

The Many Faces of RNAi

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Small non-coding RNAs, through association with Argonaute protein family members, have a variety of functions during the development of an organism. Although there is increased mechanistic understanding of the RNA interference (RNAi) pathways surrounding these small RNAs, how their effects are modulated by subcellular compartmentalization and cross-pathway functional interactions is only beginning to be explored. This review examines the current understanding of these aspects of RNAi pathways and the biological functions of these pathways.

Introduction

During the past decade, following the discovery of RNA interference (RNAi) (Fire et al., 1998), we have witnessed amazing developments in the study of small, noncoding RNA molecules (sRNAs) in animals, plants, and fungi. First noticed as products or intermediates in an experimental silencing process that was at that time poorly understood mechanistically (Hamilton and Baulcombe, 1999) and as genetically defined sRNA encoding genes (Lee et al., 1993; Wightman et al., 1993), we now know of many small RNA species (Ghildiyal and Zamore, 2009). One thing that these sRNAs all have in common is that they are embedded in a member of the Argonaute (AGO) protein family (Ender and Meister, 2010), where they act as guides to specific target molecules. While for some of these sRNAs their mode of action remains poorly understood, for others we have learned much about their biogenesis, the proteins they associate with, and the effects they can have on cells. One of the important results from these mechanistic studies is the blurring of the distinctions between various sRNA classes, as different sRNA pathways share components and exhibit crosstalk.

Perhaps the most famous class of noncoding sRNA is the microRNA. The specifics of biogenesis and functions of these short stem-loop structure-derived sRNAs are well discussed elsewhere (Bartel, 2009; Fabian et al., 2010; Kaufman and Miska, 2010). This review will instead focus on providing an overview of the great variety of other endogenous sRNA pathways, exploring their subcellular compartmentalization, functional interactions, and biological functions in the control of cell fate and development.

RNAi Pathways: A Mechanistic Overview

The one unifying theme between all RNA interference (RNAi)-related pathways is the involvement of an AGO protein. The number of AGO genes varies widely, from one in the fission yeast *Schizosaccharomyces pombe* to 27 in the nematode *Caenorhabditis elegans*. AGO proteins identify the targets of an RNAi pathway through basepairing between the AGO-bound sRNA and the target RNA. At the target, AGO proteins can induce a number of effects. Some AGO proteins have a catalytically active RNaseH-like domain and can cleave the targeted RNA molecule. Other AGOs do not rely on target cleavage, either due to the absence of key catalytic residues in their active sites

or because of slow enzyme kinetics. These AGOs employ different mechanisms to affect the activity of their targets, often involving the recruitment of additional factors (Ender and Meister, 2010; Hutvagner and Simard, 2008). The AGO proteins and their associated sRNAs that will be discussed in this Review are detailed in Table 1. In this section, a number of RNAi pathways from different organisms will be discussed using the mechanism by which the relevant sRNA is generated as a guide (Figure 1).

Dicer-Dependent Pathways

A number of RNAi pathways utilize double-stranded RNA (dsRNA) to generate sRNAs through the action of the enzyme Dicer (Bernstein et al., 2001) (Figure 1A). Sources of the dsRNA can vary between pathways. For example, most miRNAs derive from Dicer activity on intramolecular “fold-back” structures, or hairpins. Endogenous siRNAs (endo-siRNAs), another sRNA species (Table 1), can derive from more extended hairpin structures or from dsRNA assembled through intermolecular basepairing between transcripts from bidirectional transcription at a single locus or between transcripts produced from distinct loci. These types of endo-siRNAs have been described in mouse and in *Drosophila* (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008b; Tam et al., 2008; Watanabe et al., 2008). In *Drosophila*, endo-siRNAs are usually loaded into the AGO protein Ago2, triggering cleavage of their targets (Czech et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008b). Interestingly, the *Drosophila* endo-siRNA pathway is characterized by a biogenesis mechanism that appears to be a hybrid between that of miRNAs exogenous dsRNA-induced RNAi (Czech et al., 2008; Okamura et al., 2008a). In both mouse and *Drosophila*, endo-siRNA pathways mainly target transposable elements, although endo-siRNAs that target genes are present as well (Czech et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008b).

Another source of dsRNA, found in *S. pombe*, plants, and *C. elegans*, is RNA-dependent RNA polymerases (RdRPs) (Figure 1B). The *S. pombe* RdRP enzyme Rdp1 synthesizes dsRNA at centromeric loci that is subsequently diced and loaded into the AGO protein Ago1 to direct the formation of pericentromeric heterochromatin (for reviews, see (Grewal, 2010; Martienssen et al., 2005; White and Allshire, 2008)). In the plant *Arabidopsis thaliana*, multiple RdRP enzymes are involved in

Table 1. Characteristics of AGO Proteins and Associated sRNAs

Organism	Argonaute	Small RNA	5' End	Sequence Bias	Dicer Dependent / Independent	Subcellular Localization	Molecular Function	Biological Function
<i>S. pombe</i>	Ago1	siRNA	P	5'U	dependent	nucleus* (chromatin associated)	heterochromatin formation	centromere function
<i>Tetrahymena</i>	Tw1p	scnRNA	P	5'U	dependen.	nucleus* (chromatin associated)	heterochromatin formation	chromosome diminution
<i>C. elegans</i>	RDE-1	22-23 nt exo-siRNA	P	?	dependent	cytoplasm	initiate 22G RNA production	virus resistance
	ALG-3	26G	P	5'G	dependent	nuage (perinuclear)	initiate 22G RNA production	spermatogenesis
	ALG-4	26G	P	5'G	dependent	?	initiate 22G RNA production	spermatogenesis
	ERGO-1	26G	P	5'G	dependent	?	initiate 22G RNA production	gene regulation
	?	22-23 nt endo-siRNA	P	-	dependent	?	?	?
	CSR-1	22G	PPP	5'G	independent	nuage (perinuclear), chromatin associated, around metaphase plate	?	centromere function, MSUC
	WAGO's	22G	PPP	5'G	independent	nuage (perinuclear)	RNA destabilization	gene regulation
	NRDE-3	22G	PPP	5'G	independent	nucleus*	inhibition of transcription elongation	gene regulation
	PRG-1	21U (piRNA)	P	5'U	independent	nuage (perinuclear)	initiate 22G RNA production	germ cell maintenance, spermatogenesis, transposon silencing
	PRG-2	21U (piRNA)	P	5'U	independent	?	initiate 22G RNA production	germ cell maintenance, spermatogenesis, transposon silencing
<i>Drosophila</i>	Ago1	miRNA (siRNA)	P	5'U	dependent	P-bodies	mRNA destabilization / translation inhibition	gene regulation
	Ago2	siRNA (miRNA)	P	-	dependent	P-bodies	target RNA cleavage	embryonic development, gene regulation, transposon silencing, anti-viral defense
	Piwi	piRNA (only primary)	P	5'U	dependent	nucleus*, nuage (perinuclear)	target RNA cleavage, heterochromatin formation	transposon silencing, gene regulation, oogenesis, spermatogenesis, embryogenesis

