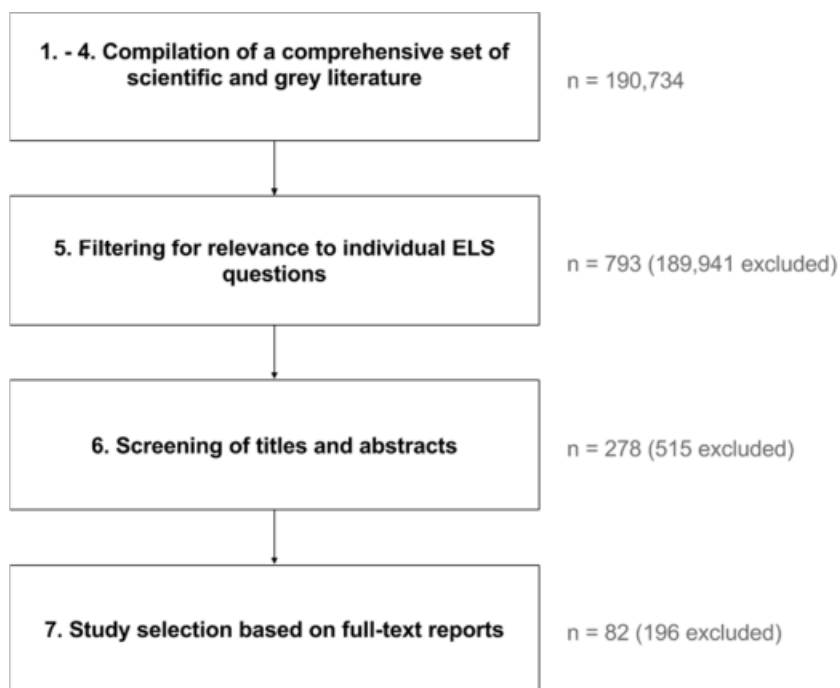
The reasons for exclusion of the majority of articles were i) completely false positives, often referring to birds but not directly concerning the studied mechanisms (bird's view, Avian flu), ii) unrelated articles concerning bird biology unrelated to the topic of the literature questions. As chickens are a common model for studying viral infections, there was a plethora of literature on viral infections in birds without a mechanistic relationship to the studied pathways. Such articles were included only when the primary dsRNA-induced factors (PKR, OAS, RIG-I) were explicitly mentioned in the abstract.

A complete set of inclusion/exclusion criteria applied in the screening of titles and abstracts is documented in the following Table 6.

Table 6 Inclusion/exclusion criteria applied in the screening of titles and abstracts.

Inclusion criteria	Exclusion criteria
Population	
Studies describing the processing of dsRNA and/or miRNA in birds	Studies describing the processing of dsRNA and/or miRNA in other organisms
Outcome	
Processing mechanisms of dsRNA and/or miRNA are described	
Study design	
All study designs are eligible	
Geography	
Studies from all countries are eligible	
Recency	
All studies are eligible regardless of the publication date	
Language	
No languages are excluded	
Publication type	
Peer-reviewed original research articles	Reviews, news, letters, brief communications, communications arising, technical reports, project reports, non-scientific media publications (newspapers, popular magazines, etc.), patents

The following Figure 9 provides the statistics of included and excluded studies in each step of the study selection process.

Figure 9 Statistics of included and excluded studies concerning dsRNA and miRNA processing in birds.

2.2.3.3 *Fish*

ELS questions

How is dsRNA and miRNA processed in fish?

What is the function of all the different components of the silencing pathways in fish?

Which dsRNA and miRNA processing mechanisms and pathway components are unique to fish?

Which pathway components are required for the normal functioning of a specific pathway in fish?

Keywords / phrases used for filtering the comprehensive set of scientific and grey literature related to RNA interference and dsRNA and miRNA pathways:

To select relevant studies concerning dsRNA and miRNA pathways in fish, the following combinations of keywords were used to select fish-related studies from the comprehensive set of literature compiled earlier:

[fish](#) OR [medaka](#) OR [carp](#) OR [halibut](#) OR [flounder](#) OR [cyprinus](#) OR [danio](#) OR [fugu](#) OR [hippoglossus](#) OR [ictalurus](#) OR [oryzias](#) OR [paralichthys](#) OR [salmon](#) OR [tetraodon](#)

Justification of the selection of taxon-representing keywords: Among the fish species, zebrafish (*Danio rerio*) stands out as the main experimental model systems for fish. There are several additional models in use, including medaka (*Oryzias latipes*), salmon (*Salmo salar*), and carp (*Cyprinus carpio*). There are nine fish species annotated in the miRBase – their genera were used for filtering fish-related entries from the main database.

Results

The initial filtering of the comprehensive set of scientific and grey literature using the above listed keywords yielded 4,029 entries. Studies passed on for screening of titles and abstracts were further limited to studies that include at least one of the following keywords in the title or abstract: miRNA, microRNA, RNAi, siRNA, Dicer, Argonaute or dsRNA. This filtering led to 1,930 entries, which were screened for relevance (title and abstract).

Upon screening of titles and abstracts and manual annotation, 1,030 entries were excluded on the basis of being false positives unrelated to the ELS questions or duplicates, and 308 were excluded because they were not directly related to studies in fish but dealt with small RNAs in other model systems. 592 entries were retained as directly relevant for the review questions. We selected studies, which contained i) any information concerning key molecular components of pathways of interest (such as Dicer, Argonaute, PKR, OAS), ii) relevant information concerning any miRNAs feature (expression, annotation, function), and iii) relevant information concerning RNA interference, including its use.

The reasons for exclusion of the majority of articles were i) completely false positives, coming from similar or identical words – FISH (fluorescence in situ hybridization), Fisher's exact test, Salmonella, pericarp, carpel, parthenocarpic, pericarpin and others, ii) unrelated articles concerning fish biology but not directly the studied mechanisms.

348 articles, which annotated and/or analyzed expression and function of miRNAs in fish were found. Almost none of these articles brought any mechanistic insight into the molecular mechanism of miRNA.

The complete set of inclusion/exclusion criteria applied in the screening of titles and abstracts is documented in the following Table 7

Table 7 Inclusion/exclusion criteria applied in the screening of titles and abstracts.

Inclusion criteria	Exclusion criteria
Population	
Studies describing the processing of dsRNA and/or miRNA in fish	Studies describing the processing of dsRNA and/or miRNA in other organisms
Outcome	
Processing mechanisms of dsRNA and/or miRNA are described	
Study design	
All study designs are eligible	
Geography	
Studies from all countries are eligible	
Recency	
All studies are eligible regardless of the publication date	
Language	
No languages are excluded	
Publication type	
Peer-reviewed original research articles	Reviews, news, letters, brief communications, communications arising, technical reports, project reports, non-scientific media publications (newspapers, popular magazines, etc.), patents

The following Figure 10 provides statistics of included and excluded studies in each step of the study selection process.

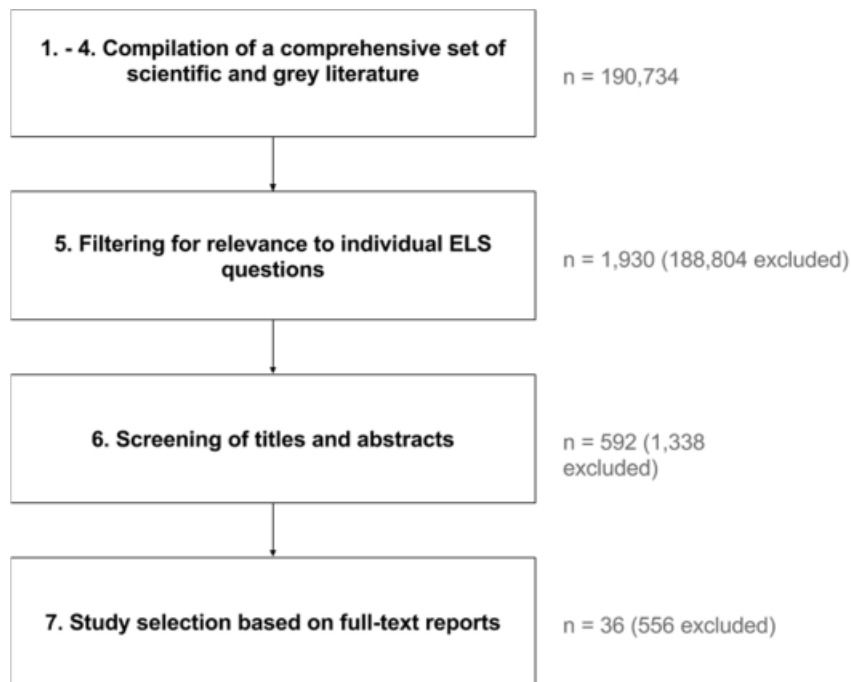


Figure 10 Statistics of included and excluded studies concerning dsRNA and miRNA processing in fish.

2.2.3.4 *Arthropods*

ELS questions

How is dsRNA and miRNA processed in arthropods?

What is the function of all the different components of the silencing pathways in arthropods?

Which dsRNA and miRNA processing mechanisms and pathway components are unique to arthropods?

Which pathway components are required for the normal functioning of a specific pathway in arthropods?

Keywords / phrases used for filtering the comprehensive set of scientific and grey literature related to RNA interference and dsRNA and miRNA pathways:

The following keywords were used for filtering of the comprehensive set of scientific and grey literature to retrieve arthropod-related entries:

[arthropods](#) OR [arthropod](#) OR [chelicerat](#) OR [horseshoe crab](#) OR [limulus](#) OR [spider](#) OR [scorpion](#) OR [crustacea](#) OR [crayfish](#) OR [lobster](#) OR [shrimp](#) OR [crab](#) OR [myriapod](#) OR [centipede](#) OR [millipede](#) OR [hexapod](#) OR [insect](#) OR [beetle](#) OR [tribolium](#) OR [drosophila](#) OR [apis melifera](#) OR [honey bee](#) OR [butterfly](#) OR [mosquito](#) OR [culex](#) OR [aedes](#) OR [anopheles](#)

Justification of the selection of taxon-specific keywords: Arthropods are the largest animal group on the planet with over a million described species (~80% of described animal species) and estimated tens of millions of species. Arthropods include four major distinct groups: *Chelicerata*, *Crustacea*, *Myriapoda*, and *Hexapoda*, each of which contains countless numbers of diverse species. Over two hundred arthropod genomes were sequenced, 13 are available through the UCSC Genome Browser (11 species of *Drosophila*, 2 species of *Anopheles*). There are 32 arthropod species (12 are from the genus *Drosophila*) having annotated miRNAs in the current edition (21) of miRBase. At the same time, there is one dominating arthropod experimental model system: *Drosophila melanogaster*, which was actually the key for deciphering the molecular mechanism of RNAi around the year 2000.

A search covering all major taxons (*Chelicerata*, *Crustacea*, *Myriapoda*, and *Hexapoda*) and their representative animals was performed. [Horseshoe crab](#), [Limulus](#), [spider](#), [scorpion](#) for *Chelicerata*, [crayfish](#), [lobster](#), [shrimp](#), [crab](#) for *Crustacea*, [centipede](#), [milipede](#), for *Miriapoda*, and [beetle](#), [honeybee](#), [honey bee](#), [butterfly](#), [mosquito](#), [insect](#) for *Hexapoda*. In addition, we added several genera to the search: the most common model system *Drosophila*, another model system, which is known for systemic RNA *Tribolium*, honey bee *Apis melifera* because of its economical importance, and three mosquito genera *Culex*, *Aedes*, *Anopheles*, because they are the critical vectors for blood borne pathogens.

Results

The initial filtering of the comprehensive set of scientific and grey literature using the above listed keywords yielded 10,718 entries. Studies passed on to the screening of titles and abstracts were further limited to studies that include at least one of the following keywords in the title or abstract: miRNA, microRNA, RNAi, siRNA, Dicer, Argonaute or dsRNA. This filtering lead to 7,085 entries, of which 1,165 were screened for relevance (title and abstract). Studies were selected for screening for relevance to the ELS questions based on the keywords listed in the following Table 8 showing the statistics of included and excluded studies – if a keyword listed in the table was present in the title or abstract, the paper was selected for screening for relevance.

Table 8 Breakdown of studies screened (titles and abstracts) for relevance to ELS questions - arthropods.

	included	excluded		total screened
		no description of dsRNA or miRNA processing	not relevant for arthropods	
TOTAL	764	325	76	1165
DGCR8	6	0	0	6
Drosha	45	2	1	48
TRBP	15	2	0	17
TARBP2	0	0	0	0
TRBP2	2	0	0	2
dsRBP	9	0	0	9
dsRBD	9	3	0	12
PACT	3	0	0	3
Dicer	289	104	9	402
Argonaut*	289	118	20	427
AGO1	88	14	6	108
AGO2	97	30	3	130
AGO3	33	1	1	35
AGO4	3	0	2	5
PAZ	22	14	4	40
PIWI	266	45	11	322
GW182	27	1	1	29
TNRC6	2	0	0	2
LIN28	2	0	0	2
RdRP	10	13	17	40
RNAi	286	138	15	439
microRNA	300	66	10	376
siRNA	535	207	30	772
dsRNA	249	107	29	385
ADAR	21	1	3	25
PKR	6	1	3	10
RIG-I	5	0	8	13
MDA5	3	0	8	11
OAS	1	5	0	6

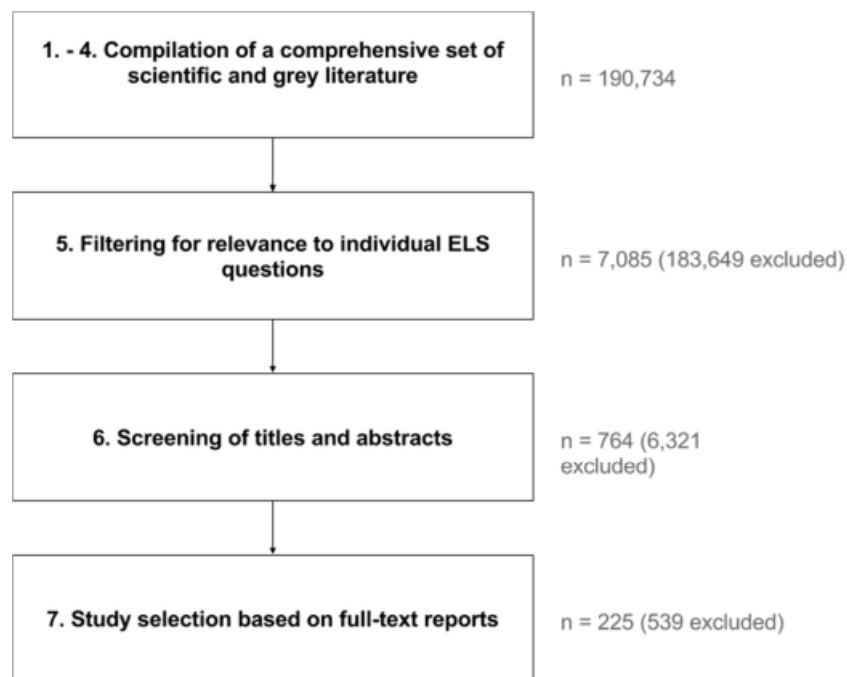
Upon screening of titles and abstracts and manual annotation, 764 entries were retained as directly relevant for the review questions. We selected entries, which contained i) any information concerning key molecular components of pathways of interest (such as Dicer, Argonaute, PKR, OAS), ii) relevant information concerning any miRNAs feature (expression, annotation, function), and iii) relevant information concerning RNA interference, including its use.

Complete set of inclusion/exclusion criteria applied in the screening of titles and abstracts is documented in the following Table 9.

Table 9 Inclusion/exclusion criteria applied in the screening of titles and abstracts.

Inclusion criteria	Exclusion criteria
Population	
Studies describing the processing of dsRNA and/or miRNA in arthropods	Studies describing the processing of dsRNA and/or miRNA in other organisms
Outcome	
Processing mechanisms of dsRNA and/or miRNA are described	
Study design	
All study designs are eligible	
Geography	
Studies from all countries are eligible	
Recency	
All studies are eligible regardless of the publication date	
Language	
No languages are excluded	
Publication type	
Peer-reviewed original research articles	Reviews, news, letters, brief communications, communications arising, technical reports, project reports, non-scientific media publications (newspapers, popular magazines, etc.), patents

The following Figure 11 provides statistics of included and excluded studies in each step of the study selection process.

Figure 11 Statistics of included and excluded studies concerning dsRNA and miRNA processing in arthropods.

2.2.3.5 *Molluscs*

ELS questions

How is dsRNA and miRNA processed in molluscs?

What is the function of all the different components of the silencing pathways in molluscs?

Which dsRNA and miRNA processing mechanisms and pathway components are unique to molluscs?

Which pathway components are required for the normal functioning of a specific pathway in molluscs?

Keywords / phrases used for filtering the comprehensive set of scientific and grey literature related to RNA interference and dsRNA and miRNA pathways:

To select relevant entries concerning dsRNA and miRNA pathways in molluscs, a diverse and extremely large group of animals, we used the following combinations of keywords to select from the main database molluscs-related entries:

molluscs OR mollusc OR oyster OR squid OR gastropod OR patella OR pinctada OR crassostrea OR argonauta OR cephalopod OR octopod OR octopus OR cuttlefish OR bivalvia OR scallop OR octopus OR conus OR corbicula OR lottia OR lymnaea OR dreissena OR mytilus OR haliotis OR crassostrea OR aplysia OR biomphalaria

Justification of the selection of taxon-representing keywords: Among the molluscs species, none is a common experimental model system. Relevant references that we surveyed before setting the search revealed that the most studied model systems in terms of RNAi appear to be oysters (genus *Crassostrea*) and squids. One mollusc genome (*Aplysia californica*) is available through the UCSC Genome Browser, although seven species have genomes sequenced according to NCBI (*Octopus bimaculoides*, *Conus tribblei*, *Corbicula fluminea*, *Lottia gigantea*, *Lymnaea stagnalis*, *Dreissena polymorpha*, *Mytilus galloprovincialis*). Three additional species have known mitochondrial genome (*Crassostrea gigas*, *Aplysia californica*, *Biomphalaria glabrata*). There are two molluscs species having annotated miRNAs in the current edition (21) of miRBase (*Haliotis rufescens*, *Lottia gigantea*).

A particular problem for filtering molluscs entries was that Snail is a name used for a family of transcription factors involved in epithelial-mesenchymal transition, which is a fundamental process of embryogenesis and cancerogenesis. Accordingly, there is a large volume of unrelated literature: Pubmed search with the term "snail" has ~24 000 hits. Therefore, we used the term [snail](#) in combination with specific molecular components – Dicer, Argonaute, dsRNA, miRNA and removed references containing [snail AND mesenchymal](#) or [snail pathway](#).

Results

The initial filtering of the comprehensive set of scientific and grey literature using the above listed taxon-representing keywords yielded 353 entries. All were screened for relevance (title and abstract). Upon screening of titles and abstracts and manual annotation, 95 entries were retained as directly relevant for the review questions.

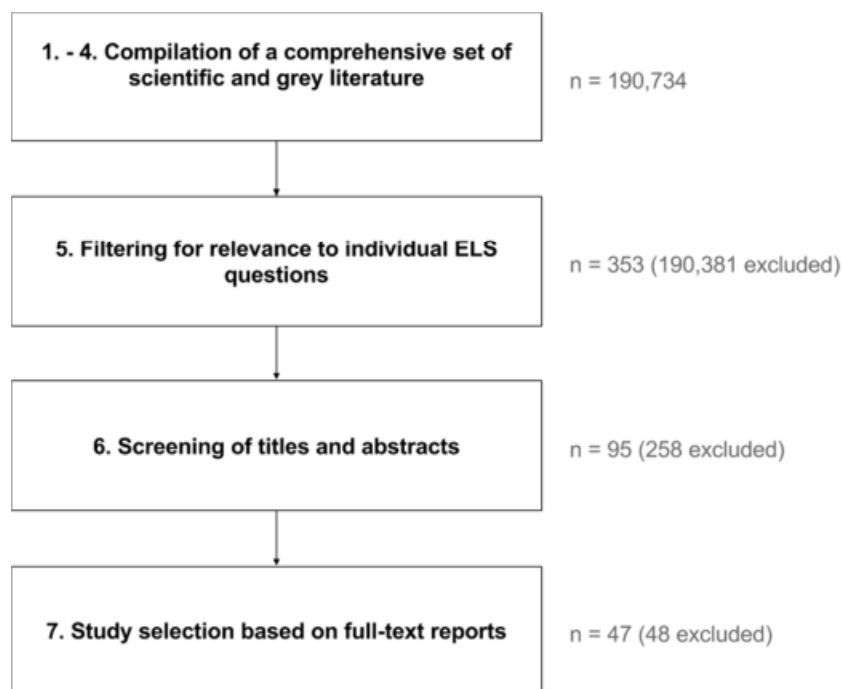
The reasons for exclusion of the majority of articles were i) completely false positives, often due to the term "[snail](#)", ii) unrelated articles concerning molluscs biology but not directly the studied mechanisms. Molluscs are a vector for schistosomal infections, hence there was a large number of literature on schistosomal infections without a mechanistic relationship to the studied pathways.

Complete set of inclusion/exclusion criteria applied in the screening of titles and abstracts is documented in the following Table 10.

Table 10 Inclusion/exclusion criteria applied in the screening of titles and abstracts.

Inclusion criteria	Exclusion criteria
Population	
Studies describing the processing of dsRNA and/or miRNA in molluscs	Studies describing the processing of dsRNA and/or miRNA in other organisms
Outcome	
Processing mechanisms of dsRNA and/or miRNA are described	
Study design	
All study designs are eligible	
Geography	
Studies from all countries are eligible	
Recency	
All studies are eligible regardless of the publication date	
Language	
No languages are excluded	
Publication type	
Peer-reviewed original research articles	Reviews, news, letters, brief communications, communications arising, technical reports, project reports, non-scientific media publications (newspapers, popular magazines, etc.), patents

The following Figure 12 provides statistics of included and excluded studies in each step of the study selection process.

Figure 12 Statistics of included and excluded studies concerning dsRNA and miRNA processing in molluscs.

2.2.3.6 *Annelids*

ELS questions

How is dsRNA and miRNA processed in annelids?

What is the function of all the different components of the silencing pathways in annelids?

Which dsRNA and miRNA processing mechanisms and pathway components are unique to annelids?

Which pathway components are required for the normal functioning of a specific pathway in annelids?

Keywords / phrases used for filtering the comprehensive set of scientific and grey literature related to RNA interference and dsRNA and miRNA pathways:

To select relevant entries concerning dsRNA and miRNA pathways in annelids, we used the following combinations of keywords to select from the main database annelid-related entries:

[Annelid](#) OR [earthworm](#) OR [polychaete](#) OR [oligochaete](#) OR [spirobranchus](#) OR [pomatoceros](#) OR [pristina](#) OR [helobdella](#) OR [platynereis](#) OR [hydroides](#) OR [enchytraeus](#) OR [myzostoma](#) OR [myzostomida](#) OR [Sipuncula](#) OR [capitella](#) OR [chaetopterus](#)

Justification of the selection of taxon-specific keywords: Among the Annelid species, none of is a truly common experimental model system, *Platynereis* is occasionally being used for evolutionary embryonic development studies. No Annelid genome is available through the UCSC Genome Browser, although seven species have genomes sequenced according to NCBI (*Helobdella robusta* and *Capitella teleta*). There are only one Annelid species having annotated miRNAs in the current edition (21) of miRBase (*Capitella teleta*).

Results

The initial filtering of the comprehensive set of scientific and grey literature using the above listed taxon-representing keywords yielded 51 entries. These were screened individually (title and abstract) for relevance to the review questions.

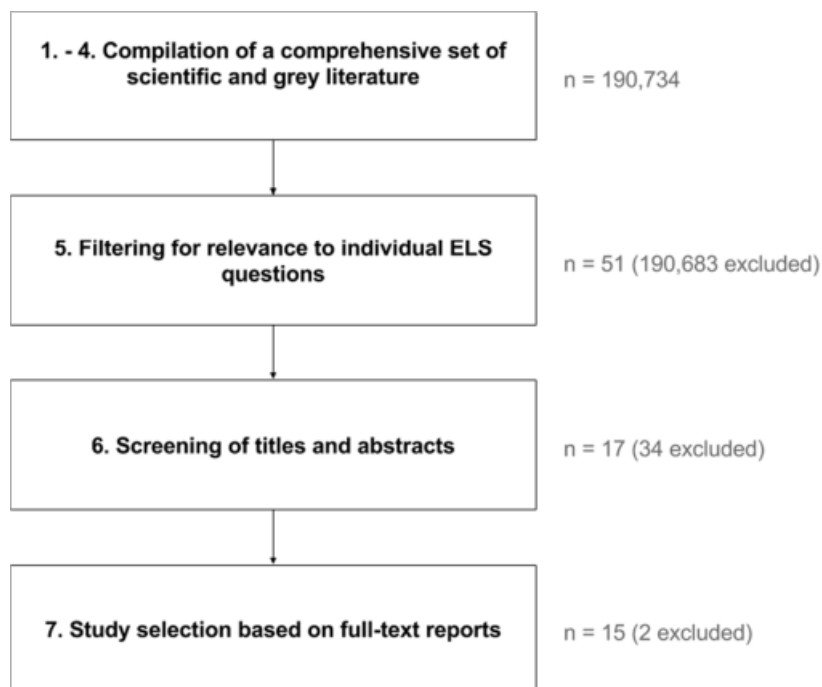
Upon screening of titles and abstracts and manual annotation, 17 entries were retained as directly relevant to the review questions.

Complete set of inclusion/exclusion criteria applied in the screening of titles and abstracts is documented in the following Table 11.

Table 11 Inclusion/exclusion criteria applied in the screening of titles and abstracts.

Inclusion criteria	Exclusion criteria
Population	
Studies describing the processing of dsRNA and/or miRNA in annelids	Studies describing the processing of dsRNA and/or miRNA in other organisms
Outcome	
Processing mechanisms of dsRNA and/or miRNA are described	
Study design	
All study designs are eligible	
Geography	
Studies from all countries are eligible	
Recency	
All studies are eligible regardless of the publication date	
Language	
No languages are excluded	
Publication type	
Peer-reviewed original research articles	Reviews, news, letters, brief communications, communications arising, technical reports, project reports, non-scientific media publications (newspapers, popular magazines, etc.), patents

The following Figure 13 provides statistics of included and excluded studies in each step of the study selection process.

Figure 13 Statistics of included and excluded studies concerning dsRNA and miRNA processing in annelids.

2.2.3.7 *Nematodes*

ELS questions

How is dsRNA and miRNA processed in fish?

What is the function of all the different components of the silencing pathways in nematodes?

Which dsRNA and miRNA processing mechanisms and pathway components are unique to nematodes?

Which pathway components are required for the normal functioning of a specific pathway in nematodes?

Keywords / phrases used for filtering the comprehensive set of scientific and grey literature related to RNA interference and dsRNA and miRNA pathways:

To select relevant studies concerning dsRNA and miRNA pathways in nematodes, the following combinations of keywords were used to select nematodes-related studies from the comprehensive set of literature compiled earlier:

Nematodes OR nematode OR roundworm OR *Caenorhabditis* OR *c. elegans* OR *ancylostoma* OR *angiostrongylus* OR *anisakis* OR *ascaris* OR *ascaris* OR *brugia* OR *bursaphelenchus* OR *cylicostephanus* OR *dictyocaulus* OR *dirofilaria* OR *ditylenchus* OR *dracunculus* OR *elaeophora* OR *enterobius* OR *globodera* OR *gongylonema* OR *haemonchus* OR *heligmosomoides* OR *heterodera* OR *heterorhabditis* OR *meloidogyne* OR *necator* OR *nippostrongylus* OR *oesophagostomum* OR *onchocerca* OR *oscheius* OR *panagrellus* OR *parascaris* OR *parastrongyloides* OR *pristionchus* OR *rhabditophanes* OR *romanermis* OR *rotylechulus* OR *soboliphyme* OR *steinernema* OR *strongyloides* OR *strongylus* OR *subanguina* OR *syphacia* OR *thelazia* OR *toxocara* OR *trichinella* OR *trichuris* OR *wuchereria*

Justification of the selection of taxon-representing keywords: Among the Nematode species, *Caenorhabditis elegans* clearly stands out as the key model organism. However, there are numerous members of the phylum, which are pathogens and, therefore, medically important. Six nematode genomes (5 of the genus *Caenorhabditis*) are available through the UCSC Genome Browser. In fact, there are 81 nematode species (46 genera) that have genomes sequenced according to NCBI. There are ten Nematode species (4 of the *Caenorhabditis* genus) having annotated miRNAs in the current edition (21) of miRBase.

Results

The initial filtering of the comprehensive set of scientific and grey literature using the above listed keywords yielded 5,324 entries. Studies passed on to the screening of titles and abstracts were further limited to studies that include at least one of the following keywords in the title or abstract: miRNA, microRNA, RNAi, siRNA, Dicer, Argonaute or dsRNA. This filtering led to 2,890 entries, of which 575 were screened for relevance (title and abstract). Studies were selected for screening for relevance to the ELS questions based on the keywords listed in the following Table 12 showing the statistics of included and excluded studies – if a keyword listed in the table was present in the title or abstract, the paper was selected for screening for relevance.

Table 12 Breakdown of studies screened (titles and abstracts) for relevance to ELS questions - nematodes.

	included	excluded		total screened
		no description of dsRNA or miRNA processing	not relevant for nematodes	
TOTAL	387	158	30	575
DGCR8	2	0	0	2
Drosha	3	0	0	3
TRBP	21	0	0	21
TARBP2	26	12	0	38
TRBP2	2	0	0	2
dsRBP	2	0	0	2
dsRBD	121	29	3	153
PACT	26	0	0	26
Dicer	147	5	102	254
Argonaut*	41	21	1	63
AGO1	11	0	0	11
AGO2	28	0	8	36
AGO3	4	0	2	6
AGO4	51	33	4	88
PAZ	11	0	0	11
PIWI	1	0	0	1
GW182	11	0	8	19
TNRC6	33	12	1	46
LIN28	186	89	9	284
RdRP	175	49	12	236
RNAi	247	100	12	359
microRNA	130	44	7	181
siRNA	17	4	0	21
dsRNA	1	0	0	1
ADAR	6	2	0	8
PKR	0	0	0	0
RIG-I	0	1	0	1
MDA5	387	158	30	575
OAS	2	0	0	2

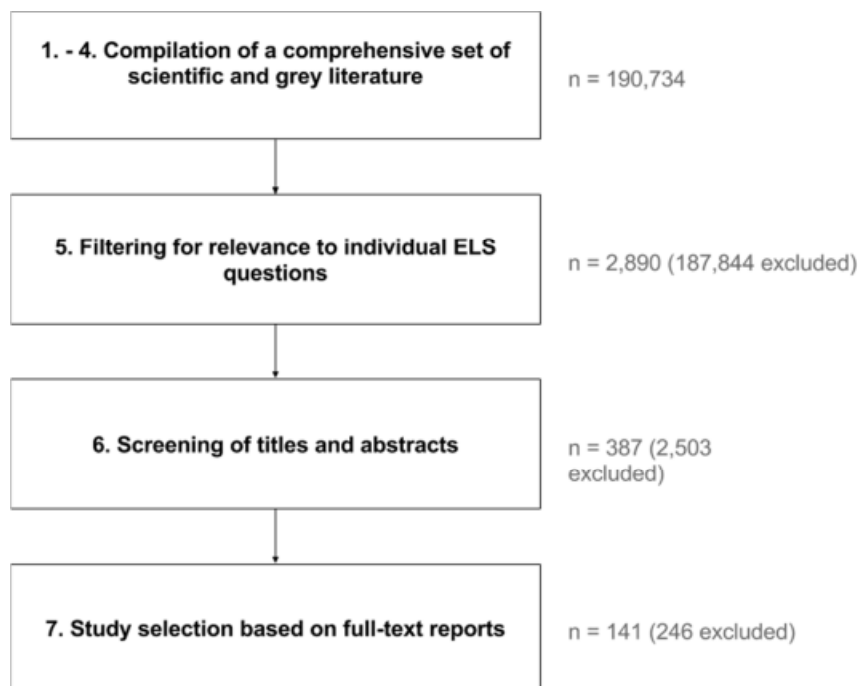
Upon screening of titles and abstracts and manual annotation, 387 entries were retained as directly relevant for the review questions.

The set of inclusion/exclusion criteria applied in the screening of titles and abstracts is documented in the following Table 13.

Table 13 Inclusion/exclusion criteria applied in the screening of titles and abstracts.

Inclusion criteria	Exclusion criteria
Population	
Studies describing the processing of dsRNA and/or miRNA in nematodes	Studies describing the processing of dsRNA and/or miRNA in other organisms
Outcome	
Processing mechanisms of dsRNA and/or miRNA are described	
Study design	
All study designs are eligible	
Geography	
Studies from all countries are eligible	
Recency	
All studies are eligible regardless of the publication date	
Language	
No languages are excluded	
Publication type	
Peer-reviewed original research articles	Reviews, news, letters, brief communications, communications arising, technical reports, project reports, non-scientific media publications (newspapers, popular magazines, etc.), patents

The following Figure 14 provides statistics of included and excluded studies in each step of the study selection process.

Figure 14 Statistics of included and excluded studies concerning dsRNA and miRNA processing in nematodes.

2.2.3.8 *Plants*

ELS questions

How is dsRNA and miRNA processed in plants?

What is the function of all the different components of the silencing pathways in plants?

Which dsRNA and miRNA processing mechanisms and pathway components are unique to plants?

Which pathway components are required for the normal functioning of a specific pathway in plants?

Keywords / phrases used for filtering the comprehensive set of scientific and grey literature related to RNA interference and dsRNA and miRNA pathways:

To select relevant studies concerning dsRNA and miRNA pathways in plants, the following combinations of keywords were used to select plants-related studies from the comprehensive set of literature compiled earlier:

[plants](#) OR [plant](#) OR [flower](#) OR [tree](#) OR [leaf](#) OR [Arabidopsis](#) OR [nicotiana](#) OR [oryza](#) OR [zea mays](#)

Justification of the selection of taxon-representing keywords: Among the plants, one stands out as a dominant experimental system: *Arabidopsis thaliana* (thale cress). The plant species list was expanded with three more taxons: genus *Nicotiana*, another dicotyledon species, which is occasionally used as a laboratory model, especially as a model for viral infections, and two monocotyledon species: *Oryza* (rice) and *Zea mays* (corn). Rice is of undisputed economical importance and it is also the leading plant species in miRBase in the number of annotated miRNAs (which is an indirect indicator of how intense is its research of small RNAs). Corn has very well-annotated miRNAs and, importantly, it is a common epigenetic and transposable element model since 1950's. Leaf and flower were included because those are anatomical parts specific for the reviewed taxonomic group – the plant kingdom.

Results

The initial filtering of the comprehensive set of scientific and grey literature using the above listed keywords yielded 16,590 entries. Studies passed on to the screening of titles and abstracts were further limited to studies that include at least one of the following keywords in the title or abstract: miRNA, microRNA, RNAi, siRNA, Dicer, Argonaute or dsRNA. This filtering led to 12,841 entries. 1,462 entries were screened for relevance (title and abstract). Studies were selected for screening for relevance to the ELS questions based on the keywords listed in the following Table 14 showing the statistics of included and excluded studies – if a keyword listed in the table was present in the title or abstract, the paper was selected for screening for relevance.

Table 14 Breakdown of studies screened (titles and abstracts) for relevance to ELS questions - plants.

	included	excluded		total screened
		no description of dsRNA or miRNA processing	not relevant for plants	
TOTAL	805	591	94	1490
DGCR8	3	0	2	5
Drosha	19	0	5	24
TRBP	5	4	2	11
TARBP2	0	0	0	0
TRBP2	0	4	0	4
dsRBP	6	2	0	8
dsRBD	10	5	1	16
PACT	1	0	1	2
Dicer	445	212	52	709
Argonaut*	372	158	53	583
AGO1	145	39	9	193
AGO2	53	8	1	62
AGO3	9	0	1	10
AGO4	47	14	2	63
PAZ	13	0	2	15
PIWI	45	6	4	55
GW182	2	0	1	3
TNRC6	2	0	0	2
LIN28	0	0	0	0
RdRP	31	106	4	141
RNAi	175	120	16	311
microRNA	420	211	28	659
siRNA	585	384	41	1010
dsRNA	233	186	17	436
ADAR	4	2	0	6
PKR	3	2	0	5
RIG-I	6	2	0	8
MDA5	0	0	0	0
OAS	0	3	0	3

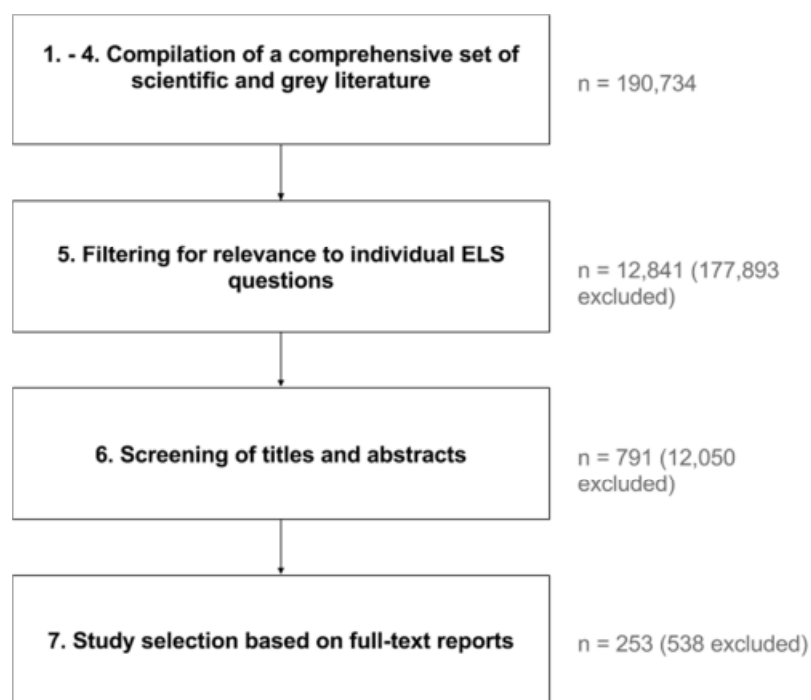
Upon screening of titles and abstracts and manual annotation, 805 entries were retained as directly relevant for the review questions.

The set of inclusion/exclusion criteria applied in the screening of titles and abstracts is documented in the following Table 15.

Table 15 Inclusion/exclusion criteria applied in the screening of titles and abstracts.

Inclusion criteria	Exclusion criteria
Population	
Studies describing the processing of dsRNA and/or miRNA in plants	Studies describing the processing of dsRNA and/or miRNA in other organisms
Outcome	
Processing mechanisms of dsRNA and/or miRNA are described	
Study design	
All study designs are eligible	
Geography	
Studies from all countries are eligible	
Recency	
All studies are eligible regardless of the publication date	
Language	
No languages are excluded	
Publication type	
Peer-reviewed original research articles	Reviews, news, letters, brief communications, communications arising, technical reports, project reports, non-scientific media publications (newspapers, popular magazines, etc.), patents

The following Figure 15 provides statistics of included and excluded studies in each step of the study selection process.

Figure 15 Statistics of included and excluded studies concerning dsRNA and miRNA processing in plants.

2.2.4. Part II: Potential for non-target gene regulation by dsRNA-derived siRNAs or miRNAs

What is the minimal sequence complementarity needed between target RNA and small RNA (siRNA or miRNA) to trigger repression of gene expression?

Which parameters influence the minimal sequence complementarity needed between target RNA and small RNA to trigger repression of gene expression?"

To what extent can mismatches between target RNA and small RNA (siRNA or miRNA) be tolerated while still allowing efficient silencing?

Which parameters influence efficient silencing through interaction between target RNA and small RNA (siRNA or miRNA)?

Keywords / phrases used for filtering of the comprehensive set of scientific and grey literature to select studies related to base pairing between a small RNA and its target:

The following combinations of keywords were used to select studies relevant for the ELS questions from the comprehensive set of literature compiled earlier:

- *target* AND prediction
- *target* AND recognition
- *target* AND off-target*
- *target* AND seed
- *target* AND siRNA AND complementar*
- *target* AND siRNA AND ("base pair*" OR basepair*)
- *target* AND miRNA AND complementar*
- *target* AND miRNA AND ("base pair*" OR basepair*)

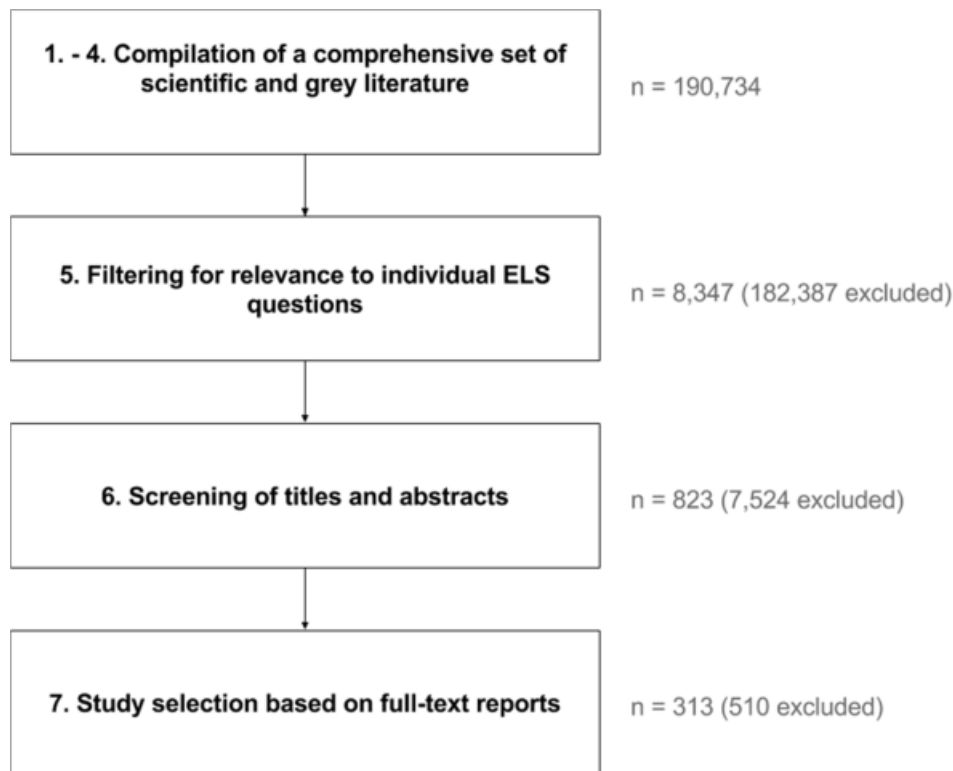
Results

The initial filtering of the comprehensive set of scientific and grey literature using the above listed keywords yielded 8,347 entries which were passed on to the screening of titles and abstracts. Upon screening of titles and abstracts, 823 entries were retained as relevant for the ELS questions. The set of inclusion/exclusion criteria applied in the screening of titles and abstracts is documented in the following Table 16 .

Table 16 Inclusion/exclusion criteria applied in the screening of titles and abstracts.

Inclusion criteria	Exclusion criteria
Population	
Studies describing minimal sequence complementarity (or parameters influencing efficient silencing through a small RNA-target RNA interaction) needed between target RNA and small RNA (siRNA or miRNA) to trigger repression in humans, mammals, birds, fish, arthropods, molluscs, annelids, nematodes and plants	Studies describing minimal sequence complementarity (or parameters influencing efficient silencing through a small RNA-target RNA interaction) needed between target RNA and small RNA (siRNA or miRNA) to trigger repression in other organisms
Outcome	
Minimal sequence complementarity (or parameters influencing efficient silencing through a small RNA-target RNA interaction) needed between target RNA and small RNA (siRNA or miRNA) to trigger repression gene expression is described	
Study design	
All study designs are eligible	
Geography	
Studies from all countries are eligible	
Recency	
All studies are eligible regardless of the publication date	
Language	
No languages are excluded	
Publication type	
Peer-reviewed original research articles, reviews	News, letters, brief communications, communications arising, technical reports, project reports, non-scientific media publications (newspapers, popular magazines, etc.), patents

Of the 823 included papers after the screening of titles and abstracts, 313 were used in the compilation of this report (included after investigation of the fulltext reports). The following Figure 16 provides statistics of included and excluded studies in each step of the study selection process.

Figure 16 Statistics of included and excluded studies.

Based on the findings of the comprehensive literature search on sequence complementarity between target RNA and small RNA, it was assessed to which extent the publicly available bioinformatic programs take into account the knowledge on sequence complementarity required to predict a silencing effect.

2.2.5. Part III: siRNA pools in plant tissues and importance of individual siRNAs

The comprehensive set of scientific and grey literature compiled according to the methodology described in section 2.2.1. (Figure 7) contains over 190,000 references. To reduce the quantity of references to a manageable number suitable for screening of titles and abstracts by experts of the project team, filtering using keywords specific to ELS questions corresponding to individual review topics was carried out. The ELS questions and the review topics were designed to address the questions posed by EFSA in the tender specifications.

ELS questions

What is the current methodology used to determine the dsRNA-derived siRNA pool in plant tissues?

What are possible advantages and biases of each method used to determine the dsRNA-derived siRNA pool in plant tissues?

What parameters influence the siRNA pools in different plant tissues?

When and how siRNA, dsRNA and miRNA move between different cells and tissues?

Keywords / phrases used for filtering of the comprehensive set of scientific and grey literature to select studies related to ELS questions:

The following combinations of keywords were used to select studies relevant for the ELS questions from the comprehensive set of literature compiled earlier:

(rice OR "oryza sativa" OR "o. sativa" OR maize OR "zea mays" OR "z.mays" OR tobacco OR "nicotiana tabacum" OR "n. tabacum" OR "arabidopsis thaliana" OR "a. thaliana" OR soy OR soybean OR "glycine max" OR "g. max") AND (mobil* OR systemic OR xenogenic OR exogenous OR population OR spread OR pool OR movement OR availability OR distribution)

Justification of selection of the keywords:

Rice, maize, tobacco, soybean and *Arabidopsis thaliana* are the key model organisms for the study of dsRNA-derived siRNA pool in plant tissues. Common as well as Latin names of these model organisms were therefore used as keywords for filtering of the comprehensive set of scientific and grey literature compiled earlier. These keywords were combined with keywords related to pools or mobility and their synonyms.

Results

The initial filtering of the comprehensive set of scientific and grey literature using the above listed keywords yielded 1,824 entries which were passed on to the screening of titles, abstracts and fulltext reports. Upon screening of titles, abstracts and fulltext reports, 160 entries were retained as relevant for the ELS questions. The set of inclusion/exclusion criteria applied in the screening of titles, abstracts and fulltext reports is documented in the following Table 16 .

Table 17 Inclusion/exclusion criteria applied in the screening of titles, abstracts and fulltext reports.

Inclusion criteria	Exclusion criteria
Population	
Studies describing methodology used to determine the dsRNA-derived siRNA pool and parameters influencing the siRNA pools in different plant tissues	Studies describing methodology used to determine the dsRNA-derived siRNA pools and parameters influencing the siRNA pools in different other organisms
Outcome	
methodology used to determine the dsRNA-derived siRNA pool in plant tissues and/or parameters influencing the siRNA pools in different plant tissues is described	
Study design	
All study designs are eligible	
Geography	
Studies from all countries are eligible	
Recency	
All studies are eligible regardless of the publication date	
Language	
No languages are excluded	
Publication type	
Peer-reviewed original research articles	Reviews, News, letters, brief communications, communications arising, technical reports, project reports, non-scientific media publications (newspapers, popular magazines, etc.), patents

The 160 included papers were used in compilation of this report. The following Figure 17 provides statistics of included and excluded studies in each step of the study selection process.

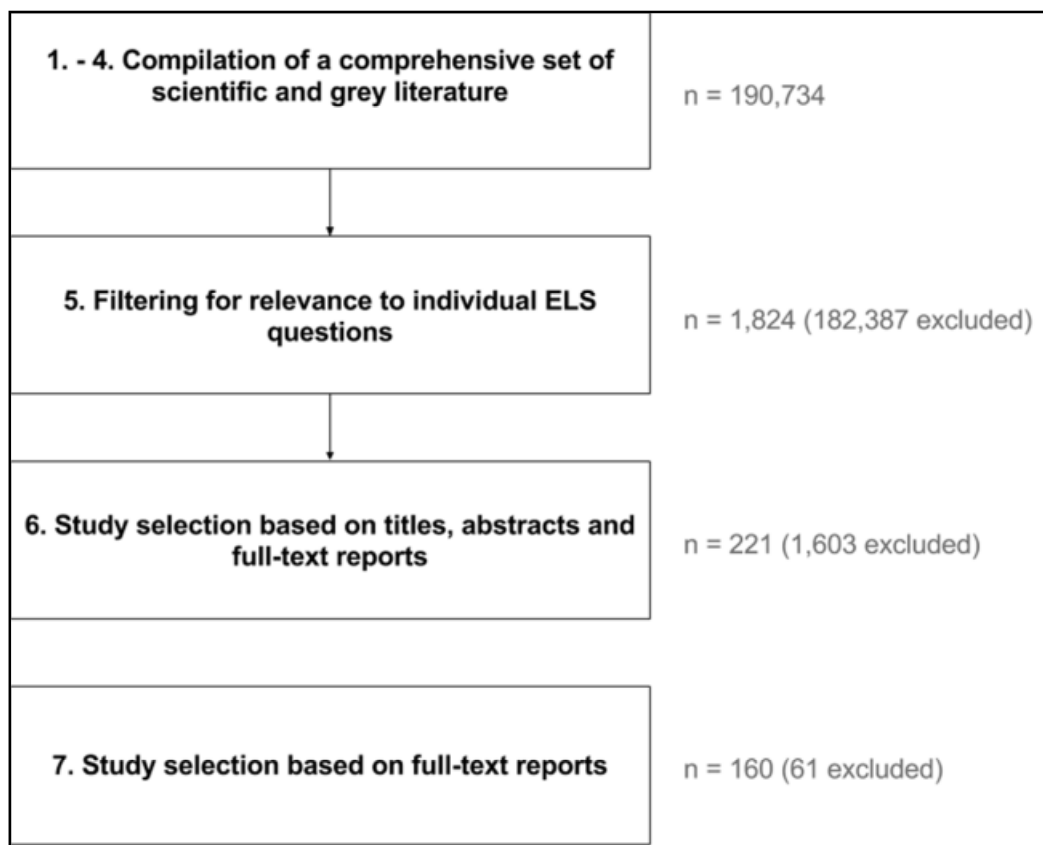


Figure 17 Statistics of included and excluded studies.

The 160 included studies were used for compilation of sections 3.3.1, 3.2.2 and 3.3.3 of this report addressing the review questions. In addition, a distinct section 3.3.4. dedicated to an intensively debated article (Zhang et al., 2012a) was prepared after a literature search using this paper as a pearl in the citation pearl growing strategy in Web of Science and subsequent screening of retrieved references.

3. Assessment/Results

3.1. Part I Mode-of-action of dsRNA and miRNA pathways

This part provides a detailed description of dsRNA and miRNA pathways in mammals (including humans), birds, fish, arthropods, annelids, molluscs, nematodes, and plants and describes functions of components of these pathways.

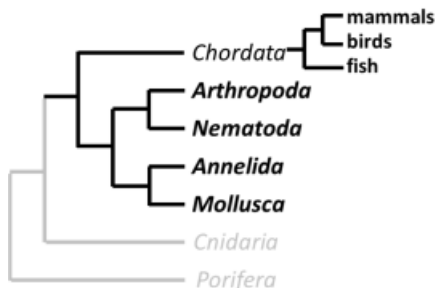


Figure 18 Simplified phylogenetic overview of the reviewed animal taxa.

In bold face are shown animal exons covered in this report: three groups (classes) of the phylum *Chordata*, which belong to *Deuterostomia*. *Protostomia* are represented by four phyla – arthropods and nematodes (belonging to the group *Lophotrochozoa*) and annelids and mollusc (belonging to the group *Ecdysozoa*).

We performed a comprehensive and systematic search of four major information databases, which yielded ~ 190 000 non-redundant records related to the area of interest, and collected into a custom-made searchable database. Subsequent hierarchical taxon-oriented and molecular mechanism-oriented filtering lead to selection of ~10,000 publications, of which titles and abstracts were individually inspected, classified and exported into eight taxon-specific files, which were further managed in the Endnote reference management system. In total, ~4600 non-redundant references were managed in eight files, which contained relevant references for mammals (including humans), birds, fish, arthropods, annelids, molluscs, nematodes, and plants. Records in the Endnote reference system were further filtered according to relevance for specific sections and subsections of the report. In total, the refined selection yielded cumulative ~1400 references in eight taxon-dedicated chapters.

The text focuses in particular on pathways utilizing small RNAs as sequence-specific guides for repression, namely RNAi, which is triggered by long dsRNA, and miRNA, which employs genome-encoded small RNAs released from short hairpin precursors. In addition, dsRNA pathways, which cross-talk with RNAi are also discussed, in particular the interferon response. Simplified overview of the reviewed components is shown below (Figure 19).

This part is divided into eight chapters (mammals (including humans), birds, fish, arthropods, annelids, molluscs, nematodes, and plants). The molecular components and mechanisms are reviewed in a taxon-specific manner. Each taxon-specific chapter is uniformly organized: a brief taxon introduction is followed by the key protein components of miRNA and RNAi pathways. Particular attention is paid to the essential key factors: Dicer and Argonaute proteins. Next, we focus on mechanistic aspects of the molecular mechanisms that employ protein factors. Particular attention is paid to miRNA and RNAi pathways and nuclear silencing in mammals and plants. Finally, additional dsRNA-responding factors and pathways are reviewed, paying particular attention to the innate immunity system known as the interferon response and to the RNA editing mediated by adenosine deaminases that act on RNA.

Our review reveals conserved and divergent aspects of small RNA pathways and dsRNA response in animals and plants. Importantly, it seems that dsRNA and miRNA pathways could be divided into four interconnected modules, which were dynamically interacting and changing during the course of the last 300 million years. There are three sequence-specific modules and there is one heterogeneous sequence-independent module:

sequence-dependent modules

- **miRNA module** – post-transcriptional regulations, which are guided by individual genome-encoded small RNAs loaded on Argonaute proteins. The key factor is that there is a [genome-encoded population of small RNAs with an identical defined sequence](#).

- **RNAi module** – degradation of RNAs by short interfering RNA pools generated from long dsRNA. The common theme is that small RNAs come as **populations of RNAs with variable sequences**.
- **transcriptional silencing module** – small RNAs loaded on Argonaute proteins **acting in the nucleus and changing chromatin structure** (DNA methylation/histone modifications). The classical example is small RNA-induced DNA methylation in plants.

sequence-independent module

sequence-independent response to dsRNA such as the interferon response and adenosine deamination. Despite the heterogeneity, the characteristic feature of the module is **coupling of a dsRNA sensor protein with sequence-independent functions**, which are coupled with signalling (e.g. innate immunity) or RNA metabolism (e.g. A-to-I editing).

Taken together, this work provides a systematic and thorough assessment of dsRNA and miRNA pathways in two eukaryotic kingdoms and can serve as a foundation for dsRNA or miRNA-related actions.

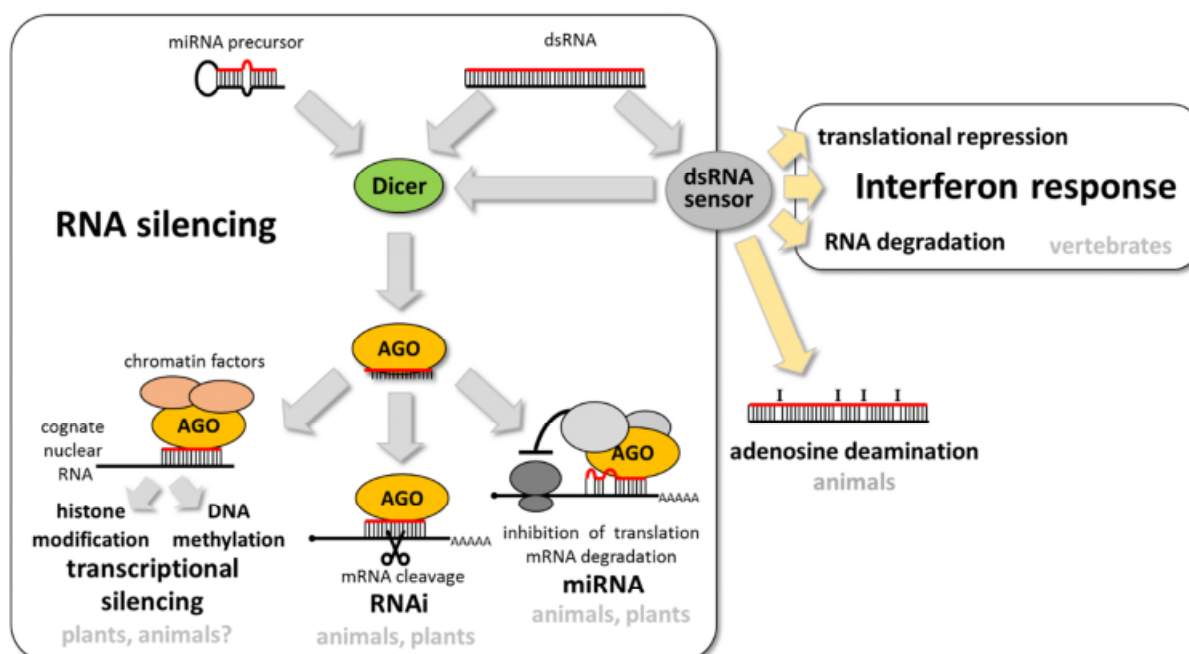


Figure 19 Overview of key dsRNA and miRNA pathways

Shown is a simplified overview of the reviewed pathways. On the left is a block of three sequence-specific pathways: **miRNA**, **RNAi**, and **small RNA-mediated transcriptional silencing**. RNAi and miRNA are found in all reviewed taxa, transcriptional silencing is well documented in some (plants, *C. elegans*) but unknown or unclear in other taxa. On the right are sequence-independent mechanisms generally known as the **interferon response** in vertebrates (components of the system are also found in invertebrates). The last mechanism displayed here is **adenosine deamination**, which is present in all animals and chemically modifies dsRNA, thereby degrading its structure. The sensor is a dsRNA binding protein, which directs dsRNA into a specific pathway. Sensors often have their own enzymatic activities – kinase, helicase, oligoadenylate synthetase, adenosine deaminase, which are integrated into signalling and RNA metabolism pathways.

3.1.1. Mammals

Mammals belong (with birds and fishes) to the group *Craniata* of the phylum *Chordata*. Mammals are homeothermic animals distinguished by mammary glands, hair, middle ear bones, and neocortex. There are ~4500 extant mammalian species, most of which are placental mammals. The synapsid animal lineage leading to mammals branched of the sauropsid lineage leading to dinosaurs and birds over 300 million years ago (MYA). The miRNA, RNAi and other dsRNA-responding pathways in mammals are intensely studied and will be used in this report to define the key principles relevant for all related pathways in Eukaryotes. The general principle of organization of each section is to first define molecular components of a pathway and then review key functional implications. The first pathway reviewed in this section is the miRNA pathway (Figure 20).

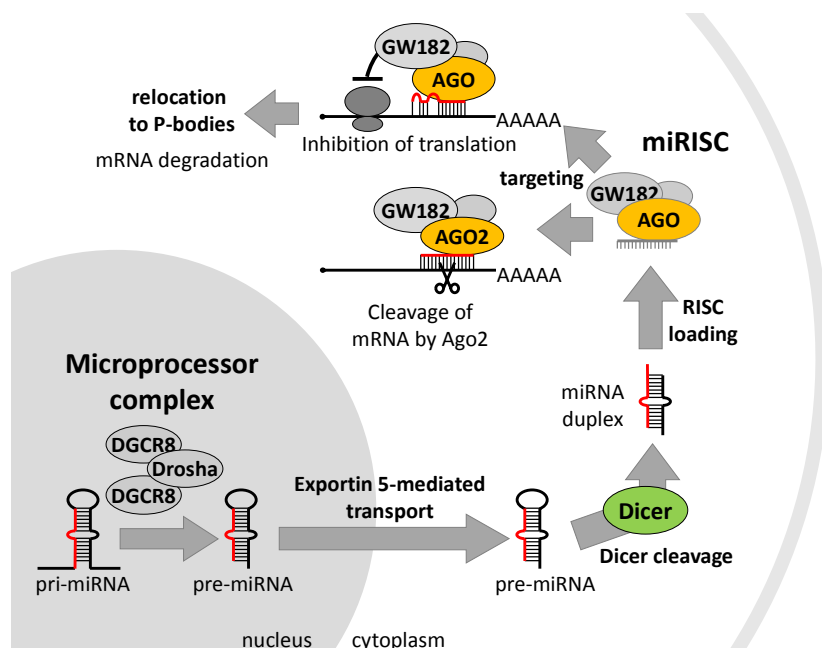


Figure 20 Overview of the mammalian miRNA pathway

3.1.1.1 Microprocessor complex – nuclear initiation of miRNA processing

The canonical primary miRNAs (non-canonical precursors are described separately further below) are transcribed by polymerase II (polymerase III-transcribed miRNA precursors are uncommon (Borchert et al., 2006; Canella et al., 2010)) and carry local hairpins, which are released in the nucleus as stem-loop precursors of approximately 70 nucleotides (pre-miRNAs) by the activity of the so-called Microprocessor complex, which is composed of RNase III Drosha and DGCR8 proteins (Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004).

Drosha

Drosha, the active component of the Microprocessor complex, was discovered in 2000 as a new member of RNase III in *Drosophila* with a conserved human homolog (Filippov et al., 2000). Structurally, Drosha protein carries a single C-terminal dsRNA-binding motif (dsRBM), tandem catalytic domains, a proline-rich region (PRR) and an RS domain (Fortin et al., 2002). Drosha was recognized as the nuclease that executes the initiation step of miRNA processing in the nucleus (Lee et al., 2003) and the Microprocessor complex was reported in a series of papers in 2004 (Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). The current understanding of the Microprocessor complex includes the resolved X-ray structure of Drosha with the C-terminal helix of DGCR8 (Kwon et al., 2016). Drosha contains two DGCR8-binding sites, one on each RNase III domain, which mediate the assembly of the Microprocessor complex. The overall structure of Drosha is similar to that of Dicer (see further below) despite no sequence homology except of the C-terminal part. This suggests that Drosha could have evolved from a Dicer homolog and would be consistent with the proposed classification as a non-canonical Dicer in a single RNase III family (Jaskiewicz and Filipowicz, 2008). In

addition, Drosha exhibits unique features, including non-canonical zinc-finger motifs, a long insertion in the first RIIID, and a kinked link between a Connector helix and RIIID explaining the 11-bp-measuring "ruler" activity of Drosha. The structural analysis is consistent with biochemical characterization of the complex, which suggested that the Microprocessor complex exists as a heterotrimeric complex (Nguyen et al., 2015; Herbert et al., 2016). In addition, Drosha and DGCR8, respectively, recognize the basal UG and apical UGU motifs in pri-miRNAs, which determines orientation of the complex (Nguyen et al., 2015). These results clarified inconsistencies and unknowns existing in the earlier literature regarding the stoichiometry of the complex and the mode of binding (Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004; Gregory et al., 2006; Ma et al., 2013; Barr and Guo, 2014).

DGCR8

While Drosha provides the catalytic site for cleavage, DGCR8 anchors substrate pri-miRNAs. A crystal structure of the human DGCR8 core (residues 493-720) showed two double-stranded RNA-binding domains (dsRBDs) arranged with pseudo two-fold symmetry tightly packed against the C-terminal helix (Sohn et al., 2007). Interestingly, DGCR8 uses heme as a co-factor. DGCR8 contains a previously uncharacterized heme-binding motif that is also required for its activity. Heme availability and biosynthesis in HeLa cells positively affect pri-miRNA processing and production of mature miRNAs while heme-binding-deficient DGCR8 mutants are defective in pri-miRNA processing (Barr and Guo, 2014; Weitz et al., 2014; Barr et al., 2015). Pri-miRNA recognition by DGCR8 seems to involve N(6)-methyladenosine mark deposited by the methyltransferase-like 3 (METTL3) (Alarcon et al., 2015). DGCR8 is also regulated by phosphorylation. There are 23 possible phosphorylation sites mapped on the full-length human DGCR8 expressed in insect or mammalian cells (Herbert et al., 2013). Subsequent analysis showed that DGCR8 phosphorylation may increase DGCR8 stability but not processing activity in response to extracellular cues (Herbert et al., 2013). DGCR8 phosphorylation by the tyrosine kinase ABL has been observed after DNA damage stimulating the processing of selective primary miRNAs (Tu et al., 2015).

Additional Microprocessor co-factors

Apart from the ABL kinase, several additional co-factors of the Microprocessor complex were identified. One of them is the RNA-Binding Protein DDX1, which appears to be a regulatory protein promoting expression of a subset of miRNAs, majority of which is induced after DNA damage (Han et al., 2014). A peculiar Microprocessor complex component is Methyl-CpG-binding protein MECP2 (Cheng et al., 2014; Tsujimura et al., 2015), which is known to stably bind methylated DNA. According to one report, MECP2 promotes the posttranscriptional processing of particular miRNAs including miR-199a, which stimulates mTOR signalling (the key pathway regulating cell metabolism, growth, and survival) by targeting inhibitors of mTOR signalling (Tsujimura et al., 2015). In contrast, Cheng et al reported that MECP2 binds directly to DGCR8 and interferes with the assembly of the Microprocessor complex, thus affecting gene expression posttranscriptionally via relieving repression of miRNA targets (Cheng et al., 2014).

Microprocessor complex localization and function(s)

The Microprocessor complex shows apparent nuclear compartmentalization. While transiently expressed pri-miRNAs accumulate in nuclear foci with splicing factor SC35 and Microprocessor components, Drosha and DGCR8. (Pawlicki and Steitz, 2008), these foci do not appear to be major sites of pri-miRNA processing, which seems to be coupled to transcription (Pawlicki and Steitz, 2009). This is consistent with live-imaging, which revealed that a large fraction of Microprocessor resides with unspliced pri-miRNAs in close proximity to their genes. This analysis also provided a direct visual evidence that DGCR8 and Drosha are targeted to pri-miRNAs as a preformed complex (Bellemer et al., 2012).

Importantly, literature review identified also reports describing additional roles of the Microprocessor complex and its components beyond miRNA biogenesis although Microprocessor expression seems to

be tuned according to pri-miRNA substrates (Barad et al., 2012). Non-canonical roles of Microprocessor (or Drosha) include: 1) mRNA cleavage (Chong et al., 2010), exemplified by Drosha-dependent cleavage of *Hoxd4* RNA (Phua et al., 2011) or destabilization of *Neurog2* mRNA, which supports neural stem cell maintenance by blocking accumulation of differentiation and determination factors (Knuckles et al., 2012), 2) processing of long non-coding RNAs restricted to the nucleus (Ganesan and Rao, 2008), 3) ribosomal RNA biogenesis (Liang and Crooke, 2011), and 4) cleavage of viral RNA (Shapiro et al., 2014). While immunoprecipitation of the Microprocessor complex followed by next-generation sequencing showed that precursors of canonical miRNAs and miRNA-like hairpins are the major substrates of the Microprocessor complex (Seong et al., 2014), high-throughput sequencing and cross-linking immunoprecipitation (HITS-CLIP) analysis of RNAs bound to DGCR8 suggest that miRNAs may not be the most abundant targets. DGCR8-bound RNAs also comprised several hundred mRNAs, small nucleolar RNAs (snoRNAs), and long noncoding RNAs (Macias et al., 2012). Interestingly, DGCR8-mediated cleavage of snoRNAs was independent of Drosha, indicating participation of DGCR8 in other RNA processing complexes (Macias et al., 2012). One of such complexes is the exosome (an hRRP6-containing nucleolar form), where DGCR8 is essential for its recruitment to snoRNAs and to the human telomerase RNA component (hTR/TERC) (Macias et al., 2015). Thus, DGCR8 acts as an adaptor recruiting the exosome complex to structured RNAs and inducing their degradation. (Macias et al., 2015).

Microprocessor complex crosstalk with other pathways

In terms of a crosstalk with other pathways, it has been established that some miRNA precursors are edited by ADARs (Yang et al., 2006a; Alon et al., 2012; Peng et al., 2012; Vesely et al., 2012; Garcia-Lopez et al., 2013; Vesely et al., 2014; Tomaselli et al., 2015) apparently as early as pri-miRNAs (Bahn et al., 2015; Chen et al., 2015). According to one model, ADAR1 interacts with Drosha and DGCR8 in the nucleus and possibly outcompetes DGCR8 in primary miRNA binding, thus enhancing mature miRNA expression. This appears dependent on ADAR1 editing activity, at least for a subset of targets (Bahn et al., 2015). According to the selective elimination model, miRNAs, such as miR-151, are edited and eliminated by Tudor-SN (a ribonuclease specific to inosine-containing dsRNAs and a reported component of RISC) during mouse preimplantation development (Garcia-Lopez et al., 2013). Similarly, pri-miR-142 editing results in suppression of its processing by Drosha while the edited pri-miR-142 is degraded by Tudor-SN. Consequently, mature miRNA-142 expression substantially increases in ADAR1^{-/-} or ADAR2^{-/-} mice (Yang et al., 2006a). According to the stimulation model, exemplified by miR-497, abundant editing event promotes processing by Drosha of the corresponding pri-miRNA (Vesely et al., 2014).

3.1.1.2 Dicer – cytoplasmic production of miRNA from pre-miRNA

A pre-miRNA produced by the Microprocessor complex is transported to the cytoplasm via Exportin 5 in a RanGTP-dependent manner. The next pre-miRNA processing step is Dicer mediated cleavage, which takes place in the cytoplasm.

Structure of Dicer

The full length mammalian Dicer has not been crystallized yet. The current understanding of the mammalian Dicer structure has thus been inferred from several different sources, which can be divided into four groups:

- (I) Biochemical studies of recombinant Dicer and individual domains (Provost et al., 2002; Zhang et al., 2002; Zhang et al., 2004; Ma et al., 2008; Park et al., 2011).
- (II) The crystal structure of *Giardia intestinalis* Dicer (serving as a comparative scaffold) (MacRae et al., 2006b; MacRae et al., 2007).
- (III) Crystallographic studies on mammalian Dicer fragments (Du et al., 2008; Wilson et al., 2015) or on individual domains (Ma et al., 2004; Takeshita et al., 2007; Tian et al., 2014; Wilson et al., 2015)

(IV) Cryo-EM studies of human Dicer and its complexes with other proteins (Lau et al., 2009; Wang et al., 2009; Lau et al., 2012; Taylor et al., 2013; Wilson et al., 2015).

Dicer is an siRNA-producing RNase III enzyme conserved across eukaryotes (Bernstein et al., 2001). Mammalian Dicer proteins are ~220 kDa multidomain proteins, which are composed of domains ordered from the N- to the C-terminus as follows: N-terminal DEXD and helicase superfamily C-terminal domains, a domain of unknown function DUF283, a PAZ domain, RNase IIIa and RNase IIIb domains, and the C-terminal dsRBD (Figure 21) (Nicholson and Nicholson, 2002). In contrast to the simplest RNase III family members (exemplified by *E. coli* RNase III), which carry only one RNase III domain and dimerize when cleaving dsRNA (Lamontagne et al., 2001; Johanson et al., 2013). Dicer proteins carry two RNase III domains, which form an intramolecular dimer (Zhang et al., 2004).

Giardia Dicer structure revealed spatial organization of the core part of eukaryotic Dicer proteins and explained how Dicer generates small RNAs of specific lengths (MacRae et al., 2006a). This crystal structure then served as a framework for deciphering the structure of other Dicer proteins, including mammalian Dicers.

The front view of the *Giardia* Dicer structure resembles an axe (Figure 21). The blade is formed of an intramolecular duplex of two RNase III domains, which are connected by a bridging domain constituting the back end of the blade. The platform domain is adjacent to the RNase IIIa domain and makes up the upper part of the handle. The PAZ domain is connected by a long helix to the RNase IIIa domain and forms the base of the handle (MacRae et al., 2006b). Altogether, the *Giardia* Dicer is formed of three rigid regions, which are linked by flexible hinges. One region is formed by RNase III domains and the bridging domain, the second by the platform domain and the connector helix, and the third by the PAZ domain. These three parts can swing relative to each other and possibly ensure accommodation of Dicer to the structure of its substrate (MacRae et al., 2006a). This conformational flexibility likely enables binding of dsRNAs with non-canonical base pairing as well as imperfect duplexes of pre-miRNAs (MacRae et al., 2006a). In addition, dsRNA binding is presumably stabilized by several positively charged patches on the surface of *Giardia* Dicer between the processing center and the PAZ domain, which are in contact with dsRNA (MacRae et al., 2006a; MacRae et al., 2007).

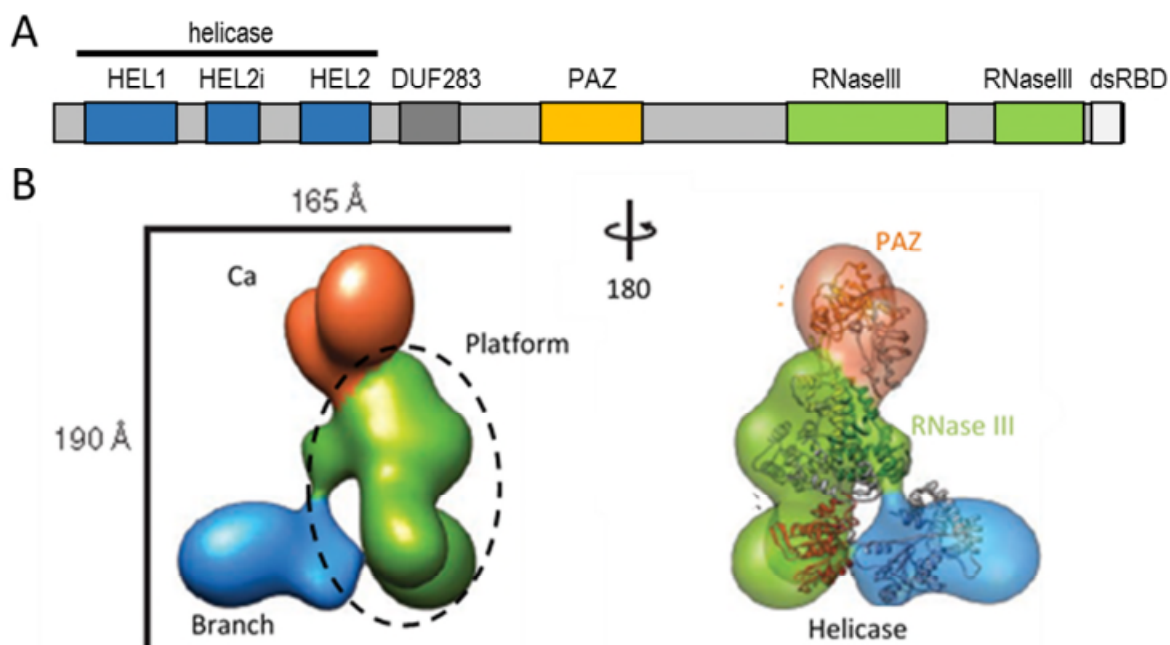


Figure 21 Domain architecture of the mammalian Dicer.

(A) Domain order. Note the position of the HEL2i domain between HEL1 and HEL2 domains as a result of blastp search, which is in contrast with the structural data from RIG-I, which place the HEL2i domain inside the HEL2 domain (Kowalinski et al., 2011; Luo et al., 2011)}. (B) An electron microscopy reconstruction of mammalian Dicer with marked regions (left panel) and several mapped domains (right panel). Images of Dicer structures were adapted from Taylor, 2013 (Taylor et al., 2013).

Mammalian Dicers are much larger and contain domains absent in the *Giardia* Dicer but follow the same organizational and functional principles. The crystal structure of *Giardia* Dicer confirmed an earlier biochemical analysis predicting that the two RNase III domains of the human Dicer form an intramolecular dimer resulting in a single processing center placed at a specific distance from the PAZ domain (Zhang et al., 2004). A structural component defining this distance is an α helix (connector helix), which directly links PAZ and RNase III domains (MacRae et al., 2006b). Thus, the key functional aspect that emerged from Dicer's structural analysis was that it functions as a molecular ruler, measuring the length of the substrate from the PAZ domain to RNase III domains where each domain cleaves one strand. Importantly, the mammalian Dicer (and metazoan Dicers in general) differ from *Giardia*'s in two main aspects. The first is the general topology reflecting the fact that the typical mammalian Dicer product is shorter (21-23 nt). The second one is that the mammalian Dicer contains additional functional domains important for substrate recognition and processing.

