Current Biology 24, R703–R715, August 4, 2014 http://dx.doi.org/10.1016/j.cub.2014.06.009

Ancient Endo-siRNA Pathways Reveal New Tricks

Review

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Endogenously produced small interfering RNAs (endo-siRNAs, 18–30 nucleotides) play a key role in gene regulatory pathways, guiding Argonaute effector proteins as a part of a functional ribonucleoprotein complex called the RISC (RNA induced silencing complex) to complementarily target nucleic acid. Enabled by the advent of high throughput sequencing, there has been an explosion in the identification of endo-siRNAs in all three kingdoms of life since the discovery of the first microRNA in 1993. Concurrently, our knowledge of the variety of cellular processes in which small RNA pathways related to RNA interference (RNAi) play key regulatory roles has also expanded dramatically. Building on the strong foundation of RNAi established over the past fifteen years, this review uses a historical context to highlight exciting recent developments in endo-siRNA pathways. Specifically, my focus will be on recent insights regarding the Argonaute effectors, their endo-siRNA guides and the functional outputs of these pathways in several model systems that have been long-standing champions of small RNA research. I will also touch on newly discovered roles for bacterial Argonautes, which have been integral in deciphering Argonaute structure and demonstrate key functions of these conserved pathways in genome defense.

Introduction

In 1993, Lee *et al.* and Wightman *et al.* described the first microRNA (miRNA) called *lin-4* in *Caenorhabditis elegans*. Their work pointed to *lin-4* as a negative regulator of the protein-coding gene *lin-14*, and the authors correctly hypothesized that this regulation occurred by an anti-sense RNA based mechanism [1,2]. These same studies identified *lin-4* in four other species of nematode, pointing to conservation of these tiny RNA-based regulators of gene expression [1,2]. Within a decade, we would learn that miRNAs (initially termed small temporal RNAs, stRNAs) were broadly conserved from plants to humans, and there would be indications of other types of endogenously produced small silencing RNAs in several organisms [3–13].

Throughout the 1990s, key parallel studies were homing in on related small RNA-mediated silencing processes that were collectively called PTGS (post-transcriptional gene silencing) in plants, quelling in fungi, and RNAi (RNA interference) in worms. Initially, researchers working in several model systems attempted to down-regulate the expression levels of endogenous genes by introducing RNAs that were antisense to a gene of interest [14–16]. Other studies attempting to increase the expression of a gene of interest by adding additional copies of that gene surprisingly obtained the opposite result: reduced expression in a process called co-suppression. One well-known example of this phenomenon resulted from studies of petunias in the early 1990s, when researchers attempted to produce more potently colored flowers by introducing extra copies of genes involved in the pigmentation pathway. Instead, they observed increasingly variegated and white flower coloration, along with a corresponding decrease in the steady state levels of mRNAs for the gene of interest [17,18]. Years later, the reason for this phenomenon was better understood: the extra copies of the genes produced structured (hairpin) RNA that could generate small RNAs to silence gene expression [19]. By the end of the decade, these results came into focus, with the identification of double-stranded RNA (dsRNA) as the initiator of gene silencing, and the characterization of small interfering RNAs (siRNAs) that were similar to miRNAs [19–23]. At this point, it became clear that phenomena such as PTGS and quelling were linked to the overarching process that we now call RNA interference.

At the same time as the small RNAs involved in gene silencing pathways were identified, the proteins necessary for small RNA pathways were also emerging from genetic and biochemical studies. One key factor, the Argonaute effector protein (Ago), was identified genetically in *Arabidopsis thaliana* in 1998, and acquired its name based on the phenotype of *ago1* mutant plants. These mutants possessed filamentous structures that resembled the tentacles of the sea creature *Argonaut*, a type of squid or octopus [24]. Shortly thereafter, Argonaute genes were genetically linked to various forms of small RNA-mediated silencing in plants, flies, fungi and worms, while biochemical studies linked Argonautes to the effector RNA induced silencing complexes (RISCs) [25–32]). Notably, loss of Argonautes in many species results in severe developmental defects, including sterility and embryonic lethality [24,33,34]. Similarly, the RNAse III type enzyme Dicer was identified biochemically and genetically as an endonuclease required for the production of small RNAs from both exogenous sources and miRNA precursors. Like Argonaute, Dicer was also shown to be essential for proper development in a number of organisms [7,12,35–38] (for review see [39]). This mounting body of evidence made it increasingly clear that endogenous small RNA pathways were involved in regulating key developmental processes and were linked to the process of RNAi.

RNAi: What's in a Name?

The term RNA interference has widely been used to describe a phenomenon in which the introduction of dsRNA complementary to a gene of interest could lead to the silencing of that gene [23,40]. Over time, this term has been expanded to encompass pathways ranging from this original phenomenon of 'classical' or 'exogenous' RNAi (exo-RNAi), to a variety of endogenous small RNA pathways that utilize overlapping factors, such as Argonautes and Dicer, to regulate gene expression at both the transcriptional and post-transcriptional level (for review see [41]).

During exo-RNAi, short siRNA duplexes are cleaved from exogenous dsRNA by Dicer and are loaded onto Argonautes by a protein complex called the RISC loading complex [42–44]. The strand of the siRNA duplex possessing the less thermodynamically stable 5′ end is retained as the guide RNA

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Table 1. endo-siRNA features across species.

Organism	Small RNA	Length (nucleotides)	5′ nucleotide	3′ modification	AGO	Biogenesis factors
Mouse	Endo-siRNA	21	A/U	-	Ago2	Dicer
Drosophila	Endo-siRNA	21	U	2′ O-methylation	Ago2	Dicer
C. elegans	26G-RNA	26	G	2′ O-methylation No methylation	ERGO-1 ALG-3/4	Dicer, RdRP: RRF-3
	22G-RNA	22	G, triphosphate	-	WAGOs CSR-1	RdRPs: EGO-1, RRF-1
Arabidopsis	*cis*-nat-RNAs	21–22	-	2′ O-methylation	-	Variable; RDR6, RDR2, DCL1
	rasiRNAs/hc siRNAs	24	A	2′ O-methylation	AGO4/6/9	RDR2 DCL3
	tasiRNAs	21–22	U	2′ O-methylation	AGO1 (AGO7)	RDR6 DCL4

This table serves as a quick guide for the basic features of each endo-siRNA pathway discussed in this review. Unless otherwise noted, 5′ nucleotides are monophosphate (Dicer products).

[45–47]. In addition, the identity of the 5′ nucleotide also influences the selection of the guide strand [48–50]. In turn, the guide RNA provides the RISC with sequence specificity in the identification of the target transcripts to be regulated.

