Small silencing RNAs: an expanding universe

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Abstract | Since the discovery in 1993 of the first small silencing RNA, a dizzying number of small RNA classes have been identified, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs). These classes differ in their biogenesis, their modes of target regulation and in the biological pathways they regulate. There is a growing realization that, despite their differences, these distinct small RNA pathways are interconnected, and that small RNA pathways compete and collaborate as they regulate genes and protect the genome from external and internal threats.

Argonaute
Argonaute proteins are the effectors of small RNA-directed silencing. Small RNAs guide Argonautes to their RNA targets; Argonautes carry out the regulation. Argonaute proteins are characterized by two domains — Piwi (a ribonuclease domain) and PAZ (a ssRNA-binding module).

The defining features of small silencing RNAs are their short length (~20–30 nucleotides), and their association with members of the Argonaute family of proteins, which they guide to their regulatory targets, typically resulting in reduced expression of target genes. Beyond these defining features, different small RNA classes guide diverse and complex schemes of gene regulation. Some small silencing RNAs, such as small interfering RNAs (siRNAs), derive from dsRNA, whereas others, such as Piwi-interacting RNAs (piRNAs), do not. These different classes of regulatory RNAs also differ in the proteins required for their biogenesis, the constitution of the Argonaute-containing complexes that execute their regulatory functions, their modes of gene regulation and the biological functions in which they participate. New small RNA classes and new examples of existing classes continue to be discovered, such as the recent identification of endogenous siRNAs (endo-siRNAs) in flies and mammals. Here, we provide an overview of small silencing RNAs from plants and metazoan animals. For each class, we describe its biogenesis, function and mode of target regulation, providing examples of the regulatory networks in which each participates (TABLE 1). Finally, we highlight several examples of unexpected, and often unexplained, complexity in the interactions between distinct small RNA pathways.

The discovery of RNAi
In 1998, Fire and Mello established dsRNA as the silencing trigger in *Caenorhabditis elegans*[1]. Their experiments overturned the contemporary view that antisense RNA induced silencing by base pairing to its mRNA counterpart, thereby preventing its translation into protein. In worms and other animals, siRNA-mediated silencing is known as RNAi. Remarkably, RNAi is systemic in both plants and nematodes, spreading from cell to cell[2]. In *C. elegans*, RNAi is also heritable: silencing can be transferred to the progeny of the worm that was originally injected with the trigger dsRNA[3]. Viral infection, inverted-repeat transgenes or aberrant transcription products all lead to the production of dsRNA. dsRNA is converted to siRNAs that direct RNAi. siRNAs were discovered in plants[4] and were later shown in animal extracts to serve as guides that direct endonucleolytic cleavage of their target RNAs[5,6]. siRNAs can be classified according to the proteins involved in their biogenesis, their mode of regulation or their size. In this Review, we differentiate the major types of siRNAs according to the molecules that trigger their production — a classification scheme that best captures the biological distinctions among small silencing RNAs.

siRNAs derived from exogenous agents. Early examples of RNAi were triggered by exogenous dsRNA. In these cases, long exogenous dsRNA is cleaved into double-stranded siRNAs by Dicer, a dsRNA-specific RNase III family ribonuclease[7] (FIG. 1). siRNA duplexes produced by Dicer comprise two ~21 nucleotide strands, each bearing a 5′ phosphate and 3′ hydroxyl group, paired in a way that leaves two-nucleotide overhangs at the 3′ ends[5,8,9]. The strand that directs silencing is called the guide, whereas the other strand, which is ultimately destroyed, is the passenger. Target regulation by siRNAs is mediated by the RNA-induced silencing complex (RISC), which is the generic name for an Argonaute–small RNA complex[6]. In addition to an Argonaute protein and a small RNA guide, the RISC might also contain auxiliary proteins that extend or modify its function; for example, proteins that redirect the target mRNA to a site of general mRNA degradation[10].

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Table 1 | **Types of small silencing RNAs**

Name	Organism	Length (nt)	Proteins	Source of trigger	Function	Refs
miRNA	Plants, algae, animals, viruses, protists	20–25	Drosha (animals only) and Dicer	Pol II transcription (pri-miRNAs)	Regulation of mRNA stability, translation	93–95, 200–202,226
casiRNA	Plants	24	DCL3	Transposons, repeats	Chromatin modification	38,44,51, 52,61–63
tasiRNA	Plants	21	DCL4	miRNA-cleaved RNAs from the *TAS* loci	Post-transcriptional regulation	64–68
natsiRNA	Plants	22	DCL1	Bidirectional transcripts induced by stress	Regulation of stress-response genes	71,72
		24	DCL2			
		21	DCL1 and DCL2			
Exo-siRNA	Animals, fungi, protists	~21	Dicer	Transgenic, viral or other exogenous dsRNA	Post-transcriptional regulation, antiviral defense	4,5,8,227
	Plants	21 and 24				
Endo-siRNA	Plants, algae, animals, fungi, protists	~21	Dicer (except secondary siRNAs in *C. elegans*, which are products of RdRP transcription, and are therefore not technically siRNAs)	Structured loci, convergent and bidirectional transcription, mRNAs paired to antisense pseudogene transcripts	Post-transcriptional regulation of transcripts and transposons; transcriptional gene silencing	75–79,82, 83,86,87, 200,201, 228
piRNA	Metazoans excluding *Trichoplax adhaerens*	24–30	Dicer-independent	Long, primary transcripts?	Transposon regulation, unknown functions	157, 163–169, 177,202
piRNA-like (soma)	*Drosophila melanogaster*	24–30	Dicer-independent	In *ago2* mutants in *Drosophila*	Unknown	76
21U-RNA piRNAs	*Caenorhabditis elegans*	21	Dicer-independent	Individual transcription of each piRNA?	Transposon regulation, unknown functions	114, 173–175
26G RNA	*Caenorhabditis elegans*	26	RdRP?	Enriched in sperm	Unknown	114