Table 1. Continued

Organism	Argonaute	Small RNA	5' End	Sequence Bias	Dicer Dependent / Independent	Subcellular Localization	Molecular Function	Biological Function
	Aub	piRNA	P	5'U	independent	nuage (perinuclear)	target RNA cleavage	transposon silencing, gene regulation, oogenesis, spermatogenesis, embryogenesis
	Ago3	piRNA	P	10A	independent	nuage (perinuclear)	target RNA cleavage	transposon silencing, gene regulation, oogenesis, spermatogenesis, embryogenesis
Mouse	Ago-2	siRNA, miRNA	P	5'U	dependent	P-bodies, nuage (chromatoid body)	target RNA cleavage	gene regulation, oogenesis, embryonic development
	Miwi	piRNA (pachytene)	P	5'U	Independent	nuage (chromatoid body)	?	spermatogenesis
	Mili	piRNA (pachytene and pre-pachytene)	P	5'U	independent	nuage (perinuclear and chromatoid body)	target RNA cleavage	transposon silencing, gene regulation?, spermatogenesis
	Miwi2	piRNA (pre-pachytene)	P	10A	independent	piP-bodies, nucleus*	target RNA cleavage, heterochromatin formation?	transposon silencing, spermatogenesis
<i>Xenopus</i>	Xiwi	piRNA	P	?	independent	nuage (balbiani body), around metaphase plate	target RNA cleavage	transposon silencing
Zebrafish	Ziwi	piRNA	P	5'U	independent	nuage (perinuclear)	target RNA cleavage	transposon silencing, germ cell maintenance
	Zili	piRNA	P	10A	independent	nucleus*, nuage (perinuclear)	target RNA cleavage	transposon silencing, germ cell differentiation

AGO proteins that are discussed in this Review are listed here, together with their bound small RNAs and a number of other characteristics. This table is not intended to be exhaustive but is meant instead to serve as a quick guide to the key characteristics of the various RNAi pathways discussed. P, 5' mono-phosphate; PPP, 5' tri-phosphate; 10A, adenosine at position 10 counted from the 5' end; nt, nucleotide.

*Only when loaded with a small RNA.

intricate networks of different RNAi pathways. In each case, the RdRP enzyme appears to make dsRNA that is then used by one of the four Dicer-like enzymes as substrate (for review, see Herr and Baulcombe, 2004; Voinnet, 2008; Xie and Qi, 2008). In animals, RdRP activity has so far only been described in *C. elegans*. In this nematode, at least one RdRP enzyme, RRF-3, may be involved in producing dsRNA that is processed by Dicer (DCR-1). RRF-3, together with DCR-1 and a number of other factors, is involved in generating a subset of the endo-siRNAs in *C. elegans*, both in the germline as well as in the soma (Gent et al., 2009, 2010; Pavelec et al., 2009). *C. elegans* endo-siRNAs include a 26-nucleotide-long species (Gent et al., 2009; Han et al., 2009; Pavelec et al., 2009), also known as 26G RNA (Ruby et al., 2006). This class of endo-siRNAs appears

to be divided into at least two subclasses. One loads into the AGO proteins ALG-3 and ALG-4 (Conine et al., 2010; Han et al., 2009) and appears to function primarily in sperm to target genes active in spermatogenesis. The other subclass is associated with the AGO protein ERGO-1 (Gent et al., 2010; Han et al., 2009; Vasale et al., 2010) and functions in both the soma as well as the germline. Apart from the 26G endo-siRNAs, there are also 22- to 23-nucleotide-long endo-siRNA that are produced by DCR-1, but the molecular characteristics of these have not yet been well defined (Welker et al., 2010).

Dicer-Independent Pathways

Some RNAi pathways function independently of Dicer. For example, in *C. elegans*, a prominent population of endo-siRNAs is, most likely, derived directly from RNA-dependent RNA

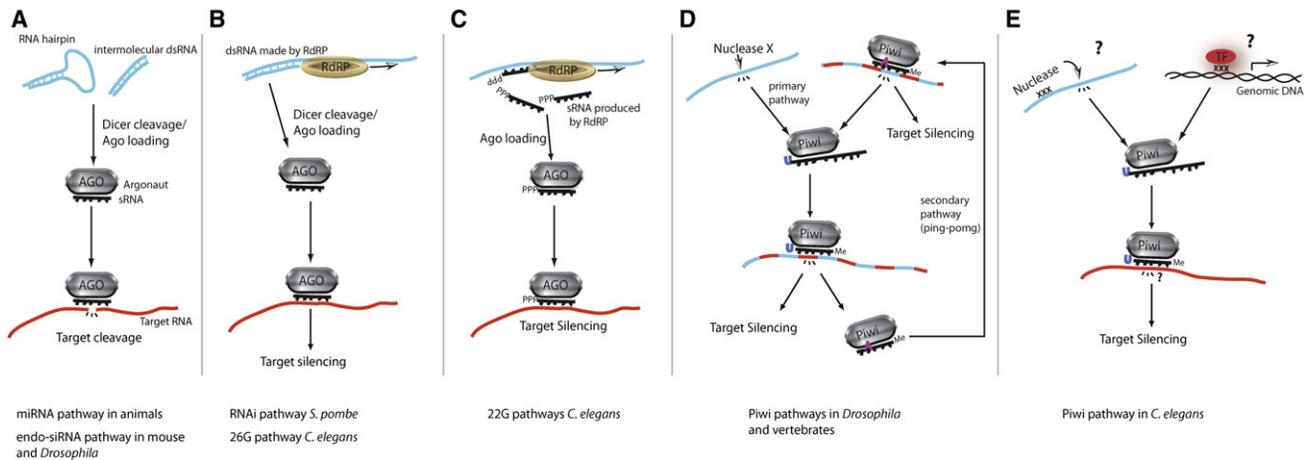


Figure 1. RNAi Pathways

Substrate RNA molecules that give rise to small RNA (sRNA) species are depicted in blue, sRNAs are depicted in black, and target RNA transcripts are depicted in red.

(A) A “canonical” RNAi pathway. Examples of this pathway are the endo-siRNA pathways in *Drosophila* and mammals. In both systems, Ago2 is most likely the AGO protein involved.

(B) dsRNA can be generated by an RNA-directed RNA polymerase (RdRP). This mechanism occurs in *S. pombe* and in *C. elegans*.

(C) RdRP enzymes can also directly generate short RNA molecules that are bound by AGO proteins. This mode of small RNA generation occurs in *C. elegans*. The AGO proteins accepting this type of small RNA do not appear to direct target RNA cleavage but do result in a drop in RNA target levels.

(D) A model of piRNA biogenesis and function. Single-stranded RNA serves as a source of small RNA and cleavage by either Piwi proteins or an unknown nuclease generates the 5' ends of the small RNAs. Primary piRNAs carry a 5'-uracil residue, whereas secondary piRNAs either have a 5'-uracil or an adenosine at position 10, due to the cleavage characteristics of AGO proteins. The processing of the 3' end is poorly understood but is finalized by methylation of the 2'OH group of the last nucleotide. Piwi proteins can cleave their targets, but whether this is required for their silencing activity is not well known. Note that it is difficult to separate target RNA molecules from piRNA substrates, due to the cyclical nature of the process.