Because small RNAs impart sequence specificity to the RISC, a major effort in the field over the past decade has been to comprehensively identify small RNA species. High throughput sequencing technologies have driven these efforts forward, and have led to the identification of three major classes of small RNAs: piRNAs (Piwi-interacting RNAs), endo-siRNAs (endogenous siRNAs) and additional miRNAs in numerous species (for review see [51]). These discrete classes of small RNAs have been categorized based on their size, first nucleotide identity, biogenesis pathways, and association with particular Argonaute proteins.

As the first endogenous small RNAs identified, and owing to their high degree of conservation, miRNAs have become the most widely studied class of small RNAs [1–3]. Generally, miRNAs are genomically encoded and transcribed by RNA Polymerase II [52]. Primary miRNA transcripts form hairpin molecules that are processed into short dsRNA by the tandem action of RNAse III enzymes Drosha (in the nucleus) and Dicer (in the cytoplasm). Mature miRNAs (21–23 nucleotides, nt) are loaded onto a group of widely conserved Argonautes to form miRISCs (miRNA-loaded RISCs) that function to regulate protein-coding transcipts. While still open to debate, a growing body of evidence supports a model in which miRISCs can induce translational inhibition, target degradation, or both, depending on the degree of complementarity between miRNA and target (for review see [53,54]).

piRNAs (24–29 nt in flies and mice, 21 nt in worms) are expressed in animal gonads and function in genome surveillance [55–60]. The mechanisms of piRNA biogenesis vary between mice, flies and worms, and many details of this process remain mysterious. However, there are several defining features of piRNAs, including that they do not require processing by Dicer, and are loaded onto a subset of Argonautes known as Piwis (P-element induced wimpy testes; for review see [61]). The targets of piRNAs are generally transposable elements and protein-coding genes, which are regulated at both the transcriptional and post-transcriptional level by this pathway (for review see [62]).

Endo-siRNAs are present in a variety of organisms, and are a somewhat diverse class of small RNAs in terms of their functions and biogenesis requirements. Endo-siRNAs act at both the transcriptional and post-transcriptional level, depending on the pathway. In general, they provide an additional layer of control on protein-coding gene expression or serve as a cellular defense system against foreign or deleterious nucleic acid, such as transposable elements (for review see [63–65]). Because of their intriguing and diverse roles, the rest of this review will focus on endo-siRNA pathways, placing emphasis on the Argonaute binding partners and functions of these pathways in mice, *Drosophila* (flies), *C. elegans* (worms), and *Arabidopsis* (plants).

Endo-siRNA Biogenesis: To Dice or Not to Dice?

Central to the biogenesis of endo-siRNAs is the production of dsRNA [37]. Endogenous sources of dsRNA vary among different organisms and result in siRNAs that are shuttled into different gene regulatory pathways. Some of these pathways require only Dicer for the production of small RNAs, while others rely on RNA-dependent RNA polymerases (RdRPs) to synthesize small RNAs, and some rely on both (Table 1) [41]. In *Drosophila* and mammals, RdRPs are not present, and long dsRNA results from events such as: (i) the bidirectional transcription of a locus (*cis* natural antisense transcripts; *cis* nat-RNA); (ii) the interaction of complementary transcripts derived from separate loci, such as transposable elements or pseudogenes (*trans* nat-RNA); and (iii) the formation of extended hairpin structures distinct from miRNAs, such as those that could result from inverted repetitive sequences) (Figure 1). In contrast, worms and plants predominantly utilize RdRPs, along with Dicer in some instances, to generate endo-siRNAs (Figures 2 and 3). Despite the differences in the sources of endo-siRNAs in various organisms, the functions can be broadly grouped into regulation of endogenous gene expression and genome protection from both endogenous and exogenous selfish genetic elements.

In *Drosophila*, the endo-siRNA pathway utilizes factors that overlap with the exo-RNA pathway, including one of two specialized Dicer proteins, Dcr2 and the Argonaute Ago2 (Table 1) [66–72]. In mice, a single Dicer protein is present, and is utilized by the endo-siRNA pathway, along with Ago2, an Argonaute that also acts in the miRNA pathway (Table 1) [73,74]. Interestingly, *Drosophila* endo-siRNAs are distinguished by a 2′-*O*-methyl modification at their 3′ end. This modification is catalyzed by the methylase Hen1, and is also found on piRNAs in animals and small RNAs in plants [66,67,69,71,72]. In both flies and mice, endo-siRNAs have a discrete 21-nt signature [66–74].

In contrast, *C. elegans* and *Arabidopsis* possess extensive and complex endo-siRNA networks, composed of

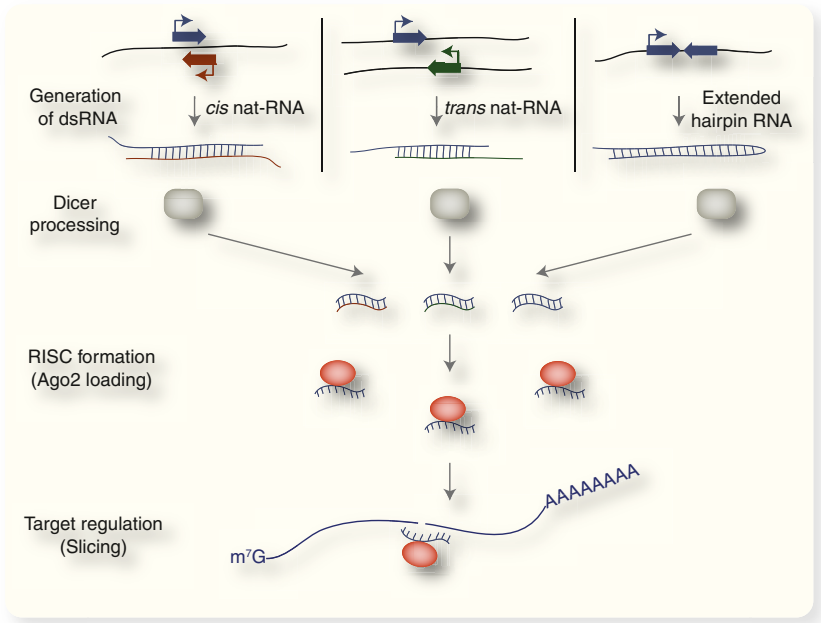

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Figure 1. Production of endo-siRNAs in *Drosophila* and mice.