ago2, Argonaute2; casiRNA, *cis*-acting siRNA; DCL, Dicer-like; endo-siRNA, endogenous small interfering RNA; exo-siRNA, exogenous small interfering RNA; miRNA, microRNA; natsiRNA, natural antisense transcript-derived siRNA; piRNA, Piwi-interacting RNA; Pol II, RNA polymerase II; pri-miRNA, primary microRNA; RdRP, RNA-dependant RNA polymerase; tasiRNA, *trans*-acting siRNA.

Mammals and *C. elegans* each have a single Dicer that makes both microRNAs (miRNAs) and siR-NAs[11–14], whereas *Drosophila* species have two Dicers: DCR-1 makes miRNAs, whereas DCR-2 is specialized for siRNA production[15]. The fly RNAi pathway defends against viral infection, and Dicer specialization might reduce competition for Dicer between precursor miR-NAs (pre-miRNAs) and viral dsRNAs. Alternatively, DCR-2 and Argonaute2 (AGO2) specialization might reflect the evolutionary pressure on the siRNA pathway to counter rapidly evolving viral strategies to escape RNAi. In fact, *dcr-2* and *ago2* are among the most rapidly evolving *Drosophila* genes[16]. *C. elegans* might achieve similar specialization with a single Dicer by using the dsRNA-binding protein RNAi defective (RDE-4) as the gatekeeper for entry into the RNAi pathway[17]. However, no natural virus infection has been documented in *C. elegans*[18]. By contrast, mammals might not use the RNAi pathway to respond to viral infection, having evolved an elaborate, protein-based immune system[19–21].

The relative thermodynamic stabilities of the 5′ ends of the two siRNA strands in the duplex determines the identity of the guide and passenger strands[22–24]. In flies, this thermodynamic difference is sensed by the dsRNA-binding protein R2D2, the partner of DCR-2 and a component of the RISC loading complex (RLC)[25,26]. The RLC recruits AGO2, to which it transfers the siRNA

duplex. AGO2 can then cleave the passenger strand as if it were a target RNA[27–31]. AGO2 always cleaves its RNA target at the phosphodiester bond between the nucleotides that are paired to nucleotides 10 and 11 of the guide strand[8,9]. Release of the passenger strand after its cleavage converts pre-RISC to mature RISC, which contains only single-stranded guide RNA. In flies, the guide strand is 2′-*O*-methylated at its 3′ end by the *S*-adenosyl methionine-dependent methyltrans-ferase HEN1 (also known as piRNA methyltransferase, PIMET), completing RISC assembly[32,33]. In plants, both miRNAs and siRNAs are terminally methylated, a modification that is crucial for their stability[34–36].