(E) Potential models of how piRNAs are generated in *C. elegans* (21U RNAs). A motif (represented as “xxx”) is found upstream of 21U RNAs. This motif may function as an RNA processing signal or as a transcriptional signal. Processing of the 3' end likely resembles the other Piwi pathways. Not much is known about how 21U RNAs silence their targets, but at least in some cases, an RdRP appears to be recruited, triggering the pathway depicted in (C).

polymerase (RdRP) activity. That is, the RdRP makes short RNA transcripts that directly bind to AGO proteins (Aoki et al., 2007; Pak and Fire, 2007; Sijen et al., 2007). These small RNAs, known as 22G, are characterized by the presence of a triphosphate group at their 5' end, possibly resulting from the first NTP residue used in their synthesis (Figure 1C). They are made in the soma, as well as in the germline, and almost any type of genomic locus, including genes, pseudogenes, transposons, and intergenic regions, can be a source of 22G RNA (Conine et al., 2010; Gent et al., 2010; Gu et al., 2009; Lee et al., 2006; Vasale et al., 2010). Many different AGO proteins associate with these small RNAs. This has been directly demonstrated for WAGO-1, -6 and -8 (Gu et al., 2009; Yigit et al., 2006), NRDE-3 (Guang et al., 2008) and CSR-1 (Claycomb et al., 2009), but also other WAGO proteins have also been suggested to bind 22G (Conine et al., 2010; Gu et al., 2009; Yigit et al., 2006). Interestingly, most of these AGO proteins are not believed to trigger target cleavage, as they do not have all the residues required to be catalytically active, implying they employ different mechanisms to affect their targets.

Another Dicer-independent pathway is the so-called Piwi-pathway (Malone and Hannon, 2009). This pathway is specific to animals and most often specifically active in the germline. The Piwi-pathway is driven by a specific subclass of the AGO proteins called Piwi proteins, which bind a type of small RNA called piRNA (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Saito et al., 2006; Vagin et al., 2006; Watanabe et al., 2006). It is generally believed that two

main pathways exist to generate piRNAs, differing in the mechanisms generating the 5' end of the piRNA. One pathway, also called the primary pathway, involves endonucleolytic cleavage of a long single stranded RNA by an unknown nuclease and generates piRNAs with a uracil at their 5' end (Figure 1D). Most likely, this pathway synthesizes the piRNAs bound by the *Drosophila* Piwi protein (Li et al., 2009; Malone et al., 2009) and the so-called pachytene piRNAs that are found in adult testis of mammals. In mice, these are bound by the Piwi proteins Miwi and Mili (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Saito et al., 2006; Watanabe et al., 2006). This primary pathway is also believed to initiate another piRNA biogenesis pathway, the secondary pathway, or the ping-pong mechanism. This pathway depends on Piwi-protein-mediated cleavages and mainly synthesizes transposon-derived piRNAs (Brennecke et al., 2007; Gunawardane et al., 2007) (Figure 1D). This mechanism allows piRNA amplification if target RNA transcripts from both strands are present, allowing an adaptive response to variable target expression. In both scenarios, mechanisms responsible for the 3' end generation have not been identified, although it has been shown that piRNA 3' ends are 2'-O-methylated by the Hen1 enzyme for increased piRNA stability (Horwich et al., 2007; Houwing et al., 2007; Kamminga et al., 2010; Kirino and Mourelatos, 2007a, 2007b; Saito et al., 2007; Vagin et al., 2006). Piwi proteins can mediate target cleavage (Gunawardane et al., 2007; Lau et al., 2006; Saito et al., 2006), but the relevance of that activity for target silencing remains unknown. At least some Piwi proteins

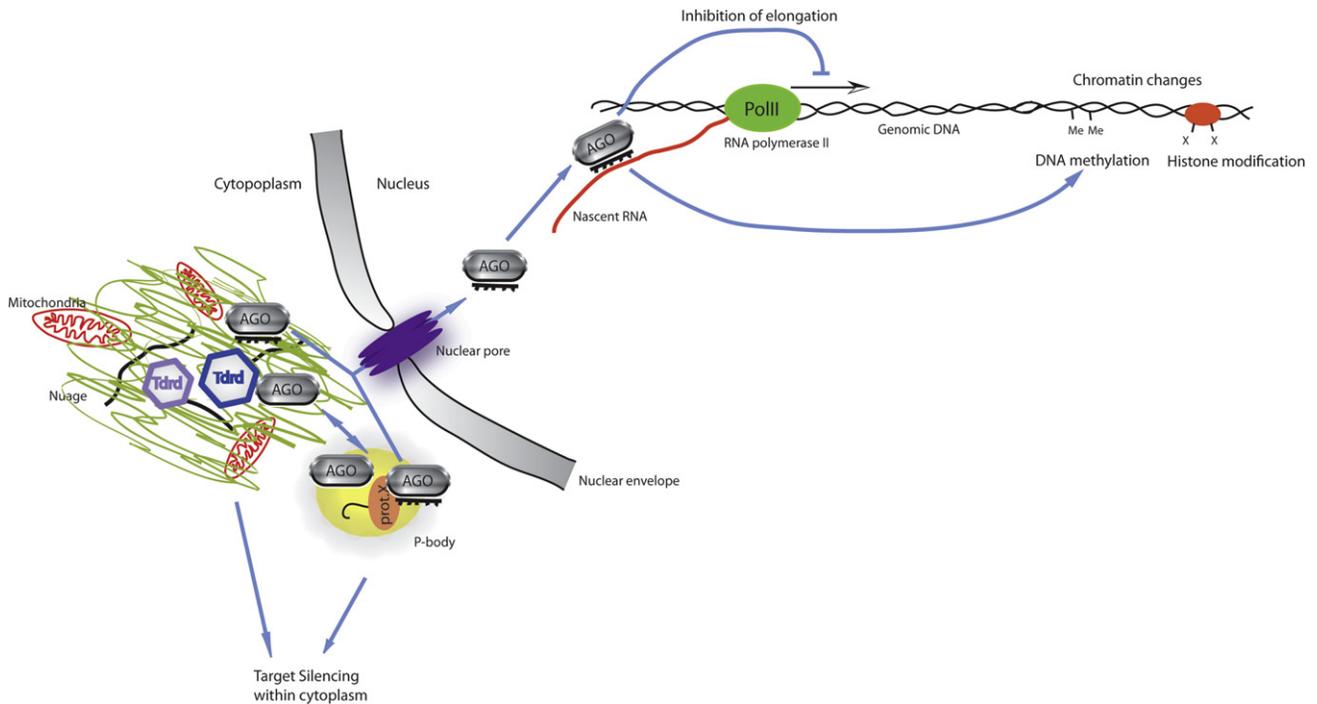


Figure 2. Subcellular Interactions of RNAi Pathways

Nuage (green) and P-bodies (yellow) have been observed to interact in multiple systems, which may result in the exchange of proteins and RNA molecules. Many AGO proteins are found within P-bodies or nuage. Some of these interactions are mediated via the binding of Tudor-domain-containing proteins (Tdrd; blue and light purple) to methylated arginine residues in AGO proteins (most notable Piwi proteins). AGO proteins associated with nuage and P-bodies can be involved directly in the silencing of target RNA molecules. Upon sRNA loading, some AGO proteins can also translocate into the nucleus through the nuclear pore (purple). Nuclear AGO proteins can affect gene expression by modulating DNA methylation (-Me) and histone (red) modification (-X), as well as RNA polymerase II (green) transcription elongation activity.

are found in the nucleus and can trigger effects at the chromatin level (see below).