dsRNA is generated from various sources, including *cis* natural antisense transcripts that overlap in their 3′ ends and result in dsRNA from bi-directional transcription; *trans* natural antisense transcripts that possess complementarity to each other but are transcribed from genes located in different regions of the genome; and transcription from inverted repetitive elements, including transposons, to produce extended hairpin molecules. dsRNA is processed by Dicer into siRNA duplexes (in *Drosophila*, this process involves Dicer2 and Loqs-PD). siRNA duplexes are loaded onto Ago2, guide strands are retained in RISC complexes, and targets with complementarity to the small RNAs, including transposon transcripts and protein-coding genes, are identified for regulation (in *Drosophila*, this step also requires the RNA binding protein R2D2).

pathways that rely on RdRPs and Dicer (Figures 2 and 3; for review see [75,76]). Complexity results, in part, from the presence of multiple RdRPs, Argonautes, and, in plants, Dicer enzymes (Table 1). This cornucopia of factors enables primary and secondary steps of endo-siRNA synthesis to amplify and specify the silencing signal. For instance, in worms, one RdRP, RRF-3, synthesizes a long complementary strand of RNA using mRNA as a template (Figure 2A). The resulting dsRNA is cleaved into 26G-RNAs (26 nt, possessing a 5′ guanine), which are loaded into a set of primary Argonautes [77–82]. In turn, the targeting of transcripts by 26G-RNA RISCs stimulates the production of a secondary set of endo-siRNAs, the 22G-RNAs (22 nt, possessing a 5′ guanine), by two additional RdRPs, EGO-1 and RRF-1, which act independently of Dicer [77–82]. 22G-RNAs are loaded into secondary Argonautes, and represent the predominant species of endo-siRNAs in *C. elegans*. It should also be noted that there are subsets of 22G-RNAs that do not require 26G-RNAs for their biogenesis [77,82–84]. This cascade of endo-siRNA production is a common theme in *C. elegans*, as a similar approach is utilized in the exo-RNAi pathway, and is likely to play a role in amplifying and reinforcing the gene regulatory signal [85–88].

As in worms, plant primary small RNAs are derived by Dicer processing from a dsRNA precursor and can lead to the production of secondary endo-siRNAs. In plants, primary small RNAs can include miRNAs or endo-siRNAs generated from extended hairpins, *cis* nat-RNAs, or RdRP activity on a template transcript (Figure 3; for review see [89]). For instance, the tasiRNAs (*trans*-acting siRNAs) are secondary endo-siRNAs produced when transcripts derived from a handful of specific loci throughout the genome (*TAS* loci) are targeted and cleaved by particular miRISCs. Cleavage of the *TAS* transcript triggers the synthesis of dsRNA by the RdRP RDR6, which is processed by Dicer DCL4 into tasiRNAs (21 nt) [90–96].

In contrast to other model organisms, where the majority of endo-siRNA regulation occurs in the cytoplasm, the largest subset of plant endo-siRNAs acts in the nucleus (Figure 3C). One type of nuclear-directed endo-siRNA is the Repeat-associated siRNA group (rasiRNAs, 24 nt, also called heterochromatic siRNAs). RasiRNAs are derived from heterochromatic transcripts synthesized by a specialized RNA Polymerase, Pol-IV. These transcripts are converted to dsRNA by the RdRP RDR2 and then processed by the Dicer DCL3 before being loaded onto a subset of Argonautes to target distinct genomic loci for repression in a process called RNA-directed DNA methylation (RdDM) (for review see [97]). Another recently discovered group of nuclear endo-siRNAs is the double-strand-break-induced RNAs (diRNAs, 21 nt and 21 nt). Although their biogenesis and function are still mysterious, as their name implies, they appear to play a role in repairing double-stranded DNA breaks [98].

Argonaute Effectors: To Slice or Not to Slice?

Argonautes have been identified and studied for their gene regulatory roles in organisms from all three domains of life (for review see [99]). The number of Argonautes per species varies greatly and does not necessarily correlate with organismal complexity; for instance, humans possess eight Argonautes, while *C. elegans* has 27, and *Arabidopsis* has 10 (for review see [100]). One extreme example is *S. cerevisiae*, which has lost the RNAi silencing machinery in lieu of maintaining a cytoplasmic dsRNA viral system [101,102]. This viral system, called ‘killer’, produces a fungicidal toxin that kills off competing fungi while providing immunity to its host, thus conferring a competitive advantage (for review see [103]). At the other extreme is the pig and human parasite *Trichinella spiralis,* which possesses hundreds of putative Argonaute genes [104]. While work in model organisms has shown that the increased numbers of Argonautes can lead to functional specialization and redundancy in key gene regulatory processes, the evolutionary trade-offs driving Argonaute expansion and retention in specific organisms are less understood.

Argonaute proteins are characterized by three conserved domains: PAZ (Piwi-Argonaute-Zwille), Mid (Middle), and

