INTRODUCTION

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ABSTRACT

RNA silencing denotes a group of pathways, which utilize small RNAs as sequence-specific guides for repressing gene expression. Two related RNA silencing pathways exist in animals and plants: RNA interference (RNAi) and microRNA (miRNA) pathway. While the miRNA pathway regulates endogenous protein-coding gene expression, RNAi serves as a form of innate immunity targeting viruses and mobile elements, although it occasionally also acquired function in protein-coding gene regulation. The aim of the following text is to provide an elementary introduction into RNAi and miRNA pathways for a series of taxon-specific and feature-specific reviews, which follow. 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 that are presented in taxon-oriented reviews. The entire review series was derived from an expert report for the European Food and Safety Agency, which was reorganized to be more accessible for the scientific community.

Introduction to mechanistic principles and roles of RNA silencing

The volume of the RNA silencing and double-stranded RNA (dsRNA)-related published data is stunning. In 2016, me and my colleagues did literature assessment for the European Food and Safety Agency, which identified over 200 000 publications (Paces et al., 2017) and which served as a foundation for this article series. While selected taxons are reviewed separately, I thought the collection would benefit from introducing the core molecular mechanisms of RNAi and miRNA pathways (admittedly animal-centric).

As mentioned in the abstract, RNA silencing (reviewed in Ketting, 2011) designates repression guided by small RNA molecules (20–30 nucleotides long) and includes diverse silencing mechanisms including RNA degradation, translational repression, induction of repressive chromatin, and even DNA deletions. RNA silencing research evolved from parallel studies in several different model systems, primarily flowering plant models and animal

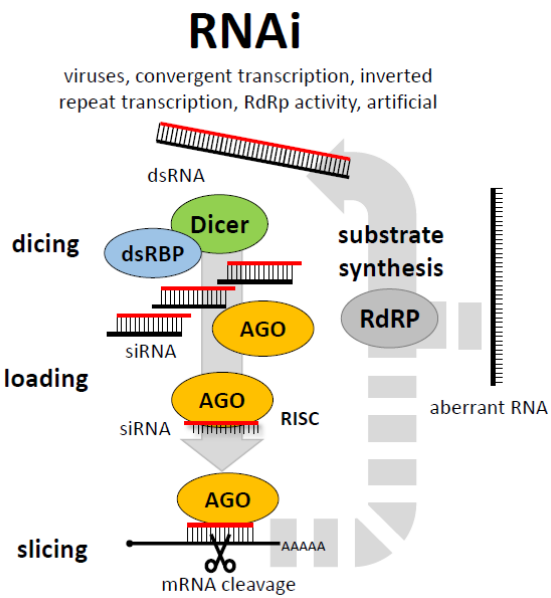

Figure 1 RNAi pathway overview
Canonical RNAi is triggered by some form of long dsRNA. dsRNA can originate from various sources including viruses and their replication intermediates or base pairing if RNAs transcribed in the genome (either as an intramolecular duplex (hairpin dsRNA), or by base pairing 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 from AGO subfamily 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 performs 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 converting cognate RNAs into dsRNA.

models including *Caenorhabditis elegans*, *Drosophila melanogaster*, zebrafish, mouse, and humans. Some form of RNA silencing exists in almost every eukaryote. Here, the primary focus will be on the RNA interference (RNAi) and microRNA (miRNA) pathways (Fig. 1 and 2). 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). Here, I will use the term RNAi strictly in its original connotation. miRNAs are genome-encoded short RNAs that regulate gene expression by translational repression and/or degradation of cognate mRNAs.