C. elegans piRNAs, also known as 21U RNAs, have different characteristics than described above (Batista et al., 2008; Das et al., 2008) (Figure 1E). They do not participate in a ping-pong-type amplification mechanism and are characterized by a consensus motif that is located just upstream of their coding sequence in the genome (Ruby et al., 2006). This consensus sequence is highly predictive of 21U RNA species, but its function in 21U production is unresolved: it may represent a transcriptional or RNA processing signal. Like other piRNAs, 21U RNAs are 2'-O-methylated at their 3' end. A few 21U RNAs are involved in transposon silencing (Batista et al., 2008; Das et al., 2008), but assuming that 21U RNAs, like piRNAs in other systems, bind their targets through extensive basepairing, the majority of 21U RNAs lack endogenous targets as they have no close sequence matches in the genome apart from their own locus (Batista et al., 2008; Das et al., 2008; Ruby et al., 2006). It is thus an unsolved puzzle as to what the majority of 21U RNAs are meant to recognize as target. Nonetheless, defects in the 21U RNA pathway have strong effects on spermatogenesis (see below), suggesting that endogenous targets do exist. Another intriguing possibility for 21U RNA targets comes from the fact that most piRNAs in other organisms are directed at parasitic DNA elements, such as transposons. It is conceivable that the *C. elegans* 21U RNAs have similar, although nonendogenous, targets. Obvious possibilities would be nucleic acids from

bacteria or viruses, but given the limited availability of biologically relevant experimental systems to study infections in *C. elegans*, this hypothesis remains difficult to test.

Compartmentalization of RNAi Pathways: RNAi in the Nucleus

Although RNAi was initially described as a cytoplasmic mechanism, genetic analysis suggested the existence of nuclear effects of RNAi, as many RNAi defective mutants displayed defects in chromosome segregation. While these defects could be secondary to loss of cytoplasmic RNAi, it is becoming clear that nuclear functions for RNAi pathways indeed exist. In many cases, nuclear RNAi pathways direct the formation of specific chromatin structures, (also see Figure 2).

Single-Cell Eukaryotes

In *S. pombe*, a nuclear RNAi pathway plays an important role in centromere function. This RNAi pathway functions in heterochromatin formation at the pericentromeric regions flanking the core centromere; components of the pathway also physically associate with these regions (Grewal, 2010; Martienssen et al., 2005; Verdell et al., 2009). Pericentromeric heterochromatin, required for centromere function, limits the deposition of centromere-specific chromatin components, such as CENPA, to the core centromere. Interestingly, the process of RNAi itself is actually completely dispensable for centromere function, as it can be bypassed by tethering RNAi components to chromosomal loci in an RNAi-independent manner and even by inducing

heterochromatin in complete absence of RNAi (Buhler et al., 2006; Kagansky et al., 2009). Thus, pericentromeric heterochromatin is the key component relevant to centromere behavior, and RNAi has evolved as a mechanism to initiate and maintain it.

Interestingly, Dicer and other RNAi components in *S. pombe* have been found to physically associate with noncentromeric loci as well, where they are active in heterochromatin-independent gene silencing (Woolcock et al., 2011). In addition, Dicer has been found to exhibit an interesting nuclear-cytoplasmic shuttling behavior and to affect gene expression when nuclear localization is prevented (Emmerth et al., 2010). These observations significantly expand our view on the role of RNAi in this organism and raise the question whether similar processes are active in other species.

Nuclear-RNAi-directed heterochromatin formation also plays a role during sexual reproduction in the ciliate *Tetrahymena* (*T. thermophila*) (Mochizuki and Gorovsky, 2004). In this binucleated organism, a micronucleus is responsible for generating both a new micronucleus and a macronucleus after a fertilization event. The development of the new macronucleus involves loss of many repetitive sequences from the micronuclear genome, a massive DNA elimination event targeted to specific regions by an RNAi pathway. The small RNAs involved, scan (scn) RNAs, bind to the AGO protein Twi1p, which enters the nucleus only when loaded with a single-stranded scnRNA (Noto et al., 2010). Twi1p marks the regions to be deleted by inducing heterochromatin formation at those loci (Liu et al., 2004; Liu et al., 2007), possibly through a mechanism similar to that observed in *S. pombe*. How this change in chromatin structure specifically triggers elimination of the marked sequences is not well understood.

Animals

It is clear that nuclear RNAi is not restricted to lower eukaryotes like *S. pombe* and *Tetrahymena*. In *C. elegans*, NRDE-3 has been identified as an AGO protein that localizes to the nucleus (Guang et al., 2008). Despite its nuclear localization signal, NRDE-3 only is localized to the nucleus upon sRNA loading, suggesting that the nucleus is the ultimate site of action for this protein. Although the physiological function of NRDE-3 remains unclear, it has been shown that nuclear NRDE-3 inhibits the elongation phase of engaged RNA polymerase II (Guang et al., 2010). This transcriptional interference depends on another evolutionary conserved protein, NRDE-2, which is recruited to nascent transcripts via NRDE-3. The NRDE-2 protein only contains poorly characterized protein domains, making it difficult to speculate about its precise molecular function. *C. elegans* also possesses an RNAi mechanism that is involved in the meiotic silencing of unpaired chromatin (MSUC) (Maine et al., 2005; She et al., 2009) and in chromosome segregation in the early embryo (Claycomb et al., 2009; van Wolfswinkel et al., 2009; Yigit et al., 2006). This system involves an AGO protein (CSR-1), an RdRP (EGO-1), an uridylyl transferase (CDE-1), an RNA helicase (DRH-3), and a Tudor domain protein (EKL-1). The sRNAs associated with this system are part of the 22G RNA family and are most likely generated directly through activity of the RdRP enzyme EGO-1 (Claycomb et al., 2009; Gu et al., 2009). CSR-1 associates with chromatin via RNA, and defects in CSR-1 or any of the aforementioned components of the pathway trigger defects in chromosome alignment during mitosis in early embryogenesis

(Claycomb et al., 2009; van Wolfswinkel et al., 2009). Puzzlingly, although CSR-1 is loaded with 22G RNAs that target genes expressed in the germline, it does not affect the expression of those genes (Claycomb et al., 2009). How exactly the CSR-1 pathway affects both MSUC and chromosome segregation is not quite clear. The genetic interactions between the components of the pathway during MSUC are complex (She et al., 2009), making it difficult to derive a clear pathway. We have previously suggested that this RNAi pathway could act to repel a default, uncharacterized chromatin silencing activity from active genes (van Wolfswinkel and Ketting, 2010).