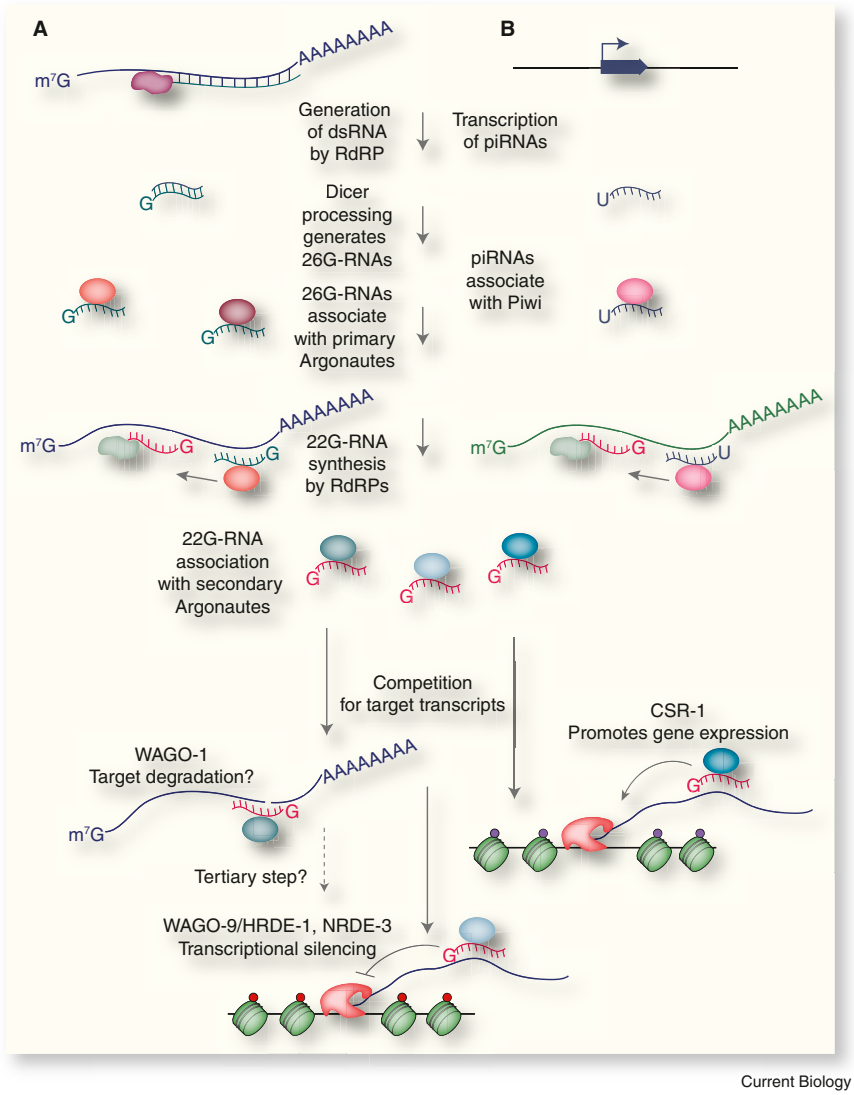

Figure 2. Production of endo-siRNAs in *C. elegans*.

(A) Protein-coding genes, pseudogenes, transposable elements, and poorly annotated genes are transcribed and serve as a template for the RdRP RRF-3 to synthesize long dsRNA. Dicer then cleaves the dsRNA into 26G-RNAs, and the anti-sense strand guide RNA is loaded into the primary Argonautes ALG-3, ALG-4 or ERGO-1 depending on the tissue and type of target transcript. Argonautes loaded with 26G-RNAs lead to the production of 22G-RNAs from target transcripts, dependent on the RdRPs EGO-1 and RRF-1. The 22G-RNAs are loaded into secondary Argonautes of the expanded worm family, including WAGO-1, WAGO-9/HRDE-1, NRDE-3, and CSR-1. The secondary Argonautes elicit a variety of effects in the cytoplasm and nucleus to impact gene expression. (B) Foreign nucleic acid is recognized as 'non-self' by PRG-1 and its 21U-RNA/piRNA binding partners. This recognition sets off a cascade whereby 22G-RNAs antisense to the foreign nucleic acid are generated and loaded into the WAGO-type Argonautes WAGO-1, WAGO-9/HRDE-1, and WAGO-10. The WAGOs induce silencing of the transcript at both the transcriptional and post-transcriptional level. CSR-1 acts in a manner that is antagonistic to the genome surveillance pathway, to license the expression of 'self' transcripts.

PIWI (P element induced wimpy testes). In addition to these domains, a globular but variable amino-terminal region is present, and two structured linker regions (L1, L2) join the amino terminus to PAZ, and PAZ to MID, respectively (Figure 4A) (for review see [105]). Although it was clear from the outset that the RISC possessed endonucleolytic activity, the protein(s) responsible for this activity remained mysterious for several years [106,107]. The first full-length structure of an Argonaute from *Pyroccocus furiosis* revealed that the active site within the PIWI domain adopted a fold similar to that of the enzyme RNAse H, and highlighted that both enzymes possess three key catalytic residues (DDH) for endonucleolytic cleavage [108]. Biochemical studies focusing on mammalian Ago2 supported this observation and demonstrated that Ago2 was capable of 'slicing', cleaving an mRNA target in a manner dependent on a complementary small RNA and the three conserved catalytic residues (along with magnesium cations) [107,109–111].

In subsequent years, conserved features of the Argonautes have been revealed by studies of both full-length proteins and particular Argonaute domains from archaebacteria, eubacteria and fungi, in complex with guide nucleic acids. For instance, the small RNA 5′ end is anchored in a binding pocket of the MID domain, with 5′ nucleotide preference being conferred by key residues of a nucleotide specificity loop [112–116]. The 3′ end of the small RNA is positioned in the oligonucleotide binding fold of the PAZ domain, facilitating the alignment of key portions of the small RNA–target-RNA duplex along the active site of the PIWI domain [116–121].

While a full-length eukaryotic Argonaute structure remained elusive for some time, within the past two years the structures of human Ago2 and Ago1 and a yeast Ago (*Kluyveromyces polysporus* Argonaute, KpAgo) in complex with small RNAs have been solved (Figure 4B) [122–126]. Overall, these structures show striking similarity to their prokaryotic counterparts and to each other (Figure 4B–D). Importantly, the structure of KpAgo revealed that an additional glutamic acid in a particular configuration is required to form an active site that is a tetrad (DEDD for KpAgo, DEDH for human Ago2), as opposed to a triad (Figure 4E) [125]. This particular glutamic acid configuration is stabilized by hydrogen bonding from several other conserved residues and correlates with release of the guide RNA 3′ end by PAZ, thus indicating that conformational changes are necessary to produce a complete active site for slicer Agos [123,125,126].

Although the presence of the catalytic tetrad is necessary for Argonaute slicing activity, it is apparently not sufficient. Of the four human Argonautes, only Ago2 has been shown to be capable of slicing *in vitro*, in spite of the fact that Ago3