Plants exhibit a surprising diversity of small RNA types and the proteins that generate them. The diver-sification of RNA silencing pathways in plants might reflect the need of a sessile organism to cope with biotic and abiotic stress. The number of RNA silencing pro-teins can vary enormously among animals too, with *C. elegans* producing 27 distinct Argonaute proteins compared with 5 in flies. Phylogenetic data suggest that nearly all of these 'extra' *C. elegans* Argonautes act in the secondary siRNA pathway[37], perhaps because endog-enous secondary siRNAs are so plentiful in worms. *Arabidopsis thaliana* has four Dicer-like (DCL) proteins and 10 Argonautes, with both unique and redundant functions. In plants, inverted-repeat transgenes or co-expressed sense and antisense transcripts produce two
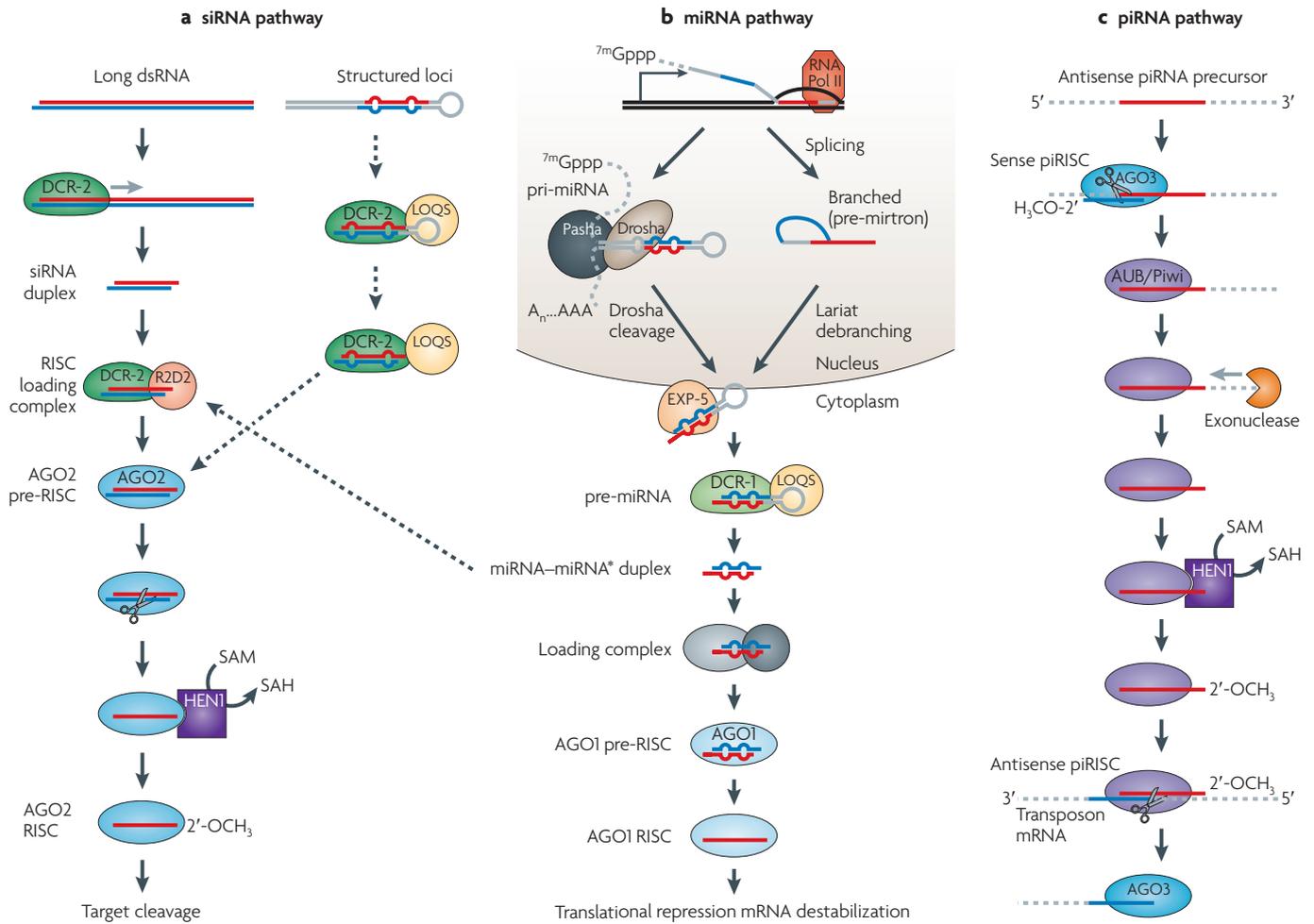
a siRNA pathway

b miRNA pathway

c piRNA pathway

Figure 1 | **Small RNA silencing pathways in *Drosophila*.** The three small RNA silencing pathways in flies are the small interfering RNA (siRNA), microRNA (miRNA) and Piwi-interacting RNA (piRNA) pathways. These pathways differ in their substrates, biogenesis, effector proteins and modes of target regulation. **a** | dsRNA precursors are processed by Dicer-2 (DCR-2) to generate siRNA duplexes containing guide and passenger strands. DCR-2 and the dsRNA-binding protein R2D2 (which together form the RISC-loading complex, RLC) load the duplex into Argonaute2 (AGO2). A subset of endogenous siRNAs (endo-siRNAs) exhibits dependence on dsRNA-binding protein Loquacious (LOQS), rather than on R2D2. The passenger strand is later destroyed and the guide strand directs AGO2 to the target RNA. **b** | miRNAs are encoded in the genome and are transcribed to yield a primary miRNA (pri-miRNA) transcript, which is cleaved by Drosha to yield a short precursor miRNA (pre-miRNA). Alternatively, miRNAs can be present in introns (termed mirtrons) that are liberated following splicing to yield authentic pre-miRNAs. pre-miRNAs are exported from the nucleus to the cytoplasm, where they are further processed by DCR-1 to generate a duplex containing two strands, miRNA and miRNA*. Once loaded into AGO1, the miRNA strand guides translational repression of target RNAs. **c** | piRNAs are thought to derive from ssRNA precursors and are made without a dicing step. piRNAs are mostly antisense, but a small fraction is in the sense orientation. Antisense piRNAs are preferentially loaded into Piwi or Aubergine (AUB), whereas sense piRNAs associate with AGO3. The methyltransferase HEN1 adds the 2′-*O*-methyl modification at the 3′ end. Piwi and AUB collaborate with AGO3 to mediate an interdependent amplification cycle that generates additional piRNAs, preserving the bias towards antisense. The antisense piRNAs probably direct cleavage of transposon mRNA or chromatin modification at transposon loci. SAH, *S*-adenosyl homocysteine; SAM, *S*-adenosyl methionine.

sizes of siRNAs: 21 and 24 nucleotides[38,39]. The 21-nucleotide siRNAs are produced by DCL4, but in the absence of DCL4, DCL2 can substitute, producing 22-nucleotide siRNAs[40–44]. The DCL4-produced 21-mers typically associate with AGO1 and guide mRNA cleavage. The 24-mers associate with AGO4 (in the major pathway) and AGO6 (in the surrogate pathway), and promote the formation of repressive chromatin[45].