As indicated above, the architecture of the human Dicer and positions of its domains and interacting partners have been inferred by cryo-EM of the full length protein and its mutants (Lau et al., 2009; Wang et al., 2009; Lau et al., 2012; Taylor et al., 2013; Wilson et al., 2015). The overall shape of the human Dicer resembles the letter L; the shape is further divided into a head, a body and a base (Figure 3 and 21). The PAZ domain is adjacent to the platform domain in the head of the protein while the RNase IIIb is located in the body. Thus, the head of the human Dicer is a topological equivalent of the base of the handle in *Giardia*'s Dicer. The helicase domain constitutes the base, which has no equivalent in *Giardia*'s Dicer. The position of the processing center relative to the PAZ domains differs between human and *Giardia* Dicers, which explains the fact that the human Dicer produces siRNA about four nucleotides shorter than the *Giardia* Dicer, which corresponds to \sim one-third of a dsRNA helical turn (Lau et al., 2012). Therefore, the processing center has to access the cleavage site of dsRNA from the different angle relative to the dsRNA helical end in comparison with *Giardia* Dicer (Lau et al., 2012).

For understanding substrate selection and processing, two areas of Dicer's structure deserve special attention: the PAZ and the N-terminal domains, which are described below. The following text represents exhaustive literature survey focused on the structural and functional aspects of the two domains.

The PAZ domain

The PAZ domain found in Dicer and Argonaute proteins is a dsRNA-terminus binding module (Ma et al., 2004; MacRae et al., 2006b). The PAZ domain has a 3' overhang binding pocket but only the PAZ domain of Dicer has an extra loop enriched in basic amino acids, changing electrostatic potential and molecular surface of the pocket. These changes may influence RNA binding by Dicer and handing-off the substrate to other proteins complexes (MacRae et al., 2006b). The PAZ domain of metazoan Dicers also recognizes phosphorylated 5' end of a pre-miRNA. A mutation of the 5' binding pocket leads to dysregulation of miRNA biogenesis *in vivo* (Park et al., 2011). The 5' binding pocket is conserved in *Drosophila* DCR-1 and human Dicer but not in *Giardia* Dicer (Park et al., 2011). Importantly, the 5' binding pocket appears conserved in Dicer proteins functioning in miRNA biogenesis (human Dicer, *Drosophila* DCR-1) but not in Dicer proteins dedicated to long dsRNA processing (*Giardia*, *Schizosaccharomyces*, *Drosophila* DCR-2). Accordingly, simultaneous fixing of 3' and 5' ends emerges as a feature important for fidelity of miRNA biogenesis but not for siRNAs (Park et al., 2011).

The N-terminal helicase domain

The N-terminus of metazoan Dicers harbours a complex helicase structure, which is adjacent to RNase III catalytic domains (Lau et al., 2012). Although the helicase must come into contact with the substrate, its functional significance is still only partially understood. However, it is clear that the N-

terminal helicase region is the key for the substrate preference. In mammals (and in most metazoan phyla), a single gene encodes Dicer, which has to process both: miRNA precursors into miRNAs as well as long double-stranded RNAs into small interfering RNAs (siRNAs). Different taxons apparently differ in how much they employ both types of Dicer activities. As we will describe here, the mammalian Dicer is mainly dedicated to the miRNA pathway while its natural production is very limited.

The N-terminal helicase belongs to the RIG-I-like helicase family (Zou et al., 2009) and consists of a proximal DExD/H domain and an adjacent helicase superfamily c-terminal domain (Figure 21). A conventional helicase domain has an ATPase activity. Indeed, invertebrate Dicers bind and hydrolyze ATP (Zamore et al., 2000; Bernstein et al., 2001; Ketting et al., 2001; Nykanen et al., 2001). However, despite the N-terminal helicase with conserved motifs important for ATP binding and hydrolysis is present in mammalian Dicers, there is no evidence of ATP requirement for the human Dicer activity (Provost et al., 2002; Zhang et al., 2002). The human Dicer has the same processing efficiency in the presence or absence of ATP. Moreover, the rate of cleavage is not influenced by addition of other nucleotides, non-cleavable ATP analogues or a mutation in the Walker A motif of ATPase/helicase domain (Provost et al., 2002; Zhang et al., 2002). Notably, these experiments were performed using a long dsRNA substrate with blunt ends, whose processing by invertebrates Dicers is ATP-dependent (Zamore et al., 2000; Bernstein et al., 2001; Ketting et al., 2001; Nykanen et al., 2001; Zhang et al., 2002). Remarkably, deletion of the helicase domain results in high cleavage rate of long dsRNAs by human Dicer *in vitro* (Ma et al., 2008) as well as *in vivo* in murine and human cells (Flemer et al., 2013; Kennedy et al., 2015). Thus, the N-terminal helicase in mammalian Dicers has a different role in substrate recognition and processing than the helicase in invertebrate Dicers although the overall shapes of human and *Drosophila* Dicer proteins are similar (Lau et al., 2012).

The crystal structure of the N-terminal helicase has not been obtained. Thus, based on the cryo-EM-based modelling, the N-terminal helicase is composed of three globular subdomains (HEL1, HEL2, HEL2i) where the DExD/H domain corresponds to HEL1 and the helicase superfamily c terminal domain to HEL2 and HEL2i. All three parts of the helicase form a clamp near the RNase III domain active site. Interestingly, the N-terminal helicase was found in two distinct conformations, with respect to the body of the enzyme (Lau et al., 2012), similar to the RIG-I helicase which was used as a template for modelling (Kowalinski et al., 2011).

Analysis of substrate-specific structural rearrangements proposed that human Dicer exists in three states depending on presence and type of substrate (Taylor et al., 2013). Unbound Dicer existing in "canonical state" rearranges upon substrate binding that involves the PAZ domain as well as the helicase domain. Substrate-bound Dicer exists either in an "open" or "closed" state. The open state is cleavage-competent and it is typical for pre-miRNA binding. It is characterized by binding of a pre-miRNA along the platform, bending of the helicase domain, and access of RNase IIIa and IIIb sites to the substrate (Taylor et al., 2013). The closed state has been observed for a 35 bp A-form RNA duplex, which represents a siRNA precursor. In this state, the substrate is trapped between the PAZ and helicase domains away from the catalytic sites (Taylor et al., 2013). This provides a structural explanation for previous observations that Dicer poorly processes longer perfect duplexes *in vitro* and *in vivo* (Kim et al., 2005; Nejebinska et al., 2012b).

Taken together, it is apparent that miRNA biogenesis has been the preferred role for Dicer during vertebrate evolution. The helicase domain in mammalian Dicers provides a structural basis for substrate specificity, namely distinguishing pre-miRNAs as the preferred substrate. In addition, a natural Dicer isoform has been found in mouse oocytes, which lacks the N-terminal helicase domain, can efficiently generate siRNAs from long dsRNAs, and is sufficient for enhancing RNAi in cultured cells. This isoform is a consequence of a rodent-specific retrotransposon insertion and is present in *Muridae* family (Flemer et al., 2013). This demonstrates that, while the mammalian Dicer primarily dedicated to the miRNA pathway, a small change in a mammalian Dicer gene can restore RNAi activity.

Substrate processing by mammalian Dicer proteins

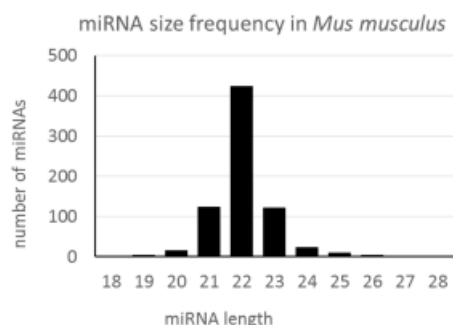
The first *in vitro* studies of recombinant human Dicer showed that substrate cleavage is dependent on Mg²⁺ but not on ATP presence (Provost et al., 2002; Zhang et al., 2002). Subsequently, it was reported that Dicer can cleave long dsRNAs and pre-miRNAs with different efficiency, which stems from substrate's structural properties (Ma et al., 2008; Flores-Jasso et al., 2009; Chakravarthy et al., 2010; Feng et al., 2012). Therefore, cleavage of miRNA precursors and long dsRNAs will be discussed in separate sections.

Canonical miRNA substrates

Canonical miRNAs of ~22 nt in length (Figure 22) are the dominant Dicer products in mammalian cells. Dicer mutagenesis showed that inactivation of the RNase IIIA domain results in complete loss of 3p-derived mature miRNAs, but only partial reduction in 5p-derived mature miRNAs (Gurtan et al., 2012). Conversely, inactivation of the RNase IIIB domain by mutation of D1709, a residue mutated in some cancers, produced complete loss of 5p-derived mature miRNAs, but only partial reduction in 3p-derived mature miRNAs (Gurtan et al., 2012). Mutation of the PAZ domain caused global reduction of miRNA processing, while mutation of the Walker A motif in the helicase domain of Dicer did not alter miRNA processing (Gurtan et al., 2012). These results are consistent with the above mentioned structural features of Dicer.

Figure 22 Mammalian miRNA size distribution

Distribution of mature miRNA lengths according to miRNA annotations in miRBase (release 21)



Pre-miRNAs are the most efficiently cleaved Dicer substrates *in vitro*. In contrast to long dsRNA, a canonical pre-miRNA is cleaved only once and releases a single small RNA duplex. Human Dicer alone cleaves pre-miRNAs much faster than pre-siRNA substrates under both single and multiple turnover conditions; with more than 100-fold difference in maximal cleavage rates (V_{max}) under multiple turnover conditions (Chakravarthy et al., 2010). This indicates that the mammalian Dicer is optimized for miRNA biogenesis and several specific structural adaptations discussed below support this notion. Dicer seems to interact directly with the terminal loop region of a pre-miRNA (Feng et al., 2012; Gu et al., 2012b) while a large pre-miRNA terminal loop further enhances pre-miRNA cleavage (Feng et al., 2012). A large-scale *in vitro* analysis and mutagenesis study of 161 human pre-miRNAs showed that human Dicer tolerates remarkable structural variation in pre-miRNA substrates (Feng et al., 2012). The dsRNA structure in the stem region and the 2-nt 3'-overhang structure in a pre-miRNA contribute to binding and cleavage by Dicer (Feng et al., 2012).

A characteristic feature of the pre-miRNA hairpin, which is accessed by the PAZ domain of Dicer, is a 2 nt 3' overhang generated by the nuclear Microprocessor complex (Gregory et al., 2004). Pre-miRNAs with the 2 nt 3' overhang at the 3' terminus are bound by Dicer with higher affinity than pre-miRNAs with different ends (Feng et al., 2012). Moreover, the 2 nt 3' end overhang leads to a higher substrate processing, which was shown on both, pre-miRNAs and perfect duplexes (Zhang et al., 2004; Park et al., 2011; Feng et al., 2012). Such preference is likely conferred by/due to simultaneous binding of pre-miRNA end by both 5' and 3' binding pockets in the PAZ domain (Park et al., 2011). Importantly, fidelity of miRNA biogenesis is critical for miRNA functionality because a single nucleotide shift at the 5' end of a miRNA would redefine its target repertoire. In contrast, RNAi, which typically involves perfect complementarity between a small RNA and its target, would be essentially insensitive to a precise cleavage positioning as long as it would not affect Argonaute loading. Thus, the simultaneous recognition of both strands at the 2 nt 3' overhang terminus by Dicer can be seen as an adaptation driven by miRNA biogenesis (Park et al., 2011).

The second structural adaptation of mammalian Dicer supporting miRNA biogenesis is the N-terminal helicase, which forms a clamp-like structure adjacent to RNase III domains, hence it is positioned to bind the stem loop of a pre-miRNA (Lau et al., 2012). While the loss of the entire N-terminal helicase only slightly increases pre-miRNA processing activity *in vitro* (Ma et al., 2008), pre-miRNA-processing by recombinant Dicer *in vitro* is much faster than that of a perfect duplex (Ma et al., 2008; Chakravarthy et al., 2010). *In vivo*, a naturally occurring N-terminally truncated Dicer isoform can rescue miRNA biogenesis in Dicer^{-/-} embryonic stem cells (ESCs) (Flemer et al., 2013). This suggests that the N-terminal helicase domain in mammalian Dicers is not important for miRNA biogenesis per se; it rather provides constraints for substrate selectivity favouring pre-miRNAs.

This is consistent with the model where pre-miRNA binding is associated with the cleavage-competent open conformation. In the open state, a pre-miRNA is bound along the platform, the helicase domain is bent, and RNase IIIa and IIIb sites have access to the substrate (Taylor et al., 2013). It has been proposed that the loop of a pre-miRNA may prevent adoption of the closed conformation by Dicer by interacting with HEL1 and HEL2i domains and possibly stabilizing the open conformation of Dicer (Feng et al., 2012; Lau et al., 2012; Ma et al., 2012). This also indicates that the N-terminal helicase had acquired distinct roles in Dicer function in RNA silencing during evolution. In mammalian cells, the N-terminal helicase has a gatekeeper function where pre-miRNA loops appear to be a key keeping the gate open.

Dicer-dependent non-canonical miRNA substrates

Apart from canonical miRNA substrates mentioned above, Dicer is processing additional miRNA-like substrates, which are independent of the Microprocessor complex (described in a separate section below). Some non-canonical miRNAs are produced by Dicer in a Microprocessor-independent fashion, including mirtrons, which utilize the splicing machinery to bypass the Microprocessor complex. Mirtrons are substantially longer than Microprocessor-generated pre-miRNAs and exhibit 3' uridylation and 5' heterogeneity (Wen et al., 2015). A recent analysis yielded ~500 novel mouse and human introns that generate Dicer-dependent small RNA duplexes (Wen et al., 2015). These represent nearly 1000 loci distributed in four splicing-mediated biogenesis subclasses, with 5'-tailed mirtrons being the dominant subtype (Wen et al., 2015). Another example of non-canonical miRNAs found in the literature are Microprocessor-independent miRNAs which were originally described as small interfering RNAs derived from a unique hairpin formed from short interspersed nuclear elements (SINEs) (Babiarz et al., 2008; Castellano and Stebbing, 2013).

While a typical pre-miRNA is a hairpin RNA with 2-nt 3' overhangs, production of a mature miRNA from an endogenous hairpin RNA with 5' overhangs has also been reported; mouse pre-mir-1982 is a mirtron with an 11 nt tail at the 5' end (Babiarz et al., 2008). A possible mechanism for processing such templates has been provided by an *in vitro* study which showed that Dicer can produce such miRNAs in a two-step cleavage, which releases dsRNAs after the first cleavage and binds them again in the inverse direction for a second cleavage (Ando et al., 2011a).

Long dsRNA substrates

In addition to pre-miRNA, Dicer can process long dsRNAs coming from different sources (Figure 2). Exogenous sources of dsRNA include viral dsRNAs and imply function of RNAi in eukaryotic antiviral immune response (Vance and Vaucheret, 2001; Wilkins et al., 2005; Wang et al., 2006). Endogenous dsRNAs have variable length and termini, and are generated by transcription of inverted repeats, by convergent transcription or by pairing of complementary RNAs in trans. Importantly, mammals lack an ortholog of RNA-dependent-RNA polymerase (RdRP), which is a conserved component of RNAi-related mechanisms in plants, fungi and invertebrates (see the separate RdRP section). Endogenous RNAi in mouse oocytes, the best documented mammalian endogenous RNAi example, works independently of RdRP activity (Stein et al., 2003).

The human Dicer binds long dsRNA but not siRNAs *in vitro* (Provost et al., 2002). Long dsRNA binding is independent both on Mg²⁺ and ATP. The human Dicer preferentially binds and cleaves long dsRNA from the end, due to inefficient binding of internal regions of dsRNA (Zhang et al., 2002). In comparison to pre-miRNA processing, human Dicer exhibits lower cleavage activity on perfect dsRNA

substrates (Ma et al., 2008). An explanation was proposed that a closed conformation of the N-terminal helicase domain disturbs the RNase III catalytic core and inhibits cleavage of perfect dsRNAs (Lau et al., 2012). As it was mentioned, *in vitro* deletion of the N-terminal helicase domain increases cleavage activity of human recombinant Dicer (~65-fold). Authors hypothesize that DExD/H-box domain mainly inhibits the functionality of the Dicer active site, but not RNA binding (Ma et al., 2008). This model is supported by previously mentioned structural data, where Dicer is in a closed state with a 35 bp A-form RNA duplex trapped between PAZ and helicase domains away from the catalytic center (Taylor et al., 2013).

The complexity of the differential substrate processing by Dicer is illustrated by a Dicer mutant carrying an in-frame 43-amino-acid insertion immediately adjacent to the DExH box. This Dicer exhibits defects in the processing of most, but not all, endogenous pre-miRNAs into mature miRNA but enhanced processing efficiency and concomitant RNA interference when thermodynamically stable, long-hairpin RNAs are used (Soifer et al., 2008). This result implies an important function for the helicase domain in the processing of thermodynamically unstable hairpin structures (Soifer et al., 2008).

Dicer-mediated cleavage of dsRNA can be stimulated *in vitro* by TARBP2. However, it is not clear if TARBP2 stimulation could be sufficient to induce endogenous RNAi *in vivo* (Chakravarthy et al., 2010). So far, the evidence for endogenous RNAi (including attempts to induce RNAi with exogenous substrates) is scarce (reviewed in detail in Nejepinska et al., 2012a; Svoboda, 2014). The only tissue type, where abundant endogenous siRNAs are present and where long dsRNA readily induces RNAi are mouse oocytes, which express an oocyte-specific Dicer isoform lacking a part of the N-terminal helicase domain (Flemr et al., 2013), thus mimicking some of the Dicer mutants tested *in vitro* (Ma et al., 2008). Taken together, long dsRNA, the typical endogenous RNAi substrate, is poorly processed by endogenous full-length Dicer. This is due to the gatekeeper role of the N-terminal helicase domain, which does not open upon binding long dsRNA.

Off note is that the human Dicer can bind 21-nt ssRNAs *in vitro*, independent of their sequence and secondary structure. Dicer binds ssRNAs having a 5'-phosphate with greater affinity versus those with a 5'-hydroxyl. (Kini and Walton, 2007).

3.1.1.3 Dicer-interacting dsRBPs: TARBP2 and PACT

A common Dicer interacting partner found across Metazoa is a dsRBP with tandemly arrayed dsRBDs. Mammals have four dsRBP with tandemly arrayed dsRBDs proteins: trans-activation responsive RNA-binding protein 2 (TARBP2), protein activator of PKR (PACT), Staufen 1 (STAU1), and Staufen 2 (STAU2). However, only TARBP2 (also known as TRBP or TRBP2) and PACT were identified as Dicer binding partners (Chendrimada et al., 2005; Haase et al., 2005).

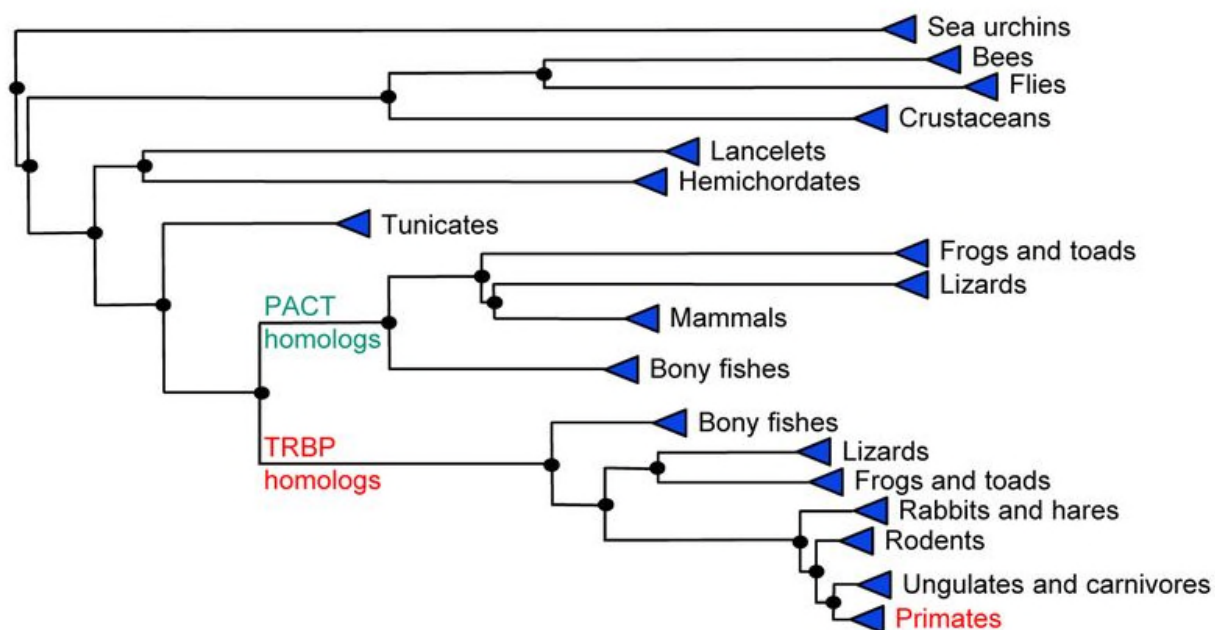


Figure 23 Phylogeny of PACT and TARBP2

Phylogenetic tree of TARBP2 from the literature (Daniels and Gatignol, 2012) obtained from COBALT multiple alignments. The lineage **PACT homologs** represent proteins with higher homology with PACT than with TARBP2. Proteins in the lineage **TRBP homologs** are more related to TARBP2. Solo proteins between sea urchins and tunicates are related to both PACT and TARBP2.

TARBP2 and PACT are paralogs, which evolved through a gene duplication event in an ancestral chordate (Daniels and Gatignol, 2012) (Figure 23). The structure of human TARBP2 has been partially resolved (Benoit and Plevin, 2013). Each protein consists of three dsRBDs, where the first two domains can bind dsRNA (or miRNA) while the third domain has a partial homology to dsRBD and does not bind dsRNA. Instead, it mediates protein-protein interactions and is a part of a larger protein-protein interacting C-terminal region referred to as Medipal domain as it interacts with Merlin, Dicer, and PACT (reviewed in Daniels and Gatignol, 2012). TARBP2 and PACT can also form homodimers and heterodimers through the Medipal domain (Laraki et al., 2008).

The binding site of TARBP2 and PACT on Dicer was recently determined using cryo-EM and crystallography (Wilson et al., 2015). Homology-based modelling showed that Dicer-binding residues are conserved in TARBP2 and PACT, implicating that binding of TARBP2 and PACT to Dicer is mutually exclusive (Wilson et al., 2015).

TARBP has a positive effect on Dicer activity. Human Dicer is much faster at processing a pre-miRNA substrate compared to a pre-siRNA substrate under both single and multiple turnover conditions. Maximal cleavage rates (V_{max}) calculated by Michaelis-Menten analysis differed by more than 100-fold under multiple turnover conditions. TARBP2 was found *in vitro* to stimulate Dicer-mediated cleavage of both, pre-miRNA and pre-siRNA substrates; this stimulation requires the two N-terminal dsRBDs (Chakravarthy et al., 2010). Thus, while the structure of the substrate affects the rate at which Dicer generates small RNAs, TARBP2 stimulates dicing by presumably enhancing the stability of Dicer-substrate complexes (Chakravarthy et al., 2010).

When compared to Dicer and Dicer:TARBP2 complex, PACT inhibits Dicer processing of pre-siRNA substrates (Lee et al., 2013). The two N-terminal dsRBDs contribute to the observed differences in dsRNA substrate recognition and processing behaviour of Dicer:dsRNA-binding protein complexes (Lee et al., 2013). In addition, PACT and TARBP2 have non-redundant effects on the generation of

different-sized miRNAs (isomiRs) (Lee et al., 2013; Kim et al., 2014; Wilson et al., 2015). Cells lacking TARBP2 exhibit altered cleavage sites in a subset of miRNAs but no effect on general miRNA abundance or Argonaute loading (Kim et al., 2014). Thus, impact of TARBP2 and PACT on miRNAs biogenesis *in vivo* seems to be relatively minor (Kim et al., 2014; Wilson et al., 2015). However, it should be pointed out that any change in the 5' end position of any miRNA will have a strong effect on its target repertoire. Taken together, TARBP2 and PACT are regulatory factors that contribute to the substrate specificity and cleavage fidelity during miRNA and siRNA production.

Moreover, TARBP2 and PACT have an additional role in a cross-talk of the interferon (IFN) response and small RNA pathways (reviewed in Daniels and Gatignol, 2012). The IFN response is the major antiviral branch of innate immunity in mammals, which deals with threats associated with long dsRNA. Among the key components sensing dsRNA in the IFN response are protein kinase R (PKR) and helicase RIG-I (reviewed in Gantier and Williams, 2007). The two N-terminal dsRBDs of PACT and TARBP2 bind PKR through the same residues (Wilson et al., 2015) while the (C-terminal) Medipal domain of PACT is needed for PKR activation (Huang et al., 2002). In contrast, the Medipal domain of TARBP2 has an inhibitory effect (Gupta et al., 2003). Furthermore, sequestering of PACT by TARBP2 has negative effect on PKR phosphorylation and activation. PKR inhibition by TARBP2 is released in stress conditions, leading to IFN response activation (Daher et al., 2009). Therefore, absolute and/or relative expression levels of TARBP2 and PACT might be buffering or sensitizing the IFN response to dsRNA. One could envision that suppression of the IFN response might result in increased RNAi. However, there is no evidence, so far that TARBP2 would redirect long dsRNA to Dicer and stimulate RNAi *in vivo* enough to achieve a robust sequence-specific mRNA knock-down.

Taken together, while TARBP2 and PACT are clearly associated with dsRNA binding, Dicer and the miRNA pathway. However, the full extent of biological roles of TARBP2 and PACT in dsRNA response, in the miRNA pathway, and elsewhere is still not fully understood. Since TARBP2 also interacts with and inhibits PKR (Park et al., 1994; Cosentino et al., 1995), it was speculated that TARBP2 could be a component of a network of protein-protein interactions underlying a reciprocal regulation of RNAi/miRNA and IFN-PKR pathway (Haase et al. 2005). This notion is further supported by PACT, a paralog of TARBP2, which exerts the opposite role on PKR. PACT was shown to interact with TARBP2 and Dicer and to facilitate siRNA production (Kok et al., 2007). At the same time, the role of TARBP2 in RNA silencing has been studied in cells where the physiological substrate for Dicer processing and RISC loading are miRNA precursors and where long dsRNA readily activates the protein kinase R (PKR) and interferons (IFN). Thus, while the RISC loading role of TARBP2 may be common for miRNA and RNAi pathway, it is not clear if an isoform of TARBP2 plays any specific role in recognition and processing of long dsRNA in the canonical mammalian RNAi pathway. Accordingly, one of the unexplored areas, which deserve further attention is the contribution of different splice variants to TARBP2 biology (Bannwarth et al., 2001). Likewise, it needs to be tested if TARBP2 plays a role in directing long dsRNA into RNAi. Finally, there is RNAi silencing-independent function of TARBP2 to explore. TARBP2 can also bind mRNA hairpins and it has been proposed that TARBP promotes metastasis by destabilizing metastasis suppressor through binding of mRNA structural elements (Goodarzi et al., 2014).

3.1.1.4 A mammalian RNA-dependent RNA polymerase?

RNA dependent RNA polymerases were found in RNA silencing in plants and lower invertebrates where they can either produce long dsRNA or short RNAs serving as an initiator or amplifier of the response (reviewed in Maida and Masutomi, 2011). Importantly, these independently discovered RdRPs that act in RNA silencing in plants, fungi and nematodes are homologs. Furthermore, homologs of these RdRPs exist in metazoan phyla, including *Nematoda* (e.g. *C. elegans*), *Cnidaria* (hydra), *Chelicerata* (tick), *Hemichordata* (acorn worm), *Urochordata* (sea squirt), and *Cephalochordata* (lancelet) but appear absent in others, including *Platyhelminthes* (planaria), *Hexapoda* (*Drosophila*), and *Craniata* (vertebrates). Phylogenetic analysis suggests that RdRPs in RNA silencing pathways have a monophyletic origin, i.e. evolved once from an ancestral RdRP presumably acting in a common ancestral RNA silencing mechanism (Cerutti and Casas-Mollano, 2006; Murphy et al., 2008). At the same time, mammalian genomes do not contain a homolog of these RdRPs, suggesting that it was

lost during evolution and is absent in RNA silencing in mammals. Whether RdRP activity completely disappeared from RNAi in mammals is unclear. One could speculate that RdRP orthologs in RNA silencing in vertebrates could be replaced by another RdRP, for example by exaptation of some viral RdRP.

The literature search for mammalian RdRPs related to RNA silencing yielded ten original research papers related to mammalian RdRPs that could be put in some context of RNA silencing. Of these, four articles actually dealt with viral RdRPs and silencing of viruses by exogenous siRNAs and not with an RdRP possibly involved in RNAi (Lee et al., 2002; Meng et al., 2006; Nygardas et al., 2009; Moon et al., 2016).

One article reported that ectopic expression of plant RdRP has no effect on RNAi in the mouse model (De Wit et al., 2002). Another report described Aquarius (AQR), a murine protein of unknown function with a weak homology to viral RdRPs expressed in embryos and ESCs (Sam et al., 1998). A follow up search for AQR-related data (28 papers citing the original one and text search by gene name) suggested that Aquarius is an RNA/DNA helicase involved in R-loop processing (Sollier et al., 2014).

An important contribution to RdRP function in mammalian RNAi was provided by a report demonstrating the lack of RdRP activity in RNAi in mouse oocytes (Stein et al., 2003). This report is of a particular importance because mouse oocytes are the only mammalian cell type, with strong endogenous RNAi pathway (reviewed in Svoboda, 2014).

Two reports proposed that two other RdRP activities could generate dsRNA that could be processed by Dicer. An indirect support for a putative RdRP emerged from *Drosophila*, where it was discovered ELP1, a non-canonical RdRP conserved in all eukaryotes, which associates with DCR-2 and its loss results in reduction of endo-siRNAs and upregulation of transposon transcripts (Lipardi and Paterson, 2009). However, a follow up search revealed the lack of data supporting that notion in mammals.

The second proposed mammalian RdRP acting in RNAi is a ribonucleoprotein complex of the human telomerase reverse transcriptase (TERT) and the RNA component of mitochondrial RNA processing endoribonuclease (RMRP). RMRP shows a strong preference for substrates that have 3' fold-back structures and produces dsRNA that can be processed by Dicer yielding self-targeting endogenous siRNAs (Maida et al., 2009). A follow-up analysis of these siRNAs revealed that these off-target effects of these endo-siRNAs would mimic miRNA activities (Maida et al., 2013). The latest report shows that TERT RdRP generates short RNAs that are complementary to template RNAs and have 5'-triphosphorylated ends, which indicates de novo synthesis of the RNAs (Maida et al., 2016).

Taken together, all available data suggest that mammalian RNAi does not employ an RdRP in a canonical way known from plants or nematodes. There are two candidate mechanisms that might involve RdRP in RNA silencing – one of them is based just on existence of a homolog of ELP1, so further experimental evidence is needed to confirm whether mammalian ELP1 homologs participate in RNAi at all. This would include analysis of small RNAs in mammalian cells lacking *Elp1* and further characterization of complexes containing ELP1. The second candidate mechanism, the TERT RdRP system, seems to produce a unique small population self-targeting endo-siRNAs in a highly localized manner, i.e. does not appear to be an RdRP acting in RNAi *in trans*.

3.1.1.5 Argonaute proteins

Once a small RNA duplex is produced by Dicer, one of the strands of the duplex is selected and loaded onto an Argonaute protein. However, before summarizing AGO loading and RISC assembly, we first review structure of AGO proteins, their covalent modifications and binding partners.

AGO2 structure and function

Argonaute proteins are the key components of miRNA and RNAi pathways as they bridge the small RNA, its target and the silencing effect. According to the model (Yuan et al., 2005), AGO proteins exist in four basic states according to the bound RNAs: apo (no substrate), pre-RISC (with a passenger and a guide), activated RISC (with a guide), and RISC targeting (with a guide and a target). Transitions

between these four states correspond to RISC loading, RISC activation and target recognition, which will be discussed later.

Argonaute proteins are divided into two subfamilies: AGO proteins, which accommodate miRNAs and siRNAs (and are in the focus of this section), and PIWI proteins, which accommodate piRNAs. As piRNAs are produced in the germline in a Dicer-independent manner from single-stranded RNAs, hence they are not systematically covered in this report and neither are PIWI proteins.

Mammalian genomes encode four AGO proteins, where AGO1, 3, and 4 are encoded within one locus on the human chromosome 1 (chromosome 4 in mice) and AGO2 is encoded separately on chromosome 8 (chromosome 15 in mice). Independent studies have revealed that mammalian Argonautes were originally discovered through screening of antibodies generated against intracellular membrane fractions from rat pancreas, which yielded a 95-kDa protein that localizing to the Golgi complex or the endoplasmic reticulum (Cikaluk et al., 1999). This initial observation was later refined by analysis of compartmentalization of miRNA- and siRNA-loaded AGO2 populations that co-sediment almost exclusively with the rough endoplasmic reticulum membranes, together with Dicer, TARBP2, and PACT (Stalder et al., 2013). The identified protein of unknown function was originally named GERP95 (Golgi ER protein 95 kDa) and was identified as a member of a family of highly conserved proteins in metazoans which function in the germline (Cikaluk et al., 1999). Apart from the original GERP95 reference, there are three others identified with the GERP95 keyword search. In one of them, GERP95 was found to bind Hsp90 chaperon (Tahbaz et al., 2001) before it was finally linked to RNAi and RISC complex and became classified as an Argonaute family member (Martinez et al., 2002; Thonberg et al., 2004).

AGO2 is the catalytical engine of RNAi and all four AGO proteins operate in the miRNA pathway. The key for understanding functional significance of AGO proteins for RNA silencing and for summarizing the molecular mechanisms mediated by AGO proteins are their structural analyses.

Unlike Dicer, mammalian AGO proteins were successfully crystalized. The core of the structural reports is thus formed by six articles reporting crystal structures of human Argonaute proteins (Elkayam et al., 2012; Schirle and MacRae, 2012; Faehnle et al., 2013; Nakanishi et al., 2013; Schirle et al., 2014; Schirle et al., 2015). Four articles describe crystal structures of AGO2 (Elkayam et al., 2012; Schirle and MacRae, 2012; Schirle et al., 2014; Schirle et al., 2015), which is capable of cleaving cognate transcripts and two describe miRNA-pathway dedicated AGO1 (Faehnle et al., 2013; Nakanishi et al., 2013).

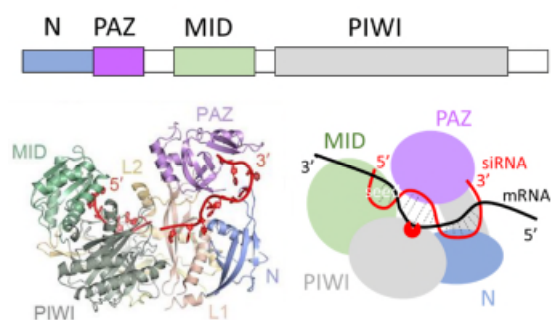


Figure 24 AGO2 structure

The figure shows domain composition and structure of human AGO2. Different colours indicate positions of the central PAZ domain and the c-terminal PIWI domain, which has an RNase H fold (Song et al., 2004). Two additional domains are recognized, the N-terminal domain and the MID domain between PAZ and PIWI domains. The ribbon model was taken from (Schirle et al., 2014).

There is a number of additional articles, which provided partial insights into AGO functional structure. These could be divided, as in the case of Dicer, into:

(I) Biochemical studies of recombinant human AGO proteins and individual domains (Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005; Lima et al., 2009; Tan et al., 2009a; Deleavey et al., 2013; Kalia et al., 2016).

(II) The pioneering AGO crystal structures from archaea *Pyrococcus furiosus* (Song et al., 2004) and *Aquifex aeolicus* (Yuan et al., 2005) that served as a comparative scaffold.

(III) Crystallographic studies on individual domains of human AGO proteins (PAZ of human AGO 1 (Ma et al., 2004), or MID domain of human AGO2 (Frank et al., 2010; Frank et al., 2011)) or modelling (Kinch and Grishin, 2009; Deerberg et al., 2013; Kandeel and Kitade, 2013; Gan and Gunsalus, 2015; Jiang et al., 2015).

Human AGO1 and AGO2 structures

Among the four mammalian AGO proteins associating with 21-23 nt long small RNAs, only AGO2 was identified to have the „slicer“ activity (Liu et al., 2004; Meister et al., 2004). The crystal structure of human AGO2 revealed a bilobed molecule with a central cleft for binding guide and target RNAs (Elkayam et al., 2012; Schirle and MacRae, 2012; Schirle et al., 2014; Schirle et al., 2015) (Figure 24). This domain organization is consistent with that found in archaeal AGO proteins (Song et al., 2004; Yuan et al., 2005). The 3' end of a short RNA is anchored by the PAZ domain in a sequence-independent manner (Ma et al., 2004). The 5' phosphate of the siRNA is buried in a pocket at the interface between the MID domain and the PIWI domain. Structural studies of archaeal AGO homologs showed that the PIWI domain has an RNase H-like fold and possess the endonucleolytic “slicer” activity (Song et al., 2004; Yuan et al., 2005). The 5' end of the base pairing cognate mRNA enters between the N-terminal and PAZ domains and its 3' end exits between the PAZ and MID domains.

Structural analysis provided a model for recognition of target RNAs, including the first nucleotide in the cognate site, which does not base pair with the loaded RNA. Yet, interaction with the cognate site is enhanced by adenosine in the position 1 of miRNA binding site; the structural analysis revealed that it is recognized indirectly by AGO2 (Schirle et al., 2015). Importantly, N6 adenosine methylation blocks recognition of the adenosine, which might reflect a possible mechanism for regulating of miRNA binding through covalent modification of the binding site (Schirle et al., 2015). Nucleotides 2 to 6 of a heterogeneous mixture of guide RNAs are positioned in an A-form conformation for base pairing with target messenger RNAs. Between nucleotides 6 and 7, there is a kink that may function in miRNA target recognition or release of sliced RNA products. (Schirle and MacRae, 2012). Crystallization of loaded human AGO2 in the presence of target RNA sequences suggested a stepwise mechanism for interaction with cognate RNAs. First, AGO2 exposes guide nucleotides (nt) 2 to 5 for initial target pairing, which then promotes conformational changes that expose nt 2 to 8 and 13 to 16 for further target recognition (Schirle et al., 2014). miRNA binding seem to lock otherwise flexible AGO2 enzyme in a stable conformation (Elkayam et al., 2012). The structure of human Ago2 bound to miRNA-20a implies that the miRNA is anchored at both ends by the MID and PAZ domains with several kinks and turns along the binding groove (Elkayam et al., 2012). Spurious slicing of miRNA targets is avoided through an inhibitory coordination of one catalytic magnesium ion (Schirle et al., 2014). Importantly, the PIWI domain contains tandem tryptophan-binding pockets, which function in recruitment of glycine-tryptophan-182 (GW182) or other tryptophan-rich cofactors (Schirle and MacRae, 2012).

Structures of human AGO1 bound to endogenous co-purified RNAs or loaded with let-7 miRNA are strikingly similar to the structures of AGO2 (Faehnle et al., 2013; Nakanishi et al., 2013). Evolutionary changes that rendered hAGO1 inactive included a mutation of a catalytic tetrad residue and mutations on a loop near the active site (Faehnle et al., 2013; Nakanishi et al., 2013).

Taken together, crystal structures of AGO2 explained the nucleotide-pairing patterns that emerged during previous studies of miRNA sequences, namely analyses of conservations of miRNA binding sites and biochemical analyses of target recognition, which are discussed later.

3.1.1.6 Post-translational modifications of AGO proteins

Annotation of Argonaute-related literature yielded a number of reports describing covalent modifications of AGO proteins implicated in post-translational regulations, namely phosphorylation (Zeng et al., 2008; Rudel et al., 2011; Horman et al., 2013; Mazumder et al., 2013; Shen et al., 2013; Lopez-Orozco et al., 2015; Patranabis and Bhattacharyya, 2016), prolyl 4-hydroxylation (Qi et al., 2008; Wu et al., 2011), sumoylation (Josa-Prado et al., 2015), ubiquitination (Rybak et al., 2009;

Johnston et al., 2010; Bronevetsky et al., 2013; Smibert et al., 2013), and poly-ADP-ribosylation (Leung et al., 2011; Seo et al., 2013).

Phosphorylation

Phosphorylation of AGO occurs at multiple positions and published data imply that phosphorylation operates through multiple different mechanisms. Phosphorylation has been detected on AGO1 and AGO2 in the PAZ domain (pS253, pT303, pT307), the PIWI domain (pS798), in the L2 linker region (pS387, pY393) and in the MID domain (pY529) (Rudel et al., 2011).

S387 phosphorylation - Serine-387 (S387) was found to be the major Ago2 phosphorylation site *in vivo* (Zeng et al., 2008). Phosphorylation of Ago2 at S387 was significantly induced upon stress in a mitogen-activated protein kinase (MAPK)-dependent manner but, apparently independently of JNK and MEK kinases (Zeng et al., 2008). Another kinase implicated in S387 phosphorylation was AKT3 (Horman et al., 2013). S387A mutation or treatment with a p38 MAPK inhibitor reduced the localization of Ago2 to cytoplasmic P-bodies suggesting a potential regulatory mechanism (Zeng et al., 2008). Conversely, S387 phosphorylation downregulated RNAi-like cleavage and increased miRNA-mediated translational repression (Horman et al., 2013). Furthermore, AGO2 phosphorylation at S387 facilitated interaction with GW182 and localization to P bodies (Horman et al., 2013).

S798 phosphorylation - Serine-798 (S798) phosphorylation has been also associated with P-body localization as mutation analysis of phospho-residues within AGO2 revealed that S798D completely abrogated association of Ago2 with P-bodies and stress granules (Lopez-Orozco et al., 2015).

Y529 phosphorylation - Tyrosine 529 (Y529), which is conserved in all other species that have been analyzed. is located in the small RNA 5'-end-binding pocket of Ago proteins was found to be phosphorylated *in vivo* (Rudel et al., 2011). Y529E phosphomimicking mutation strongly reduced small RNA binding suggesting a potential regulatory role (Rudel et al., 2011). Y529 phosphorylation causing impaired binding miRNAs was subsequently implicated as a mechanisms transiently relieving miRNA repression during macrophage function (Mazumder et al., 2013). Y529 phosphorylation was also proposed as a mechanism relieving Let-7-mediated repression during neuronal differentiation (Patranabis and Bhattacharyya, 2016)

Y393 phosphorylation - Tyrosine 393 (Y393) was implicated in EGFR-mediated repression of miRNA biogenesis during hypoxia (Shen et al., 2013). According to the model, Y393 negatively impacts the interaction between AGO2 and Dicer and inhibits maturation of long-loop pre-miRNAs carrying tumour-suppressor-like miRNAs (Shen et al., 2013).

Prolyl 4-hydroxylation

Prolyl 4- hydroxylation has been implicated in AGO stabilization and increased RNAi. Mass spectrometry analysis hydroxylation of the endogenous AGO2 at proline 700 (P700) and P700A mutation resulted in destabilization of AGO2 (Qi et al., 2008). Prolyl hydroxylation was observed under hypoxic conditions, where it lead to increased AGO2 stability (Wu et al., 2011). AGO2 hydroxylation correlated with increased miRNA levels as well as the endonuclease activity of AGO2 (Wu et al., 2011). Conversely, human cells depleted and mouse embryonic fibroblast cells depleted of a specific prolyl-4-hydroxylase showed reduced stability of AGO2 and impaired RISC activity (Qi et al., 2008). Hydroxylation of AGO2 was required for its association with HSP90 (see further below), which is implicated in the RISC loading with miRNAs and translocation to stress granules (Wu et al., 2011).

SUMOylation

The small ubiquitin-like modifier (SUMO) regulates various cellular processes. AGO2 was identified as a substrate for SUMO E3 ligase PIAS3. AGO2 was SUMOylated in mammalian cells by both SUMO1 and SUMO2 primarily at lysine 402. Mutation of the SUMO consensus site reduced RNAi activity of AGO2, suggesting that SUMOylation might regulate endonucleolytic activity of AGO2 (Josa-Prado et al., 2015)

Ubiquitination

Ubiquitin-proteasome apparently tunes AGO levels to adjust miRNA, AGO and Dicer stoichiometry (Smibert et al., 2013). It was found that levels of AGO1 are adjusted according to miRNA expression in a ubiquitin-proteasome-dependent manner (Smibert et al., 2013). Similarly, lower stability of AGO2 in Dicer-knockout cells could be rescued by proteasome inhibition or Dicer expression (Smibert et al., 2013). AGO and GW182 protein levels also depend on HSP90 availability (Johnston et al., 2010). Two studies show examples of developmentally regulated ubiquitination, which is apparently used to suppress AGO activities during developmental transitions. First, the let-7 target *Lin-41* gene in mice is a stem cell specific E3 ubiquitin ligase targeting AGO1, AGO2, and AGO4 proteins (Rybak et al., 2009). Second, AGO proteins are downregulated in a proteasome-dependent manner during T cell differentiation, presumably as a part of gene expression reprogramming (Bronevetsky et al., 2013).

Poly-ADP-ribosylation

This AGO modifications seems to be linked to suppression of RNA silencing. Poly(ADP-ribose) has been associated with the assembly of stress granules, which accumulate RNA-binding proteins regulating mRNAs stability and translation upon stress. Stress granule proteins modified by poly(ADP-ribose) include AGO1–4 (Leung et al., 2011). Interestingly, poly-ADP-ribosylation of RISC associated with reduced RISC activity has been observed upon viral infection (Seo et al., 2013). According to the model, poly-ADP-ribosylation after viral infection releases miRNA-mediated repression of interferon-stimulated genes, hence boosting innate antiviral pathways (Seo et al., 2013).

3.1.1.7 Other Dicer and AGO interacting proteins

Apart from the RISC-loading complex and miRISC components such as GW182/TNRC6 or DDX6 and others mentioned above and elsewhere, the literature survey revealed a number of reports describing other Dicer and AGO interacting proteins. A large number of AGO-interacting partners has been identified in the past and reported individually (see further below) or comprehensively (Meister et al., 2005). Here, we provide overview of the interacting partners, which were identified to date

DDX3 – DEAD-box helicase 3 is one of the helicases sensing viral double-stranded RNAs. DDX3 was also among the P-body components recruited to the West Nile virus replication sites and regulating viral replication (Chahar et al., 2013). DDX3 was also identified by an RNAi screen as an essential factor involved in RNAi pathway (Kasim et al., 2013). DDX3 is co-localized with AGO2 and a dominant negative mutant of DDX3 affected the RNAi activity (Kasim et al., 2013).

CLIMP-63 - The cytoskeleton-linking endoplasmic reticulum (ER) membrane protein of 63 kDa (CLIMP-63) was identified as a novel Dicer-interacting protein through a yeast two-hybrid screening. CLIMP-63 interacts with Dicer to form a high molecular weight complex, which is catalytically active in pre-miRNA processing (Pepin et al., 2012). These results are consistent with analysis of Dicer compartmentalization, which showed that loading of small RNAs into RISC, cognate mRNA binding, and Ago2-mediated mRNA slicing in mammalian cells are nucleated at the rough endoplasmic reticulum (Stalder et al., 2013). While the major RNAi pathway proteins are found in most subcellular compartments, the miRNA- and siRNA-loaded AGO2 populations co-sediment almost exclusively with the rough endoplasmic reticulum membranes, together with Dicer, TARBP2, and PACT (Stalder et al., 2013).

NUP153 - The nuclear pore complex protein NUP1 was found to associate with human Dicer protein. The association was detected mainly in the cytoplasm but was also apparent at the nuclear periphery. Accordingly, it has been suggested that NUP153 plays a role in the nuclear localization of Dicer (Ando et al., 2011b)

FMRP - X mental retardation protein (FMRP) is included in the list despite its questionable role in mammalian RNA silencing. In any case, our literature search revealed a number of articles dealing with mammalian FMRPs because FMRP is a highly conserved protein and its *Drosophila* ortholog dFXR was implicated in RNAi (Caudy et al., 2002; Ishizuka et al., 2002) and will be mentioned in the Arthropod section. According to the available data, FMRP is associated with RNA silencing factors.

FMRP co-localized with AGO2 (Goodier et al., 2007) and immunoprecipitation suggested that a portion of Dicer and AGO were associated with each other and with FMRP (Lugli et al., 2005). *In vitro* data using recombinant proteins, suggested that human FMRP can act as a miRNA acceptor protein for Dicer and facilitate the assembly of miRNAs on specific target RNA sequences (Plante et al., 2006). The requirement of FMRP for efficient RNAi was also supported *in vivo* by reporter assays supporting the role of FMRP in the mammalian RISC (Plante et al., 2006). However, the loss of mammalian FMRP did not reveal any apparent direct impact on RISC function (Didiot et al., 2009; Madsen et al., 2009).

Huntingtin – AGO2 was found as one of the Huntingtin associated proteins by co-immunoprecipitation. Furthermore, Huntingtin and AGO2 co-localized in P-bodies and, importantly, depletion of Huntingtin compromised RNA-mediated gene silencing (Savas et al., 2008). However, the molecular mechanism by which Huntingtin would influence RNA silencing remain unknown.

14-3-3 - Cell cycle regulating 14-3-3 proteins were reported to bind the amino terminus of AGO1 and AGO2 (Stoica et al., 2006). Overexpression of the Ago1 amino terminus in yeast resulted in cell cycle delay at the G(2)/M boundary prompting a hypothesis that 14-3-3 proteins contribute to Argonaute protein functions in cell cycle and/or gene-silencing pathways (Stoica et al., 2006).

UPF1 – mRNA surveillance protein appears to provide a nexus between three different mechanisms of RNA metabolism: adenosine deamination, mRNA surveillance (non-sense-mediated decay) and RNA silencing. Both, human ADAR1 and UPF1 were found associated within nuclear RNA-splicing complexes (Agranat et al., 2008). At the same time, UPF1 was connected to RNA silencing (Jin et al., 2009). UPF1 interacts with human AGO1 and AGO2 and co-localizes with them into P-bodies. UPF knock-down yielded upregulation of miRNA targets while its overexpression resulted in their downregulation (Jin et al., 2009). This would suggest that UPF may contribute to RNA silencing, maybe at the level of RISC binding to its targets and accelerating their decay (Jin et al., 2009).

RBM4 - The RNA-binding motif protein 4 (RBM4) plays multiple roles in mRNA metabolism. RBM4 was found during proteomic analysis of AGO-containing miRNPs (i.e. miRISC) and RBM4 knockdown showed that it is required for miRNA-guided gene regulation (Hock et al., 2007). It was also found to co-localize with AGO2 during muscle cell differentiation (Lin and Tarn, 2009). RBM4 interacts directly with AGO2 and may selectively enhance miRISC association with target mRNAs (Lin and Tarn, 2009). RBM4 was also implicated in miRNA-mediated repression in inflammation where inflammation-induced miRNA-146 promotes a feed-forward loop that modifies through phosphorylation the subcellular localization RBM4 and promotes its interaction with AGO2 and, subsequently, tames an excessive acute inflammatory response (Brudecki et al., 2013)

TRIM32 - TRIM-NHL 32 protein regulates protein degradation and miRNA activity in neural progenitor cells to control the balance between differentiating neurons and daughter cells retaining the progenitor fate. TRIM32 was shown to bind AGO1 and increase the activity of specific miRNAs, such as Let-7 (Schwamborn et al., 2009)

QKI-6 – QKI-6 is one of the protein isoforms encoded by the qkI gene in mice. QKI-6 was found to interact with AGO2 and to co-localize with AGO2 into stress granules (Wang et al., 2010). At the same time QKI-6 depletion lead to increased miR-7 expression while QKI-6 presence inhibits processing of pri-miR-7 into miR-7 in glioblastoma cells (Wang et al., 2013b). It has been suggested that QKI-6 mediates selective nuclear retention of pri-miR-7, hence preventing its processing (Wang et al., 2013b). Further research is needed to clarify these two seemingly distant activities of QKI-6.

RACK - receptor for activated protein kinase C (RACK1), a constituent of the eukaryotic 40S subunit, was reported to be important for miRNA-mediated gene regulation in *C. elegans* and humans, essentially linking miRISC with the ribosome (Jannot et al., 2011). RACK1 was also identified as a gene necessary for full miRNA function a screen for genes regulating miRNA function (Otsuka et al., 2011). RACK1 interacts with components of the miRISC in nematodes and mammals; the alteration of RACK1 expression alters miRNA function and impairs the association of the miRNA complex with the translating ribosomes (Jannot et al., 2011). Another study found that RACK1 binds to KH-type splicing regulatory protein (KSRP) and is required for the recruitment of mature miRNAs to RISC (Otsuka et al., 2011)

PTB - Polypyrimidine Tract Binding Protein (hnRNP I) was found during a search for proteins involved in let-7 mediated gene regulation. (Engels et al., 2012). PTB interacts with miRNAs and human AGO2 through RNA and there is a population of cellular targets that are co-regulated by PTB and AGO2 (Engels et al., 2012).

LRRK2 - leucine-rich repeat kinase 2 (LRRK2) gain-of-function mutations cause age-dependent degeneration of dopaminergic neurons. The analysis of the molecular mechanism of pathogenesis in *Drosophila* and humans revealed that LRRK2 associates with *Drosophila* AGO1 or human AGO2 (Gehrke et al., 2010) and that the gain-of-function LRRK2 mutant antagonizes let-7, causing derepression of Let-7 targets (Gehrke et al., 2010)

APOBEC3G - the apolipoprotein-B-mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G or A3G) is cytidine deaminase. APOBEC3G is an antiviral factor is found in P-bodies (Wichroski et al., 2006; Izumi et al., 2013). APOBEC3H also inhibits miRNA-mediated repression of translation (Huang et al., 2007) by competitively inhibiting binding of MOV10 to AGO2, causing either abnormal assembly or abnormal maturation of miRISC (Liu et al., 2012a).

3.1.1.8 **AGO loading and RISC formation**

The next important step is formation of RISC, the effector complex of miRNA and RNAi pathways. It involves formation of the RISC Loading Complex (RLC), transfer of a small RNA on an AGO-protein, and RISC activation.

RISC Loading Complex (RLC)

RISC assembly was so far explored more in *Drosophila* (Pham et al., 2004; Tomari et al., 2004a; Tomari et al., 2004b; Iwasaki et al., 2010) than in mammals (Gregory et al., 2005; MacRae et al., 2008; Bernard et al., 2015) perhaps because of the robust *in vitro* system of *Drosophila* embryo lysate. Mammals differ from *Drosophila* because they do not use different Dicer and Argonaute proteins dedicated to RNAi and miRNA pathway although it is assumed that both pathways use a similar if not the same RLC. Our knowledge of the mammalian RLC comes mainly from cells where RLC normally loads miRNAs or from *in vitro* reconstitution of the RLC with purified proteins. The minimal RLC is composed of Dicer, TARBP2 and AGO2 (Gregory et al., 2005; MacRae et al., 2008). *In vitro* reconstituted mammalian RLC contains one copy of each protein and has dicing, guide-strand selection, loading, and slicing activities (Martinez et al., 2002; Gregory et al., 2005; MacRae et al., 2008; Bernard et al., 2015).

AGO interacts with Dicer through a subregion of the PIWI domain (the PIWI-box), which binds directly to the Dicer RNase III domain. (Tahbaz et al., 2004). Single-particle EM analysis suggested that Dicer's N-terminal DEXH/D domain interacts with TARBP2, whereas its C-terminal catalytic domains in the main body are proximal to AGO2 (Wang et al., 2009). Interestingly, binding of AGO to Dicer inhibits dicing activity *in vitro* (Tahbaz et al., 2004). Analysis of individual siRNA positions revealed that RNA sequences at positions 9-12 and 15-18 were associated with TARBP2 while positions 19-21 with AGO. AGO binding was enhanced by positions 15-18 (Takahashi et al., 2014). AGO2 was reported to bind primarily to the 5'- and alternatively, to the 3'-end of pre-miRNAs. (Tan et al., 2011). All four human AGO proteins show remarkably similar structural preferences for small-RNA duplexes: central mismatches promote RISC loading, and seed or 3'-mid (guide position 12-15) mismatches facilitate unwinding. All these features of human AGO proteins are highly reminiscent of fly AGO1 but not fly AGO2. (Yoda et al., 2010). Biochemical and structural analysis suggests that TARBP2 is flexibly bound to the Dicer DEXH/D domain (Daniels et al., 2009; Wang et al., 2009). TARBP2 seems to bridge release of the siRNA by Dicer and loading of the duplex onto AGO2. Binding by TARBP2 may allow the siRNA intermediate to stay associated with the RLC after release from Dicer and may also help in orientation of the siRNA for AGO2 loading. Just as in flies, human RISC assembly is uncoupled from dicing (Yoda et al., 2010).

Analysis of miRNA-carrying RISC (miRISC) yielded a similar picture. Since loading of miRNA duplexes to AGO proteins is assisted by HSP70/ HSP90 chaperones (Maniataki and Mourelatos, 2005b; Yoda et

al., 2010), HSP90 is sometimes also included as the component of miRLC (Liu et al., 2012c). At the same time, AGO2 and Dicer are sufficient for processing and loading of miRNAs into RISC (Tan et al., 2011).

Combination of *in vivo* studies in Dicer^{-/-} cells reconstituted with wild-type or catalytically inactive Dicer showed that the miRNA loading complex (miRLC) is the primary machinery linking pre-miRNA processing to miRNA loading and lead also to definition of a miRNA Precursor Deposit Complex (miPDC) for Dicer-independent RISC loading exemplified by miR-451 (Liu et al., 2012c). miPDC is formed of AGO, pre-miRNA, and HSP chaperone. It functions in Dicer-independent miRNA biogenesis (e.g. miR-451) and also promotes miRNP assembly of certain Dicer-dependent miRNAs (Liu et al., 2012c).

Earlier studies suggested a difference between fly and human systems because human RISC assembly using immunopurified or reconstituted human RLC containing AGO2, Dicer and TARBP2 did not require ATP hydrolysis, (Gregory et al., 2005; Maniataki and Mourelatos, 2005b; MacRae et al., 2008). Recent data suggest that ATP facilitates also human RISC loading while it is dispensable for unwinding (Yoda et al., 2010).

Accessory RLC factors

Apart from the three established RLC components, several proteins emerged as RLC cofactors, among which stand out HSP70/90 chaperones. Heat shock protein 90 was actually the first identified AGO-associated protein even before AGO was associated with RNA silencing (Tahbaz et al., 2001). Inhibition of HSP90 reduces AGO levels (Tahbaz et al., 2001; Johnston et al., 2010; Martinez and Gregory, 2013) as well as GW182 protein levels and abolishes P-bodies (Johnston et al., 2010). In addition, stable binding between AGO and Dicer is dependent on the activity of Hsp90 (Tahbaz et al., 2004) and association of AGO2 with HSP90 involves prolyl-hydroxylation of AGO2 (Wu et al., 2011). HSP90 activity is not required for association of AGO with intracellular membrane (Tahbaz et al., 2001) but appears to chaperon AGO proteins before binding RNA and may facilitate loading of small RNAs (Johnston et al., 2010). Interestingly, miRNA*s (miRNA* is an equivalent of the passenger strand) with fast turnover exhibited different sensitivity to HSP90 inhibition suggesting differential HSP90 requirements for different miRNA*s (Guo et al., 2015). HSP90 is also a negative regulator of PKR; it is able to bind and inhibit PKR phosphorylation and prevent apoptosis (Donze et al., 2001). Thus, HSP90 provides a factor bridging RNA silencing and innate immunity.

Furthermore, HSP90 co-chaperones FKBP4/5 control AGO2 expression and facilitate RISC assembly (Martinez et al., 2013). FKBP4/5 were identified as AGO2-associated proteins in mouse embryonic stem cells. Inhibition of FKBP4/5 lead to decreased Ago2 protein levels while overexpression stabilized AGO2 expression (Martinez et al., 2013). Another study has found that FKBP4 forms a stable complex with human AGO2 before small RNA loading in the cytoplasm and is required for efficient RNAi (Pare et al., 2013).

Another component reported to function as an RISC-loading factor is RNA helicase A (RHA, also known as DHX9) Dicer (Robb and Rana, 2007). RHA is a conserved protein with two dsRBDs (Nagata et al., 2012) with multiple roles in the gene expression of cellular and viral mRNAs. RHA recognizes highly structured nucleotides and catalytically rearranges the various interactions between RNA, DNA, and protein molecules to provide a platform for the ribonucleoprotein complex. RHA was shown in human cells to function in the RNAi pathway and interact with siRNA, AGO2, TARBP2, and Dicer (Robb and Rana, 2007). RHA-depleted cells, showed reduced RNAi, apparently as a consequence of lower active RISC suggesting that RHA functions in RISC as an siRNA-loading factor (Robb and Rana, 2007). A later structural analysis of dsRBDs showed that both dsRBDs are required for RISC association, and such association is mediated by dsRNA (Fu and Yuan, 2013).

Minimal miRNA sorting

As mentioned above, of the four AGO proteins that can be loaded with small RNAs equally well (Meister et al., 2004). All four mouse AGO proteins seem to be functionally redundant in the miRNA

pathway as shown by rescue experiments in ESCs lacking all four Argonaute genes (Su et al., 2009). Consistent with this, all four AGOs are functionally equivalent when accommodating bulged miRNA duplexes, whereas AGO1 and AGO2 appear to be more effective at utilizing perfectly matched siRNAs (Su et al., 2009). Furthermore, AGO2 can execute endonucleolytic cleavage of cognate RNAs while all four can mediate translational repression. This raises a question whether small RNAs may undergo some kind of sorting that would result in preferential loading onto specific AGO homologs.

Structural analysis showed that all four human AGO proteins showed similar structural preferences for small-RNA duplexes, which were highly reminiscent of *Drosophila* AGO1 but not of AGO2 (Yoda et al., 2010). Human AGO2 and AGO3 immunoprecipitation and subsequent sequencing of small RNAs revealed that both AGOs were associated with 21-23 nt RNAs, majority of which were miRNAs (Azuma-Mukai et al., 2008). While fifteen miRNAs showed more than 2-fold significant difference in loading onto AGO2 or AGO3, it is not clear whether this discrimination occurs also *in vivo* (Azuma-Mukai et al., 2008).

A detailed analysis of small RNAs associated with all four human AGO proteins revealed approximately equivalent amounts of sequence tags derived from miRNA loci associated with individual AGOs with some exceptions that could be coupled to specific AGOs (Burroughs et al., 2011). However, further analysis suggested existence of some sorting mechanism affecting a subset of distinct isomiRs that seemed to be differentially associated with distinct AGO proteins (Burroughs et al., 2011). This observation contrasts with another cloning and deep sequencing experiment addressing distribution of endogenous miRNAs associated with AGO1-3, which did not find evidence for miRNA sorting in human cells. (Dueck et al., 2012).

It is possible that sorting of small RNAs on AGO proteins may not be a general phenomenon while differential presence of small RNAs on AGO proteins can also emerge from selective mechanisms operating after loading. This can be illustrated on selective progressive 3' shortening of AGO2-bound miRNAs observed in the brain (Juvvuna et al., 2012). Furthermore, Dueck et al also reported that AGO identity appears to influence the length of some miRNAs, while others remain unaffected (Dueck et al., 2012). Taken together, it seems that miRNAs are generally not sorted for loading onto AGO proteins. Notable exceptions include miRNAs with unique biogenesis such as miR-451 whose biogenesis requires AGO2 slicing activity (Dueck et al., 2012).

Loading asymmetry

While both siRNA strands can guide post-transcriptional silencing in mammals (Wei et al., 2009), selection of the loaded strand exhibits a clear and long-known thermodynamic bias where the strand whose 5'-end is less thermodynamically stable is preferentially loaded onto AGO as the guide strand (Khvorova et al., 2003; Schwarz et al., 2003). Selection of the guide strand involves multiple sensors – this includes AGO2 strand selection capability (Noland and Doudna, 2013; Suzuki et al., 2015), which is enhanced in complex with Dicer and TARBP2 or PACT. In addition, strand selection for some miRNAs is enhanced in complexes containing PACT but not TARBP2 (Noland and Doudna, 2013). Notably, TARBP2 was predicted to be a sensor of the thermodynamic stability of 5' siRNA in strand selection during RISC loading, similarly to DCR-2 and R2D2 (a TARBP2 homolog) in *Drosophila* (Wang et al., 2009). However, the supporting evidence is inconclusive (Haase et al., 2005) although some argue that TARBP2 can indeed acts as a sensor (Gredell et al., 2010). Furthermore, while TARBP2 function is similar to that of R2D2, TARBP2 sequence is more closely related to Loquacious than R2D2 (Murphy et al., 2008). Finally, quantitative analysis of RISC assembly and target silencing activity in the presence or absence of Dicer suggest that the mammalian Dicer is nonessential for asymmetric RISC loading *in vivo* and *in vitro*. (Betancur and Tomari, 2012).

RISC activation

The next step after AGO loading is removal of the passenger strand from the loaded duplex RNA. In some cases, the passenger strand can be eliminated by the slicer activity where the RISC complex uses the guide siRNA to cleave the passenger strand. In other words the first cleavage actually targets the passenger strand of a loaded siRNA duplex to free the guiding strand, so it can base pair to

cognate mRNAs (Matranga et al., 2005). 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).

Slicer-independent mechanism is needed to remove the passenger strands from non-slicing AGO proteins and from miRNA duplexes bound to AGO2 where the passenger strand cannot be cleaved. As slicer-deficient hAGO1, hAGO3, and hAGO4 are able to eject the passenger strand of siRNA duplexes at 37°C, it is apparent that AGO1, 3, and 4 can be readily programmed with siRNAs at the physiological temperature (Park and Shin, 2015). This implies that a slicer-independent mechanism, which relies on the thermal dynamics of the PAZ domain (Gu et al., 2012a; Park and Shin, 2015), is likely a common feature of human AGOs.

Importantly, RISC activation has been associated with additional factors. One of them is C3PO, an endonuclease that activates RISC (Ye et al., 2011). According to the model of RISC activation that integrates the C3PO crystal structure, Ago2 directly binds duplex siRNA and nicks the passenger strand, and then C3PO activates RISC by degrading the Ago2-nicked passenger strand (Ye et al., 2011). Another factor is La, Sjogren's syndrome antigen B (SSB)/autoantigen, which is acting as an activator of the RISC-mediated mRNA cleavage activity. (Liu et al., 2011). Thus, similarly to C3PO, La is a regulatory factor helping to remove AGO2-cleaved products in order to promote active RISC formation (Liu et al., 2011).

Additional small RNAs associated with AGO proteins

The literature search brought a heterogeneous group of publications describing small RNAs loaded on AGO proteins that were clearly distinct from canonical miRNAs - small RNAs generated by the mechanism described above. A canonical miRNA is transcribed by polymerase II, the primary transcript contains a ~ 70 nt short hairpin precursor pre-miRNA, which is released by the Microprocessor complex, transported to the cytoplasm where Dicer cleaves of the loop and one of the strands of the miRNA duplex is loaded onto miRISC. However, next generation sequencing revealed existence of AGO-loaded small RNAs that were apparently generated from different substrates and by molecular mechanisms, which deviated from the canonical pathway. Below is an overview of diversity of AGO-bound RNAs, which emerged from the literature search.

Non-canonical miRNAs can be divided across two axes – (I) according to the RNA precursor and (II) according to the proteins involved in (or omitted from) their biogenesis. Non-canonical miRNAs were discovered during systematic analyses of small RNAs in different model systems, such as disease models (e.g. Xia et al., 2013) or cultured cells (Babiarz et al., 2008; Babiarz et al., 2011). A good experimental strategy to identify non-canonical miRNAs is a high throughput sequencing analysis of genetic models lacking some of the components of RNA silencing such as Dicer or DGCR8 (Babiarz et al., 2008; Babiarz et al., 2011). Phenotypic difference and differential expression of distinct miRNA-like sequences can indicate biological roles of non-canonical miRNAs while knock-out data offer an insight into the non-canonical biogenesis mechanism.

Perhaps the best known non-canonical miRNA class, which comes from unique, Microprocessor-independent precursors, are mirtrons, miRNA-like molecules arising from spliced-out introns, which are Microprocessor-independent (Berezikov et al., 2007; Babiarz et al., 2011; Ladewig et al., 2012; Schamberger et al., 2012; Sibley et al., 2012; Westholm et al., 2012). Interestingly, some predicted mirtron-like miRNAs (miR-1225 and miR-1228) are splicing-independent (simtrons) and their biogenesis involves Drosha but neither DGCR8 nor Dicer (Havens et al., 2012). Other non-canonical substrates can be, for example, 5'-Capped RNAs (Xie et al., 2013), SINE repeat-derived, (Babiarz et al., 2008; Castellano and Stebbing, 2013), small vault RNA (svtRNA2-1a) (Minones-Moyano et al., 2013), or RNase III transcripts (Maurin et al., 2012) including annotated RNAs such as snoRNAs (Ender et al., 2008; Burroughs et al., 2011; Li et al., 2012), 7SL RNA (Ren et al., 2012), tRNA fragments (Maniataki and Mourelatos, 2005a; Haussecker et al., 2010; Burroughs et al., 2011; Li et al., 2012; Maute et al., 2013; Kumar et al., 2014; Venkatesh et al., 2016). Non-canonical miRNAs can be also produced from viral RNAs (Li et al., 2009c; Xu et al., 2009; Bogerd et al., 2010; Kincaid et al., 2014). A non-canonical small RNA class of unclear significance are semi-microRNAs (smiRNAs), which

are ~ 12nt short RNA fragments apparently emerging from other miRNAs, such as let-7 or miR-223 (Plante et al., 2012).

Non-canonical miRNAs can be also classified by their biogenesis as Microprocessor, DGCR8-, or Dicer-independent. For instance, the above-mentioned mirtrons do not require the Microprocessor complex while simtrons require Drosha but neither DGCR8 nor Dicer. Non-canonical miRNAs can be also produced from bona-fide miRNA precursors, which give a rise to a small RNA in a non-canonical way, for example by a dual role of AGO protein (Diederichs and Haber, 2007). A classic example is miR-451, a Dicer-independent miRNA biogenesis pathway that requires Ago catalysis (Cheloufi et al., 2010). A unique type of non-canonical miRNAs are loop-miRs, which are released from the loop region of a pre-miRNA (Okamura et al., 2013; Winter et al., 2013).

3.1.1.9 Target recognition and modes of silencing

Target recognition

Target recognition by RISC is mediated by base pairing between RISC-loaded small RNAs and cognate RNAs. Consistently with the structural analysis of AGO proteins, target recognition by siRNAs exhibits a distinct 5' bias. Analysis of miRNA-targeted mRNAs in *Drosophila* and mammals revealed that miRNA bases 2-8 form a distinct „seed“, which base pairs perfectly to the target transcript (Enright et al., 2003; Lewis et al., 2003). This is consistent with the fact that the 5' half of a small RNA provides most of the binding energy that tethers RISC to a target RNA (Doench et al., 2003; Haley and Zamore, 2004). Structural features of the target site are only important for RISC binding, while sequence features such as the A/U content of the 3' UTR are important for mRNA degradation. (Hausser et al., 2009). According to analyses of RISC kinetics, small RNAs loaded onto AGO proteins are actually composed of five distinct domains (Figure 25): the anchor, seed, central, 3' supplementary, and tail (Wee et al., 2012).

Biochemical analysis of target recognition by mammalian RISC showed that the RISC is apparently not systematically scanning transcripts. RISC is unable to unfold structured RNA. Thus, RISC randomly transiently contacts single-stranded RNA and promotes siRNA-target base pairing where the 5' end of the loaded siRNA creates a thermodynamic threshold for stable association of RISC with its target (Ameres et al., 2007).



Figure 25 miRNA/siRNA functional domains, taken from (Wee et al., 2012)

The fact that 5' and 3' ends of a siRNA are bound by distinct binding pockets and that both ends contribute differently to binding to the target lead to a „two-state model of Argonaute function proposed based on the *Drosophila* model (Tomari et al., 2004b). In this model, the 3' end is bound in the PAZ domain and the 5' end to in a pocket at the interface between the MID and the PIWI domains. The 5' end is pre-organized to interact with the cognate mRNA and, upon binding, the 3' end is dislodged from the binding pocket to allow for base pairing of the 3' end.

Importantly, the literature search brought several reports with kinetic data, which should be taken as biochemical range for any hypotheses concerning target recognition and biological effects of small RNAs in the context of loaded RISC. Among these is a detailed kinetic study of *Drosophila* and mouse AGO2 (Wee et al., 2012). It was found that mouse AGO2, which mainly mediates miRNA-directed repression *in vivo*, dissociates rapidly and with similar rates for fully paired and seed-matched targets (Wee et al., 2012). An important conclusion from this study is that low-abundant miRNAs are unlikely to contribute much biologically meaningful regulation because they are present at a concentration less than their KD for seed-matching targets (Wee et al., 2012).

These results were subsequently corroborated by single molecule analysis. Single-molecule fluorescence experiments using a minimal RISC (a small RNA and AGO2) showed that target binding starts at the seed region of the guide RNA (Chandradoss et al., 2015; Jo et al., 2015a; Jo et al., 2015b). AGO2 initially scans for complementarity to nucleotides 2-4 of the miRNA and this interaction propagates into a stable association when target complementarity extends across the seed (Chandradoss et al., 2015). Stable RISC binding is thus efficiently established with the seed match only, providing a potential explanation for the seed-match rule of miRNA target selection (Chandradoss et al., 2015; Jo et al., 2015a; Jo et al., 2015b). Remarkably, mouse AGO2 binds tighter to miRNA targets than its RNAi cleavage product, even though the cleaved product contains more base pairs (Salomon et al., 2015). In contrast, target cleavage required extensive sequence complementarity and accelerated core-RISC dissociation for recycling (Jo et al., 2015b) and sensitively depended on the sequence (Jo et al., 2015a). RISC thus utilizes short RNAs as specificity determinants with thermodynamic and kinetic properties more typical of RNA-binding proteins while a small RNA loaded on AGO no longer follows rules by which sole oligonucleotides find, bind, and dissociate from complementary nucleic acid sequences (Salomon et al., 2015). Importantly, target site recognition is coupled to lateral diffusion of RISC along the target RNA, which facilitates recognition of target sites within the cellular transcriptome space (Chandradoss et al., 2015).

To put the aforementioned kinetic data into comparison with laboratory practice, the table below shows on ten randomly selected examples of RNAi experiments that an effective repression by exo-RNAi in cultured cells usually employs siRNA transfection in 20-50 nM range. Thus, a robust biological effect of a specifically designed perfectly complementary siRNA acting through the AGO2-mediated endonucleolytic cleavage is usually observed with a nanomolar concentration of siRNA.

Table 18 Random examples of RNAi experiments in cultured cells

concentration	method	cells	exposure time	knockdown efficiency	reference - doi
5 nM	transfection	mouse preadipocytes 3T3-L1	48 h	70 %	10.1128/MCB.01856-08
20 nM	transfection	human HUVEC	0-5 d	60-80 %	10.1016/j.bone.2014.12.060
20 µM	transfection	human breast carcinoma cell lines	48 h	60 %	10.1038/onc.2014.421
20 µM	transfection	murine breast epithelial cell	48 h	60 %	10.1038/onc.2014.421
25 nM	transfection	mouse kidney	7 d	20 %	10.1152/ajprenal.00052.2014
20 - 40 nM	transfection	human LA-4	24 h	60-80 %	10.1155/2015/473742
50 nM	transfection	mouse cardiomyocyte	48 h	60 %	10.5582/bst.2015.01159.
80 nM	transfection	mouse osteoblasts	0-5 d	40-50 %	10.1016/j.bone.2014.12.060
100 nM	transfection	human endothelial cells	72 h	80%	10.1152/ajplung.00263.2009
500 nM	electroporation	human monocytic cell	24 h	60-90%	10.1111/jdi.12434

Silencing by direct endonucleolytic cleavage – RNAi-like silencing

Base pairing in the middle of siRNA results in correct positioning of the cognate strand and its cleavage in the active site of the PIWI domain of AGO2 - out of four mammalian AGO proteins, which bind small RNAs, only AGO2 has the „slicer“ activity (Liu et al., 2004; Meister et al., 2004). These simple facts have several important consequences. First, siRNAs loaded on AGO1, AGO3, and AGO4 will repress their perfectly complementary targets by other means (translational repression/deadenylation/decapping) than by cleavage. Conversely, AGO2 loaded miRNA binding perfectly complementary targets will behave as siRNAs and mediate RNAi-like endonucleolytic cleavage. Third, imperfect complementarity in the middle of the base pairing site of AGO-loaded siRNAs will result in miRNA-like effects (Doench et al., 2003). Thus, RISC-loaded siRNAs have additional silencing effects due to 2-8 nucleotide seed matches to other RNAs (which is the molecular foundation of the off-targeting phenomenon).

Silencing by translational repression and RNA degradation – common miRNA silencing

There is extensive literature on the molecular mechanism of miRNA repression involving translational repression followed by mRNA degradation as this issue was extensively studied over more than a decade and represents conjunction of several complex processes - translational repression and RNA degradation involving deadenylation and decapping. We focused our search on molecular component

of the RISC complex, namely AGO proteins and protein GW182/TNRC6, which was identified as the key component of the effector complex, essentially bridging AGO with the silencing effects.