Finally, some Piwi proteins exhibit nuclear localization. For example, *Drosophila* Piwi is nuclear (Cox et al., 2000) when loaded with a piRNA (Olivieri et al., 2010; Qi et al., 2010; Saito et al., 2010), and may play a role in heterochromatin formation (Pal-Bhadra et al., 2004). In mouse, the Piwi homolog Miwi2 transits to the nucleus when loaded with a piRNA (Aravin et al., 2008), which occurs upon interaction with Mili, another Piwi homolog. While mechanistic details of Miwi2 function in the nucleus are lacking, the ultimate effect of Miwi2 activity is the remethylation of certain genomic regions that were demethylated during mouse primordial germ cell (PGC) development (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). It is hypothesized, largely based on the understanding of the *S. pombe* RNAi pathway, that Miwi2 is involved in altering chromatin structures, which is in turn interpreted by the de novo DNA methylation machinery (Aravin and Bourc'his, 2008).

Overall, a common theme in the role of nuclear RNAi appears to be the specific modulation of chromatin structure that in turn determines activity, or some other characteristic of the targeted genomic region. Intriguingly, similar to the above observations, other RNAi pathway components, such as mammalian Ago2 and Dicer (Sinkkonen et al., 2010; Weinmann et al., 2009), have been found in the nucleus. The biological significance of these nuclear protein pools awaits further study.

Compartmentalization of RNAi Pathways: Cytoplasmic Domains

Many RNAi pathways appear to be compartmentalized in the cytoplasm, possibly facilitating distinction of RNAi pathways that are active in parallel. One cytoplasmic site where AGO proteins have been found to localize is on the cytoplasmic surface of multivesicular bodies (MVBs) (Gibbings et al., 2009; Lee et al., 2009), membrane-rich structures in the endocytic pathway that relate to endosomes and lysosomes. This interaction has been found to stimulate the silencing effects of miRNAs and siRNAs, perhaps by enhancing the loading of AGO complexes with sRNAs. It has also been suggested that this interaction may aid in the dynamics of cytoplasmic RNAi pathways by promoting continuous assembly and disassembly of AGO complexes (Gibbings et al., 2009; Lee et al., 2009).

Processing bodies (P-bodies), cytoplasmic aggregates of RNA and protein, marked by the presence of multiple RNA-processing activities, such as deadenylation, decapping, and RNA breakdown (Parker and Sheth, 2007), have also been described as centers for sRNA activity, including miRNA activity (Liu et al., 2005; Rossi, 2005). In the case of miRNAs, P-bodies are not required for silencing activity, suggesting that these sites represent storage sites or end-stages of the pathway rather than

locations where the silencing process is actively taking place (Eulalio et al., 2007). Despite these findings, aggregation of other RNAi pathways into these bodies may still be relevant for their activities. For example, Miwi2, and not its close homolog Mili, associates with a subset of the P-bodies marked by the presence of a number of other Piwi-pathway factors, including Maelstrom and Tdrd9 (Aravin et al., 2009), two proteins required for proper Miwi2 function (Shoji et al., 2009; Soper et al., 2008). These P-bodies have been dubbed piP-bodies, to distinguish them from the P-bodies lacking Piwi pathway components. Interestingly, piP-bodies have been shown to be in close physical contact with another subcellular structure, nuage (see below), and it has been suggested that these two structures exchange materials (Aravin et al., 2009) (Figure 2). In *C. elegans*, P-bodies have also been found to interact with nuage-like structures, named P-granules (Gallo et al., 2008).

Biochemically related to P-bodies are RNA-rich subcellular structures named nuage. Found in the germ cells of many, if not all, animals, this material is very electron dense and can be easily recognized by electron microscopy (EM). Besides its electron density, nuage is distinguishable from P-bodies through its close proximity to mitochondria (Clark and Eddy, 1975); a number of proteins, such as the Tudor domain protein Tdrd1, are also uniquely found in nuage (Chuma et al., 2003; Parker and Sheth, 2007). There are many different types of nuage (reviewed in Chuma et al., 2009; Kotaja et al., 2006; Mahowald, 2001; Thomson and Lasko, 2005; Updike and Strome, 2010). For example, two types of nuage, perinuclear nuage and polar granules, are found in *Drosophila*. Perinuclear nuage is present in oocytes and nurse cells, whereas polar granules are present only in the oocyte, where it is involved in axis specification. In *Drosophila*, embryo polar granules become part of the pole plasm, which functions in defining germ cells (Mahowald, 2001; Thomson and Lasko, 2005). Vertebrate oocytes similarly have at least two types of nuage: a cytoplasmic nuage structure called the Balbiani body and perinuclear nuage (de Sousa Lopes and Roelen, 2010). Perinuclear nuage is also observed in vertebrate male germ cells, and in mammals this material seems to collapse into a single body (the chromatoid body) during later stages of spermatogenesis (Kotaja et al., 2006). For sake of simplicity, all of these structures will be collectively referred to here as nuage.

In *Drosophila* and *C. elegans*, there is clear interaction between nuage and Piwi pathway components. Mutations that disrupt nuage hamper Piwi protein activity, and defects in Piwi pathway activity affect the subcellular localization of many other Piwi pathway components, as well as nuage formation (Batista et al., 2008; Harris and Macdonald, 2001; Li et al., 2009; Lim and Kai, 2007; Malone et al., 2009). In this light, it is interesting to note that a protein named Tejas, which is required for sufficient piRNAs production, seems to be required to tether the Piwi pathway to nuage (Patil and Kai, 2010). It is therefore thought that activity of the Piwi pathway is an important driving force in establishing nuage and that nuage is an important site for piRNA production and activity. Not much is known about the intermolecular interactions that drive nuage formation, but arginine methylation of Piwi proteins and other nuage components, such as the RNA helicase Vasa, plays a role in establishing interactions with tudor-domain-containing nuage

components (Kirino et al., 2009, 2010; Nishida et al., 2009; Vagin et al., 2009). It bears mentioning that given the differences between nuage as defined by EM and as defined by immunofluorescence in mice (see discussion below), further study of the effects of Piwi pathway components on nuage using EM techniques will be important to complement the current, mainly immunofluorescence-based analyses.

Nuage-like structures are also found in the somatic follicle cells surrounding the *Drosophila* oocyte. These cells execute a Piwi pathway that associates with electron-dense and mitochondria-associated structures, named Yb bodies (Olivieri et al., 2010; Qi et al., 2010; Saito et al., 2010; Szakmary et al., 2009). Yb bodies are found close to P-bodies in nurse cells, similar to the close association between nuage and piP-bodies in germ cells; Yb bodies have also been identified as a site of piRNA biogenesis (Olivieri et al., 2010; Qi et al., 2010; Saito et al., 2010).