In plants, exogenous sources of siRNAs are not confined to dsRNAs. Single-stranded sense transcripts from tandemly repeated or highly expressed single-copy transgenes are converted to dsRNA by RDR6, a member of the RNA-dependent RNA polymerase (RdRP) family that transcribe ssRNAs from an RNA template[46] (BOX 1). RDR6 and RDR1 also convert viral ssRNA into dsRNA, initiating an antiviral RNAi response[47]. The

Box 1 | **Amplifying silencing**
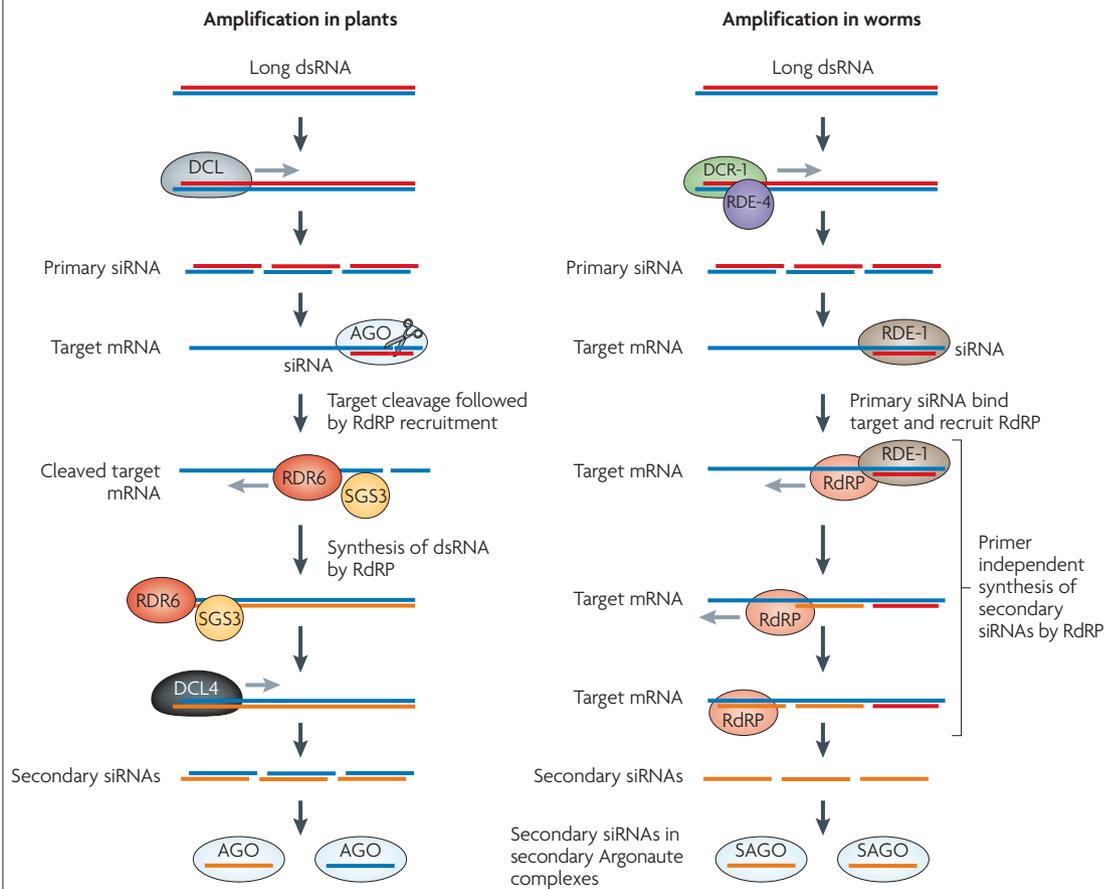
RNA-dependent RNA polymerases (RdRPs) amplify the silencing response. Primary small interfering RNAs (siRNAs), which are derived from exogenous triggers by Dicer processing, bind their mRNA targets and direct cleavage by Argonaute (AGO) complexes[204]. In plants, RdRPs (including RDR6) use these cleaved transcript fragments as templates to synthesize long dsRNA; the dsRNA is then diced into secondary siRNAs[41,42,44,46,205–207] (see figure). Secondary siRNAs are formed by DICER-LIKE 4 (DCL4) both 5′ and 3′ of the primary targeted interval, suggesting that mRNA cleavage, rather than priming of RdRP by primary siRNAs, is the signal for siRNA amplification. Other data suggest that production of secondary siRNAs in *Arabidopsis* might sometimes be primed[208]. RdRP amplification of siRNAs is especially important in defending plants against viral infection.

In *Caenorhabditis elegans*, primary siRNAs are amplified into secondary siRNAs by a different mechanism[204]. In worms, primary siRNAs are bound to RDE-1, a 'primary Argonaute' (REFS 209,210). The primary siRNAs guide RDE-1 to the target mRNA, to which it recruits RdRPs that synthesize secondary siRNAs[211,212] (see figure). Worm secondary siRNAs have a 5′ diphosphate or triphosphate, indicating that they are produced by transcription rather than by dicing[209,210,213] and, at least *in vitro*, secondary siRNA production does not require Dicer[212]. How the length of siRNA transcription is controlled is perplexing but, *in vitro*, the *Neurospora crassa* RdRP, QDE1, can directly transcribe short RNA oligomers of ~22 nucleotides from a much longer template[213]. As a consequence of their production by an RdRP, secondary siRNAs in *C. elegans* are exclusively antisense to their mRNA targets[209,214]. Secondary siRNAs function when bound to secondary Argonautes (SAGOs), such as CSR-1, which can cleave its mRNA targets in the same way as fly and human AGO2 proteins[212].