In order to understand the molecular mechanism of repression mediated by small RNA-loaded AGO, many AGO-associated proteins were identified in mammalian cells (reviewed in detail (Peters and Meister, 2007). These include MOV10, DDX6 (Rck/p54), DDX20 (Gemin3), TNRC6A (GW182), and many others (Meister et al., 2005; Hauptmann et al., 2015). Over a decade of research focused on understanding how miRNA-guided RISC suppresses gene expression, it became clear that miRNAs provide the guide while the repression is mediated through AGO and associated proteins as shown by suppression occurring upon tethering AGO proteins (Pillai et al., 2004). Building a model of silencing, was a lengthy and convoluted process (which is not over yet) from which emerged GW182 as a key docking factor integrating miRNA-mediated silencing.

GW182 proteins

Mammalian GW182 proteins carry at the N-terminus multiple glycine-tryptophan (GW) repeats, a central ubiquitin-associated (UBA) domain, a glutamine-rich (Q-rich) domain, and an RNA recognition motif (RRM) (Figure 26). Interestingly, GW182 seems to be largely unstructured protein, including functionally essential domains, such as the N-terminus, which is interacting with AGO2 (Behm-Ansmant et al., 2006; Lazzaretti et al., 2009; Lian et al., 2009; Takimoto et al., 2009). Similarly, the silencing domain is also predicted to be disordered (Lazzaretti et al., 2009; Zipprich et al., 2009; Huntzinger et al., 2010).

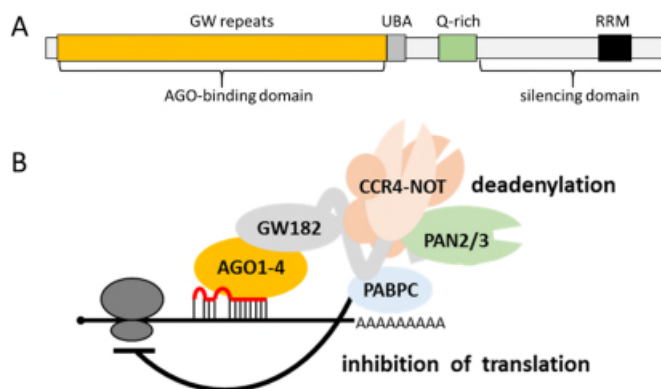


Figure 26
W182 domain structure and function.

(A) Domain composition: UBA, ubiquitin-associated motif; RRM, RNA recognition motif. (B) A schematic depiction of GW182-mediated bridging of AGO and translational repression and mRNA degradation. See the text below for details.

The N-terminal part of GW182 can interact with any human (and presumably all mammalian) AGO proteins through multiple GW repeats, which bind tryptophan binding pockets on AGO and contribute to the interaction in an additive manner (Jakymiw et al., 2005; Liu et al., 2005a; El-Shami et al., 2007; Lazzaretti et al., 2009; Lian et al., 2009; Takimoto et al., 2009; Zipprich et al., 2009; Yao et al., 2011; Schirle and MacRae, 2012). The role of AGO is most likely to anchor through miRNA-mediated interaction GW182 to the silenced RNA as tethering of GW182 alone or even of (its silencing domain) is sufficient to mediate repression (Chekulaeva et al., 2011). The silencing domain directly binds PolyA Binding protein (PABP) as well as with PAN3 and NOT1 components of PAN2/PAN3 and CCR4–NOT deadenylase complexes, respectively. (Fabian et al., 2009; Zekri et al., 2009; Huntzinger et al., 2010; Jinek et al., 2010; Chekulaeva et al., 2011).

The general, somewhat simplistic model of miRNA-mediated silencing proposes that GW182 interacts with PABP of the cognate RNA and recruit PAN2/3 and CCR4–NOT deadenylases, which would result in translational repression likely occurring at the level of initiation and the repressed RNA would be deadenylated (Braun et al., 2011; Chekulaeva et al., 2011; Fabian et al., 2011a; Fabian et al., 2011b; Christie et al., 2013; Huntzinger et al., 2013; Zekri et al., 2013; Kuzuoglu-Ozturk et al., 2016). Deadenylated RNAs in mammalian cells are generally decapped and degraded by XRN1 exonuclease (Schoenberg and Maquat, 2012).

Several mechanisms have been proposed how the GW182–PABPC interaction contributes to silencing (Fabian et al., 2009; Zekri et al., 2009; Jinek et al., 2010) although it is not clear how critical this role is as mRNAs lacking polyA tail (i.e. neither circularize nor are deadenylated) are silenced nonetheless

(Pillai et al., 2005; Chekulaeva et al., 2011; Zekri et al., 2013). This would suggest for a non-essential role of PABPC/GW182 interaction.

There are additional RISC interactions, which are important for miRNA-mediated silencing, such as DDX6/RCKp54 (Chu and Rana, 2006; Mathys et al., 2014; Kuzuoglu-Ozturk et al., 2016) or LIM domain proteins LIMD1, Ajuba, and WTIP, which are required for miRNA-mediated, but not siRNA-mediated gene silencing (James et al., 2010). According to the model, the LIM proteins facilitate miRNA-mediated gene silencing by creating an inhibitory closed-loop complex where they bridge the translationally inhibited cap structure and AGO1/2 within the miRISC complex bound to the 3'-UTR (James et al., 2010). The list of proteins associated with RISC or the target repression is longer, and includes, for example, also Pumilio/FBF, a miRNA targeting cofactor (Friend et al., 2012) or eIF4GI (Ryu et al., 2013). However, for understanding the miRNA mechanism, their detailed review is would be overreaching.

P-bodies

Studies on localization of miRNA pathways components revealed their presence in distinct cytoplasmic foci, known as P-bodies (or GW-bodies) (Liu et al., 2005a; Liu et al., 2005b; Pillai et al., 2005; Yu et al., 2005). P-bodies (reviewed in detail Jain and Parker, 2013) are distinct cytoplasmic foci, which contain proteins associated miRNA-mediated repression (Liu et al., 2005b; Rehwinkel et al., 2005; Yu et al., 2005; Behm-Ansmant et al., 2006; Chu and Rana, 2006; Zhou et al., 2009; James et al., 2010; Johnston et al., 2010; Pare et al., 2011; Chahar et al., 2013; Ozgur and Stoecklin, 2013). P-body association has been observed for the mature RISC components and RNA degradation pathway proteins but not for Dicer or TARBP2, indicating that P-bodies are associated with miRNA-mediated suppression but not biogenesis. This is supported by the presence of miRNA-targeted mRNAs in P-bodies (Liu et al., 2005b; Shih et al., 2011). P-bodies are highly dynamic (Kedersha et al., 2005; Aizer et al., 2014) but not an essential structure for miRNA-mediated repression (Eulalio et al., 2007). P-bodies probably should be seen as aggregating foci, which may facilitate miRNA-mediated repression. Since inhibition of the miRNA pathway prevents P-body formation, it was proposed that aggregation of miRNA pathway factors to P-bodies is not required for miRNA function and mRNA degradation *per se* but rather is a consequence of miRNA activity (Eulalio et al., 2007).

3.1.1.10 miRNA-mediated control of gene expression – important functional aspects

One of the major categories of small RNA publications represent studies of expressed miRNAs and their effects. At the same time, individual reports on miRNA expression and biological functions are not much relevant for the narrative of the molecular mechanism. The number of studies on mammalian miRNAs is not that surprising considering that miRNAs are among the most common gene products one can find in mammalian cells. The current miRBase edition (21) annotates 1881 human miRNA loci that give rise to 2588 annotated miRNAs. There are 1193 precursors and 1915 mature miRNAs annotated in mouse. A simple connection of these counts with the fact that only nucleotides 2-8 of a miRNA are sufficient for target recognition and suppression implies that miRNA-mediated repression is a widespread and extremely evolvable regulatory system for gene expression. At the same time, one should not forget the above-mentioned stoichiometry between miRNAs and their target sites that is needed for efficient silencing.

Evolution of miRNAs is fast - there are only a few miRNAs conserved between *Drosophila* and mammals. Given the diversity of canonical and non-canonical miRNAs, it is conceivable that miRNAs to emerge from random formation of Drosha/Dicer substrates. Newly evolving miRNAs likely form a considerable portion of annotated miRNAs, especially in species where miRNAs are deeply sequenced and low-abundant miRNAs are identified.

According to the evolutionary theory, new miRNAs would either acquire function and become fixed during evolution or they would be lost. In addition, the target repertoire of existing miRNAs can also rapidly evolve since a single point mutation can weaken an existing regulation or create a new one. This idea is consistent with the data showing that mammalian mRNAs are under selective pressure to maintain and/or avoid specific 7-nucleotide seeding regions (Farh et al., 2005). It can be nicely

exemplified on the Texel sheep phenotype where a single mutation creating a novel miRNA target site in myostatin causes the exceptional meatiness of this breed (Clop et al., 2006).

The set of miRNAs in each cell type forms a combinatorial post-transcriptional regulation system stabilizing gene expression pattern. miRNAs have widespread impact on expression and evolution of protein-coding genes (Farh et al., 2005). The number of mRNAs that have functionally important interaction with miRNAs (i.e. suppression of this interaction yields a phenotype) in a studied model system is presumably small and certainly difficult to discern among the possible interactions. Thus, every search for functionally important interactions between miRNAs and their targets has to face the fact that miRNAs represent a dynamically evolving system with countless random interactions, which are not biologically relevant.

3.1.1.11 Extracellular microRNAs

One important phenomenon, which was recognized by the literature search, was the release of miRNAs from cells, detection of extracellular miRNAs, and transfer between cells. Importantly, the vast majority of the references provided descriptive and correlative data documenting presence of circulating miRNAs under different conditions (e.g. (Luo et al., 2009; Arroyo et al., 2011; Turchinovich et al., 2011; Bellingham et al., 2012; Novellino et al., 2012; Huang et al., 2013b)). We have omitted the bulk of the circulating RNA literature, which provides data concerning biomarker potential of circulating miRNAs, which is undoubtedly of extreme clinical relevance but of minimal relevance for this report. Below, we summarize the current state of knowledge, which admittedly raises more questions than provides satisfactory answers.

Small RNAs can be transmitted from one cell to another under physiological conditions, as evidenced, for example, by systemic RNAi in arthropods or plants. Small RNAs can utilize dedicated transporters, common communication channels, or secretory vehicles. It was also reported that Gap junctions can serve for miRNA transfer from microvascular endothelial cells to colon cancer cells (Thuringer et al., 2016). Circulating mammalian miRNAs were reported 2008 when they were found in serum of lymphoma patients; they were immediately recognized as potential non-invasive biomarkers for cancer diagnostics and treatment (Lawrie et al., 2008). The same year, placental miRNAs were found circulating in maternal plasma (Chim et al., 2008), which was one of the discoveries leading to the notion that miRNAs could be a mobile regulating molecule (Iguchi et al., 2010) and that could even mediate transgenerational epigenetic inheritance (Sharma, 2015) or be transmitted across species (Zhang et al., 2012; Buck et al., 2014). Since then, extracellular miRNAs were identified in a broad range of biological fluids, including plasma, aqueous humour, cerebrospinal fluid, nasal mucus, or milk (Pegtel et al., 2011; Huang et al., 2013b; Kropp et al., 2014; Baglio et al., 2015; Dismuke et al., 2015; Izumi et al., 2015; Wu et al., 2015a). miRNAs were identified in the cargo of exosomes, membranous vesicles 40 to 100 nm in diameter, which are constitutively released by almost all cell type and are found essentially in every biological fluid (reviewed, for example, in (Rak, 2013; Yoon et al., 2014)

However, extracellular miRNAs do not need to be necessarily encapsulated in extracellular vesicles, as two studies showed that 95–99% of extracellular miRNA are not in extracellular vesicles but associated with AGO proteins in serum and cell culture media (Arroyo et al., 2011; Turchinovich et al., 2011). Furthermore, most individual exosomes in standard preparations do not seem to contain biologically significant numbers of miRNAs (Chevillet et al., 2014). The molecular mechanism of miRNA release, either as a cargo in a vesicle or free, is poorly understood and the current knowledge does not allow for building a coherent model as the literature is scarce. Non-templated nucleotide additions were found to distinguish between cellular miRNAs, which were 3' end adenylated in cells whereas 3' end uridylylated isoforms appeared overrepresented in exosomes suggesting a possible role of 3' terminal modifications in sorting miRNAs into extracellular vesicles (Koppers-Lalic et al., 2014). Recently, ALIX, an accessory protein of the endosomal sorting complex, it has been implicated in sorting miRNAs into extracellular vesicles based on its interaction with AGO2 and reduced miRNAs levels in extracellular vesicles upon *Alix* knock-down (Iavello et al., 2016).

Importantly, any model where miRNAs would be carried over to regulate gene expression by the canonical miRNA activity must face the kinetic data mentioned above (Wee et al., 2012). While one

cannot exclude a non-canonical signalling function of circulating miRNAs (which has not been conclusively demonstrated yet), the literature on circulating RNAs often includes statements, which are not supported by strong experimental evidence and may be misleading; several examples, taken from published abstracts, are shown below:

MicroRNAs can also be secreted from the cells in exosomes and act as paracrine signalling molecules. (Kuosmanen et al., 2015)

Secreted microRNAs (miRNAs) enclosed within extracellular vesicles (EVs) play a pivotal role in intercellular communication by regulating recipient cell gene expression and affecting target cell function (Ji et al., 2014).

Exosomal miRNAs act as an effector during communication with appropriate recipient (Momose et al., 2016).

Taken together, while existence of circulating miRNAs has been demonstrated beyond a doubt, experimental evidence for their function (if any) is not conclusive. Exosomal vesicles can carry miRNAs and siRNAs – in the latter case, exosomes were adapted for a delivery tool for siRNAs, which has a good potential for further development of siRNA therapy (El-Andaloussi et al., 2012; Lasser, 2012; Lee et al., 2012; Nguyen and Szoka, 2012; Wahlgren et al., 2012; Shtam et al., 2013; Kumar et al., 2015; Wahlgren et al., 2016).

3.1.1.12 RNAi pathway in mammals– important functional aspects

It should reiterated that the, “so-called” RNAi knock-down with siRNAs in mammalian cells is essentially using the miRNA pathway with retained the ability to cleave perfectly complementary targets by AGO2. The mammalian canonical RNAi (i.e. long dsRNA-driven) is a dormant pathway, at best. By that is meant that the protein factors present in every mammalian cells (Dicer, TARBP2, and AGO2) are competent to support RNAi but long dsRNA does not efficiently induce RNAi in most mammalian cells (Nejepsinska et al., 2012b). This notion is supported by the reconstitution of human RNA interference in budding yeast demonstrates that Dicer, TARBP2, and AGO2 are sufficient to functionally reconstitute RNAi (Suk et al., 2011). This demonstrates that these three proteins constitute the essential core of RNAi mechanism although RNAi is not properly reconstituted when bona fide RNAi precursors were co-expressed (Wang et al., 2013a). The problem is apparently at the level of Dicer processing as the human slicer AGO2 RNAi role is so conserved that it could function in RNAi in the early divergent protozoan *Trypanosoma brucei*, demonstrating conservation of basic features of the RNAi mechanism (Shi et al., 2006). In an analogous experiment, human AGO2 could not replace *Arabidopsis thaliana* AGO1 in the miRNA pathway (Deveson et al., 2013). In a sense, these different results are not that surprising considering the minimal requirements for RNAi and the complexity of the miRNA pathway, which provides a larger space for evolution of incompatible adaptations. However, there are some cases indicating that RNAi is still active in mammals and, under unique circumstances, may be even an essential pathway.

The main bottleneck for canonical RNAi in mammals is efficient production of siRNAs from long dsRNA, which is poor in most mammalian cells (Nejepsinska et al., 2012b; Flemr et al., 2013). However, several reports showed that induction of RNAi with intracellular expression of long dsRNA can be achieved in transformed and primary somatic cells (Elbashir et al., 2001; Gan et al., 2002; Diallo et al., 2003; Shinagawa and Ishii, 2003; Yi et al., 2003; Tran et al., 2004). These data imply that RNAi can occur if there is a sufficient amount of long dsRNA, which is directed preferentially to RNAi but not into other dsRNA pathways. Under these circumstances, the limiting factor is just Dicer’s ability to produce siRNA (Flemr et al., 2013).

Endogenous RNAi in the germline

Retrotransposon repression

RNAi-mediated mobile element silencing has also been documented in the mouse germline (Watanabe et al., 2006; Tam et al., 2008; Watanabe et al., 2008). Mutations in the piRNA pathway components are detrimental to sperm development, suggesting that piRNAs are the dominant class of small RNAs controlling mobile element activity in the male germline (reviewed in Toth et al., 2016). In contrast, female mice lacking functional piRNA pathway are fertile with no obvious defects in oocytes (Carmell et al., 2007). Endo-siRNAs suppress TE silencing in mammalian oocytes as documented by derepression of some retrotransposons in oocytes depleted of Dicer or AGO2 (Murchison et al., 2007; Watanabe et al., 2008). As already proposed for invertebrates, the piRNA and endo-siRNA pathways likely cooperate in creating a complex silencing network against mobile elements in the mammalian germline. Long terminal repeat MT elements and SINE elements are strongly upregulated in *Dicer*^{-/-} oocytes, while the levels of IAP transposon are elevated in the absence of MILI protein but not in *Dicer*^{-/-} oocytes (Murchison et al., 2007; Watanabe et al., 2008). Still many loci composed of other types of TEs, e.g. LINE retrotransposons, give rise to both piRNAs and endo-siRNAs, again suggesting that the biogenesis of these small RNAs is interdependent. The role of endogenous RNAi in TE silencing extends from germ cells to preimplantation embryo stages. Apart from maternally derived piRNAs and endo-siRNAs, which persist in the embryos for a large part of preimplantation development, zygotic endo-siRNAs are generated *de novo* mainly to control the activity of zygotically activated MuERV-L retrotransposon (Svoboda et al., 2004; Ohnishi et al., 2010). SINE-derived endo-siRNAs also increase in abundance in early embryo stages, which is consistent with the observation that B1/Alu SINE endo-siRNAs account for a vast majority of endo-siRNAs sequenced from mouse ES cells (mESCs) (Babiarz et al., 2008). Whether these SINE endo-siRNAs play an active role in TE silencing in mESCs similarly to other TE-derived endo-siRNAs in oocytes remains to be determined. RNAi-dependent silencing of LINE transposons has also been described in cultured HeLa cells, where endo-siRNAs derived from bidirectional transcripts of sense and antisense L1 promoter were proposed to control L1 activity (Yang and Kazazian, 2006). Although some evidence for retrotransposon-derived endo-siRNAs from mammalian somatic cells was obtained from deep sequencing data (Kawaji et al., 2008), a convincing support for the function of endo-siRNAs in TE silencing in mammalian somatic tissues, has yet to be provided.

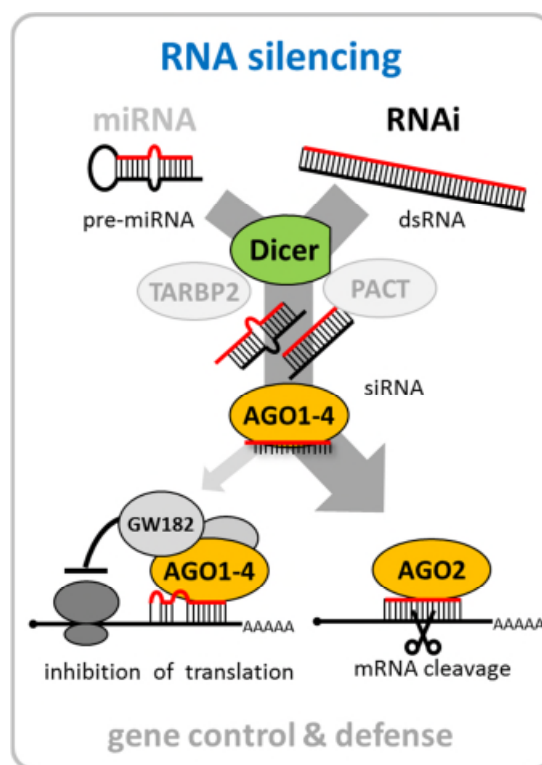


Figure 27 RNA silencing in mouse oocytes

Control of endogenous genes

In mice, perturbation of the endo-siRNA pathway in oocytes is responsible for severe meiotic defects and resulting female infertility. Targeted oocyte-specific knockout of both *Dicer* and *Ago2* lead to similar phenotypes including chromosome misalignment and defective spindle (Murchison et al., 2007; Tang et al., 2007; Kaneda et al., 2009). These effects were originally attributed to the loss of maternal miRNAs. However, miRNA pathway is suppressed in mouse oocytes and oocytes lacking *Dgcr8*, which is required for canonical miRNA biogenesis, can be fertilized and do not show any significant disturbance of the transcriptome (Ma et al., 2010; Suh et al., 2010). This means that the canonical miRNA pathway is non-essential and largely inactive in mouse oocytes. In fact, the spindle

phenotype is caused by the loss of a highly active RNAi pathway in mouse oocytes (Figure 27). High-throughput analysis of small RNAs in mouse oocytes revealed a unique class of endo-siRNAs derived from processed pseudogenes (Tam et al., 2008; Watanabe et al., 2008). Transcriptomes of oocytes lacking *Dicer* and *Ago2* (including oocytes expressing catalytically-dead AGO2) are similarly affected (Kaneda et al., 2009; Stein et al., 2015). At the same time, genes matching pseudogene-derived endo-siRNAs are enriched in the group of upregulated genes in both knockouts (Tam et al., 2008; Watanabe et al., 2008; Kaneda et al., 2009; Stein et al., 2015).

In addition, putative endo-siRNA targets are enriched in cell cycle regulators and genes involved in microtubule organization and dynamics (Tam et al., 2008). These findings suggest that regulation of protein-coding genes by endo-siRNAs controls the equilibrium of protein factors required for proper spindle formation, chromosome segregation and meiosis progression in mouse oocytes. As pseudogenes are rapidly evolving source of dsRNA for endo-siRNA production, it will be interesting to investigate whether the role of RNAi in spindle formation during meiotic maturation of oocytes is conserved in mammals.

The reason for high levels of endo-siRNAs and the high RNAi activity in mouse oocytes is the aforementioned truncated *Dicer* isoform that lacks the N-terminal helicase domain (Flemer et al., 2013). It efficiently generates siRNAs from long dsRNAs, and is sufficient for enhancing RNAi in cultured cells while its loss in mouse oocytes yields the same phenotype as conditional knock-outs of *Dicer* or *Ago2* (Flemer et al., 2013).

Endo-siRNAs have also been proposed to contribute to the self-renewal and proliferation of mouse embryonic stem cells (mESCs), since the proliferation and differentiation defects observed in *Dicer*^{-/-} mESCs are more dramatic than in *Dgcr8*^{-/-} mESCs (Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007). A population of endo-siRNAs derived mostly from hairpin forming B1/Alu subclass of SINE elements was identified in mESCs (Babiarz et al., 2008). Fragments of SINE elements are commonly present in untranslated regions of protein-coding transcripts and it is therefore possible that SINE-derived endo-siRNAs participate in posttranscriptional gene silencing in mESCs. However, this hypothesis has not been tested experimentally.

Endogenous RNAi in the soma

Little evidence is available for potential role of endo-siRNAs in the regulation of protein-coding mRNAs in mammalian somatic tissues. The natural antisense transcription in somatic cells, which has a potential to generate dsRNA, yields low levels of endo-siRNAs, whose biological relevance is questionable. At the same time, endo-siRNAs derived from natural antisense transcripts of *Slc34a* gene were identified in mouse kidney, where Na/phosphate cotransporter exerts its physiological function (Carlile et al., 2009). However, changes in expression levels of *Slc34a* upon suppression of the endo-siRNA pathway have not been addressed. In mouse hippocampus, deep sequencing revealed a set of potential endo-siRNAs generated from overlapping sense/antisense transcripts and from hairpin structures within introns of protein-coding genes (Smalheiser et al., 2011). The most abundant endo-siRNAs from *SynGAP1* gene locus were also found in complexes with AGO proteins and FMRP *in vivo*. Interestingly, a large part of potential hippocampal endo-siRNA targets encode for proteins involved in the control of synaptic plasticity and the number of endo-siRNAs derived from these gene loci increased significantly during olfactory discrimination training (Smalheiser et al., 2011). Given the fact that vast majority of identified endo-siRNA sequences mapped to intronic regions, the endo-siRNAs could act co-transcriptionally on nuclear pre-mRNAs, perhaps similarly to the mechanism of RNAi-mediated inhibition of RNA Pol II elongation described in *C. elegans* (Guang et al., 2010). Alternatively, endo-siRNAs could control correct distribution of target mRNAs as unspliced pre-mRNA can be exported from the neuronal nucleus and transported to dendrites for processing (Glanzer et al., 2005). In any case, these findings open an attractive hypothesis that endo-siRNAs participate in synaptic plasticity during learning process and the neuronal endo-siRNA pathway might be also linked to various neurodegenerative disorders (Smalheiser et al., 2011).

Antiviral RNAi?

In contrast to nematodes and insects, data supporting involvement of mammalian RNAi in antiviral defense is weak (reviewed in detail (Cullen, 2006; Cullen et al., 2013)). It is unlikely that RNAi substantially acts as an antiviral mechanism in mammals where long dsRNA induces a complex sequence-independent antiviral response, commonly known as the interferon response (reviewed in (Gantier and Williams, 2007)). Consistent with this, no siRNAs of viral origin have been found in human cells infected with a wide range of viruses (Pfeffer et al., 2005). Occasional observations, such as detection of a single siRNA in HIV-1 infected cells (Bennasser et al., 2005) does not provide any conclusive evidence that RNAi is processing viral dsRNA and suppresses viruses under physiological conditions *in vivo*.

It must be stressed that circumstantial evidence suggesting the role of RNAi in viral suppression must be critically examined and interpreted. One has to keep in mind, for example, that data, which appear as evidence for viral suppression by RNAi, could reflect miRNA-mediated effects. Since viruses co-evolve with different hosts and explore all possible strategies to maintain and increase their fitness, it is not surprising that viral reproductive strategies come into contact with mammalian RNA silencing pathways, particularly the miRNA pathway, which shares components with the RNAi pathway. For example, Epstein-Barr virus (EBV) and several other viruses encode their own miRNAs (Pfeffer et al., 2004; Pfeffer et al., 2005; Sullivan et al., 2005; Parameswaran et al., 2010) or take advantage of host cell miRNAs to enhance their replication (Jopling et al., 2005).

Another evidence for an interaction between viruses and RNA silencing is the presence of putative suppressors of RNA silencing (SRS) in various viruses. As viral genomes rapidly evolve, SRS should be functionally relevant. For example, B2 protein in Nodaviruses (e.g. FHV) is essential for replication, inhibits Dicer function, and B2-deficient FHV can be rescued by artificial inhibition of RNAi response (Li et al., 2002). B2 protein also enhances the accumulation of Nodaviral RNA in infected mammalian cells (Johnson et al., 2004; Fenner et al., 2006). Other potential SRS molecules have been identified in viruses infecting vertebrates, such as Adenovirus VA1 noncoding RNA (Lu and Cullen, 2004), Influenza NS1 protein (Li et al., 2004), Vaccinia virus E3L protein (Li et al., 2004), Ebola virus VP35 protein (Haasnoot et al., 2007), TAS protein in primate foamy virus (Lecellier et al., 2005), or HIV-1 TAT protein (Bennasser et al., 2005).

The existence of SRS in viruses infecting mammals does not prove that these viruses are targeted by mammalian RNAi. First, viruses may have a broader range of hosts (or vectors), including, e.g. blood sucking insects. Thus, a virus can be targeted by RNAi in one host and by another defense mechanism in another one. For example, the Dengue virus, whose life cycle takes place in humans and mosquitoes, is targeted by RNAi in mosquitoes and it likely evolved an adaptation to circumvent RNAi (Sanchez-Vargas et al., 2009). Second, viral SRS in mammalian cells may have other purpose than counteracting viral suppression by RNAi. Since biogenesis and mechanism of action of mammalian miRNAs overlaps with RNAi, it is possible that the role of such SRS is to modify cellular gene expression by suppressing the activity of miRNAs. Third, the main effect of SRS may be aimed at other defense mechanisms recognizing and responding to dsRNA and, as a consequence, SRS effects on RNAi are observed.

Systemic RNAi in mammals

Non-cell autonomous RNA with an extent similar to that of *C. elegans* or in some insects is highly unlikely to function in vertebrates. However, a limited environmental or systemic RNAi may exist there as the homologs of *sid-1* have been found in all sequenced vertebrate genomes (Jose and Hunter, 2007). Two *sid-1* homologs (SidT1 and SidT2) are present in mice and humans with a documented role for SidT1 in dsRNA uptake in humans (Duxbury et al., 2005; Wolfrum et al., 2007). Furthermore, experimental overexpression of human SidT1 significantly facilitated cellular uptake of siRNAs and resulted in increased RNAi efficacy (Duxbury et al., 2005). As it will be discussed later, the mammalian immune system employs a number of proteins responding to dsRNA independently of RNAi (Gantier and Williams, 2007), while RNAi does not seem to participate in the innate immunity (Cullen, 2006;

Cullen et al., 2013). Thus, the primary role of a dsRNA uptake mechanism in mammals is likely not involving RNAi even though it could have served such a role in an ancestral organism.

3.1.1.13 Nuclear function of small RNAs

The literature search yielded a large heterogeneous group of publications concerning nuclear localization of Dicer and AGO proteins as well as nuclear effects, including transcriptional gene silencing. Some of these observations might come from physiologically relevant nuclear silencing mechanisms. However, when critically evaluating published studies, not enough evidence was found, to establish a model for transcriptional silencing in mammals; except of the PIWI-induced transcriptional silencing in the germline (REF). Here, we will provide an overview of nuclear aspects of RNA silencing and highlight those observations which might be related to the miRNA pathway or long dsRNA response.

Homology-dependent phenomena and observations that may reflect nuclear mechanisms involving small RNAs can be sorted into several areas, which will be discussed further below:

Indirect effects of miRNAs on chromatin

Nuclear RNAi (nuclear post-transcriptional silencing)

Transcriptional regulations (stimulation/repression) by exogenous small RNAs

Transcriptional regulations (stimulation/repression) by miRNAs

27-nt RNA – mediated regulation of endothelial nitric oxide synthase gene

Splicing regulation by small RNAs

DNA repair-associated small RNAs

First, it is necessary to discuss nuclear localization of miRNA and RNAi factors, since their nuclear localization is a pre-requisite for nuclear silencing. Pioneering RNAi work indicated that silencing occurs in the cytoplasm because dsRNA against intronic sequences had no silencing effect (Fire et al., 1998) and the RISC complex co-purified with ribosomes (Hammond et al., 2000). Early studies in mammalian cells also suggested that RNAi is cytoplasmic (Billy et al., 2001; Zeng et al., 2002). However this notion was subsequently challenged by a series of studies reporting nuclear RNAi and small RNA-induced transcriptional silencing (Morris et al., 2004; Robb et al., 2005; Ting et al., 2005).

Nuclear localization of Dicer

A number of works directly or indirectly implies nuclear localization of Dicer (Haussecker and Proudfoot, 2005; Sinkkonen et al., 2010; Gullerova and Proudfoot, 2012; Ohrt et al., 2012; Doyle et al., 2013; Drake et al., 2014; Gagnon et al., 2014a; White et al., 2014; Neve et al., 2016), which contrasts with a recent *in vivo* study on mouse Dicer where tagging of an endogenous Dicer gene with an antibody epitope yielded exclusively cytoplasmic localization in all analyzed tissues with an extremely sensitive detection limit for nuclear Dicer (Much et al., 2016). Thus, despite the collection of the reports below, one should still approach the nuclear aspect cautiously. One of the earlier implications for nuclear localization of Dicer was the reported Dicer-dependent turnover of intergenic transcripts from the human beta-globin gene cluster (Haussecker and Proudfoot, 2005). However, this study showed mostly correlation of abundance of nuclear transcripts. Nuclear Dicer processing was also implicated by several other studies (Valen et al., 2011; Flemr et al., 2013; White et al., 2014; Neve et al., 2016). In terms of function, nuclear Dicer was thought to be involved in nuclear dsRNA processing (?) (White et al., 2014), selection of alternative polyadenylation sites (Neve et al., 2016) or rRNA processing (Liang and Croke, 2011). Several studies documented Dicer nuclear localization by microscopy. Dicer was detected in cultured mammalian cells with several different antibodies in the chromatin where it resided in rDNA clusters on acrocentric human chromosomes (Sinkkonen et al., 2010). Finer mapping using chromatin immunoprecipitation suggested that Dicer localizes in the proximity of the rRNA transcribed region (Sinkkonen et al., 2010). However, this study failed to reveal any functional significance of Dicer localization and it is not clear if the localization is related to the later reported role of Dicer in pre-rRNA processing (Liang and Croke, 2011) or to rDNA-derived small RNAs (Wei et al., 2013).

Furthermore, localization of Dicer to rDNA is distinct from the nuclear localization of Dicer detected by fluorescence correlation/cross-correlation spectroscopy (FCS/ FCCS) (Ohrt et al., 2012), since FCS/ FCCS can detect diffusing and not rDNA chromatin-bound Dicer. FCS/FCCS identified Dicer in the nucleus in HeLa cells and suggested that nuclear Dicer is alone while the cytoplasmic Dicer exists in a large complex with AGO2 (presumably RLC) (Ohrt et al., 2012). This would imply that while Dicer could process nuclear substrates, they would not be loaded on AGO proteins in the nucleus. However, a later study suggested that Dicer, TARBP2, AGO2 and GW182 associate into a large complex in the nucleus although, consistently with FCS/FCCS data, loading of nuclear small RNA duplexes was not detected (Gagnon et al., 2014a). Regarding the nuclear localization mechanism of Dicer, it does not employ a canonical nuclear localization signal (NLS). A pyruvate kinase fusion system suggested that dsRBP of Dicer could function as an NLS similarly to ADAR dsRBD (Doyle et al., 2013). However, this study did not prove nuclear localization of full-length Dicer under physiological conditions. Interestingly, an additional report implied phosphorylation of Dicer in nuclear localization in nematodes, humans and mice (Drake et al., 2014). Remarkably, one of the two reported phosphorylation sites for ERK was in the dsRBD, providing a hypothetical link to analysis of the study implying dsRBD in nuclear localization of Dicer. Yet, as mentioned above, EGFP tagging of Dicer in mice does not support nuclear localization of Dicer (Much et al., 2016). Taken together, nuclear localization/function of Dicer is still poorly understood and further research is needed to build a more coherent picture from the contradictory observations.

Nuclear AGO1-4

A similarly unclear situation exists for nuclear AGO1-4 proteins. AGO1-4 proteins were observed in the nucleus under different circumstances including immunofluorescent staining, such as, for example (Robb et al., 2005; Berezhna et al., 2006; Janowski et al., 2006; Kim et al., 2006; Ohrt et al., 2008; Rudel et al., 2008; Tan et al., 2009a; Aporntewan et al., 2011; Liang and Crooke, 2011; Ahlenstiel et al., 2012; Jang et al., 2012; Kim et al., 2012; Ohrt et al., 2012; Huang et al., 2013a; Nishi et al., 2013; Allo et al., 2014; Bai et al., 2014; Gagnon et al., 2014a; Sharma et al., 2016). Notably, one should be cautious about nuclear localization detected by antibodies and cellular fractionation in the absence of appropriate controls. While antibody cross-reactivity often cannot be excluded, biochemical fractionations suffer from impurities and endoplasmic reticulum contamination is frequently not examined. In any case, if there would be any consensus about nuclear AGO localization, it seems that a small fraction of AGO proteins is indeed in the nucleus and can engage complementary RNAs (Robb et al., 2005; Berezhna et al., 2006; Ohrt et al., 2008; Gagnon et al., 2014b). The mechanism of nuclear import of AGO proteins is unclear. AGO proteins do not carry a canonical NLS. It has been proposed that AGO proteins could be imported into the nucleus by GW182 (Nishi et al., 2013) or via Importin 8 (Weinmann et al., 2009).

The next section reviews effects of small RNA mechanisms in the nucleus, starting with indirect ones.

Indirect effects of miRNAs on chromatin

Studies of miRNA targets suggest that 10 to 30% of human genes are potential miRNA targets (Lewis et al., 2003; John et al., 2004). Also, experiments with delivering miRNAs into different cell types suggest that individual miRNAs can down-regulate a large number of genes (Lim et al., 2005). Thus, it is not surprising that many genes regulating chromatin structure are directly or indirectly regulated by miRNAs. One of the recent examples of such a connection is the regulation of DNA methylation in murine embryonic stem cells (ESCs). Phenotype analysis of Dicer^{-/-} cells revealed that the loss of Dicer in ES cells leads to defects in differentiation and it may (Kanellopoulou et al., 2005) or may not (Murchison et al., 2005) lead to aberrant changes in centromeric chromatin. It has been speculated that Dicer functions in a pathway similar to that of *Schizosaccharomyces pombe* (Kanellopoulou et al., 2005). This interpretation was consistent with previous analysis of chicken-human hybrid DT40 cell line lacking Dicer, where defects in heterochromatin were also observed (Fukagawa et al., 2004). However, cloning of small RNAs from WT and Dicer^{-/-} ES cells suggests that ES cells do not naturally produce endogenous siRNAs and that Dicer exclusively produces miRNAs (Calabrese et al., 2007).

Detailed analysis of the transcriptome of murine *Dicer*^{-/-} ES cells (Sinkkonen et al., 2008) correlated changes of gene expression with the presence of binding sites for AAGUGC-seeded miRNAs (miR-290 cluster and other miRNAs), which were previously found in human and murine ES cells (Suh et al., 2004; Houbaviiy et al., 2005). The loss of *Dicer* and miRNAs resulted in down-regulation of de novo DNA methyltransferases and defects in de novo DNA methylation during differentiation. This defect could be rescued by over-expressing de novo DNA methyltransferases or by transfection of the miR-290 cluster miRNAs (Sinkkonen et al., 2008). These data were complemented by the study of Benetti et al., who showed that the loss of *Dicer* leads to decreased DNA methylation, concomitant with increased telomere recombination and telomere elongation (Benetti et al., 2008).

Regulation of de novo DNA methyltransferases by miRNAs is likely much more complex than described above because other miRNAs were also implicated in their direct regulation in other cell types (Fabbri et al., 2007; Duursma et al., 2008). Importantly, the genetic background or culturing conditions can also influence epigenetic changes in studied cells, which might explain why a third study of *Dicer*-deficient ESCs observed normal DNA methylation dynamics (Ip et al., 2012).

In any case, it is very likely that miRNAs play a similar role in other aspects of chromatin formation. Considering that up to 60% of the genes are possibly regulated by miRNAs, data from experiments which directly or indirectly affect the miRNA pathway (including siRNA off-targeting), should be handled with open mind and great caution.

Post-transcriptional regulations by small RNAs in the nucleus – nuclear RNAi

In mammalian cells, Robb et al. showed that nuclear RNAs can be targeted by RNAi (Robb et al., 2005). In addition they also provided biochemical data showing that AGO1 and AGO2 localize into the nuclear RISC (Robb et al., 2005). While these data did not make a conclusive evidence as contamination of the nuclear fraction with AGO proteins associated with outer nuclear envelope could not be excluded, nuclear localization of AGOs was later backed up by other data (Berezhna et al., 2006; Ohrt et al., 2008; Gagnon et al., 2014b). Berezhna et al. showed that siRNAs accumulate in the nucleus in a cognate mRNA dependent manner (Berezhna et al., 2006). Ohrt et al. reported that siRNAs against firefly luciferase microinjected into HeLa cells enter nucleus but are actively excluded from non-nucleolar space in Exportin-5 dependent manner (Ohrt et al., 2006).

Taken together, loaded AGO2 seems to be able to engage nuclear RNAs and, in case of perfect complementarity, it can cleave its targets. How nuclear RNAi incorporates nuclear and cytoplasmic small RNA precursors and how it is working under physiological conditions on perfectly complementary and partially complementary targets remains largely unknown.

Transcriptional regulations (stimulation/repression) by exo-siRNAs

In plants and fungi, RNA silencing mechanisms also mediate transcriptional silencing. Similar transcriptional silencing phenomena were intensely searched also in mammals. The first two reports of transcriptional silencing in mammals were published in 2004 when two groups reported siRNA-mediated transcriptional silencing coupled with DNA methylation (Kawasaki and Taira, 2004; Morris et al., 2004) and laid problematic foundations of transcriptional silencing research in mammals. The reason is that reproducibility of both reports was quickly questioned and one of them was later forced retracted, formally because “a proper data notebook is not available as evidence to support our findings, which constitutes non-adherence to ethical standards in scientific research. In accordance with the recommendations from the National Institute of Advanced Industrial Science and Technology, K.T. therefore wishes to retract this paper.” (Taira, 2006). While the second report has not been retracted, DNA methylation has been doubted as a key silencing effect (Ting et al., 2005). Furthermore, the report did not analyse induction of DNA methylation by informative bisulfite sequencing but relied on suboptimal methylation-sensitive restriction digest. Another troubling aspect of the second report is a technically impossible transcriptional silencing experiment shown in Fig. 1B, which raises a question how careful was the peer reviewing process. In any case, transcriptional misregulation by exogenous oligonucleotides complementary to promoter sequences has been reported by different laboratories for different promoters in different cells arguing that some

complementarity-based transcriptional regulation by small RNAs functions in mammalian cells. Importantly, the underlying mechanism was not conclusively revealed after a decade and is still a matter of debates.

The siRNA-induced transcriptional silencing involves changes in the chromatin structure such as loss of “active” histone modifications (H3K4 methylation, histone acetylation) (Morris et al., 2004; Janowski et al., 2006), appearance of “inactive” histone modifications (H3K9 and H3K27 methylation) (Castanotto et al., 2005; Janowski et al., 2006; Kim et al., 2006; Weinberg et al., 2006; Kim et al., 2007; Jiang et al., 2012), and occasionally DNA methylation (Morris et al., 2004). These are all common features of transcriptional repression and could be either directly induced by siRNA-containing complexes or they be a consequence of transcriptional silencing. DNA methylation is apparently a secondary effect (Ting et al., 2005) although a systematic analysis revealed a group of gene promoters whose methylation was dependent on Dicer (Ting et al., 2008).

Small RNAs used for silencing were either “classical” synthesized siRNAs (agRNAs) (Morris et al., 2004; Castanotto et al., 2005; Janowski et al., 2005a; Ting et al., 2005; Kim et al., 2007; Hawkins et al., 2009; Napoli et al., 2009; Ahlenstiel et al., 2012; Jiang et al., 2012) or shRNAs expressed from a plasmid (Castanotto et al., 2005; Kim et al., 2007). Importantly, transcriptional silencing could be induced by a variety of antisense oligomers targeting promoter sequences such as single-stranded antigene (ag) peptide nucleic acid (PNA) (Janowski et al., 2005b), PNA-peptide conjugates (Hu and Corey, 2007), locked nucleic acid (LNA) (Beane et al., 2007) or duplex RNA (siRNA) oligos (Janowski et al., 2005a). These results represent a remarkably comprehensive set of data concerning inhibition of human progesterone receptor A (hPR-A) and B (hPR-B) isoforms. Despite all approaches achieved inhibition of gene expression, these silencing oligonucleotides have radically different properties and it is questionable whether they would all operate loaded on an AGO protein in a RISC-like complex.

Furthermore, studies of oligonucleotides targeting promoter sequences revealed that some oligonucleotides have a positive effect on transcription (Li et al., 2006; Janowski et al., 2007). The activating effect of small RNAs also appeared later in other reports (Hu et al., 2012; Zhang et al., 2014; Wang et al., 2015a). “Scanning” a promoter with oligonucleotides revealed the existence of sites whose targeting results in transcriptional repression as well as sites whose targeting promotes expression (Janowski et al., 2005a; Janowski et al., 2007). According to these data, a shift by several nucleotides could have an opposite effect on gene expression that would correlate with changes in histone modifications. These data would imply existence of a still-unknown RISC-like nuclear complex acting as a switch or siRNA strand-selection onto a single RISC-like complex, where the opposing effects would stem from targeting sense and antisense RNAs in the locus. Alternatively, the observed silencing stems from the oligonucleotide binding and is not mechanistically involving AGO proteins.

To date, the active agent of the silencing has not been conclusively identified and critically assessed. It still remains a question whether there is a dedicated transcriptional silencing machinery in mammalian cells involving AGO proteins and some binding partners as proposed by some reports (Janowski et al., 2006; Kim et al., 2006; Suzuki et al., 2008; Hawkins et al., 2009; Hu et al., 2012; Cho et al., 2014), which are partially contradicting themselves and are not supported by proteomic analysis of AGO complexes (Meister et al., 2005; Hock et al., 2007; Hauptmann et al., 2015), or we are looking at various artificial disturbances of lncRNAs resulting in the observed effects.

Transcriptional regulations (stimulation/repression) by miRNAs

A peculiar phenomenon of miRNA-associated transcriptional activation was reported from cultured mammalian cells for the E-cadherin and cold-shock domain-containing protein C2 (CSDC2) promoters and the miR-373 (Place et al., 2008). The initial observation leading to discovery of this phenomenon was that transfection of siRNAs homologous to E-cadherin, p21WAF1/CIP1, and VEGF promoters lead to unexpected transcriptional activation (Li et al., 2006). Subsequent sequence analysis of the E-cadherin promoter revealed high complementarity between the miR-373 and the sequence at position -645 relative to the transcription start site (Place et al., 2008). Delivery of miR-373, pre-miR373 and a synthetic siRNA sequence targeting -640 position could stimulate E-cadherin expression by ~5-7-fold in PC-3 cells. Interestingly, intact miRNA pathway was required for the E-cadherin stimulation as

partial down-regulation of Dicer protein by morpholino oligonucleotides abolished the stimulatory effect of pre-miR-373. Since then, several other reports of miRNA-mediated transcriptional silencing appeared, including transcriptional inhibition of *HOXD4* expression by miRNA-10a (Tan et al., 2009b), miR-320-mediated transcriptional silencing of *POLR3D* (Kim et al., 2008), and others (Kim et al., 2011; Adilakshmi et al., 2012; Benhamed et al., 2012)

It remains an open question whether effects of exogenous siRNAs represent the same molecular mechanism as those attributed to nuclear activities of miRNAs. It would be supported by non-cleaving AGO1 implicated in transcriptional regulation (Janowski et al., 2006; Kim et al., 2006; Huang et al., 2013a) and transcriptional silencing by miRNA mimics targeting gene promoters (Younger and Corey, 2011). In terms of the possible miRNA-mediated nuclear silencing, it was reported that *POLR3D* silencing involves miRNA-mediated promoter association with a complex including AGO1 and EZH2 (H3K27 histone methyltransferase) (Kim et al., 2008). It was also suggested that AGO1 interacts with polymerase II (Kim et al., 2006; Huang et al., 2013a). At the same time, others implicated GW182 as the factor important for nuclear localization and function of nuclear AGO proteins (Nishi et al., 2013; Nishi et al., 2015). However, the mechanistic link between AGO-GW182 and histone modifications still remains elusive.

27nt RNAs – mediated regulation of endothelial nitric oxide synthase gene

This phenomenon has been observed in endothelial nitric oxide synthase (eNOS) where a 27nt repeat polymorphism in intron 4 was a source of predominantly nuclear 27nt small RNAs, which could be either a novel class of small RNAs, or atypical miRNAs (Zhang et al., 2008b). In any case, these 27nt RNAs were implicated in eNOS suppression by altering histone acetylation and DNA methylation in regions adjacent to the 27nt repeat element and core promoter (Zhang et al., 2008a). Whether this phenomenon is related to other ones described here remains unclear.

Regulation of splicing

In addition to transcriptional and post-transcriptional silencing, one of the reported effects of small RNAs transfected into mammalian cells was also an impact on alternative splicing (Allo et al., 2009). A mechanism was proposed, which involves AGO1 recruitment to transcriptional enhancers (Allo et al., 2014), while other reports implicated AGO2 (Liu et al., 2012b; Liu et al., 2015) or both (Ameyar-Zazoua et al., 2012).

Small RNAs associated with DNA repair

The last nuclear phenomenon associated with small RNAs is their emergence upon DNA damage. It was reported that DICER and DROSHA-dependent small RNAs emerge as DNA-damage response and are functionally associated with it through the MRE11 complex (Francia et al., 2012). In addition, knock-down of Dicer or AGO2 in human cells reduced double-stranded break repair (Wei et al., 2012). It was proposed that small RNAs emerging from DNA –damage loci may function as guide molecules directing chromatin modifications or the recruitment of protein complexes to facilitate repair (Francia et al., 2012; Wei et al., 2012). What is somewhat confusing in DNA-repair associated small RNAs is the role of miRNA-specific factors Drosha (Francia et al., 2012) or DGCR8 (Swahari et al., 2016).

Despite the heterogeneity of the nuclear effects and many unknowns, some common themes emerged, allowing for formulating testable hypotheses that could be critically evaluated. First, nuclear effects can be mediated by small RNAs provided in trans. Second, small RNAs recruit AGO proteins in a sequence-specific manner, most likely recognizing a local transcript (perhaps an ncRNA). Third, the effect involves a change in the chromatin structure. Thus, by carefully examining essential exogenous siRNA properties in previously reported nuclear effects, one should be able to demonstrate that the silencing phenomenon truly involves an AGO-loaded small RNA engaging another nuclear RNA and whether the effect requires the “slicer” activity. Detailed examination of the seed sequence would also discern between specific nuclear effects and off-targeting. Furthermore, should the effect involve small RNA loaded AGO protein, the kinetics of the phenomenon should be in agreement with known RISC kinetics discussed above. Finally, if the aforementioned phenomena rely on localized recruitment

of AGO-loaded small RNAs, one should be able to mimic those effects by tethering AGO proteins through sequence-specific DNA binding modules such as those employed by TALEN or CRISPR nucleases. These research directions should be combined with validated antibodies for chromatin immunoprecipitation and immunofluorescence (or epitope knock-in into candidate genes), more extensive use of mutants defective in RNA silencing, detailed quantitative analysis of cellular fractionation and identifications of interacting partner, studies of putative nuclear import and export signals of Dicer and AGO proteins, and advanced imaging techniques.

3.1.1.14 **Other dsRNA-associated mechanisms I – dsRNA sensing in the interferon pathway**

Long dsRNA is not a usual RNA molecule in eukaryotic cells while RNA viruses produce dsRNA during replication. A common mechanism repressing viruses in non-vertebrate species is RNA silencing (Wilkins et al., 2005; Wang et al., 2006). However, response to foreign long dsRNA in mammals is much more complex and involves a set of sequence-independent sensors triggering expression of a defined set of genes known as interferon-stimulated genes (ISGs). The interferon pathway is the most ubiquitous sequence-independent pathway induced by dsRNA in mammalian cells (reviewed in detail in de Veer et al., 2005). Among the relevant sensors recognizing cytoplasmic dsRNA are protein kinase R (PKR), the helicase RIG-I, MDA5, 2',5'-oligoadenylate synthetase (2',5'-OAS), or Toll-like receptors (TLR3, 7, 8) (reviewed in Gantier and Williams, 2007; Sadler and Williams, 2007). Notably, there are also dsRNA-independent mechanisms that can activate interferons in mammalian cells. Altogether, different stimuli are being sensed and converge on activation of overlapping but distinct sets of ISGs (Geiss et al., 2001). The situation is even more convoluted by cellular diversity as some cell types, particularly immune cells, can elicit the interferon response by additional, cell-type-specific pathways (reviewed in Schlee and Hartmann, 2010).

PKR

PKR is the oldest known mammalian dsRNA sensing protein. A pioneering work by Hunter et al. showed that different types of dsRNA can block translation in reticulocyte lysates (Hunter et al., 1975). Analysis of the phenomenon identified PKR that is activated upon binding to dsRNA and blocks translation by phosphorylating the alpha subunit of eukaryotic initiation factor 2 (eIF-2 α) (Meurs et al., 1990). Activation of PKR also includes activation of the NF κ B transcription factor and a large number of interferon-stimulated genes (ISGs) (Geiss et al., 2001). PKR response to viral dsRNA can be coordinated with other dsRNA sensors, such as RIG-I and MDA5 (Sen et al., 2011). PKR can also respond to endogenous RNAs in unique physiological regulations (Bevilacqua et al., 1998; Bommer et al., 2002). However, endogenously expressed long dsRNA does not necessarily induce canonical PKR response with interferon activation, although PKR binding to dsRNA and restricted translational repression can be observed (Nejepinska et al., 2012b; Nejepinska et al., 2014). It was believed that dsRNA <30-bp in length does not induce PKR. However, Marques et al. reported that, siRNAs can bind and activate PKR *in vitro* regardless of siRNA termini (Marques et al., 2006) arguing against the long-established 30-bp length as the minimal size-limit for PKR activation. There are also other data indicating sensitivity of PKR to dsRNA motifs shorter than 30-bp (Zheng and Bevilacqua, 2004; Puthenveetil et al., 2006; Reynolds et al., 2006).

RIG-I-like receptors (RIG-I, MDA5, LGP2)

Mammalian somatic cells can respond to dsRNA in a sequence-independent manner. In addition to PKR, several other proteins recognizing dsRNA are integrated to the interferon response, including helicases RIG-I (retinoic-acid-inducible gene-I, also known as DDX58), MDA5 (IFIH1), and LGP2 (DHX58), which sense cytoplasmic dsRNA and activate interferon expression.

RIG-I is a cytoplasmic sensor differentiating between endogenous and foreign RNAs structures. In particular, RIG-I is activated by blunt-ended dsRNAs with or without a 5'-triphosphate, by single-stranded RNA marked by a 5'-triphosphate, and by polyuridine sequences. RIG-I domains organize into a ring around dsRNA, capping one end, while contacting both strands; the structure is consistent

with dsRNA translocation without unwinding and cooperative binding to RNA (Jiang et al., 2011a; Jiang et al., 2011b). Like RIG-I and LGP2, MDA5 preferentially binds dsRNA with blunt ends (Li et al., 2009a). RIG-I, MDA5, and LGP2 exhibit differences in recognizing specific RNA structures and different types of viruses providing a broader range of coordinated sensitivity do different potential threats (Kato et al., 2006; McCartney et al., 2008; Li et al., 2009b; Slater et al., 2010; Sen et al., 2011; Wu et al., 2015b). Interestingly, RIG-I can become activated also with siRNAs lacking 2-nt 3' overhangs (Marques et al., 2006). These data imply that 2-nt 3' overhangs generated by Dicer are the structural basis for discriminating between Dicer products and other short dsRNA. Roles of MDA5 and LGP2 in siRNA-mediated interferon response remains to be addressed. Furthermore, recognition 5' triphosphate RNA ends RIG-I (Hornung et al., 2006; Pichlmair et al., 2006) highlights importance of appropriate processing of 5' termini of RNAs produced by phage polymerases when such RNAs are used in mammalian cells.

It is not clear how PKR and RIG-I pathways are integrated. RIG-I binds siRNAs (with or without 2-nt 3' overhangs) *in vitro* and it shows greater unwinding of blunt-ended siRNAs. Unwinding is then translated into the interferon activation mediated via IRF-3.

Toll-like Receptor 3 (TLR3)

TLR3 is a member of the Toll-like receptor (TLR) family and functions as a sensor of extracellular, intracellular and viral dsRNAs (Yang et al., 2006b; Amarante et al., 2011; Seo et al., 2013; Wang et al., 2015b; Wu et al., 2015b). TLR3 has distinct or complementary roles to RIG-I and related helicases in sensing foreign molecules and activating downstream responses (Livengood et al., 2007; McCartney et al., 2009; Slater et al., 2010; Wu et al., 2015b).

Oligoadenylate Synthetase (OAS)

Interferon and dsRNA also activate 2',5'-oligoadenylate synthetase (2',5'-OAS) that produces 2',5' oligoadenylates with 5'-terminal triphosphate residues that subsequently induce activation of RNase L; a protein responsible for general RNA degradation (de Veer et al., 2005).

TARBP2 and PACT

Interactions between RNAi, miRNA, and interferon response are poorly understood. There are two clear mechanistic connections between these two pathways. First, TARBP2 and PACT, two dsRNA binding proteins, which were mentioned earlier as Dicer-interacting proteins, interact also with PKR. Notably, while TARBP2 inhibits PKR (Park et al., 1994; Cosentino et al., 1995), PACT has the opposite role (Patel and Sen, 1998). While cytoplasmic long dsRNA in somatic cells apparently triggers the interferon response, it is not clear if the same dsRNA is also routed into the RNAi pathways. Experiments in oocytes and undifferentiated embryonic stem cells (Yang et al., 2001; Stein et al., 2005) suggest that RNAi dominates response to cytoplasmic long dsRNA in the absence of a strong interferon response and that the interferon pathway dominates when its relevant components are present. On the other hand, this view may be too simplistic as it does not explain the lack of both, RNAi and interferon response, in somatic cells expressing long dsRNA (Nejepinska et al., 2012b; Nejepinska et al., 2014). In any case, understanding the role of TARBP2 and PACT isoforms in routing long dsRNA into RNAi and interferon pathways requires further studies.

There is a clear evolutionary connection between RNAi and interferon response. The above-mentioned mammalian RNA helicases RIG-I, LGP2 and MDA5 are the closest homologs of helicases involved in processing of long dsRNA during RNAi in *C. elegans*. Notably, RIG-I is an established component of the interferon response to long dsRNA (Yoneyama et al., 2004). This suggests that the interferon response, which has a common trigger and evolved after the RNAi pathway, adopted several components from the latter pathway. It remains to be determined whether these and other components of RNAi lost their function in RNAi entirely or mediate some form of a cross-talk between RNAi and interferon response.

Finally, there is also a complex relationship between miRNA and interferon pathways (Xu et al., 2011; Ostermann et al., 2012; Shapiro et al., 2014; Ingle et al., 2015). One connection is exemplified by viral miRNAs, which viruses use to regulate the host response, in particular factors of the interferon pathway (Ostermann et al., 2012) or other cellular signalling (Xu et al., 2011). However endogenous cellular miRNAs may also act to suppress the interferon response factors, such as the case mir-485, which has a dual role in targeting RIG-I as well as the influenza virus H5N1 (Ingle et al., 2015).

3.1.1.15 Other dsRNA-associated mechanisms II – Adenosine deamination

A-to-I editing is a covalent RNA modification system of broad significance (reviewed in Nishikura, 2016). It is mediated by adenosine deaminases acting on RNA (ADARs), enzymes that carry two or three dsRBD and recognize both inter- and intramolecular dsRNAs longer than 20-30 bp (Nishikura et al., 1991). ADARs convert adenosines to inosines, which base pair with cytosines, which are interpreted as guanosines during translation. Thus, RNA editing affects coding potential, fidelity of RNA replication reverse transcription, or formation/stability of RNA secondary structures where a change of a single base in a sequence may result either in dsRNA destabilization (inosine-uridine pair) or stabilization (inosine-cytidine pair) (Nishikura, 2010). Such transition in the local and global stability of dsRNA structure can influence further processing of dsRNA, such as the selection of the effective miRNA strand (Bartel, 2004; Meister and Tuschl, 2004).

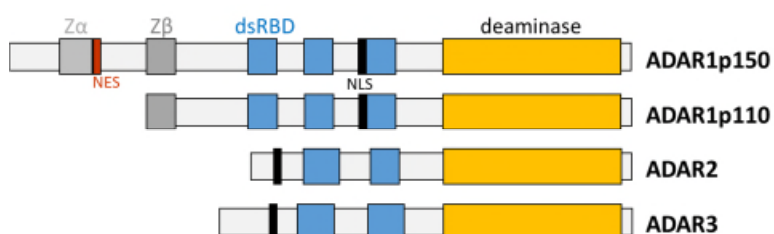


Figure 28 Domain composition of mammalian ADAR proteins

NES, nuclear export signal; NLS, nuclear localization signal; dsRBD, dsRNA binding domain.

Mammals (and vertebrates in general) have three ADAR genes (reviewed in Nishikura, 2016) (Figure 28). Two encode proteins carrying deaminase activity: ADAR1, which is interferon-inducible, and ADAR2, which is constitutively expressed. ADAR3 is mostly expressed in the brain but its editing activity has not been shown yet. The specificity of the ADAR1 and ADAR2 deaminases ranges from highly site-selective to non-selective, dependent on the duplex structure of the substrate RNA.

The complete ADAR structure has not been solved yet but structure of several domains is known - the Z alpha domain of the human editing enzyme ADAR1 (Schwartz et al., 1999) and dsRBDs of ADAR2 (Steffl et al., 2010). The analysis of dsRBDs provided an insight into editing of a specific substrate and revealed that dsRBDs of ADAR not only recognize the shape but also the sequence of the dsRNA (Steffl et al., 2010). The unexpected direct readout of the RNA primary sequence by dsRBDs is achieved via the minor groove of the dsRNA and this recognition is critical for both editing and binding affinity of edited RNA (Steffl et al., 2010). It was also shown that ADAR2 forms dimers *in vivo* and that dsRBDs are necessary and sufficient for dimerization of the enzyme (Poulsen et al., 2006).

ADARs exhibit complex regulation of localization. For example, it was shown that mouse ADAR1 isoforms are differentially localized in cellular compartments and that their localization is controlled by several independent signals, which include a nuclear localization signal (NLS), the nucleolar localization signal (NoLS), the nuclear exporter signal (NES) near the N terminus (Nie et al., 2004). ADAR1 interacts with TUDOR-SN nuclease (Scadden, 2005; Yang et al., 2006a; Nishikura, 2010; Weissbach and Scadden, 2012) and localizes to stress granules upon stress induction (Weissbach and Scadden, 2012) while tudor-SN degrades hyperedited dsRNA (Scadden, 2005).

RNA editing concerns a broad range of RNAs including viral and cellular RNAs. Many long perfect dsRNAs (>100 bp) undergo extensive editing with a conversion of approximately 50 % of adenosines to inosines (Nishikura et al., 1991; Polson and Bass, 1994). Extensive editing (hyperediting) is linked with nuclear retention (reviewed in DeCervo and Carmichael, 2005). On the other hand, short RNAs (~20-30 bp) or imperfect long dsRNAs are edited selectively; usually only a few adenines at specific

sites are deaminated (Lehmann and Bass, 1999). High throughput analyses revealed the extent of RNA editing of mammalian RNAs in terms of substrate diversity and frequency of editing in the transcriptome (Carmi et al., 2011; Peng et al., 2012). Edited endogenous RNAs (Morse et al., 2002; Dawson et al., 2004; Hundley et al., 2008; Salameh et al., 2015) include mRNAs, repetitive sequences (mainly Alu (Athanasiadis et al., 2004)), and miRNAs. It was predicted that more than 85% of pre-mRNAs may be edited, predominantly in the non-coding regions (Athanasiadis et al., 2004).

Several pri-miRNAs (e.g. miR-142) are known to undergo editing, which inhibits Drosha cleavage or even causes degradation of pri-miRNA by Tudor SN (Scadden, 2005; Yang et al., 2006a; Nishikura, 2010). In other cases, pri-miRNA editing does not influence Drosha activity but inhibits processing of pre-miRNA by Dicer (e.g. miR-151) (Kawahara et al., 2007a). Last but not least, RNA editing might also inhibit export of miRNAs from the nucleus (Nishikura, 2010). A systematic analysis of edited miRNAs in the human brain showed that editing of miRNAs affects several miRNAs but it is not widespread (Alon et al., 2012). A similar picture was obtained from analysis of embryonic miRNAs (Vesely et al., 2012; Garcia-Lopez et al., 2013)

One of the roles of ADARs in immunity is to prevent innate immune sensing of self-RNA (Heraud-Farlow and Walkley, 2016). ADARs also affect viral RNAs in various ways – ADARs are both antiviral and proviral; the effect on virus growth and persistence depends upon the specific virus. (Samuel, 2011). Viruses targeted by ADARs in mammals include HIV (Clerzius et al., 2009), herpesvirus (Gandy et al., 2007), HRSV (Martinez and Melero, 2002), HCMV (Nachmani et al., 2014), VSV (Nie et al., 2007), and HDV (Wong and Lazinski, 2002).

Crosstalk between RNA editing and other dsRNA pathways.

ADARs affect other dsRNA pathways in several ways. In RNA silencing, ADARs can compete with RNAi for dsRNA substrates (including siRNAs). The ADAR1 isoform (ADAR1p150) strongly binds siRNA and reduces thus the availability of dsRNA for RNAi, resulting in less efficient RNAi in normal cells compared to *Adar1*^{-/-} cells (Yang et al., 2005). Interestingly, injection of high doses of siRNAs enhances ADAR1 expression, suggesting a role of ADAR1 in a cellular feedback mechanism in response to siRNA (Hong et al., 2005).

Editing affects base pairing quality of dsRNA substrates as well as target recognition since a single nucleotide mismatch between siRNA and target mRNA can reduce RNAi efficacy (Scadden and Smith, 2001) or modify target specificity, especially when occurring in the seed sequence (Kawahara et al., 2007b). MiRNAs would be affected in a similar way. A moderate deamination (one I-U pair per siRNA) does not prevent Dicer processing to siRNAs (Zamore et al., 2000) but, hyperediting (~50 % of deaminated adenosines) can make dsRNA resistant to Dicer processing (Scadden and Smith, 2001).

Thus, ADARs are factors conferring to formation of RNAi resistance (Hong et al., 2005), which may be one of the viral strategies to avoid being targeted through a dsRNA-responding pathway (Zheng et al., 2005). ADARs influence the innate immunity either indirectly by preventing sensing of self-RNA (Heraud-Farlow and Walkley, 2016) or by interacting with innate immunity factors, such as PKR (Clerzius et al., 2009). The immunosuppressive role of ADAR1 could explain the phenotype of the Aicardi-Goutieres syndrome (AGS, OMIM# 225750), an autoimmune disorder caused by ADAR1 mutations (Rice et al., 2012). It has been proposed that in the absence of ADAR1, accumulation of cytoplasmic dsRNA may provoke interferon signalling and cause upregulation of interferon-stimulated genes, which is observed in AGS (Rice et al., 2012).

3.1.1.16 Summary

The miRNA pathway seems to be the dominant small RNA pathway in the soma while the existence and functionality of endogenous RNAi remains unclear. The only cell type with well documented and mechanistically characterised endo-RNAi is the mouse oocyte. Somatic cells typically respond to long dsRNA with a sequence-independent interferon response, which is employing multiple dsRNA sensors, which trigger a complex interferon response.

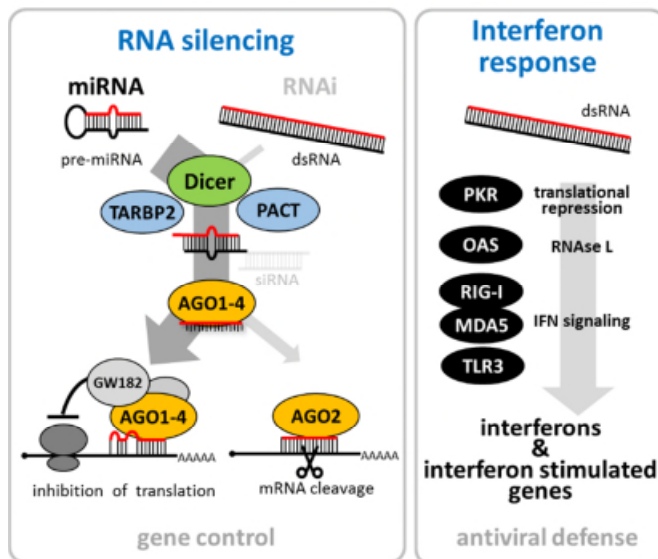


Figure 29 Overview of mammalian RNA silencing and dsRNA response pathways

3.1.1.17 References

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3.1.2. Birds

Birds (*Aves*) belong together with mammals and fishes to the group *Craniata* within chordates. Birds are essentially feathered reptiles as they are direct descendants of dinosaurs. There are ~9000 bird species, some of which are of high economic importance (food industry) or medical relevance (viral vectors causing zoonoses). Bird ancestors branched off mammalian ancestors over 300 MYA when the synapsid lineage leading to mammals branched off the sauropsid lineage leading to dinosaurs and birds. During their evolution, birds evolved numerous physiological adaptations in which they differ from mammals, including feathers, shelled eggs with external development, or different sex chromosome system, to name a few. At the same time, they are the closest mammal-related group covered in this report, in terms of synteny and sequence similarity. This is helpful for assessing features of dsRNA and miRNA pathways. The available literature on RNA silencing in birds is limited but some of the facts can be deduced from the genomic data in the public domain.

In general, miRNA, RNAi and other dsRNA-responding pathways in birds are very much like those in mammals. The literature does not report an important bird-specific feature in RNA silencing pathways.

3.1.2.1 Dicer

According to the complete genome sequences of chicken and Zebra Finch, birds have one Dicer protein. Chicken Dicer has been assigned to the chromosome 5 according to the radiation hybrid mapping (Tian et al., 2007) which is in agreement with the current chicken genome map. There is no detailed analysis of avian Dicer specificity and activity, which have to be inferred indirectly from other results. Chicken Dicer can process both, long dsRNA and miRNA precursors, as evidenced by induction of RNAi with long dsRNA (Pekarik et al., 2003; Mauti et al., 2008) and hundreds of avian miRNAs in the miRBase.

The common Dicer product size seems to be 21-23nt with a typical size of 22nt. This information can be inferred from available miRBase data (Figure 30).

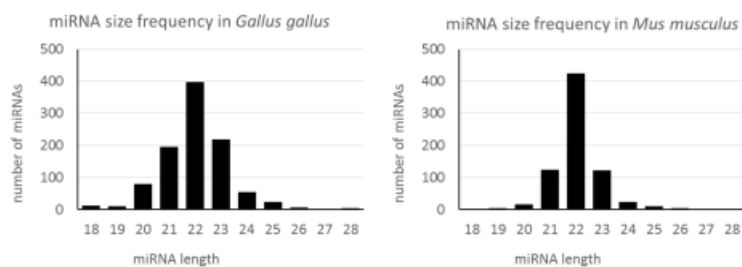


Figure 30 Avian miRNA lengths

The left graph depicts size distribution of all 994 chicken miRNAs deposited in the miRBase (release 21). For comparison, the right graph shows size distribution of 721 high-confidence murine miRNAs.

Thus, the avian Dicer produces small RNAs with the same sizes as the mammalian Dicer (Figure 30). Another possible substrate of Dicer in birds might be snoRNAs, although the biological significance of this observation remains unclear (Taft et al., 2009).

It is unclear if there are functionally different avian Dicer isoforms as is the case in murine oocytes and somatic cells (Flemer et al., 2013). There is one report of different Dicer splice variant in goose (*Anser cygnoides*) where one variant lacks a linker between DEAD box and helicase C domains at the N-terminus (gDicer-b) (Hu et al., 2014). The shorter isoform gDicer-b is present in multiple tissues, however its functional significance is unclear. The truncation is found in the N-terminus, which is associated with substrate selectivity and efficient processing. Therefore, one might speculate about some functional divergence in substrate processing between the two isoforms. However, there is no experimental evidence at the moment. The only available data, so far, concern cloning of the short isoform and expression analysis of several tissues and follicular stages by RT-PCR (Hu et al., 2014).

3.1.2.2 dsRBPs

dsRBP binding partners of Dicer have not been studied, so far. Interestingly, the chicken genome contains a dsRBP, which is related to TARBP2 and PACT, suggesting a more ancestral vertebrate state and a reduced crosstalk between RNAi and the interferon pathway.

3.1.2.3 Argonaute proteins

Argonaute family proteins are effectors of RNA silencing mechanisms. They are divided into two subfamilies: AGO proteins, which accommodate miRNAs and siRNAs, and PIWI proteins, which accommodate piRNAs. Avian AGO proteins have not been characterized in a published report but public chicken genome data show that the setup is the same as in mammals: Studies in chicken revealed four AGO proteins, where AGO1, 3, and 4 are encoded within one locus on chromosome 23 and AGO2 is encoded separately on chromosome 2. This arrangement appears to be shared within mammals and birds (Zhou et al., 2010). Additional information about avian AGOs can be inferred indirectly from the existence of functional RNAi and miRNA pathways (discussed below), which implies that at least one AGO protein is a “slicer” (presumably AGO2, given its conserved role as a slicer from *Drosophila* to mammals). Avian AGO proteins can also mediate post-transcriptional silencing guided by imperfectly base paired miRNAs.

In addition, there were two publications found, which mention avian PIWI proteins, which primarily control genome integrity in the germline and are not within the scope of this report (Kim et al., 2012; Lim et al., 2013).

3.1.2.4 Other factors

Birds have additional proteins involved in other dsRNA responses, which are either associated with adenosine deamination (Herbert et al., 1995) or interferon response. Interferon response factors, which recognize some form of dsRNA and are also found in mammals, include MDA5 (Lee et al., 2012; Hayashi et al., 2014; Lee et al., 2014), RIG-I (Li et al., 2014a; Chen et al., 2015; Xu et al., 2015), and PKR (Gonzalez-Lopez et al., 2003; Zhang et al., 2014; Lostale-Seijo et al., 2016). Interestingly, chicken lack the RHA/DHX9 homolog (Sato et al., 2015). The antiviral response to dsRNA will be discussed further below in the section 3.1.2.7. Other dsRNA response pathways.

3.1.2.5 miRNA

According to miRBase, bird genomes encode hundreds of miRNAs:

species [genome annotation]	miRNA precursors	mature miRNA
<i>Gallus gallus</i> [Gallus-gallus-4.0]	740	994
<i>Taeniopygia guttata</i> [taeGlu3.2.4]	247	334

During the systematic literature review, miRNA-related publications lacking a mechanistic molecular insight into the miRNA pathway were the most common class of annotated publications for birds (~50% of all selected publications). These publications fall into four basic categories:

- annotations of novel miRNAs, including high-throughput expression analyses (for example (Taft et al., 2009; Luo et al., 2012; Godnic et al., 2013) and many others). This category also includes the original chicken and Zebra Finch genome annotation papers (International Chicken Genome Sequencing, 2004; Warren et al., 2010).
- studies of miRNAs in different biological contexts, including reproduction (Lee et al., 2011; Lee et al., 2015), skeletomuscular apparatus (Chen et al., 2009a), bird song physiology (Gunaratne et al., 2011), growth/weight gain (Li et al., 2013), and many others; their comprehensive listing would be beyond the scope of this report.
- studies of relationship between miRNAs and the immune system, especially antiviral – these will be discussed further below in the section 3.1.2.7. Other dsRNA response pathways
- false positives of the search- reports describing mRNA knock-down through short hairpin RNAs adopting miRNA-like appearance. There is a series of nearly identical methodological papers, apparently published twice in 2006 and 2013, which fall in this category (Lin et al., 2006a; Lin et al., 2006b; Lin and Ying, 2006; Ying and Lin, 2009; Ying et al., 2010; Lin et al., 2013a; Lin et al., 2013b; Lin and Ying, 2013; Deng et al., 2015) and several other publications concerning

development and adaptations of shRNA systems (e.g. Das et al., 2006; Chen et al., 2011; Andermatt et al., 2014). These articles actually belong to the RNAi section below but due to the confusing use of nomenclature, they would also fall into the miRNA category.

Taken together, essentially all miRNA-related publications dealt with miRNA annotation, analysis of biological functions of miRNAs, and adoption of miRNAs for artificial knock-down systems allowing for suppressing any gene of interest. Avian miRNA-related publications did not reveal any avian-specific mechanistic insight into miRNA biogenesis, in which birds would differ from the general consensus for mammals, or other vertebrates in general. The complete list of all miRNA-related publications is available in a library accompanying this section.

3.1.2.6 RNAi

Avian RNAi-related literature deals mainly with experimental knock-down of gene expression, which does not reveal much about the physiological role of RNAi pathway in birds. These studies cannot all be included in the report due to the high number, but they are available in the reference library accompanying this section). What can be inferred from those studies is that birds have the complete molecular mechanisms for canonical RNAi and can efficiently execute it. This is evidenced by efficient knock-downs with long dsRNA (Pekarik et al., 2003; Mauti et al., 2008).

Published exogenous RNAi data provide insights into possible routes nucleic acids can become biologically active in birds and concern areas of EFSA main interests as various forms of RNAi technology (siRNAs or transgenic) were considered a way for preventing virulent strain circulation in poultry (O'Neill, 2007) although results of these efforts were relatively modest, being typically developed in cultured cells (Yin et al., 2010; Stewart et al., 2011; Hutcheson et al., 2015; Sahare et al., 2015). Exogenous RNAi in vivo required non-physiological manipulations such as 1) plasmid or siRNA electroporation (Pekarik et al., 2003; Sato et al., 2004; Baeriswyl et al., 2008; Mauti et al., 2008; Wilson and Stoeckli, 2011, 2012; Andermatt et al., 2014), 2) transfection (Dai et al., 2005; Lin et al., 2006a; Lin et al., 2013a; Wei et al., 2015), 3) recombinant virus (Lambeth et al., 2009b), or 4) recombinant lentivirus delivery (Haesler et al., 2007; Chen et al., 2009b). Altogether, these data suggest that exogenous RNAi would not be achieved by just exposing birds to small RNAs or their precursors in the environment or food.