The Piwi pathway connects to nuage in vertebrates as well. The Piwi protein Xiwi associates with the Balbiani body in *Xenopus* oocytes (Lau et al., 2009) and in zebrafish, Piwi proteins have been observed in both cortical granules (which derive from the Balbiani body) and perinuclear nuage (Houwing et al., 2008; Houwing et al., 2007). Notably, these associations can change during development. In the mammalian male germline many miRNA and piRNA pathway components are found in the chromatoid body (Grivna et al., 2006; Kotaja et al., 2006; Unhavaithaya et al., 2009; Wang et al., 2009), and Piwi proteins have been found in perinuclear nuage structures (Aravin et al., 2008, 2009). As in *Drosophila*, methylated arginines in vertebrate Piwi proteins interact with tudor domain proteins in nuage to establish full activity of the RNAi pathway (Chen et al., 2009; Reuter et al., 2009; Vagin et al., 2009; Wang et al., 2009). As eluded to earlier, the presence of nuage, as defined by EM, does not always correlate well with nuage as identified through immunofluorescence. For example, based on EM analysis, perinuclear nuage appears essentially gone in mouse cells lacking the tudor domain protein Tdrd1 (Chuma et al., 2006), while immunostainings for Mili in the same mutant still shows Mili accumulation in perinuclear foci (Vagin et al., 2009). Interestingly, in *Drosophila*, polar granule formation occurs in steps with the Piwi protein Aubergine (Aub) acting upstream of a tudor-domain-containing protein (Thomson and Lasko, 2005). Similarly, the Mili-positive granules that are observed in Tdrd1 mutant mice could be precursors of the electron-dense nuage structures seen in wild-type mice.

Other examples of interactions between RNAi and nuage can be found in *C. elegans*. Many different AGO proteins associate with nuage in *C. elegans* (Batista et al., 2008; Conine et al., 2010; Gu et al., 2009; Wang and Reinke, 2008). In addition, some RNAi pathways actually affect the appearance of nuage. For example, nuage is normally strongly associated with nuclear pores in germ cells (Updike and Strome, 2010), but it changes in appearance and no longer associates with germ cell nuclei when the AGO protein CSR-1 is absent (Claycomb et al., 2009; Updike and Strome, 2009). While not well understood, detached nuage in *csr-1* mutants resembles the detached appearance of nuage during early embryogenesis in *C. elegans* and zebrafish, illustrating the complex nature of nuage and its interactions with RNAi pathways and with other cellular compartments.

The recent analysis of the physical qualities of nuage in *C. elegans* (Brangwynne et al., 2009) has interesting implications for RNAi processes proposed to occur within them. The proposed liquid-like structure of the nuage that is separated from the surrounding through surface-tension forces would provide a very dynamic and concentrated environment for efficient interaction of proteins. Thus, defects in the formation of such “liquid droplets” would be expected to have effects on the efficiency of the processes taking place within them, but not to lead to absolute ablation of these processes. This may explain why the mouse *tdrd1* and *tdrd6* mutants, in which nuage is severely affected, still have an active, albeit crippled, Piwi pathway (Reuter et al., 2009; Vagin et al., 2009; Vasileva et al., 2009; Wang et al., 2009). Furthermore, the ectopic formation of nuage-like structures may be expected to provide sites for aberrant RNAi and, thus, result in hyper-activation of RNAi activity. Indeed, mutations that result in the formation of nuage-like structures in *C. elegans* somatic cells have been shown to correlate with increased RNAi sensitivity (Wang et al., 2005).

Interactions between RNAi Pathways

Many of the RNAi pathways discussed here operate in parallel, and there is evidence for both competition between pathways and mechanisms that allow effective separation of pathways. In *C. elegans*, where DCR-1 appears to be present in limiting amounts, different RNAi pathways compete for its activity. Indeed, disruption of one pathway can result in hyperactivity of other pathways (Duchaine et al., 2006; Lee et al., 2006; Yigit et al., 2006). To separate distinct RNAi pathways acting in one shared environment, AGO proteins, or the machineries that load sRNAs into AGO proteins, can exhibit differing affinities for certain RNA structures. For example, *Drosophila* endo-siRNAs are derived from extended dsRNA duplexes and load into the Ago2 protein, whereas miRNAs are derived from imperfectly basepaired short hairpin structures and preferentially load into Ago1 (reviewed in Ghildiyal and Zamore, 2009). In addition, it has been proposed for the CSR-1-mediated RNAi pathway in *C. elegans* that reduced 22G RNA stability ensures that CSR-1-associated sRNAs do not flow into RNAi pathways mediated by other AGO proteins, where they may have detrimental effects. This destabilization of 22G RNAs depends on an enzyme, CDE-1, that uridylylates 22G RNAs at their 3' ends (van Wolfswinkel et al., 2009). Work in *Drosophila* and human cells has shown that such uridylation activity depends on target RNA recognition (Ameres et al., 2010). Indeed, uridylylated 22G RNAs are found in the context of CSR-1, consistent with the possibility that they become modified by CDE-1 upon target recognition by CSR-1.

In addition to mechanisms enforcing strong separation of pathways, examples also exist where different RNAi pathways exhibit functional interactions and synergy. Such pathway interdependence can be observed in *C. elegans*. For instance, 22G RNA production can be triggered through recognition by a 21U RNA (Batista et al., 2008; Das et al., 2008), suggesting that this piRNA pathway functions upstream of 22G RNA biogenesis. Similarly, a miRNA has been found to trigger 22G RNA production (Correa et al., 2010), and 26G RNAs function upstream of certain 22G RNA pathways as well (Conine et al., 2010; Gent et al., 2010; Han et al., 2009; Vasale et al., 2010).

Finally, RNAi triggered through the administration of exogenous dsRNA results in a two-step RNAi process in *C. elegans*. First, sRNAs generated by DCR-1 load into the AGO protein RDE-1. RDE-1 then triggers the generation of secondary sRNAs (22G RNAs) that load into other AGO proteins, collectively called WAGO (Gu et al., 2009; Yigit et al., 2006). How these various pathways couple together is not known, but a recurring theme seems to be that a “primary” AGO protein recruits an RdRP enzyme that then uses the target of the primary AGO protein as a template to generate secondary sRNAs. Indeed, while primary endo-siRNAs are found in both sense and antisense polarity (Welker et al., 2010), reflecting their dsRNA origin, secondary endo-siRNAs are mainly antisense and derived almost exclusively from exonic sequences, reflecting their synthesis on sense RNA templates (Correa et al., 2010; Gent et al., 2010; Gu et al., 2009). Together, these data support a broad role for *C. elegans* 22G RNAs in amplifying silencing responses that are first primed by more specialized RNAi pathways.

Amplification of primary silencing responses is certainly not a peculiarity of *C. elegans* RNAi. Amplification strategies similar to those used by *C. elegans* have been described in plants (Voinnet, 2008), and as discussed above, the Piwi pathways in *Drosophila*, mice, and zebrafish also amplify sRNA pools, albeit through different mechanisms. Why amplification is such a common theme in RNAi pathways is not completely clear, but it may offer additional possibilities for regulation and diversification of silencing responses.