Historically, the first discovered RNA silencing pathway was plant co-suppression, 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

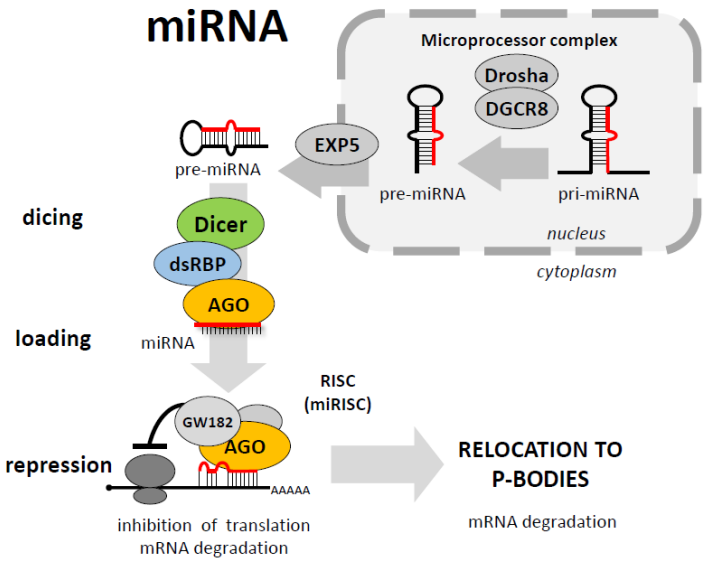

Figure 2 Canonical animal miRNA pathway overview
miRNAs are genome-encoded. Their synthesis starts with Pol II-mediated transcription of long primary miRNA transcripts (pri-miRNAs), which carry one or more local short hairpins, which are released as precursor miRNAs (pre-miRNAs) by the activity of the nuclear "Microprocessor complex". Pre-miRNAs are transported into the cytoplasm via Exportin 5. In the cytoplasm, Dicer cleaves a 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 and 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

the nematode *Caenorhabditis elegans* (Lee et al., 1993). The idea of a conserved miRNA pathway emerged upon discovery of Let-7 miRNA in 2000, which was is conserved from *Caenorhabditis elegans* to mammals (Pasquinelli et al., 2000). In the meantime, RNAi was found in *Caenorhabditis* as well (Fire et al., 1998). Around the year 2000, it became apparent that earlier observations, which included the aforementioned plant co-suppression, quelling in fungi, and animal RNAi and miRNA pathways (Lee et al., 1993; Napoli et al., 1990; Romano and Macino, 1992; van der Krol et al., 1990), belong to one group of related molecular mechanisms commonly called RNA silencing.

The core principle of RNA silencing (repression mediated by a ribonucleoprotein complex guided by a small RNA) 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 (Tuschl et al., 1999; Zamore et al., 2000) and genetic studies in *Caenorhabditis elegans* and plants (e.g. (Bohmert et al., 1998; Dalmay et al., 2000; Fagard et al., 2000; Grishok et al., 2000; Lynn et al., 1999; Mourrain et al., 2000; Smardon et al., 2000; Tabara et al., 1999)). The last discovery, which arguably closed the era of deciphering the key principles of RNA silencing, was the structural

	mammals				birds		fish		arthropods		mollusks		annelids		nematodes		plants	
	Homo sapiens		*Mus musculus*		*Gallus gallus*		*Danio rerio*		*Drosophila melanogaster*		*Crassostrea gigas*		*Helobdella robusta*		*Caenorhabditis elegans*		*Arabidopsis thaliana*	
	#	protein	#	protein	#	protein	#	protein	#	protein	#	protein	#	protein	#	protein	#	protein
DROSHA	1	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	dgcr8	1	pasha	1	Dgcr8	1	DGCR8	1	DGCR8	0	
DICER	1	DICER1	1	Dicer1	1	DICER1	1	dicer1	2	dcr-1, dcr-2	1	Dicer-like1	1	HELRODRAFT_103772	1	dcr-1	4	DCL1, DCL2, DCL3, DCL4
dsRBP partner	2	TARBP2, PACT		Tarbp2, PACT	1	TARBP2	2	tarbp2, prkra	2	r2d2, loqs	1	*BRAFLDRAFT_242885*	1	HELRODRAFT_102635	1	rde-4	7	DRB1/HYL1, DRB2, DRB3, DRB4, DRB5, DRB6, DRB7
ARGONAUTE	8	AGO1, AGO2, AGO3, AGO4, PIWIL1, PIWIL2, PIWIL3, PIWIL4	7	AGO1, AGO2, AGO3, AGO4, MIWI, MIWI2, MILI	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	4	HELRODRAFT_65165, HELRODRAFT_182062, HELRODRAFT_75625, HELRODRAFT_65566	26	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		0		0		6?	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