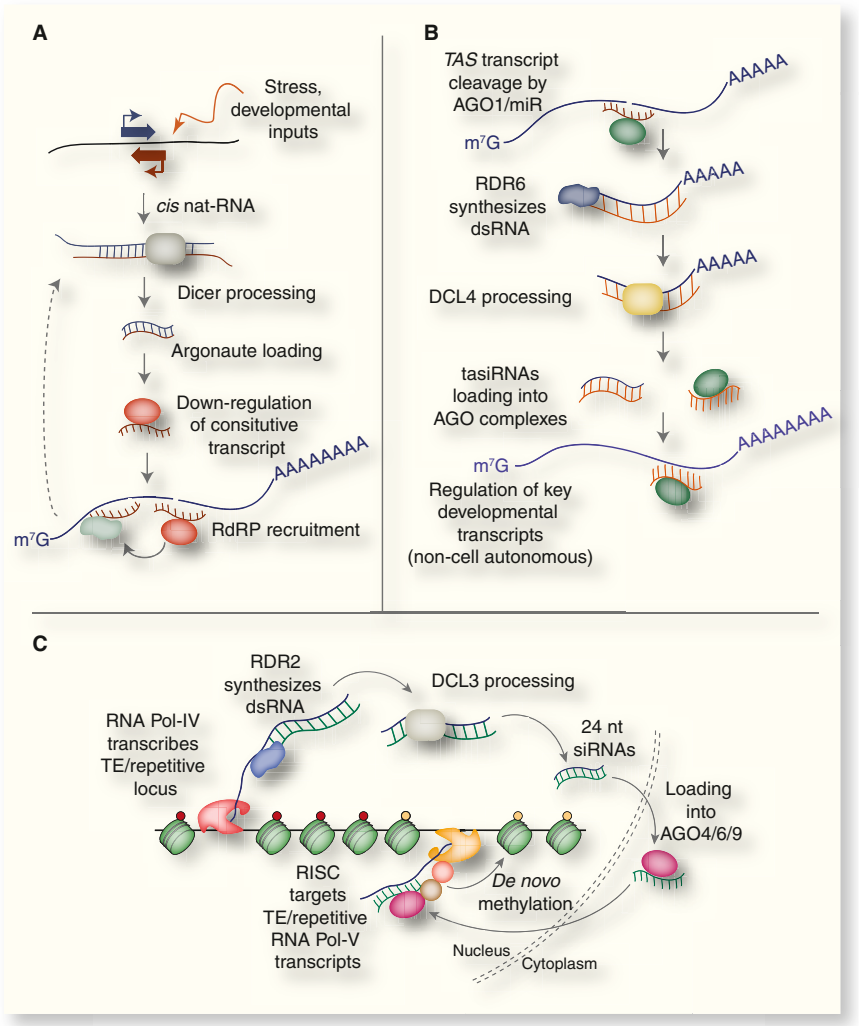

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Figure 3. Endo-siRNA pathways in *Arabidopsis*.

(A) *cis* nat-RNAs are generated from overlapping transcript pairs, one of which is constitutively expressed and the other of which is induced during development or stress. Some of these loci form endo-siRNAs that down-regulate the constitutively expressed gene and enable the plant to better tolerate the stress. (B) *TAS* RNAs are generated from several loci throughout the genome. These transcripts are targeted by particular miRNAs loaded into AGO1 or AGO7, and cleaved. In the mode of tasiRNA biogenesis depicted here, a single miRNA site is utilized, but there are instances in which two sites are utilized (AGO7/mi390). RDR6 and DCL4 produce tasiRNAs from the cleaved *TAS* transcripts. tasiRNAs can then act non-cell autonomously to target key developmental regulators. (C) In the process of RdDM, RNA Pol-IV produces transcripts that are used by RDR2 and DCL3 to produce rasiRNAs. The rasiRNAs are loaded into AGO4/6/9 and then recruited to other repetitive or TE loci transcripts generated by another RNA polymerase, RNA Pol-V. Other chromatin modifying factors are recruited to induce *de novo* DNA methylation.

also possesses a DEDH motif [110]. Several groups recently demonstrated that restoring the catalytic tetrad in Ago1 (from DEDR to DEDH) did not convert Ago1 into a slicer [123,126,127]. Instead, in a series of domain swaps and deletion studies, the presence of the Ago2 amino-terminal domain stimulated slicer activity of Ago1 and Ago3 in conjunction with an intact catalytic tetrad [123,127]. Site-directed mutagenesis studies also identified several key residues in a loop of the protein adjacent to the catalytic motif that were necessary for Ago2 to slice and, when introduced into Ago1, stimulated slicer activity [123,126,127]. One additional factor that contributes to slicing capacity but is independent of Argonaute structure is the degree of base-pairing between guide and target RNAs, with more extensive base-pairing leading to endonucleolytic activity [128,129] (for review see [130]).

Clearly, slicer activity is a more complicated affair than was previously thought, and additional studies will identify other key residues and conformational changes that contribute to catalytic activity going forward. It is also important to remember that, like Ago1, not all Agos possess catalytic activity, yet they are still capable of influencing gene expression by various means, ranging from translational inhibition to transcriptional silencing. Such activities are likely to be influenced by Argonaute binding partners and post-translational regulation of RISC components, many of which remain to be characterized (for review see [131]).

Everything in Moderation: Regulating the Expression of Endogenous Genes

One key role for endo-siRNA pathways is in modulating the levels of endogenous genes. In *Drosophila*, endo-siRNAs derived from *cis* nat-RNA transcripts were shown to silence, in *trans*, distinct protein-coding genes (Figure 1). Accordingly, loss of *ago2* or *dcr2* led to increases in transcript levels for these *trans*-targeted genes [66–72]. Similarly, in mouse embryonic stem cells (mESCs) and oocytes, endo-siRNAs derived from mRNA/pseudogene dsRNAs (*trans* nat-RNAs) play a negative gene regulatory role, as loss of *Dicer* or *Ago2* leads to an up-regulation of target mRNAs [73,74,132]. In addition to the role of *Drosophila* Ago2 in post-transcriptional gene regulation, Ago2 has been shown to associate with chromatin, possibly via promoter-derived endo-siRNAs, to regulate transcriptional responses under conditions of stress, such as heat shock [133]. Together, these studies highlight roles for endo-siRNAs in both transcriptional and post-transcriptional gene regulatory functions during development and in response to stress.

In worms, the endo-siRNA network plays a major role in regulating germline gene expression (Table 1). The 26G-RNA pathway can be broken into two branches, one of which targets spermatogenesis-related mRNAs during gametogenesis, and another which regulates extended gene families, poorly annotated loci, and pseudogenes that are expressed in oocytes and embryos [80,81]. While loss of the Argonautes in the spermatogenesis pathway leads to

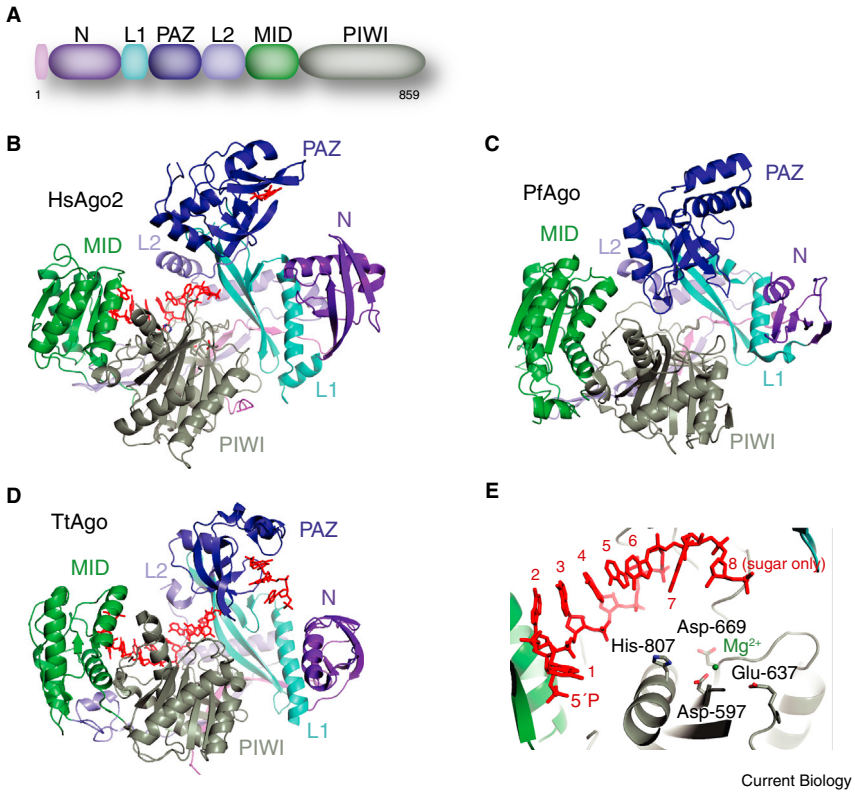

Figure 4. Argonaute proteins are the effectors of RNAi pathways.