The presence of siRNA amplification in plants, worms, fungi and, according to some early reports, flies, led to the speculation that RdRPs are a universal feature of RNAi. An amplification step in human RNAi could produce secondary siRNAs bearing homology to other genes — a significant impediment to the use of RNAi as a target discovery tool or as therapy for human diseases. However, the success of allele-specific RNAi in cultured human cells and in mice makes it unlikely that an RdRP-catalysed amplification step occurs in mammals[215–219]. Similarly, extensive biochemical and genetic studies have shown that the fly RNAi pathway does not use an RdRP enzyme[5,39,220–223].

resulting dsRNA is cleaved by Dicer into siRNAs that are terminally 2′-*O*-methylated by HEN1 (REF. 36). It is poorly understood why plant RNAs that are expressed from transgenes are converted by RDR6 into dsRNA but abundant endogenous mRNAs are not. Recent evidence that some housekeeping exonucleases compete with plant RNA silencing pathways for aberrant RNAs suggests that substandard RNA transcripts — for
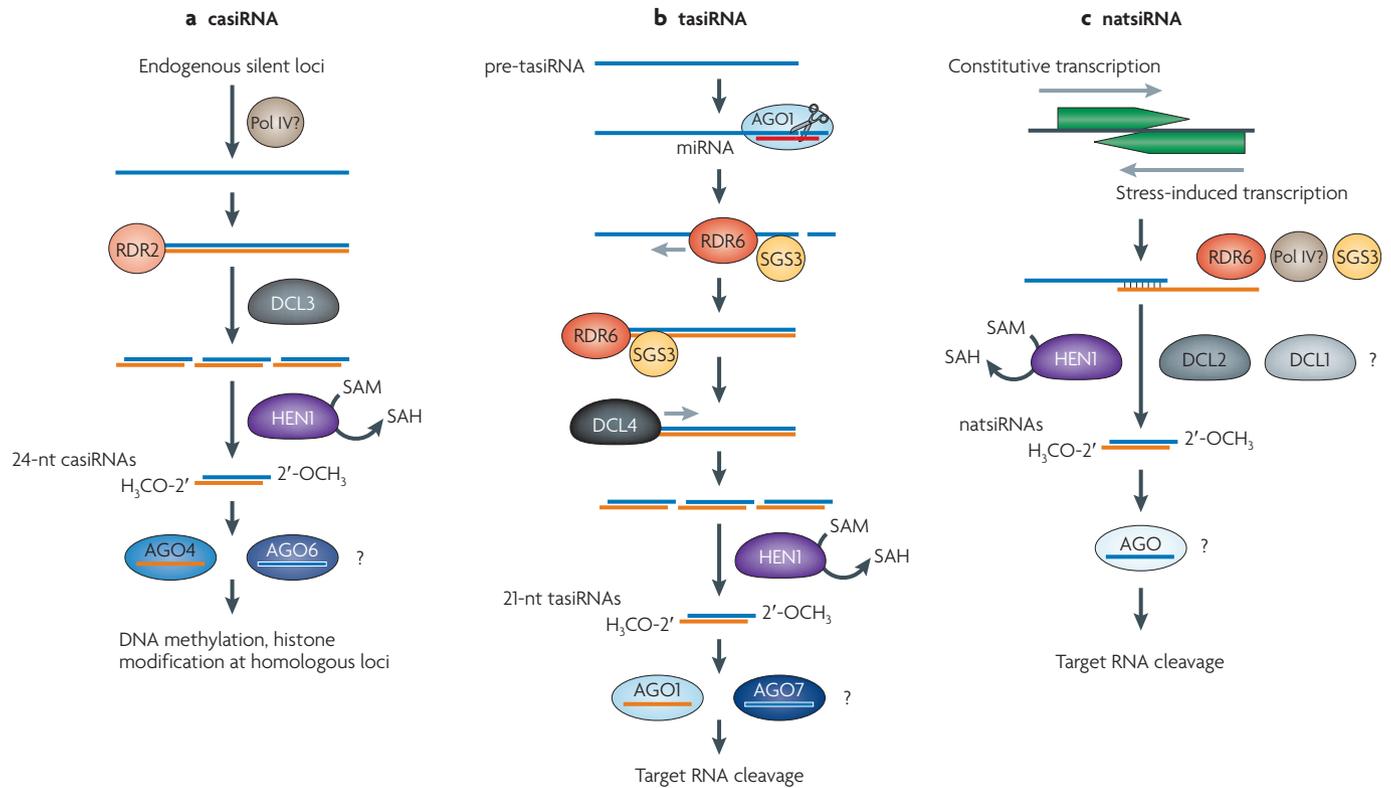
a casiRNA

Endogenous silent loci

Pol IV?

RDR2

DCL3

SAM
SAH

24-nt casiRNAs
H₃CO-2'   2'-OCH₃

AGO4   AGO6   ?