Table 1 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.

analysis of Argonaute 2, which revealed the last missing piece of the big puzzle: this protein carries the endonucleolytic activity executing (slicing) targeted mRNAs (Liu et al., 2004; Meister et al., 2004; Song et al., 2004).

RNAi and miRNA pathway components

Substrates

RNA silencing employs different types of substrates, which give rise to different kinds of small RNA populations (Fig. 3). Canonical precursors of miRNAs contain short hairpin structures, which are released by the Microprocessor complex and processed by Dicer in the cytoplasm (reviewed in Kim et al., 2009; Winter et al., 2009). However, additional substrates can produce non-canonical miRNAs, which can be generated in Drosha- and/or Dicer-independent manner. 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 repeats. The most efficient long dsRNA formation comes from RdRP synthesis followed by efficient forming intramolecular duplexes. Sense and antisense base pairing is, in principle, 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.

Dicer – structure and function

Dicer is the central enzyme for producing small RNAs in miRNA and RNAi pathways. It was discovered in 2001 as the enzyme processing long dsRNA into siRNAs (Bernstein et al., 2001). Dicer 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. It carries two RNase III domains and several other domains that are typically found in Dicer-like proteins in eukaryotes (Fig. 4). 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).

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 structure sets 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; Ma et al., 2004; Song et al., 2003; Yan et al., 2003);

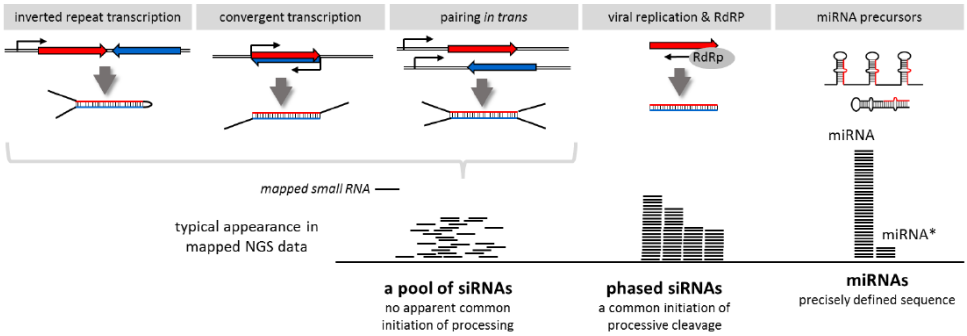

Figure 3 Substrate types in RNA silencing pathways.
The lower part schematically depicts typical appearance of small RNAs in mapped RNA-sequencing data.

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., 2007; MacRae et al., 2006), 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 (Dlakic, 2006) and perhaps mediates protein-protein interaction (Qin et al., 2010). Full-length Dicer proteins from animals and plants were not crystalized. However, recent advances in cryo-electron microscopy (cryo-EM) provide additional insights into Dicer structure and function in other models (Fig. 4), which utilize more complex Dicer proteins. These results will be summarized in corresponding taxon-specific reviews.

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. In plants, some Dicer proteins have a dedicated nuclear localization.

dsRNA binding proteins (dsRBPs)

Dicer interacts with many proteins of which two protein types stand out: (I) Argonaute proteins, which receive small RNAs produced by Dicer, and (II) dsRNA-binding proteins