In addition to pathway interdependence, different RNAi pathways can also display functional overlap. The WAGO proteins just discussed display redundancy as they can partially fulfill each others' functions (Yigit et al., 2006). In mice, oocytes contain piRNAs and endo-siRNAs derived from the same genomic elements (Tam et al., 2008; Watanabe et al., 2008). It seems that the siRNA pathway may largely be responsible for the lack of phenotype in Piwi-deficient mouse oocytes, as Dicer (Murchison et al., 2007; Tang et al., 2007)- and Ago2 (Kaneda et al., 2009)-defective oocytes display strong meiotic phenotypes, while miRNA activity in oocytes seems to be of limited importance (Ma et al., 2010). Likewise, in *Drosophila*, siRNAs and piRNAs derived from the same transposable elements can be found (Chung et al., 2008; Czech et al., 2008; Ghildiyal and Zamore, 2009). Why two or more RNAi pathways would evolve to silence largely overlapping targets is unknown. However, cell-type compatibility may play a role, as, for example, *Drosophila* endo-siRNAs appear to be mainly active in somatic cells while piRNAs act in the germ cells (Czech et al., 2008; Ghildiyal et al., 2008; Okamura and Lai, 2008). It is also possible that different RNAi pathways function at different stages of the response to a challenge (for example, transposon invasion), with one pathway acting as a primary response to the challenge and another pathway acting as a more stable system to follow up the initial silencing response.

Biological Functions for RNAi Pathways

In many RNAi mutants across different species, effects on the integrity of the germline are evident. For example, defects in sperm development were noted in the first RNAi defective mutants isolated from *C. elegans* (Ketting et al., 1999; Tabara

et al., 1999), and similar phenotypes have since then been described in many additional RNAi pathway mutants, including mutants in the 21U and certain 26G and 22G pathways (Conine et al., 2010; Gent et al., 2009; Han et al., 2009; Pavelec et al., 2009; Wang and Reinke, 2008). The exact causes of the sperm defects are unknown, but it is clear that the complete gene expression pattern in sperm is affected by changes in these RNAi pathways. Interestingly, temperature sensitivity is a common feature of these phenotypes, suggesting that RNAi pathways contribute to the stability, or robustness, of gene expression programs during sperm development. The following section will discuss a number of germ-cell-related processes that are affected by RNAi pathways.

Meiosis

Defects in X chromosome segregation during oogenesis have been reported in *C. elegans* RNAi mutants (Ketting et al., 1999; Tabara et al., 1999; van Wolfswinkel et al., 2009), suggesting that RNAi pathways are involved in meiotic progression, although the molecular details remain unclear. Interestingly, meiotic defects are also associated with RNAi in vertebrates. As just discussed, Dicer- and Ago2-defective oocytes in mice exhibit meiotic problems, suggesting endo-siRNAs play a role in female meiosis. And although mouse Piwi mutants do not display defects in oocytes, meiotic defects have been linked to the Piwi pathway through a piRNA-pathway factor called Maelstrom (A. Bortvin, personal communication). Furthermore, in zebrafish, a specific mutation in Zili results in a meiotic block in oocyte maturation (Houwing et al., 2008). Perhaps related to this, in *Xenopus* oocytes, Xiwi has been found around the metaphase plate (Lau et al., 2009), similar to what has been described for the *C. elegans* AGO protein CSR-1 during mitosis (Claycomb et al., 2009). It remains, however, unclear whether nuclear RNAi mechanisms similar to that described in *S. pombe* underlie these requirements for RNAi pathways during meiosis in oocytes.

In mouse testis, defects in all three Piwi proteins, Miwi, Miwi2, and Mili, have strong effects on meiosis (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). As Mili binds transposon-derived piRNAs and pachytene piRNAs (discussed above), the defects in *mili* mutant animals are more difficult to interpret. Miwi and Miwi2 are more specific for pachytene- and transposon-derived piRNAs, respectively. The role of pachytene piRNAs in testis biology is poorly understood, but the strong spermatogenesis phenotype of *miwi* mutant mice just after pachytene stage (Deng and Lin, 2002)—when homologous chromosomes align and form bivalent structures—suggests an important function for these pachytene piRNAs. The chromosomal origin of pachytene piRNAs appears to be essential to their function, as their chromosomal locations, but not their sequences, are conserved between pachytene piRNAs from different mammals (Girard et al., 2006).

Mutation of *miwi2* triggers spermatogenesis phenotypes just before pachytene stage (Carmell et al., 2007; Kuramochi-Miyagawa et al., 2004; Unhavaithaya et al., 2009), as does disruption of the Miwi2-associated factor Tdrd9 (a tudor-domain containing RNA helicase and the mouse homolog of *Drosophila* SpnE). These defects have been best described for the loss of *tldr9* (Shoji et al., 2009). In the *tldr9* mutant, spermatogenesis does not proceed beyond leptotene-like stages, the stage just preceding pachytene. The homologous recombination

machinery still associates with the chromosomes in *tldr9* mutants but fails to assemble into clear foci on paired chromosomes. Continuous staining for SCP1, a marker for proper synapsis between the homologous chromosomes, was also not observed. Puzzlingly, Miwi2 is no longer expressed when the first germ cells enter leptotene stages (Carmell et al., 2007), suggesting an indirect mechanism by which Miwi2 defects affect meiosis. Interestingly, transposons are activated in *miwi2* mutant germ cells due to DNA hypomethylation (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). This likely results in the activation of DNA-damage checkpoint programs. Activation of DNA-damage checkpoints has been shown to trigger secondary phenotypes associated with Piwi pathway mutations in *Drosophila* (Klattenhoff et al., 2007), raising the possibility that the meiotic phenotypes in mouse *miwi2* pathway mutants may be indeed triggered through similar secondary effects (Figure 3). More detailed analysis of these mutants will be required to solve these issues.

Germ Cell Specification and Differentiation

Apart from meiosis, Piwi proteins play a role in germ cell specification and differentiation during early development. In *Drosophila*, removal of maternal Piwi affects germ cell specification by affecting the maintenance of pole plasm (Megosh et al., 2006), and in zebrafish, the disruption of the Piwi protein Zili affects PGC differentiation (Houwing et al., 2008). Furthermore, nuage structures as a whole have been shown to be relevant in the context of germ cell specification. This is exemplified in species where maternally derived nuage material is involved in the specification of germ cell fate. In zebrafish and *Drosophila*, nuage appears to be both required and sufficient for germ cell specification (Bontems et al., 2009; Hashimoto et al., 2004; Thomson and Lasko, 2005). This suggests that nuage and Piwi pathway activity can be vital components in the process of establishing a germ cell state. However, Piwi activity in mouse PGCs only begins after the specification of PGC fate (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004), suggesting that Piwi activity is not an absolute prerequisite for PGC cell-fate specification. Rather, it may be more related to the maintenance of a newly established PGC population, similar to its role in maintaining germ cells at later stages of development (see below). This is consistent with a recent report that suggests that P-granule assembly in *C. elegans* is a consequence, rather than a cause, of establishing a germ cell fate (Gallo et al., 2010).

Piwi activity has also been associated with germ cell maintenance at later stages of germline development. In *C. elegans*, the Piwi proteins PRG-1 and PRG-2 play a role in the maintenance of the mitotic germ cell population (Cox et al., 1998). Piwi proteins play a similar role in mice, as the testis of *mili* and *miwi2* mutant animals become depleted of spermatogonia, suggesting a defect in spermatogonial stem cell maintenance (Carmell et al., 2007; Unhavaithaya et al., 2009).