Regarding the endogenous RNAi, it remains what its physiological role is. There are three possible roles for endogenous RNAi: antiviral defense, genome defense against retrotransposons and control of gene expression. These roles would be associated with production of viral siRNAs, retrotransposon siRNAs and mRNA-targeting siRNAs in vivo. However, an unequivocal evidence for existence of these classes and their function was not provided yet.

One report attempted to examine the role of Dicer in retrotransposon repression. It was shown that the loss of Dicer in chicken cells does not result in accumulation of chicken CR1 retrotransposon while introduction of a human L1 element into cells lacking Dicer results in accumulation of L1 transcripts and increased retrotransposition (Lee et al., 2009). However, these data are difficult to interpret as different scenarios could lead to the same observations, especially downstream effects of a perturbed miRNA pathway and chromatin-mediated silencing of CR1.

3.1.2.7 Other dsRNA response pathways

Chromatin regulation by small RNAs

Two studies involving bird models brought up a possible nuclear function of Dicer and its link to chromatin regulation, which is of the unsettled issues in vertebrate models. Despite a decade of research, there is still no proposed molecular mechanism explaining these phenomena while the literature contains a number of contradicting observations.

Fukagawa et al. produced a conditional loss-of-function Dicer mutant in a chicken-human hybrid DT40 cell line that contains human chromosome 21. The loss of Dicer resulted in cell death and accumulation of premature sister chromatid separation. Furthermore, aberrant accumulation of

transcripts from human centromeric repeats was also found suggesting loss of heterochromatin at centromeres. While localization of two heterochromatin proteins (Rad21 and BubR1) was abnormal, localization of core centromeric heterochromatin proteins CENP-A and -C was normal (Fukagawa et al., 2004). Although the article is highly cited (335 times up to date according to WOS core collection), the molecular mechanism of the effect remains elusive. It is possible that the phenomenon is an indirect consequence of perturbing the miRNA pathway. Furthermore, the model system is unique and human heterochromatin sequences might exhibit unusual behaviour in the chicken nuclear environment.

Giles et al. examined a 16 kilobase (kb) heterochromatin domain in the chicken erythroid progenitor cell line 6C2. RNAi-mediated downregulation of the enzyme Dicer resulted in increased histone acetylation and transcript levels from the heterochromatin locus while compact chromatin structure became more accessible to restriction endonucleases. It was also shown that chicken AGO2 homolog binds the 16 kb region in a Dicer-dependent manner and is necessary for a condensed chromatin structure (Giles et al., 2010). The article has been cited 26 times up to date according to WOS (core collection), yet there was no follow up providing any mechanistic explanation of the phenomenon. It is possible that the observed effects could be an indirect effect of suppression of the miRNA pathway or even an experimental artefact. Additional controls and experiments would be needed to address these concerns and clarify inconsistencies with other reports. Therefore, this report should be considered an interesting observation without a clear mechanistic explanation.

Taken together, small RNA-mediated chromatin changes in birds remain an open question. Without knowing the molecular mechanism, especially that of biogenesis of small RNAs regulating chromatin and their mode of action, there is simply not enough information for qualified conclusions.

Antiviral defense - interferon response and crosstalk with RNA silencing

Many studies deal with various aspects of viral infections in birds or avian cells. The most studied model for viral infections in birds is Marek's disease, which is a consequence of a Herpesvirus infection in poultry. Publications linked to Marek's disease addressed virus encoded miRNAs (Morgan et al., 2008; Yao et al., 2008; Zhao et al., 2009; Muylkens et al., 2010; Luo et al., 2011; Xu et al., 2011; Zhao et al., 2011; Coupeau et al., 2012; Strassheim et al., 2012), changes in host miRNA expression during infection (Yao et al., 2008; Lambeth et al., 2009a; Xu et al., 2010; Tian et al., 2012; Stik et al., 2013; Dinh et al., 2014; Li et al., 2014b; Li et al., 2014c; Lian et al., 2015a; Lian et al., 2015b; Han et al., 2016), or attempts to block the virus with RNAi (Chen et al., 2008; Chen et al., 2009b; Lambeth et al., 2009b). A similar set of articles has been found for other studied viruses infecting birds – e.g. avian influenza virus H5N1 and H9N2, bursal disease virus, subgroup J avian leucosis virus. The complete list is available in the library accompanying this section.

Reports concerning host and virus-encoded miRNAs generally represent adaptations manipulating the miRNA pathway for the benefit of the pathogen. At the same time, these articles did not reveal some unique adaptation of the chicken miRNA pathway, which would differ from molecular mechanisms and principles described in the previous section.

The last group of articles reviewed here represent publications covering the interferon system, the common antiviral system induced by dsRNA and other RNA species (Karpala et al., 2008; Kint et al., 2015; Lostale-Sejjo et al., 2016). Birds generally utilize the same antiviral interferon system including its key dsRNA sensing proteins: PKR (Gonzalez-Lopez et al., 2003; Zhang et al., 2014; Lostale-Sejjo et al., 2016), RIG-I (Li et al., 2014a; Chen et al., 2015; Xu et al., 2015), and MDA5 (Lee et al., 2012; Hayashi et al., 2014; Lee et al., 2014), 2',5'-OAS (Villanueva et al., 2011; Lee et al., 2014). However, there seem to be some species-specific variations. For example, RIG-I is found in some birds, such as ducks or pigeons (Chen et al., 2015; Xu et al., 2015) but not in chicken, which lack RIG-I and the RNA sensing RHA/DHX9 helicase homolog (Sato et al., 2015). Although the lack of RIG-I is partially compensated by chicken MDA5 activity (Karpala et al., 2011; Hayashi et al., 2014) the absence of RIG-I-like function may contribute to the chicken's susceptibility to highly pathogenic influenza (Karpala et al., 2011; Li et al., 2014a).

Adenosine deamination

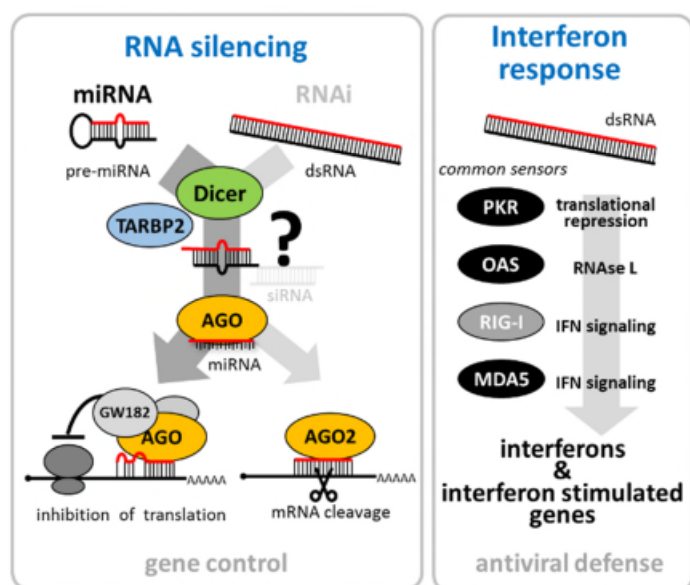
Birds have also adenosine deaminases that act on RNA (Herbert et al., 1995) but their physiological significance in birds is unknown at the moment.

3.1.2.8 Summary

In terms of the mode-of-action of dsRNA and miRNA pathways, birds are closely resembling mammals despite over 300 millions of years of separate evolution. The molecular mechanism of RNAi and miRNA pathways seems to be essentially identical to that of mammals except of a single dsRBD instead of two different ones. The significance of this difference is unclear. The miRNA pathway seems to be the dominant small RNA pathway while the existence and functionality of endogenous RNAi remains unclear. Some variations were found in the interferon system (lack of RIG-I in chicken), which appears to be the main antiviral system in birds.

Figure 31 Overview of avian pathways

dsRNA and miRNA pathways in birds are very much similar to the mammalian ones with some minor exceptions. Birds have only a single dsRBP homologous to TARBP2, and lack PACT ortholog.



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3.1.3. Fish

Fish are an aquatic heterogeneous paraphyletic group with the majority of the species belonging to the bony fish class (*Osteichthyes*) group, which has ~25000 species, the highest species diversity than any other vertebrate group. We focused our search on the *Osteichthyes* Class, as it is the same taxonomic level as mammals and birds.

Literature search for publications on fish RNAi and miRNA pathways in reference databases has been described in detail in the section 2. Notably, the search in this particular section encountered many false positives because of FISH (fluorescence in situ hybridization), a common approach to study RNA localization, and Fischer's exact test, a statistical analysis method.

Overall, the organization of small RNA pathways in fish is very similar to that of mammals. Next generation sequencing analysis of zebrafish small RNAs identified miRNAs and germline piRNAs as common small RNAs. The dominant small RNA pathway in fish is the miRNA pathway. Studies of roles of miRNAs account for the vast majority of the literature. At the same time, the molecular mechanism of miRNA and RNAi pathways was seldom studied in the fish model. In addition, several reports studied the piRNA pathway, which is an RNA silencing pathway distinct from RNAi and miRNA pathways. The piRNA pathway protects the germline from mobile elements (Houwing et al., 2007; Kamminga et al., 2010; Huang et al., 2011). However, in contrast to mammals, where oocytes lacking piRNAs develop normally, piRNAs in the zebrafish are required for normal oocyte development (Houwing et al., 2007; Kamminga et al., 2010).

3.1.3.1 Dicer

Fish genomes carry a single gene for Dicer, which is an ortholog of the mammalian Dicer and Dicer-1 in *Drosophila* (Murphy et al., 2008). This is supported by annotated fish genome data in the UCSC genome browser (<https://genome.ucsc.edu/>), and tblastn search of fish sequences at NCBI (e.g. *Salmo salar*, *Danio rerio*, *Takifugu rubripes*, *Gasterosteus aculeatus* (stickleback), *Oryzias latipes* (medaka)). Existence of a single Dicer gene in fish genomes is remarkable in teleost fish species, which underwent genome duplication. This suggests selective pressure for maintaining a single Dicer gene in fish. Zebrafish Dicer is essential for development and its primary role seems to be miRNA biogenesis (Wienholds et al., 2003). The role of Dicer in endogenous RNAi in fish has not been addressed in much detail. A study of Dicer in grass carp *Ctenopharyngodon idellar* revealed a CDS encoding Dicer protein carrying all known functional domains found typically in other Dicers (Shen et al., 2013). *Ctenopharyngodon idellar* Dicer is abundantly expressed in brain, gill, head kidney, liver, spleen, heart, muscle and intestine. A positive correlation was found between *Ctenopharyngodon idellar* Dicer mRNA expression and infection with grass carp reovirus (GCRV) infection in cultured kidney cells and in the liver (8.46-fold, $P < 0.01$, 12 h post-infection) and spleen *in vivo* (Shen et al., 2013). This suggests that *Ctenopharyngodon idellar* Dicer is an inducible gene responding to viral infection. At the same time, this is not a direct evidence for antiviral RNAi in fish because the evidence for virus-derived endogenous siRNAs has not been provided.

3.1.3.2 dsRBPs

Fish genomes typically contain orthologs of TRBP2 and PACT, which are mammalian Dicer-interacting dsRBPs (Murphy et al., 2008). However, they were not studied in the fish model, so far.

3.1.3.3 Argonaute proteins

Fish AGO proteins are orthologs of AGO proteins observed in other vertebrates (Murphy et al., 2008). However, teleost fish clade contains an additional AGO paralog, which emerged from a fish-specific genome duplication event that occurred approximately 350 million years ago (McFarlane et al., 2011). All five Ago genomic loci in teleosts contain specific, conserved sequence elements in non-coding regions indicating that the teleost AGO paralogs are differentially regulated, which is consistent with expression analysis in the zebrafish model. Multiple sequence alignments show that teleost homologs

possess critical aminoacid residues for AGO function, yet they also contain ortholog-specific features, retained throughout the vertebrate lineage (McFarlane et al., 2011).

3.1.3.4 miRNA pathway

In terms of small RNA research, fish models are typically used for exploring miRNA populations and studying biological roles of different miRNAs. Articles annotating miRNAs and analyzing their expression and function represent the bulk of the fish-related references identified through the systematic literature search. We have found 348 articles, which annotated and/or analyzed expression and function of miRNAs in fish. Almost none of these articles brought any mechanistic insight into the molecular mechanism of miRNA.

According to the miRBase (release 21), miRNA population in fish appears less complex than in mammals - fish models have less annotated miRNAs although there are dozens of reports on next generation sequencing analysis and miRNA annotation. In any case, zebrafish is an experimentally easily accessible model for exploring conserved roles of miRNAs in different tissues, which is also reflected in the number of references. The current top numbers of miRBase-annotated miRNAs in fish species are as follows:

species	miRNA precursors	mature miRNA
<i>Cyprinus carpio</i>	134	146
<i>Danio rerio</i>	346	350
<i>Fugu rubripes</i>	131	108
<i>Hippoglossus hippoglossus</i>	40	37
<i>Ictalurus punctatus</i>	281	205
<i>Oryzias latipes</i>	168	146
<i>Paralichthys olivaceus</i>	20	38
<i>Salmo salar</i>	371	498
<i>Tetraodon nigroviridis</i>	132	109

In terms of the molecular mechanism of RNA silencing, studies in the zebrafish and other fish models brought several interesting discoveries concerning specific miRNA functions and unique adaptations in fish. As a detailed overview would be beyond the purpose of this text, we will mention only two, examples, which have at least some relevance for the research of the molecular mechanism of miRNA pathway:

The first example is the biology of the miR-430 family of miRNAs. While miRNAs in mice are essentially irrelevant for the oocyte-to-embryo transition, the zebrafish oocyte-to-embryo transition incorporates zygotically-expressed miR-430 family in maternal mRNA degradation (Giraldez et al., 2006; Mishima et al., 2006). Furthermore, the onset of miR-430 activity in the zebrafish zygote allowed for addressing the relationship between miRNA-induced translational repression and mRNA degradation (Bazzini et al., 2012; Mishima et al., 2012). Using ribosome profiling of zygotic stages, Antonio Giraldez group showed that miR-430 reduces translation before causing mRNA decay (Bazzini et al., 2012). A significant technological outcome of these studies was development of target protectors, morpholino oligonucleotides specifically disrupting miRNA-mediated repression via hybridizing to and masking miRNA-binding sites (Choi et al., 2007).

Another contribution to small RNA biology coming from fish model was discovery of one of the non-canonical miRNA biogenesis mechanisms, namely Dicer-independent miRNA biogenesis of miR-451, which uses AGO2 slicing activity followed by uridylation and trimming (Cifuentes et al., 2010). The process also employs translation initiation factor eIF1A, which directly interacts with AGO2 and promotes miR-451 biogenesis (Yi et al., 2015). Data from zebrafish also contributed to understanding other miRNA regulations. Two related terminal uridyl transferases (TUTases), Zcchc6 (TUT7) and Zcchc11 (TUT4), selectively 3' monouridylate a subset of miRNAs (Thornton et al., 2014). TUTase inhibition in zebrafish embryos causes developmental defects and aberrant *Hox* gene expression

(Thornton et al., 2014). Another miRNA regulator discovered in the zebrafish is dead end 1 (DND1), which is negatively regulating miRNA targeting. DND1 is an evolutionary conserved RNA-binding protein (RBP) that counteracts the function of several miRNAs in zebrafish primordial germ cells as well as in human cells. DND1 binds mRNAs and prohibits miRNAs from binding cognate mRNAs. DND1 effects involve uridine-rich regions present in the miRNA-targeted mRNAs (Kedde et al., 2007)

Taken together studies of molecular mechanism of miRNA-mediated repression in fish did not reveal any notable deviation from what has been observed in mammals. While the protein machinery appears to be well-conserved, miRNA pathways among vertebrates differ in the sets of miRNAs and their targets, which dynamically evolve over time. This was for example demonstrated for zebrafish miR-430 and murine miR-290-295 miRNA clusters. They share common ancestry, both are associated with early development but do not regulate the same genes although some targets seem to be conserved (Svoboda and Flemr, 2010).

3.1.3.5 RNAi

The presence of RNAi response was examined in zebrafish at the same as in other animal models during the turn of the century. However, unlike specific RNAi observed in mouse oocytes and early embryos (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000), long dsRNA injection into zebrafish had been yielding inconsistent results (Li et al., 2000; Oates et al., 2000; Mangos et al., 2001; Zhao et al., 2001). While some reported specific knock-down effects (Li et al., 2000; Mangos et al., 2001), others observed non-specific effects (Oates et al., 2000; Zhao et al., 2001). Non-specific effects remained a recurring theme also in later studies (Zhao et al., 2008; Wang et al., 2010) although some authors were able to achieve specific RNAi effects (Ying et al., 2010; De Rienzo et al., 2012; Dong et al., 2013). Non-specific effects in zebrafish embryos were not remedied by the use of siRNAs and it was later shown that the basis of the non-specific effects is interference with miRNA function (Zhao et al., 2008). Injection of zebrafish zygotes with siRNA caused a significant reduction in miR-430 levels leading to unspecific developmental defects (Zhao et al., 2008). Interestingly, literature survey revealed that experiments with the parasitic sea lamprey (*Petromyzon marinus*), a jawless fish relative, showed that uptake of free siRNA at 5µg/ml did not trigger an RNAi response (Heath et al., 2014). In any case, RNAi did not become a favourite knock-down strategy to study genes during zebrafish development; microinjection of morpholino oligonucleotides (Eisen and Smith, 2008; Blum et al., 2015) became the preferred approach instead.

Taken together, successful RNAi experiments with long dsRNA demonstrate that zebrafish holds the molecular machinery for executing RNAi: Dicer, TARBP2, and AGO2. However, its capacity for mediating specific knock-down effects is limited because the same machinery is being simultaneously utilized by the miRNA pathway. Importantly, the availability of the machinery above the minimum capacity sustaining the miRNA pathway functionality likely differs during development and among different cell types.

While zebrafish holds the molecular machinery for executing RNAi, the question remains whether the endogenous RNAi has any significant role in fish. Available data do not provide unequivocal evidence for significant endogenous RNAi in fish. Next generation sequencing of small RNAs contains fractions of non-miRNA small RNAs of endo-siRNA size, yet it is not clear if these fragments truly represent *bona fide* endo-siRNAs. Some other data indirectly point to a possible antiviral role, namely GCRV-induced transient upregulation of Ago2 in rare minnow (*Gobio cypris rarus*) and Dicer upregulation in grass carp (*Ctenopharyngodon idella*) (Guo et al., 2012; Shen et al., 2013). Interaction of GCRV with the small RNA machinery (the miRNA pathway should not be excluded) has been suggested based on the observation in grass carp kidney cells that GCRV dsRNA could be processed into siRNAs but GCRV infection did not yield GCRV-derived siRNAs while Dicer upregulation occurred (Gotesman et al., 2014). It has been thus proposed that an unidentified RNAi suppressor might contribute to the survival of the viral genome and efficient viral replication.

(Gotesman et al., 2014). The presence of a virus-derived inhibitor of RNA silencing in a fish RNA virus would be indicative of an existing antiviral role of small RNAs, which is being suppressed. However, an alternative scenario that should be considered as well is that dsRNA formed during viral replication is

not accessible for Dicer-mediated cleavage. Therefore, further research is needed to address this issue.

3.1.3.6 Other notable silencing phenomena

There are two phenomena, which clearly overlap with RNA silencing but their underlying molecular mechanism remains unclear and will require further investigation.

Andrews et al found that introduction of transgenes containing convergent transcription units in zebrafish embryos induced stable transcriptional gene silencing in *cis* and *trans*. The silencing was suppressed upon Dicer knockdown, indicating processing of double stranded RNA. ChIP revealed that silencing was accompanied by enrichment of the constitutive heterochromatin mark H3K9me3 (Andrews et al., 2014). While small RNA-induced transcriptional silencing is well established in fungi and plants (and seems to be a product of convergent evolution), the molecular mechanism underlying seemingly related observations in vertebrates is unclear.

The second phenomenon deals with the role of small RNAs during DNA-damage response, which has been discussed above in the mammalian section (Francia et al., 2012). It was shown in zebrafish that DICER and DROSHA, but not downstream elements of the RNAi pathway, are necessary to activate the DDR upon exogenous DNA damage and oncogene-induced genotoxic stress, as studied by DDR foci formation and by checkpoint assays. It was also reported that formation of DDR foci requires site-specific DICER- and DROSHA-dependent small RNAs, which are sufficient to restore the DDR in RNase-A-treated cells (Francia et al., 2012).

3.1.3.7 Summary

The molecular mechanism of RNAi and miRNA pathways in fish seems to be essentially identical to that of mammals. While there are two dsRBPs (Figure 23), the role of PACT in RNA silencing remains to be determined. miRNA pathway is the dominant small RNA pathway while the existence and functionality of endogenous RNAi are unclear. Some variations (DNA-binding PKR homologs (Rothenburg et al., 2008)) were found in the interferon system, which is the main fish antiviral system.

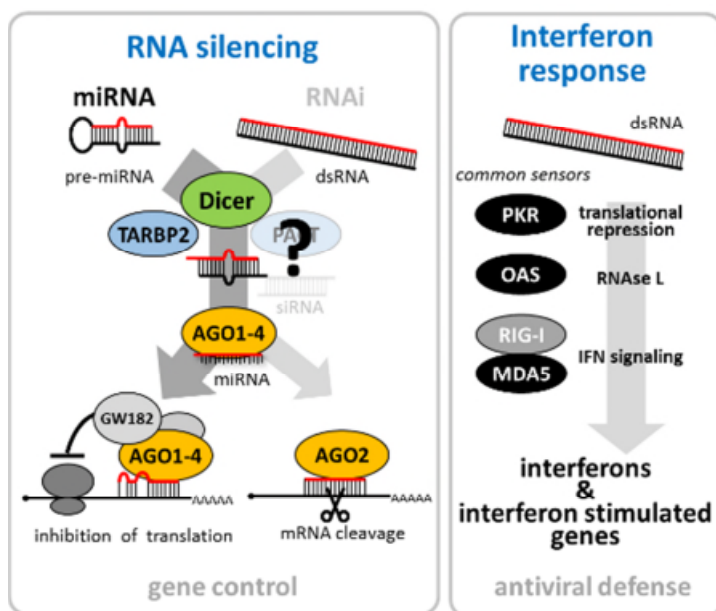


Figure 32 Overview of RNA silencing and dsRNA response in FISH

dsRNA and miRNA pathways in bony fishes are very much similar to the mammalian ones. Fishes have homologs of TARBP2 and PACT (Figure 23).

3.1.3.8 References

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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.

The formal structure of the report will be as in other taxons – upon reviewing molecular features of key individual components of reviewed mechanisms, we will discuss the reviewed mechanisms and their biological roles. Importantly, to provide an overview of miRNA and dsRNA mechanisms in arthropods, we 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. However, the sake of the length of the report, we will not comprehensively cover every report documenting miRNA and RNAi pathway in all arthropod species.

3.1.4.2 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) (Filippov et al., 2000; Denli et al., 2004; 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 (Martin et al., 2009; Flynt et al., 2010; 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 (Xu et al., 2013; Ghosh et al., 2014; Wynant et al., 2015), 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).

3.1.4.3 Dicer

Drosophila utilizes two Dicer proteins (Figure 35), 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, 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 and (ii) host-pathogen arms race where Dicer evolves to avoid viral proteins interfering with its function (Figure 36).

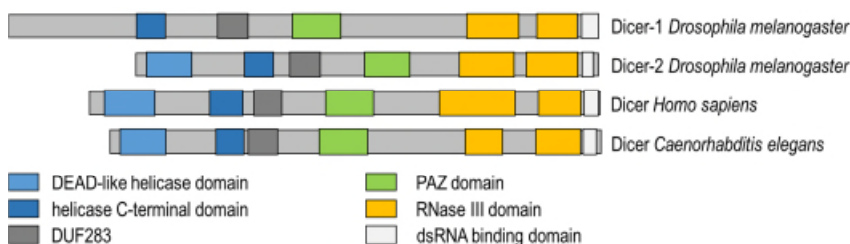


Figure 35 Comparison of *C. elegans*, human and *Drosophila* Dicer proteins

Domain composition was adopted from (Jaskiewicz and Filipowicz, 2008).

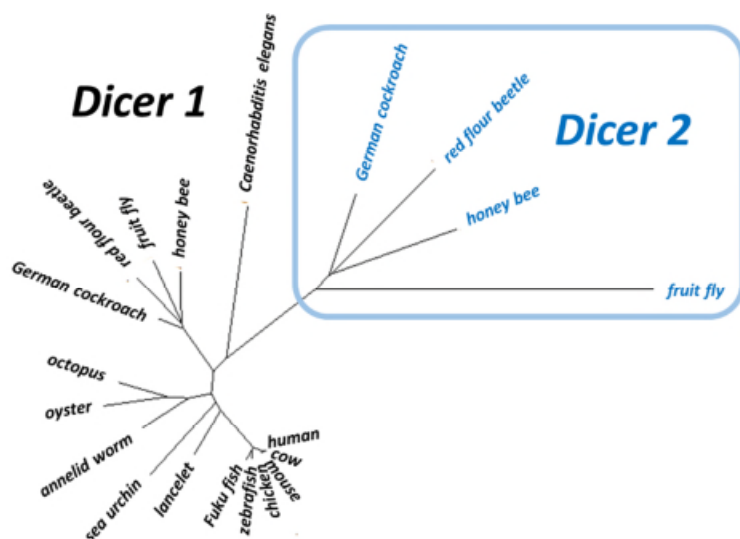


Figure 36 Metazoan Dicer phylogeny

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.

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 (Figure 35). 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 (Ye et al., 2007; Tsutsumi et al., 2011), the crystal structure of *Giardia intestinalis* Dicer (MacRae et al., 2006; MacRae et al., 2007), 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 (Figure 37). DCR-1 also functions in biogenesis of mirtron class of miRNAs (Okamura et al., 2007).

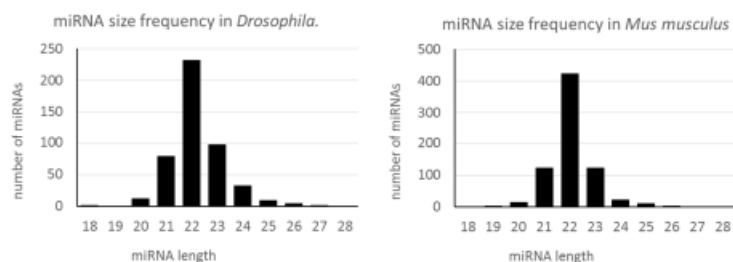


Figure 37 *D. melanogaster* miRNA lengths

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.

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 (Nykanen et al., 2001; Provost et al., 2002; Zhang et al., 2002; Liu et al., 2003; Welker et al., 2011). 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)

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).

3.1.4.4 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) (Hartig et al., 2009; Zhou et al., 2009; Miyoshi et al., 2010a; Fukunaga et al., 2012).

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 (Marques et al., 2010; Miyoshi et al., 2010a; Hartig and Forstemann, 2011). 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 (Tomari et al., 2004b; Nishida et al., 2013).

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).

3.1.4.5 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 (Pham et al., 2004; Kim et al., 2007), 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 (Miyoshi et al., 2010b; Iwasaki et al., 2015).

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 (Pham et al., 2004; Tomari et al., 2004a; Kawamata et al., 2009). 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* 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 (Matranga et al., 2005; Miyoshi et al., 2005;

Kim et al., 2007). 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; Okamura et al., 2009; Ghildiyal et al., 2010). 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 (Iwasaki et al., 2009; Fukaya et al., 2014). 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 (Rehwinkel et al., 2005; Behm-Ansmant et al., 2006; Eulalio et al., 2008; Huntzinger et al., 2010; Chekulaeva et al., 2011; Fukaya and Tomari, 2011; Moretti et al., 2012; Huntzinger et al., 2013). 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; Phetrungnapha et al., 2013; Yang et al., 2014b; Leebonoi et al., 2015). 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).

3.1.4.6 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 (Han et al., 2011; Liu et al., 2011; Yang et al., 2014a; Feltzin et al., 2015; Wang et al., 2016). 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 (Horwich et al., 2007; Abe et al., 2014; 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 adenylation enzyme in *Drosophila*, adenylation did not appear to affect miRNA stability on a genome-wide scale (Burroughs et al., 2010). Another non-canonical adenylation enzyme 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 (Westholm et al., 2012; Reimao-Pinto et al., 2015).

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

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 (Nagao et al., 2010; Saito et al., 2010; Qi et al., 2011; Huang et al., 2014; Murota et al., 2014).

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). dFMR1 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 no 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)

3.1.4.7 **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 could be 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 (Schwarz et al., 2002; Roignant et al., 2003), 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.

3.1.4.8 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* (Yang et al., 2007; Azzam et al., 2012) 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).

While miRNAs are a dominant small RNA category found in RNA-seq of small RNAs in *Drosophila*, individual reports on miRNA expression and biological functions are not much relevant for the narrative of the molecular mechanism. Insects have the most detailed miRNA annotations available. The current miRBase edition (21) has the following miRNA annotations for arthropods:

subphylum	species	miRNA precursors	mature miRNA
<i>Chelicerata</i>	<i>Ixodes scapularis</i>	49	49
	<i>Rhipicephalus microplus</i>	24	24
	<i>Tetranychus urticae</i>	52	92
<i>Myriapoda</i>	<i>Strigamia maritima</i>	3	4
<i>Crustacea</i>	<i>Daphnia pulex</i>	44	45
	<i>Marsupenaeus japonicus</i>	5	7
<i>Hexapoda</i>	<i>Aedes aegypti</i>	101	124
	<i>Apis mellifera</i>	254	259
	<i>Acyrtosiphon pisum</i>	123	103
	<i>Bombyx mori</i>	487	563
	<i>Drosophila melanogaster</i>	256	466
	<i>Drosophila simulans</i>	135	178
	<i>Drosophila virilis</i>	134	237
	<i>Plutella xylostella</i>	133	128
	<i>Tribolium castaneum</i>	220	430

note: only species with >100 annotated miRNA precursors are shown for *Hexapoda*

Drosophila's miRNA annotations is likely the most thoroughly done. Mt includes meta-analysis of > 10⁹ 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 (Okamura et al., 2007; Ruby et al., 2007; Martin et al., 2009). 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).

3.1.4.9 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 (Lee et al., 2004; Xu et al., 2004; Deshpande et al., 2005; Li et al., 2013), 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; Lucchetta et al., 2009; Li et al., 2013).

RNAi is functional across other arthropods subphyla, including *Chelicerata* (ticks and mites (Kurscheid et al., 2009; Schnettler et al., 2014; Hoy et al., 2016)) and *Crustacea* (shrimps (Chen et al., 2011; Huang and Zhang, 2013; Sabin and Cherry, 2013; Yang et al., 2014b; Jariyapong et al., 2015; Maralit et al., 2015; Hoy et al., 2016)). 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 (Zamboni 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 mosquitoes (Vodovar et al., 2012; Leger et al., 2013; Schnettler et al., 2013a). A number of studies provided ample mechanistic evidence that RNAi plays an essential role in antiviral response in *Drosophila* (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Nayak et al., 2010).

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 mosquitoes, 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 (Campbell et al., 2008; Adelman et al., 2012), 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), Mosinivirus (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 (Schnettler et al., 2014; Hoy et al., 2016)) or *Crustacea* (shrimps (Huang and Zhang, 2013; Sabin and Cherry, 2013; Yang et al., 2014b; Jariyapong et al., 2015; Maralit et al., 2015; Hoy et al., 2016)).

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).

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 posttranscriptional 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 (Gerbası 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 *Tribalium*, 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; Shih and Hunter, 2011; Mon et al., 2013).

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 *Tribalium* (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).

3.1.4.10 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 Sato and Siomi, 2013; Fu and Wang, 2014; Han and Zamore, 2014; Haase, 2016). 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 (Pressman et al., 2012; Ghosh et al., 2014).

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).

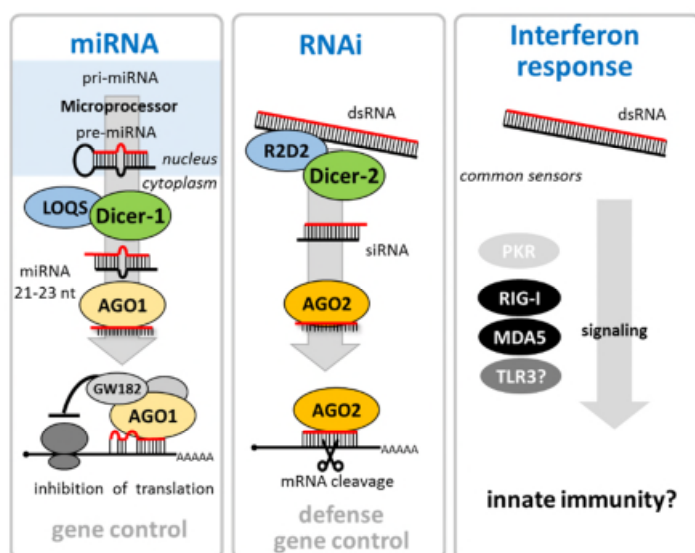
3.1.4.11 Other dsRNA response pathways in arthropods

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).

3.1.4.12 Summary



Taken together, arthropods are an extremely large and diverse taxon, characterized by an extended genetic separation of miRNA and RNAi pathways. 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*.

Figure 38 Overview of 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.

3.1.4.13 References

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3.1.5. Molluscs

Molluscs are a large and extremely diverse group of coelomate protostomes, which have an unsegmented soft body, internal or external shell, and a muscular foot. There are ~ 50 000 described species, which makes molluscs the second largest phylum after *Arthropoda* (third if *Chelicerata* and *Hexapoda* would be considered separate phyla). Apart from the complex classification of molluscs into 7-10 classes, three groups of molluscs are commonly recognized: *Cephalopoda* (squid, octopus), *Gastropoda* (snails and slugs), and *Bivalvia* (clams, mussels, scallops, oysters). Literature on RNA silencing and dsRNA pathways is limited. There were 92 publications dealing with small RNAs, most of which was related to use of RNAi as an experimental tool for suppressing gene expression.

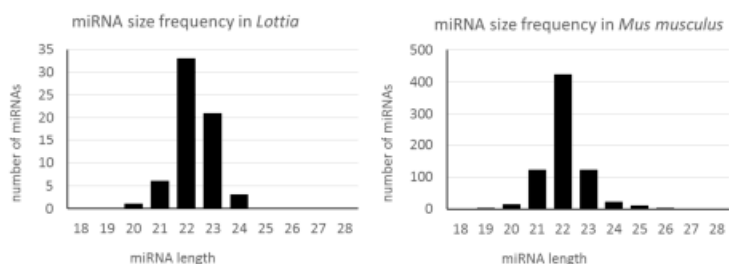
3.1.5.1 Dicer

There was no specific functional analysis of Dicer in molluscs. There is one study from 2016, which identified and bioinformatically analyzed Dicer and other components of miRNA and RNAi pathways in marine bivalves with a focus on a mussel *Mytilus galloprovincialis* and oyster *Cassostrea gigas* (Rosani et al., 2016). Their results show that all examined molluscs (>30 species of cephalopods, gastropods, and bivalves) have a single Dicer protein, which participates in both, RNAi and miRNA pathways and that Dicer of *Mytilus galloprovincialis* and *Cassostrea gigas* has a common structure found in *Metazoa*.

Additional information regarding Dicer structure was extracted from genomic databases. Blast search of molluscs entries in Genbank (query: murine Dicer protein, algorithm: tblastn, database: Nucleotide collection nr/nt, results restricted to molluscs (taxid:6447)) identified transcripts from *Mytilus*, *Crasostrea*, *Lottia*, *Aplysia*, *Miomphalaria*, and *Octopus* that apparently encoded full-length Dicer orthologs. Molluscs Dicer also produces miRNAs with median length of 22 nucleotides as estimated from the miRBase data. Interestingly, the incidence of 23 nt long miRNAs seems to be higher in molluscs. However, given the low number of miRNAs (64) and unexplored diversity of molluscs, it should not be considered a significant feature.

Figure 39 Molluscs miRNA lengths

The left graph depicts size distribution of all 64 miRNAs of sea snail *Lottia gigantea* deposited in the miRBase (version 21). For comparison, the right graph shows size distribution of 721 high-confidence murine miRNAs.



3.1.5.2 dsRBPs

There is no literature concerning dsRBP proteins participating in RNA silencing in molluscs. The above-mentioned analysis of miRNA and RNAi pathway components identified only a single dsRBP (TARBP2) homolog (Rosani et al., 2016).

3.1.5.3 Argonaute proteins

Likewise, there is essentially no information regarding the AGO subfamily of Argonaute proteins. The above-mentioned analysis of miRNA and RNAi pathway components identified one to four Argonaute proteins from both, AGO and PIWI clades (Rosani et al., 2016). However, general and derived roles of AGO proteins in molluscs remain unknown at the moment. Data from *Mytilus galloprovincialis* indicate presence of one AGO and two PIWI proteins (Rosani et al., 2016). This is remarkable because this AGO protein would act in both, RNAi and miRNA pathways like AGO2 in mammals.

In addition, there were two articles concerning the PIWI clade, which is acting in the piRNA pathway in the germline. In one of them, authors reported differential proteomic responses to generic dsRNA (poly I:C and poly A:U) in two oyster species (*Saccostrea glomerata* and *Crassostrea gigas*), which have differential susceptibility to ostreid herpesvirus infection. Interestingly, *Saccostrea glomerata*, which is not susceptible, showed production of proteins implicated in the TLR signalling pathway and

PIWI protein was also found in *Saccostrea glomerata* but not in *Crassostrea gigas* when challenged with dsRNA (Masood et al., 2016). Although it is unclear whether PIWI could be mistaken for AGO, it is possible that piRNAs might have acquired additional roles in molluscs, perhaps including also immunity. This notion would be supported by the second report, which identified 28nt piRNAs in brain (while piRNAs are generally restricted to gonads if not into germline cells only). These piRNAs had unique biogenesis patterns, nuclear localization, sensitivity to serotonin, and were implicated in stable long-term changes in neurons associated with memory (Rajasethupathy et al., 2012).

3.1.5.4 Other factors

According to the genome analysis of *Mytilus galloprovincialis*, Precambrian molluscs/mammalian ancestors must have shared all ancestral proteins in the miRNA pathway, including DROSHA, DGCR8 and GW182 (Rosani et al., 2016). There are no published mechanistic data suggesting that there would be any difference in activity of any of these factors in molluscs.

Importantly, we examined if molluscs genomes also contain an RdRP, which is found in RNA silencing in plants and Nematodes but not in insects or mammals: query: *C. elegans* RRF-1 NP_001250555, algorithm: tblastn, database: Nucleotide collection nr/nt, results restricted to molluscs (taxid:6447) identified transcripts six different transcripts from *Crassostrea gigas* (XM_011450789, XM_011427600, XR_900019, XR_902698, XM_011450791, XR_900018) suggesting that molluscs might indeed employ RdRPs in RNAi. This would be a significant observation, making molluscs RNA silencing an intermediate type between those found in nematodes, arthropods, and mammals.

3.1.5.5 miRNA

There is two molluscs species, which have annotated miRNAs in the miRBase (release 21):

species	miRNA precursors	mature miRNA
<i>Haliotis rufescens</i>	5	5
<i>Lottia gigantean</i>	59	64

Annelid miRNAs have are ~ 22 nt long like other animal miRNAs (Figure 39). Literature search revealed 15 reports on molluscs miRNA identification/annotation and/or analysis of expression/function (Rajasethupathy et al., 2009; Millan, 2011; Biggar et al., 2012; Bitel et al., 2012; Chen et al., 2014; Jiao et al., 2014; Martin-Gomez et al., 2014; Xu et al., 2014; Zhou et al., 2014; Jiao et al., 2015; Kenny et al., 2015; Tian et al., 2015; Zhao et al., 2016; Zheng et al., 2016a; Zheng et al., 2016b).

3.1.5.6 RNAi

RNAi is functional in molluscs as evidenced by 18 reports, which employed RNAi in different molluscs species (see the table below). The canonical RNAi (i.e. using long dsRNA) has been observed in the following species upon various forms of delivery including larva soaking, animal injection (adductor muscle, gonad, brain, larva, post-renal sinus etc.), polyethyleneimin-mediated delivery, or cell injection. Collectively, these data imply that different molluscs have an intact machinery to execute RNAi. Effects of injection into body cavity would suggest that molluscs might have some cellular uptake mechanism for dsRNA or systemic RNAi but the direct evidence for any of that is lacking at the moment.

RNAi in molluscs induced with short dsRNA molecules

species	siRNA amount	delivery method & effect	reference
<i>Chlamys farreri</i>	1µg/g	adductor muscle siRNA injection 70% KD at 72h	(Miao et al., 2016)
<i>Biomphalaria glabrata</i>	772 ng/250 µl	polyethyleneimine-mediated delivery >50-90% KD at 72h by ELISA	(Knight et al., 2011)
<i>Lymnaea stagnalis</i>	5 µl of 200 ng/µl	gonad injection, 27-mer 30-50% KD	(Fei et al., 2007)
<i>Lymnaea stagnalis</i>	2 µl of 20 µM	head injection above central ganglia, 27-mer,	(Hui et al., 2007)

RNAi in molluscs induced with long dsRNA molecules

species	long dsRNA		delivery method	reference
	concentration	length		
<i>Haliotis diversicolor</i>	5 µg/ml	136-819 bp	larva soaking	(Wang et al., 2016a)
<i>Haliotis diversicolor</i>	5 µg/ml	136-819 bp	larva soaking	(Wang et al., 2015a)
<i>Crassostrea gigas</i>	150-750 µg/ml 10 µg/g	652 bp	2x 100 µl injection ~50% KD at 48h	(Huvet et al., 2015)
<i>Crassostrea gigas</i>	50 µg/oyster	723 bp	adductor muscle inject. 1-7 days, good effect	(Choi et al., 2013)
<i>Crassostrea gigas</i>	530 µg/ml 53 µg/oyster	425 bp	gonad injection 77.52% KD	(Huvet et al., 2012)
<i>Nipponacmea fuscoviridis</i>	5 µg/ml	947 bp 667 bp	larva injection	(Hashimoto et al., 2012)
<i>Aplysia</i>	500 ng/µl	316 bp	sensory cell injection protein not decreased	(Lyles et al., 2006)
<i>Lymnaea stagnalis</i>	500 ng/µl 2 µg/oyster	~300 bp	central ring ganglia 60% KD at 24 h	(Guo et al., 2010)
<i>Lymnaea stagnalis</i>	5 µl of 200 ng/µl	321 bp	snail ganglia injection 30-50% KD	(Fei et al., 2007)
<i>Crassostrea gigas.</i>	20 or 100 µg/oyster	525 bp 877 bp	gonad injection 39% & 87% KD	(Fabioux et al., 2009)
<i>Biomphalaria glabrata</i>	0.1, 1.0 and 5.0 µg/snail	537 bp 541 bp	post-renal sinus inj. 70-80% KD	(Jiang et al., 2006)
<i>Biomphalaria glabrata</i>	120 ng/250 µl	397 bp	polyethyleneimine-mediated delivery >50% KD at 72h by ELISA.	(Knight et al., 2011)
<i>Aplysia</i>	500 ng/µl	316 bp	sensory cell injection protein not decreased	(Lyles et al., 2006)
<i>Aplysia</i>	up to 700 µg/mL	N.A.	~20 giant neurons of the abdominal ganglion inj., 80-95% KD	(Lee et al., 2001)
<i>Aplysia</i>	500 µg/mL	800 bp	sensory neuron injection decrease by microscopy	(Ormond et al., 2004)

3.1.5.7 Other dsRNA responding pathways

Molluscs seem to have a complex dsRNA response, which includes interferon-like response functioning in antiviral response (reviewed in Green et al., 2015a; Wang et al., 2015b). 21 articles dealt with dsRNA-induced interferon-like response. Several studies in Oyster have reported that dsRNA mimic poly(I:C) can strongly induce non-specific antiviral immune responses (De Zoysa et al., 2007; Green

and Barnes, 2009; Green and Montagnani, 2013; Green et al., 2015b; Masood et al., 2016; Wang et al., 2016b; Wang et al., 2016c).

Molluscs have 2'5'- oligoadenylate synthetases (Kjaer et al., 2009; Pari et al., 2014), RIG-I-like protein (Zhang et al., 2014), MDA5 (Green et al., 2014), and PKR (Green and Montagnani, 2013; Green et al., 2014; Green et al., 2015b). Poly I:C can be also bound by Leucine-rich repeat (LRR)-only protein found in scallop *Chlamys farreri* (Wang et al., 2016b; Wang et al., 2016c). Another gene, which is induced by poly I:C or sodium alginate is Myxovirus resistance (Mx) protein, which has been found in Abalone (De Zoysa et al., 2007; Cheng et al., 2012). Mx is one of intensely studied antiviral proteins, which is induced by the type I interferon system (IFN alpha/beta).

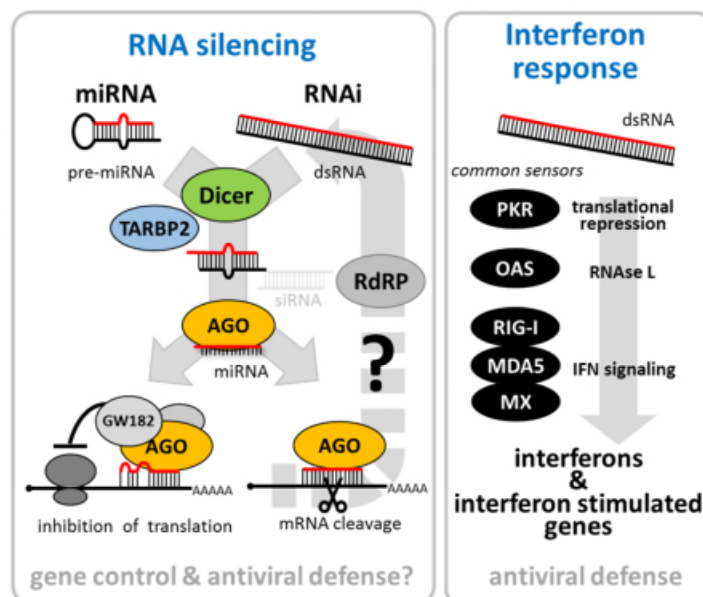
However, it is important to recognize that molluscs are a heterogeneous group with distinct antiviral adaptations. For example, proteomic profiling of two oyster species with differential susceptibility to ostreid herpesviruses showed that the resistant species has a stronger manifestation of the interferon-like response in the proteome upon induction with poly I:C (Masood et al., 2016).

3.1.5.8 Summary

Taken together, molluscs are an interesting taxon, which appears to have a unique setup of RNA silencing and its nexus with antiviral responses, which warrant further investigation.

Figure 40 Overview of mollusc pathways

dsRNA and miRNA pathways in molluscs are poorly understood. Among the missing pieces of information are: 1) degree of separation of miRNA and RNAi pathways (dedicated AGO proteins, co-existence of Dicer isoforms). 2) existence and functionality of RdRPs in molluscs, 3) derived roles of piRNAs in gene regulation and antiviral defense. Proteins from other dsRNA pathways related to innate immunity include PKR, OAS, RIG-I and MDA5 homolog, Mx protein and others.



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3.1.6. Annelids

Annelids are coelomate protostome worms whose body is organized into a series of ring-shaped repetitive segments. ~15 000 annelid species are grouped into three classes: Polychaeta (bristle worms, e.g. *Platynereis*), Oligochaeta (earthworms, e.g. *Lumbricus*), and *Hirudinea* (leeches, e.g. *Hirudo*). Literature on RNA silencing and dsRNA pathways is extremely limited. There were only 16 publications dealing with small RNAs, most of which was related to detection of miRNAs.

3.1.6.1 Dicer

There was no publication concerning Dicer structure or function in annelids, thus all information provided here had to be extracted from genomic databases. Blast search of annelid entries in Genbank (query: murine Dicer protein, algorithm: tblastn, database: Nucleotide collection nr/nt, results restricted to *Annelida* (taxid:6340)) identified a single mRNA XM_009031272 from the leech *Helobdella robusta* encoding a 1316 aa Dicer protein which lacked ~ 400 aminoacids at the N-terminus. The protein contained HELICc domain at the N-terminus but lacked the N-terminal DExD domain. Thus, this Dicer is structurally reminiscent of the N-terminally truncated Dicer capable of producing endo-siRNAs (Flemer et al., 2013). Importantly, analysis of *Helobdella robusta* genomic sequence identified a single Dicer gene on a contig ref|NW_008705401.1|, which also carried the entire N-terminus, which was lacking in the identified mRNA. This would imply a similar scenario as observed in mouse oocytes – Dicer encodes two protein isoforms, where the longer one is adapted for the production of miRNAs, while the shorter can produce both, miRNAs and siRNAs. However, this information would need to be validated experimentally and it needs to be also tested how common would be this scenario for annelids in general. Finally, annelid Dicer also produces miRNAs with median length of 22 nucleotides as estimated from the miRBase data.

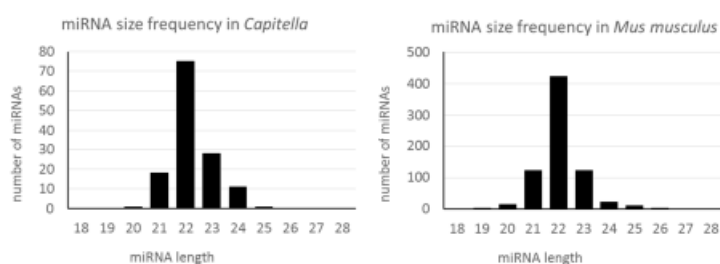


Figure 41 Annelid miRNA lengths

The left graph depicts size distribution of all 134 miRNAs of a polychaete worm *Capitella teleta* deposited in the miRBase (version 21). For comparison, the right graph shows size distribution of 721 high-confidence murine miRNAs.

3.1.6.2 dsRBPs

There is no literature concerning dsRBP proteins participating in RNA silencing, Rosani et al. suggest that annelids employ a single TARBP2 homolog (Rosani et al., 2016).

3.1.6.3 Argonaute proteins

Likewise, there is essentially no information regarding the AGO subfamily of Argonaute proteins. There is a study of protein components in molluscs, which included two annelid species and found that annelids have three (*Capitella*) and four (*Helobdella*) Argonaute proteins from both, PIWI and AGO clades (Rosani et al., 2016). There are five papers concerning Argonaute, however of the PIWI clade, which is functioning in the germline (Sugio et al., 2008; Giani et al., 2011; Weigert et al., 2013; Kozin and Kostyuchenko, 2015; Ozpolat and Bely, 2015). Blast search of *Helobdella* genome identified two AGO paralogs (XM_009021176.1 and XM_009031816.1) and two possible PIWI proteins (XM_009015681.1 and XM_009021522.1). The two paralogs of PIWI proteins would be consistent with the aforementioned analysis of *Myzostoma cirriferum* PIWI proteins (Weigert et al., 2013). Whether the two AGO paralogs are functionally dedicated to RNAi and miRNA pathways like AGO proteins in *Drosophila* is unknown. Furthermore, if annelids would have two PIWI proteins, it is possible that *Capitella* might have only one AGO protein serving in RNAi and miRNA pathways.

3.1.6.4 other factors

No other proteins factors from miRNA or RNAi pathway have been specifically reported. Data from Rosani et al. show that annelids employ microprocessor complex (Rosani et al., 2016)

3.1.6.5 miRNA

There is one annelid species, which has annotated miRNAs in the miRBase: *Capitella teleta* -129 precursor miRNAs and 134 mature miRNAs. Literature search revealed seven publications reporting annelid miRNA identification and or expression (Tessmar-Raible et al., 2007; Sperling et al., 2009; Christodoulou et al., 2010; Gong et al., 2010; Helm et al., 2012; Huang et al., 2012; Kenny et al., 2015). As shown above, annelid miRNAs have an average length 22 nucleotides like other animal miRNAs.

3.1.6.6 RNAi

The only information available concerning RNAi is that it is functional (Takeo et al., 2010; Yoshida-Noro and Tochinai, 2010). The only one published experimental paper reporting RNAi employed long dsRNA that was injected into the coelom (1 µg/µl, 100 nl per worm, i.e. 100 ng of dsRNA/worm).

3.1.6.7 Other dsRNA responding pathways

Only one reference mentioned another? protein involved in dsRNA response - OAS (Kjaer et al., 2009). We performed Blast search for murine PKR, RIG-I, and MDA5 (algorithm: tblastn, database: Nucleotide collection nr/nt, results restricted to *Annelida* (taxid:6340)). Regarding PKR, the analysis revealed many sequences of *Helobdella*, and six of *Platynereis*, which were similar to the second half of the protein, suggesting they were not orthologs. RIG-I and MDA5 searches identified two hypothetical proteins in *Helobdella* (ref|XM_009026626.1| and XM_009014668.1) with ~35% identity and ~50% similarity, which could be orthologs.

3.1.6.8 Summary

In terms of the molecular mechanism of dsRNA and miRNA pathways, annelids remain largely unexplored. While, the available information points towards coexistence of miRNA and RNAi pathways, their integration or genetic separation remain unclear.

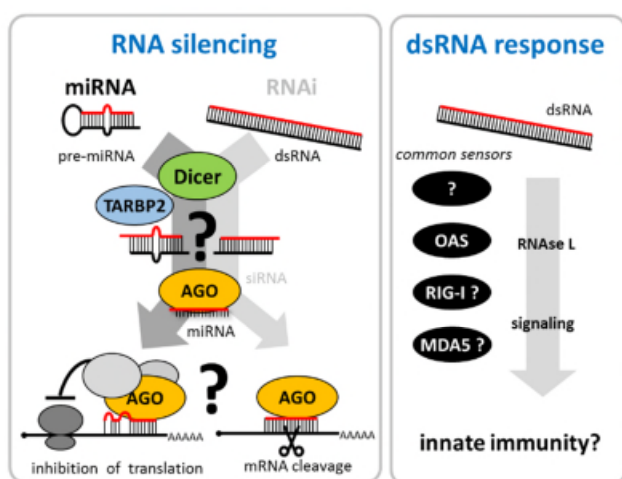


Figure 42

Overview of annelid pathways

dsRNA and miRNA pathways in annelids are poorly understood. Among the missing pieces of information are: 1) degree of separation of miRNA and RNAi pathways (dedicated AGO proteins, co-existence of Dicer isoforms). Proteins from other dsRNA pathways related to innate immunity include OAS, most likely RIG-I and MDA5 homologs but probably not PKR.

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3.1.7. Nematodes

Nematodes are free living or parasitic unsegmented pseudocoelomate worms. There is ~80 000 described species, estimates of the total number of species are reaching 1 million. Parasitic nematodes, cause disease in animals and plants and are thus of medical and economical importance. In addition, a free-living soil nematode *Caenorhabditis elegans* became a laboratory workhorse for the last half a century that contributed to our understanding of every major molecular mechanism in multicellular organisms. This is neatly documented on both reviewed topics – miRNA and RNAi pathways were first discovered in *C. elegans* as well.

RNA silencing in *C. elegans* is very complex despite the organism has only one Dicer gene. The complexity is created by the extreme diversity of downstream pathways, which employ miRNAs as well as primary and secondary siRNA and other small RNAs that are loaded on 26 Argonaute proteins of three subfamilies, which have different biological functions in the cytoplasm and nucleus. For the purpose of the report, we focused on the miRNA pathway and pathways initiated by long dsRNA. This part of the report is organized similarly to the other taxon reports – first we introduce the key molecular players of RNA silencing in nematodes and then we summarize miRNA and dsRNA-induced mechanisms, in this case RNAi (exogenous = exo-RNAi and endogenous = endo-RNAi) and adenosine deamination (nematodes do not have the interferon system described for vertebrate groups).

3.1.7.1 The Microprocessor complex

C. elegans utilizes the same Microprocessor complex as the earlier discussed *Metazoa*, i.e. a complex of Drosha (*drsh-1*) and DGCR8 homologs, the latter being named Pasha (partner of Drosha, *pash-1*) (Denli et al., 2004). The complex cleaves the pri-miRNA into pre-miRNA in the nucleus. Suppression of Pasha expression in *C. elegans* interferes with pri-miRNA processing, leading to an accumulation of pri-miRNAs and a reduction in mature miRNAs (Denli et al., 2004). Furthermore, *pash-1* mutation relieves let-7-mediated repression and the phenotype overlaps with those observed in *dcr-1* or *drsh-1* mutants (Denli et al., 2004). NGS of small RNAs showed that Drosha cleavage sites were enriched with specific nucleotides (mainly uridine) and structural distortions resulting in reduced stability on the 5' arm (Warf et al., 2011).

3.1.7.2 Dicer

C. elegans genome contains a single Dicer gene (*dcr-1*), which was discovered as a miRNA and RNAi biogenesis factor in 2001 (Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Dicer is the central component in biogenesis miRNAs, endo-siRNAs and exo-siRNA. The loss of Dicer results in sterility, abnormal oocytes, and heterochronic phenotypes (Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001).

Most nematodes possessed only one Dicer gene (Gao et al., 2014). Dicer cleaves long dsRNA into 23 bp siRNAs *in vitro* in the presence of ATP (Ketting et al., 2001). However the length of the primary cleave product is retained only in miRNAs, where *C. elegans* miRNAs deposited in the miRBase are 22-23 nt long (Figure 43) and in RDE-1-bound siRNAs in the exo-RNAi. The endogenous siRNAs are longer and belong to the class of 26G RNAs whose biogenesis requires additional factors beyond Dicer.

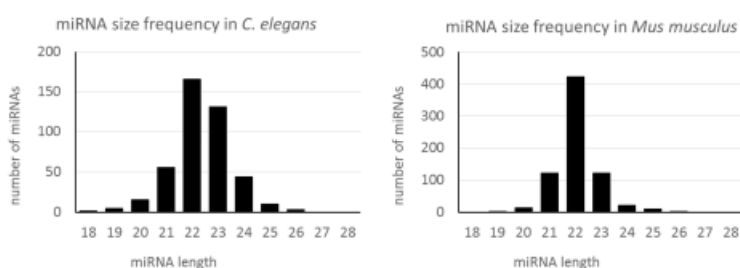


Figure 43 Nematode miRNA lengths

The left graph depicts size distribution of all 334 miRNAs of a *C. elegans* deposited in the miRBase (version 21). For comparison, the right graph shows size distribution of 721 high-confidence murine miRNAs.



Figure 44 Comparison of *C. elegans*, human and *Drosophila* Dicer proteins

Domain composition was adopted from (Jaskiewicz and Filipowicz, 2008).

There is neither crystal structure of *C. elegans* Dicer nor a cryo-EM analysis of the shape of the molecule. However, the general domain composition of *C. elegans* Dicer is very similar to that of human Dicer (Figure 44). Thus, some information can be inferred from sequence comparison as well as from biochemical analyses of isolated domains.

Analysis of the PAZ domain showed that the 5' and 3' pockets are conserved across members of the Dicer1 subfamily, with the exception of the 5' pocket in nematode *Bursaphelenchus xylophilus*. The helicase domain of Dicer is not necessary for miRNA processing, or exo-RNAi. Comparisons of wild-type and helicase-defective strains showed that the helicase domain is required by a subset of annotated endo-siRNAs, in particular, 26G RNAs (Welker et al., 2010). Consistently with these observations, the helicase domain is essential for cleaving dsRNA with blunt or 5'-overhangs but not with 3' overhangs, such as miRNA precursors (Welker et al., 2011). Further, blunt termini, but not 3' overhangs yield siRNAs from internal regions of dsRNA (Welker et al., 2011). RNase IIIb and dsRNA binding domains contain two conserved phosphorylation sites, which are phosphorylated by ERK. The phosphorylation is necessary and sufficient to trigger Dicer's nuclear translocation (Drake et al., 2014) and plays a role during oocyte-to-embryo transition (Drake et al., 2014).

C. elegans evolved a system where Dicer processes substrates for different pathways (endogenous RNAi, exogenous RNAi, antiviral defense) into ~22 nt small RNAs. Thus, needs to DCR-1 function as a hub for several small RNA pathways where it contributes to sorting of small RNAs into different pathways. This contrasts with mammals, where there is minimal, if any, sorting during loading of Dicer-produced small RNAs, and with *Drosophila*, which employs two dedicated Dicer proteins for RNAi and miRNA pathways. Sorting involves substrate structure and distinct protein factors (Jannot et al., 2008). Pre-miRNAs have unique structures that facilitate loading onto ALG-1/2 Argonaute proteins. Pre-miRNA analysis showed structural distortions adjacent to Dicer cleavage sites (Warf et al., 2011) and common internal bulges/mismatches. Remarkably, nucleotide changes in the pre-let-7 miRNA precursor that create a perfectly complementary stem cause loading of resulting small RNAs on RDE-1, which is the Argonaute protein acting in the exogenous RNAi pathway (Steiner et al., 2007). Reintroducing mismatches into the stem restores loading onto ALG-1 (Steiner et al., 2007). The second sorting system relies of proteins recognizing Dicer substrates and presenting it to Dicer, such as RDE-4 discussed in the next section, or Dicer associated proteins, which modify DCR-1 products and direct them on distinct Argonaute proteins (see further below).

DCR-1 also binds RNAs without generating small RNAs. Analysis of Dicer-associated RNAs in *C. elegans* by PAR-CLIP (Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) revealed ~2500 Dicer binding sites in the *C. elegans* transcriptome (Rybak-Wolf et al., 2014). Apart from miRNA precursors, DCR-1 also binds a variety of RNA molecules (structural RNAs, promoter RNAs, and mitochondrial transcript). However, Dicer-binding sites beyond miRNAs mostly reside on mRNAs/lncRNAs that are not significantly processed into small RNAs. These passive binding (i.e. without dicing) typically involves small, Dicer-bound hairpins within intact transcripts and generally stabilizes target expression (Rybak-Wolf et al., 2014). It was proposed that passive binding might be one of the mechanisms balancing siRNA/miRNA production since it was shown that passive binding sites can sequester Dicer and reduce microRNA expression (Rybak-Wolf et al., 2014). For example, a noncoding RNA *rncs-1* is a non-cleaved Dicer substrate, which appears to reduce Dicer activity in hypodermis and intestine (Hellwig and Bass, 2008)

Remarkably, two distinct truncated Dicer isoforms produced by proteolytic cleavage were reported from *C. elegans*. While both isoforms are C-terminal fragments created by proteolytic cleavage, they

show profound functional differences. The first one is created by cleavage of DCR-1 by CED-3 caspase. The released ~43 kDa C-terminal fragment changes its RNase activity into DNase, which fragments chromosomal DNA during apoptosis (Nakagawa et al., 2010). Subsequent molecular analysis proposed a model where the N-terminal helicase domain is suppressing DNase activity and release of the C-terminal part upon cleavage at Asp1472 (within the RNase IIIa domain) by CED-3 activates a DNA-binding activity and enables DNA cleavage (Ge et al., 2014). The second truncated form of Dicer exists as a ~ 95 kDa C-terminal Dicer fragment generated by proteolytic cleavage upstream of RNase IIIa domain. It is abundant in adult *C. elegans* cells where it might enhance exogenous and antiviral RNAi while negatively regulating miRNA biogenesis (Sawh and Duchaine, 2013). The 95 kDa Dicer fragment does not interact with RDE-4 nor with the ERI complex but it was found to bind miRNA-associated AGO proteins ALG-1/2 suggesting that it sequesters ALG-1/2 from the full-length DCR-1, thereby acting as a competitive inhibitor of miRNA processing (Sawh and Duchaine, 2013).

There has been a number of identified Dicer binding partners, which form different complexes, which act in different types of RNAi pathways (Duchaine et al., 2006; Lee et al., 2006). One of them is the exo-RNAi Dicer complex composed of RDE-4 (dsRBP), DCR-1, RDE-1 (Argonaute protein), and DRH-1/2 (Dicer-related helicases) (Tabara et al., 2002). Another complex is ERI/Dicer complex, which was reported for endo-RNAi during sperm development (Pavelec et al., 2009). It is composed of ERI-1/3/5, DCR-1, and RRF-3 (RdRP) and produces 26G primary endo-siRNAs, which then drive production of secondary 22G RNAs (Duchaine et al., 2006; Gent et al., 2009; Han et al., 2009). ERI-1 is an exoribonuclease that degrades siRNAs *in vitro* (Kennedy et al., 2004) and acts in 26G biogenesis *in vivo* (Duchaine et al., 2006; Gent et al., 2009; Han et al., 2009), ERI-3 has no known domain (Duchaine et al., 2006), and ERI-5 is a tudor domain protein, which tethers the RdRP to Dicer (Thivierge et al., 2012). An additional genetic screen identified ERI-9 as an additional component of ERI/DCR complex acting in the endo-RNAi pathway (Pavelec et al., 2009). The miRNA pathway-dedicated complex involves interaction between Dicer and ALG-1/2 and, eventually, LIN-41 regulating let-7 miRNA (Duchaine et al., 2006). The list of Dicer-binding proteins in *C. elegans* is even longer and involves proteins with roles in different aspects of silencing or with yet undetermined functions (Duchaine et al., 2006; Racen et al., 2008; Beshore et al., 2009; Beshore et al., 2011).

3.1.7.3 dsRBPs – RDE-4 and homologs

RDE-4 is a dsRBP partner of Dicer in *C. elegans*. It is a 385-amino acid protein carrying two N-terminal dsRBDs and a third degenerate dsRBD at the C-terminus. A similar organization is found in other dsRBDs mentioned above - R2D2 and Loquacious in *Drosophila* and TRBP and PACT in mammals. RDE-4 was identified by a systematic screen for *C. elegans* RNAi-deficient mutants (Tabara et al., 1999). The *rde-4* mutant was completely deficient in RNAi but failed to show any discernible phenotype, including the absence of transposon activation, which was observed in some other *rde* mutants (Tabara et al., 1999). The loss of RDE-4 function can be compensated with injection of synthetic siRNA (Parrish and Fire, 2001) or with high amounts of dsRNA and siRNAs (Habig et al., 2008).

Mutants and biochemical analyses support a model where RDE-4 dimerizes through the C-terminal domain; dimers cooperatively bind long dsRNA, interacts with Dicer through the linker region, and forms a complex initiating the RNAi together with Dicer, RDE-1, and DRH-1/2 (Parrish and Fire, 2001; Tabara et al., 2002; Parker et al., 2006; Parker et al., 2008; Blanchard et al., 2011; Chiliveri and Deshmukh, 2014). The presence of an Argonaute protein in the complex suggests that in exo-RNAi, dsRNA recognition, processing into siRNA and loading of the Argonaute-containing effector complex is integrated in a one complex. Importantly, while RDE-4 is involved in siRNA production from exogenous and endogenous dsRNAs, RDE4, Dicer, RDE-1 and DRH-1/2 act in the exo-RNAi (Lee et al., 2006). The endogenous RNAi pathway (endo-RNAi), which targets endogenous genes, employs a distinct mechanism of siRNA production involving DCR-1 and RDE-4 but not RDE-1 and DRH-1/2 (Lee et al., 2006; Gent et al., 2010).

Importantly, RDE-4 differs from TARBP2 role in RNAi. While RDE-4 is involved in siRNA production from dsRNA but is not essential for later steps of RNAi because RDE-4 immunoprecipitates with trigger dsRNA but not siRNA (Tabara et al., 2002), TARBP2 functions during RISC formation and AGO loading. Binding properties *in vitro* correspond to different roles of the two proteins *in vivo*. RDE-4 preferentially binds long dsRNA, while TRBP binds siRNA with an affinity that is independent of dsRNA length (Parker et al., 2008). This stems from the fact that RDE-4 binds cooperatively, via contributions from multiple domains, while TRBP binds non-cooperatively (Parker et al., 2008).

3.1.7.4 Argonaute proteins

Caenorhabditis has the largest diversity of Argonaute proteins of all studied model systems – 25-27 Argonaute family members were described to carry primary and secondary small RNAs that act in soma and germline in multiple inter-related pathways (Yigit et al., 2006; Buck and Blaxter, 2013) (*Arabidopsis*, which has complex RNA silencing has 10 Argonaute proteins (reviewed in Buck and Blaxter, 2013). In addition to the above-described AGO and PIWI clades of the Argonaute family, nematodes utilize a unique Argonaute subfamily termed WAGO, which are “Worm-specific AGO” proteins, which bind secondary 22G small RNAs (summarized in Buck and Blaxter, 2013). The complete description of small RNA pathways in *C. elegans* is beyond the scope of this report. Here, we will mainly focus on Argonaute proteins acting as the primary recipients of Dicer-generated small RNAs and will include the downstream pathways when directly related to the miRNA pathway or dsRNA response.

Figure 45 Key catalytic residues for the slicer activity in *C. elegans* Argonaute proteins.

Alignment of 26 *C. elegans* AGO proteins in three regions with similarity to the catalytic center of RNase H. Within these regions two key aspartic acid residues (highlighted in red) and a histidine residue (highlighted in dark blue) coordinate a magnesium ion at the catalytic center of the RNase H enzyme. Substitutions compatible with metal binding are indicated in orange. The alignment was directly taken from (Yigit et al., 2006).

ZK757.3	TMVVGI	DV...IVYR	DGVS...IPTPVYYA	DLVAT
T22B3.2	TMVVGI	DV...IVYR	DGVS...IPTPVYYA	DLVAT
T23D8.7	VLFIGC	HL...IIYR	AGIA...IPSPVYYA	KLVAQ
ALG-1	VIFPGC	DI...VVYR	DGVS...IPAPAYYA	BLVAF
ALG-2	VIFLGC	DI...VVYR	DGVS...IPAPAYYA	HLVAF
ERGO-1	TLVLGI	DV...VVYR	DGLS...LPAPVLYA	HLAAK
RDE-1	TMVVGI	DV...VVYR	DGVS...LPAPVLYA	HLBCE
PRG-1	TMIVGY	DL...ILYR	DGAG...VPAPQYYA	HLKLF
PRG-2	TMIVGY	DL...ILYR	DGAG...VPAPQYYA	HLKLF
CSR-1	TFVIGM	DV...IIFR	DGVS...IPTPVYYA	HLLAK
C04F12.1	TLIISY	DV...VILR	DGVS...LPESIYAA	DEYAK
M03D4.6	LLLIGL	ST...VIYL	CGMS...LPAPLYLT	AEMAR
SAGO-1	RLIIGF	T...LIYF	SGVS...LPVPLHIA	GTYSR
SAGO-2	RLIVGF	VT...LLYF	NGVS...LPVPLYIA	DRYSQ
PPW-1	RLIVGF	VT...LLYF	NGVS...LPVPLYIA	DRYSQ
F58G1.1	HLIIGV	GI...IVYR	TGTS...LPTPLYVA	NEYAK
C06A1.4	HLIIGV	GI...IVYR	TETS...LPTSLYVA	NEYAK
EK1248.7	QLIIGV	GV...IIYR	SGAS...IPTPLYVA	NEYAK
PPW-2	HLIIGV	GI...TIYR	SGSS...IPTPLYVA	NEYAK
R06C7.1	QLIIGV	GV...IIYR	SGAS...IPTPLYVA	NEYAK
F55A12.1	QLIIGV	GV...IIYR	SGAS...IPTPLYVA	NEYAK
B04A9.2	TQFIGF	EM...VVYR	VGSG...IPNVSYAA	QNLAK
Y49F6A.1	TQFIGF	EM...VIYR	TGAG...VPHILYAA	QNLAK
C16C10.3	VQFIGF	LI...VIYR	VGAG...VPDVLVYAA	NLAK
C14B1.7	VQFIGF	LI...VIYR	VGAG...VPDVLVYAA	NLAK
T22H9.3	VQFIGF	LI...VIYR	IGAG...PPDVLVYAA	NLAK

ALG-1/2 - miRNA-binding Argonaute proteins

There are two Argonaute proteins carrying canonical miRNAs in *C. elegans*: ALG-1 and ALG-2 (collectively designated ALG-1/2). They were identified as RDE-1 homologs, which cause heterochronic phenotypes similar to *lin-4* and *let-7* mutations and which are (together with *dcr-1*) necessary for the maturation and activity of the *lin-4* and *let-7* miRNAs. (Grishok et al., 2001). ALG-1/2 are required for the miRNA function, but not for the siRNA-directed gene silencing (Jannot et al., 2008). Although ALG-1 and ALG-2 are almost identical and highly redundant (only loss of both genes leads to embryonic lethality), several non-redundant functions have been observed in processing pre-miRNA processing/miRISC formation (Tops et al., 2006; Steiner et al., 2007; Kuzuoglu-Oeztuerk et al., 2012; Vasquez-Rifo et al., 2012). Furthermore, while ALG-1/2 spatio-temporal expression profiles overlap, there is variability in dominating ALG expression in various cells and differential association to specific miRNAs (Vasquez-Rifo et al., 2012).

Interestingly, ALG-1/2 have conserved key residues for the slicing activity (Figure 45) suggesting that they might function as active slicers despite the general lack of miRNA-mediated site-specific cleavage of cognate mRNAs. In any case, the slicing activity of ALG-1/2 has been demonstrated *in vitro* and *in*

vivo (Bouasker and Simard, 2012). The loss of the slicing activity results in the accumulation of truncated miRNA precursors and altered miRISC formation suggesting that the slicing activity is necessary during miRISC formation (Bouasker and Simard, 2012). This notion is further supported by mutations in ALG-1, which separate miRISC loading and repression of cognate RNAs (Zinovyeva et al., 2014)

Canonical miRNA-mediated repression involves AIN-1 (ALG-1 Interacting protein), GW182 homolog, which interacts with miRISC and has been implicated in targeting ALG-1 to P-bodies (Ding et al., 2005; Ding and Grosshans, 2009). It was reported that lin-4-mediated repression acts at the level of translation initiation and frequently but not always coincides with transcript degradation (Ding and Grosshans, 2009). Remarkably, despite AIN-1/2 (two redundant GW182 homologs (Zhang et al., 2007)) lack most of the domains found in vertebrate proteins, they still interact with ALG-1/2 (Kuzuoglu-Oeztuerk et al., 2012). The interaction is mediated by GW repeats in the central part of AIN-1/2 but only AIN-1 interacts with PABPC1, PAN3, NOT1 and NOT2 (Kuzuoglu-Oeztuerk et al., 2012). This implies that the key principle of miRNA mediated repression, GW182-mediated tethering of PABPC1 and deadenylase complexes to AGO, is broadly conserved across metazoa.

The key determinant of target recognition is the seed binding as described in the mammalian section. Direct probing of the RNA backbone in isolated native *C. elegans* miRISC that the seed region is uniquely constrained, while the rest of the microRNA structure is conformationally flexible. Probing the Watson-Crick edges of the bases shows that bases 2-4 are largely inaccessible to solvent, while seed region bases 5-8 are readily modified (Lambert et al., 2011). These data are consistent with structural analyses of AGO proteins in mammals and elsewhere.

RDE-1 – primary siRNA-binding Argonaute in exo-RNAi and antiviral RNAi

RDE-1 has the central role in RNAi. It was identified through a screen for RNA-deficient mutants (Tabara et al., 1999). *Rde-1* mutants are strongly resistant to RNAi but without obvious defects in growth or development (Tabara et al., 1999). As mentioned above, RDE-1 associates with RDE-4, Dicer and DRH1/2 acting in the exo-RNAi and antiviral RNAi (Parrish and Fire, 2001; Tabara et al., 2002; Lu et al., 2005; Wilkins et al., 2005). As mentioned above, RDE-1 accommodates perfect siRNA duplexes, which includes artificially modified miRNA precursors (Steiner et al., 2007). It was proposed that RDE-1 functions as a scavenger taking up small RNAs from different sources (unlike ALG-1/2), which exclusively bind miRNAs (Correa et al., 2010).

Interestingly, the bulk of the slicing activity in *C. elegans* extracts *in vitro* comes from CSR-1 loaded with secondary 22G RNAs and not from RDE-1 loaded with primary siRNA (Aoki et al., 2007). While RDE-1 is a "slicer", it uses its endonucleolytic activity for removal of the passenger strand of the siRNA duplex and not for cleaving cognate RNAs, suggesting uncoupled roles of the RDE-1 slicer activity in siRNA maturation from and cognate RNA cleavage (Steiner et al., 2009). It was found that RDE-1 recruits RDE-8 endoribonuclease to target RNA (Tsai et al., 2015). According to the model, RDE-8 cleaves cognate RNAs and is needed for the production of 3' uridylated fragments of target mRNA, which provide templates for RdRP-mediated production of secondary siRNAs (Tsai et al., 2015).