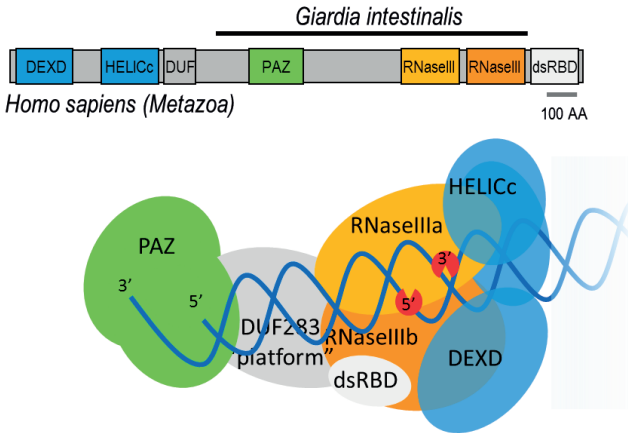

Figure 4 Dicer structure
The first Dicer structure was inferred from the crystal structure of *Giardia intestinalis*, which corresponds to a fragment of larger metazoan Dicer proteins. The schematic structure of human Dicer was subsequently derived from cryo-electron microscopy analysis. Dicers typically cleave dsRNA from an end, which are bound by the PAZ domain. Each of the RNase III domains cleaves one strand at a defined distance from the PAZ domain.

(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 will be discussed for each taxon separately.

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 (Fig. 5). The PAZ domain binds the 3' end of a short RNA in a sequence-independent manner (Lingel et al., 2003, 2004; Ma et al., 2004; Song et al., 2003). Structural studies of archaeal Argonaute homologs showed that the PIWI domain has an RNase H-like fold (Ma et al., 2005; Parker et al., 2004; Song et al., 2004; 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.

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

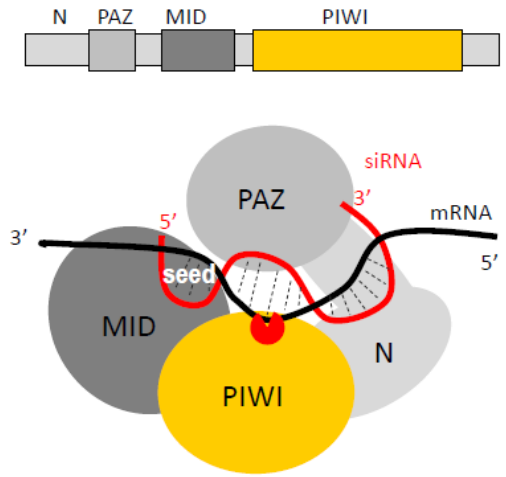

Figure 5 Argonaute protein structure
The order of domains in an Argonaute protein. The scheme shows how Argonaute cleaves a perfectly complementary RNA, which becomes accessible by the catalytical center in the PIWI domain upon base pairing with a small RNA. Nucleotides 2–8 of the small RNA initiate 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.

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 *Caenorhabditis 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 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 (Fig. 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 reviews.

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 (Martinez et al., 2002; Mourelatos et al., 2002; Nykanen et al., 2001; 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.

RNA-dependent RNA polymerase (RdRPs) proteins

RdRPs can contribute to RNA silencing in two ways – either by converting single-stranded RNA to dsRNA (Fig. 1) 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 *Caenorhabditis elegans* (Grishok et al., 2001; Smardon et al., 2000), SDE1/SGS2 in *Arabidopsis* (Dalmay et al., 2001; Mourrain et al., 2000), 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. *Caenorhabditis 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 (Roignant et al., 2003; Schwarz et al., 2002; Stein et al., 2003).

Diversity of RNAi and miRNA pathway functions

Some taxons, exemplified by 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 in the Table 1. Other pathways diversified, often at the level of Argonaute protein adaptation for different pathways (Table 2). In mammals, 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 *Caenorhabditis 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.