DNA methylation, histone
modification at homologous loci

b tasiRNA

pre-tasiRNA

AGO1

miRNA

RDR6   SGS3

RDR6   SGS3

DCL4

SAM
SAH

HEN1

21-nt tasiRNAs
H₃CO-2'   2'-OCH₃

AGO1   AGO7   ?

Target RNA cleavage

c natsiRNA

Constitutive transcription

Stress-induced transcription

RDR6   Pol IV?   SGS3

SAM
SAH   HEN1   DCL2   DCL1   ?

natsiRNAs
H₃CO-2'   2'-OCH₃

AGO   ?

Target RNA cleavage

Figure 2 | **Plant endogenous small interfering RNA (endo-siRNA) biogenesis.** *Cis*-acting siRNAs (casiRNAs), *trans*-acting siRNAs (tasiRNAs) and natural antisense transcript-derived siRNAs (natsiRNAs) are derived from distinct loci. Several of the proteins involved in their biogenesis are genetically redundant, whereas others have specialized roles. **a** | casiRNAs are the most abundant endogenously produced siRNAs in plants. The RNA polymerases POL IV and RDR2 are proposed to generate dsRNA precursors, which are then diced by DICER-LIKE 3 (DCL3) to generate 24-nucleotide casiRNAs. The methyltransferase HEN1 adds the 2'-O-methyl modification at the 3' end. These small RNAs load into ARGONAUTE 4 (AGO4) and perhaps AGO6, and promote heterochromatin assembly by targeting DNA methylation and histone modification at the corresponding loci. **b** | tasiRNA biogenesis requires microRNA (miRNA)-mediated cleavage of transcripts from the *TAS* loci (pre-tasiRNA), which triggers the production of dsRNA by RDR6. The dsRNA is diced into 21-nucleotide tasiRNAs by DCL4 and acts through either AGO1 or AGO7. **c** | natsiRNAs are derived from overlapping regions of convergent transcripts and require DCL1 or DCL2, POL IV, RDR6 and SUPPRESSOR OF GENE SILENCING 3 (SGS3) for their biogenesis.

example, those lacking a 5' cap or 3' poly(A) tail — act as substrates for RdRPs. Highly expressed transgenes might overwhelm normal RNA quality-control pathways, escape destruction and be converted to dsRNA by RdRPs[47–49].

endo-siRNAs
The first endo-siRNAs were detected in plants and *C. elegans*[38,50,51], and the recent discovery of endo-siRNAs in flies and mammals suggests that endo-siRNAs are ubiquitous among higher eukaryotes.

Plant endo-siRNAs. In plants, *cis*-acting siRNAs (casiR-NAs) originate from transposons, repetitive elements and tandem repeats such as 5S ribosomal RNA genes, and comprise the bulk of endo-siRNAs[44] (FIG. 2). casiRNAs are predominantly 24 nucleotides and are methylated by HEN1. Their accumulation requires DCL3 and the RNA polymerases RDR2 and POL IV, and either AGO6 (primarily) or AGO4, which acts redundantly[44,51–60]. casiRNAs promote heterochromatin formation by

directing DNA methylation and histone modification at the loci from which they originate[38,44,51,52,61–63].

Another class of plant endo-siRNAs illustrates how distinct small RNA pathways interact. *Trans*-acting siRNAs (tasiRNAs) are endo-siRNAs generated by the convergence of the miRNA and siRNA pathways in plants[64–68] (FIG. 2). miRNA-directed cleavage of certain transcripts recruits the RdRP enzyme RDR6. RDR6 then copies the cleaved transcript into dsRNA, which DCL4 dices into tasiRNAs that are phased. This phasing suggests that DCL4 begins dicing precisely at the miRNA cleavage site, making a tasiRNA every 21 nucleotides[68]. The site of miRNA cleavage is crucial, because in determining the entry point for Dicer it establishes the target specificity of the tasiRNA produced. One of the determinants that seems to predispose a transcript to produce tasiRNAs after its cleavage by a miRNA is the presence of a second miRNA- or siRNA-complementary site on the transcript. Of particular note is the *TAS3* locus, the RNA transcript of which has two binding sites for miR-390. Only one of these sites is efficiently cleaved
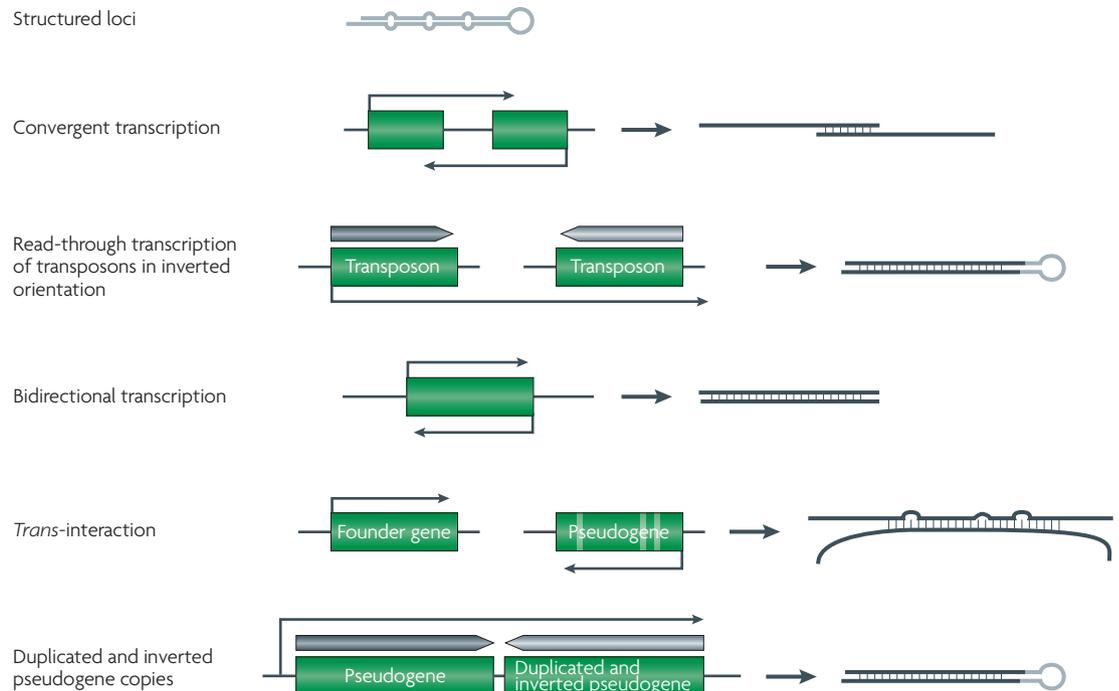
Figure 3 | **Genomic sources of dsRNA triggers for endogenous small interfering RNAs (endo-siRNAs) in flies and mammals.** siRNAs are derived from dsRNA precursors. endo-siRNAs can arise from structured loci that can pair intramolecularly to produce long dsRNA, complementary overlapping transcripts and bidirectionally transcribed loci. endo-siRNAs might also originate from protein-coding genes that can pair with their cognate pseudogenes and from regions of pseudogenes that can form inverted-repeat structures. Solid grey arrows indicate orientation, whereas black arrows indicate transcription.