Taken together, RDE-1 loaded with primary siRNAs stands at the beginning of an amplification cascade, while other Argonaute proteins loaded with secondary siRNAs mediate downstream silencing (Yigit et al., 2006). A remarkable feature of the downstream AGOs loaded with 22G siRNAs is the lack the slicer activity (Figure 45). RDE-1 does not initiate only post-transcriptional repression; it has also been implicated in initiation (but not maintenance) of long-term silencing (>25 generations) induced by long dsRNA in neurons (Buckley et al., 2012)

26G Argonaute effectors in RNAi - ERGO-1 - primary 26G siRNA-binding Argonaute in endo-RNAi

ERGO-1 is a PIWI-clade effector that binds 26G RNAs generated in the endo-RNAi pathway in female germline and embryo (Pavelec et al., 2009; Gent et al., 2010; Vasale et al., 2010). ERGO-1 targets exhibit a non-random distribution in the genome and appear to include many gene duplications,

suggesting that this pathway may control overexpression resulting from gene expansion (Vasale et al., 2010). In endo-RNAi in the soma, ERGO-1 accommodates 26G small RNAs in a process involving the ERI/Dicer complex and leading to production of more abundant secondary 22G small RNAs (Duchaine et al., 2006; Gent et al., 2009; Han et al., 2009). The biogenesis of primary 26G RNAs involves RdRP RRF-3 and RDE-4 while the biogenesis of secondary 22G siRNAs involves a second RdRP (RRF-1 or EGO-1) and loading on WAGOs. Additional proteins implicated in ERGO-1-mediated repression include the helicase ERI-6/7 (Armitage helicase homolog) (Fischer et al., 2011), MUT-2, MUT-7, MUT-16 (Zhang et al., 2011), and RDE10/RDE-11 complex (Zhang et al., 2012), and RDE-12 (Shirayama et al., 2014).

CSR-1 - secondary 22G siRNA-binding Argonautes

The “executive arm” of RNAi in *C. elegans* are secondary 22G RNAs. One of the proteins binding 22G RNAs is CSR-1, which was identified in *in vitro* experiments with *C. elegans* lysates as a dominant slicer activity. However, CSR-1 was shown to rather associate with chromatin to promote proper organization or assembly of targets within the holocentric chromosomes (Claycomb et al., 2009; Seth et al., 2013; Wedeles et al., 2013b, a; Cecere et al., 2014; Campbell and Updike, 2015; Tu et al., 2015). CSR-1 22G RNAs are produced in the germline by a complex of DRH-3 (helicase), EKL-1 (tudor protein), and EGO-1 (RdRP) similarly to WAGO 22G RNAs, which utilize RRF-1 RdRP (Claycomb et al., 2009).

WAGO proteins - secondary 22G siRNA-binding Argonautes

WAGO proteins are loaded with 22G RNAs, which were produced by RdRPs. They represent secondary effectors, which execute silencing in slicer-independent fashion in the soma and/or germline in the cytoplasm and the nucleus. NRDE-3 and HRDE-1 were identified as WAGO proteins mediating nuclear silencing (Allo and Kornblihtt, 2010; Gent et al., 2010; Burton et al., 2011; Buckley et al., 2012). HRDE-1 has been implicated in initiation and maintenance of long-term silencing (>25 generations) induced by long dsRNA in neurons (Burton et al., 2011; Buckley et al., 2012). For example, WAGO-1 silences genes, transposons, pseudogenes, and cryptic loci at the chromatin level by directing H3K27me3 through the NRDE pathway (Mao et al., 2015) and possibly in some association with components of the nonsense-mediated decay (NMD) (Gu et al., 2009). A *C. elegans* isolate from Hawaii had a natural defect in RNAi that was specific to the germline and was a result of multiple mutations the WAGO protein PPW-1 (Tijsterman et al., 2002).

3.1.7.5 RdRPs

C. elegans is the main animal model for studying RdRPs. While an earlier study of RdRP in *C. elegans* suggested that dsRNA synthesis can be primed by primary siRNAs (a model of “degradative PCR”) (Sijen et al., 2001), later studies demonstrated that RdRPs do not require the priming by primary siRNAs and produce short RNAs using RISC-targeted mRNAs as templates (Pak and Fire, 2007; Sijen et al., 2007). Surprisingly, sequencing of small RNAs associated with ongoing RNAi in *C. elegans* showed that Dicer-independent secondary siRNAs constitute the majority of cloned siRNAs (Pak and Fire, 2007). These secondary siRNAs are only antisense, carry 5' di- or triphosphates, and are not bound by RDE-1 but by other Argonaute proteins (Pak and Fire, 2007; Sijen et al., 2007). *C. elegans* genome four putative RdRPs, three of which were implicated in RNA silencing (Smardon et al., 2000; Sijen et al., 2001; Duchaine et al., 2006; Lee et al., 2006).

Of the three RdRPs, RRF-3 was implicated in the upstream part of RNAi, i.e. production of primary 26G siRNAs associated with ERGO-1 or ALG-3/4 Argonaute proteins (Duchaine et al., 2006; Gent et al., 2009; Han et al., 2009; Pavelec et al., 2009; Conine et al., 2010; Gent et al., 2010; Vasale et al., 2010; Conine et al., 2013) while EGO-1 and RRF-1 produce 22G RNAs (Duchaine et al., 2006; Gent et al., 2009; Han et al., 2009; Pavelec et al., 2009; Gent et al., 2010; Vasale et al., 2010; Jose et al., 2011).

3.1.7.6 miRNA pathway-specific features in nematodes

C. elegans was the first organism, in which the first miRNA (*lin-4*) was identified (Lee et al., 1993) and which revealed that a conserved *let-7* miRNA is conserved up to humans (Pasquinelli et al., 2000; Reinhart et al., 2000). The first two miRNAs were identified as regulators of developmental timing. Remarkably, phenotype of *dcr-1* mutants is similar to those of *lin-4* and *let-7* mutants (Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001) suggesting that miRNAs are the main Dicer product in *C. elegans* and that *lin-4* and *let-7* are functionally among the most significant miRNAs of the 434 annotated *C. elegans* mature miRNAs. *C. elegans* also produces non-canonical miRNAs, such as mirtrons (Ruby et al., 2007). High throughput analyses provided estimates of ~ 3500-5000 of binding sites bound by miRNAs in *C. elegans* (Zhang et al., 2007; Zisoulis et al., 2010).

C. elegans miRNAs are exposed to different regulations, some of which are also found in other model systems – this includes regulation of *let-7* by LIN-28 and sequence uridylation (Morita and Han, 2006; Newman et al., 2008; Lehrbach et al., 2009; Van Wynsberghe et al., 2011; Vadla et al., 2012; Ouchi et al., 2014; Stefani et al., 2015), regulation of *let-7* by RACK1 (Chu et al., 2014), and miRNA turnover by XRN-2 (Chatterjee and Grosshans, 2009) and DCS-1-XRN-1 complex (Bossé et al., 2013). miRNA-mediated repression is further regulated at the cellular level by autophagy (Zhang and Zhang, 2013) and other regulatory factors, such as the Golgi-associated retrograde protein (GARP) complex (Vasquez-Rifo et al., 2013). At the same time, specific miRNAs influence other RNA silencing pathways – they may interfere with RNAi (Massirer and Pasquinelli, 2013) or direct siRNA biogenesis (Correa et al., 2010).

3.1.7.7 RNAi pathway-specific features in nematodes

As it could be expected from diversity of factors mentioned above, RNAi in *C. elegans* is complex – different variants of RNAi operate in the germline and soma, RNAi recognizes different substrates and has a sequential production of small RNAs, which are the main executors of RNAi. We will further deal with two flavors of RNAi in *C. elegans* – endo-RNAi and exo-RNAi.

Endogenous RNAi in the germline and soma

The role of endogenous RNAi in shaping the transcriptomes of protein-coding genes during development has been challenged by mutant worms and flies lacking essential components of the RNAi pathway, which were viable and produced healthy offspring (Tabara et al., 1999; Lee et al., 2004; Okamura et al., 2004). Because of that, RNAi had been viewed as a defense mechanism against invasive nucleic acids. However, deep sequencing analyses revealed that endo-siRNAs with sequence complementarity to hundreds of protein-coding mRNAs are present in *C. elegans* (Ambros et al., 2003; Ruby et al., 2006). Endo-RNAi contributes to control of gene expression. This was revealed by microarray analysis of mutant worms lacking various RNAi-related factors, which found non-overlapping sets of differentially expressed genes, supporting the idea of multiple functionally distinct RNAi pathways in nematodes (Lee et al., 2006). At the same time, the multiple functionality also brings competition between small RNA pathways, which are manifested in different mutants – e.g. endo-siRNA mutants have increased expression of miRNA-regulated stage-specific developmental genes (Zhuang and Hunter, 2012).

Endo-RNAi pathway is initiated by endogenous dsRNAs, which are processed into primary siRNAs (26G RNAs associated with ERGO-1). Their biogenesis involves RDE-4, ERI-1/3/5/9, and RdRP RRF-3. Primary 26G RNAs recognize their targets, leading to production of secondary 22G RNAs, which will be antisense to the target and represent the main repressive force of endo-RNAi. 22G RNA biogenesis involves EGO-1 and RRF-1 RdRPs, RNAs are loaded mainly on CSR-1 or WAGO proteins. Secondary siRNAs are generated by RdRPs thus serve as amplifiers of the endogenous RNAi in both soma and germline.

A number of endo-siRNA genes were identified in a genetic screen for factors silencing a multicopy transgene. They included genes required for siRNA biogenesis or stability in the oocyte-specific ERGO-1 pathway, including *eri-12*, encoding an interactor of the RNAi-defective protein RDE-10, and *ntl-*

9/CNOT9, one of several identified CCR4-NOT complex genes, and a conserved ARF-like small Cell ARL-8 required for primary siRNA biogenesis or stability in the sperm-specific ALG-3/4 endogenous RNAi pathway (Fischer et al., 2013). Additional studies revealed that mutations in RNAi-related genes, result in defects in meiotic chromosome disjunction, spindle formation or microtubule organization during sperm development and ultimately lead to male sterility or embryonic lethality of the offspring (Han et al., 2008; Gent et al., 2009; Han et al., 2009; Pavelec et al., 2009).

In search for the cellular and developmental processes, which might be controlled by endo-siRNAs in *C. elegans*, spermatogenesis-associated genes were found enriched in the group of transcripts matching endo-siRNAs (Ruby et al., 2006). Analysis of endo-siRNA target suggested a difference between primary and secondary endo-siRNA targets as 18- to 22-mer siRNAs associated with genes required for embryonic development, 23-mers associated uniquely with post-embryonic development, and 24-26-mers associated with phosphorus metabolism or protein modification (Asikainen et al., 2008). It was also shown that in oocytes and embryos, ERGO-1-associated 26G siRNAs and NRDE-3-associated 22G siRNAs silence recently duplicated genes (Vasale et al., 2010; Fischer et al., 2011).

Exogenous RNAi – systemic and environmental

Exo-RNAi is triggered by exogenous dsRNA, which can come in many forms; RNAi in *C. elegans* can be triggered by injecting dsRNA essentially anywhere in the animal, soaking the animal in dsRNA solution or even by feeding it bacteria expressing long dsRNA (Tabara et al., 1998). This allows not only experimental manipulations in the lab but also target parasitic nematodes by creating nematode resistant plants (Yang et al., 2013). However, there is a heterogeneity in the nature in the presence of non-cell autonomous RNAi. For example, only one of eight tested *Caenorhabditis* species showed efficient environmental RNAi (Winston et al., 2007). Remarkably, RNAi also operates in zero gravity in space (Etheridge et al., 2011). It has zero significance for the report, but worth of mentioning.

The spreading of the silencing signal among cells is controlled by dsRNA-transporting channels encoded by *sid-1* and *sid-2* genes, which were identified in forward genetic screen to be responsible for systemic RNAi in *C. elegans* (Winston et al., 2002). SID-1 (systemic RNAi deficient-1) is a conserved transmembrane protein that forms a dsRNA channel (Feinberg and Hunter, 2003; Shih et al., 2009; Shih and Hunter, 2011). *Sid-1* has homologs in a wide range of animals, including mammals. *Sid-1* mutants have intact cell autonomous RNAi, but cannot perform neither systemic RNAi nor environmental RNAi in response to feeding, soaking, or injection of dsRNA (Winston et al., 2002). SID-2 is a transmembrane protein localized to an apical membrane of intestinal cells. It is necessary for the initial import of dsRNA from gut lumen, but not for the systemic spread of silencing signals among cells (McEwan et al., 2012). *Sid-2* homologs have been identified only in two other *Caenorhabditis* species (Winston et al., 2007). SID-3 is a conserved tyrosine kinase required for the efficient import of dsRNA. Without SID-3, cells perform RNA silencing well but import dsRNA poorly (Jose et al., 2012). The next and distinct systemic RNAi SID factor is SID-5 (SID-4 does not exist), an endosome-associated protein, which promotes transport of RNA silencing signals between cells and act differently than SID1-3 (Hinas et al., 2012). Remarkably, SID-1 sensitizes *Drosophila* and silkworm cells to RNAi induced by soaking by enabling concentration-dependent cellular uptake of dsRNA (Feinberg and Hunter, 2003; Shih et al., 2009) (Shih and Hunter, 2011; Mon et al., 2012; Mon et al., 2013; Xu et al., 2013a; Xu et al., 2013b).

In cells, exo-RNAi involves the above-mentioned RDE-4/Dicer/DRH-1 complex in processing dsRNA into primary siRNAs, which are loaded on RDE-1 Argonaute protein. RDE-1 itself does not cleave cognate RNAs. Instead, it recruits RDE-ribonuclease, which is involved in production of templates for RdRP-mediated production of secondary siRNAs (Tsai et al., 2015). In addition, genetic, proteomic, and biochemical data suggested that downstream of RDE-1 operates RDE-10/RDE-11 complex, which is recruited for degradation of target mRNA and RRF-1-dependent secondary siRNA synthesis (Zhang et al., 2012). The link between the RDE-10/RDE-11 complex and the aforementioned RDE-8 nuclease is unknown at the moment. Finally, several negative regulators of exo-RNAi were identified in genetic screens, including the ERI-1 nuclease (Kennedy et al., 2004), the ERI-2/RRF-3 RdRP (Simmer et al., 2002), ERI-3 (Duchaine et al., 2006), ERI-9, and the Argonaute ERI-8/ERGO-1 (Pavelec et al., 2009).

Antiviral RNAi

RNAi functions as an antiviral defense in *C. elegans*. This role of RNAi was experimentally addressed even though endogenous viral pathogens of *C. elegans* were unknown. The problem of absent endogenous *C. elegans* viruses was bypassed by using an “artificial” infection with viruses, which had a broad host range and could infect *C. elegans* under laboratory conditions. Model viral infections were based on the (+)ssRNA flock house virus (FHV) (Lu et al., 2005) or the (-)ssRNA vesicular stomatitis virus (VSV) (Schott et al., 2005; Wilkins et al., 2005). Infection with the recombinant VSV was augmented in strong RNAi mutant animals (*rde-1* and *rde-4*), which produced higher viral titers. Furthermore, VSV infection was attenuated in *rrf-3* and *eri-1* mutants that are hypersensitive to RNAi (Wilkins et al., 2005). Similar results were obtained from infected cultured cells (Schott et al., 2005) and FHV infection of *rde-1* mutants (Lu et al., 2005). Not surprisingly, genes misregulated in *C. elegans* deficient in Dicer, RDE-4, or RDE-1 are enriched for innate immunity genes (Welker et al., 2007). Antiviral RNAi involves also other genes, such as *rsd-2* (RNAi spreading defective 2), which was implicated in secondary viral siRNA biogenesis in RDE-4 independent manner (Guo et al., 2013b).

The antiviral role of exo-RNAi was subsequently confirmed in a natural viral infection of *C. elegans* and *C. briggsae* by Orsay virus (Felix et al., 2011). Analysis of gene expression in *C. elegans* infected with Orsay virus revealed competition of the antiviral response with endogenous small RNA pathways, which was manifested as redirection of RDE-1 from its endogenous small RNA cofactors, leading to loss of repression of endogenous RDE-1 targets and reduction of miRNA levels and an up-regulation of their target genes (Sarkies et al., 2013). Interestingly, genome-wide association study in *C. elegans* wild populations identified as a major determinant of viral sensitivity DRH-1, the aforementioned RIG-1 homolog (Ashe et al., 2013). Similarly, a genetic analysis revealed an essential role for both DRH-1 and DRH-3 in antiviral RNAi (Guo et al., 2013a). DRH-3 is another Dicer-RIG-I family protein that is essential for RNA silencing and germline development (Nakamura et al., 2007). DRH-3 binds both single-stranded and double-stranded RNAs with high affinity. However, the ATPase activity of DRH-3 is stimulated only by double-stranded RNA (Matranga and Pyle, 2010). Analysis of DRH-1 and DRH-3 in infected worms suggested distinct roles where DRH-1 was involved in production of viral primary siRNAs, whereas DRH-3 is involved in biogenesis of 22 nt secondary siRNAs (Guo et al., 2013a; Fitzgerald et al., 2014) (Figure 46). Taken together, RIG-I homologs function in innate immunity through evolution of Metazoa where they adapting to specific molecular mechanisms – primary and secondary siRNA biogenesis in RNAi in nematodes and stimulation of the interferon response in mammals.

Importantly, innate immunity operates beyond viruses and RNAi; *Bacillus thuringiensis* strain DB27 is virulent bacterium for *C. elegans* but not another nematode species. An analysis of virulence factors revealed that *dcr-1* mutant alleles deficient in microRNA (miRNA) processing, but not those deficient only in RNAi, are resistant to *B. thuringiensis* DB27 (Iatsenko et al., 2013). This suggests that Dicer is a part of a system balancing small RNA pathways and antiviral immunity. This model is further extended with the miR-35-41 family, regulates the efficiency of RNAi (Massirer and Pasquinelli, 2013). The loss of miR-35-41 results in enhanced exo-RNAi sensitivity and reduced endo-RNAi effectiveness suggesting that these miRNAs normally help balance the RNAi pathways (Massirer and Pasquinelli, 2013).

3.1.7.8 Adenosine deamination

A connection between A-to-I editing and RNAi has been revealed in *C. elegans*. A transcriptome-wide analysis of A-to-I editing sites identified as many as 664 editing-enriched regions, which represent the core of A-to-I editing substrates in *C. elegans*. Among the known substrates are mRNAs with hairpin structures in 3' UTRs; editing however does not necessarily prevent nuclear export and presence on polysomes (Morse and Bass, 1999; Morse et al., 2002; Hundley et al., 2008). Approximately 1.7% of *C. elegans* mRNAs contained such editing-enriched regions (Whipple et al., 2015). A-to-I editing also affects small RNAs.

In contrast to mice, where the loss of A-to-I editing is lethal, in *Drosophila* and *C. elegans* Adar null phenotype causes only weak phenotypic alterations (Palladino et al., 2000; Tonkin et al., 2002; Tonkin

and Bass, 2003). Importantly, *adr-1* or *adr-2* mutant worms exhibit a defective chemotaxis but the phenotype is reverted when worms lacking Adar are crossed with RNAi-defective strains (Tonkin and Bass, 2003). Analysis of ADAR mutants showed that A-to-I editing affects microRNAs as well as 26G endo-siRNAs (Warf et al., 2012). That ADAR and RNAi pathways are competing for common targets is supported by common loci from which originate edited transcripts and in which are found RNAi-dependent short RNAs upregulated in ADAR mutants (Wu et al., 2011).

3.1.7.9 Summary

The principles of primary and secondary small RNA production adopted during *C. elegans* evolution provide an explanation how such a complex of small RNA pathway can rely on a single Dicer protein? The key apparently lies in structural differences among Dicer substrates, Dicer co-factors facilitating loading onto correct Argonaute proteins, and cognate RNAs (targeted by primary small RNA), which serve as templates for secondary small RNAs.

Given the complexity of the Nematode phylum, one could ask how representative is *C. elegans* model of the phylum. There has been a survey for orthologs of 77 *C. elegans* RNAi pathway proteins in 13 nematode species, which revealed that while proteins responsible for uptake and spread of exogenously applied dsRNA are absent from parasitic species. Furthermore, Argonaute proteins regulating gene expression but not exo-RNAi Argonautes are broadly conserved; secondary Argonautes (SAGO/WAGO) are poorly conserved, and the nuclear AGO NRDE-3 was not identified in any parasite (Dalzell et al., 2011). Taken together, *Caenorhabditis* possess an expanded RNAi effector repertoire relative to the parasitic nematodes while all nematode species displayed qualitatively similar coverage of functional protein groups (Dalzell et al., 2011).

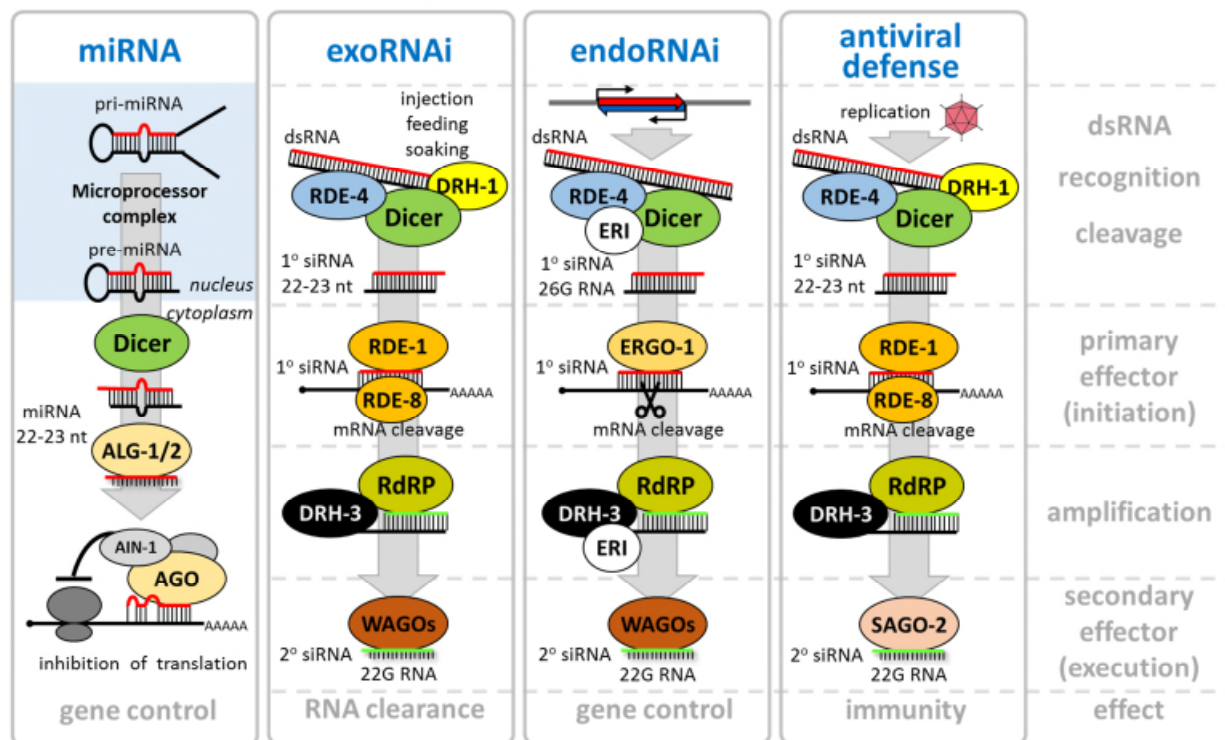


Figure 46 Schematic overview of reviewed small RNA pathways in *C. elegans*

C. elegans has a number of outstanding features, which make it the best available biosensor for environmental exposure to dsRNA, siRNA, and perhaps even miRNA in Europe.:

Geographical coverage: *C. elegans* is found worldwide, predominantly in temperate areas, in decomposing plant material, which provides abundant bacterial food (Frezal and Felix, 2015).

Accessibility: *C. elegans* can be easily isolated, maintained, and analyzed. Its genome is reasonably small and known, its biology is the best understood of all multicellular organisms.

Penetrability: *C. elegans* exhibits both, environmental and systemic RNAi, so it can readily uptake and distribute across its cells dsRNAs and siRNAs.

Sensitivity: *C. elegans* has highly complex RNAi, which can amplify the response to the original trigger, typically based on interaction with cognate transcripts. The amplification of the response would allow for easier detection of any effect caused by exogenous long dsRNA or small RNAs. Furthermore, given the interaction of small RNA pathways, a perturbed small RNA flux and/or environmentally induced exo-RNAi in wild-type animals, may impact development via effects on the endo-RNAi and microRNA pathways, as suggested by Zhuang et al (Zhuang and Hunter, 2012).

Cost: *C. elegans* arguably offers the best cost efficiency of genome-wide transcriptional analysis among all multicellular models.

3.1.7.10 References

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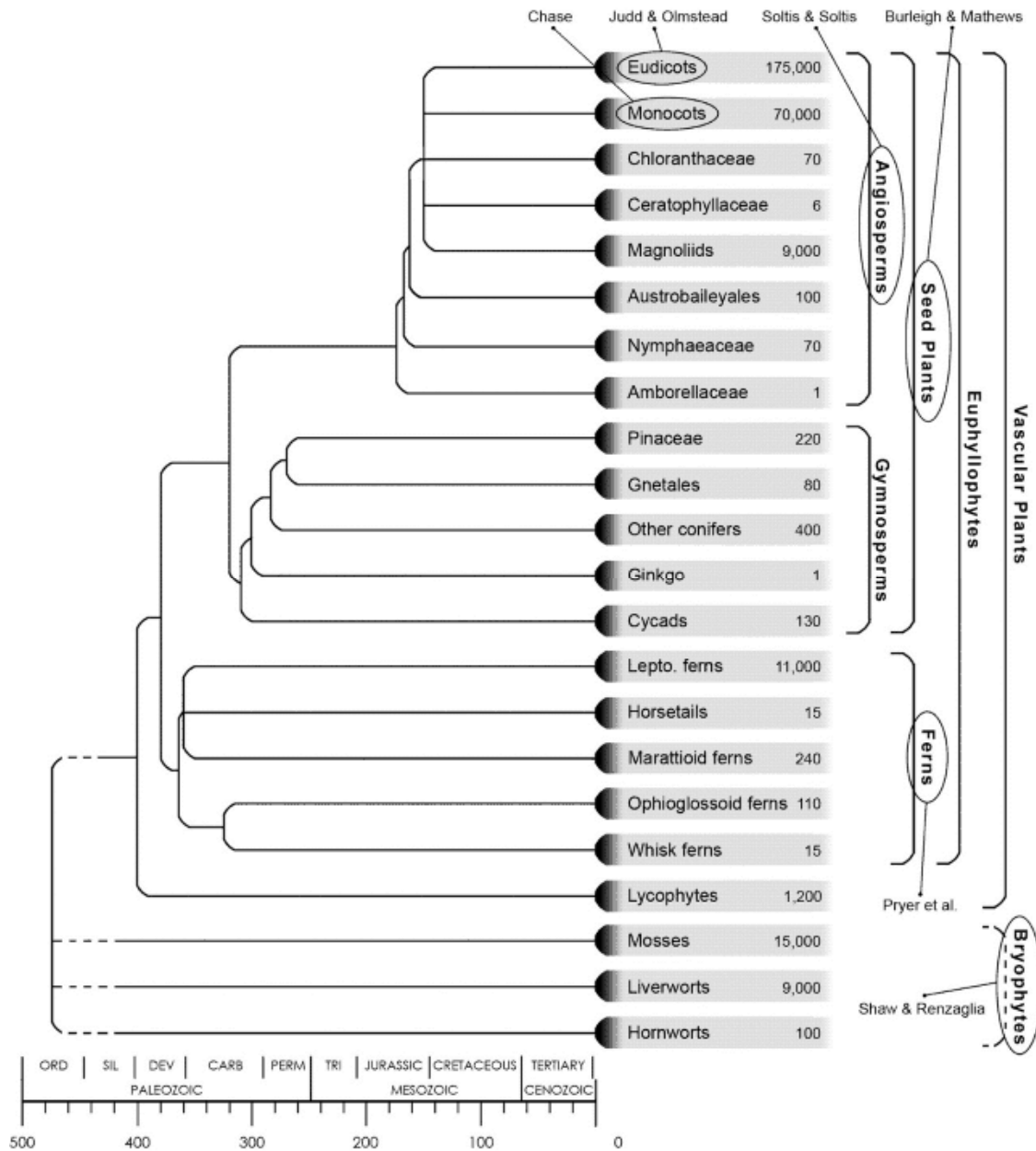
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3.1.8. Plants

3.1.8.1 Intro to the taxon

Plants are another extremely large and diverse group of multicellular organisms (Figure 47). Among their defining features is the ability to perform photosynthesis, which takes place in chloroplasts, double membrane organelles containing chlorophyll a/b. Their cell walls are made of cellulose and



they use starch to store photosynthetic products.

Figure 47 Phylogenetic classification of land plants, taken from (Palmer et al., 2004)

There are varying taxonomical concepts of plant classification. Here we will follow the concept of land plants as depicted in Figure 47 (Palmer et al., 2004) and which is consistent with Five kingdoms by Lynn Margulis and Karlene V. Schwartz (W.F. Freeman Printing in New York in 1999).

There are currently over 300 000 plant species, which are classified in 10-12 phyla. At the same time, virtually all model systems used to study the subject of this report (among which dominates *Arabidopsis thaliana*), belong to so-called angiosperms (phylum *Magnoliophyta* or *Anthophyta*), which is also the most populous phylogenetic group, representing perhaps up to 90% of plant species.

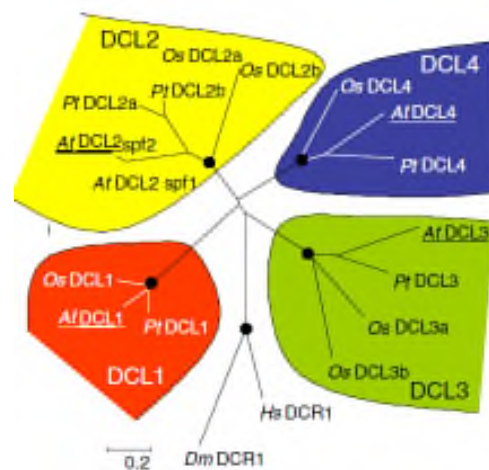
RNA silencing is extremely convoluted in plants. It is the most complex RNA silencing among the reviewed taxons thanks to existence of multiple Dicers and Argonautes, which operate in an intricate network of primary and secondary small RNAs, which mediate transcriptional and post-transcriptional effects. It can be divided in three to four main systems: miRNA, RNAi/ post-transcriptional gene silencing (PTGS), antiviral defense/virus-induced gene silencing (VIGS), and transcriptional gene silencing (TGS) (Galun, 2005; Bonnet et al., 2006; Vazquez, 2006; Mallory et al., 2008; Chen, 2009; Van Ex et al., 2011; Bologna and Voinnet, 2014; Borges and Martienssen, 2015; Carbonell and Carrington, 2015; Zhang et al., 2015)⁸. Therefore, with respect to the goal of the report, we will focus on the data from *Arabidopsis thaliana* and first review the molecular features of key individual components of RNA silencing related to the miRNA pathway and dsRNA response – Dicers, Argonautes, and RdRPs. Subsequently, we will discuss the reviewed mechanisms themselves and their biological roles, with a particular focus on the miRNA pathway, PTGS/VIG – plant's equivalent of RNAi, and transcriptional gene silencing (TGS), which is a unique adaptation found in plants. Unless specifically stated, presented data come from *A. thaliana*.

3.1.8.2 Absenting Microprocessor complex in plants

Plant miRNAs differ from animal miRNAs in several aspects. That includes the biogenesis, which does not use the two-step nuclear-cytoplasmic process employing Drosha and Dicer. Instead, plant miRNAs are produced in the nucleus in a two-step process involving a single Dicer protein – DCL1 whose function is described in the next section.

Figure 48 Phylogenetic analysis of plant Dicers

Shown is a published consensus phylogenetic tree (directly taken from Margis et al., 2006) constructed by neighbor-joining method with pairwise deletion, using the Dayhof matrix model for amino acid substitution, presented in radial format for the entire DCL proteins. *At* = *Arabidopsis thaliana*, *Os* = *Oryza sativa* (rice), *Pt* = *Populus trichocarpa* (polar tree). Note that the phylogenetic tree contains six DCL genes in rice but a thorough genome search yielded total of eight DCL genes in the genome (Kapoor et al., 2008).



3.1.8.3 Dicer proteins – DCL1-4 and additional Dicer family members

Plants have specialized and compartmentalized Dicer (Dicer-like – DCL) proteins that act partially redundantly and hierarchically in small RNA production in different pathways – miRNA, antiviral defense, endo-RNAi pathways, or chromatin remodelling (Xie et al., 2005; Moissiard and Voinnet, 2006; Liu et al., 2009b). The “basal” plant state found in *Arabidopsis* (or, for example, cotton (Silva et al., 2011)) is four Dicers, which presumably evolved through duplications of an ancestral Dicer (Margis et al., 2006) (Figure 48). Some plants (such as monocots) have higher counts of Dicer homologs – there are five in poplar and eight in rice; the additional Dicers evolved through duplications of one of the four “basic” plant Dicers (Margis et al., 2006; Kapoor et al., 2008; Mukherjee et al., 2013).

⁸ nomenclature of small RNAs in plants is complex and arbitrary. These small RNAs include miRNAs and various types of siRNAs produced from dsRNA, which are distinguished by their origin (viral siRNA), biogenesis (phased siRNAs) or their effect (heterochromatinizing siRNAs).

Plant Dicer proteins are large multidomain proteins, which have essentially the same domain organization as animal Dicers (Figure 49), i.e. they are composed of domains ordered from the N- to the C-terminus as follows: N-terminal DExD and helicase superfamily C-terminal domains, a domain of unknown function DUF283 (not annotated in DCL-3 of *Arabidopsis thaliana* but annotated in poplar and rice DCL3), a PAZ domain, RNase IIIa and RNase IIIb domains, and the C-terminal dsRBD. The four plant Dicers differ in size and the number of dsRBD domains – DCL1, DCL3, and DCL4 have an additional dsRBD (Figure 49). Although none of the plant Dicers has been either crystallized or analyzed by cryo-EM, the conserved domain organization and available biochemical data suggest that plant Dicers operate under the same principles as animal Dicers despite their functional diversification. In general, DCL1 is mainly functioning in the miRNA pathway while DCL2, 3, 4 produce siRNAs for silencing mobile elements or in antiviral defense where DCL3 represents the nuclear arm of the defense, which is distinguished by production of longer siRNAs (24 nt). Detailed review of individual DCL proteins is provided further below:

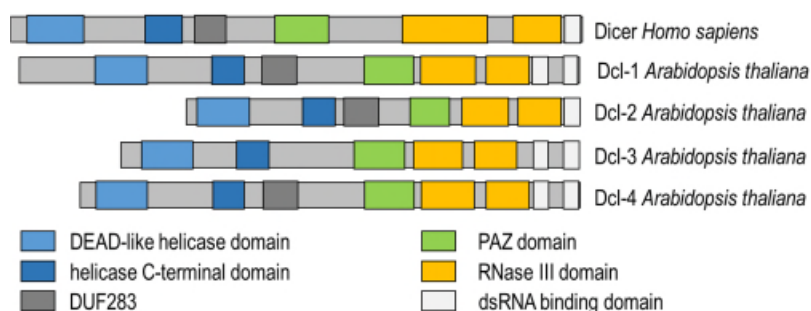


Figure 49 Comparison Dicer protein domain composition among Dicer proteins in *A. thaliana*, and *C. elegans*

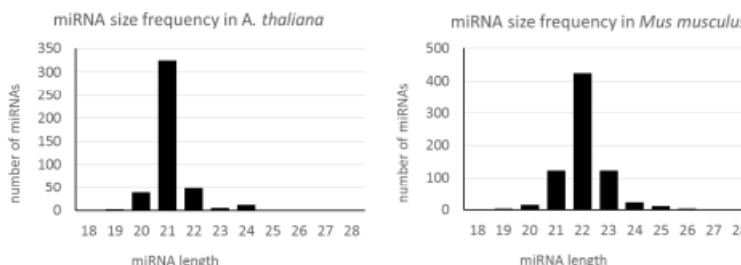
Domain composition was adopted from (Jaskiewicz and Filipowicz, 2008)

DCL1 – miRNA biogenesis & PTGS

DCL1 is the oldest known and possibly best characterized member of the DCL gene family in *Arabidopsis*. DCL1 was repeatedly recovered from mutation screens in plants since early 90's and it has been given several names (EMBRYO DEFECTIVE76 (EMB76), SHORT INTEGUMENTS1 (SIN1), SUSPENSOR1 (SUS1) and CARPEL FACTORY (CAF)) prior recognizing that it is a factor closely related to Dicer proteins acting in RNA silencing in animals (Schauer et al., 2002). DCL1 was first shown to be required for biogenesis of miRNAs but not siRNAs (Park et al., 2002; Reinhart et al., 2002). The size of the cleavage product is 21 nt (Figure 50).

Figure 50 *Arabidopsis* miRNA size distribution

Distribution of *Arabidopsis* mature miRNA lengths according to miRNA annotations in miRBase



DCL1 contains two putative nuclear localization signals and it localizes to the nucleus where it produces small RNAs (Papp et al., 2003). Furthermore, it was shown that DCL1 is also able to produce 21 nt from a transgenic inverted repeat (Papp et al., 2003).

DCL1 operates with a dsRBD binding partners DRB1 and DRB2 (Curtin et al., 2008; Reis et al., 2015c; Reis et al., 2016). Interestingly, DCL1 represses antiviral RNA silencing through negatively regulating the expression of DCL4 and DCL3 (Qu et al., 2008). DCL1 is an essential gene in *Nicotiana attenuata* (Bozorov et al., 2012) and its miRNA function is conserved in tomato (Kravchik et al., 2014b).

DCL2

DCL2 functions in the antiviral response (Fusaro et al., 2006; Curtin et al., 2008; Donaire et al., 2008; Urayama et al., 2010; Zhang et al., 2012; Ogwok et al., 2016) where it produces viral siRNAs without

requiring assistance from any dsRBP. (Curtin et al., 2008). DCL2 was also implicated in 21/22 nt siRNA production from longer intronic hairpins (sirtrons) (Chen et al., 2011). DCL2 acts hierarchically with DCL4 to produce 22- and 21-nt siRNAs in antiviral resistance and amplification of silencing mediated by RNA-dependent RNA polymerase RDR6 (Deleris et al., 2006; Brosnan et al., 2007; Di Serio et al., 2009; Garcia-Ruiz et al., 2010; Parent et al., 2015; Ogowok et al., 2016). DCL2 can substitute DCL4 and produce 22nt viral siRNAs even in the absence of DCL4, (Bouche et al., 2006; Moissiard et al., 2007; Wang et al., 2011). Under some circumstances, DCL2 can antagonize production of miRNAs and siRNAs by DCL1 (Bouche et al., 2006).

DCL2 is required for transitive cell-autonomous post-transcriptional silencing of transgenes (Mlotshwa et al., 2008) and it was implicated in the transgenerational stress memory (Boyko and Kovalchuk, 2010; Migicovsky and Kovalchuk, 2014; Migicovsky et al., 2014; Migicovsky and Kovalchuk, 2015). Two paralogs of DCL2 were found in *Medicago truncatula* where are DCL genes differentially expressed during symbiosis with nitrogen fixing bacteria and upon pathogen infection (Tworak et al., 2016). Two paralogs of DCL2 were also found in soybean where DCL2 showed the strongest transcriptional response to stress (Curtin et al., 2012).

DCL3

DCL3 is producing longer siRNAs (24 nt) than the other three DCL proteins in *Arabidopsis* and other plants, including tomato, rice, medick, or moss (Kravchik et al., 2014a; Wei et al., 2014; Coruh et al., 2015; Tworak et al., 2016). DCL3 preferentially cleaves dsRNAs with 5' phosphorylated adenosine or uridine and a 1 nt 3' overhang (Nagano et al., 2014) and produces 24 nt RNA duplexes with 2 nt 3' overhangs; inorganic phosphate, NaCl and KCl enhance DCL3 activity (Kravchik et al., 2014a).

DCL3 long dsRNA substrates are typically generated from RNA polymerase IVa/IV and IVb/V (Pol IV and Pol V hereafter) transcripts by RDR2 (Zhang et al., 2007; Daxinger et al., 2009). DCL3 co-localizes with RDR2, AGO4, NRPD1b (the largest Pol V subunit) and siRNAs within the nucleolus (Pontes et al., 2006).

DCL3 was implicated in 24 nt siRNA production from longer intronic hairpins (sirtrons), which were associated with AGO4 and could mediate RNA-dependent DNA methylation (RdDM) (Chen et al., 2011), and in production of viral 24 nt siRNAs (Akbergenov et al., 2006; Fusaro et al., 2006; Diaz-Pendon et al., 2007; Curtin et al., 2008; Doinaire et al., 2008; Raja et al., 2014). DCL3 can produce viral siRNAs without requiring assistance from any dsRBP (Curtin et al., 2008) but, it was also shown that it can function with DRB3 and AGO4 in methylation-mediated antiviral defense (Raja et al., 2014). Interestingly, the loss of DCL3 is partially complemented by DCL4 and DCL2, which produce 21/22 nt small RNAs (Kravchik et al., 2014a) indicating partial functional redundancy of DCL2/3/4 in recognition of dsRNA and silencing.

DCL3 and RDR2 were also implicated in production of a pseudogene-derived 24 nt siRNAs in rice (Guo et al., 2009). DCL3b paralog in rice functions in processing of 24 nt phased small RNAs in miRNA targeted loci suggesting functional divergence of DCL3 paralogs in rice (Song et al., 2012). DCL3 was suggested to participate also in the transgenerational stress memory (Boyko and Kovalchuk, 2010; Migicovsky and Kovalchuk, 2014; Migicovsky et al., 2014; Migicovsky and Kovalchuk, 2015).

Finally, DCL3, RDR2 and Pol IV, also operate in production of 24 nt small RNAs from miRNA loci, which are loaded on AGO4 and mediate RdDM (Chellappan et al., 2010; Wu et al., 2010). DCL3 may be absent in conifers, which radiated from other seed-bearing plants approximately 260 million years ago; there were no significant amounts of 24 nt siRNAs in growing shoot tissue while no evidence for DCL3 was found (Dolgosheina et al., 2008).

DCL4

DCL4 cleaves long dsRNAs with blunt ends or with a 1 or 2 nt 3' overhang with similar efficiency; inorganic phosphate, NaCl and KCl inhibit DCL4 activity (Nagano et al., 2014). DCL4 operates with DRB4 to produce 21 nt trans-acting siRNAs (tasiRNAs, they are 21 nt siRNAs produced from discrete loci (TAS genes).and siRNAs from viral RNA. (Fusaro et al., 2006; Nakazawa et al., 2007; Curtin et al.,

2008; Qu et al., 2008). DCL4 alone was sufficient for antiviral silencing in leaves inoculated with Turnip mosaic virus (Garcia-Ruiz et al., 2010). While DCL4 is important for biogenesis of tasiRNA and antiviral response, it does not participate in the miRNA pathway (Xie et al., 2005).

DCL4 acts hierarchically with DCL2 to produce 21- and 22-nt siRNAs and in antiviral resistance and amplification of silencing mediated by RDR6 (Deleris et al., 2006; Brosnan et al., 2007; Howell et al., 2007; Liu et al., 2007; Qu et al., 2008; Di Serio et al., 2009; Garcia-Ruiz et al., 2010; Parent et al., 2015; Ogowok et al., 2016). The *dcl4-2* mutants lack each of three families of 21-nt tasiRNAs, have elevated levels of tasiRNA target transcripts, and display heterochronic defects similar to RDR6 mutants (Xie et al., 2005). Furthermore, different double mutant phenotypes also suggested hierarchical redundancy among DCL activities leading to alternative tasiRNA biogenesis in the absence of DCL4 (Xie et al., 2005).

DCL4 also operates (again, hierarchically with DCL2) in biogenesis of secondary siRNAs in transitive RNAi (Moissiard et al., 2007; Mlotshwa et al., 2008) and it was implicated in 21/22 nt siRNA production from longer intronic hairpins (sirtrons) (Chen et al., 2011)

Two paralogs of DCL4 exist in *Medicago truncatula* where DCL genes are differentially expressed during symbiosis with nitrogen fixing bacteria and upon pathogen infection (Tworak et al., 2016).

3.1.8.4 dsRBPs –DRB1-5

While DCLs act redundantly and hierarchically, there is little if any redundancy or hierarchy among DRBPs in their Dicer-associated functions. *Arabidopsis* genome encodes five DRBs composed of two types of dsRBDs that form a distinct clade (Clavel et al., 2016). Two additional proteins carrying dsRBDs (At1g80650 and At4g00420, renamed AtDRB7.1 and AtDRB7.2, respectively) were localized in the *Arabidopsis* genome. They differ from DRB1-5 in terms of dsRBD composition (Clavel et al., 2016). DRB1 and DRB2 associate with DCL1, DRB4 with DCL4, while DCL2 and DCL3 produce viral siRNAs without requiring assistance from any dsRBP. (Curtin et al., 2008) below we will briefly summarize information about individual DRBs.

DRB1/HYL1

DRB1 has two dsRBDs separated by a linker of ~ 20 amino acids (Clavel et al., 2016). DRB1 is a nuclear dsRBP exclusively functioning together with DCL1 and a small RNA methyltransferase HEN1 in miRNA biogenesis (Vazquez et al., 2004; Curtin et al., 2008). This role seems conserved across plants; its homologs were found in all tested plant genomes (You et al., 2014; Clavel et al., 2016). In association with DCL1 it directs the guide strand selection for AGO loading (Eamens et al., 2009) and determines the slicing mode of action of the miRNA-loaded AGO1 (Reis et al., 2015c). It also interacts with a hairpin in short interspersed element SB1 RNA and facilitates DCL1-mediated production of small RNAs from these repetitive elements (Pouch-Pelissier et al., 2008). DRB1 is phosphorylated by mitogen activated protein kinase MPK3 in both rice and *Arabidopsis* (Raghuram et al., 2015).

DRB2

DRB2 proteins possess two dsRBDs separated by a linker of 19 amino acids (Clavel et al., 2016). DRB2 is involved in miRNA biogenesis (Eamens et al., 2012a) where it, in association with DCL1, determines the translational repression of miRNA-loaded AGO1 (Reis et al., 2015c). DRB2 is involved in the processing stage of the biogenesis of non-canonical miRNA subsets while DRB3 and DRB5 are somehow required downstream to mediate RNA silencing of DRB2-associated miRNA target genes (Eamens et al., 2012b). The role of DRB2 in the miRNA pathway is distinct from that of DRB1 as shown by proteomic analysis of *drb1* and *drb2* mutants, which suggested that DRB2-associated translational inhibition appears to be less ubiquitous and specifically aimed toward responses against environmental stimuli (Reis et al., 2015b). DRB2 acts redundantly with DRB3 and DRB5 during development and appears unnecessary for other types of plant small RNAs, such as miRNA, tasiRNA, viral siRNA, or heterochromatinising siRNA production (Curtin et al., 2008). The loss of DRB2 protein in *Arabidopsis* results in increased levels of Pol IV dependent siRNAs, which are involved in RdDM. It

was proposed that DRB2 is part of epigenetic regulation suppressing transcription of transposable elements (Pelissier et al., 2011; Clavel et al., 2015).

DRB3

DRB3 proteins possess two dsRBDs separated by a linker of 19 amino acids (Clavel et al., 2016). DRB3 participates to the RdDM defense against Geminiviruses (Raja et al., 2014). At the same time, it appears unnecessary for miRNA, tasiRNA, viral siRNA, or heterochromatinising siRNA production but acts redundantly with DRB2 and DRB5 during development (Curtin et al., 2008). DRB3 participates in RNA silencing of target genes of DRB2-associated non-canonical miRNAs (Eamens et al., 2012b)

DRB4

DRB4 protein and its relatives carry three dsRBDs. They are found in all vascular plants but were absent in the tested bryophyte and lycophyte genomes (Clavel et al., 2016). DRB4 operates with DCL4 to produce 21 nt tasiRNAs and 21nt siRNAs from viral RNA (Curtin et al., 2008; Qu et al., 2008; Shivaprasad et al., 2008; Jakubiec et al., 2012). DRB4 expression is regulated by E3 ubiquitin ligase APC/C (Anaphase Promoting Complex or Cyclosome). APC10 interacts with DRB4 through the second dsRBD of DRB4, which is also required for its homodimerization and binding to DCL4 (Marrocco et al., 2012). In contrast to the loss of DRB2 protein in *Arabidopsis*, the loss of DRB4 results in reduced levels of Pol IV dependent siRNAs, which are involved in RdDM (Pelissier et al., 2011)

DRB5

DRB5 proteins possess two dsRBDs separated by a linker of 19 amino acids (Clavel et al., 2016). DRB5 appears unnecessary for miRNA, tasiRNA, viral siRNA, or heterochromatinising siRNA production but acts redundantly with DRB2 and DRB3 during development (Curtin et al., 2008). DRB5 is somehow required downstream to mediate RNA silencing of target genes of DRB2-associated non-canonical miRNAs (Eamens et al., 2012b). DRB3 was shown to associate with DCL2 and AGO4 in the RdDM arm of antiviral defense against Geminiviruses (Raja et al., 2014)

DRB6

DRB6 proteins carry two dsRBDs and are present in all vascular plants except for bryophytes, lycophytes and *Brassicaceae* species (Clavel et al., 2016). Their biological significance is unknown.

DRB7

The DRB7 family has a single dsRBD; there are two members AtDRB7.1 and AtDRB7.2. Their role is largely unknown but it was found that they interact with DRB4 but not with DCL4 in *Arabidopsis* (Clavel et al., 2016).

3.1.8.5 Argonaute proteins

Plants show quite some variability in the AGO homolog pool (Figure 51). For example, *Oryza sativa* has 19 AGO genes, *Glycine max* (soybean) has 22 (Zhang et al., 2015), *Solanum lycopersicum* 15 (Bai et al., 2012). A dicot plant *Sativa miltiorhiza* (red sage) has 10 AGO genes of which AGO1, 2, 3, 7, and 10 were proposed to function similarly to their *A. thaliana* counterparts (Shao and Lu, 2013). A systematic survey of 32 plant genomes showed that plants have 6-24 AGO homologs per genome, most often more than 10 (Mirzaei et al., 2014). AGO proteins are phylogenetically divided into three clades (Figure 51), the clades also reflect different classes of bound small RNAs – clades I and II bind 21-22 nt small RNAs while the clade III accommodates longer (~24 nt small RNAs) (Zhang et al., 2015). The phylogenetic analysis shows that the varying number of homologs in each species stems from multiple duplication events, which occurred during evolution of different taxons such that some species contain multiple paralogs of an ancestral AGO, which can be present in a single copy in another plant species (Figure 51 – compare, for example AGO1 and AGO10 paralogs in *A. thaliana* and *G. max*).

AGO proteins loaded with different types of small RNAs play diverse roles in terms of molecular mode of action mechanisms and biological function. Small RNAs are sorted onto specific AGO proteins based on different factors, which might include subcellular localization or the DCL protein that is producing a particular small RNA. One of the key factors contributing to sorting of small RNAs onto AGO proteins is also the 5' terminal nucleotide. For example, AGO1 favors miRNAs with a 5' terminal uridine, AGO2 and AGO4 prefer small RNAs with a 5' terminal adenosine, AGO5 with a 5' terminal cytosine (Mi et al., 2008; Takeda et al., 2008).

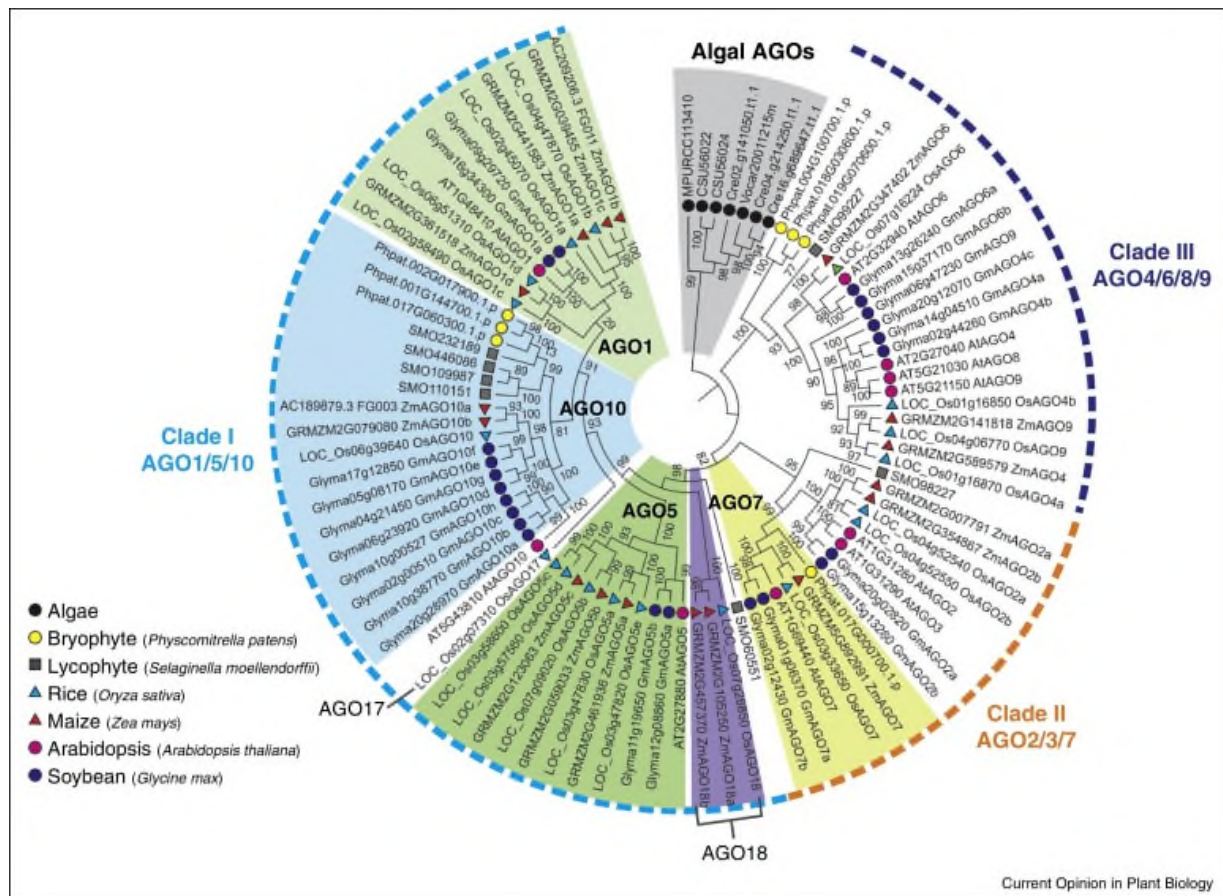


Figure 51 Phylogeny of plant AGO proteins, taken from (Zhang et al., 2015)

AGO1

Arabidopsis AGO1 gave the name to the entire Argonaute protein family because *ago1* mutants were having a phenotype reminiscent of the tentacles of an *Argonauta* squid (Bohmert et al., 1998). *Arabidopsis* has a single AGO1 gene, rice has four paralogs (Wu et al., 2009). AGO1 preferentially binds miRNAs and small RNAs with a 5' uridine (Bohmert et al., 1998; Vaucheret et al., 2004; Mi et al., 2008; Wu et al., 2009; Jeong et al., 2013; Rogers and Chen, 2013). Analysis of miRNAs loaded onto AGO1 paralogs in rice suggested that a subset of miRNAs is specifically incorporated into or excluded from one of these paralogs suggesting they have both redundant and specialized roles in rice (Wu et al., 2009). AGO1 also associates with tasiRNAs. In contrast to miRNAs, tasiRNAs involve RdRP-mediated conversion of cleaved TAS RNA into dsRNA followed by production of phased tasiRNAs (i.e. secondary small RNAs) by DCL-4, which are loaded onto AGO1 (Vaucheret, 2005)

AGO2

AGO2 is a slicing AGO (Carbonell et al., 2012) but can also directly repress translation (Fatyol et al., 2016). It favors small RNAs with a 5' terminal adenosine (Mi et al., 2008; Takeda et al., 2008), which

include miR393*, regulating antibacterial innate immunity (Zhang et al., 2011). AGO2 plays a role in the natural cis-antisense (natsiRNA) pathway (Oliver et al., 2014). AGO2 also mediates antiviral defense (Jaubert et al., 2011; Odokonyero et al., 2015) and was implicated (together with a plant-specific GW protein NERD) in nuclear silencing of a set of non-conserved genomic loci (Pontier et al., 2012). In addition, AGO2 was also associated with diRNAs, small RNAs emerging during double-stranded break repair (Wei et al., 2012; Oliver et al., 2014).

AGO3

Drosophila AGO3 is a close paralog apparently emerging through a genome duplication of the AGO2 locus (Vaucheret, 2008). Interestingly, rice and maize lack the AGO3 ortholog but have a pair of AGO2 genes instead (Kapoor et al., 2008; Zhai et al., 2014).

AGO4

AGO4 preferentially recruits 24 nt small RNAs with a 5' terminal adenosine (Mi et al., 2008; Havecker et al., 2010) and mediates RNA-directed DNA methylation (Zilberman et al., 2004; He et al., 2009; Havecker et al., 2010; Wu et al., 2010; Xie and Yu, 2015). AGO4-loaded small RNAs are often DCL-3 produced repeat and heterochromatin-associated siRNAs from introns and intergenic regions (Zilberman et al., 2004; He et al., 2009; Zheng et al., 2009; Chen et al., 2011; Xie and Yu, 2015) but can also be DCL-4 produced tasiRNAs (Wu et al., 2012). In rice, it was even found that RNA-directed DNA methylation can be guided by a specific miRNA class produced by DCL3 (Wu et al., 2010). AGO4 is also coupled to antibacterial (Agorio and Vera, 2007) and antiviral innate immunity (Jones et al., 2006; Minoia et al., 2014; Raja et al., 2014; Ma et al., 2015).

AGO5

AGO5 is highly enriched in the germline (Oliver et al., 2014). It binds preferentially 21 nt small siRNAs (phased small RNAs, phasiRNAs) with a 5' terminal cytosine (Mi et al., 2008; Takeda et al., 2008; Komiyama et al., 2014). It also binds miRNAs and mediates miRNA-directed target cleavage (Oliver et al., 2014). *Arabidopsis* AGO5 has been implicated in female gametogenesis (Tucker et al., 2012) and in antiviral defense (Brosseau and Moffett, 2015). MEL1, AGO5 homolog in rice, has specific functions in the development of pre-meiotic germ cells and the progression of meiosis (Komiyama et al., 2014).

AGO6

AGO6 preferentially recruits 24 nt RNAs with a 5' terminal adenosine (Havecker et al., 2010). AGO6 mediates RNA-directed DNA methylation (Zheng et al., 2007; Havecker et al., 2010; Eun et al., 2011; McCue et al., 2015). AGO6 participates in RNA-directed DNA methylation of transcriptionally active transposable elements through incorporation of fragments of PTGS-targeted transcripts of transposable elements onto AGO6 (McCue et al., 2015). AGO6 can also accommodate DCL4-produced produced tasiRNAs (Wu et al., 2012)

AGO7

AGO7 was originally named ZIPPY and was primarily associated with the regulation of developmental timing and did not have a significant role in transgene silencing (Hunter et al., 2003). It was subsequently found that AGO7 is preferentially loaded with miR390 and triggers production of tasiRNAs from the TAS3 locus (Garcia et al., 2006; Montgomery et al., 2008). TAS3-derived tasiRNA target AUXIN RESPONSE FACTORS and regulate leaf patterning and lateral organ separation (Montgomery et al., 2008; Zhou et al., 2013). This role of AGO7 appears conserved across plants as it has been also reported from rice (Nagasaki et al., 2007; Shi et al., 2007) and maize (Douglas et al., 2010).

AGO8 - seems to be a pseudogene in A. thaliana (Zhang et al., 2015)

AGO9

AGO9 is involved in RNA-directed DNA methylation and is highly enriched in the germline (Oliver et al., 2014) where it controls female gamete formation by repressing the specification of germ cell fate through epigenetic reprogramming in companion somatic cells (Olmedo-Monfil et al., 2010). *Zea mays* AGO2 homolog (originally designated ZmAGO104) is also specifically expressed in the somatic cells surrounding future gametes but it had an opposing function – it suppresses the somatic cell fate in germ cells (Singh et al., 2011).

AGO10

AGO10 is the closest homolog of AGO1 and its main role appears to be sequestration of miR156/166 miRNA family from AGO1 (Zhu et al., 2011; Zhou et al., 2015). The targets of miR166/165 are class III HOMEODOMAIN-LEUCINE ZIPPER transcription factors, which determine the shoot apical meristem fate. According to the model, AGO10 uses its higher binding affinity for miR166/165 and functions as a decoy, preventing loading of miR166/165 onto AGO1, hence preventing their suppression and allowing for proper regulation of the shoot apical meristem (Liu et al., 2009a; Ji et al., 2011; Zhu et al., 2011; Brandt et al., 2013; Tucker et al., 2013; Roodbarkelari et al., 2015; Zhou et al., 2015).

Additional relevant homologs

Monocot genomes encode for AGO18 homologs, which are not found in dicots, such as *A. thaliana* (Figure 51) (Zhang et al., 2015). In rice, AGO18 is important for antiviral defense (Wu et al., 2015).

3.1.8.6 RdRPs

The first RdRP homolog found in plants was RdRP from tomato (Schiebel et al., 1993; Schiebel et al., 1998). *Arabidopsis* genome carries six RdRP genes: RDR1-RDR6 (Yu et al., 2003). There are five RdRPs in *Salvia miltiorrhiza* (Shao and Lu, 2014) and six in potato *Solanum lycopersicum* (Lin et al., 2016). Plant RdRPs are homologs of RdRPs acting in RNA silencing in other kingdoms, such as QDE-3 in *Neurospora* (Salgado et al., 2006; Wassenegger and Krczal, 2006), suggesting that they evolved from a single ancestral RdRP acting in RNA silencing. RdRPs produce dsRNA that can enter the RNA silencing pathway. Hence, they either initiate RNA silencing or function as an amplifier of an already present dsRNA response.

RDR1

RDR1 (also known as SDE1/SGS2) in *Arabidopsis* and its homologs in other plant species contribute to RNA silencing-based resistance to virus infection (Vaistij et al., 2002; Yu et al., 2003; Muangsan et al., 2004; Yang et al., 2004; Diaz-Pendon et al., 2007; Garcia-Ruiz et al., 2010; Blevins et al., 2011; Leibman et al., 2011; Cao et al., 2014). It was also implicated in biogenesis of tasiRNAs during juvenile development (Peragine et al., 2004)

RDR2

RDR2 has been linked with transcriptional silencing in the nucleus and RdDM of specific loci (Chan et al., 2004; Xie et al., 2004). It participates in biogenesis of endogenous siRNAs (natsiRNAs) (Brosnan et al., 2007; Borges and Martienssen, 2015). RDR2 is not required for production of viral siRNAs from the Cauliflower mosaic pararetrovirus (Blevins et al., 2011), Cabbage leaf curl geminivirus (Aregger et al., 2012). RDR3 converts PolIV transcripts into dsRNA, which is processed by DCL3 into 24 nt siRNAs loaded onto AGO4 (Pontes et al., 2006; Zhang et al., 2007). RDR2 and DCL3 were implicated in production of pseudogene-derived 24 nt siRNAs in rice (Guo et al., 2009).

RDR3 – no functional information available

RDR4

RDR4 has been linked to biogenesis of endogenous siRNAs (natsiRNAs) (Borges and Martienssen, 2015)

RDR5 – no functional information obtained

RDR6

RDR6 is necessary for sense-transgene mediated silencing and is important in antiviral defense against certain viruses (Dalmay et al., 2000; Mourrain et al., 2000; Beclin et al., 2002). RDR6-dependent antiviral response includes the cucumber mosaic virus in *Arabidopsis* (Wang et al., 2010) or tobacco mosaic virus in *Nicotiana benthamina* (Qu et al., 2005) but not the cauliflower mosaic virus in *Arabidopsis* (Blevins et al., 2011). RDR6 was also implicated in the biogenesis of tasiRNAs and development (Peragine et al., 2004; Li et al., 2005a; Vaucheret, 2005). RDR6-generated dsRNA is being processed by DCL4 (Howell et al., 2007; Qu et al., 2008).

3.1.8.7 miRNA module

miRNA biogenesis

Biogenesis initiates with recognition and cleavage of a primary miRNA (pri-miRNA), which is transcribed by polymerase II (Xie et al., 2010; Zhao et al., 2013). A plant pri-miRNA is a single-stranded RNA carrying a local hairpin structure. Many miRNAs in plants apparently originate from longer inverted repeats carrying sequences of their targets, which were generated by sequence duplications (Allen et al., 2004). These long inverted repeats subsequently eroded during evolution and only a short stem in the pri-miRNA persists as a functional remnant of the original long hairpin. The inverted repeat duplication hypothesis provides an explanation for the evolution of perfectly pairing miRNAs in plants.

miRNA biogenesis in plants occurs as a two-step process entirely in the nucleus and employs a single RNase III – DCL1 (see the scheme below). DCL1 requires additional cofactors, including DRB1/HYL1 (a nuclear dsRNA binding protein (Vazquez et al., 2004)), HEN1 (HUA ENHANCER1, a small RNA methyltransferase (Yu et al., 2005)), SE (SERRATE, C2H2-type zinc finger, (Lobbes et al., 2006; Yang et al., 2006)). DCL1 resides in a complex, in which physically interacts with DRB1 and HEN1 (Baranuskas et al., 2015). DCL1, DRB1 and SE co-localize in the nucleus in so-called dicing bodies (D-bodies) (Fang and Spector, 2007). D-body function and assembly is not fully understood and there is a number of additional components which need to be functionally analyzed to unravel the complex connections between the D-body, signalling cascades, and responses to the environment (Reis et al., 2015a).

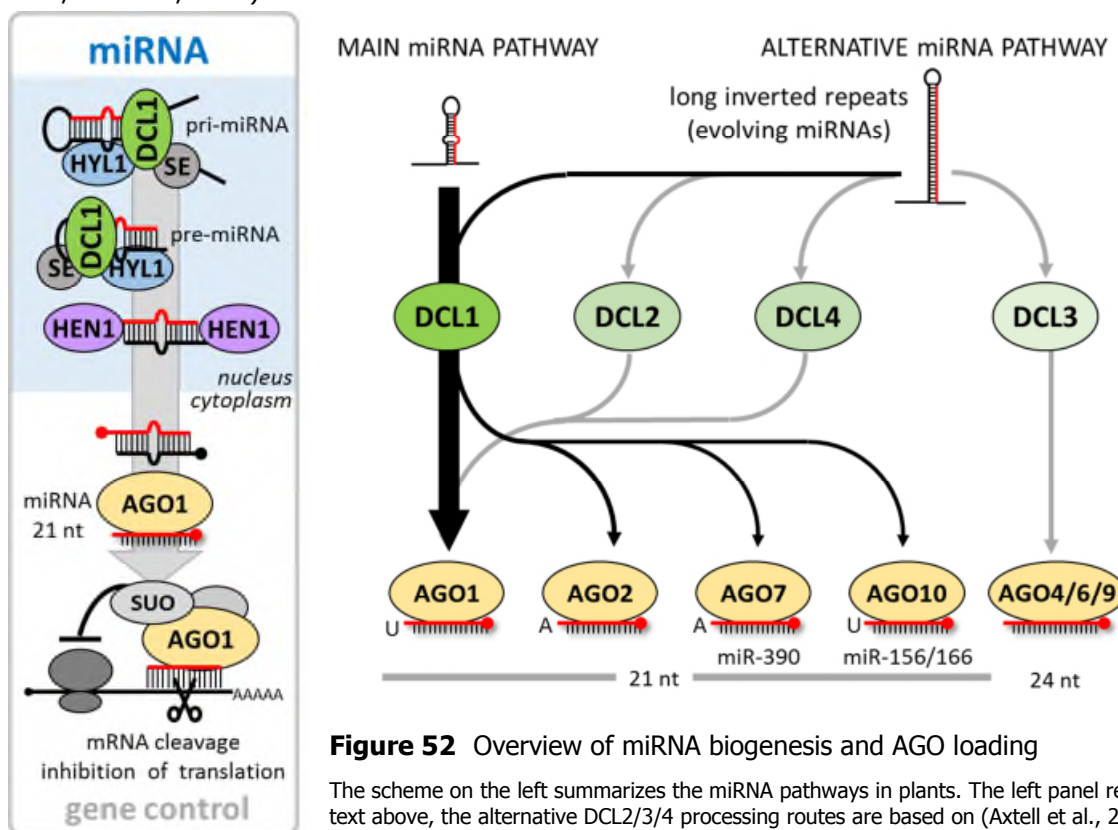
In the first step of miRNA biogenesis, DCL1 excises the miRNA/miRNA* duplex processing pri-miRNA from the base of the hairpin toward the loop (base-to-loop) (Park et al., 2002; Reinhart et al., 2002). Loop-to-base processing occurs in specific cases, such as miR159 and miR319 (Bologna et al., 2009). A unique case of bidirectional processing was observed for miR166, where it seems to play a regulatory role (Zhu et al., 2013). Plants also have non-canonical miRNAs, such as mirtrons, which skip the first cleavage step by DCL1 (Meng and Shao, 2012).

The stem loop structure of pri-miRNA is recognized and processed by the DCL1-DRB1-SE complex (Finnegan et al., 2003; Vazquez et al., 2004; Lobbes et al., 2006; Yang et al., 2006). Other DRB2 proteins (DRB2, 3, 5) also participate in biogenesis of miRNAs (Eamens et al., 2012a; Eamens et al., 2012b). Some pri-miRNA stems produce a single miRNA, some are longer and two or three additional ones on phase, i.e. require additional DCL1 cuts (Bologna et al., 2009; Zhang et al., 2010). Long hairpins can be processed by a diversity of Dicers to generate either miRNAs or small interfering RNAs

(siRNAs). The subcellular location for dicing by DCL2 and DCL4, and subsequent AGO loading of the resulting siRNAs, is not completely understood (Axtell et al., 2011).

The 3' termini of the miRNA/miRNA* duplex are modified by HEN1 which adds a 2'-O-methyl group to the miRNA (Yu et al., 2005). This modification distinguishes plant miRNAs from animal miRNAs, which are not methylated (perhaps except of some minor population of miRNAs in arthropods (Horwich et al., 2007), which are discussed in the *Arthropoda* section). HEN1 was first identified genetically as a miRNA biogenesis co-factor, which was shown to localize into the nucleus (Park et al., 2002; Vaucheret et al., 2004; Vazquez et al., 2004). The HEN1 ortholog in rice was identified as WAF (WAVY LEAF), a crucial developmental factor (Abe et al., 2010). Structural analysis of HEN1 and its homologs showed that the catalytic domain of HEN1 is not closely related to any known RNA:2'-OH methyltransferases, but rather to small-molecule methyltransferases (Tkaczuk et al., 2006). One of the functions of the methylation is that it protects miRNAs from uridylation by an AGO1-associated uridylyase that uridylyates 5' RNA fragments generated by AGO1 cleavage (Li et al., 2005b; Ren et al., 2014).

miRNAs are exported from the nucleus with the assistance of hasty (HST), a plant homolog of Exportin 5 (Park et al., 2005). The subcellular location for dicing by DCL2 and DCL4, and subsequent AGO loading of the resulting siRNAs, is not yet clear. Loading of AGO1 with DCL1 products is assumed to take place in the cytoplasm (Park et al., 2005; Axtell et al., 2011). This loading onto AGO proteins involves sorting miRNAs according to the 5' terminal nucleotide and other factors (Mi et al., 2008; Montgomery et al., 2008; Zhu et al., 2011). Analysis of strand selection suggest that the strand with a lower 5'-end thermostability is preferentially loaded into AGO1 (Eamens et al., 2009), which shows that plants employ the same loading asymmetry rule as animals. The selective loading of miRNA guide strand is directed by DRB1 (Eamens et al., 2009). DRB1 needs to be dephosphorylated for optimal activity; dephosphorylation is ensured by CPL1 (C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1/FIERY2 (FRY2)) (Manavella et al., 2012). AGO1 miRISC loading also involves cyclophilin40 and HSP90; ATP hydrolysis by HSP helps to release the AGO1-miRNA complex (Smith et al., 2009; Earley and Poethig, 2011; Iki et al., 2012).



Plant miRNAs are mainly loaded onto AGO1, which has an endonuclease activity and is able to suppress gene expression through both target cleavage and translational inhibition (Mourrain et al., 2000; Baumberger and Baulcombe, 2005; Wu et al., 2009). AGO1 shows a preferential loading for miRNAs carrying uridine at their 5' end. A change in the 5' terminal nucleotide of an miRNA predictably redirects it into a different AGO complex and alters its biological activity (Mi et al., 2008). Remarkably, the DCL1 partnering with DRB1 or DRB2 will determine the mode of action of a loaded miRNA: DRB1 is associated with dicer cleavage while DRB2 with translational repression (Reis et al., 2015c). Interestingly, a subset of miRNAs is only 20 nt long – their length appears to be determined by asymmetric bulges and mismatches at specific positions of the precursor (Lee et al., 2015).

Modes of miRNA action in plant cells

Similarly to animals, miRNAs loaded on AGO proteins serve as a guide for sequence-specific repression. Similarly to mammalian miRNAs, plant miRNAs can also mediate translational repression and sequence-specific cleavage of cognate mRNAs (e.g. Rhoades et al., 2002; Brodersen et al., 2008; Mallory and Bouche, 2008; Beauclair et al., 2010; Li et al., 2014). At the same time, activities of plant mRNAs differ from their animal counterparts in several aspects. First, unlike animal miRNAs, many plant miRNAs frequently exhibit perfect or nearly perfect complementarity to their substrates resulting in RNAi-like cleavage of their targets (e.g. (Llave et al., 2002; Rhoades et al., 2002; Xie et al., 2003; Allen et al., 2004; Bowman, 2004; Kidner and Martienssen, 2004; Mallory et al., 2004; McHale and Koning, 2004; German et al., 2008; Liu et al., 2014). The high complementarity interactions are also easier to predict and this predictive value is being used for identification of putative plant miRNA targets. (Rhoades et al., 2002; Bonnet et al., 2010; Shao et al., 2013; Kumar et al., 2014). While animal miRNA binding sites are typically localized to 3'UTRs, plant miRNA recognition sites can be found in 5'UTRs, ORFs, or 3' UTRS as well as in non-coding RNAs (e.g. TAS3 RNA (Montgomery et al., 2008)). At the same time, functional features of miRNA binding are still incompletely understood as showed analysis of miR159 sites in MYB33/MYB65 (Li et al., 2014). Importantly, as mentioned above, the mode of miRNA action can be also influenced by a DRB partner during its biogenesis, i.e. independently of the target binding site (Reis et al., 2015c). Finally, plant miRNAs can also mediate transcriptional repression through RNA-dependent DNA methylation. In this section, we will review all three types of miRNA-mediated repression.

miRNA-mediated target cleavage

miRNA-mediated target cleavage by AGO1 is a functionally important silencing mode as evidenced by the requirement of catalytically active AGO1 in ago1 mutant complementation experiments (Carbonell et al., 2012). AGO1, guided by a miRNA, cleaves in the middle of the base paired sequence (Llave et al., 2002; German et al., 2008). Similarly to other eukaryotes, exposing a free 5' fragment with a 3' hydroxyl and a 3' fragment with a 5' phosphate from a cleaved mRNA leads to decay, which involves a 5'-3' exonuclease (AtXrn4 in *Arabidopsis*), which attacks the 3' cleaved fragments (Souret et al., 2004). The 5' fragments are uridylated at 3' ends by HESO1 terminal uridylase; uridylation seem to be coupled with their final demise (Ren et al., 2014).

miRNA-mediated translational inhibition

Some plant miRNA:mRNA target base pairing could have central mismatches, preventing AGO-mediated cleavage. Other observations also suggest that plant miRNA-target interaction does not always result in AGO-catalyzed slicing but leads to translational repression (Schwab et al., 2005; Axtell et al., 2006; Franco-Zorrilla et al., 2007; Brodersen et al., 2008; Li et al., 2013). The molecular mechanism of miRNA-mediated translational repression in plants is less well understood than in animals. In any case, some similarities emerged. For example, AGO1 localizes to P-bodies, dynamic cytoplasmic foci containing many proteins involved in translational repression and mRNA degradation (Brodersen et al., 2008; Yang et al., 2012).

Another interesting factor, resembling GW182 bridging of target recognition and recruitment of mRNA degrading mechanisms, is SUO, which was identified through a mutation screen for factors contributing to miRNA-mediated repression (Yang et al., 2012). SUO encodes a large protein with N-

terminal bromo-adjacent homology and transcription elongation factor S-II domains and, importantly, two C-terminal GW repeats (Yang et al., 2012). The SUO loss-of-function phenotype is a consequence of a defect in miRNA-mediated translational repression and it is reminiscent of plant phenotypes with reduced AGO1 activity (Yang et al., 2012). SUO is present in the nucleus, and co-localizes with DCP1 in the cytoplasm (Yang et al., 2012). An independent study of miRNA-mediated repression showed a functionally important link to decapping through the decapping component VARICOSE (VCS) (Ge-1 homolog), further suggesting that mechanisms underlying miRNA-mediated translational repression in animals and plants are related (Brodersen et al., 2008).