Finally, RNAi activity, in particular the Piwi pathway, has been connected to stem cell function in flatworms (De Mulder et al., 2009; Reddien et al., 2005), where it is required for proper stem cell differentiation and maintenance. Whether such functions for the Piwi pathway are to be found in other animals is questionable, as the expression of Piwi proteins is very germline specific in most animals and only germline-specific phenotypes have been observed in the available mutants (see above).

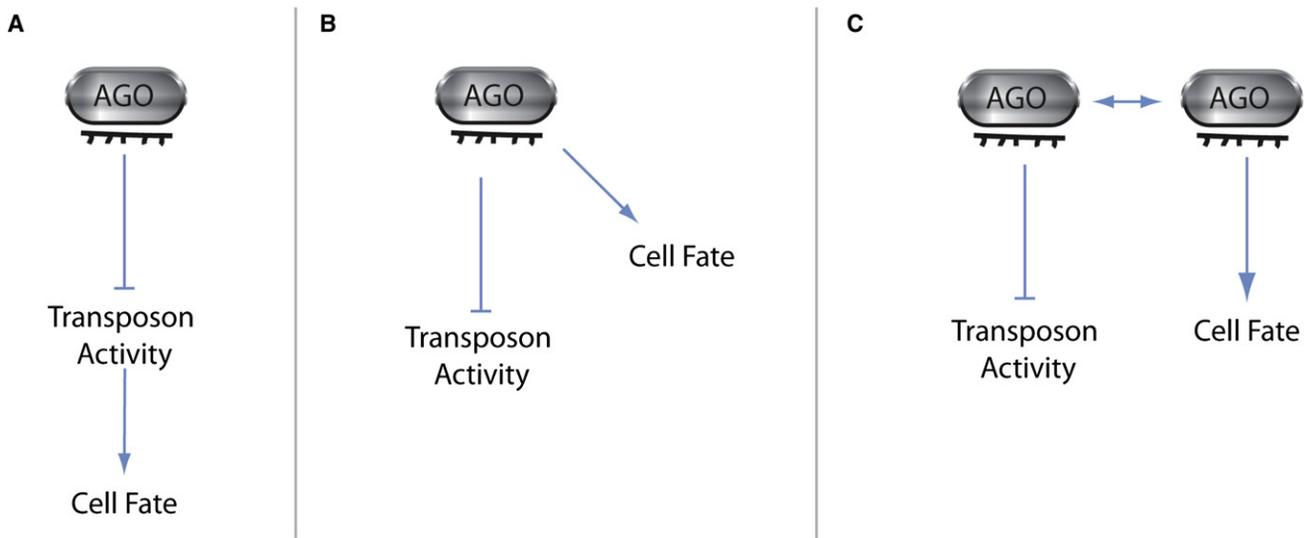


Figure 3. Potential Links between Transposon Silencing and Developmental Phenotypes

Three possible, mutually nonexclusive models of how the regulation of transposon activity may affect seemingly unrelated processes, such as meiosis and germ cell differentiation, which are collectively referred to here as cell fate.

(A) Transposon activity may directly influence these processes, perhaps via activation of DNA-damage checkpoint mechanisms.

(B) RNAi pathways may have acquired silencing targets other than transposons, and these may be involved cell-fate-related processes.

(C) Multiple RNAi-like pathways may be active, of which only a subset target transposons. As RNAi-pathways can affect each other, disruption of the transposon-related pathways could affect the regulation of other targets as well.

Still, these findings may relate to the germ cell maintenance defects that were discussed above, and further dissection of the Piwi pathways in flatworms would be very informative for understanding Piwi pathway biology in general.

Impact of Transposon Silencing on Developmental Phenotypes

As transposons constitute important targets for many, but not all, RNAi pathways (Ghildiyal and Zamore, 2009; Malone and Hanon, 2009; Okamura and Lai, 2008), activation of transposable elements could be the basis for many of the observed phenotypes when pathway components are disrupted (Figure 3). For example, this could be through the activation of DNA-damage checkpoints (as discussed above). It has recently also been suggested that the reduced silencing of transposons could play an essential role in meiosis (van der Heijden and Bortvin, 2009), and it has been proposed that transposon directed piRNAs have direct effects on gene expression in the early embryo (Rouget et al., 2010). Thus, transposon silencing may be more intertwined with other cellular processes than expected. However, it should be kept in mind that few of the RNAi pathways target transposons exclusively. Indeed, small RNA molecules with sequences derived from genes are incorporated into these pathways through which they can affect gene expression (Robine et al., 2009; Saito et al., 2009). Future studies will have to reveal to what extent transposon- and nontransposon-derived sRNA in a given RNAi pathway are relevant to the biological impact of that RNAi mechanism.

Concluding Remarks

As sketched above, RNAi pathways are involved in a great variety of processes, with a range of targets and mechanisms of action at both chromatin and posttranscriptional levels.

Transposons and other types of repetitive sequences constitute a significant portion of these targets, but nontransposon targets most definitely exist as well. It is striking, however, that while broadly evolutionary conserved, most RNAi pathways differ significantly from each other in their mechanistic details. For example, as discussed above, RdRP activity can be coupled to Dicer activity for sRNA generation or can independently generate products that may enter RNAi complexes directly. Furthermore, Piwi pathways are found either with or without the ping-pong amplification mechanism, and in the case of *C. elegans*, it is not even clear that the primary piRNA biogenesis mechanism resembles Piwi pathways of other organisms at all. The localization and sRNA association of RNAi pathway components also show major variations. The mouse Mili protein is usually not nuclear and can associate with primary piRNAs (Aravin et al., 2008), while its zebrafish ortholog Zili can be nuclear and is primarily loaded with secondary piRNAs (Houwing et al., 2008). On the other hand, Ziwi is cytoplasmic in zebrafish while Miwi2's main function appears to be in the nucleus. The only mechanistic consistency appears to be that Mili and Zili bind piRNAs derived mainly from sense transcripts, whereas their paralogs, Miwi2 and Ziwi, mainly bind antisense fragments.

We can only speculate as to why RNAi pathways appear to be so mechanistically flexible across different organisms. One answer could be that, as RNAi pathways often seem to be derived from defense systems, each organism has optimized its RNAi system(s) to efficiently battle the challenges it faces. Developmental programs could also have influenced the shape of RNAi pathways. For example, in comparing the Piwi pathways in mice and zebrafish, it is important to consider that germline development in the two systems differs substantially. In zebrafish, PGCs are specified early on, with major contribution of

antisense piRNAs from the oocyte to the PGCs as potential initiators of the Piwi pathway. In contrast, mouse PGCs are formed significantly later, in absence of maternally provided material. Thus, mouse PGCs most likely require a complete de novo initiation of the Piwi pathway and use sense transposon transcripts as a starting point. Such differences in the components that initiate an RNAi pathway may well be a contributing factor to the variations between homologous RNAi pathways in different organisms, though there is too little known at this point to support these speculations.

While we are beginning to grasp the mechanistic details of various RNAi pathways, it is clear that we still understand very little about the ways these mechanisms interact with other cellular programs and their functional significance. In addition, further study of various conserved RNAi pathways will be important to reveal their truly conserved properties. These insights will be essential to get at the heart of what the various RNAi pathways actually do and see and to truly understand their influence on the cells in which they are active.

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