(A) Diagram of the domains of human Ago2. This domain arrangement is conserved across species. Colors correspond to the colors used in subsequent crystal structures. (B) Crystal structure of human Ago2 in complex with small RNAs (solved by [124]). (C) Crystal structure of *Pyrococcus furiosis* Ago (solved by [108]). (D) Crystal structure of *Thermus thermophilus* Ago in complex with guide DNA (solved by [116]). (E) Close-up of active site of human Ago2, with small RNA in red. Nucleotide positions are numbered. Four catalytic residues, D597, E637, D669, H807, and a Mg^{2+} ion are present in the active site. All crystal structure images are courtesy of Dr Ian MacRae.

temperature-sensitive male sterility and alterations in the levels of target genes, loss of the Argonaute in the oocyte/embryo pathway has no overt phenotype despite displaying de-repression of target transcripts [77–82].

The 26G-RNA pathways feed into two major branches of the 22G-RNA pathway (Figure 2A). In one branch, the Argonaute WAGO-1 and its 22G-RNAs function downstream of both 26G-RNA pathways [77,80]. Loss of *wago-1* alone leads to no overt developmental defects, but loss of twelve *wago*s (worm Argonautes) that functionally overlap with *wago-1* leads to a loss of WAGO-1 associated 22G-RNAs and causes a temperature-sensitive decrease in fertility [83]. Several of these WAGOs, including NRDE-3 (nuclear RNAi deficient) and WAGO-9/HRDE-1 (heritable RNAi deficient) interact with subsets of WAGO-1 22G-RNAs and their recruitment to target gene loci correlates with accumulation of histone H3 lysine 9 methylation, RNA Polymerase II attenuation, and decreases in pre-mRNA levels [134–140].

In contrast to *wago-1*, loss of the Argonaute in the second branch of the 22G-RNA pathway, *csr-1*, leads to chromatin alterations, embryonic lethality, and sterility [84,86,141]. Although it was not initially evident that the CSR-1 22G-RNA pathway functioned secondarily to either 26G-RNA pathway, a key role for CSR-1 downstream of the spermatogenesis pathway in promoting, as opposed to silencing, the expression of a subset of spermatogenesis genes was recently revealed [82]. CSR-1 is recruited to its target gene loci and appears to promote euchromatin modifications and RNA Polymerase II activity, as the loss of this pathway leads to decreases in target gene pre-mRNA, mRNA, and protein steady state levels [82,84,142].

In plants, *cis* nat-RNAs are generated from a number of genomic loci under stressful conditions (Figure 3A). At these loci, one member of the dsRNA pair tends to be constitutively expressed, while the other is induced by stress. Endo-siRNAs derived from a subset of these loci target the constitutively expressed transcript to down-regulate its expression, thus conferring a tolerance to the stress [143,144]. The genetic requirements for *cis* nat-RNA-derived endo-siRNAs are varied, suggesting that they may not belong to a single group.

tasiRNAs serve as a diverse set of endo-siRNAs that are capable of regulating multiple members of extended gene families (Figure 3B). Key targets of tasiRNAs include genes such as those encoding transcription factors (such as auxin response factor, ARF) that serve as master regulators during differentiation [92,145,146]. In addition, tasiRNAs can act in a non-cell autonomous manner, enabling them to regulate targets in distant tissues and form regulatory gradients, akin to morphogens. At some loci, tasiRNA targeting has been linked to RdDM, thus indicating that tasiRNAs can act both post-transcriptionally and in the nucleus (for review see [147]).

Protection from Enemies Within: Transposon Defense

Early genetic studies in plants, flies, and *C. elegans* pointed to transposon silencing as a key function of endogenous small RNA pathways (for review see [148]). Transposable elements (TEs) of various types serve as a major threat to genome integrity, as their mobilization and reinsertion elsewhere in the genome can result in mutations in essential genes, activation of DNA damage responses, and other potentially mutagenic or deleterious outcomes [149].

Although the piRNA pathway serves as a key defense against repetitive transposable elements in animal gonads, endo-siRNA pathways have also been implicated in silencing TEs. In flies, TE transcripts are de-repressed in a manner consistent with post-transcriptional regulation in *ago2* or *dcr2* mutants [66–71]. In *Drosophila* somatic cells, the endo-siRNA pathway appears to be the main small RNA-mediated control mechanism over transposons, supported by the observation that *ago2* mutants show increased levels of TE expression in neurons, leading to several behavioral defects and a shortened lifespan [150].

In mice, retrotransposons are the targets of the piRNA pathway in both the male and female germline [151]. Loss of piRNA pathway components in males leads to DNA damage, transposon mobilization and sterility, but this is not the case for females [73,74,149,152]. Interestingly, in the female germline (but not the male) transposable elements are also an abundant source of dsRNAs, and these are shuttled into the endo-siRNA pathway [73,74,152]. As in flies, loss of *Ago2* or *Dicer* results in transposon de-repression [73,132,152]. Thus, the endo-siRNA pathway functions in an overlapping role with the piRNA pathway to repress TEs and protect the female germline.

In worms, the piRNA pathway has been shown to regulate only a small number of TEs [59,60]. Instead, the bulk of transposable and repetitive element silencing in the germline is executed by the WAGO-1 22G-RNA pathway [59,60,83]. Accordingly, loss of WAGO-1 pathway factors leads to de-repression of transposon transcripts and results in increased transposition [83,153].

In plants, repetitive and transposable elements are silenced predominantly via RdDM (Figure 3C). Rasi-RNAs are generated and loaded into a group of AGO4-related Argonautes, which are then recruited to repetitive loci throughout the genome via an interaction between the rasi-RNA and a nascent transcript (for review see [154,155]). Once at these target loci, the Argonaute recruits chromatin-modifying factors that induce the formation of heterochromatin. Ultimately, this process is self-reinforcing and leads to robust and heritable repression of transposable elements.