by miR-390, but binding of the miRNA to both sites seems to be required to initiate conversion of the *TAS3* transcript to dsRNA by RDR6 (REFS 69,70).

Natural antisense transcript-derived siRNAs (nat-siRNAs) are produced in response to stress in plants[71,72] (FIG. 2). They are generated from a pair of convergently transcribed RNAs: typically, one transcript is expressed constitutively, whereas the complementary RNA is transcribed only when the plant is subject to environmental stress, such as high salt levels. Production of 21- and 24-nucleotide siRNAs from region of overlap between the two transcripts requires DCL2 and/or DCL1, RDR6, SGS3 (SUPPRESSOR OF GENE SILENCING 3, probably an RNA-binding protein)[73] and Pol IV[71,72]. The natsiRNAs then direct cleavage of one of the mRNAs of the pair, and in one such case, trigger the DCL1-dependent production of 21-nucleotide secondary siRNAs[72]. In addition to natsiRNAs, 'long' siRNAs (lsiRNAs) in *Arabidopsis* species also originate from natural antisense transcript pairs and are stress induced. Unlike natsi-RNAs, lsiRNAs are 30–40 nucleotides and require DCL1, DCL4, AGO7, RDR6 and Pol IV for their production[74].

Animal endo-siRNAs. Plant and worm endo-siRNAs are typically produced through the action of RdRPs (BOX 1). The genomes of flies and mammals do not seem to encode such RdRP proteins, so the recent discovery of endo-siRNAs in flies and mice was unexpected.

The first mammalian endo-siRNAs to be reported corresponded to the long interspersed nuclear element (L1) retrotransposon and were detected in cultured human cells[75]. Full-length L1 contains both sense and antisense promoters in its 5′ UTR that could, in principle, drive bidirectional transcription of L1, producing overlapping complementary transcripts to be processed into siRNAs by Dicer. However, the precise mechanism by which transposons trigger siRNA production in mammals remains unknown.

More recently, endo-siRNAs have been detected in somatic and germ cells of *Drosophila* species and in mouse oocytes. High-throughput sequencing of small RNAs from germline and somatic tissues of *Drosophila* and of AGO2 immunoprecipitates revealed a population of small RNAs that could readily be distinguished from miRNAs and piRNAs[76–81]. These small RNAs are nearly always exactly 21 nucleotides, are present in both sense and antisense orientations, have modified 3′ ends and, unlike miRNAs and piRNAs, are not biased towards beginning with uracil. Production of the 21-mers requires DCR-2, although in the absence of DCR-2 a remnant of the endo-siRNA population inexplicably persists.

Fly endo-siRNAs derive from transposons, heterochromatic sequences, intergenic regions, long RNA transcripts with extensive structure and, most interestingly, from mRNAs (FIG. 3). Expression of transposon mRNAs increases in both *dcr-2* and *ago-2* mutants,

Retrotransposon
A transposable element that replicates via an RNA intermediate, which is converted by reverse transcriptase to cDNA. The cDNA can be inserted into genomic DNA, increasing the number of copies of the retrotransposon in the genome.

implicating an endogenous RNAi pathway in the silencing of transposons in flies, as reported previously for *C. elegans*[82,83]. siRNAs derived from mRNAs are over ten times more likely to come from regions that are predicted to produce overlapping convergent transcripts than expected by chance[76], suggesting that endo-siRNAs originate from the endogenous dsRNA that is formed when these complementary transcripts pair.