Another line of evidence linking AGO1 and GW-mediated recruitment of downstream repressing factors came from the analysis of viral inhibitors encoded by plant viruses – the P1 protein from the Sweet potato mild mottle virus targets AGO1 and inhibits RISC activity through the N-terminal half containing region three WG/GW motifs (Giner et al., 2010).

miRNA-mediated transcriptional silencing/DNA methylation

In addition to the two usual post-transcriptional modes of action, plant miRNAs can be also plugged into the RNA-dependent DNA methylation mechanism. In this case, miRNA precursors would be processed by DCL3 into longer (24 nt) species and would be loaded onto AGO4/6/9 system (Axtell et al., 2011). A specific example of such miRNA has been discovered in rice where a class of miRNAs (denoted long miRNA, lmiRNAs) is processed by DCL3, loaded onto AGO4, and directs DNA methylation (Wu et al., 2010) (the molecular mechanism of RNA-dependent DNA methylation is described later).

Physiological roles of plant miRNAs

Plant miRNAs have much more “focused” roles than animal miRNAs, in particular mammalian ones. This likely reflects their evolutionary origin, which is connected with their target genes, and the common slicing mode of action (discussed for example in {Svoboda, 2006 #875}). Briefly, many plant miRNAs seem to originate from inverted repeats, which formed from sequences of their target genes, e.g. through duplication or recombination involving genes and pseudogenes. An interesting aspect of plant miRNA-mediated regulations is the targeting of various transcription factor families, which is translated phenotypic alterations. For example, during miRNA target prediction, of the 49 predicted targets, 34 were members of these transcription factor gene families involved in developmental patterning or cell differentiation (Rhoades et al., 2002). Validated miRNA-targeted transcription factor include the Class III HD-Zip gene family (Bowman, 2004), GRF transcription factors (Debernardi et al., 2014), Scarecrow-like (SCL) family of putative transcription factors (Llave et al., 2002) or MYB33/MYB65 (Li et al., 2014).

Mutants of miRNA factors yielded a whole array of phenotypes suggesting a number of different roles of miRNAs in cell proliferation (Rodriguez et al., 2010; Debernardi et al., 2012; Debernardi et al., 2014), plant development (Schauer et al., 2002; Abe et al., 2010; Jover-Gil et al., 2012; Datta and Paul, 2015), or in response to various physiological conditions, including environmental stress (Sunkar and Zhu, 2004; Huang et al., 2009). One of the notable features of plant miRNAs, which is analogous but distinct from circulating miRNAs in mammals, is that some plant miRNAs can cross cellular boundaries through plasmodesmata to adjacent cells (Marin-Gonzalez and Suarez-Lopez, 2012).

Interestingly, miRNA pathways in *Arabidopsis* are regulated by a negative feedback loop targeting DCL1 by miR162-guided mRNA cleavage (Xie et al., 2003). An analogous negative feedback loop was observed in *Arabidopsis* for AGO1, which is targeted by miR168 during development (Vaucheret et al., 2004).

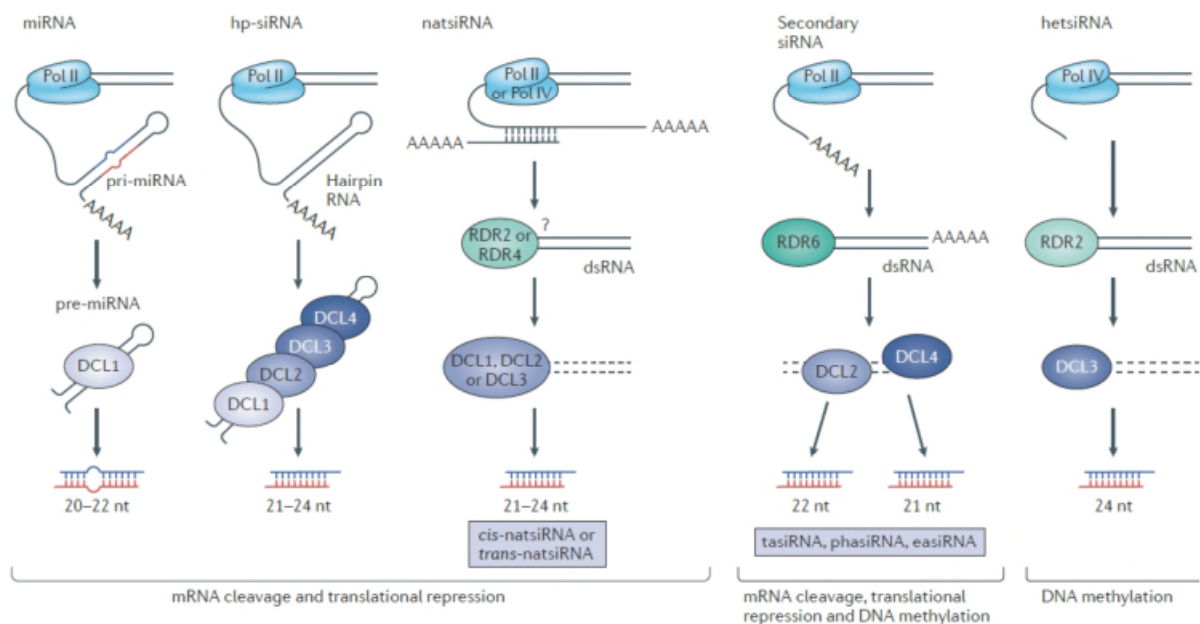
3.1.8.8 dsRNA-induced post-transcriptional silencing in PTGS & VIG pathways

The complexity of RNA silencing in plants comes from multiple layers, which are integrated to provide specific functions of specific small RNA pathway. These layers are (I) structural – e.g. processing of different types of substrates, sorting of small RNAs onto AGO proteins, and molecular effects – endonucleolytic cleavage, translational repression etc. (II) functional/conceptual – e.g. distinguishing

between defensive mechanisms and physiological gene regulations, (III) spatiotemporal at multiple levels – including cellular compartmentalization, distinct genomic loci, parts of a plant or its life-cycle, differentiating between somatic and germ cells, leaves, flowering etc. This explains that despite four Dicer proteins, which produce two main classes of small RNAs (21/22 and 24 nt), there is over ten different names for AGO-bound small RNAs (Axtell, 2013; Borges and Martienssen, 2015), some of which were already mentioned: miRNA, lmiRNA, hp-siRNA, natsiRNA, cis-natsiRNA, trans-natsiRNA, tasiRNA, phasiRNA, easiRNA, hetsiRNA, diRNAs, ... (Figure 51).

However, while there seems to be an unpenetrable thicket of substrates, DCLs, AGOs, RDRs, small RNAs, and biological effects, RNA silencing in plants is built from three main mechanistic modules. The three modules are (I) **the miRNA module** reviewed above, (II) **the "RNAi" module**, into which we include dsRNA-induced post-transcriptional silencing in PTGS & VIG pathways, and the (III) **the transcriptional silencing RdDM module**, which encompasses 24 nt small RNA-driven *de novo* DNA methylation and associated chromatin changes and which will be discussed in the next section. In this module, we will thus focus on long dsRNA processing into small RNAs, which is the key step in the dsRNA response.

Many different substrates give a rise to primary and secondary small RNAs that are 21/22 or 24 nt long. Deep sequencing showed that plants have relative to other eukaryotes extraordinarily large and complex populations of small RNAs (Lu et al., 2005; Henderson et al., 2006; Rajagopalan et al., 2006; Howell et al., 2007; Kasschau et al., 2007). More than the half of the small RNAs are 24 nt long "heterochromatic" siRNAs, which map to intergenic regions, particularly to the proximal and distal pericentromeric regions (Rajagopalan et al., 2006). Notably, plant small RNAs are typically methylated at the 2'-hydroxyl group of the 3' terminal nucleotide by the methylase HEN1 (Li et al., 2005b; Yu et



al., 2005).

Figure 53 Complexity of small RNAs in plants. The scheme is from (Borges and Martienssen, 2015)

Structurally, the three main types of primary substrates for DCL proteins are short, miRNA-like hairpins, long dsRNA hairpins, and long dsRNA (e.g. formed during viral replication). In addition, activity of RDRs generates long dsRNA to produce either primary siRNAs (i.e. long dsRNA origin is more or less independent of AGO proteins activity) or secondary siRNAs, where an RDR converts an AGO-targeted transcript into dsRNA. In terms of silencing effects, AGO-loaded small RNAs can induce

mRNA cleavage, translational repression, and *de novo* DNA methylation (RdDM). Biogenesis of small RNAs and the first two modes of action were described above, RdDM will be described in the next section. Thus, in the rest of this section, we will provide an overview of the main routes of dsRNA synthesis and degradation involved in post-transcriptional silencing (Figure 52).

The main Dicer proteins producing small RNAs in the RNAi-like module are DCL2/3/4. All of them participate in antiviral response and in processing various other substrates, which were described above in the section reviewing individual Dicer proteins. Importantly, there is a hierarchical and functional separation of the three DCL proteins, such that small RNAs produced by DCL3 can be channelled into the RdDM module. DCL4 and DCL2 act hierarchically on viral and endogenous substrates. DCL4 seems to act earlier while DCL2 later. Both act in a loop involving DRD6, which amplifies the effect (Xie et al., 2005; Deleris et al., 2006; Brosnan et al., 2007; Howell et al., 2007; Liu et al., 2007; Moissiard et al., 2007; Mlotshwa et al., 2008; Qu et al., 2008; Di Serio et al., 2009; Garcia-Ruiz et al., 2010; Chen et al., 2011; Parent et al., 2015; Ogwok et al., 2016).

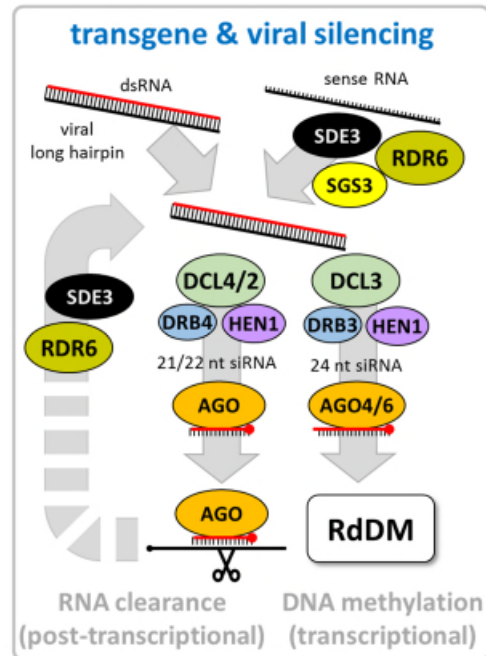
Small RNAs produced by DCL2/3/4 are sorted onto different AGO proteins, which execute the silencing and, eventually, mark cognate RNAs for RDR6 for amplification. Some of the sorting rules were described above. AGO4/6 accommodate longer 24 nt small RNAs produced by DCL3 and can induce RdDM. Smaller RNAs are loaded onto other AGO proteins depending on the sorting rules and AGO availability – please, refer to the AGO section for more details.

Systemic silencing

RNAi in higher plants can be non-autonomous (Himber et al., 2003; Dunoyer et al., 2005). It was found that exogenous and endogenous DCL4-dependent 21 nt siRNAs can act as mobile silencing signals between plant cells in a process which likely involves siRNA duplexes rather than loaded AGO1 proteins (Dunoyer et al., 2010). Follow up studies confirmed the core conclusions: graft transmission of endogenous siRNAs inducing silencing (Liang et al., 2012; Ali et al., 2013). The molecular mechanism of systemic RNAi through the vasculature is not completely understood. It seems that small RNAs are transported from cell to cell via plasmodesmata rather than diffusing from their source in the phloem (Liang et al., 2012).

Figure 54 “RNAi module” of RNA silencing in *Arabidopsis*.

The key step in the RNAi module is conversion of dsRNA into small RNAs by one of the DCL proteins, among which dominate DCL2/3/4. However, mentioned above, DCL1 is also able to produce 21 nt siRNAs from a transgenic inverted repeat (Papp et al., 2003). Various dsRNA substrates can enter the RNAi module. Some of them are produced by RDR6 either as the initial trigger or as an amplification step where AGO-targeted RNAs are converted to dsRNA, which is processed into secondary siRNAs. If the targeting of AGO is precisely defined (e.g. by miRNA), the secondary siRNAs would be phased. DCL3-generated 24 nt siRNAs can induce RdDM – for details about the RdDM mechanism, see the next section.



3.1.8.9 Transcriptional Gene Silencing

RdDM was actually the first discovered small RNA-controlled epigenetic mechanism. It was originally found in tobacco plants where viroid cDNA, integrated into the genome, became specifically

methyated in the presence of autonomous viroid RNA-RNA replication (Wassenegger et al., 1994). It was subsequently shown that as little as 30bp of targeted DNA is sufficient for RdDM and that dsRNA complementary to promoter region can induce promoter methylation and transcriptional silencing (Pelissier et al., 1999; Mette et al., 2000; Pelissier and Wassenegger, 2000; Jones et al., 2001; Thomas et al., 2001).

RdDM not only affects cytosine residues within canonical, symmetrical CpG dinucleotides, but also CpNpG and other non-CpG asymmetric targets (Pelissier et al., 1999; Aufsatz et al., 2002a). Since 21-24 nt small RNAs were produced from the original trigger (Mette et al., 2000), RdDM was recognized as one of RNA silencing pathways in plants.

The canonical RdDM pathway (Figure 53) is initiated by plant-specific RNA polymerase Pol IV that produces single-stranded RNA transcripts from genomic loci to be silenced (Herr et al., 2005; Onodera et al., 2005). RNA transcripts are transported into the nucleolus where they are converted into dsRNA by RNA-dependent RNA polymerase RDR2 and processed by DCL3 into siRNAs, which are then methylated by the methylase HEN1. AGO4, DCL3, RDR2 and 24-nt siRNAs complementary to the heterochromatin regions co-localize in nucleolar processing centers (Li et al., 2006; Pontes et al., 2006). Importantly, AGO4 is not the only AGO mediating RdDM, RdDM can be induced by AGO4, 6, and 9, which are functionally diverged, largely due to their differential expression (Havecker et al., 2010)

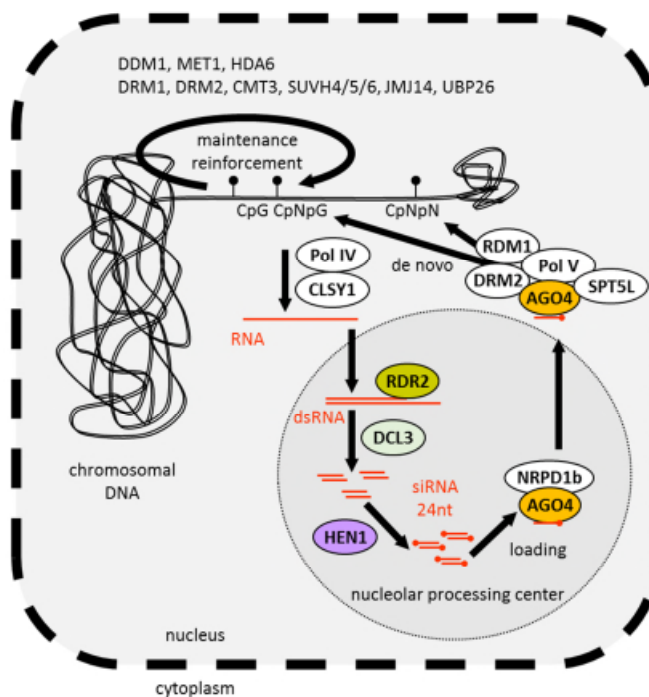


Figure 55 Schematic model of canonical RdDM in *A. thaliana*

Core components of RNA silencing are colored. Target locus is transcribed by RNA polymerase Pol IV into RNA, which is relocated into a nucleolar processing center where it is converted into dsRNA by RNA-dependent RNA polymerase RDR2 and further processed. Priming of RDR2 may involve cleavage of pol IV transcripts by an AGO protein (AGO4?). dsRNA is cleaved by DCL3 into siRNAs, which are methylated by the methylase HEN1. siRNAs are then loaded onto AGO4, which interacts with Pol V subunit NRPD1b. The complex moves into the nucleoplasm and forms functional Pol V complex, which, in co-operation with *de novo* DNA methyltransferase DRM2, and SNF2-like chromatin remodelling protein DRD1, facilitates in a sequence-specific manner *de novo* DNA methylation of cytosines (black circles on a stalk) in all sequence contexts at homologous loci. Recognition of a target locus probably occurs via binding to an RNA from the silenced locus. CpG methylation is maintained during the replication by complexes containing DDM1, a maintenance DNA methyltransferase MET1, and histone deacetylase HDA6. Methylation at non-CpG nucleotides is dependent on DNA methyltransferase CMT3, *de novo* cytosine methyltransferases DRM1 and DRM2, and a H3K9 methyltransferase SUVH4/KRYPTONITE.

Processing centers are located at a distance from source/target loci and siRNAs trafficking between processing bodies and target regions has to take place. However, mechanisms regulating this process remains unknown at present. Nucleolus-associated Cajal bodies are also centers for AGO1- and DCL1-dependent miRNA processing and are the sites of nonsense-mediated decay indicating closer relationship between RNAi-dependent and other RNA regulating pathways.

Methylated siRNAs associate with AGO4, which interacts with Pol V subunit NRPD1b and the complex moves into the nucleoplasm where it associates with NRPD2a and forms functional Pol V complex. In co-operation with *de novo* DNA methyltransferase DRM2, and SNF2-like chromatin remodelling protein DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), the Pol V complex facilitates *de novo*

DNA methylation of cytosines in all sequence contexts at the targeted locus (Kanno et al., 2004; Kanno et al., 2005a; Kanno et al., 2005b; Li et al., 2006; Pontes et al., 2006).

CpG methylation in the targeted locus is subsequently maintained during the replication by complexes containing DECREASE IN DNA METHYLATION 1 (DDM1) (Vongs et al., 1993), a maintenance DNA methyltransferase MET1 and histone deacetylase HDA6 (Jones et al., 2001; Aufsatz et al., 2002b; Aufsatz et al., 2004). Methylation at non-CpG nucleotides depends on DNA methyltransferase CHROMOMEHTYLASE3 (CMT3), *de novo* cytosine methyltransferases DRM1 and DRM2 (DOMAINS REARRANGED METHYLTRANSFERASE), *Arabidopsis thaliana* homologs of mammalian DNMT3, and a lysine 9 on histone 3 (H3K9) methyltransferase SUVH4/KRYPTONITE (Bartee et al., 2001; Lindroth et al., 2001; Cao and Jacobsen, 2002; Jackson et al., 2002; Chan et al., 2004). Notably, SUVH4 is dispensable for *de novo* DNA methylation and silencing (Jackson et al., 2002; Malagnac et al., 2002) suggesting that H3K9 methylation in plants does not precede DNA methylation. It has been speculated that the difference in mechanistic relationships between H3K9 methylation and DNA methylation in plants and other model organisms may perhaps reflect dependence and independence of RNA silencing and histone modifications in these models (Matzke and Birchler, 2005).

RdDM is induced by different types of sequences and has a number of targets. RdDM was studied using two types of dsRNA as inducers of methylation (a) transgenic hairpin constructs (Pelissier et al., 1999; Aufsatz et al., 2002a), and (b) dsRNA viruses (Hall et al., 2002; Vaistij et al., 2002). Notably, these two triggers elicit somewhat different responses. In contrast to the RdDM silencing induced by hairpin constructs, silencing induced by homologous viral transgenes can spread from the region of homology both upstream and downstream (Vaistij et al., 2002). This process depends on RNA-dependent RNA polymerase RDR6, a maintenance methyltransferase MET1, and AGO1 (Jones et al., 2001; Morel et al., 2002). Although histone modifications were not examined in the original paper of Vaistij et al., spreading of silencing resembles RNAi-dependent heterochromatin formation in *Schizosaccharomyces pombe* (Vaistij et al., 2002). In addition to these exogenous triggers, analysis of endogenous small RNAs and DNA methylation studies revealed that major targets of RdDM are transposons and repeats in constitutive and facultative heterochromatin but not all transposons are repressed by RdDM; only a limited number of targets of RdDM are endogenous genes (Borges and Martienssen, 2015). As RdDM can be reversed by demethylation (Penterman et al., 2007), a picture emerges in which RdDM is not only a repressive mechanism controlling repetitive and viral sequences but also a part of regulatory networks controlling gene expression, which includes other chromatin modifications.

In addition, there is a second RdDM pathway in flowering plants, designated non-canonical RdDM (Matzke et al., 2015), which is initiated by pol II transcripts, which are channeled through DCL3 and AGO4/6 in the "RNAi module" into RdDM (Figure 54).

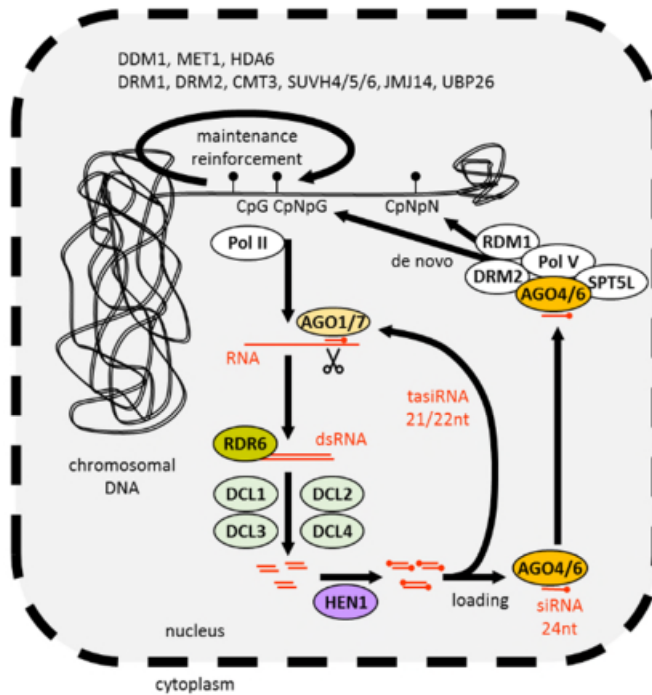


Figure 56 Schematic model of non-canonical RdDM in *A. thaliana*

The target/trigger locus is transcribed by RNA polymerase II into RNA, which is recognized by an AGO-loaded small RNA, is converted into dsRNA by RNA-dependent RNA polymerase RDR6, and it is processed by one of DCL proteins where AGO4/6 bound small RNAs can enter RdDM module, 21/22 nt small RNAs can operate within the RNAi module and amplify the silencing response.

3.1.8.10 References

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3.1.9. Conclusions - Part I

Based on available scientific literature, we provided in this report a detailed description of the processing of dsRNA and miRNA in plants, humans, mammals, birds, fish, arthropods, annelids, nematodes, and molluscs; the function of all the different components of the silencing pathways in these organisms is described. We identified dsRNA and miRNA processing mechanisms and pathway components that are common or unique to plants, humans, mammals, birds, fish, arthropods, annelids, nematodes, and molluscs and are required for normal functioning of the pathway in these specific organisms. In this section, we highlight the key similarities and differences and provide comparative graphical representations of the reviewed pathways in addition to those at the end of taxon-specific sections.

3.1.9.1 Small RNA pathways and dsRNA response – common themes

The main focus of the report is on the pathways utilizing small RNAs as sequence-specific guides for repression, namely RNAi, which is triggered by long dsRNA, and miRNA, which employs genome-encoded short hairpin precursors. In addition, dsRNA pathways cross-talking with RNAi, were also discussed, in particular the interferon response. While there is an overwhelming complexity of the pathways and their components, it is possible to divide them into four basic mechanistic modules.

The first three are the sequence-specific modules for small RNA-guided mechanisms namely miRNA, RNAi, and transcriptional silencing. These modules are distinguished by the origin of small RNAs and their silencing effect. The miRNA module uses genome-encoded precursors, which guide post-transcriptional repression of protein-coding genes, typically imperfectly base pairing and causing translational repression coupled with mRNA degradation (animals); plant miRNAs (occasionally animal ones) induce sequence-specific cleavage like RNAi. The RNAi module is characterized by long dsRNA-induced sequence-specific mRNA degradation. Some organisms (*C. elegans*, plants) employ also RdRP, either to produce dsRNA to initiate RNAi or to function as an amplifier of the response. Transcriptional silencing employs small RNAs loaded on AGO proteins, which base pair with chromatin associated RNAs and recruit chromatin modifying mechanisms, including DNA methylation (RNA-dependent DNA methylation, RdDM) and/or histone modifications.

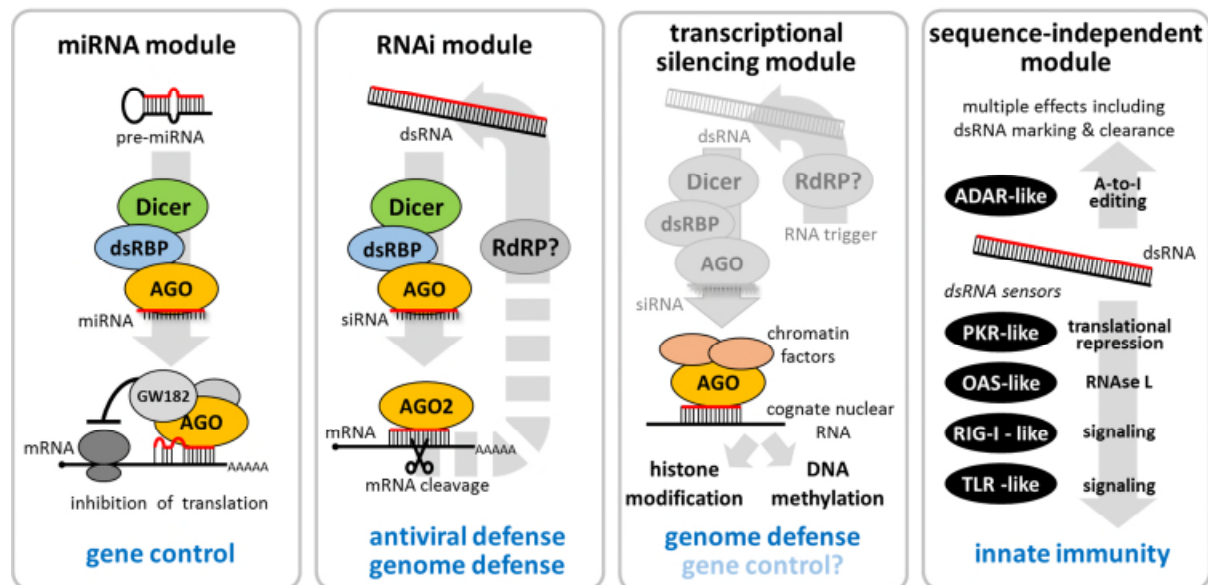


Figure 57 Overview of dsRNA and miRNA pathways/elementary modules

Shown are schemes of the four mechanistic modules, which are adapted and interconnected in different ways in the reviewed taxons. From the left, three sequence-specific modules: **miRNA module**, **RNAi module**, **transcriptional silencing module**; on the far right the sequence-independent pathways are combined into one **sequence-independent module**. See the text for more details.

The fourth module, is the sequence-independent and includes two distinct molecular mechanisms: (i) the adenosine deamination, which is found in animals, where ADAR enzymes edit dsRNA by converting adenosines to inosines; this marks dsRNA with inosines and disrupts U-A base pairing and (ii) the interferon response, where multiple sensors trigger increased expression of interferon-stimulated genes and induce global silencing effects involving translational repression and RNA degradation.

miRNA module

Of the four modules, the miRNA module is the most conserved across all taxons. In all species, the following principle apply: miRNAs are genome-encoded small RNAs with precisely defined sequences, which function as suppressors of gene expression. Each miRNA has specific repertoire of target mRNAs. The effector complex mediating silencing has been given different names such as RISC, miRISC, or microRNP. It contains a miRNA, an AGO protein, and, typically additional protein factors contributing to the repression. While the degree of complementarity between miRNAs and their targets varies, the key component of miRNA:mRNA interaction is the seed sequence at the 5' end of miRNA. miRNA mediated repression can involve RNAi-like cleavage if there is a high degree of complementarity and a miRNA is loaded on an AGO protein with slicing activity, which would be the minimal effector complex (which may be called holoRISC, minimal RISC, or minimal miRISC). In other situations, repression is achieved through the activity of proteins associated with AGO in the effector complex. The repression may include translational repression, deadenylation, and decapping. Effector complexes and targeted mRNAs localize in cells to cytoplasmic foci called P-bodies; the significance of this compartmentalization is still debated.

RNAi module

The RNAi module employs siRNAs generated from dsRNA. Importantly, siRNAs are produced as a population of small RNAs with many different sequences. This makes them different from miRNAs, which have defined sequences that can be annotated in miRBase. It appears that all reviewed taxons have a functional RNAi module. They all have a Dicer and an AGO protein with the slicing activity. Thus, theoretically they should all be able to cleave long dsRNA into siRNAs, which could guide a slicing AGO to perfectly complementary cognate RNAs. Canonical RNAi can be observed in all organisms but in some only under specific circumstances (discussed in the section 3.1.9.2).

Transcriptional silencing module

The transcriptional silencing module is a diverse collection of observations on different molecular mechanisms and is therefore discussed separately in section 3.1.9.2.

Sequence-independent module

This module does represent a single common theme for reviewed taxons. It refers to cases such as the interferon response found only in a subset of animals, or to ADARs, which are absent in plants.

3.1.9.2 Taxon-specific features on small RNA pathways and the response to dsRNA

In this section, we summarize key differences between reviewed taxons and highlight those of particular functional importance. One of the more general differences among the reviewed taxons is the diversity of RNA silencing pathways. Some taxons, such as vertebrates, utilize a relatively simple setup of RNA silencing pathways, which is also reflected by the low numbers of homologs of the key genes, listed on the next page. The dominant somatic RNA silencing pathway in vertebrates is the miRNA pathway, which employs a single Dicer, one or two associated dsRBPs, and four AGO proteins, one of which retains the slicing activity. There is no protein uniquely dedicated to RNAi and no RdRP. The other three Argonaute proteins in vertebrates function in the piRNA pathway, present in the germline. This simple setup contrasts with that in *C. elegans* where 26 Argonaute proteins and three RdRPs exist, as well as with that in *A. thaliana* where there are four Dicer proteins, seven dsRBPs, ten Argonaute proteins and six RdRPs. Consequently, nematodes and plants have highly complex RNA silencing system adapted to many different biological roles.

Table 19 Overview of homologs of key RNAi and miRNA factors across reviewed taxons

The counts of the homologs and their names were collected from the literature and genome annotations. When there were more names given to a particular gene, synonymous gene names are separated by /, e.g. PIWIL2/ZIWI in *Danio rerio*. Argonaute proteins with names in grey function in the piRNA pathway.

	mammals		birds		fish		arthropods		mollusks		annelids		nematodes		plants	
	#	protein	#	protein	#	protein	#	protein	#	protein	#	protein	#	protein	#	protein
DROSHA	1	DROSHA	1	DROSHA	1	DROSHA	1	DROSHA	1	DROSHA	1	DROSHA	1	drsh-1	0	
DGCR8	1	DGCR8	1	DGCR8	1	dgcr8	1	pasha	1	Dgcr8	1	DGCR8	1	DGCR8	0	
DICER	1	DICER1	1	DICER1	1	dicer1	1	dcr-1, dcr-2	1	Dicer-likel	1	HELODRAFT_103772	1	dcr-1	4	DCL1, DCL2, DCL3, DCL4
dsRBP partner	2	TARBP2, PACT	1	TARBP2	1	tarbp2, prkra	2	r2d2, loqs	1	BRAFDRAFT_242885	1	HELODRAFT_102635	1	rde-4	7	DRB1/HYL1, DRB2, DRB3, DRB4, DRB5, DRB6, DRB7
ARGONAUTE	8	AGO1, AGO2, AGO3, AGO4, PIWIL1, PIWIL2, PIWIL3, PIWIL4	6	AGO1, AGO2, AGO3, AGO4, PIWIL1, PIWIL2	6	AGO1, AGO2, AGO3, AGO4, PIWIL1, PIWIL2/ZIWI	5	AGO1, AGO2, AGO3, PIWI, AUB	4	AGO1, AGO2, PIWI-like1, PIWI-like2	26	HELODRAFT_182062, HELODRAFT_75625, HELODRAFT_65566	1	ALG-1, ALG-2, ALG-3, ALG-4, RDE-1, ERGO-1, CSR-1, C06A1.4, H10D12.2, C14B1.7, T23D8.7, C04F12.1, PRG-1, PRG-2, WAGO-1, WAGO-2, PPW-2/WAGO-3, WAGO-4, WAGO-5, SAGO-2/WAGO-6/8, PPW-1/WAGO-7, SAGO-1/WAGO-8/6, HRDE-1/WAGO-9, WAGO-10, WAGO-11, NRDE-3/WAGO-12	10	AGO1, AGO2, AGO3, AGO4, AGO5, AGO6, AGO7, AGO8, AGO9, AGO10
RdRP	0		0		0		6?		0	XM_011450789, XM_011427600, XR_900019, XR_902698, XM_011450791, XR_900018	0		3	EGO-1, RRF-1, RRF-3	6	RDR1/SDE1/SGS2, RDR2, RDR3, RDR4, RDR5, RDR6

miRNA module

There are only minor differences in the miRNA module across animals. The main one is genetic separation between miRNA and RNAi pathways in arthropods, which utilize miRNA-dedicated Dicer, dsRBP, and AGO while other animals use one Dicer to produce miRNAs and siRNAs. There is a clear difference between animals and plants. Plants employ a single RNase III, one of their Dicer paralogs, to process pri-miRNA into pre-miRNA and then into miRNA duplex in the nucleus. These miRNAs are 2'-O-methylated at their 3' termini. This modification is absent in animal miRNAs (but found at piRNAs in the germline). In addition, animals employ two distinct RNase III enzymes – Drosha in the Microprocessor complex in the nucleus, which releases pre-miRNA from pri-miRNA, and Dicer, which produces miRNA duplex in the cytoplasm.

RNAi module

As mentioned above, the role of RNAi varies among different organisms. Vertebrates replaced the antiviral defense system provided by RNAi by an array of innate immune sensors of dsRNA molecules, whose activation converges on a sequence-independent interferon response. Thus, the canonical RNAi is generally not a ubiquitous primary mechanism in response to dsRNA in vertebrates although it is observed in specific cases. In species, which still use RNAi as the primary antiviral immunity pathway (such as plants, nematodes, arthropods), it is frequently observed that viruses overcome the RNAi response with various protein inhibitors. Whether the more complex interferon system in vertebrates provides a stronger defense barrier is unclear as the interferon pathway is just a part of a highly complex immune system. One interesting aspect of RNAi and interferon response evolution is the rewiring of the RIG-I helicase family, which is associated with RNAi in *C. elegans* and interferon response in mammals. Understanding the role of the RNAi module in immunity of molluscs and annelids requires further research. Molluscs are a particularly interesting case, because their genome carries homologs of the genes involved in the interferon response, and studying them might provide an insight into how the interferon response has replaced RNAi, as the main antiviral response.

Transcriptional silencing module

The transcriptional silencing module is a diverse collection of different molecular mechanisms and observations. There are four distinct endogenous small RNA mechanisms involved in transcriptional silencing, which were explored into a considerable detail: transcriptional silencing in plants, transcriptional silencing in fungi (not covered in this report), nuclear silencing in *C. elegans*, and piRNA pathways in animals (not covered in this report). For these four pathways, biogenesis of endogenous small RNAs and effector complexes were more or less determined. These four mechanisms are so distinct that it appears they evolved through convergent evolution, which would find different solutions to connect two conserved mechanisms: small RNA pathways and heterochromatin formation. Transcriptional silencing by siRNA/miRNA-like molecules in vertebrates is poorly understood. While there is a number of interesting observations and experimental data, there is no clear consensus regarding the endogenous molecular mechanism of transcriptional silencing, including the origin and biogenesis of small RNAs or the composition and mode of action of the effector complex.

Sequence-independent module

In terms of sequence-independent module, animals differ from plants by utilizing ADARs. The other dsRNA-responding proteins vary among animals where the interferon-response factors are present in vertebrates and possibly in molluscs while arthropods, nematodes and possibly annelids have RIG-I helicase family homologs, which are integrated into RNA silencing.

3.1.9.3 Final remarks

There are still many open questions concerning RNA silencing and dsRNA response waiting to be answered. Importantly, unknown mechanisms and emerging concepts must be confronted with two key facts. First, for an RNA silencing mechanism, which utilizes a small RNA bound to an AGO protein, recognition of a small RNA target may require as little as the seed sequence, which is a hexamer or heptamer motif. Thus, for predicting targets and estimating a potential impact of a small RNA, one has to consider all potential binding sites. A small RNA could have hundreds to thousands of putative binding sites in the transcriptome. Accordingly, reliability of target prediction remains an unsolved issue and needs to be complemented with experimental validation. Second, stoichiometry between the loaded AGO and cognate RNA is important for effective silencing while structural analyses and single molecule studies provide a framework for estimating how effective a target recognition could be. Accordingly, target validation assays ideally need to operate with physiological levels of a small RNA and titrate the levels of a targeted reporter to estimate the silencing potential of a small RNA.

At the moment, knowing the concentration and the seed sequence, one can predict possible targets of a specific small RNA but it is not possible to reliably predict all effects a small RNA would have. Thus, for assessment of effects of a specific small RNA, it is important to use biosensors and test biological effects *in vivo*. Since miRNA-mediated repression follows the same principles and some of the key kinetic parameters were estimated (Figure 23), one could use whole animal models to test a given concentration if there is any detectable effect, where the most sensitive monitoring would analyze transcriptome changes. Such analysis would simply show, which small RNA concentration has a measurable effect on gene expression *in vivo*. There are currently two species, which could serve as good animal biosensors: zebrafish and *C. elegans*. Zebrafish is a model vertebrate with a genetic makeup of RNA silencing and sequence-independent responses similar to those of humans. *C. elegans* is phylogenetically more distant from humans than zebrafish but has a number of advantages, which make it the best available biosensor for environmental exposure. Some of these features are shared with the zebrafish model, such as **accessibility** to a number of tools (including well-annotated genome and cell fate map), and **low cost**. However, *C. elegans* offers several additional benefits such as **availability** in many habitats in temperate areas where it is found in decomposing plant material, which makes it a possible model for field studies. Furthermore, *C. elegans* also offers “**penetrability**” through the environmental and systemic RNAi and increased **sensitivity**, due to ability to amplify a small RNA pathway response through an RdRP.

To conclude, any proposed molecular mechanism involving a small RNA loaded on an AGO protein must be either consistent with the known effective small RNA:target stoichiometry or must provide a testable explanation why it is not. In other words, without showing, for example, that subpicomolar concentration of a specific miRNA has a significant impact on silencing its predicted targets, one cannot simply conclude that subpicomolar amounts of that miRNA have biologically significant effects through the miRNA pathway. This is a recurring problem of proposed biological roles of circulating, environmental and xenogenic small RNAs, which would deserve their own systematic review, since it is beyond the scope of this report.

3.2. Part II Potential for non-target (off-target) gene regulation by dsRNA-derived siRNAs or miRNAs

This part provides an overview of the potential for non-target (off-target) gene regulation by dsRNA-derived siRNAs or miRNAs. This part capitalizes on the previous comprehensive report of molecular mechanisms (Part 1 - Mode-of-action of dsRNA and miRNA pathways) and focuses on the analysis of base pairing between a small RNA and its cognate RNA in the three most explored experimental model systems. This is reflected in the structure of the report, which was conceived as a stand-alone text.

The core of the report (section 3.2.2.) is divided into four main parts:

- 3.2.2.1. Small RNA:target RNA base pairing
- 3.2.2.2. Other key factors influencing target recognition and repression
- 3.2.2.3. Off-targeting – causes and remedies
- 3.2.2.4. Target identification

This report structure was chosen to organize the literature search and to address the following topics:

- sequence complementarity between target RNA and small RNA (siRNA or miRNA)
- extent to which mismatches between target RNA and small RNA (siRNA or miRNA) can be tolerated
- parameters influencing sequence complementarity (and target recognition and repression)
- target recognition by small RNAs – specific targeting by miRNAs and off-targeting by siRNAs
- assessment of publicly available bioinformatic programs predicting target recognition
- discussion on reliable identification of target RNAs (and, eventually, biological effects)

The last section Conclusions summarizes the usefulness of sequence complementarity search and brings up the question of reliable determination of biological effects of small RNAs in animals and plants

3.2.1. Principles of targeting by small RNAs

The aim of this section is to provide an introduction into the molecular mechanism of silencing by small RNAs in plants and animals before the detailed literature survey concerning sequence complementarity between small RNAs and their targets. The idea is to introduce common general principles to produce a stand-alone report with all necessary information for understanding the literature survey part. The section 3.1. capitalizes on the literature review done for our first report (*Mode-of-action of dsRNA and miRNA pathways*) with a particular focus on the execution step of RNAi and miRNA pathways.

3.2.1.1 miRNA and RNAi pathways in animals and plants

Within the complex world of RNA silencing, two related yet distinct pathways exist in animals and plants: RNA interference (RNAi) and microRNA (miRNA) pathways. The essential difference between these two pathways is that miRNA pathways employ genome-encoded small RNAs with defined sequences (i.e. they can be annotated) while RNAi is initiated by processing long double-stranded RNA into a mixture of short interfering RNAs (siRNAs). Thus, the miRNA pathway in a specific cell employs a population of miRNA molecules that can be clustered based on unique sequences, corresponding to predictable positions in miRNA precursors. In other words, the major distinction between RNAi and miRNA pathways is the origin of small RNAs and their information content. In terms of their mode of action, siRNAs and miRNAs can be in some cases essentially indistinguishable.

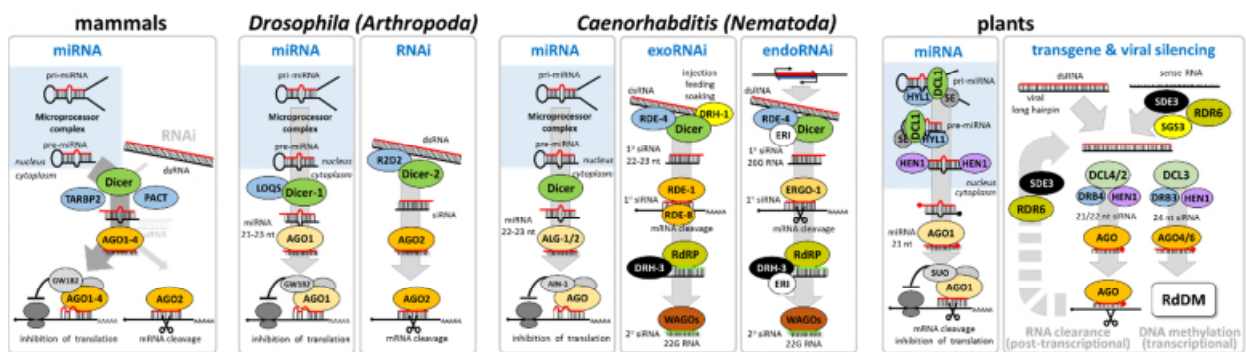


Figure 58 miRNA and RNAi pathways in animals and plants

The schemes depict key components of miRNA and RNAi pathways in the main eukaryotic model systems.

In the section 3.1. (*Part I - Mode-of-action of dsRNA and miRNA pathways*), we showed that many of the mechanistic aspects of RNAi and miRNA pathways are shared across animals and plants. Briefly, the miRNA pathway, which is primarily a gene-regulating pathway, is highly conserved in animals. The canonical miRNA biogenesis in animals is a spatially separated into two-steps. The first step occurs in the nucleus where RNase III Drosha, a component of the Microprocessor complex, releases a precursor miRNA (pre-miRNA) from a primary miRNA transcript (pri-miRNA). Next, a pre-miRNA is transported to the cytoplasm where it is cleaved by a second RNase III Dicer. Dicer releases a miRNA duplex of which one strand will be loaded on an Argonaute protein where it will form the guide for the effector complex. The miRNA pathway in plants operates similarly but employs only a single nuclear Dicer-like 1 (DCL1) RNase III to produce pre-miRNAs and miRNAs. The second important difference is the methylation of plant miRNAs at their 3' end mediated by HEN1 methyltransferase (section 3.1.8.7.).

The RNAi pathway is much more diverse across animals and plants. It is conceivable given the antiviral role of RNAi where the parasite:host interactions can accelerate evolution of RNAi pathways in different taxons. At least four types of RNAi can be recognized in animals and plants:

- 1) **overlapping miRNA and RNAi pathways with a single-set of Dicer and Argonaute proteins and without RNA-dependent RNA polymerase (RdRP)** – typical for vertebrates and, especially mammals. The molecular machinery in the cell primarily produces miRNAs but it can also support canonical RNAi, which can be observed under rare circumstances. The term RNAi in mammals is commonly used for RNAi-like cleavage mediated by a siRNA loaded by AGO2. However, siRNAs are being loaded on all four mammalian AGO protein (Meister et al., 2004), and once loaded, their behavior is indistinguishable from miRNAs.
- 2) **distinct miRNA and RNAi pathways with pathways dedicated Dicer and Argonaute proteins and without RdRP.** This set-up is observed in Arthropods.
- 3) **distinct miRNA pathway and a complex RNAi system employing RdRp(s) but with a single Dicer.** This set-up is observed in nematodes where expansion of Argonaute proteins created a highly complex RNA silencing system
- 4) **distinct miRNA pathway and a complex RNAi system employing RdRp(s) with more Dicer proteins.** This set-up is observed in plants.

Despite the differences, RNAi and miRNA pathways share several common features, which include biogenesis of small RNAs involving Dicer and effector complexes containing an Argonaute protein carrying a small RNA. Argonaute proteins are particularly important for this report because they facilitate recognition of target RNAs and mediate silencing effects.

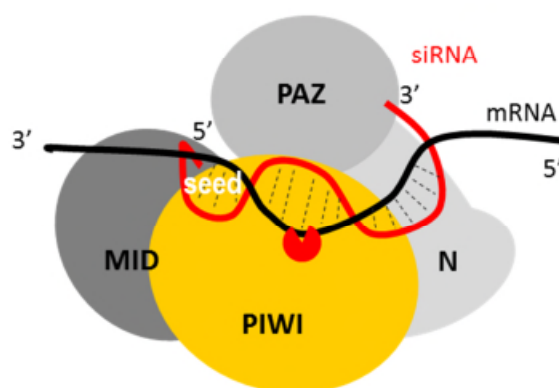
3.2.1.2 Argonaute proteins – structure and function

Argonaute proteins are the key mediators of RNA silencing. They are composed of four main domains: the central PAZ domain, the C-terminal PIWI (P-element induced wimpy testis), the N-terminal domain, and the MID domain between PAZ and PIWI domains (Figure 59). A small RNA is anchored with its 3' end in the PAZ domain. The 5' phosphate of the small RNA is buried in a pocket at the interface between the MID domain and the PIWI domain (reviewed in (Jinek and Doudna, 2009)). The 5' end of the bound cognate RNA enters between the N-terminal and PAZ domains and its 3' end exits between the PAZ and MID domains. The PIWI domain has an RNase H-like fold and carries the slicer activity (Parker et al., 2004; Song et al., 2004; Ma et al., 2005; Yuan et al., 2005). Argonaute proteins fall into three distinct groups (reviewed in (Faehnle and Joshua-Tor, 2007)): (1) AGO proteins, found in all kingdoms, (2) PIWI proteins found in animals, and (3) WAGO proteins found only in nematodes.



Figure 59 Argonaute protein structure

Schematic domain organization of an Argonaute protein. The scheme shows how a siRNA-loaded Argonaute cleaves a perfectly complementary RNA, which becomes accessible by the catalytical center in the PIWI domain upon base pairing with a small RNA. Nucleotides 2-8 of the small RNA initiate the interaction with the cognate RNA and form the so-called "seed", which has a highly predictive value for miRNA binding sites and siRNA off-targeting. The cognate mRNA is cleaved in the middle of the base paired sequence by the slicer activity depicted as a red pac-man.



3.2.1.3 Target recognition and modes of silencing

From the mechanistic perspective, post-transcriptional repression by small RNAs employs two distinct yet related (and often overlapping) modes of action:

(I) **direct endonucleolytic RNA cleavage** mediated by the so-called “slicer” activity of an Argonaute protein. This mode of action needs two conditions to be met: (i) the Argonaute protein has the Slicer activity (not all family members have it) and (ii) there needs to be extensive base pairing between the Argonaute-bound small RNA and the cognate RNA. Extensive base pairing positions the cognate RNA such that it can be sliced in the position corresponding the middle of the guiding small RNA. This mode of action has been traditionally associated with RNAi and we will refer to it as “RNAi-like” targeting. However, it should be pointed out that the two conditions for RNAi-like targeting do not exclude miRNAs and, in fact, it is well established that miRNAs loaded on a slicing Argonaute would guide slicing of perfectly complementary cognate RNAs.

(II) **indirect mRNA destabilization**, which is found when an Argonaute protein lacks the slicing activity or the base pairing is incomplete and prevents positioning of the cognate RNA (typically lack of base pairing in the middle of the small RNA:target RNA duplex. In these cases, Argonaute-bound small RNAs provide sufficiently stable interaction for target recognition while the repression is mediated by Argonaute-interacting partners. While the precise mode of action is still debated and may vary between different cell types and model systems, it seems to be coupled with common mechanisms of mRNA destabilization, i.e. deadenylation and decapping.

3.2.2. Potential for non-target (off-target) gene regulation by dsRNA-derived siRNAs or miRNAs

The following text systematically covers sequence-specific gene regulation by miRNAs and dsRNA-derived siRNAs in animals and plants from the perspective of target RNA recognition and potential for non-target (off-target) effects. This report structure was chosen in order to systematically organize the literature search and to address tender goals, namely to review:

- sequence complementarity between target RNA and small RNA (siRNA or miRNA)
- extent to which mismatches between target RNA and small RNA (siRNA or miRNA) can be tolerated
- parameters influencing sequence complementarity (and target recognition and repression)
- target recognition by small RNAs – specific targeting by miRNAs and off-targeting by siRNAs
- assess publicly available bioinformatic programs predicting target recognition
- discuss reliable identification of target RNAs (and, eventually, biological effects)

Accordingly, the section 3.2. is divided into four sections:

- 3.2.1. small RNA:target RNA base pairing
- 3.2.2. Other key factors influencing target recognition and repression
- 3.2.3. Off-targeting – causes and remedies
- 3.2.4. Target identification

In the first section, we discuss requirements for base pairing, which emerged from the literature search with a focus on the concept of the “seed”, nucleotides 2-7, which have a unique role in mediating suppressing effect. However, it is clear that base pairing is only one of several key factors, which determine whether a potential base pairing interaction will occur and has a repressive effect. Thus, we included the section 3.2.2., which discusses accessibility of the target and stoichiometry/kinetics of the repression. It seemed essential to add this to the report besides the required base pairing analysis because it is apparent that repression can occur only if a small loaded on an Argonaute protein is sufficiently abundant. Base pairing itself has a minimal predictive value for

estimating a potential silencing effect. The section 3.2.3. is focused on siRNA off-targeting as it is a well-established phenomenon of repression mediated by a small RNA beyond its perfect complementarity with a desired target. The last section 3.2.4. deals with miRNA target prediction tools, which were designed to identify potential target RNAs recognized through minimal functional complementarity. This section also includes a review of available strategies for experimental high-throughput detection of miRNA targets.

Table 20 Overview of Argonaute proteins and their associated RNAs in key model organisms

model organism	common name	subfamily	slicer	associated small RNA				
				type	length	5' nt	5' end	3' end
<i>Homo sapiens</i>	AGO1	AGO	-	miRNA, siRNA	21-23	N (U)	mono-P	-OH
	AGO2	AGO	+	miRNA, siRNA	21-23	N (U)	mono-P	-OH
	AGO3	AGO	-	miRNA, siRNA	21-23	N (U)	mono-P	-OH
	AGO4	AGO	-	miRNA, siRNA	21-23	N (U)	mono-P	-OH
	PIWIL1	PIWI	+	piRNA	29 - 31	N	mono-P	2'-O-met
	PIWIL2	PIWI	+	piRNA	24 - 28	U	mono-P	2'-O-met
	PIWIL3	PIWI	+	piRNA	??	??	mono-P	2'-O-met
<i>Mus musculus</i>	PIWIL4	PIWI	+	piRNA	27 - 29	U	mono-P	2'-O-met
	AGO1	AGO	-	miRNA, siRNA	21-23	N (U)	mono-P	-OH
	AGO2	AGO	+	miRNA, siRNA	21-23	N (U)	mono-P	-OH
	AGO3	AGO	-	miRNA, siRNA	21-23	N (U)	mono-P	-OH
	AGO4	AGO	-	miRNA, siRNA	21-23	N (U)	mono-P	-OH
	MIWI (PIWIL1)	PIWI	+	piRNA	29-31	N	mono-P	2'-O-met
	MIWI2 (PIWIL4)	PIWI	+	piRNA	27 - 29	U	mono-P	2'-O-met
<i>Drosophila melanogaster</i>	MILI (PIWIL2)	PIWI	+	piRNA	24 - 28	U	mono-P	2'-O-met
	AGO1	AGO	+	miRNA	21-23	N (U)	mono-P	-OH
	AGO2	AGO	+	siRNA	~ 21	N	mono-P	-OH
	AGO3	PIWI	+	piRNA	24 - 27	N	mono-P	2'-O-met
	PIWI	PIWI	+	piRNA	24 - 29	U	mono-P	2'-O-met
<i>Caenorhabditis elegans</i>	AUB	PIWI	+	piRNA	23 - 27	U	mono-P	2'-O-met
	ALG-1, F48F7.1	AGO	+	miRNA	22-23		mono-P	-OH
	ALG-2, unknown	AGO	+	miRNA	22-23		mono-P	-OH
	ALG-3, T2B3.2	AGO	+	26G siRNA	26	G	mono-P	-OH
	ALG-4, ZK757.3	AGO	+	26G siRNA	26	G	mono-P	-OH
	RDE-1, K08H10.7	AGO	+	primary siRNA	22-23		mono-P	-OH
	ERGO-1, R09A1.1	AGO	+	26G siRNA	26	G	mono-P	2'-O-met
	CSR-1, F20D12.1	AGO	+	22G siRNA	22	G	tri-P	-OH
	C06A1.4	AGO?	-					
	H10D12.2, M03D4.6	AGO?	-					
	C14B1.7	PIWI?	-					
	T23D8.7, HPO-24	PIWI?	-					
	C04F12.1	PIWI?	+					
	PRG-1, D2030.6	PIWI	+	21U piRNA	21	U	mono-P	2'-O-met
	PRG-2, C01G5.2	PIWI	+	21U piRNA	21	U		
	WAGO-1, R06C7.1	WAGO branch1	-	22G siRNA	22	G	tri-P	-OH
	WAGO-2, F55A12.1	WAGO branch1	-	22G siRNA	22	G	tri-P	-OH
	PPW-2, WAGO-3, Y110A7A.18	WAGO branch1	-	22G siRNA	22	G	tri-P	-OH
	WAGO-4, F58G1.1	WAGO branch1	-	22G siRNA	22	G	tri-P	-OH
	WAGO-5, ZK1248.7	WAGO branch1	-	22G siRNA	22	G	tri-P	-OH
	SAGO-2, WAGO-6/8, F56A6.1	WAGO branch2	-	22G siRNA	22	G	tri-P	-OH
	PPW-1, WAGO-7, C18E3.7	WAGO branch2	-	22G siRNA	22	G	tri-P	-OH
	SAGO-1, WAGO-8/6, K12B6.1	WAGO branch2	-	22G siRNA	22	G	tri-P	-OH
	HRDE-1, WAGO-9, C16C10.3	WAGO branch 3	-	22G siRNA	22	G	tri-P	-OH
	WAGO-10, T22H9.3	WAGO branch 3	-	22G siRNA	22	G	tri-P	-OH
	WAGO-11, Y49F6A.1	WAGO branch 3	-	22G siRNA	22	G	tri-P	-OH
	NRDE-3, WAGO-12, R04A9.2	WAGO branch 3	-	22G siRNA	22	G	tri-P	-OH
<i>Arabidopsis thaliana</i>	AGO1	plant AGO clade I	+	miRNA	21	U	mono-P	2'-O-met
	AGO2	plant AGO clade II	+	miRNAs, tasiRNAs, rasiRNAs	21	A	mono-P	2'-O-met
	AGO3	plant AGO clade II	+	siRNAs	24	A	mono-P	2'-O-met
	AGO4	plant AGO clade III	+	intergenic siRNAs, rasiRNAs	23 - 24	A	mono-P	2'-O-met
	AGO5	plant AGO clade I	+	intergenic siRNA	21,22,24	C	mono-P	2'-O-met
	AGO6	plant AGO clade III	+	siRNAs	24	A	mono-P	2'-O-met
	AGO7	plant AGO clade II	+	miRNA (mir390), ta-siRNA	21	A	mono-P	2'-O-met
	AGO8	plant AGO clade III	+	-	-	-	-	-
	AGO9	plant AGO clade III	+	rasiRNAs	24	A	mono-P	2'-O-met
	AGO10	plant AGO clade I	+	miRNA (mir165/166)	21	U	mono-P	2'-O-met

The table was compiled from the following literature (Tijsterman et al., 2002a; Tijsterman et al., 2002b; Yigit et al., 2006; Forsternann et al., 2007; Zheng et al., 2007; Batista et al., 2008; Das et al., 2008; Wang and Reinke, 2008; Liu et al., 2009; Duran-Figueroa and Vienne-Calzada, 2010; Vasale et al., 2010; Fischer et al., 2011; Buckley et al., 2012; Vourekas et al., 2012; Iwasaki et al., 2015; Zhang et al., 2016b). Slicer activity "+" indicates that a given Argonaute protein has potential to act as a slicer, not that slicing is its primary mode of action. In some case, slicing potential has been inferred from the sequence, i.e. it is not supported with experimental evidence.

3.2.2.1 Small RNA:target RNA base pairing

A small RNA loaded on an Argonaute protein functions as a guide selectively recognizing cognate RNAs through sequence complementarity. Sequence complementarity can be high (full or almost full) or partial. High sequence complementarity operates in RNAi-mediated innate immunity and genome defense where it is desirable to degrade all nucleic acids with highly similar sequences. High sequence complementarity is also observed for many plant miRNAs, which could be, at least in part, a consequence of their evolution (Llave et al., 2002; Allen et al., 2004). Animal miRNAs and some plant miRNAs have typically partial sequence complementarity, which seems to be non-randomly distributed along a small RNA (reviewed, for example in (Bartel, 2009)). Partial complementarity could be seen as a minimal requirement for functional target recognition formed by natural selection. However, before addressing small RNA:target RNA complementarity, we will review the structure of Argonaute proteins and the implications for base pairing and target recognition. The reason is that structural analyses of Argonaute proteins provided important insights into the mechanism of how an Argonaute-loaded small RNA recognizes and binds its target.

Structural insights into target recognition by Argonaute-bound small RNAs

The one of the fundamental steps in deciphering rules governing target recognition and repression in RNA silencing is understanding the structure of a cognate RNA bound to a guide RNA loaded on an Argonaute protein. There is a large volume of literature on Argonaute proteins in different taxons (Table 20), which was reviewed in the section 3.2. of the report (*Part 1 - Mode-of-action of dsRNA and miRNA pathways*). These include structural analyses of individual Argonaute domains as well as several structures of whole Argonaute proteins. The pioneering structural analysis of full-length Argonaute proteins has been carried out on crystalized archaeal proteins from *Pyrococcus furiosus* (Song et al., 2004), *Aquifex aeolicus* (Yuan et al., 2005), *Archaeoglobus fulgidus* (Ma et al., 2005; Parker et al., 2005), and *Thermus thermophilus* (Wang et al., 2008; Wang et al., 2009) and, subsequently on human AGO1 and AGO2 proteins (Elkayam et al., 2012; Schirle and MacRae, 2012; Faehnle et al., 2013; Nakanishi et al., 2013; Schirle et al., 2014; Schirle et al., 2015; Schirle et al., 2016). These analyses have been complemented with numerous biochemical studies of Argonautes and their domains, which are beyond the scope of this report. Here, we summarize those insights into the structure and function of eukaryotic Argonaute proteins, which are important for small RNA:target RNA interaction.

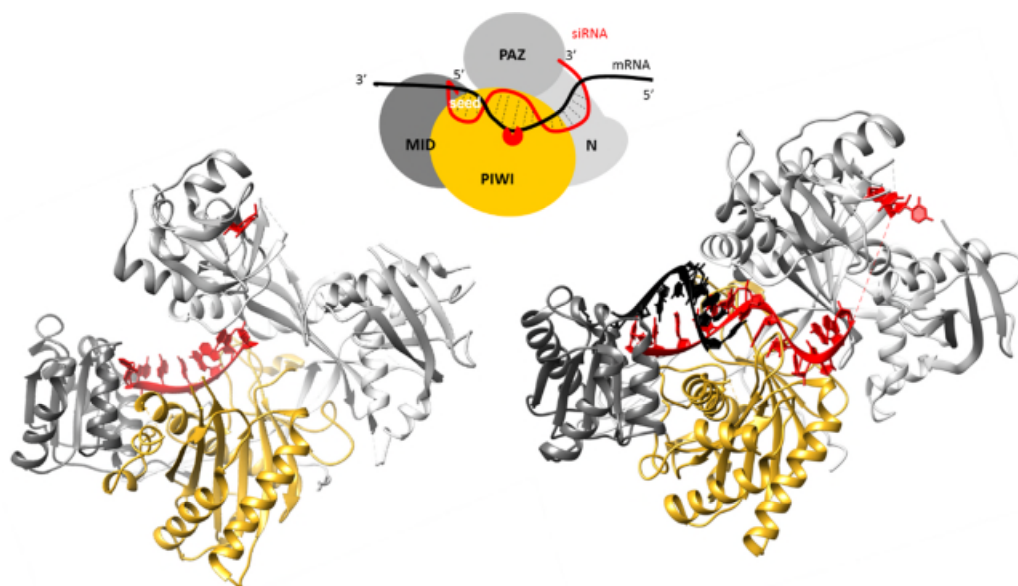


Figure 60 Crystal structures of Argonautes with bound RNAs

Left: AGO2 with bound small RNA (in red), visible is the seed in A conformation (Schirle et al., 2014). Right: AGO2 loaded with a small RNA (in red) interacting with a target RNA (in black) (Schirle et al., 2014). Data for visualization were obtained from wwPDB and displayed in UCSF Chimera.

Human AGO1 and AGO2 as prototypes of eukaryotic Argonaute proteins

According to the data deposited in the world-wide Protein Data Bank (<http://wwpdb.org/>), human AGO1 and AGO2 proteins are the only deciphered structures of eukaryotic Argonaute proteins of the AGO subfamily. While both proteins accommodate siRNAs and miRNAs, only AGO2 has the slicer activity (Liu et al., 2004; Meister et al., 2004). As mentioned above, structural analysis of archaeal proteins revealed that Argonaute proteins are composed of four main domains: the central PAZ domain, the C-terminal PIWI, the N-terminal domain, and the MID domain between PAZ and PIWI domains. A small RNA is anchored with its 3' end in the PAZ domain and the 5' end in a binding pocket between the MID domain and the PIWI domain (Figure 60). Human AGO1 and AGO2 proteins also show this organization (Elkayam et al., 2012; Schirle and MacRae, 2012; Faehnle et al., 2013; Nakanishi et al., 2013; Schirle et al., 2014; Schirle et al., 2015; Schirle et al., 2016). The crystal structure of human AGO2 revealed a bilobed molecule with a central cleft for binding guide and target RNAs (Elkayam et al., 2012; Schirle and MacRae, 2012; Schirle et al., 2014; Schirle et al., 2015). The crystal structures of human AGO1 bound to endogenous co-purified RNAs or loaded with miRNA (let-7) are very similar to the structures of AGO2 despite the fact that AGO1 lacks the slicer activity (Faehnle et al., 2013; Nakanishi et al., 2013).

The key observation coming from the structural analysis is that nucleotides 2 to 6 of a guide RNA are positioned in an A-form conformation for base pairing with target messenger RNAs (Elkayam et al., 2012; Schirle and MacRae, 2012; Faehnle et al., 2013; Nakanishi et al., 2013; Schirle et al., 2014; Schirle et al., 2015; Schirle et al., 2016) (Figure 60). An RNA molecule can occur in many three dimensional conformations because there are multiple angles along which it can rotate its parts. Accordingly, initiation of base pairing requires proper conformation of two RNA molecules in order to initiate formation of hydrogen bonds between two complementary molecules. An Argonaute protein facilitates base pairing between a small RNA and a complementary RNA (= target recognition) by exposing nucleotides 2-6 arranged in a conformation needed for proper base pairing.

Between nucleotides 6 and 7, there is a kink that may function in miRNA target recognition or release of sliced RNA products. (Schirle and MacRae, 2012). Crystallization of loaded human AGO2 in the presence of target RNA sequences suggested a stepwise mechanism for interaction with cognate RNAs. First, AGO2 exposes guide nucleotides (nt) 2 to 5 for initial target pairing, which then promotes

conformational changes that expose nt 2 to 8 and 13 to 16 for further target recognition (Schirle et al., 2014). miRNA binding seem to lock the otherwise flexible AGO2 enzyme in a stable conformation (Elkayam et al., 2012). The structure of human Ago2 bound to miRNA-20a implies that the miRNA is anchored at both ends by the MID and PAZ domains with several kinks and turns along the binding groove (Elkayam et al., 2012). Spurious slicing of miRNA targets is avoided through an inhibitory coordination of one catalytic magnesium ion (Schirle et al., 2014). Evolutionary changes that rendered hAGO1 inactive included a mutation of a catalytic tetrad residue and mutations on a loop near the active site (Faehnle et al., 2013; Nakanishi et al., 2013). Importantly, the PIWI domain contains tandem tryptophan-binding pockets, that function in recruitment of glycine-tryptophan-182 (GW182) or other tryptophan-rich cofactors (Schirle and MacRae, 2012). Computer simulation of the structural and functional dynamics of human Ago2 and the interaction mechanism with siRNAs confirmed that Ago2 adopts two conformations such as "open" and "close" and the PAZ is a highly flexible region. (Bhandare and Ramaswamy, 2016). Models of miRNA-loaded Argonautes imply that Argonautes adopt variable conformations at distinct target sites that generate distorted, imperfect miRNA-target duplexes where structural distortions are better tolerated in solvent-exposed seed and 3'-end regions than in the central duplex region (Gan and Gunsalus, 2015).

Structural analysis also clarified the effect of the first nucleotide in the cognate site, which does not base pair with the loaded small RNA because the first nucleotide of the small RNA (frequently U) is buried in the 5' end-binding pocket. Yet, it was observed that interaction with the cognate site is enhanced by adenosine in the position 1 of a miRNA binding site; the structural analysis revealed that the adenosine in the mRNA is recognized indirectly by AGO2 through a hydrogen-bonding network of water molecules that preferentially interacts with the N6 amine on the adenine base (Schirle et al., 2015). Importantly, N6 adenosine methylation blocks recognition of the adenosine, which might reflect a possible mechanism for regulating of miRNA binding through covalent modification of miRNA binding sites (Schirle et al., 2015).

These data provide structural foundations of many features of target recognition and can be used for computer simulations of miRNA-target interaction in the context of the loaded Argonaute structure. In fact, an algorithm MiREN, which builds and scores three-dimensional models of the ternary complex formed by AGO, a miRNA and 22 nt of a target mRNA, can be used to assess the likelihood that an RNA molecule is the target of a given miRNA (Leoni and Tramontano, 2016).

Importantly, they also explain features associated with different regions of miRNA and siRNA sequences that were identified in kinetic and bioinformatics studies. Taken together, crystal structures of AGO2 explain the nucleotide-pairing patterns that emerged during previous studies of miRNA sequences, namely analyses of conservations of miRNA binding sites and biochemical analyses of target recognition, which are discussed later.

small RNA:target RNA base pairing

As mentioned above, sequence complementarity between a small RNA and its target RNA can be full (or almost full) and partial. Full complementarity is typically associated with siRNAs while partial with miRNAs although imperfect base pairing of siRNAs and perfect base pairing of miRNAs also occur. To provide a framework for this section, we first review the full-complementarity, then the partial complementarity involving base pairing of 5' small RNA nucleotides (the seed) and then seedless (non-canonical, non-seed) interactions and their implications on target recognition, prediction and effective repression. Importantly, target mRNAs are as efficiently repressed by microRNA-binding sites in the 5' UTR as in the 3' UTR as shown in experiments in cultured human cells (Lytle et al., 2007).

siRNA complementarity and sequence features

RNAi efficiency correlates well with the binding energy of a siRNA to its mRNA target (Muckstein et al., 2006). While full complementarity yields a perfect duplex in which all nucleotides participate seemingly equally, some positive correlations were identified between positions of specific nucleotides and siRNA suppressing efficiency. These features may reflect positive effects on Argonaute loading (strand selection) as well as on target recognition. Analysis of the efficiency of ~600 siRNAs suggested

higher siRNA efficiency with A/U at positions 10 and 19, a G/C at position 1, and more than three A/Us between positions 13 and 19, in the sense strand of the siRNA sequence (Jagla et al., 2005). Furthermore, specific residues at every third position of an siRNA influence its efficient RNAi activity, which might reflect interaction with TARBP2 during formation of the RNA-induced silencing complex (RISC) (Kato and Suzuki, 2007).

Target recognition by siRNAs is highly specific. However, discrimination of RNAi between two sequences differing by a single nucleotide varies according to the position of the mismatch. A systematic analysis of single-nucleotide mutations in target sites of a functionally validated siRNA showed that the position of the mismatched base pair and the identity of the nucleotides forming the mismatch matter for effective silencing (Du et al., 2005). A:C mismatches were, in addition to the G:U wobble base pairs, surprisingly well tolerated and target sites containing such mismatches were silenced almost as efficiently as with full complementarity (Du et al., 2005). G:U wobble base pairing in the central part of the antisense strand caused a pronounced decrease in activity, while mutations at the 5' and 3' ends were well-tolerated (Holen et al., 2005). Interestingly, analysis of siRNA selectivity suggested that siRNAs with G:U wobble base pairs or a mismatches located in the "seed" are discriminating less between perfect and mismatched target than those in which the mismatch was located 3' to the seed (nucleotides 9-14); this region is critical for target cleavage but not siRNA binding (Schwarz et al., 2006).

miRNAs with extensive base pairing

Target recognition by miRNAs in plants is commonly thought to involve extensive base pairing and RNAi-like cleavage of the target (reviewed in (Axtell, 2013; Wang et al., 2015a)). This notion stems from the perfect complementarity between miR171 and its SCARECROW-LIKE (SCL) mRNA target, which was the first identified miRNA:mRNA interaction in plants (Llave et al., 2002). However, the perfect complementarity is rather an exception as most of the identified miRNA targets in plant cells have some imperfect base pairing (summarized in (Jones-Rhoades and Bartel, 2004; Jones-Rhoades et al., 2006)). Extensive base pairing and microRNA-directed RNAi-like cleavage exists also in animals but it is rare; one of the exceptional cases is HOXB8 mRNA cleavage by miR-196 (Yekta et al., 2004)

Mismatches to the miRNA 5' regions strongly reduce repression but are found in several natural miRNA-binding sites while miRNA binding with a few mismatches to the miRNA 3' regions are common in plants and are often equally (or even more) effective as perfectly matched sites (Liu et al., 2014b). Central mismatches interfere with repression (Liu et al., 2014b). However, miR398 in *Arabidopsis* binds 5'UTR of the blue copper-binding protein mRNA with a bulge of six nucleotides opposite to the 5' region of the miRNA (Brousse et al., 2014). These and other studies led to consensus base pairing rules for a functional plant miRNA-target interaction: little tolerance for mismatches at positions 2–13, with especially little tolerance of mismatches at positions 9–11, and more tolerance of mismatches at positions 1, and 14–21 (Wang et al., 2015a). This is in contrast with animal miRNAs where pairing at positions 2–7 can be sufficient for a functional interaction (Bartel, 2009).

High sequence complementarity in mammals may be coupled with Argonaute "unloading". It was found that highly complementary target RNAs significantly accelerate release of the guide RNA from Ago2. Unloading can be enhanced by mismatches between the target and miRNA's 5' end and attenuated by mismatches to miRNA's 3' end (De et al., 2013).

Imperfect base pairing of miRNAs

Animal miRNAs typically base pair imperfectly with their targets. Target sites can be grouped into two broad categories. 5' dominant sites have sufficient complementarity to the miRNA 5' end to function with little or no support from pairing to the miRNA 3' end. Indeed, sites with 3' pairing below the random noise level are functional given a strong 5' end. In contrast, 3' compensatory sites have insufficient 5' pairing and require strong 3' pairing for function (Brennecke et al., 2005). Accordingly, we will separately discuss the canonical base pairing involving miRNA's 5' end (the seed) and the non-canonical (seedless) interactions. We will start with the canonical interaction involving base pairing of the seed because it is the most studied and integrates knowledge from structural studies as well as sequence analyses.

Seed-involving interactions

The seed sequence concept emerged already during pioneering work on miRNA annotation where it became apparent that miRNAs form families sharing 5' sequences (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Then it became clear that the seed sequence is a strong predictor for miRNA targets (Lewis et al., 2005; Sood et al., 2006) as well as for siRNA off-targeting (Jackson et al., 2006b). An analysis of more than 18,000 high-confidence miRNA-mRNA interactions suggested that binding of most miRNAs includes the 5' seed region, while around 60% of seed interactions contained bulged or mismatched nucleotides (Helwak et al., 2013). The molecular mechanism of miRNA and target recognition (reviewed in (Bartel, 2009)) provides an explanation for the significance of the seed sequence and, while there are also small RNA:target mRNA interactions that do not involve the seed sequence, the concept of the seed is sufficient to explain that any AGO-loaded small RNA in any cell type has the potential to interact with hundreds and thousands of different mRNAs. In fact the estimates for human mRNAs targeted by miRNAs are between 30 and >60% (Lewis et al., 2005; Friedman et al., 2009).

The seed region is generally defined as a 7nt region mapping to positions 2-8 and it strongly confers specificities of animal miRNAs to their mRNA targets. There is a high functional cost of even single nucleotide changes within seed regions, which is consistent with their high sequence conservation among miRNA families both within and between species and suggests processes that may underlie the evolution of miRNA regulatory control (Hill et al., 2014). The target specificity determined by the seed has evolutionary and biological implications because single nucleotide polymorphisms in canonical miRNA binding sites would affect miRNA-mediated regulations, a notion supported also by experimental data (Afonso-Grunz and Muller, 2015; Vosa et al., 2015). The canonical 7nt seed can be divided into several types (Ellwanger et al., 2011). More specifically, the core seeds have been described as a 6-mer (bases 2–7), 7-mer ("7-mer-A1" being bases 1–7, and "7-mer-m8" being bases 2–8), and 8-mer (bases 1–8); sometimes the 7-mer-A1 and 8-mer seeds are required to have an adenine, 'A', as the first nucleotide types (Bartel, 2009; Ellwanger et al., 2011).

Longer seeds, i.e. seeds of 7 or 8 nucleotides in length are more evolutionarily conserved than shorter ones (Ellwanger et al., 2011). Longer seeds confer higher specificity and repression. It was reported that the extent of the seed match has a strong impact on resulting target repression: single 8 mer seed match mediates down-regulation comparable to two 7 mer seed matches (Nielsen et al., 2007). However, others did not observe a linear relationship between seed length and miRNA expression dysregulation, which does not support the hypothesis the seed region length alone influences mRNA repression. (Mullany et al., 2016)

In any case, the majority of functional target sites seems formed by less specific seeds of only 6 nt indicating a crucial biological role of this type (Ellwanger et al., 2011). In fact, pairing at positions 2–7 is sufficient for a functional interaction of animal miRNAs with their targets (Bartel, 2009). In contrast, seed pairing does not appear to be critical for land plant miRNAs (Liu et al., 2014b). The minimal requirement for miRNA:mRNA interactions in animals explains the large numbers of targets of animal miRNAs and the fact that, the majority of functional sites is poorly detected by common prediction methods (Ellwanger et al., 2011). While the initial studies suggested that average miRNAs have approximately 100 target sites (Brennecke et al., 2005), subsequent bioinformatics and experimental identification of miRNA targets suggest even higher number of target sites.

There are several targeting determinants that enhance seed match-associated mRNA repression, including the presence of adenosine opposite miRNA base 1 (this functionality is explained by Argonaute protein structure (Schirle et al., 2015)) and of adenosine or uridine opposite miRNA base 9, independent of complementarity to the siRNA/miRNA (Lewis et al., 2005; Nielsen et al., 2007). Furthermore, seed-based canonical target recognition was dependent on the GC content of the miRNA seed - low GC content in the seed was coupled with non-canonical target recognition. (Wang, 2014). Additional reported determinants beyond seed pairing include: AU-rich nucleotide composition near the site, proximity to sites for co-expressed miRNAs (which leads to cooperative action), proximity to residues pairing to miRNA nucleotides 13-16, positioning within the 3'UTR at least 15 nt from the stop codon, and positioning away from the center of long UTRs (Grimson et al., 2007).