RNAi pathway

The RNAi pathway (Fig. 1) has three mains 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

model organism	common name	subfamily	slicer	associated small RNA				
				type	length	5' nt	5' end	3' end
Homo sapiens	**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
	PIWIL4	PIWI	+	piRNA	27 - 29	U	mono-P	2'-O-met
Mus musculus	**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
	MILI (PIWIL2)	PIWI	+	piRNA	24 - 28	U	mono-P	2'-O-met
Drosophila melanogaster	**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
	AUB	PIWI	+	piRNA	23 - 27	U	mono-P	2'-O-met
Caenorhabditis elegans	**ALG-1, F48F7.1**	AGO	+	miRNA	22-23		mono-P	-OH
	ALG-2, unknown	AGO	+	miRNA	22-23		mono-P	-OH
	ALG-3, T22B3.2	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
Arabidopsis thaliana	**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	+	intergentic 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

Table 2 Overview of Argonaute proteins and associated RNAs in key model organisms
The table was compiled form the following literature (Batista et al., 2008; Buckley et al., 2012; Das et al., 2008; Duran-Figueroa and Vielle-Calzada, 2010; Fischer et al., 2011; Forstemann et al., 2007; Iwasaki et al., 2015; Liu et al., 2009; Tijsterman et al., 2002a; Tijsterman et al., 2002b; Vasale et al., 2010; Vourekas et al., 2012; Wang and Reinke, 2008; Yigit et al., 2006; Zhang et al., 2016; Zheng et al., 2007). 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.

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 Marques and Carthew, 2007; Xie and Guo, 2006): 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).

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 *Caenorhabditis 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.

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. 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. Two pathways for dsRNA uptake were described: (1) a specific transmembrane channel-mediated uptake and (2) an alternative endocytosis-mediated uptake (reviewed in Huvenne and Smagghe, 2010; Whangbo and Hunter, 2008).

The non-cell autonomous RNAi was observed already during the first RNAi experiments in *Caenorhabditis 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).

miRNA pathway

Unlike siRNAs, miRNAs are genome-encoded short RNAs with defined sequences 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. 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.

Thousands of miRNAs have been annotated. The central miRNA database miRBase (http://www.mirbase.org, (Kozomara and Griffiths-Jones, 2014) includes 2654 human, 1978 murine, 469 *Drosophila melanogaster* , 437 *Caenorhabditis elegans*, and 428 *Arabidopsis thaliana* mature miRNAs (release 22.1). Remarkably, 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.

Animal miRNAs 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 (Fig. 2).

The AGO-containing effector complex has been given different names; here it will be referred to 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 (Doench et al., 2003; Hutvagner and Zamore, 2002), 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 (Chen et al., 2014; Djuranovic et al., 2012; Nishihara et al., 2013; 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).

There are only minor differences in miRNA pathways 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 in piRNA small RNAs 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.

Other relevant pathways in Metazoa

Adenosine deamination

A-to-I editing is mediated by Adenosine Deaminases Acting on RNA (ADAR) enzymes, which contain dsRBD domains and recognize both inter- and intramolecular dsRNAs longer than 20–30 bp (Nishikura et al., 1991). ADARs convert adenosines to inosines, which translation and reverse transcription interpret as guanosines. ADARs were found in animals (including earliest branching groups) but not plants, yeasts or protozoa (Grice and Degnan, 2015; Nishikura, 2010). It was predicted that more than 85% of pre-mRNAs could be edited, predominantly in the non-coding regions (Athanasiadis et al., 2004).

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; Du and Zamore, 2005; Meister and Tuschl, 2004). 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). ADAR mutants in *Caenorhabditis elegans* exhibit defective chemotaxis while the phenotype can be rescued by RNAi-deficiency(Tonkin and Bass, 2003). In mammalian cells, ADAR1 limits siRNA efficiency (Yang et al., 2005). Editing can affect 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). Several pri-miRNAs (e.g. miR-142) undergo editing, which inhibits miRNA biogenesis or causes even degradation of pri-miRNA by TSN (Kawahara et al., 2007a; Nishikura, 2010; Scadden, 2005; Yang et al., 2006).

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 (eIF2α) (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 NFκB transcription factor and many 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 *Caenorhabditis 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. Notably, there is also connection between interferon pathway and A-to-I editing; analysis of mutant mice showed mice suggested that *Adar1* targets dsRNA and prevents MDA5-mediated interferon response (Liddicoat et al., 2015).

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