Defending Against Invaders: Anti-Viral Endo-siRNA Systems

It has long been thought that the variety of RNAi pathways present in various organisms evolved from cellular 'innate immune' defenses against RNA viruses. Although these activities have been well described in flies and plants, until recently these activities were not entirely well characterized in mammals or worms. RNA viruses produce dsRNA intermediates that can be shuttled into RNAi pathways during replication, and to a lesser extent, during viral transcription [156]. Many viruses also express viral suppressors of RNAi (VSRs), which inhibit various proteins in RNAi pathways, including Dicer (for review see [157]). Thus, the use of viruses possessing VSRs in differentiated cells may have prevented the identification of anti-viral RNAi responses in previous studies. Furthermore, in mammals, the protein-mediated interferon response in differentiated cells has long been thought to provide the predominant anti-viral response, as opposed to RNAi (for review see [158]). However, the interferon response is absent or attenuated in stem and germ cells, and in these cases the RNAi pathway appears to predominate as a cellular immune system, as highlighted by several recent papers [159] (for review see [160]).

Plants and flies were the first organisms in which anti-viral responses by RNAi pathways were demonstrated. Several types of viruses have been shown to be capable of infecting flies and fly-derived cell lines, and infection results in the production of 21-nt viral small RNAs [161–163]. Loss of the exo- and endo-siRNA pathway factors, including Dicer2 and Ago2, results in increased sensitivity to viral infection [163–166]. In plants, AGO1 is the predominant Argonaute that functions in anti-viral responses and mutants of *ago1* are hypersensitive to viral infection [25,167].

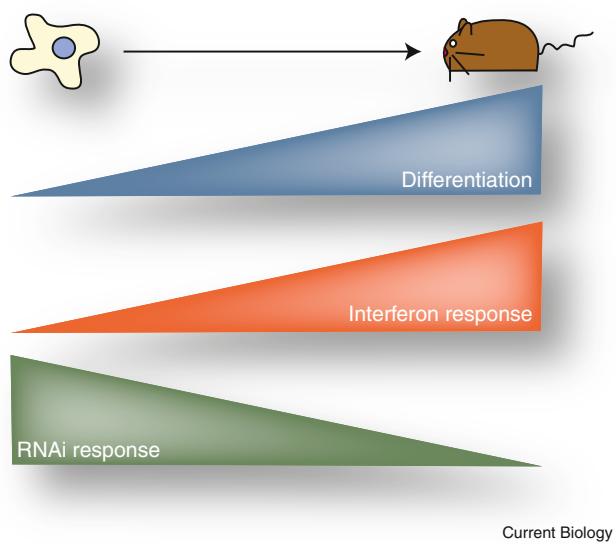

Figure 5. Anti-viral responses in mice.
In totipotent and pluripotent cells, RNAi responses predominate as an anti-viral response. As differentiation proceeds, the protein-mediated interferon response prevails.

In two recent studies, Maillard *et al.* and Li *et al.* showed that infection of mESCs, baby hamster kidney cells (BHK21 cells), and suckling mice with two types of virus, Nodamura virus (NoV) and encephalomyocarditis virus (EMCV), led to a potent RNAi response [168,169]. In these studies, mESCs infected with EMCV produced 21–23-nt viral small RNAs (vsRNAs), which were generated in a Dicer-dependent manner and loaded into Ago2 complexes [168,169]. Although a VSR for EMCV has not been identified, NoV has a potent VSR called B2, and only infection of mESCs and BHK21 cells with viruses in which the B2 protein was mutated led to the accumulation of vsRNAs [168,169]. Consistent with the notion that the interferon response is active in differentiated cells, upon differentiation of mESCs, vsRNAs were significantly depleted [168]. NoV rapidly kills young mammals, but surprisingly, when suckling mice were infected with a B2-deficient NoV, vsRNAs accumulated and the mice survived for the duration of the experiment (one month) [169].

It will be useful to discern which cell types or tissues are capable of maintaining RNAi-mediated anti-viral activity in young mice and why this occurs. Because induction of the interferon response is frequently detrimental to cell viability, perhaps it is logical that pluripotent cells would retain cell-autonomous defenses such as RNAi, where loss of individual cells would be deleterious, whereas differentiated cells could favor interferon responses to alert neighboring cells of infection (Figure 5). It should also be noted that the miRNA pathway has been shown to play a role in modulation of gene expression in response to viral infection in mammals. Together, these recent studies have provided the foundation for understanding a pathway that derives small RNAs from viral RNAs and targets them for degradation in mammals.

Surprisingly, there were no known viruses that infected *C. elegans* until the identification of the *C. elegans* Orsay virus in 2011 [170]. Instead, transgenic viral models had

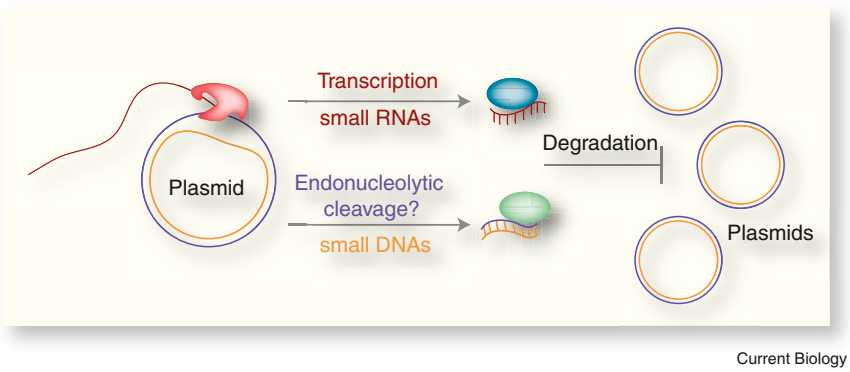

Figure 6. Bacterial RNAi pathways.

Argonautes in *Thermus thermophilus* and *Rhodobacter sphaeroides* have been shown to interact with guide DNAs (both species) and guide RNAs (*R. sphaeroides)* derived from plasmid sequences ('non-self' sequences). These Argonaute complexes are then capable of silencing plasmids to protect the bacterial genome.

been utilized to implicate RNAi factors and small RNAs in anti-viral responses [171–173]. It is a common practice for *C. elegans* researchers to 'bleach' worm strains during propagation in the lab, which is one likely reason why the first intact virus shown to infect lab strains of *C. elegans*, the positive strand RNA Orsay virus, was only first cultured from strains of *Caenorhabditis* nematodes taken from the wild. Notably, mutation of exo-RNAi pathway components, including the Argonaute required for exo-RNAi, RDE-1, and WAGO-1 22G-RNA pathway components supported increased levels of viral replication, implicating small RNA pathways in anti-viral responses [170,174]. Small RNAs complementary to viral sequences and bearing characteristics of 22G-RNAs were identified by deep sequencing studies from infected populations of worms, but were not found in worm populations exposed to bleach [170]. Together, these studies lay out a convincing case for RNAi responses as a potent and conserved anti-viral defense in various animal species.