Non-canonical – non-seed interactions

There is large variety of miRNA-target duplex structures, which include seedless interactions (reviewed in (Cipolla, 2014; Seok et al., 2016a)). The existence of seedless interactions explains reports that perfect seed pairing is not a generally reliable predictor for miRNA-target interactions (Didiano and Hobert, 2006). Despite attempts to classify non-canonical interactions (Xu et al., 2014b) and tertiary structure-based modelling of miRNA interactions (Gan and Gunsalus, 2015), bioinformatic prediction of non-canonical interactions is far from ideal. A solution is integration of bioinformatic target prediction with biochemically identified miRNA binding sites. Such analyses suggested that most miRNA targets were of a non-canonical type, i.e. not involving perfect complementarity in the seed region (Khorshid et al., 2013; Wang, 2014). Importantly, analysis of AGO-associated mRNAs that lack seed complementarity with miRNAs suggested that AGO might have its own binding preference within target mRNAs, independent of guide miRNAs (Li et al., 2014). A structurally accessible and evolutionarily conserved region (~10 nucleotides in length) was identified that alone can accurately predict AGO-mRNA associations, independent of the presence of miRNA binding sites (Li et al., 2014). In any case, the impact of non-canonical targeting regarding target downregulation is not fully resolved (Khorshid et al., 2013; Martin et al., 2014; Wang, 2014).

3.2.2.2 Other key factors influencing target recognition and repression

It is important to recognize that sequence complementarity between a small RNA and its putative target is not sufficient to make any prediction about silencing of the target because there are other important factors at play. The two most important are discussed in the next two sections are (I) the binding site accessibility and (II) stoichiometry between a small RNA and its target (or binding kinetics). Other factors, which might contribute to silencing in a context-dependent manner are, for example, alternative polyadenylation and arrangement of miRNA binding sites in 3'UTRs might cause different effects in different cells (Hon and Zhang, 2007; Majoros and Ohler, 2007; Nam et al., 2014a). In particular, there was a strong preference reported for targets to be located in close vicinity of the stop codon and the polyadenylation sites. (Majoros and Ohler, 2007).

Binding site accessibility

Mere sequence complementarity is not a sufficient predictor whether base pairing will occur *in vivo*. RNA molecules always form secondary structures and, in the cellular context, a number of proteins interacts with RNA molecules. Accordingly, secondary structures or RNA binding proteins may prevent base pairing of two complementary sequences. The issue of sequence accessibility was recognized during early RNAi experiments with stochastic knockdown efficiency. When searching for factors influencing knock-down efficiency, attention turned to the local RNA structure at siRNA target sites and it was demonstrated that local RNA target structure is an important factor for siRNA efficacy (Schubert et al., 2005). Accordingly, siRNA design tools started to accommodate not only properties of siRNAs but also properties of the target site because it strongly increased efficiency of designed siRNAs (Heale et al., 2005, 2006; Shao et al., 2007; Tafer et al., 2008).

Systematic investigation of siRNA:target RNA interactions and the effect of local secondary structures provided also insights into the molecular mechanism of target recognition. It was shown *in vitro* and *in vivo*, that the accessibility of the target site correlates directly with the efficiency of cleavage, demonstrating that RISC is not unfolding structured RNA (Ameres et al., 2007). During target recognition, RISC transiently contacts single-stranded RNA nonspecifically and promotes siRNA-target RNA annealing (Ameres et al., 2007). The seed of Argonaute-associated siRNA creates a thermodynamic threshold that determines the stable association of RISC and the target RNA (Ameres et al., 2007).

The same principles apparently apply for miRNA-mediated repression (Long et al., 2007; Xu et al., 2014b). Mutations diminishing target accessibility substantially reduce microRNA-mediated translational repression, with effects comparable to those of mutations that disrupt sequence complementarity (Kertesz et al., 2007).

small RNA:target RNA stoichiometry and binding kinetics

The second critical factor for target repression is stoichiometry between a small RNA and its target. This is especially important for the miRNA-like type of target repression because a miRNA must remain associated with its target RNA in order to induce its translational repression and degradation. Thus, suppression of a specific mRNA by a miRNA requires enough miRNA molecules that would assure enough interactions with binding sites in that particular RNA while these binding sites essentially compete with all binding sites for that miRNA in the transcriptome.

Biochemical analyses of stoichiometry and kinetics

Kinetic data should be taken as a biochemical range for any hypotheses concerning target recognition and biological effects of small RNAs in the context of loaded RISC. Among these is a detailed kinetic study of *Drosophila* and mouse AGO2 RISCs (Wee et al., 2012).

It was shown that siRNA-programmed RISC is a classical Michaelis-Menten enzyme in the presence of ATP (Haley and Zamore, 2004). In the absence of ATP, the rate of multiple rounds of catalysis is limited by release of the cleaved products (Haley and Zamore, 2004). Kinetic analysis suggests that different regions of the siRNA play distinct roles in the cycle of target recognition, cleavage, and product release (Haley and Zamore, 2004). Later, it was shown that Argonaute divides its RNA guide into domains with distinct functions and RNA-binding properties. (Wee et al., 2012) According to this analysis, small RNAs loaded onto AGO proteins are actually composed of five distinct modules: the anchor, seed, central, 3' supplementary, and tail (Figure 25) (Wee et al., 2012). Bases near the siRNA 5' end disproportionately contribute to target RNA-binding energy, whereas base pairs formed by the central and 3' regions of the siRNA provide a helical geometry required for catalysis (Haley and Zamore, 2004). Mouse AGO2, which mainly mediates miRNA-directed repression in vivo, dissociates rapidly and with similar rates for fully paired and seed-matched targets (Wee et al., 2012). An important conclusion from this study is that low-abundant miRNAs are unlikely to contribute much biologically meaningful regulation because they are present at a concentration less than their KD for seed-matching targets, which are in a picomolar range (Figure 61) (Wee et al., 2012). Another study characterized siRNA binding, target RNA recognition, sequence-specific cleavage and product release by recombinant human Ago 2 (hAgo2). This yielded a minimal mechanistic model describing fundamental steps during RNAi, which is consistent with a "two-state" model of RISC action (Deerberg et al., 2013). Finally, it was found that Mg²⁺ concentration, influences AGO2 structural flexibility and is important for its catalytic/functional activity, with low [Mg²⁺] favoring greater Ago2 flexibility (e.g., greater entropy) and less miRNA/mRNA duplex stability, thus favoring slicing (Nam et al., 2014b).

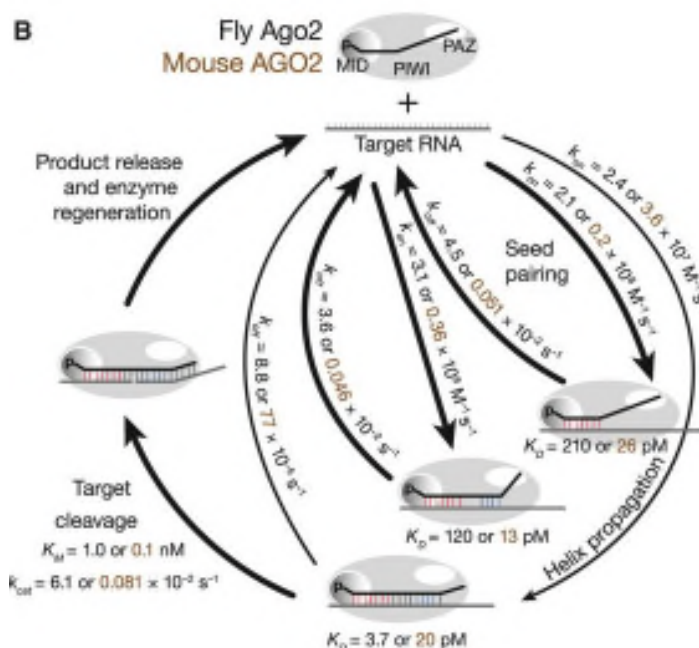


Figure 61 Targeting by small RNAs

A model for RISC binding and cleavage. Data show that nearly every fly Ago2-RISC that reaches this conformation cleaves its RNA target rather than releasing it. For mouse AGO2-RISC, a slow catalytic rate often allows the target to escape before being sliced. In contrast, most miRNA:Argonaute complexes rapidly bind to and dissociate from their RNA targets via their seed. Even when RISC binds a target through both its seed and 3' supplementary regions, it dissociates nearly as rapidly as for seed-only binding. Thus, the properties of RISC are essentially the same for both the typical seed-only and the less common seed plus 3' supplementary pairing targets. That the rates of association and dissociation are so similar for these two binding modes suggests that pairing between a target and the 3' supplementary region of a miRNA does not require winding the target RNA around the guide, side-stepping the topological problem that must be solved for siRNAs to direct RISC to cleave a target. Taken from (Wee et al., 2012)

Importantly, it seems that miRNA:mRNA stoichiometry cannot be simply determined by quantifying RNAs. Quantification of Argonaute-associated endogenous miRNAs or exogenous siRNAs in cultured cells suggested that only a small proportion (even <10%) of such small RNAs is loaded on Argonautes (Janas et al., 2012; Stalder et al., 2013). Furthermore, a substantial percentage of the miRNA pool associated with mRNAs without Argonautes (Janas et al., 2012; Stalder et al., 2013). It was also found that endogenous human miRNAs vary widely, by >100-fold, in their level of RISC association and show that the level of Ago binding is a better indicator of inhibitory potential than is the total level of miRNA expression (Flores et al., 2014). Together, these data indicate that the level of RISC association of a given endogenous miRNA is regulated by the available RNA targetome and predicts miRNA function. (Flores et al., 2014).

Small RNA:target RNA binding single-molecule analysis

Recent advances in single-molecule analysis brought also single-molecule data about RISC:target interaction, which is consistent with other biochemical data and the two state model for Argonaute action (Li and Zhang, 2012; Zander et al., 2014).

Loaded AGO2 utilizes short RNAs as specificity determinants with thermodynamic and kinetic properties more typical of RNA-binding proteins. A small RNA loaded on Argonaute does not follow rules by which free oligonucleotides find, bind, and dissociate from complementary nucleic acid sequences (Salomon et al., 2015). This is conceivable given the fixed "A" conformation of the seed of a small RNA loaded on an Argonaute protein.

Single-molecule fluorescence experiments using a minimal RISC (a small RNA and AGO2) showed that target binding starts at the seed region of the guide RNA (Chandradoss et al., 2015; Jo et al., 2015a; Jo et al., 2015b). AGO2 initially scans for target sites with complementarity to nucleotides 2-4 of the miRNA. This initial transient interaction propagates into a stable association when target complementarity extends to nucleotides 2-8. This stepwise recognition process is coupled to lateral diffusion of AGO2 along the target RNA, which promotes the target search by enhancing the retention of AGO2 on the RNA (Chandradoss et al., 2015). Stable RISC binding is thus efficiently established with the seed match only, providing a potential explanation for the seed-match rule of miRNA target selection (Chandradoss et al., 2015; Jo et al., 2015a; Jo et al., 2015b). Mouse AGO2 binds tighter to miRNA targets than its RNAi cleavage product, even though the cleaved product contains more base pairs (Salomon et al., 2015). Annealing between miRNA and its target with poor seed match proceeds in a stepwise way, which is in accordance with the increase in the number of conformational states of miRNA-target duplex accommodated by the miRISC, suggesting the structural plasticity of human miRISC to conciliate the mismatches in seed region (Li and Zhang, 2012)

Target cleavage required extensive sequence complementarity and accelerated core-RISC dissociation for recycling (Jo et al., 2015a) and sensitively depended on the sequence (Jo et al., 2015b). While RISC generally releases the 5' cleavage fragment from the guide 3' supplementary region first and then the 3' fragment from the seed region. This order can be reversed by extreme stabilization of the 3' supplementary region or mismatches in the seed region. Therefore, the release order of the two cleavage fragments is influenced by the stability in each region, in contrast to the unidirectional base pairing propagation from the seed to the 3' supplementary region upon target recognition. (Yao et al., 2015).

3.2.2.3 Off-targeting – causes and remedies

Off-targeting effects surfaced as a major issue in RNAi experiments when the effects of RNAi treatment were systematically analyzed (Jackson et al., 2003; Scacheri et al., 2004; Snove and Holen, 2004; Lin et al., 2005; Fedorov et al., 2006). One of the most revealing data came from mammalian cells transfected with different siRNAs targeting the same gene, which were systematically analyzed using microarrays (Jackson et al., 2003). Using 16 different siRNAs against IGF1R and 8 different siRNAs against MAPK14, strong siRNA-specific expression changes were found in transfected cells with only a few genes regulated in common by siRNAs targeting the same gene. Off-targeting effects were

also found also in other animal models (Ma et al., 2006) and plants (Xu et al., 2006). In fact, off-targeting causes a significant bias in high-throughput RNAi screens (Ma et al., 2006)

Off-targeting is concentration dependent, it could be attributed to both siRNA strands, and a portion of off-targeting appears to be caused by partial complementarity between a siRNA and its target, reminiscent of the 5' seed regions of miRNAs (Jackson et al., 2003; Birmingham et al., 2006; Jackson et al., 2006b; Aleman et al., 2007; Qiu et al., 2007). In some cases of off-targeting, no correlation between predicted and actual off-target effects was reported (Hanning et al., 2013). However, this probably reflects problems of accurate miRNA target prediction rather than the absence of miRNA-like off-targeting. In any case, a recent systematic analysis of off-targeting effects confirmed that strength of base pairing in the siRNA seed region is the primary factor determining the efficiency of off-target silencing (Kamola et al., 2015)

The main cause of off-targeting is miRNA-like behavior of siRNAs. It was experimentally demonstrated in mammalian cells that siRNAs can function as miRNAs (Doench et al., 2003) and that siRNAs imperfectly matching endogenous mRNAs repress translation (Saxena et al., 2003; Martin and Caplen, 2006) suggesting that miRNAs and siRNAs use similar if not identical, mechanisms for target repression (Zeng et al., 2003). The current view of mammalian RNAi is that experimental RNAi induced with a siRNA or shRNA hijacks the molecular machinery dedicated to the miRNA pathway (reviewed in (Svoboda, 2014)). Consequently, some degree of off-targeting likely occurs in every RNAi experiment.

Importantly, experimental RNAi can also cause artifacts through saturation of the miRNA pathway, which essentially suppresses normal miRNA function (Khan et al., 2009). Exportin 5 seems to be a bottleneck for an effective RNA silencing (Lu and Cullen, 2004; Yi et al., 2005). Indeed, lethal non-specific effects observed with type I shRNAs delivered to the mouse liver by a viral vector were linked to the saturation of Exportin 5 (Grimm et al., 2006). Inhibition of Exportin 5 could also provide an explanation to early lethality defects observed during generation transgenic mice carrying class I shRNA expression cassette (Cao et al., 2005).

Suppression and by-passing off-targeting

Off-targeting has been a recurring problem with RNAi experiments, especially in RNAi screens searching for novel regulators. Off-targeting was frequently causing false-positive results in such screens although this issue has been partially remedied (reviewed in (Petri and Meister, 2013; Mohr et al., 2014)). Below, we list options for dealing with off-targeting, which emerged from the literature review.

Appropriate experimental design

This is actually a simple solution, which emerged from initial experiments detecting off-targeting (reviewed in (Svoboda, 2007)), which suggested that off-targeting operates through miRNA-like behavior of siRNAs and is concentration-dependent. Thus, a proper practice is to use the minimal effective siRNA concentration. Importantly, this step strongly reduces off-targeting but it does not eliminate it as the targeting siRNA is still present and functions as a miRNA (Jackson et al., 2003; Jackson et al., 2006b).

Pools of siRNA

An extension of a strategy to lower siRNA concentration to the point that off-targeting effects in the model system become very low or even undetectable. If a pool of 10 siRNAs is used at the same total siRNA concentration, a single siRNA is having ten times lower concentration and causes lower off-targeting effects. One can produce an siRNA pool by an enzymatic digest of long dsRNA with Dicer or simply purchase a number of siRNAs targeting a single mRNA. In fact, some companies offer pre-made siRNA pools. A unique type of siRNA pools are siPools, which are produced by in vitro transcription of tandemly arrayed siRNA sequences (Hannus et al., 2014)

Bioinformatics filtering

Since the siRNA seed region is strongly associated with off-target silencing (Jackson et al., 2006b; Kamola et al., 2015), it could be used to filter RNAi screening data to reduce off-target rates (Yilmazel et al., 2014; Zhong et al., 2014). In fact, revised analysis of RNAi screens could identify functionally relevant genes suppressed by off-targeting (Lin et al., 2007; Adams et al., 2015; Singh et al., 2015).

Better small RNA design

Understanding of the molecular mechanism of RNAi is also reflected in constantly improving siRNA design which aims at providing siRNAs specifically silencing a gene of interest with little or no off-target effects and no cell toxicity (reviewed in (Tafer, 2014; Ahmed et al., 2015)). Improved siRNA design can reduce off-targeting in several ways. First, siRNAs designed for efficient strand selection would have reduced off-targeting caused by AGO-loaded passenger strand. It was also found that increased siRNA duplex stability correlates with reduced off-target and elevated on-target effects (Petri et al., 2011). This can be, for example, influenced by the seed binding energy and seed composition, which would determine the pool of potential binding sites in the transcriptome and the difference between on-target and off-target RNAs (Das et al., 2013a; Das et al., 2013b). Adaptations of siRNA/shRNA design to reduce off-target effects include weak base pairing in both seed and 3' regions (Gu et al., 2014) and evaluation of potential cross-hybridization candidates (Yamada and Morishita, 2005; Anderson et al., 2008). Reduced off-targeting features were subsequently integrated into siRNA design tools such as siDirect (Naito et al., 2009; Naito and Ui-Tei, 2013).

Mismatch introduction

Mismatch introduction into siRNA at the positions 2 of the base pairing also weakens off-targeting (Dua et al., 2011, Li et al., 2015)

Chemical modifications of small RNAs

The discovery that off-targeting involves miRNA-like behavior of siRNAs prompted research on chemical modifications that would reduce miRNA-like behavior while not interfering with desired RNAi effects (Chiu and Rana, 2003). A thorough review of the chemical modifications is beyond the scope of this report but can be found elsewhere (Snove and Rossi, 2006; Peacock et al., 2011; Engels, 2013; Nolte et al., 2013). There are two common strategies, to reduce off-targeting – (I) Chemical modifications on the passenger strand preventing its loading, hence eliminating off-targeting caused by the passenger strand (Chen et al., 2008; Snead et al., 2013) and (II) Chemical modifications in the seed region, which interfere with miRNA-like target recognition but do not prevent specific RNAi targeting. Different chemistry was used for chemical modification of siRNAs with reduced off-targeting effects including unlocked nucleic acid (UNA) modification (Snead et al., 2013), locked nucleic acid (LNA) modification (Fluiter et al., 2009), 2'-O-methyl ribosyl (Jackson et al., 2006a; Chen et al., 2008), or abasic nucleotides (Seok et al., 2016b).

Position of the modification on the guiding strand is important for reduced off-targeting. While it is usually involving seed, the modified nucleotide may vary. For example, 2'-O-methyl ribosyl substitution at position 2 in the guide strand reduces most off-target effects caused by complementarity to the seed region of the siRNA guide strand (Jackson et al., 2006a). At the same time, an abasic nucleotide at the position 6 in the guide strand also eliminates miRNA-like off-target repression but preserves near-perfect on-target activity (80-100%) (Seok et al., 2016b).

Discerning specific RNAi phenotypes from off-targeting effects

While strategies for suppressing off-targeting effects clearly reduce experimental artifacts, off-targeting should be seen as a type of noise in RNAi experiments that cannot be completely eliminated. Assuming that some off-targeting occurs in every RNAi experiment, one can focus on a more important issue: how to identify biologically relevant effects of off-targeting (phenotype) and separate them from the specific RNAi effect caused by knock-down of the desired gene. The idea is simple – while one can try to minimize off-targeting effects, the risk cannot be completely eliminated. Thus, it

is equally important to use an appropriate experimental design, which allows to distinguish between off-targeting and specific RNAi effects. The two possible strategies were proposed a decade ago and were named “the two R’s”: rescue and redundancy (Echeverri et al., 2006).

The principle of the rescue strategy is expressing an RNAi-resistant version of the targeted gene. If a phenotype is caused by the gene knock-down, it should be rescued. It can be either mutated such that the base pairing with a short RNA is eliminated. One can, for example target 3’UTR and use a different one in the rescue construct or mutate/degenerate appropriate codon positions if targeting CDS. This strategy is powerful because it accepts all effects in an RNAi experiment and tests the contribution of the specific gene knock-down. For recent information on design of the rescue system see, for example, (Kumar, 2015)

The second strategy is based on phenotype redundancy. Two or more RNAi triggers with different sequences (i.e. specific siRNAs or shRNAs) producing the same phenotype decrease the probability that a phenotype would be caused by off-targeting. However, some common phenotypes (e.g. slower growth, apoptosis, and developmental arrest) may be a frequent off-targeting phenotype induced by different RNAi triggers, so the redundancy strategy would be less powerful than the rescue strategy described above. However, for some purposes (e.g. high-throughput RNAi screening), it might be easier to implement the redundancy strategy as a control for off-targeting than the rescue strategy.

Importantly, “non-targeting” controls (e.g. siRNAs with a random sequence or targeting non-expressed genes such as EGFP or Luciferase) cannot be used controls for off-targeting for reasons mentioned above. It is a frequent misconception ignoring the fact that off-targeting is individual to each RNAi trigger because it is sequence-specific. “Non-targeting” siRNAs or shRNAs RNAs may serve as controls for the sequence-independent effects, such as interferon response and saturation of RNA silencing with an excess of exogenous short RNAs. If a small RNA is needed as a control for off-targeting, one may only use a pool of scrambled small RNAs, which would have highly diluted off-targeting effects.

3.2.2.4 Target identification – in silico & experimental approaches

Target identification is a common issue in the small RNA field (reviewed for example in (Pasquinelli, 2012; Tarang and Weston, 2014)). Target identification can utilize bioinformatic analysis, experimental analysis or their combination. We provide an overview of in silico approaches in the section 3.2.4.1. and experimental approaches in the section 3.2.4.2. Briefly, bioinformatic analysis primarily implements the canonical seed-match model, evolutionary conservation, and binding energy, which are often complemented by neural networks trained on sets of experimental data in order to optimize filtering parameters. Some bioinformatics tools also consider non-canonical binding sites. Importantly, a mere presence of a miRNA binding site is insufficient for predicting target regulation as additional factors influence the regulation, including the above-mentioned accessibility of a binding site and stoichiometry between a miRNA and its targets. In the end, experimental verification of microRNA targets is essential, prediction alone is insufficient (Law et al., 2013).

Target prediction in silico

Identification of many miRNAs in model organisms prompted development of bioinformatics tools for prediction of targeted mRNAs (Enright et al., 2003; Lewis et al., 2003; Stark et al., 2003; John et al., 2004; Kiriakidou et al., 2004; Grun et al., 2005; Krek et al., 2005). A number of bioinformatics tools emerged for miRNA analysis and target prediction (a comprehensive overview of all miRNA analysis tools is provided at <https://tools4mirs.org/>, for recent reviews on bioinformatic target prediction see, for example, (Elton and Yalowich, 2015; Lagana, 2015; Li and Zhang, 2015; Ristevski, 2015). Our literature search revealed an overwhelming amount of miRNA analysis tools. In the Table 21, we provide summary of the tools, which were identified during the literature search. However, their detailed annotation is beyond the scope of this report. A searchable database of systematically annotated miRNA tools can be found here: https://tools4mirs.org/software/target_prediction/.

Table 21 Overview of bioinformatics tools for miRNA analysis

Tool	Description	www	URL	Reference
ActMiR	identifying active miRNAs and miRNA-mediated regulatory mechanisms.		http://research.mssm.edu/integrative-network-biology/Software.html	(Lee et al., 2016)
Avishkar	MicroRNA target prediction using thermodynamic and sequence curves		https://bitbucket.org/cellsandmachines/avishkar	(Ghoshal et al., 2015)
BiLTR	A semi-supervised tensor regression model for siRNA efficacy prediction.		http://www.jaist.ac.jp/~bao/BiLTR/	(Thang et al., 2015)
Chu2015	two score-based local causal learning algorithms that utilized the Markov blanket search			(Chu et al., 2015)
CIDER	prediction of miRNA-mRNA interactions. CIDER (Causal miRNA target Discovery with Expression profile and Regulatory knowledge) is able to utilise a variety of gene regulation knowledge, including transcriptional and post-transcriptional knowledge, and to exploit gene expression data for the discovery of miRNA-mRNA regulatory relationships			(Zhang et al., 2016a)
ComiRNet	ComiRNet (Co-clustered miRNA Regulatory Networks);web-based system for the analysis of miRNA-gene regulatory networks	fail	http://comirnet.di.uniba.it	(Pio et al., 2015)
CRCmiRTar	disease-specific algorithm (colorectal carcinoma)			(Amirkhah et al., 2015)
Cupid	simultaneous prediction of microRNA-target interactions and their mediated competitive endogenous RNA (ceRNA) interactions.		http://cupidtool.sourceforge.net	(Chiu et al., 2015)
DIANA-LncBase	database of experimentally supported and in silico predicted miRNA Recognition Elements (MREs) on lncRNAs	yes	http://www.microma.gr/LncBase	(Paraskevopoulou et al., 2016)
DIANA-microT-ANN	DIANA-microT-ANN combines multiple novel target site features through an artificial neural network (ANN)			(Reczko et al., 2011)
ECCA	identification of shared miRNAs of different species			NA
ensemble	ensemble based on Borda count election on Pearson+IDA+Lasso methods			(Le et al., 2015a)
GenMiR++	Generative model for miRNA regulation			(Huang et al., 2007)
gesper	statistical model for deconvoluting off-target-confounded RNA interference screens		http://icb.helmholtz-muenchen.de/mirlastic	(Schmich et al., 2015)
Hong2016				NA
IDA	Pearson+IDA+Lasso			(Le et al., 2015a)
ImiRP	target disruption by mutation		https://github.com/imirp	(Ryan et al., 2016)
ImiRP			https://github.com/imirp	(Ryan et al., 2016)
imiRTP				(Ding et al., 2012b)
isomiR-SEA	miRNA expression levels		http://eda.polito.it/isomir-sea	(Urgese et al., 2016)
isomiR-SEA			http://eda.polito.it/isomir-sea/	(Urgese et al., 2016)
Katanforoush2015	alignment information and gene expression profiles			(Katanforoush and Mahdavi, 2015)
Korfiati2015	three distinct steps: a filtering step, a novel hybrid classification methodology and an advanced methodology to extract interpretable fuzzy rules from the final prediction model. The classification methodology			(Korfiati et al., 2015)

Tool	Description	www	URL	Reference
	relies on a combination of Genetic Algorithms and Support Vector Machines.			
MAGIA 2		yes	http://gencomp.bio.unipd.it/magia2	(Bisognin et al., 2012)
MBSTAR (Multiple instance learning of Binding Sites of miRNA TARGETS)	microRNA binding site prediction tool	yes	http://www.isical.ac.in/~bioinfo_miu/MBStar30.htm	(Bandyopadhyay et al., 2015)
miRanda	Predicted microRNA targets & target downregulation scores. Experimentally observed expression patterns.	yes	http://www.microna.org/microna/getGeneForm.do	(Betel et al., 2008)
MiRBook	simulates the miRNA and mRNA hybridization competition that occurs in given cellular conditions, and derives the whole set of miRNA:mRNA interactions at equilibrium (microtargetome)	no	http://major.irc.ca/Web/miRBook	(Weill et al., 2015)
miRComb	find potential miRNA-mRNA targets that are more reliable to occur in a specific physiological or disease context		http://mircomb.sourceforge.net	(Vila-Casadesus et al., 2016)
miRDB	online resource for microRNA target prediction and functional annotations.	yes	http://mirdb.org	(Wong and Wang, 2015)
miRecords	integrated resource for microRNA-target interactions	fail	http://miRecords.umn.edu/miRecords	(Xiao et al., 2009)
miREE	miRNA recognition elements ensemble	fail	http://didattica-online.polito.it/eda/miREE/	(Reyes-Herrera et al., 2011)
MiREN				(Leoni and Tramontano, 2016)
miRepress	modelling gene expression regulation by microRNA with non-conventional binding sites			(Ghosal et al., 2016)
miRGate	curated database of human, mouse and rat miRNA-mRNA targets	yes	http://mirgate.bioinfo.cnio.es	(Andres-Leon et al., 2015)
miRGator	miRGator integrates the target prediction, functional analysis, gene expression data and genome annotation.			(Nam et al., 2008)
miRGen				(Megraw et al., 2007)
miRINT				NA
miRLAB	R Based Dry Lab for Exploring miRNA-mRNA Regulatory Relationships.			(Le et al., 2015b)
miRlastic	MicroRNA-Target Network Inference and Local Network Enrichment Analysis			(Sass et al., 2015)
miRNA Digger	screening the cleavage signals, secondary structure prediction, originally from <i>Arabidopsis</i>		http://www.bioinfolab.cn/miRNA_Digger/index.html	NA
miRNA Lasso	matlab source, regularized regression approach that is based on the adaptive Lasso procedure		http://nba.uth.tmc.edu/homepage/liu/miRNALasso	(Wang et al., 2015b)
miRNA Map		yes	http://mirnamap.mbc.nctu.edu.tw	(Hsu et al., 2006)

Tool	Description	www	URL	Reference
MiRror		yes	http://www.proto.cs.huji.ac.il/mirror/	(Friedman et al., 2010)
MIRRO R (miRNA Occupancy Rate predictor)			http://bioinfo.au.tsinghua.edu.cn/member/xwwang/MIROR	(Xie et al., 2014)
miRSEQ				NA
MirTarget/CLIP L	identification of genes targeted by miRNAs based on CLIP experimental data	yes	http://mirdb.org	(Wang, 2016)
miRTarget2		yes	http://mirdb.org	(Wang and El Naqa, 2008)
miRTar Vis	interactive visual analysis tool that predicts targets of miRNAs from miRNA-mRNA expression profile data and visualizes the resulting miRNA-target interaction network		http://hcil.snu.ac.kr/~rati/miRTarVis/index.html	(Jung et al., 2015)
MirTDL	miRNA target prediction algorithm based on convolutional neural network (CNN)	fail	http://nclab.hit.edu.cn/ccrm	(Cheng et al., 2015)
MirZ (+versions)		yes	http://www.mirz.unibas.ch	(Hausser et al., 2009)
ML	Ensemble Pruning and Rotation Forest (EP-RTF), Genetic Algorithm (GA)			(Mousavi et al., 2015)
MMIA		fail	http://cancer.informatics.indiana.edu/mmia	(Nam et al., 2009)
MMiRNA-Tar		yes	http://bioinf1.indstate.edu/MMiRNA-Tar	(Liu et al., 2015)
Mitibase	database for decoding microRNA target sites located within CDS and 5' UTR regions from CLIP-Seq and expression profile datasets	yes	http://mitibase.sysu.edu.cn	(Guo et al., 2015)
NBmiRTar		fail	http://wotan.wistar.upenn.edu/NBmiRTar/	(Yousef et al., 2007)
ncRNAClass	A Web Platform for Non-Coding RNA Feature Calculation and MicroRNAs and Targets Prediction	fail	http://biotools.ceid.upatras.gr/ncrnaclass/	(Karathanou et al., 2015)
Nithin2015				(Nithin et al., 2015)
PACCMIT/PACCMIT-CDS	Prediction of Accessible and/or Conserved MicroRNA Targets)	yes	http://paccmitepfl.ch	(Sulc et al., 2015)
PAREsnip	a tool for rapid genome-wide discovery of small RNA/target interactions evidenced through degradome sequencing			(Folkes et al., 2012)
PicTar		yes	http://www.pictar.org/	(Krek et al., 2005)
PITA				(Kertesz et al., 2007)
PlantMirnaT	miRNA-mRNA integrated analysis system		https://sites.google.com/site/biohealthinformatics/resources	(Rhee et al., 2015)
ProMIS e				NA

Tool	Description	www	URL	Reference
RDDpred	A condition-specific RNA-editing prediction model from RNA-seq data.		http://biohealth.snu.ac.kr/software/RDDpred	(Kim et al., 2016)
R-G2015				(Rabiee-Ghahfarrokhi et al., 2015)
RNAHybrid				(Huang et al., 2016)
SIM	target prediction + effect of not just copy number, but also of other molecular profiles such as methylation changes and loss-of-heterozygosity		CRAN	(Menezes et al., 2016)
SMILE	stacking-based miRNA interaction learner ensemble			(Yu et al., 2014)
SPICE	shRNA target prediction informed by comprehensive enquiry	yes	http://www.spice.sugysun.org/	NA
SPICE (shRNA target prediction informed by comprehensive enquiry)	sequence identification, searching the target genes in the database, retrieve biological information from the database	yes	http://www.spice.sugysun.org/	NA
sRNAtoolbox	integrated collection of small RNA research tools	yes	http://bioinfo5.ugr.es/srnaatoolbox	(Rueda et al., 2015)
SubmiRine	assessing variants in microRNA targets using clinical genomic data sets			(Maxwell et al., 2015)
TargetBoost		fail	http://www.interagon.com/demo/	(Saetrom et al., 2005)
TargetMine	Chen Y.-A., Tripathi L. P., Mizuguchi K. TargetMine, an integrated data warehouse for candidate gene prioritisation and target discovery			NA
TargetS	canonical seed matching and non-canonical seed pairing	yes	http://liubiointfolab.org/targetS/mirna.html	(Xu et al., 2014a)
Target Scan				(Agarwal et al., 2015)
TargetSpy		fail	http://www.targetspy.org	(Sturm et al., 2010)
ToppMiR	rank interactants as a function of their potential to impact a given biological system	yes	http://toppmir.cchmc.org	(Wu et al., 2014)

Importantly, accurate bioinformatic prediction of miRNA-mediated repression is still problematic. This was shown, for example, during experiments with systematically generated artificial miRNAs targeting a desired gene (Arroyo et al., 2014). It turned out that seed-based artificial miRNA design was highly inefficient, as the majority of miRNAs with even perfect seed matches did not repress either target. Moreover, commonly used target prediction programs had problems to discriminate effective artificial miRNAs from ineffective ones, indicating that current algorithms do not fully accommodate important miRNA features allowing for designing artificial miRNAs (Arroyo et al., 2014). Another unresolved issue is reliable prediction of non-canonical (non-seed) miRNA binding sites as most algorithms are based on detection of seed-based miRNA binding sites.

Common target prediction tools

Among the prediction tools, several can be highlighted. These include Targetscan, miRanda, DIANA-microT, PicTAR, whose predictions were integrated into the miRBase, the central annotation database for miRNAs (Kozomara and Griffiths-Jones, 2014) and were also repeatedly evaluated in benchmark studies (Alexiou et al., 2009; Ding et al., 2012a; Majoros et al., 2013; Peterson et al., 2014; Xu et al., 2014a). We add to these also MIRZA as it is one of the most recent algorithms, which in many aspects outperforms the other ones (Gumienny and Zavolan, 2015).

Targetscan (http://www.targetscan.org/vert_71/)

Targetscan is one of the most popular miRNA target prediction tools and its predictions are integrated in the miRBase (Kozomara and Griffiths-Jones, 2014). It is being developed in David Bartel's laboratory as a tool for miRNA target prediction for over a decade (Lewis et al., 2003; Lewis et al., 2005). It predicts biological targets of miRNAs by searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA but there is also an optional search for poorly conserved sites. Its development included also scoring for binding sites with mismatches in the seed region that are compensated by 3' end pairing (Friedman et al., 2009) an improved quantitative model of canonical targeting (Agarwal et al., 2015) and addition other features. The current version considers a site type and fourteen other features and, according to authors, it outperforms other tools and matches high-throughput in vivo crosslinking approaches (Agarwal et al., 2015).

DIANA-MicroT (<http://diana.imis.athena-innovation.gr/DianaTools/index.php>)

DIANA-MicroT target prediction tools are another popular source for miRNA target prediction whose predictions are integrated with miRBase (Kozomara and Griffiths-Jones, 2014). DIANA-MicroT tools are being developed in Artemis Hatzigeorgiou's laboratory for over a decade (Kiriakidou et al., 2004; Sethupathy et al., 2006; Megraw et al., 2007; Maragkakis et al., 2009; Alexiou et al., 2010; Maragkakis et al., 2011; Reczko et al., 2011; Vergoulis et al., 2012; Vlachos et al., 2012; Paraskevopoulou et al., 2013a; Paraskevopoulou et al., 2013b; Paraskevopoulou et al., 2016). MicroT is specifically trained on a positive and a negative set of miRNA binding sites located in 3'-UTR and CDS regions. DIANA Tools offer target prediction algorithms (microT v4 and microT-CDS), databases of experimentally verified miRNA targets on coding and non-coding RNAs (TarBase v7.0 and LncBase), and tools for assessment of biological impacts of miRNAs (mirPath). In addition, the Web Server (v5.0) supports workflows enabling to perform complex functional miRNA analyses.

Pictar (<http://www.pictar.org/>)

Pictar is an algorithm for the identification of microRNA targets from Nikolaus Rajewsky's laboratory (Grun et al., 2005; Krek et al., 2005). Its predictions are also integrated with miRBase (Kozomara and Griffiths-Jones, 2014). Pictar offers for searching of targets of annotated miRNAs or mRNAs. Pictar predicts targets based on complementarity in a 7nt seed region, takes into account conservation and uses hidden Markov model approach to produce the final score. In contrast to Targetscan and DIANA-MicroT, Pictar has not been intensely developed. While it represents one of the older and simpler target prediction algorithms it is quite accurate prediction tool (Alexiou et al., 2009).

miRanda at microRNA.org - Targets and Expression (<http://www.microrna.org/>)

miRanda belongs among the pioneering target prediction algorithms (Enright et al., 2003; John et al., 2004). Its latest version miRanda-miRSVR (Betel et al., 2008; Betel et al., 2010) is integrated into target predictions at <http://www.microrna.org> where one can search predictions for annotated miRNAs in the main experimental model organisms. These predictions are also integrated with miRBase (Kozomara and Griffiths-Jones, 2014). miRanda analyses miRNA sequence complementarity with 3'UTRs and evaluates binding energy, conservation and binding site position in the 3'UTR. miRanda-miRSVR also identifies non-canonical and non-conserved sites (Betel et al., 2008; Betel et al., 2010).

MIRZA & MIRZA-G (<http://www.sib.swiss/zavolan-mihaela/services>)

These tools are being developed in Mihaela Zavolan's lab and can be used for the prediction of miRNA targets and siRNA off-targets on a genome-wide scale. MIRZA is biophysical model of microRNA-target interaction that enables accurate identification of microRNA targets, particularly from Argonaute-CLIP data (Khorshid et al., 2013). MIRZA-G employs both the MIRZA biophysical model as well as other features to predict microRNA target sites genome-wide (Gumienny and Zavolan, 2015). MIRZA-G performed better on a benchmark test than Targetscan Context+ and DIANA-microT-v3 (Gumienny and Zavolan, 2015) making it a good choice for predicting canonical and non-canonical miRNA target sites as well as siRNA off-target sites.

Target prediction for plant miRNAs

This section highlights a specific situation concerning prediction of highly complementary targets of plant miRNAs, which is distinct from prediction of miRNA targets in animals. A pioneering study of miRNA-mediated repression in plants revealed near-perfect complementarity between *Arabidopsis* miRNAs and their targets suggesting that many plant miRNAs act similarly to siRNAs and direct mRNA cleavage (Rhoades et al., 2002). Consequently, miRNA target prediction in plants (for a recent review, see, for example, (Mishra et al., 2015)) is routinely performed as a relatively simple search for highly complementary mRNA sequences without a specialized target prediction algorithm. For example, Singh et al. (Singh et al., 2016) used for miRNA target prediction in ginger (*Zingiber officinale*) the following three simple criteria, which could be written into a simple search script:

- 1) not more than four mismatches allowed between predicted mRNAs and target gene.
- 2) no mismatches allowed for 10th and 11th positions of complementary site (a cleavage site).
- 3) maximum 4 GU pair was allowed in the complimentary alignment.

Some authors even use for searching sequence similarity between a plant miRNA and mRNAs the Basic Local Alignment Search Tool algorithm (Huang et al., 2014a). A specific plant-miRNA-target analysis server is psRNATarget: a plant small RNA target analysis server (Dai and Zhao, 2011), which can be used not only for miRNAs but also for other plant small RNA analysis (Guzman et al., 2013; Huang et al., 2014b; Kumar et al., 2014). Other authors use general target prediction algorithms such as Miranda or RNAhybrid either alone (Shweta and Khan, 2014) or in more complex arrangements (Kurubanjerjit et al., 2013).

A systematic evaluation of tools to predict targets of miRNAs and siRNAs in plants was provided by Srivastava et al. who compared 11 computational tools in identifying genome-wide targets in

Arabidopsis and other plants. Among them, Targetfinder was the most efficient in predicting 'true-positive' targets in *Arabidopsis* miRNA-mRNA interactions but performed much worse when analyzing data from non-*Arabidopsis* species. (Srivastava et al., 2014). Furthermore, combination of Targetfinder and psRNATarget provides high true positive coverage, whereas the intersection of psRNATarget and Tapirhybrid outputs deliver highly 'precise' predictions. All evaluated tools yielded a large number of 'false negative' predictions in non-*Arabidopsis* datasets (Srivastava et al., 2014).

Targets of plant miRNAs, that induce sequence-specific RNAi-like cleavage, can be further identified by employing degradome sequencing, a method determining RNA termini. Thus, in mRNAs cleaved by a miRNA after its 10th nucleotide, one would observe alignment of RNA termini matching the predicted miRNA binding site. This strategy complementing bioinformatics description is further described in the following section.

Common experimental approaches for identification of targets of small RNAs

Bioinformatic target prediction is probabilistic. In other words, bioinformatics predictions identifies a set of putative small RNA targets, which fit certain set of criteria and are assigned a certain probability of being targeted by a specific small RNA. At the same time, each prediction yields positive and false negative results. A common problem in bioinformatic prediction is reliable prediction of non-canonical targets, whose recognition does not involve a complete seed match and, to a lesser extent, prediction of targets recognized through non-conserved binding sites. It is common that researchers aiming at target identification start with bioinformatics prediction and become entangled in the net of prediction tools and generate partially overlapping lists of predicted targets. This strategy is inherently biased towards canonical conserved miRNA binding sites and the highest scoring targets will have more than one such a site. However, this strategy is problematic for identification of the full set of targets.

Accordingly, more reliable identification of small RNA targets usually combines bioinformatics and experimental approaches. This section provides an overview of experimental strategies for identification of small RNA targets that were found during keyword search for target prediction. It is beyond the original EFSA tender requirement but we feel it is useful to include it into the report because it provides an insight into how one could complement the bioinformatics prediction. More detailed reviews of this topic can be found elsewhere (e.g. (Thomson et al., 2011; Tarang and Weston, 2014; Chen et al., 2015)).

High throughput expression analysis

High throughput analysis (expression arrays, RNA sequencing or high-throughput proteomics) can complement target prediction in different ways. One can manipulate the miRNA pathway by various means (reviewed, for example in (Svoboda, 2015)), such as miRNA overexpression, knock-out or inhibition by complementary oligonucleotides (so-called antagomirs) and then identify correlations between target prediction and their actual behavior (e.g. (Krutzfeldt et al., 2005; Lim et al., 2005; Sood et al., 2006)). However, these strategies yield only correlative results, i.e. do not directly detect smallRNA:targetRNA interaction.

Small RNA capture strategies

These strategies can be used to identify either mRNAs bound by a small RNA or small RNAs bound to a selected mRNA. Identification of targets of a small RNA employs delivery of a tagged small RNA (e.g. biotinylated miRNA) followed by an affinity capture to co-purify targets (Orom and Lund, 2007; Baigude et al., 2012; Tan and Lieberman, 2016). These strategies are prone to artifacts because delivery of biotinylated small RNAs can create nonphysiological conditions and affinity purification could be influenced by the binding site context.

Identification of small RNAs bound to a selected mRNA (e.g. miR-CATCH or miRIP methods) employs capture of a selected RNA with associated small RNA using a complementary oligonucleotide, which can be used for affinity capture (e.g. a biotinylated complementary oligonucleotide or a complementary oligonucleotide covalently bound to a magnetic bead) (Su et al., 2015; Vencken et al., 2015). This strategy is suitable for detailed analysis of miRNA-mediated regulation of a specific mRNA

but not for a transcriptome-wide target assessment. One variant employs a tested mRNA fused with a common sequence (EGFP) allowing for using the same set of biotinylated DNA anti-sense oligonucleotides for analyzing different mRNAs allowing for increasing the throughput (Wei et al., 2014).

miRNA extension

This strategy employs miRNA extension with a reverse transcriptase on endogenous target mRNAs. Purified hybrid 3'-cDNA-miRNA-5' molecules are used in a second round of reverse transcription and sequenced (Vatolin et al., 2006). However, this method is prone to artifacts stemming from the variability of miRNA:target mRNA base pairing, which would result in highly variable efficiency of reverse transcription priming.

Immunoprecipitation of small RNA:target RNA complexes

There is a large number of immunoprecipitation strategies aimed at purifying small RNA:target RNA complexes, usually by immunoprecipitating them through an Argonaute protein. Initial experiments immunoprecipitated native Argonaute complexes without including a cross-linking step; immunopurified RNAs were analyzed on microarrays (Easow et al., 2007; Karginov et al., 2007; Hendrickson et al., 2008). An adaptation of Argonaute immunoprecipitation for detection of specific miRNA targets is a RIP competition assay wherein anti-miR is titrated into cytosolic extracts prior to Argonaute immunoprecipitation. Direct target transcripts displaced by anti-miR are then identified based on their depletion from IP fraction (Androsavich and Chau, 2014)

The immunoprecipitation strategy was further developed into a number of methods for isolation of small RNAs bound to their targets, which include high throughput sequencing of crosslinking immunoprecipitation (HITS-CLIP) and crosslinking ligation and sequencing of hybrids (CLASH) methods (reviewed more detail in (Jaskiewicz et al., 2012; Broughton and Pasquinelli, 2016)).

There are several modifications of the basic HITS-CLIP. An improvement of the basic HITS-CLIP approach (Chi et al., 2009) (Zisoulis et al., 2010) came with crosslinking based on photoactivatable nucleosides such as 4-thiouridine, a CLIP modification known as photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) (Hafner et al., 2010; Hafner et al., 2012). PAR-CLIP offers more efficient crosslinking, hence up to three orders of magnitude better RNA recovery than HITS-CLIP (Hafner et al., 2010). Furthermore, PAR-CLIP also allows for precise localization of miRNA binding site as cross-linked 4-thiouridine marks the cross-linked site with frequent thymidine to cytidine change, which is revealed by deep sequencing (Hafner et al., 2010).

Another modified strategy is covalent ligation of endogenous Argonaute-bound RNAs crosslinking immunoprecipitation (CLEAR-CLIP) which enriches miRNAs ligated to their endogenous mRNA targets (Moore et al., 2015). CLEAR-CLIP approach is in principle the same as the above-mentioned CLASH (Helwak et al., 2013; Helwak and Tollervey, 2014). Adding ligation of miRNAs to their mRNA targets yields chimeric reads allowing for robust detection miRNA:target RNA interactions occurring *in vivo*.

Data mining of CLIP data provides not only a comprehensive list of miRNA:target mRNA interactions but also provides insights into the principles governing these interactions, which in turn facilitate further improvement of target prediction algorithms. For example, an *in vivo C. elegans* data set and reanalysis of published mammalian AGO-CLIP data yielded approximately 17,000 miRNA:target site interactions. This strategy identified canonical, noncanonical, and nonconserved miRNA:targets with about 80% of miRNA interactions having perfect or partial seed complementarity (Grosswendt et al., 2014). Another comprehensive analysis of 34 Argonaute HITS-CLIP datasets from human and mouse cells revealed that many heteroduplexes are "non-canonical" i.e. their seed region comprises G:U and bulge combinations (Clark et al., 2014).

CLIP strategies are nowadays popular for high-throughput analysis of physiological miRNA targets (Chi et al., 2009; Hafner et al., 2010; Zisoulis et al., 2010; Leung et al., 2011; Chi et al., 2012; Marin et al., 2012; Clark et al., 2014; Grosswendt et al., 2014; Haecker and Renne, 2014; Liu et al., 2014a; Imig et al., 2015) and it is accompanied with a number of algorithms and databases facilitating identification of miRNA targets in high-throughput CLIP data (Hsieh and Wang, 2011; Yang et al., 2011; Balaga et

al., 2012; Chou et al., 2013; Erhard et al., 2013; Liu et al., 2013; Paraskevopoulou et al., 2013a; Wang et al., 2013; Rennie et al., 2014; Wang et al., 2014; Xie et al., 2014; Bandyopadhyay et al., 2015; Guo et al., 2015; Hsu et al., 2015).

Degradome analysis

It was mentioned above in the section discussing target prediction for plant miRNAs that when small RNA-target RNA interaction results in RNAi-like cleavage, identification of targets can be experimentally augmented by degradome sequencing. This sequencing allows for identification of RNA termini, including those created by RNAi-like cleavage. Thus, if there are mRNAs cleaved by a miRNA after its 10th nucleotide, one would observe alignment of RNA termini matching the predicted miRNA binding site. Degradome analysis usually defines a category of transcripts predicted to be endonucleolytically cleaved and then are cleavage positions compared to predicted miRNA binding sites (Li and Sunkar, 2013; Shao et al., 2013; Xing et al., 2014; Ding et al., 2016; Fan et al., 2016; Wang et al., 2016). Degradome analysis and target prediction has been integrated in to a web resource compARE for plant miRNA target analysis (Kakrana et al., 2014). Degradome analysis can be also used in animals to identify rare miRNA targets suppressed by slicing (Park et al., 2013).

3.2.3. Conclusions – Part II

Given the nature of the interaction between small RNAs and their target RNAs, target prediction will always yield putative targets with partial complementarity. For seed-mediated interactions, there can easily be hundreds of targets predicted for any small RNA acting as a miRNA. This is due to the combination of the following facts: a) a specific hexamer sequence occurs in a random sequence with a theoretical frequency of 1/4096 and b) exons of protein-coding genes constitute 70-80 megabases of well-annotated mammalian genomes (and exome size of eukaryotic genomes might not be dramatically smaller than that). Therefore, a hexamer would occur in a mammalian exonic sequence on average ~20 000x and if 1% of these hexamers would fit other target site prediction criteria, that would leave on average 200 potential binding sites.

The bottom line is that applying a minimal base pairing criterion for miRNA-like interaction will identify a number of potential targets in any eukaryotic organism. At the same time, sequence based target prediction is insufficient to assess whether there will be target repression induced by a specific small RNA when introduced into an animal or mammal because there is a number of other critical parameters, which must be considered.

Two of them stand out above anything else: 1) the amount of the specific small RNA loaded on Argonaute proteins, and 2) target site accessibility. Thus, target assessment of small RNAs needs to address these two parameters. While target accessibility can be considered a relatively common feature for all organisms since the same rules would apply for RNA folding and interference caused by RNA binding proteins (translation machinery etc.), loading of a small RNA onto Argonaute proteins depends on factors which may dramatically differ between different organisms. For example, organisms that exhibit environmental RNAi would be much more prone to the uptake of small RNAs. Factors such as length, chemical modifications, or terminal nucleotides of a small RNA, could underlie differences in sorting and loading to the various Argonaute proteins. Thus, possible fates of a specific small RNA in a specific organism are difficult to predict and should be tested experimentally. The most informative parameter is the amount (number of molecules) of a small RNA in question, which would be loaded on an Argonaute protein (e.g. AGO1 and AGO2 in animals), because it could be compared with known kinetic data to assess the strength of potential repressive effects it could achieve *in vivo*.

The function/effect of a specific small RNA may differ among different organisms unless its target sequences are conserved through evolution; this should be considered when comparing homologs in animals from lineages that branched during the Pre-Cambrian period. Additionally, biological effects of two different small RNAs in one organism would be different because they would have different sets of targets.

To conclude, assessment of the effect of xenogenic small RNAs cannot rely only on bioinformatics prediction strategy based only on sequence homology. Ideally, it should include implementation of the following two strategies:

Determining a safe threshold for environmental concentration of small RNAs. A threshold, below which small RNAs would be considered safe in terms of inducing canonical miRNA and siRNA effects, should reflect (i) known kinetic parameters estimated for small RNAs bound to Argonaute proteins (ii) experimental assessment of efficiency of transfer of environmental small RNAs on Argonaute proteins and (iii) abundance of biologically active small RNA-loaded Argonaute proteins *in vivo*. Defining such a threshold would allow for using measured small RNA concentrations from environmental samples for evaluating their potential effects.

Monitoring effects in vivo in biosensors. Biosensors could be model organisms or cultured cells. As we discussed in the conclusions of part I, *C. elegans* would make an excellent biosensor because it has systemic RNAi and its RNA silencing employs amplification of the response through RNA-dependent RNA polymerases. Importantly, a biosensor will not predict biological effects on other organisms. Biosensor can only reveal that a specific xenogenic small RNA can provoke a biological response in that particular biosensor.

3.2.4. References

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3.3. Part III Small RNA pools in plants and mobility of small RNAs

This part reviews (I) the current methodology used to determine the dsRNA-derived siRNA pool in plant tissues (II) dsRNA-derived siRNA pool in plant tissues and movement of small RNAs in plants, and (III) transfer of small RNAs between species and circulating miRNAs in mammals.

3.3.1. Introduction to small RNA pools

Two distinct types of small RNAs can be recognized when considering their sequence prediction: The first type, exemplified by miRNAs, comprises small RNAs, which occur in cells in many identical copies (i.e. with the same sequence). Biogenesis of miRNAs requires precise sequence-specific positioning of the biogenesis machinery on the miRNA precursor. miRNAs can be annotated. Their biological effect on gene expression does not necessarily require perfect sequence complementarity and miRNA targets are partially predictable as there is typically perfect base pairing at small RNA's 5' end

The second type, exemplified by siRNAs derived from dsRNA, is characterized by existence of populations of small RNAs with variable sequences, which originate from a longer sequence. Although their sequences could be determined, their individual annotation is not very useful. To illustrate this point: one kilobase of dsRNA has the potential to produce nearly thousand small RNAs differing at their 5' ends where each one could have a different seed sequence and regulate a different set of genes. siRNAs usually function as a defense system operating on the basis of (nearly) perfect complementarity.

However, the two types of RNA silencing should not be seen as two entirely separated categories, but rather as two distinct prototypes where specific small RNA categories, recognized in different species may be matching one or the other or fall somewhere in between them (Figure 62).

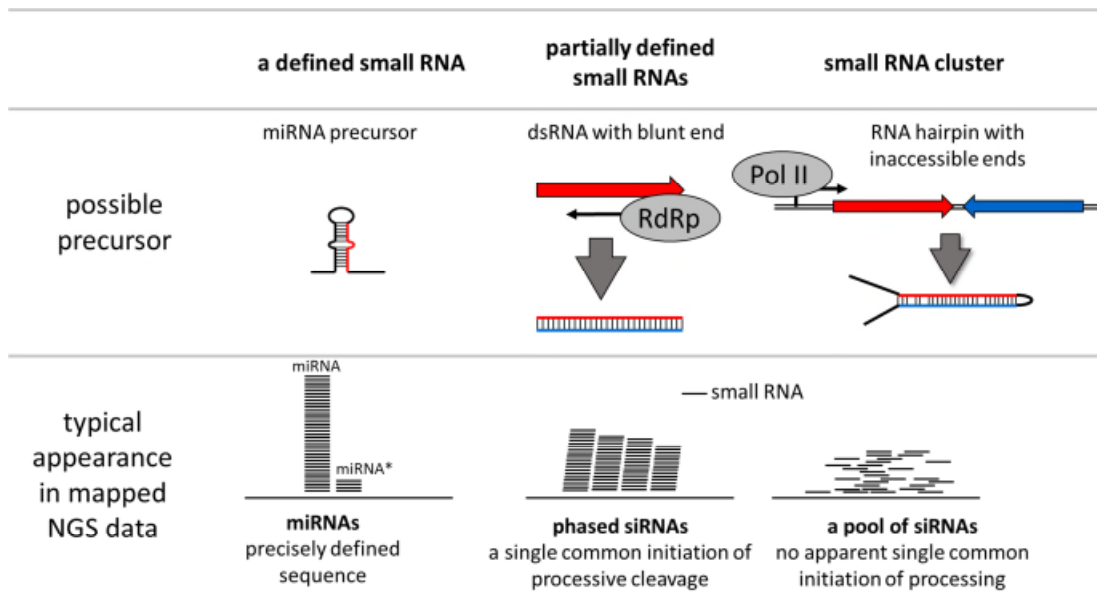


Figure 62 Distinct types of small RNAs differing in occurrence of RNAs with identical sequences

During eukaryotic evolution, a broad range of scenarios evolved in different lineages, which differ in co-occurrence and relationships of the two RNA silencing types. Some taxons, such as mammals, have a relatively simple set-up in the soma where the first type dominates (section 3.1.1.). Other taxons, such as Nematodes (section 3.1.7.), have more complex systems consisting of primary and secondary small RNAs, which are sorted in cells into specific effector complexes based on a number of features. Plants have highly complex small RNA biology (reviewed in the section 3.1.8.). In this section, we will review the analysis of small RNA populations in plants and discuss their mobility within plants and

across species, with a particular focus on small RNA transfer from plants to animals and existence of small RNAs in the mammalian circulation system.

3.3.2. Determining dsRNA-derived siRNA pools in plant tissues

Analysis of the 160 original research articles obtained as described in the section 2.2.5 showed that there are essentially just two basic methods to determine a dsRNA-derived siRNA pool in plants (plant tissues): Northern blotting and next generation sequencing (NGS) (Table 22).

Table 22 Methods used to determine dsRNA-derived siRNA pools in plant tissues

The table is built from method sections of 72 research articles studying small RNA pools in plants during (2005-2016)

reference	method	platform	library preparation	sequencing	doi
Niu, D., et al. (2016)	RNA-Seq	Illumina	standard by Illumina Inc.	Illumina	10.1111/jipb.12446
Kubota, K. and J. C. K. Ng (2016).	northern blot				10.1094/PHYTO-09-15-0219-R
Lewsey, M. G., et al. (2016)	MethylC-Seq, RNA-Seq	Illumina	ScriptSeq RNA-Seq Library Preparation Kit (Epicentre)	Illumina HiSeq 2000 and 2500, 100 cycles	10.1073/pnas.1515072113
Chen, H., et al. (2016)	sRNA-Seq	Illumina	TruSeq™ small RNA sample prep kit (Illumina)	Illumina Miseq platform	10.1371/journal.pone.0150582
Cabrera, J., et al. (2016)	miRNA-Seq	Illumina	standard Illumina at Beijing Genomics Institute (Shenzhen, China)	Illumina HiSeq 2000	10.1111/nph.13735
Zywicki, M., et al. (2015)	RNA-Seq	Illumina	TruSeq SmallRNA Preparation Kit (Illumina)	Genome Analyzer Iix (Illumina), 72 cycles	10.1007/s11738-015-2022-5
Zavallo, D., et al. (2015)	RNA-Seq	Illumina	standard Illumina at LC Sciences, LLC (Houston, Texas, USA)	Illumina HiSeq 2000	10.1371/journal.pone.0134719
Zahid, K., et al. (2015).	RNA-Seq	Illumina	standard Illumina at Beijing Genomics Institute (Shenzhen, China)	Illumina HiSeq 2000	10.1371/journal.pgen.1004906
Yin, F., et al. (2015).	RNA-Seq	Illumina	Illumina gene expression sample preparation kit, Small RNA Sample Prep Kit (Illumina, San Diego, CA, USA)	35 cycles	10.3390/ijms16035714
Srivastava, S., et al. (2015)	ncRNA-Seq	Illumina	J.L. Reyes, C. Arenas-Huertero, R. Sunkar. <i>Methods Mol. Biol.</i> , 639 (2010), pp. 239–251	Illumina Genome Analyzer II, 36 cycles	10.1016/j.plantsci.2015.03.002
Patil, B. L. and C. M. Fauquet (2015)	microarray				10.1111/mpp.12205
Patil, B. L. and C. M. Fauquet (2015).	microarray				10.1007/s11262-015-1184-y
Meyer, R. C., et al. (2015)	RNA-Seq	Illumina	Illumina TruSeq smallRNA sample prep kit	Illumina	10.3389/fpls.2015.00531
Kansal, S., et al. (2015).	RNA-Seq	Illumina	Mutum RD, Balyan SC, Kansal S et al (2013) <i>FEBS J</i> 280:1717–1730	Illumina Genome Analyzer II	10.1007/s00425-015-2279-3

reference	method	platform	library preparation	sequencing	doi
Li, H., et al. (2015).	sRNA-Seq	Illumina	Illumina TruSeq Small RNA Sample Prep Kit (Illumina, Inc., San Diego, CA, USA)	Illumina HiSeq	10.1111/jen.12224
Le, D. T., et al. (2015).	northern blot				10.1080/21645698.2015.1025188
Lam, P., et al. (2015)	RNA-Seq	Illumina	Illumina Tru-Seq Small RNA Sample Prep Kit	Illumina	10.1104/pp.114.252825
Hong, H., et al. (2015).	miRNA-Seq	Illumina	standard Illumina by CapitalBio (Beijing, China)	Illumina Genome Analyzer II, 33 cycles	10.1371/journal.pone.0137360
He, D., et al. (2015)	miRNA-Seq	Illumina	L C, Meyers BC, Green PJ. Methods. 2007; 43: 110–117.	Illumina Genome Analyzer, 23 cycles	10.1371/journal.pone.0145424
Han, Y., et al. (2015).	RNA-Seq	Illumina	Guo Y et al. PLoS One. 2014; 9: e98783.	Illumina HiSeq 2500 (51 cycles)	10.1371/journal.pone.0144909
Garcia-Ruiz, H., et al. (2015)	RNA-Seq	Illumina	Gilbert K, et al. Bioprotocol. 2014; 4(21).	Illumina High Seq 2000	10.1371/journal.ppat.1004755
Chen, S., et al. (2015)	RNA-Seq	Illumina	standard Illumina at Beijing Genomics Institute (Shenzhen, China)	Illumina HiSeq-2000	10.1186/s12985-015-0384-3
Chen, H., et al. (2015).	RNA-Seq	Illumina	TruSeq™ small RNA sample prep kit (Illumina)	Illumina Miseq	10.1016/j.virol.2014.12.034
Almasi, R., et al. (2015)	northern blot				10.1016/j.virusres.2015.06.020
Zhou, G., et al. (2014)	miRNA-Seq	Illumina	standard Solexa sRNA library protocol	Illumina Genome Analyzer, 51 cycles	10.1371/journal.pone.0103041
Xiao, B., et al. (2014).	miRNA-Seq	Illumina	Yang X et al. PLoS One 2011,6(2):e16928. 10.1371/journal.pone.0016928	Illumina Genome Analyzer, 36 cycles	10.1186/1471-2229-14-60
Sohn, S. H., et al. (2014)	RNA-Seq	Illumina	NA	HiSeq 2000, 101 cycles	10.1093/jxb/eru200
Shen, W. X., et al. (2014).	northern blot				10.1016/S2095-3119(13)60525-0
Naveed, K., et al. (2014)	RNA-Seq	Illumina	standard by Beijing Genomics Institute, Hong Kong	NA	10.1016/j.virusres.2014.07.005
Li, Y., et al. (2014)	RNA-Seq	Illumina	standard by Keck center (UIUC)	NA	10.3389/fpls.2014.00156
Dadami, E., et al. (2014)	northern blot				10.4161/rna.29623
Creasey, K. M., et al. (2014).	RNA-Seq	Illumina	Illumina Tru-Seq Small RNA preparation kit	Illumina HiSeq 2000, 50 cycles	10.1038/nature13069
Celton, J. M., et al. (2014).	directional PE RNA-Seq	Illumina	FASTERIS Stranded Protocol (Fasteris, Plan-les-Ouates, Switzerland)	Illumina HiSeq, 2x101 cycles	10.1111/nph.12787

reference	method	platform	library preparation	sequencing	doi
Cao, M., et al. (2014)	RNA-Seq	Illumina	Baumberger N, Baulcombe DC (2005) Proc Natl Acad Sci USA 102(33):11928–11933.	Illumina	10.1073/pnas.1407131111
Shimizu, T., et al. (2013).	northern blot				10.1094/Phyto-07-12-0165-R
Mitter, N., et al. (2013).	RNA-Seq	Illumina	standard by BGI (Americas, Cambridge, MA)	Illumina (50 cycles)	10.1371/journal.pone.0076276
Mittal, D., et al. (2013)	RNA-Seq	Illumina	standard	GAll sequencer (Illumina)	10.1002/jcb.24552
Kallman, T., et al. (2013).	RNA-Seq	Illumina	Illumina Small RNA kit version A	Illumina	10.1104/pp.113.214643
Huang, Y., et al. (2013)	RNA-Seq	Illumina	Mosher, R.A et al. PLoS One 2011, 6, e25756.	Illumina	10.3390/biology2041210
Hu, Z., et al. (2013).	RNA-Seq	Illumina	German, MA, et al. (2008) Nat. Biotech. 26, 941–946.	Illumina Genome Analyzer	10.1111/jipb.12002
Zhang, X. M., et al. (2012).	RNA-Seq	Illumina	standard by BGI (Americas, Cambridge, MA)	Genome Analyzer-II and HiSeq-2000 (Illumina)	10.1186/gb-2012-13-3-r20
Xu, Y., et al. (2012).	RNA-Seq	Illumina	Mi S et al. Cell 133: 116–127.	Illumina HiSeq 2000 (91 cycles)	10.1371/journal.pone.0046238
Velten, J., et al. (2012).	RNA-Seq	SOLiD	The Quant-iT™ RNA assay kit	SOLiD	10.1371/journal.pone.0030141
Tang, S., et al. (2012).	RNA-Seq	Illumina	Small RNA Sample Prep Kit (Illumina)	Illumina GA II	10.1186/1471-2229-12-28
Park, H. M., et al. (2012).	hybridization				10.1007/s10059-012-2185-5
Lu, T., et al. (2012).	ssRNA-seq	Illumina	standard Illumina	Illumina GA IIX (2 × 120 cycles)	10.1186/1471-2164-13-721
Wei, L. Q., et al. (2011)	sRNA-Seq	Illumina	Illumina TruSeq Small RNA (sRNA) preparation kit	1G Genome Analyzer (Illumina)	10.1186/gb-2011-12-6-r53
Wang, H., et al. (2011).	smRNA-Seq	Illumina	standard Illumina	Illumina Genome Analyzer II	10.1111/j.1365-313X.2011.04594.x
de Felippes, F. F., et al. (2011).	RNA-Seq	Illumina	Mosher, R.A. et al. (2009) Nature, 460, 283–286.		10.1093/nar/gkq1240
Sun, Z. N., et al. (2010)	Northern Blotting				10.1007/s12010-010-8968-2
MacLean, D., et al. (2010)	sRNA-Seq	Illumina	small RNA sequencing protocol	Illumina	10.1371/journal.pone.0009901
Chen, D., et al. (2010).	in-silico				10.1093/bioinformatics/btq150

reference	method	platform	library preparation	sequencing	doi
Boccaro, M., et al. (2010)	microarray	Affymetrix			10.1007/978-1-60761-646-7_8
Zhou, X., et al. (2009).	transcriptome sequencing	454	standard 454 by 454 Life Sciences	454 GS	10.1101/gr.084806.108
Wang, X. F., et al. (2009).	mRNASeq	Illumina	standard Illumina	Illumina Genome Analyzer	10.1105/tpc.109.065714
Qi, X., et al. (2009).	sRNA-Seq	Illumina	Kasschau KD et al. (2007) PLoS Biol 5: e57.	Illumina Genome Analyzer II	10.1371/journal.pone.0004971
Jia, Y., et al. (2009).	RNA-Seq	Illumina	Ohtsu K et al. (2007) Plant J 52: 391–404.	Solexa 1G Genome Analyzer	10.1371/journal.pgen.1000737
Ha, M., et al. (2009)	siRNA-Seq, microarray	454, Affymetrix	Kasschau KD, et al. (2007) PLoS Biol 5:e57.	454 GS	10.1073/pnas.0907003106
Grant-Downton, R., et al. (2009)	RNA-Seq	454	standard 454 by Eurofins MWG (Ebersberg, Germany)	454 Life Sciences	10.1186/1471-2164-10-643
Elling, A. A. and X. W. Deng (2009)	in-silico				10.1105/tpc.109.065714
Zhu, Q. H., et al. (2008)	RNA-Seq	454	standard 454	454 GS	10.1101/gr.075572.107
Vogler, H., et al. (2008).	northern blot				10.1371/journal.ppat.1000038
Nobuta, K., et al. (2008)	sRNA-Seq	Illumina	Lu C et al.(2007) Methods 43:110–117.	Illumina GS	10.1073/pnas.0808066105
Mlotshwa, S., et al. (2008)	northern blot				10.1371/journal.pone.0001755
Heisel, S. E., et al. (2008)	RNA-Seq	454	Llave C et al. (2002) Plant Cell 14: 1605–19.	454 GS	10.1371/journal.pone.0002871
Brodersen, P., et al. (2008).	northern blot				10.1126/science.1159151
Nobuta, K., et al. (2007)	MPSS	MPSS	Lu, C. et al. Science 309, 1567-1569 (2005)	MPSS	10.1038/nbt1291
Kasschau, K. D., et al. (2007).	sRNA-Seq	454	Llave C et al. (2002) Plant Cell 14: 1605–19.	454 GS	10.1371/journal.pbio.0050057
Hournard, N. M., et al. (2007)	sRNA-Seq	454	Llave, C. et al. (2002) Plant Cell, 14, 1605–1619.	454 GS	10.1111/j.1467-7652.2007.00265.x
Lu, C., et al. (2006)	sRNA-Seq	454	Lu, C. et al. Science 309, 1567-1569 (2005)	454 GS	10.1101/gr.5530106
Molnar, A., et al. (2005).	Sanger	Sanger	cloning and sanger sequencing	Sanger	10.1128/JVI.79.12.7812-7818.2005
Chellappan, P., et al. (2005)	northern blot				10.1104/pp.105.066563

3.3.2.1 Small RNA analysis by Northern blotting

Northern blot analysis of small RNAs (for an example of a small RNA Northern blot protocol see (Rio, 2014)) is common in the older RNA silencing literature but it is still being used, especially when a specific siRNA pool is being analyzed (e.g. transgene-derived siRNAs). It is also a common approach for detecting miRNAs.⁹ However, small RNA Northern blot analysis has several disadvantages:

- It identifies only small RNAs complementary to the used hybridization probe. Thus, one lacks information about other small RNAs.
- Northern blot reveals lengths of small RNAs complementary to the hybridization probe but does not reveal variability of their sequences.
- Small RNA Northern blot analysis is a labor-intensive, low throughput method, which requires work with radioactivity to achieve the maximum sensitivity. In addition, the subsequent quantitative analysis of siRNA pool dynamics is not trivial.
- A small RNA Northern blot requires a considerable amount of material and is less sensitive than NGS.

3.3.2.2 Next generation sequencing of small RNAs

NGS of small RNAs revolutionized RNA analysis. Before NGS, small RNAs were cloned and sequenced using the classical Sanger sequencing. This was a labor-intensive approach suitable for analysis of microRNAs but not for studying populations of small RNAs. A classic example of such a small RNA analysis is the landmark resource paper by Landgraf et al. (Landgraf et al., 2007), which was based on >330 000 independent small RNA sequences from 256 libraries where each library was covered by ~1300 clones and contains ~65% of miRNA sequences. However, this analysis was too costly and too labor-intensive for thorough analyses of small RNA populations in different cell types. These issues were resolved with introduction of NGS, which offers a high throughput analysis (typically 10^6 - 10^8 sequence reads/sample). However, it should be noted that the cost of NGS analyses might be a limiting factor when a large number of samples needs to be analyzed. In any case, NGS is a good technique to analyze small RNAs in a sample. In contrast to Northern blotting, NGS reveals the composition of the entire small RNA population in a model system/tissue/cell of interest. It requires less material than Northern blotting and the benchwork part of the analysis is much simpler thanks to available kits for preparing sequencing libraries for NGS. Furthermore, some sequencing services offer analysis of isolated RNA. However, NGS analysis has also several caveats:

- NGS is demanding for hardware in terms of data processing and storage. Accurate data analysis requires expertise in bioinformatics.
- Although the cost of sequencing is constantly going down, the price of the analysis needs to be taken into consideration (see Appendix D for pricing in January 2017). When detecting a particular small RNA population derived from one sequence in a number of samples, a Northern blot analysis may be a cost-effective alternative.
- Small RNA abundance might be distorted during library preparation resulting in artifacts (Linsen et al., 2009; Hafner et al., 2011). Thus, an NGS experiment should be carefully designed including external controls (spiking). One should ideally, complement the analysis with Northern blot or qPCR analyses whenever possible.

For NGS, there are several different NGS platforms available (for a comparison, see for example (Li et al., 2014)). A literature survey (Table 22) showed that different Illumina sequencing platforms account for the vast majority of NGS analyses of plant small RNAs. Because of the constantly changing landscape of NGS, comments made on sequencing platforms used in the past may become obsolete fast. Thus, one needs to survey presently available options when considering NGS analysis.

⁹ for miRNA and small RNA Northern blot protocols online, see, for example the following websites:

http://bartellab.wi.mit.edu/protocols/smallRNA_northern.pdf, http://www.narrykim.org/legacy/Northern_blot_analysis_for_microRNA.pdf

For normalization purposes, spiking controls should be included whenever possible because having an external normalization standard is invaluable for data interpretation. The desired sequencing depth depends on the desired sensitivity. For example, 10^5 sequencing reads could be enough for the analysis of miRNAs in a homogeneous population of cells while detection of a small siRNA pool in a small fraction of cells of a tissue may require depth over ten million reads. At the same time, increased NGS depth also increases depth of the background formed of degradation fragments and stochastically generated small RNAs without a biological role. Distinguishing then between the biologically relevant signal and non-functional noise is a major challenge where bioinformatics offers only limited means and has to be complemented with carefully designed experimental validation strategies. Additional information on NGS of small RNA services can be found, for example, here:

<https://www.scienceexchange.com/services/small-rna-sequencing>

An important general consideration for NGS analysis of small RNAs is accurate data analysis. There is a number of bioinformatics tools for NGS data analysis, including identification of siRNA loci (Hardcastle et al., 2012). It is important to establish functional data management and bioinformatics analysis. NGS providers typically offer data analysis (usually for extra charge), which should satisfy common needs for simple differential expression analysis. A recommended solution, however, is to obtain own local bioinformatics support.

An interesting variation of NGS analysis represents detection of small RNAs bound to specific Argonaute proteins. In such a case, small RNAs are not directly isolated from the tissue of interest but they are immunoprecipitated together with the Argonaute protein of interest. While this strategy is laborious and requires a good antibody, it allows for identifying small RNAs loaded on specific Argonautes. This has been exemplified by the study of miRNA loading on Argonautes in *Arabidopsis* (Jeong et al., 2013) or by a study of small RNAs bound by *Arabidopsis* AGO1 and AGO4 (Wang et al., 2011). Furthermore, this strategy would be very useful for analyzing the molecular mechanism of activity of xenogenic miRNAs.

3.3.2.3 Analysis of small RNA by microarrays

While small RNA profiling by microarrays is a common strategy for miRNA analysis (reviewed in (Svoboda, 2015)), it is a marginal approach for dsRNA-derived siRNA pools. Since miRNAs have well-defined sequences, it is possible to optimize specific microarray probes for optimal hybridization. For siRNAs, one cannot design specific probes for individual siRNAs in the pool. Instead, the probe needs to correspond to a genomic region/sequence, from which those siRNAs are derived. Our literature search identified one report, which employed a custom-made DNA tiling array covering *Arabidopsis* chromosome 4 and showed that hybridization signals along chromosome 4 were in good agreement with small RNA abundance determined by NGS (Boccaro et al., 2010). However, microarray analysis does not seem to offer any significant advantage over NGS, which is much more informative about the composition of a siRNA pool in terms of small RNA lengths and specific sequences.

3.3.3. Features of dsRNA-derived siRNA pools in plant tissues

3.3.3.1 Plant anatomy and features relevant for movement of molecules

Plants are unique in several aspects when compared to cells of other eukaryotic organisms. Plant cell features that are important for intercellular exchange of molecules include a polysaccharide cell wall and plasmodesmata. The latter are microscopic channels traversing cell walls of plant cells allowing for movement (symplastic movement or symplast) of molecules between adjacent cells (reviewed in (Maule, 2008; Maule et al., 2011)). An alternative to the symplastic movement, which involves the cytoplasm, is apoplastic movement (apoplast) where molecules move through cell walls and intercellular space.

A flowering plant (Figure 63) consists of three anatomical systems – root, shoot, and inflorescence. It is anchored by a root, absorbs water and minerals from the grounds and transports them through the

xylem vasculature through the plant and into leaves, which are the main photosynthetic organs. Sugars produced in leaves are transported around through the phloem vasculature.