Not in My Backyard: Transgenes and Plasmids Set Off Alarms

Much like viruses, foreign nucleic acid in the form of transgenes and plasmids is capable of initiating potent RNAi responses. *C. elegans* is the model in which this process has been studied most intensively in recent years, although the process has been studied extensively in plants, as well as in flies and mice [27,175]. Several recent studies have revealed that the silencing of foreign nucleic acid, such as single copy transgenes, is due to recognition by the piRNA pathway that leads to the production of secondary 22G-RNAs, akin to 26G-RNA pathways (Figure 5) [137,139,176–179]. The 22G-RNAs are loaded into WAGO-1 and WAGO-9/HRDE-1 complexes, which cause transcriptional and post-transcriptional silencing of the foreign nucleic acid [137–139,176,178]. The CSR-1 pathway counteracts piRNA-mediated silencing to license the expression of germline transgenes, likely at the transcriptional level [82,180,181]. Activating roles for small RNA pathways are unusual; however, there are several indications for such processes in mammals and *Drosophila*, and most of these involve chromatin-mediated activities [182–184]. Clearly the interplay between these opposing pathways is complex and will be a topic of intense interest going forward.

Although they have been incredibly useful for understanding Argonaute protein structure, until very recently the functions of prokaryotic Agos were unclear. Two recent reports from Olovnikov *et al.* and Swarts *et al.* revealed that two eubacterial Agos defend bacteria cells against foreign plasmid DNA (Figure 6) [185,186]. Notably, despite playing a similar role in cellular defense, these Agos, from *Thermus thermophilus* and *Rhodobacter sphaeroides*, act in distinct ways. For instance, TtAgo possesses an intact catalytic site and was shown to prefer to bind single-stranded DNA guides (siDNAs) derived from plasmids [186]. Loss of TtAgo led to an increase in plasmid yields and natural competency, indicating that TtAgo functions in defense against foreign sequences [186]. Furthermore, TtAgo (loaded *in vivo* with siDNAs) is capable of cleaving target plasmid DNA *in vitro* [186]. In contrast, the process in *R. sphaeroides* appears to be more complex, as RsAgo is associated with both RNA and DNA guides [185]. Notably, RsAgo appears to sample the bacterial transcriptome, as it is associated with sense-stranded RNA guides derived from protein coding genes and a plasmid present in the strain examined [185]. The majority of the small DNA complement associated with RsAgo was predominantly antisense and matching to the RNA guides for protein coding genes but possessed a significant bias toward repetitive, transposable elements, and plasmid sequences [185]. Expression of RsAgo in *Escherichia coli* led to a decreased yield of plasmid DNA, and loss of RsAgo resulted in increased expression of plasmid transcripts (but not endogenous genes), indicating that the system down-regulates foreign sequences [185]. RsAgo does not possess key catalytic residues, thus it may silence its targets without cleaving them or it may recruit an as yet unknown nuclease to do so [185]. These pathways, despite their distinctions, highlight a consistent role for Argonaute endo-siRNA pathways in the sequence-specific recognition of foreign nucleic acid as a cellular defense mechanism.

Pass It On: Transgenerational Inheritance

Several small RNA pathways have been shown to provide a cellular epigenetic memory of past gene expression and exposure to foreign or deleterious nucleic acids that can be transmitted to the next generation. This 'memory' is encoded by two major components: transmission of small RNAs through the germline, and inheritance of patterns of chromatin modification (histone modifications and DNA methylation in organisms where this process occurs) induced by the activity of nuclear small RNA pathways (for review see [187]). For instance, the piRNA pathway in mice and *Drosophila* provides a record of past exposure to various transposable elements [188–190]. Likewise, in *C. elegans* the piRNA/WAGO pathway (described above) transmits a memory of exposure to transgenes and foreign nucleic acid which should be silenced, while the CSR-1 pathway transmits a memory of which germline genes should be expressed [82,137–139,176–178,180,181]. Similarly, the

NRDE-3 Argonaute pathway has been shown to transmit a memory of exposure to dsRNA during exo-RNAi [136,140]. In each of these cases, it appears that small RNAs themselves play a key role in passing along a gene regulatory signal through the germline, which may then reinforce patterns of transcription and chromatin modification in the next generation to maintain a proper balance of gene expression [136,140,180,189,191].

It is important to recognize that, although the chromatin-mediated effects of small RNA pathways are coming into focus in animals, an extensive amount of work in fungi and plants has laid the foundation for these studies (for reviews see [192,193]). In plants, stress and bacterial infection have been shown to lead to increased disease resistance in subsequent generations [194–198]. Such stresses are correlated with the hypo-methylation of genes that are normally silenced by RdDM and encode proteins beneficial for tolerating stress or infection. Consistent with a role for hypomethylation in disease resistance, loss of factors in the RdDM pathway also leads to increased disease tolerance in progeny [195]. Thus, in this case endo-siRNAs normally silence genes that are beneficial for stress resistance, and the transgenerational effect is in fact loss of silencing of these loci.

Ultimately, we still have much to learn about the mechanisms by which transgenerational effects of small RNA pathways are executed, and how such pathways could act in mammals. However, like many other activities for endogenous small RNA pathways described herein, lessons learned from plants, flies and worms are likely to be of impact for mammals.

Concluding Remarks

Clearly, over the past 15 years, the field of RNAi research has made tremendous progress in understanding the molecular mechanisms by which Argonautes and their small RNA binding partners regulate gene expression and defend against foreign nucleic acids. These endogenous siRNA pathways play key roles in both the cytoplasm and nucleus to balance gene expression, silence selfish elements, and distinguish self versus nonself, across species. Model organisms, including *Drosophila*, mice, *C. elegans*, and *Arabidopsis*, have played an integral role in the identification and characterization of these pathways, and the 'rules' learned in each system have influenced discovery in other organisms to drive the field ever forward. New roles are being defined for these pathways with each passing year, and if there is one lesson that continues to hold true for the field of small RNA research it is that these pathways will not cease to surprise us.

Acknowledgements

I would like to thank Pedro Batista, Alexander Ensminger, Nelson Lau and Martin Simard, as well as all members of my lab for critical reading and engaging discussions regarding this review. I am also grateful to Ian MacRae for providing images of Argonaute structures for use in Figure 1. My lab is funded by the Canada Research Chairs program, the CIHR (Grants MOP-274660, CAP-262134), NSERC (RGPIN-418), and the University of Toronto Dept. of Molecular Genetics and Connaught Fund.

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