Structurally, plant tissues are divided into meristems (containing actively dividing cells, thus being the primary place of plant growth) and permanent tissues classified according to their shapes and intercellular space.

Long distance movement of molecules occurs through the aforementioned vascular system, which transports water, mineral, signaling molecules, nutrients, and other molecules. In particular, phloem is the main avenue for transporting organic molecules (discussed in (Turgeon and Wolf, 2009; Atkins et al., 2011; De Schepper et al., 2013)). There is a large number of mobile macromolecules in plants. In an effort to provide a systematic catalogue of mobile macromolecules, a database PlaMoM was compiled, which provides convenient and interactive search tools allowing users to retrieve, to analyze and also to predict mobile RNAs/proteins (Guan et al., 2017). The current version compiles a total of 17,991 mobile macromolecules from 14 plant species/ecotypes and is available at:

<http://www.systembioinfo.org/plamom/>

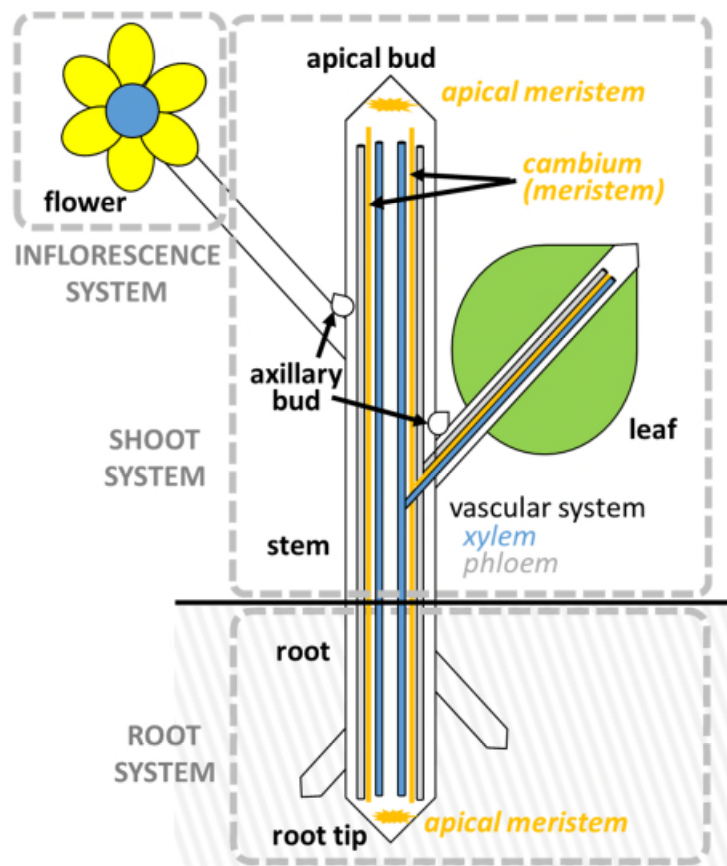


Figure 63 A schematic overview of plant body organization.

3.3.3.2 Distribution of RNA silencing pathways across plant tissues

Plants express multiple AGOs, DCLs and RDRs supporting different types of RNA silencing pathways (reviewed in the section 3.1.8). miRNA and related post-transcriptional pathways (section 3.1.8.7.) utilize genome-encoded substrates and include the canonical pathway (employing DCL1 and AGO1-dependent 21 nt miRNAs) and pathways employing longer hairpin substrates and other DCL and AGO proteins. siRNA pathways use for silencing either exogenous substrates (antiviral defense and transgene silencing) or various genome-derived dsRNAs derived from genomic sequences (summarized in sections 3.1.8.8 and 3.1.8.9). Various biogenesis pathways produce primary and secondary siRNAs of various types and lengths (21, 22, or 24 nt).

There is a large volume of literature concerning NGS analysis of small RNA populations from different tissues in different plant species. A large volume of NGS data can be accessed through Plant MPSS (massively parallel signature sequencing) databases website (<https://mpss.danforthcenter.org/>), which was introduced in 2005 (Nakano et al., 2006) and has been continuously updated and expanded. It currently contains small RNA data from 19 plant species. Most datasets in the database (six) come from analysis of small RNAs in rice:

https://mpss.danforthcenter.org/dbs/index.php?SITE=rice_sRNA (unpublished)

https://mpss.danforthcenter.org/dbs/index.php?SITE=rice_sRNA2 (Jeong et al., 2011)

https://mpss.danforthcenter.org/dbs/index.php?SITE=rice_sRNA3 (unpublished)

https://mpss.danforthcenter.org/dbs/index.php?SITE=rice_sRNA4 (Fei et al., 2016)

https://mpss.danforthcenter.org/dbs/index.php?SITE=rice_glab_sRNA (OMAP project: www.omap.org/)

https://mpss.danforthcenter.org/rice/mpss_index.php (Nobuta et al., 2007)

These rice datasets combine the expression atlas of rice mRNAs and small RNAs (Nobuta et al., 2007), with analysis of small RNAs during development where different pools of phasiRNAs were identified (Fei et al., 2016). A comprehensive analysis of small RNAs in different tissues under normal conditions and stress (Jeong et al., 2011) and unpublished NGS data from different rice tissues and AGO immunoprecipitates can also be found in these databases.

These and other data from rice (e.g. (Heisel et al., 2008)) reveal common and tissue-specific populations of small RNAs. The existence of tissue-specific small RNAs shows that systemic RNA silencing co-exists with RNA silencing mechanisms restricted to specific tissues. Several factors may underlie tissue specificity of particular small RNA mechanisms. These include (i) tissue-restricted expression of proteins involved in biogenesis and activity of particular small RNA class, (ii) tissue-restricted presence of substrates for biogenesis of small RNAs, and (iii) restriction of mobility/spreading of small RNAs, which could differ between different cell types.

In the next paragraphs, we will briefly discuss selected specific examples, which provide an insight into the complexity of the issue:

Tissue-restricted expression of proteins acting in small RNA biogenesis and activity

While many genes encoding factors involved in small RNA biogenesis (e.g. Dicers, RDRs) and function (e.g. Argonautes) of small RNAs are ubiquitously expressed, some exhibit preferential or tissue restricted expression. For example, a systematic analysis of gene expression was done in *Arabidopsis* and rice (Kapoor et al., 2008), where microarray profiling identified several differentially expressed genes encoding RNA silencing factors during development and in different tissues (Figure 64). Similar data could also be extracted from available NGS profiling of mRNAs at the aforementioned website (<https://mpss.danforthcenter.org/>). In general, these expression data have predictive value mainly for specific Argonaute paralogs, which host unique small RNA pools and have highly restricted expression patterns.

Tissue-restricted presence of substrates for small RNAs

This factor involves localization of exogenous small RNA substrates to different tissues (i.e. viral infections and transgene expression) or expression of endogenous small RNA substrates. This allows, for example to differentiate expression of subpopulations of small RNAs in time and space. A classic example is tissue-specific expression miRNAs, whose precursors are transcribed by polII polymerase and thus can exhibit tissue-restricted expression as mRNAs. As there is a large volume of the literature on miRNA expression in plants, which is beyond the scope of this report, we only select illustrative examples of analyses of tissue-specific expression of miRNAs in rice (Zhu et al., 2008; Mittal et al., 2013), *Nicotiana* (Valoczi et al., 2006), and *Arabidopsis* (Valoczi et al., 2006; Grant-Downton et al., 2009). Tissue-specific expression of small RNAs can be observed for tasiRNAs, which originate from specific loci (Marin et al., 2010; Zabala et al., 2012) or phasiRNA, where a specific miRNA initiates production of phasiRNAs (Fei et al., 2016).

Restriction of spreading of small RNAs in specific cell types

Restriction of mobility assures that tissue/cell-specific expression will remain contained. There are several cell types, from which small RNAs do not seem to be spreading around. These include guard cells of stomata (Voinnet et al., 1998), endosperm (energy storage of the seed) (Hournard et al., 2007) or the seed coat (Tuteja et al., 2009).

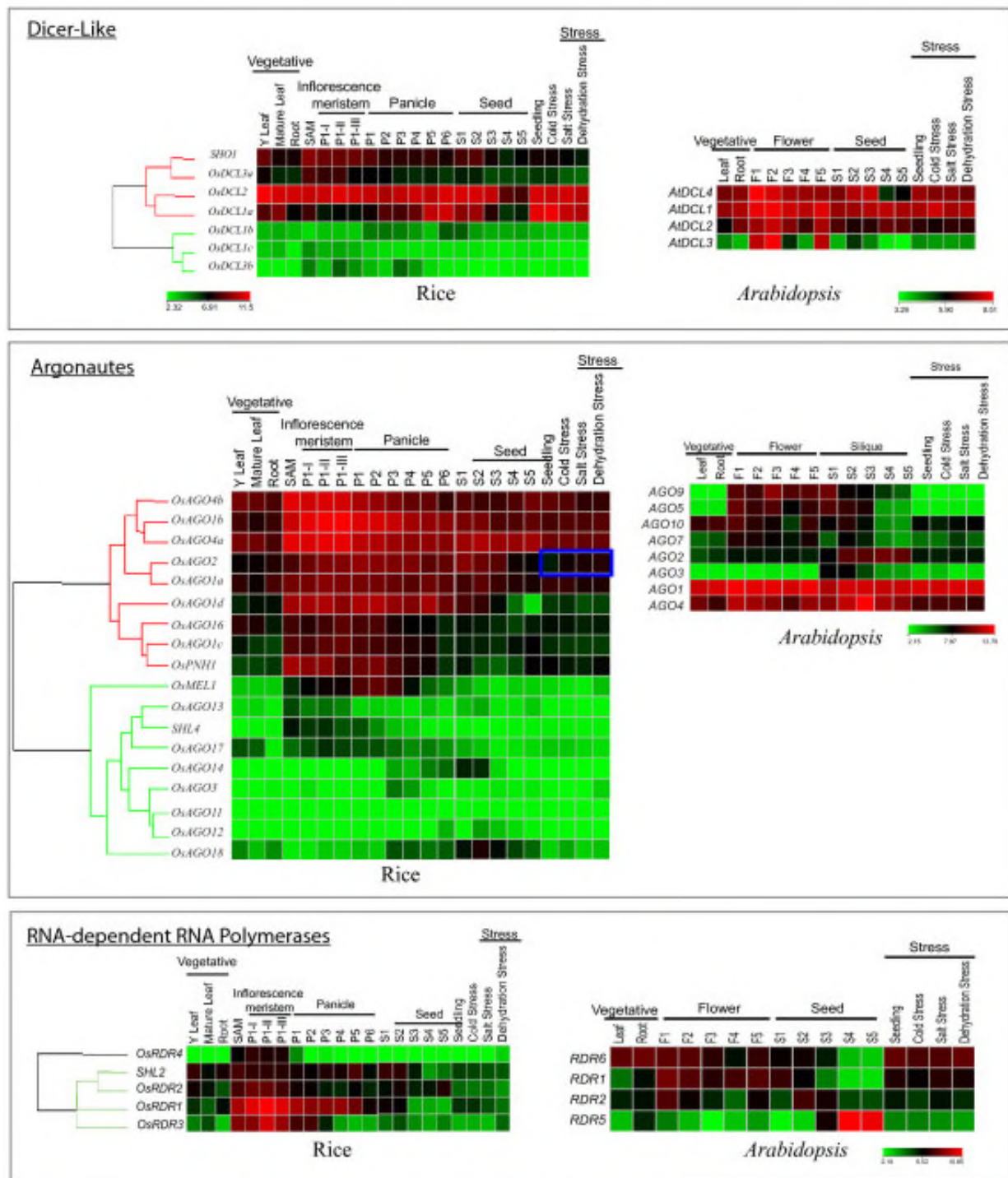


Figure 64 Dicer-like, Argonaute and RDR expression in rice and *Arabidopsis*.

Microarray expression profiles of rice genes were analyzed in vegetative tissues (Y leaf, mature leaf, roots), Shoot Apical Meristem (SAM) and six stages of panicle development (P1 to P6) along with 3 substages of P1 i.e. P1-I, P1-II and P1-III, five stages of seed development (S1 to S5) and under three abiotic stress conditions (cold, salt and dehydration). *Arabidopsis*, samples included two vegetative stages (leaf and root), five stages of flower development (F1–F5), five stages of silique development and three stress treatments (cold, salt and dehydration) have been compiled. The color bar in each panel represents log₂ expression values. Developmental stages used for expression profiling are shown on top of each column. Expression of OsAGO2 that shows two-fold up-regulation in response to all three stresses is boxed in blue in the middle panel. The figure was adopted from Kapoor et al., 2008 (Kapoor et al., 2008).

3.3.3.3 Movement of small RNAs between cells and tissues – systemic RNAi in plants

As mentioned in the introduction (section 1.4.3.1.), RNAi can either act in a cell autonomous manner, i.e. affecting only cells directly exposed to dsRNA, or can propagate across cell boundaries (Figure 65). Early observations of co-suppression in petunia (Napoli et al., 1990; Jorgensen, 1995) and antiviral resistance in *Nicotiana benthamiana* (Ratcliff et al., 1997) suggested that RNA silencing in plants includes a mobile silencing signal. Several studies subsequently confirmed that transgene-induced silencing is mobile (Palauqui et al., 1997; Voinnet and Baulcombe, 1997; Voinnet et al., 1998, 2016). These experiments used grafting and agroinfiltration strategies to show that silencing spreads from the lower silenced leaves to the upper non-silenced leaves (Palauqui et al., 1997; Voinnet and Baulcombe, 1997). Based on the distance of silencing spread, short range and long range/systemic spread can be distinguished (Figure 65).

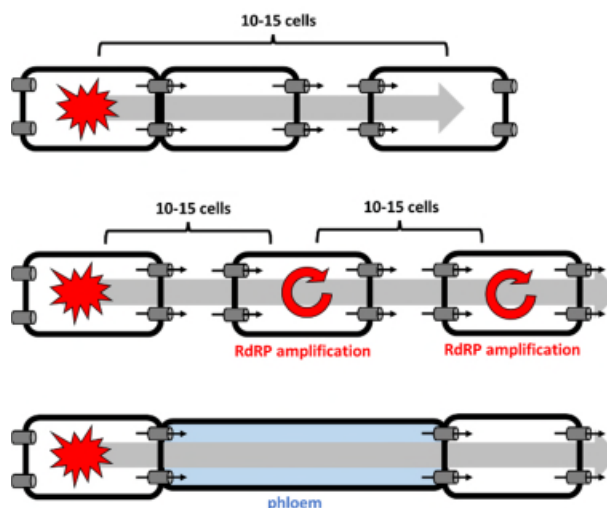


Figure 65 Movement of RNA silencing in plants.

In short-distance cell-to-cell movement, small RNAs move from the source cells where silencing was initiated (explosion pictogram) through plasmodesmata (symplastic route). Plasmodesmata are schematically depicted as tunnels in cell walls. Mobility is limited to 10–15 cells. In long-range cell–cell movement, small RNA move again via plasmodesmata. Amplification (cycling arrow) of small RNAs by transitive small RNA production utilizing an RdRP activity extends the range of mobility far beyond the first 10–15 cells. In systemic movement, small RNAs enter the phloem and are transported to distant tissues

Short range silencing

Short range silencing manifests as RNA silencing in a defined area. It was shown that a short-distance spreading of RNA silencing, once initiated from a small group of cells, can extend over 10–15 cells independently of the presence of cognate transcripts (Himber et al., 2003). Short range silencing was observed for both transgenes and endogenous genes (Ryabov et al., 2004; Schwach et al., 2005; Kalantidis et al., 2006; Dunoyer et al., 2007; Smith et al., 2007).

Regarding the nature of the short-range mobile signal, it is still a matter of debate. Initially, it was proposed that short range spreading involves DCL3-dependent 24 nt siRNAs (Hamilton et al., 2002; Molnar et al., 2010). Subsequently, DCL4-dependent 21 nt siRNA were also implicated in short-range spreading (Himber et al., 2003; Dunoyer et al., 2005; Dunoyer et al., 2007; Smith et al., 2007). It was shown that they are sufficient for the limited moving of the silencing and that the short range silencing is independent of an RdRP SDE1 and helicase SDE3 (Himber et al., 2003). Furthermore, it was suggested that the mobile signal are siRNAs themselves and not their longer precursors or AGO-bound single strand molecules. However, this evidence was first provided in one of the recently retracted papers (discussed in the section 3.3.3.5.) and should be interpreted with caution.

Besides 21 siRNAs, several other types of plant small RNAs can spread from cell-to-cell. These include miRNAs (discussed separately further below), tasiRNAs of the TAS3 locus (Chitwood et al., 2009; Schwab et al., 2009; Marin et al., 2010; de Felippes et al., 2011).

In terms of the mechanism of cell-to-cell silencing movement, early studies implied that post-transcriptional silencing spreads through plasmodesmata (Palauqui et al., 1997; Voinnet et al., 1998). However, the amount of evidence for cell-to-cell silencing movement through plasmodesmata is rather moderate. Support for the symplastic movement through plasmodesmata comes from an observation

that symplastically isolated guard cells of stomata escape short-range spreading (Voinnet et al., 1998; Himber et al., 2003; Kalantidis et al., 2006). Furthermore, analysis of short-range spreading in *Arabidopsis* embryos, suggested that the spread is affected by and positively correlates with plasmodesma aperture (Kobayashi and Zambryski, 2007). While these results are in agreement with the assumption that short-range spreading of silencing occurs through diffusion and plasmodesmata, alternative routes, such as secretory vesicles (which were reported from animals, see section 3.1.1.11) should still not be excluded.

In addition, genetic analysis revealed several factors involved for cell-to-cell silencing movement (summarized in Table 24). Some of these factors were already mentioned above. Interestingly, among the factors whose mutations reduce cell-to-cell silencing movements were also RDR2, CLSY1, and NRPD1a, which are required for 24 nt siRNA-mediated transcriptional silencing (Dunoyer et al., 2007; Smith et al., 2007). It was suggested that these factors might function in reception and/or downstream functional integration of mobile siRNAs in recipient cells (Brosnan and Voinnet, 2011). Another nuclear factor whose mutation affects cell-to-cell silencing movement is JM14, a H3K4 histone demethylase implicated in non-CpG DNA methylation (Searle et al., 2010). However, short-range movement of tasiRNAs does not seem to require RDR2, CLSY1, NRPD1a, and JM14.

Taken together, while there is evidence that DCL4-dependent 21 nt siRNAs are the mobile signal in cell-to-cell movement, cell-to-cell silencing mobility might also concern DCL3-dependent 24 nt siRNAs and other RNA molecules.

Extended short-range silencing

In addition to short-range spreading of RNA silencing, a long-range cell-to-cell movement process was also reported that occurs as a relay amplification, involving the combined activity of an RdRP SDE1 and helicase SDE3 (Himber et al., 2003) (Figure 65). This is essentially an extended version of short-range silencing described above where the mobile signal is amplified along the way through production of secondary small RNAs. This amplification process, is known as “transitivity” (Melnik et al., 2011b). Thus, extensive and limited cell-to-cell movements of silencing are triggered by the same molecules, occur within the same tissues and likely recruit the same plasmodesmata channels (Himber et al., 2003).

Long range/systemic silencing

Plants exhibit systemic RNA silencing, which can move over long distance. This implies that a mobile silencing signal from the source tissue enters the vascular system, it is transported, and then enters the recipient tissue or cells (Figure 66). Early studies implied that post-transcriptional silencing spreads over long distance through phloem (Palauqui et al., 1997; Voinnet et al., 1998) (of note is that xylem sap, which transports water and ions, does not carry RNA (Buhtz et al., 2008)).

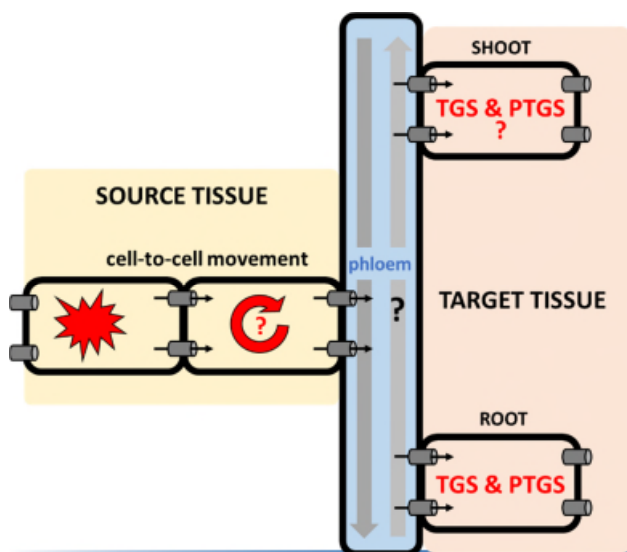


Figure 66 Systemic silencing mobility

Systemic silencing is initiated in the source tissue. The mobile silencing signal enters phloem and moves to the target tissue. Systemic silencing can occur in both directions, i.e. shoot-to-root and root-to-shoot. The shoot-to-root direction is following the common path of sugars to “sink” tissues. However, there is also evidence that systemic silencing can propagate in root-to-shoot direction.

Table 23 Mutations that affect the cell-to-cell RNA silencing spreading phenotype in *Arabidopsis* adopted from (Melnyk et al., 2011b)

Mutations that affect the cell-to-cell RNA silencing spreading phenotype in <i>Arabidopsis</i>				
Mutations that reduce RNA silencing spread				
mutation	type	gene	note	reference
<i>ago1</i>	<i>inhib.</i>	AGO1	Argonaute, 21 nt siRNA pathway	(Dunoyer et al., 2007)
<i>clsy1</i>	<i>inhib.</i>	CLSY1	SNF2-domain-containing protein	(Smith et al., 2007)
<i>dcl1</i>	<i>inhib.</i>	DCL1	Dicer, miRNA & 21 nt siRNA pathway	(Dunoyer et al., 2007)
<i>dcl4</i>	<i>inhib.</i>	DCL4	Dicer, 21 nt siRNA pathway	(Dunoyer et al., 2005; Smith et al., 2007)
<i>fca</i>	<i>inhib.</i>	FCA	flowering control, RNA binding domain, interacts with FY	(Baurle et al., 2007)
<i>fpa</i>	<i>inhib.</i>	FPA	flowering control	(Baurle et al., 2007)
<i>fy</i>	<i>inhib.</i>	FY	flowering control, RNA 3' end-processing factor	(Manzano et al., 2009)
<i>jmj14</i>	<i>inhib.</i>	JMJ14	histone H3K4 demethylase linked to non-CpG methylation	(Searle et al., 2010)
<i>hen1</i>	<i>inhib.</i>	HEN1	small RNA methylase	(Dunoyer et al., 2007)
<i>hpr1</i>	<i>inhib.</i>	HPR1	RNA trafficking protein	(Jauvion et al., 2010)
<i>nRPD1/smd1</i>	<i>inhib.</i>	NRPD1	plant RNA pol-IV component	(Dunoyer et al., 2005; Brosnan et al., 2007; Dunoyer et al., 2007; Smith et al., 2007)
<i>nRPD2a</i>	<i>inhib.</i>	NRPD2A	plant RNA pol-IV component	(Smith et al., 2007)
<i>rdr2/smd2</i>	<i>inhib.</i>	RDR2	plant RNA pol-IV component	(Dunoyer et al., 2005; Brosnan et al., 2007; Dunoyer et al., 2007; Smith et al., 2007)
<i>smd3</i>	<i>inhib.</i>	SMD3	silencing movement deficient	(Dunoyer et al., 2005)
<i>tex1</i>	<i>stim</i>	TEX1	mRNA export complex	(Yelina et al., 2010)
<i>ago4</i>	<i>stim</i>	AGO4	Argonaute,	(Smith et al., 2007; Searle et al., 2010)
	<i>inhib</i>		partially compromised transmission of silencing	(Brosnan et al., 2007)
<i>dcl3^b</i>	<i>stim</i>	DCL3	Dicer	(Smith et al., 2007)
	<i>inhib</i>		Dicer	(Brosnan et al., 2007)

It was shown that transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions (Palauqui et al., 1997). The transmission of co-suppression occurs when silenced stocks and non-silenced target scions are physically separated by up to 30 cm of stem lacking cognate RNAs (Palauqui et al., 1997). The systemic movement of the silencing signal takes days and typically moves from leaves (photosynthetic source) to roots and growing points (sucrose sinks) (Voinnet et al., 1998); this flow is characteristic of phloem (discussed in (Turgeon and Wolf, 2009; De Schepper et al., 2013)). Furthermore, phloem transport blocked by cadmium inhibits systemic silencing spread (Beclin et al., 1998; Ghoshroy et al., 1998). Later, it was also shown that phloem flow strongly influences the systemic spread of silencing in *Nicotiana benthamiana* and that the direction of systemic spread of silencing from inducer to sensor can be manipulated by altering sink/source relations in the plant (Tournier et al., 2006). Thus, systemic silencing is not an accidental

stochastic process, but can be predicted according to the phloem flow direction (Tournier et al., 2006).

The systemic mobile signal could be various forms of RNA – small RNAs or their precursors. Identification of the systemic RNA silencing signal included analyses of the phloem sap and RNA behavior upon various grafting experiments

Analysis of phloem sap found presence of siRNAs and miRNAs (Yoo et al., 2004; Buhtz et al., 2008). Subsequent grafting experiments suggested that all classes of small RNAs could be mobile while the systemic silencing signal was proposed to be the 24 nt siRNA species (Himber et al., 2003; Molnar et al., 2010; Melnyk et al., 2011a). However, grafting experiment with rootstocks lacking DCL2, 3, and 4 showed that such rootstocks still transmit the systemic silencing signal (Brosnan et al., 2007). This suggests that the silencing signal could be either long dsRNA precursors or siRNAs generated by DCL1, which normally generates miRNAs. Mobility of small RNAs was supported by NGS analysis of tissues lacking DCL2, 3, and 4, which could identify long dsRNA-derived 21, 22, and 24 nt siRNAs (Molnar et al., 2010). Furthermore, the situation is complicated by secondary siRNAs, which emerge in the systemically silenced scion and degrade the target mRNA (Shimamura et al., 2007).

In any case, it is generally accepted that 24 nt siRNA species are associated with systemic RNA silencing in plants but little is known concerning the exact form of systematically moving silencing RNAs. Since phloem transport can accommodate relatively large molecules including mRNAs (reviewed in (Atkins et al., 2011; Hannapel et al., 2013)) mobility of small RNA precursors should not be excluded.

Protein factors involved in systemic RNA silencing

Consistent with involvement of 24 nt siRNAs, three genes involved in a chromatin silencing pathway: NRPD1a, RDR2, and DCL3, are required for reception of long-distance mRNA silencing and an AGO4 mutant is also partially compromised in the reception of silencing (Brosnan et al., 2007). These data were corroborated by another study, which linked CLSY1, AGO4, RDR2, and NRPD1a to silencing spread (Smith et al., 2007). DCL3 appears to be the only DCL protein essential for systemic silencing (Melnyk et al., 2011a). In addition, RDR6 was implicated in systemic silencing where it seems to be involved in the perception of the silencing signal (Schwach et al., 2005) and its maintenance (Vaistij et al., 2002; Himber et al., 2003). RDR6 is amplifying decapped mRNAs cleaved by 24 nt siRNAs. Subsequently, DCL4 and DCL2 act hierarchically to produce 21- and 22 nt siRNAs guiding mRNA cleavage mRNA degradation (Brosnan et al., 2007).

Interestingly, biochemical analysis of pumpkin (*Cucurbita maxima*) identified CmPSRP1 (PHLOEM SMALL RNA BINDING PROTEIN 1), which binds 25 nt single-strand small RNAs in pumpkin and is a candidate protein for trafficking small single-strand RNA molecules (Yoo et al., 2004). Although there is additional supporting biochemical evidence for the role of CmPSRP1 in systemic silencing, there is no genetic support and its homologs across the plant kingdom are yet to be identified (Ham et al., 2014).

Taken together, it seems that the systemic silencing employs both, post-transcriptional and transcriptional RNA silencing mechanisms. However, their integration in the systemic silencing process is still a matter of debate.

Directionality of systemic RNA silencing

Systemic silencing frequently flows in the common source-to-sink direction. This, however, does not preclude spreading in the opposite direction, which has also been observed. Below, we will discuss directionality of spreading between main plant parts.

Shoot-to-root

Depletion of shoot small RNAs corresponded to reduction of 24 nt small RNAs in wild-type roots, indicating that shoot-derived small RNAs contribute to the total root small RNA population as small RNAs in these grafted plants move predominantly from shoot to root following source–sink gradients (Molnar et al., 2010). A later study showed in *Arabidopsis thaliana* that 24 nt small RNAs are mobile from shoot to root (Melnyk et al., 2011b). This was further extended by showing that RNA-dependent DNA methylation in root tissues (occurring predominantly in non-CpG contexts) depends on mobile small RNAs from the shoot (Lewsey et al., 2016).

Root-to-shoot

Upward (root-to-shoot) systemic silencing has also been reported from both *Nicotiana* and *Arabidopsis* (Kasai et al., 2011; Liang et al., 2012; Kasai et al., 2013). For example, post-transcriptional silencing of an endogenous gene in the shoot was observed in *Nicotiana* when a wild-type scion was grafted on a rootstock synthesizing a siRNA signal (Kasai et al., 2011). The signal can spread from a rootstock can travel quite far as demonstrated in cherry, where siRNAs were detected 1.2 m from the graft union (Zhao and Song, 2014).

To what extent this type of spreading employs phloem is not clear. As mentioned above, while phloem flow influences the direction of systemic spread, it can be manipulated (Tournier et al., 2006). However, a study in *Arabidopsis* suggested that root-to-shoot signal movement might occur through plasmodesmata and not through the phloem (Liang et al., 2012).

Importantly, root-to-shoot spreading of RNA silencing is not a universal plant mechanism *in vivo*. It was shown that by RNA-mediated gene silencing signals are not graft transmissible from the rootstock to the scion in greenhouse-grown apple plants *Malus* sp. (Flachowsky et al., 2012). Further research is needed to reveal the molecular mechanisms and biological role(s) of the root-to-shoot (rootstock–scion) transmission of RNA silencing.

Flower/seed concerning silencing spreading

Some of the references addressed spreading of silencing in reproductive organs and seeds. It was shown that gamete formation requires AGO9, which controls female gamete formation in a non-cell autonomous manner (Olmedo-Monfil et al., 2010). In grafted *Arabidopsis* and *Nicotiana* the systemic silencing penetrated male sporogenic tissues suggesting that plants harbor an endogenous long-distance small RNA transport pathway facilitating siRNA signalling into meiotically active cells. (Zhang et al., 2014). Regarding seeds, an experiment targeting RNAi to the endosperm showed that RNAi was restricted to the endosperm tissue and that transitive RNAi did not occur (Hournard et al., 2007). Similarly, RNAi remained contained in the seed coat and did not spread to cotyledons and vegetative tissues in *Glycine max* (Tuteja et al., 2009).

3.3.3.4 miRNA mobility

miRNAs can also be transported over long distances but this is not a universal rule for all miRNAs (Buhtz et al., 2008; Lin et al., 2008; de Felippes et al., 2011; Knauer et al., 2013). Analysis of the phloem sap of oilseed rape *Brassica napus* identified 32 annotated microRNAs (miRNAs) from 18 different families (Buhtz et al., 2008). In addition, the levels of three mature miRNAs known to respond to nutrient deprivation in non-vascular tissue, MIR395 (sulphate), MIR398 (copper) and MIR399 (phosphate), were increased in phloem sap during the growth of plants under the respective nutrient deprivation (Buhtz et al., 2008). Other known mobile miRNAs in *Arabidopsis* are MIR394, which is produced by the surface cell layer and contributes to shoot meristem formation (Knauer et al., 2013), and MIR165, which regulates differentiation (Miyashima et al., 2011).

MIR395 and MIR399 mobility through phloem was demonstrated in grafting experiments while MIR171 was not transported (Pant et al., 2008; Buhtz et al., 2010). Consistent with phloem movement, another analysis of miR399 movement using reciprocal grafting in *Arabidopsis* suggested that it moves from shoots to roots (Lin et al., 2008).

The basis of selectivity underlying miRNA mobility in plants remains unclear. It was shown that 22 nt artificial miRNAs derived from asymmetric duplexes mediate widespread silencing of their cognate gene more efficiently than 21 nt siRNAs from symmetric duplexes (McHale et al., 2013).

3.3.3.5 A note on papers authored by Olivier Voinnet

In January 2015, a number of publications authored by Olivier Voinnet was brought up at a server PubPeer.com as potentially containing manipulated images. Among these were highly cited publications that laid foundations of RNA silencing research in plants (four of them are among the highly-cited articles used in the search (Appendix C)). A subsequent ethical committee concluded that publications contained manipulated figures. Since then, a number of the publications were corrected or retracted (as of November 2016):

Retractions:

1. Brigneti G. et al., EMBO J (1998)
2. Voinnet, O. et al., Plant J. 33: 949-56 (2003)
3. Dunoyer P. et al., Plant Cell 16: 1235-50 (2004)
4. Moissiard G. and Voinnet O., Proc. Nat. Acad. Sci. USA 103 (51): 19593 (2006)
5. Dunoyer P. et al., EMBO J (2010)
6. Dunoyer P. et al., Science 328 (5980), 912 (2010)
7. Ciaudo C. et al., PLoS Genetics 9(11): e1003791 (2013)
8. Sansregret R. et al., PLoS Pathogens 1(9): e1005207 (2013)

Corrections:

1. Ruiz MT et al, Plant Cell 10: 937–946 (1998)
2. Voinnet O, et al., Nat. Acad. Sci. USA 96:14147–14152; (1999)
4. Dunoyer P. et al., Plant J. 29: 555-67 (2002)
3. Hamilton A. et al., EMBO J (2002)
5. Parizotto E.A. et al., Genes & Development 18: 2237-42 (2004) - 2nd Correction
8. Deleris A. et al, Science 313(5783): 68-71 (2006)
6. Dunoyer P. et al., Nature Genetics 38: 258-63 (2006)
7. Navarro L. et al., Science 312(5772): 436-439 (2006)
9. Moissiard et al, RNA 13: 1268–1278 (2007)
10. Haas G. et al, EMBO J 27, 2102-2112 (2008)
11. Ciaudo C. et al., PLoS Genetics 5(8): e1000620 (2009)
12. Bennasser Y et al., Nat. Struct. Mol. Biol. 18, 323–327 (2011)
13. Jay F, et al. (2011) PLoS Pathog 7(5): e1002035
14. Brodersen P. et al., Proc. Nat. Acad. Sci. USA 109: 1778-83 (2012)
15. Gibbings D. et al, Nature Cell Biology 14, 1314–1321 (2012)
16. Schott G. et al, EMBO J 31, 2553-2565 (2012)
17. Boccara M., et al., PLoS Pathogens 10(1): e1003883 (2014)

The authors of this report were aware of the above-mentioned issue and it was, where possible, taken into the account in the reporting of the available literature on this topic.

3.3.3.6 Summary

Taken together, it seems that there is not an exclusive mobile small RNA species. Rather, there may be several forms of small mobile RNAs. At the moment, there is no definite consensus on specific mechanism(s) regulating sorting of small RNAs into mobile and cell-autonomous small RNAs.

3.3.4. Horizontal transfer of small RNAs and its possible consequences

3.3.4.1 Plant-pathogen transfer & plant artificial plant resistance

RNA silencing can spread out of a plant as demonstrated by RNAi-inducing transgenes, which can extend their effects into nematodes (Huang et al., 2006), insects (Baum et al., 2007; Mao et al., 2007), parasitic fungi (Nowara et al., 2010) or another plant through a parasitic plant intermediate (Melnik et al., 2011b). Thus, systemic RNAi has biotechnological implications as it allows for producing a genetically engineered plant, which can be resistant to a specific virus or even induce an RNAi effect in a selected recipient (Mansoor et al., 2006). This can be explored, for example, for producing plants expressing dsRNA and selectively targeting RNAi-sensitive pests with an outcome of choice, e.g. repelling the pest, immobilizing it, sterilizing it (Bhatia et al., 2012), or killing it (Baum et al., 2007; Kola et al., 2016). Given the genome sequence diversity and relatively high sequence-specificity of RNAi, RNAi offers an adjustable selectivity for pest control. At the same time, this new technology might raise safety concerns. If the small RNAs can spread, could an RNAi-inducing transgene in a plant affect also non-targeted organisms?

The issue is essentially equivalent to off-targeting effects of RNAi brought to a species level. RNAi-off-targeting (reviewed in the section 3.2.2.3.) is a manifestation of the fact that partial base pairing can also induce RNA silencing. Consequently, some level of off-targeting in RNA silencing is inevitable. However, off-target effects can be suppressed to the point that they are not phenotypically manifested.

The key factors reducing RNAi off-targeting effects are the structure of the small RNA population and amount of small RNAs loaded on effector complexes, which would exhibit miRNA-like reduced sequence-specificity (i.e. seed match).

It can be inferred directly from the molecular mechanism of RNAi and miRNA pathways that a homogeneous small RNA population where all small RNAs have the same sequence (e.g. represented by specific siRNA delivery) has a higher off-target risk than a mixture of small RNAs with different sequences (e.g. produced from a long dsRNA).

In transmission of RNA silencing from one species to another, the amount of small RNAs loaded on effector complexes in the recipient species will be influenced by (i) the nature of the transferred silencing molecule (small RNA, long dsRNA), (ii) efficiency of the transfer, and (iii) efficiency of effector complex formation in the recipient species. Thus, species, which can absorb long dsRNA and have systemic RNAi like *C. elegans* (Tabara et al., 1998) are more prone to exhibit specific silencing effects as well as off-target effects. At the same time, significant off-target effects require specific conditions in terms of stoichiometry between a small RNAs and their targets, which are unlikely to be met upon RNAi induction with long dsRNA.

3.3.4.2 Extracellular miRNAs in mammals and their transfer

Release of miRNAs from mammalian cells is a well-known phenomenon. Circulating mammalian miRNAs were reviewed in the section 3.1.1.11. Here, we will highlight features of circulating miRNAs, which are important in the context of the section 3.3.4.

Extracellular circulating mammalian miRNAs were reported in 2008 when they were found in serum of lymphoma patients; they were immediately recognized as potential non-invasive biomarkers for cancer diagnostics and treatment (Lawrie et al., 2008). In fact, the bulk of the circulating RNA literature concerns biomarker potential of circulating miRNAs, which is of high clinical relevance but outside the scope of this report. Extracellular miRNAs were identified in a broad range of biological fluids (Pegtel et al., 2011; Huang et al., 2013; Kropp et al., 2014; Baglio et al., 2015; Dismuke et al., 2015; Izumi et al., 2015; Wu et al., 2015). Apart from plasma, circulating miRNAs in milk are also intensely studied (Izumi et al., 2012; Kuruppath et al., 2013; Sun et al., 2013; Izumi et al., 2015; Alsaweed et al., 2016c, b, a; Benmoussa et al., 2016; Xi et al., 2016).

miRNAs were identified in the cargo of exosomes, membranous vesicles 40 to 100 nm in diameter, which are constitutively released by almost all cell type and are found essentially in every biological fluid (reviewed, for example, in (Rak, 2013; Yoon et al., 2014)). Exosomal vesicles can carry miRNAs as well as siRNAs (reviewed in (Lasser, 2012)) – in the latter case, exosomes were adapted as delivery tool for siRNAs (reviewed in (Kumar et al., 2015)). However, most individual exosomes in standard preparations do not seem to contain biologically significant numbers of miRNAs (Chevillet et al., 2014) and 95–99% of circulating miRNA were reported to be associated with AGO proteins and not in extracellular vesicles (Arroyo et al., 2011; Turchinovich et al., 2011).

The molecular mechanism of miRNA release, whether as a cargo in a vesicle or not, is unclear and so is the function of circulating miRNAs, if there is any. Two distinct hypotheses were formulated concerning existence of circulating miRNAs (for the most recent detailed review, see (Turchinovich et al., 2016)):

Cell-to-cell communication hypothesis: According to this hypothesis, miRNAs can mediate cell-to-cell signalling. While the hypothesis is supported by circumstantial and correlative evidence (reviewed in (Turchinovich et al., 2016)), a mechanism for sorting, mobility, and targeting by mobile miRNAs and their function *in vivo* are unclear. This hypothesis has to face the kinetic data mentioned earlier (Wee et al., 2012) either by providing reliable evidence that sufficient quantities of miRNAs reach recipient cells or by identifying some non-canonical signalling function.

Cellular by-product hypothesis: This hypothesis states that circulating miRNAs are a non-specific noise resulting from cell physiology and turnover of biological material in the organism. As such, circulating miRNAs do not have a specific cell-to-cell communication function and their role in regulating gene expression in other cells is negligible or even non-existent. While this hypothesis might be consistent with the kinetic data (Wee et al., 2012), one cannot exclude a possibility that the “noise” of circulating miRNAs could yield specific biological roles during evolution. In fact, emergence of such an adaptation could be expected.

In fact, the scenario where the bulk of circulating miRNAs would represent a consequence of cell physiology while a limited number of circulating miRNAs would represent functionally relevant adaptations might reflect the real situation. In any case, extensive and rigorous research is needed to address the problem. This means in particular designing experiments aimed at proving either hypothesis wrong by deciphering underlying molecular mechanisms.

Finally, extracellular mammalian miRNAs can be transmitted from one animal to another or even between species. For example, placental miRNAs were found circulating in maternal plasma (Chim et al., 2008). It has also been reported that food-borne plant miRNAs are able to regulate gene expression in the liver (Zhang et al., 2012a) or miRNAs from consumed milk might contribute to circulating miRNAs in plasma (Baier et al., 2014) or that exosomes secreted by nematode parasites transfer small RNAs to mammalian cells (Buck et al., 2014). The issue of xenogenic miRNAs is addressed in detail in the following section.

3.3.4.3 Exogenic (nutritional, xenogenic) miRNAs in mammals

In 2012, the article by Zhang et al. proposed that miRNAs from ingested plants could traverse into the bloodstream and suppress genes in the liver (Zhang et al., 2012a). The report sparked an ongoing debate because of potential implications these data could have.

It should be pointed out that, while the article reported unexpected and surprising results, it was not breaking any conceptual dogma. The idea that information could be transmitted from food in a form of a large organic molecule that would traverse into the human organism has been an integral part of the prion hypothesis, which brought a concept of food-borne infectious particles made only of proteins (prions, reviewed, for example in (Peggion et al., 2016)). The prion hypothesis, for which Stanley Prusiner received a Nobel Prize in 1997, is nowadays a biology textbook knowledge. Furthermore, cross-kingdom regulation by small RNAs was discovered in RNA silencing field already in its early years – long dsRNA expressed in bacteria could induce repression of worm genes with complementary sequences when worms were fed with such bacteria (Timmons and Fire, 1998). Furthermore, in 2012

it was already well known that feeding on a plant carrying an RNAi-inducing transgene can induce RNAi in nematodes, insects, or fungi (Huang et al., 2006; Baum et al., 2007; Mao et al., 2007; Nowara et al., 2010). Thus, the article by Zhang et al. was not bringing any major shift in existing paradigms. The article essentially extended knowledge of RNA silencing spreading by reporting an example of a miRNA activity transferred from plants to mammals through feeding.

The critical issues

The main controversy of the field of mammalian nutritional miRNAs is that its claims are inconsistent with the molecular mechanism of the mammalian miRNA pathway. Namely, there are three areas, which need to be further investigated and receive solid experimental support:

(i) **Mechanism of the transport.** It is unknown how plant miRNAs would be transported from the intestinal lumen into liver cells across cell membranes. Mammals have no systemic RNAi. miRNA pathway mobility is also limited. It is known that miRNAs can be released from cells and be detected in blood plasma (discussed in 3.1.1.11.). While circulating miRNAs offer interesting biomarkers, their functional significance is still unclear and the mechanism of their sorting and release is also not understood. What is missing is the molecular mechanism transporting miRNAs from the intestinal lumen into the blood and into target cells.

(ii) **Effector complex structure.** It is unknown how plant miRNAs could regulate mammalian genes in mammalian cells. miRNA-mediated repression requires miRNAs to be loaded on AGO proteins. Thus, a plant miRNA operating in mammalian cells must either remain with its plant AGO protein, which would have to be compatible with mammalian components required for miRNA function, or be unloaded and reloaded onto a mammalian Argonaute protein. Importantly, plant miRNAs are methylated at their 3' end while mammalian miRNAs are not. Data by Zhang et al. suggest that plant miRNAs detected in mammalian serum are indeed methylated (Figure 1 in (Zhang et al., 2012a)). However, mammalian 3' small RNA methylation concerns only piRNAs, a distinct class of small RNAs produced from sense and antisense RNAs. piRNAs are longer (24-30 nt) than siRNAs or miRNAs and are loaded onto a distinct subfamily of Argonaute proteins named PIWI (reviewed in (Peters and Meister, 2007)). PIWI proteins are expressed in the germline but not in mammalian somatic cells. In contrast, miRNA and siRNA-binding Argonaute proteins (AGO subfamily) do not bind small RNAs methylated at the 3' end. (Tian et al., 2011). Yet, it has been reported that MIR168 is bound by murine AGO2.

(iii) **Targeting stoichiometry.** The canonical miRNA-mediated post-transcriptional regulation requires appropriate stoichiometry between miRNAs and their targets (section 3.2.2.1.). However, the evidence that plant miRNAs would reach such physiologically active levels in liver cells is questionable. Plant miRNAs were detected in the serum at femtomolar concentrations (~3-16 fM), which was one order of magnitude less than the serum level of endogenous miRNAs (Zhang et al., 2012a). Total circulating miRNA concentration in human plasma ranges were estimated to be 68-250 fM (Williams et al., 2013). At this concentration, human circulating miRNAs are considered unlikely to regulate gene expression by canonical miRNA activity as miRNAs are thought to require intracellular levels greater than 1000 copies per cell to exert measurable activity (Brown et al., 2007; Hafner et al., 2011). In fact, miR-122, a highly abundant miRNA regulating gene expression in the liver, has been estimated to be expressed at 120,000 molecules/hepatocyte (one of the highest miRNA amounts/cell) while less expressed miR-33 and miR-16 were estimated to be expressed at 1,200 and 11,000 copies/hepatocyte, respectively (Bissels et al., 2009; Denzler et al., 2014). These numbers contrasts with estimates for xenogenic miRNAs. As 10 femtomoles of miRNAs are approximately 6×10^9 molecules, an extracellular concentration of 10 fM corresponds to 6 molecules per nanoliter of serum. A somatic cell volume is up to several picoliters. Thus, unless there is yet unknown active transport of circulating miRNAs into hepatocytes, xenogenic miRNAs entering the circulatory system have no capacity to regulate gene expression by canonical miRNA activity in the liver. Yet, Zhang et al. argue that MIR168a abundance in the liver tissue is equivalent to 853 copies per cell. The ongoing debate on stoichiometry, which is discussed later, has been inconclusive. Furthermore, a critical review of the

results is compromised by the fact that some of the critical NGS datasets were not publicly released; hence, their analysis cannot be independently validated.

Exogenic miRNA transfer literature

As of December 2016, there were 227 citations of the original article, which were reviewed for this section. Among the 227, we identified 51 original research articles, which were related to xenogenic and circulating miRNAs. From these, we focused on those directly relevant to the concept of plant-mammal miRNA transmission. Literature concerning the existence and function of xenogenic miRNAs includes:

- The original study (Zhang et al., 2012a). Using NGS, they identified plant miRNAs in mammalian sera. Unfortunately, the information provided is only partial. The entire data normalization is described as: *For normalization, the sequencing frequency of each plant miRNA was normalized to the total amount of mammalian miRNAs.* (Zhang et al., 2012a) while the availability of original sequencing data in a public repository was not indicated, thus not allowing for an independent assessment of sequence analysis and quantification. Different quantification strategy could be a potential source of discordance with other published data, which emerged in the literature (Chen et al., 2013).
- The patent: WO 2012135820A2 Edible transgenic plants as oral delivery vehicles for RNA-based therapeutics filed by Eric Lam, Rutgers University. The claim is based on an experiment where feeding rabbits or mice with transgenic tomato producing siRNA against HCV yielded detectable HCV siRNAs and tomato RNAs in mammalian blood and tissues. This work was not published in a peer review journal, the patent documentation contains a small RNA northern blot with a faint signal, RT-PCR detection of tomato RNAs in mammalian samples and analysis of RNAi-mediated repression in cultured HepG2 cells.
- A survey of human plasma for microRNA biomarkers in NGS datasets from human plasma revealed circulating RNAs appearing to originate from exogenous species including bacteria, fungi, and other species (Wang et al., 2012). Interestingly, authors could detect RNA fragments from common food where American samples exhibited higher abundance of corn over rice sequences while a control Chinese sample (SRR332232) showed a reversed trend (Wang et al., 2012). Authors also report detection of minuscule amounts of plant miRNAs in human samples 6-16 reads of MIR168 per sample were observed at total sequencing depths of 18-28 million reads; public availability of raw data was not indicated. Notably, authors also indicate low fraction of mappable sequences – they observed less than 1.5% of processed reads mapping to human miRNAs (Wang et al., 2012). Thus, calculating miRNA RPM values (reads per million) from the mapped reads or miRNA reads could yield two orders of magnitude differences.
- The same group, which published the original xenomiR study, recently published another article proposing that a plant miRNA MIR2911 from honeysuckle (*Lonicera japonica*), a traditional Chinese medicine herb, can traverse into mouse lungs and inhibit Influenza A (Zhou et al., 2015). In this case, MIR2911 concentration was estimated to reach 1 pM in plasma after feeding mice honeysuckle decoction, which is the range of endogenous circulating miRNAs. MIR2911 was detected in the mouse by NGS (acc. no. GSE55268), qPCR, and in situ hybridization. The estimated amount of MIR2911 in lung cell was 300-400, suggesting that MIR2911 operates within the physiological range of the canonical miRNA pathway (Zhou et al., 2015).
- Vicki Vance's group reported that oral administration of 2'-O--methylated mammalian miRNAs, which have tumor suppressor properties (miR-34a, miR-143, and miR145) results in reduced intestinal tumor burden in mice (Mlotshwa et al., 2015). This work is also linked to two issued patents and one pending patent application, all entitled "Compositions and Methods for the Modulation of Gene Expression in Plants": US patent application no. 13/282,680, Canadian patent no. 2,276,233 and Australian patent no. AU 2003/254052 B2.
- A third report of xenogenic miRNA regulation suggests that plant miRNA MIR159 is present in human plasma and its level inversely correlates with breast cancer incidence. MIR159 was identified

in the serum by NGS (GSE55268). A synthetic mimic of MIR159 was capable of inhibiting cell proliferation in breast cancer cells but not non-cancerous mammary epithelial cells (Chin et al., 2016). Authors claim, "These results demonstrate for the first time that a plant miRNA can inhibit cancer growth in mammals."

- Work by Zhang et al. used miRNA target prediction in *Arabidopsis* and humans to assemble a hypothetical cross-species regulatory network, which they further analyzed. They proposed that the cross-species regulatory network contains three core module functionally linked to ion transport, metabolic process and stress response (Zhang et al., 2016). However, this study provides a strategy to explore possible cross-species miRNA regulations rather than providing evidence that such regulations exist.

However, next to these publications, there is also a number of reports, which question the idea of functional xenogenic miRNAs. For example:

- Analysis of 83 NGS datasets (identified in the report) revealed presence of plant-derived MIRNAs among which dominated MIR168 from monocot plants. However, the highest observed ratio of plant miRNAs/animal miRNAs is 0.456%, which is 10 times lower than a figure of ~5% reported by Zhang et al. (Zhang et al., 2012a). These data suggests that the observed plant miRNAs in animal small RNA datasets can originate in the process of sequencing, and that accumulation of plant miRNAs via dietary exposure is not universal in animals (Zhang et al., 2012b).
- Snow et al. analyzed miRNA content in diets of humans, mice, and honeybees and examined transfer of several different miRNAs into the recipient organism: conserved and highly-expressed plant miRNAs (MIR156a, MIR159a and MIR169a) and conserved, abundant, and ubiquitous animal miRNA miR-21. Healthy human subjects routinely eating fruits replete in MIR156a, MIR159a and MIR169a had undetectable levels of plant-derived miRNAs in their plasma (as measured by qPCR). Similarly, miR-21 levels were negligible in plasma or tissues of miR-21^{-/-} mice after oral diets containing miR-21. The same was observed for MIR156a, MIR159a and MIR169a in mice and honeybee tissues. This study concludes that: "*horizontal delivery of microRNAs via typical dietary ingestion is neither a robust nor a frequent mechanism to maintain steady-state microRNA levels in a variety of model animal organisms, thus defining the biological limits of these molecules in vivo.*" (Snow et al., 2013)
- Importantly, an attempt to replicate the claims in the original xenogenic miRNA paper failed (Dickinson et al., 2013). This article prompted a comment by authors of the original xenogenic paper pointing out several discrepancies in data and that experimental design used by Dickinson et al. for NGS analysis was suboptimal and could produce artifacts (Chen et al., 2013). Unfortunately, one cannot reanalyze both datasets because only the one from Dickinson et al. was made available (SRP028401).
- A feeding study on *Macaca nemestrina* failed to provide support for xenogenic miRNAs in blood plasma in response to dietary intake of a plant miRNA-rich food source. Putative xenogenic miRNAs were detected real-time quantitative PCR and droplet digital PCR (Witwer et al., 2013). Detected levels of miRNAs were minimal and/or amplification was non-specific. Detection was largely unreliable for plant miRNAs 156, 160, 166, 167, 168 and 172 despite the relative abundance of all but miR172 in a soy- and fruit-substance that was administered to the macaque subjects (Witwer et al., 2013).
- Bioinformatic analysis of NGS data for diet-derived miRNAs in deep-sequencing libraries also did not provide a support for the "xenomiR hypothesis". Authors provide evidence that cross-contamination during library preparation can be a source of exogenous RNAs and that such a cross-contamination could be identified in an NGS study of *Amphioxus* performed by the group, which published the first xenogenic miRNA article in 2012 (Tosar et al., 2014). The original data from the Zhang et al. 2012 article could not be examined for cross-contamination. Tossar et al. conclude that cross-contamination between samples from the same organism can go completely unnoticed, possibly affecting conclusions derived from NGS transcriptomics (Tosar et al., 2014).

Conclusions

Thus, it is apparent that four years after the original report (Zhang et al., 2012a), the field remains split. The essential questions concerning the existence of the proposed mechanism emerged already in 2012. Further research is necessary to clarify the basis of the aforementioned contradictory observations. At the moment, there is no consensus regarding any aspect of the xenomiR hypothesis, i.e.:

- the mechanism of transport of a plant miRNAs from food into the blood.
- amounts of plant miRNAs reaching the mammalian circulation system and tissues
- the nature of the ribonucleoprotein complexes, in which they exist in the plasma
- the mechanism of entrance (accumulation?) of xenomiRs into cells
- the nature of the ribonucleoprotein complexes, in which they exist in the cell

Remarkably, the simplicity of the xenomiR hypothesis where an oral uptake of miRNAs in food results in post-transcriptional repression in tissues contrasts with more than a decade of development of small RNA-based therapeutics, which still struggles with efficiency of uptake, distribution and targeting. However, the current stage of (lack of) knowledge precludes strong conclusions regarding the future of the xenomiR hypothesis.

3.3.5. Conclusions - Part III

Mobile RNA silencing exists in plants and some animals. RNA silencing, and RNAi in particular, operates with unique (though not absolute) selectivity and is being explored in many different ways in biomedicine and biotechnology. In some cases, RNA silencing can spread across cellular boundaries, which can extend into systemic silencing. Systemic silencing is restricted to specific taxons and RNA silencing pathways. While the framework for understanding the molecular basis of systemic silencing in different model systems was established, the knowledge is still fragmented and far from complete. Even less clear is the horizontal transmission of RNA silencing, especially into species, where primarily cell-autonomous silencing is expected.

3.3.6. References

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Glossary and Abbreviations

Glossary

BioGPS	gene annotation portal, http://biogps.org
Cajal body	nuclear membraneless organelle, the site of assembly of several snRNPs
Dicer	protein producing short RNAs of defined lengths from dsRNA precursors
endo-RNAi	endogenous RNAi
holo-RISC	the minimal RISC effector complex = small RNA + AGO2
lncRNA	long non-coding RNA, an RNA >200 nt in length not coding for a protein
Microprocessor	nuclear complex processing miRNA precursors
miRBase	miRNA database, http://www.mirbase.org
P-body	cytoplasmic membraneless organelle, contains proteins involved in mRNA turnover
PTGS	post-transcriptional gene silencing, a term common for research in plants
RNA silencing	repression guided by small RNA molecules (~20-30 nucleotides long).
RNA interference	sequence-specific mRNA degradation induced by long dsRNA
Slicer	protein or endonucleolytic activity cleaving mRNAs in RNAi
systemic RNAi	RNAi that spreads across cellular boundaries (non-cell-autonomous RNAi)
taxon	a (taxonomically) classified group of organisms

small RNA types

22G-RNA	a class of small RNAs in <i>C. elegans</i> , which is 22nt long and starts with guanosine
diRNA	small RNA generated at a site of DNA damage
easiRNA	epigenetically activated 21 nt small interfering RNA, a type of siRNA
endo-siRNA	endogenous siRNA, produced from endogenous dsRNA
exo-siRNAs	exogenous siRNA
het-siRNA	heterochromatic 24 nt siRNA
hp-siRNA	hairpin RNA-derived 21-24 nt siRNA
microRNA	genome-encoded small RNA regulating gene expression
mirtron	a non-canonical, splicing-dependent intronic microRNA
natsiRNA	natural antisense small interfering RNA, a type of siRNA
phasRNA	phased 21-22 nt siRNA, a type of secondary siRNA
pre-miRNA	miRNA precursor - a small hairpin precursor before Dicer cleavage
pre-siRNA	siRNA precursor
pri-miRNA	primary miRNA – a primary miRNA transcript before Microprocessor cleavage
siRNA	short interfering RNA (sometimes also small interfering RNA)
shRNA	small hairpin RNA
tasiRNA	genome-encoded trans-acting siRNA that repress gene expression

Abbreviations:

22G-RNA	a class of small RNAs in <i>C. elegans</i> , which is 22nt long and starts with guanosine
ABL	Abelson murine leukemia viral oncogene homolog tyrosine kinase
ADAR	adenosine deaminase acting on RNA, and RNA editing enzyme
AGO	Argonaute protein
AGS	Aicardi-Goutieres syndrome, OMIM# 225750
AIN	ALG-1 interacting protein, a GW182 homolog in <i>C. elegans</i>
ALG	Argonaute-like gene, an AGO homolog in <i>C. elegans</i>
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
A-to-I	adenosine to inosine conversion
C3PO	Mg ²⁺ -dependent endoribonuclease promoting RISC activation
CCR4-NOT	RNA deadenylase complex
cDNA	complementary DNA, DNA produced through reverse transcription of RNA
CED-3	cell-death gene 3, a caspase in <i>C. elegans</i>
CMT3	chromomethylase 3 or cytosine methyltransferase 3
CpG	cytosine-guanine dinucleotide
CpNpG	cytosine-any nucleotide-guanine trinucleotide
CR1	chicken repeat 1 retrotransposon
cryo-EM	cryo electron microscopy
CSR-1	chromosome-segregation and RNAi deficient, an AGO homolog in <i>C. elegans</i>
DCL	Dicer-like proteins in plants
DCP	Decapping protein
DCS-1	Decapping Scavenger enzyme
DDM1	decrease in DNA methylation, a nucleosome remodeler in <i>Arabidopsis</i>
DDR	putative chromatin-remodelling complex in TGS in <i>Arabidopsis</i>
DDX	DEAD-box helicase gene family member
DEAD	helicase family specifying sequence motif
DEAH	helicase family specifying sequence motif
DExD	helicase family specifying sequence motif
DExH	helicase family specifying sequence motif
dFMR1	<i>Drosophila</i> FMRP homolog
DGCR8	DiGeorge syndrome critical region 8 protein
DHX	helicase gene family member
diRNA	small RNA generated at a site of DNA damage
DMP68	<i>Drosophila</i> homolog of p68 RNA helicase
DND	Dean end protein

DOI	digital object identifier
DRB	dsRNA binding protein family in plants
DRD1	Defective in RNA-directed DNA methylation protein
DRH	Dicer-related helicase protein
DRM	domains rearranged methyltransferase, a plant <i>de novo</i> DNA methyltransferase
dsRBD	double-stranded RNA binding domain
dsRBM	double-stranded RNA binding motif
dsRBP	double-stranded RNA binding protein
dsRNA	double-stranded RNA
DT40	chicken B cell line DT40
DUF283	domain of unknown function 283
DXV	<i>Drosophila X virus</i>
easiRNA	epigenetically activated small interfering RNA, a type of siRNA
EGO-1	enhancer of Glp-One, an RdRP homolog in <i>C. elegans</i>
eIF1A	eukaryotic translation initiation factor 1A
eIF2	eukaryotic translation initiation factor 2
eIF4GI	eukaryotic translation initiation factor 4GI
EKL-1	enhancer of ksr-1 lethality protein, a Tudor-domain protein required for RNAi
ELISA	enzyme-linked immunosorbent assay
ELP1	elongator complex protein 1 in <i>Drosophila</i>
endo-RNAi	endogenous small interfering RNA
eNOS	endothelial nitric oxide synthase
ERGO	endogenous-RNAi deficient Argonaute, an AGO homolog in <i>C. elegans</i>
ERI	enhancer of RNA interference – a mutation type and a corresponding gene name
ERK	extracellular signal–regulated kinases
ESC	embryonic stem cells
exo-RNAi	exogenous RNAi, RNAi induced with exogenous dsRNA
exo-siRNAs	exogenous siRNA
FCCS	fluorescence cross-correlation spectroscopy – a microscopic method
FCS	fluorescence correlation spectroscopy – a microscopic method
FHV	feline herpesvirus
FISH	fluorescence in situ hybridization – a microscopic method
FKBP4	FK506 binding protein 4
FMRP	Fragile X mental retardation protein
GARP	Golgi-associated retrograde protein, a protein complex

GCRV	grass carp reovirus
GERp95	Golgi-endoplasmic reticulum protein 95 kDa, an old name for AGO2
GLH-1	germline RNA helicase in <i>C. elegans</i>
GRF	growth-regulating factor, a transcription factor family in <i>Arabidopsis</i>
GW182	glycine-tryptophan-rich protein, an AGO-associated protein
H3	histone H3
H3K27	histone H3, lysine 27
H3K27me3	trimethylated histone H3, lysine 27
H3K9	histone H3, lysine 9
H5N1	influenza A virus subtype H5N1
H9N2	influenza A virus subtype H9N2
HCMV	human cytomegalovirus
HDA6	plant histone deacetylase 6
HeLa	human cervical cancer cell line
HEN1	small RNA 2'-O-methyltransferase
het-siRNA	heterochromatic siRNA
HITS-CLIP	high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation
HIV-1	human immunodeficiency virus 1
hnRNP	heterogeneous nuclear ribonucleoprotein
HOXD4	Homeobox D4 protein
hp-siRNA	hairpin RNA-derived siRNA
HRDE-1	heritable RNAi defective 1, an AGO homolog in <i>C. elegans</i>
HSP70	heat shock protein 70
HSP90	heat shock protein 90
HYL1	dsRNA-binding domain-like superfamily protein
IAP	intracisternal A particle, a murine endogenous retrovirus
IFN	interferon
IGFBP7	insulin-like growth factor-binding protein 7
L1	long interspersed element L1, a mammalian autonomous retrotransposon
Let-7	a conserved animal microRNA
LGP2	laboratory of genetics and physiology 2, a synonym for DHX58
LIM	Lin11, Isl-1 & Mec-3 domain
LIN	lumpy infections, a mutation type and a corresponding gene/protein name
LINE	long interspersed element
lncRNA	long non-coding RNA
LOQS	Loquacious, a dsRNA binding protein in <i>D. melanogaster</i>

LRRK2	leucine-rich repeat kinase 2
MDA5	melanoma differentiation-associated protein 5, a RIG-I-like receptor dsRNA helicase
MECP2	Methyl-CpG binding protein 2
mESCs	mouse embryonic stem cells
MET1	DNA (cytosine-5)-methyltransferase 1 in plants
miPDC	miRNA precursor deposit complex
miRBase	miRNA database
miRISC	miRNA RNA-induced silencing complex
miRLC	miRISC loading complex
miRNA	microRNA
miRNPs	microRNA ribonucleoproteins
MOV10	Moloney leukemia virus 10 protein
mTOR	mammalian target of rapamycin, a phosphatidylinositol 3-kinase-related kinase
MuERV-L	mouse endogenous retrovirus type L
MYA	million years ago
natsiRNA	natural antisense small interfering RNA, a type of siRNA
NCBI	National Center for Biotechnology Information
NERD	nuclease-related domain, a plant-specific GW protein
NES	nuclear export signal
NGS	next generation sequencing
NLS	nuclear localization signal
non-CpG	any other dinucleotide than CpG
OAS	oligoadenylate synthetase
OMIM	Online Mendelian Inheritance in Man, a database of human genetic traits
PABP	PolyA-binding protein
PABPC	PolyA-binding protein, cytoplasmic
PACT	Protein ACTivator of the interferon-induced protein kinase, a mammalian dsRBP
PAN2/3	PAN2-PAN3 deadenylase
PAZ	Piwi-Argonaute-Zwille domain
PCR	polymerase chain reaction
phasiRNA	phased small interfering RNAs, a type of secondary siRNA
piRNA	PIWI-associated RNA
PIWI	P-element induced wimpy testis, a domain in AGO proteins
PKR	protein-kinase R
PPW-1	PAZ/PIWI domain-containing, an AGO homolog in <i>C. elegans</i>
pre-miRNA	miRNA precursor - a small hairpin precursor before Dicer cleavage

pre-mRNA	nascent mRNA, unspliced mRNA
pre-siRNA	siRNA precursor
pri-miRNA	primary miRNA – a primary miRNA transcript before Microprocessor cleavage
PTGS	post-transcriptional gene silencing
R2D2	dsRNA binding protein in <i>D. Melanogaster</i>
RACK1	receptor for activated C kinase 1
RdDM	RNA-dependent DNA methylation
RDE	RNA-defective – a mutation type and a corresponding gene/protein name
rDNA	ribosomal DNA – a part of the genome encoding ribosomal RNA
RDR	RNA-dependent RNA polymerase genes in plants
RdRP	RNA-dependent RNA polymerase
RHA	RNA helicase A
RIG-I	RIG-I-like receptor dsRNA helicase enzyme encoded by the DDX58 gene
RISC	RNA-induced silencing complex, an effector complex of RNA silencing
RLC	RISC loading complex, a complex formed during RISC formation
RNAi	RNA interference
RNA-seq	high-throughput sequencing of RNA
RRF	RNA-dependent RNA polymerase Family, RdRP homologs in <i>C. elegans</i>
RRM	RNA recognition motif domain
rRNA	ribosomal RNA
SC35	Splicing component, 35 kDa protein
self-RNA	own/endogenous RNA
SGS	Suppressor of Gene Silencing, a mutation type and a corresponding gene/protein name
shRNA	small hairpin RNA
SID	Systemic RNAi defective – a mutation type and a corresponding gene/protein name
SINE	short interspersed element
siRNA	short interfering RNA (sometimes also small interfering RNA)
snoRNAs	small nucleolar RNA
SRS	suppressors of RNA silencing, proteins found in viruses
ssRNA	single-stranded RNA
SUMO	Small ubiquitin-like modifier protein
SUO	plant GW protein acting in microRNA-mediated translational repression
TARBP	HIV-1 TAR RNA binding protein (same as TRBP)
TAS	ta-siRNA-generating gene
tasiRNA	genome-encoded trans-acting siRNA that repress gene expression
TBLASTN	translated nucleotide query basic local alignment search tool

TERT	telomerase reverse transcriptase
TGS	transcriptional gene silencing
TLR	Toll-like receptor
TNRC6	trinucleotide repeat containing 6 gene
TRIM	tripartite motif
TRIM-NHL	Tripartite motif-NCL-1/HT2A/LIN-41 domains-containing protein
tRNA	transfer RNA
TSN	Tudor staphylococcal nuclease (same as Tudor-SN)
TUT	terminal uridylyltransferase
UBA	ubiquitin-associated domain
UPF	Up-frameshift protein, a protein involved in non-sense mediated decay
VA1	viral associated 1, Adenovirus non-coding RNA
VIGS	virus-induced gene silencing
VP35	Polymerase cofactor VP35, an Ebola virus protein
VSV	vesicular stomatitis virus
WAGO	nematode (worm)-specific subfamily of AGO proteins
WAF	WAVY LEAF protein, a rice homolog of HEN1
WG/GW	glycine-tryptophan-rich protein domain
WOS	Web of Science, scientific citation indexing service maintained by Thomson Reuters
WTIP	Wilms tumor protein 1-interacting protein
XRN1	5'-3' exoribonuclease 1
Zcchc	Zinc fingers CCHC-type gene family
Z-DNA	Z-DNA is a left-handed double helical DNA conformation
ZIPPY	AGO7 homolog in <i>Arabidopsis</i>

Appendix A – Search protocol for compilation of a comprehensive set of scientific and grey literature related to RNA interference and dsRNA and miRNA pathways

Study retrieval

Study retrieval table		
Information source	PubMed	
Searched fields	All Fields	
Search parameters	None	
Search date	1 April 2016 – 29 April 2016	
Keywords / phrases	Keyword / phrase	No. of results
	"21u rna"	11
	"double stranded rna"	11,394
	"rna interference"	48,861
	"rnase 1"	85
	"ago1"	519
	"ago2"	794
	"Argonaute"	2,626
	"dicer"	2,463
	"dsrna"	19,100
	"gene silencing"	29,256
	"mirna or microRNA"	51,671
	"oligoadenylate"	2,292
	"piwi"	1,160
	"pkr"	2,349
	"ptgs"	554
	"r2d2"	139
	"rasirna"	17
	"rdeg1 or rde1"	55
	"rnai"	19,283
"sirna"	74,930	
"tarbp2"	44	
"trbp2"	17	
"wago"	79	
Information source	Web of Science	
Searched fields	Topic (TS)	
Searched collections	Web of Science™ Core Collection	
Search parameters	None	
Search date	1 April 2016 – 29 April 2016	
Keywords / phrases	Keyword / phrase	No. of results
	"21u rna"	9
	"double strand* rna"	16,309
	"gene silenc*"	41,908
	"rna interference"	28,549
	"rnase 1"	67
	"ago1"	446
	"ago2"	776
	"argonau*"	6,067
	"dicer"	6,925
	"dsrna"	6,649
	"gene silenc*"	41,908

	"mirna or microrna"	45,522
	"oligoadenylate"	1,473
	"piwi"	1,419
	"pkr"	3,210
	"ptgs"	678
	"r2d2"	108
	"rasirna"	23
	"rdeg1 or rde1"	4
	"rnai"	20,679
	"sirna"	40,518
	"tarbp2"	51
	"trbp2"	18
	"wago"	22
Information source	Scopus	
Searched fields	Title, Abstract, Keywords	
Search parameters	Search limited to Document Type: Article	
Search date	1 April 2016 – 29 April 2016	
Keywords / phrases	Keyword / phrase	No. of results
	"21u rna"	19
	"double strand* rna"	12,555
	"rna interference"	52,759
	"rnase 1"	81
	"ago1"	566
	"ago2"	754
	"argonau*"	2,613
	"dicer"	2,558
	"dsrna"	6,751
	"gene silenc*"	56,413
	"mirna or microrna"	38,691
	"oligoadenylate"	2,303
	"piwi"	1,139
	"pkr"	2,497
	"ptgs"	611
	"r2d2"	91
	"rasirna"	23
	"rdeg1 or rde1"	4
	"rnai"	18,812
"sirna"	39,201	
"tarbp2"	35	
"trbp2"	19	
"wago"	20	
Information source	ProQuest Dissertations & Theses Full Text: The Sciences and Engineering Collection	
Searched fields	Title (TI), Abstract (AB), Key Fields (KF)	
Search parameters	None	
Search date	1 April 2016 – 29 April 2016	
Keywords / phrases	Keyword / phrase	No. of results
	"21u rna"	1
	"double stranded rna"	643
	"rna interference"	1,301
	"rnase 1"	7
"ago1"	36	

	"ago2"	69
	"argonau*"	180
	"dicer"	205
	"dsrna"	717
	"mirna or microrna"	1,757
	"oligoadenylate"	67
	"piwi"	88
	"pkr"	274
	"ptgs"	43
	"r2d2"	15
	"rasirna"	2
	"rdeg1 or rde1"	0
	"rnaï"	2,004
	"sirna"	2,587
	"tarbp2"	4
	"trbp2"	0
	"wago"	0

Appendix B – P(E)ICO characterisation of ELS questions

Extensive Literature Search question	Characteristics of the question	Is the question answerable using systematic literature search?
<p>How is dsRNA and miRNA processed in different organisms of interest?</p>	<p>Specific, Open-framed, type: PO</p> <p>Population = the organisms of interest (plants, humans, mammals, insects, birds, fish, arthropods, annelids, nematodes and molluscs)</p> <p>Outcome = processing mechanisms of dsRNA and miRNA (to be determined)</p>	Yes
<p>What is the function of all the different components of the silencing pathways in the organisms of interest?</p>	<p>Specific, Open-framed, type: PO</p> <p>Population = the organisms of interest (plants, humans, mammals, insects, birds, fish, arthropods, annelids, nematodes and molluscs)</p> <p>Outcome = function of the silencing pathways components (to be determined)</p>	Yes
<p>Which dsRNA and miRNA processing mechanisms and pathway components are unique to any of the organisms of interest?</p>	<p>Specific, Open-framed, type: PO</p> <p>Population = the organisms of interest (plants, humans, mammals, insects, birds, fish, arthropods, annelids, nematodes and molluscs)</p> <p>Outcome = description of dsRNA and miRNA processing mechanisms and pathway components unique to any of the organisms of interest (to be determined)</p>	Yes
<p>Which pathway components are required for the normal functioning of a specific pathway in the organisms of interest?</p>	<p>Specific, Open-framed, type: PO</p> <p>Population = the organisms of interest (plants, humans, mammals, insects, birds, fish, arthropods, annelids, nematodes and molluscs)</p> <p>Outcome = identification of pathway components required for the normal functioning of a specific pathway in the organisms of interest (to be determined)</p>	Yes

Appendix C – References used as pearls in citation pearl growing search strategy

For the core reference list, we selected (I) primary references, which provided fundamental insights into deciphering the molecular mechanism of RNAi and miRNA pathways and (II) reviews, which covered broad and specific aspects of the reviewed pathways. The inclusion criteria were importance of the field, number of citations, and coverage of as many as possible aspects of RNA silencing in animals and plants.

ORIGINAL RESEARCH PAPERS

1. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*. 2001;409(6818):363-6.

[This is the first work identifying Dicer as the key small RNA producing enzyme. Cited 2,581 times.](#)

2. Denli AM, Tops BBJ, Plasterk RHA, Ketting RF, Hannon GJ. Processing of primary microRNAs by the Microprocessor complex. *Nature*. 2004;432(7014):231-5.

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Appendix D – Overview of costs of NGS analysis of small RNA pools (January 2017)

The price quotes are intended to provide a general idea about the current (January 2017) range of costs of various small RNA sequencing services and may not be directly comparable as the price per sample is influenced by many variables.

company	product/service name	platform	depth	price per sample	note
Applied Biological Materials	miRNA/Small RNA Sequencing (10 million, 1x75bp SE)		10M	485 USD	
BGI-Europe	BGI-seq500 small RNA sequencing, <100 samples	BGI-seq500	20M	350 USD	
	BGI-seq500 small RNA sequencing, >100 samples	BGI-seq500	20M	300 USD	
Biomedical Sequencing Facility	HiSeq 3000/4000: 1 lane of 1x50bp (single read)			883 EUR	
	HiSeq 2000: 1 lane of 50bp SR			832 EUR	
CRG-CNAG (Centre for Genomic Regulation)	Sequencing Lane (1x50, v3, HiSeq highoutput mode)	Illumina		805 EUR	public institution price
	Sequencing Lane (1x50, v4, HiSeq highoutput mode)	Illumina		789 EUR	
	Sequencing Lane (1x50, HiSeq 3000/4000)	Illumina		859 EUR	
	Sequencing lane (50cycles, v2, MiSeq)	Illumina		788 EUR	private company price
	Sequencing Lane (1x50, v3, HiSeq highoutput mode)	Illumina		928 EUR	
	Sequencing Lane (1x50, v4, HiSeq highoutput mode)	Illumina		908 EUR	
Girihlet Inc	Sequencing Lane (1x50, HiSeq 3000/4000)	Illumina		985 EUR	
	Sequencing lane (50cycles, v2, MiSeq)	Illumina		924 EUR	
				from \$300 USD	
Omegabioservices	Bioo Scientific Small RNA (SR 1x50)	Illumina	10M	438 USD	
	Bioo Scientific Small RNA (SR 1x50)	Illumina	20M	500 USD	
RNA Sequencing Core	small-RNAseq Packages : Sequencing (50+nt); Add'l 10M reads	Illumina	>= 10M	136 USD	
Seqmatic				from \$219 USD	