A subset of fly endo-siRNAs derives from 'structured loci', RNA transcripts of which can fold into long intramolecularly paired hairpins[77–79]. Accumulation of these siRNAs requires DCR-2 and the dsRNA-binding protein Loquacious (LOQS) — which is typically considered the partner of DCR-1, the Dicer that produces miRNA — rather than R2D2 (REF. 84), the usual partner of DCR-2 (FIG. 1). Although surprising, a role for LOQS in the biogenesis of endo-siRNAs from structured loci was anticipated by the earlier finding that LOQS has a role in the production of siRNAs from transgenes that are designed to produce long intramolecularly paired inverted-repeat transcripts that trigger RNAi in flies[85].

endo-siRNAs have also been identified in mouse oocytes[86,87]. As in flies, mouse endo-siRNAs are 21 nucleotides, Dicer-dependent and derived from a variety of genomic sources (FIG. 3). The mouse endo-siRNAs were bound to AGO2, the only mammalian Argonaute protein thought to mediate target cleavage, although it is not known if they also associate with any of the other three mouse Argonaute proteins. Mammalian AGO2 is not, however, the orthologue of fly AGO2, the sequence of which is considerably diverged from other Argonaute proteins.

A subset of mouse oocyte endo-siRNAs maps to regions of protein-coding genes that are capable of pairing to their cognate pseudogenes, and to regions of pseudogenes that are capable of forming inverted-repeat structures (FIG. 3). Pseudogenes can no longer encode proteins, but they drift from their ancestral sequence more slowly than would be expected if they were simply junk DNA. Perhaps some pseudogene sequences are under evolutionary selection to retain the ability to produce antisense transcripts that can pair with their cognate genes to produce endo-siRNAs[88].

A key challenge for the future will be to understand the biological function of endo-siRNAs, especially those that can pair with protein-coding mRNAs. Do they regulate mRNA expression? And can endo-siRNAs act like miRNAs, tuning the expression of large numbers of genes?

miRNAs

The first miRNA to be discovered, *lin-4*, was identified in a screen for genes that are required for postembryonic development in *C. elegans*[89]. The *lin-4* locus produces a 22-nucleotide RNA that is partially complementary to sequences in the 3′ UTR of its regulatory target, the *lin-14* mRNA[90–92]. miRNA binding to partially complementary sites in mRNA 3′ UTRs is now considered to be a hallmark of animal miRNA

regulation. In 2001, tens of miRNAs were identified in humans, flies and worms by small RNA cloning and sequencing, thereby establishing miRNAs as a new class of small silencing RNAs[93–95]. miRBase (release 12.0), the registry that coordinates miRNA naming, now lists 1,638 distinct miRNA genes in plants and 6,930 in animals and their viruses[96].

miRNA biogenesis. miRNAs derive from precursor transcripts called primary miRNAs (pri-miRNAs), which are typically transcribed by RNA polymerase II (RNA Pol II)[97–100]. Several miRNA genes are present as clusters in the genome and probably derive from a common pri-miRNA transcript. Liberating a 20–24-nucleotide miRNA from its pri-miRNA requires the sequential action of two RNase III endonucleases, assisted by their dsRNA-binding domain (dsRBD) partner proteins (FIG. 1). First, the pri-miRNA is processed in the nucleus into a 60–70-nucleotide pre-miRNA by Drosha, acting with its dsRBD partner — DGCR8 in mammals and Pasha in flies[97,101–105]. The resulting pre-miRNA has a hairpin structure: a loop flanked by base-paired arms that form a stem. Pre-miRNAs have a two-nucleotide overhang at their 3′ ends and a 5′ phosphate group, which are indicative of their production by an RNase III. The nuclear export protein Exportin 5 carries the pre-miRNA to the cytoplasm bound to Ran, a GTPase that moves RNA and proteins through the nuclear pore[106–109].

In the cytoplasm, Dicer and its dsRBD partner protein, TRBP in mammals and LOQS in flies, cleaves the pre-miRNA[7,11–13,85,110–113]. Dicer — like Argonaute proteins but unlike Drosha — contains a PAZ domain, presumably allowing it to bind the two-nucleotide 3′-overhanging end left by Drosha. Dicer cleavage generates a duplex containing two strands, termed miRNA and miRNA*, corresponding to the two sides of the base of the stem. These correspond to the guide and passenger strands of an siRNA, and similar thermodynamic criteria influence the choice of miRNA versus miRNA*[22,23]. miRNAs can arise from either arm of the pre-miRNA stem, and some pre-miRNAs produce mature miRNAs from both arms, whereas others show such pronounced asymmetry that the miRNA* is rarely detected even in high-throughput sequencing experiments (BOX 2).

In flies, worms and mammals, a few pre-miRNAs are produced by the nuclear pre-mRNA splicing pathway instead of through processing by Drosha[115–119]. These pre-miRNA-like introns, termed mirtrons, are spliced out of mRNA precursors. The spliced introns first accumulate as lariat products that require 2′–5′ debranching by a lariat-debranching enzyme. Debranching yields an authentic pre-miRNA, which can then enter the standard miRNA biogenesis pathway.

In plants, DCL1 fills the roles of both Drosha and Dicer, converting pri-miRNAs to miRNA–miRNA* duplexes[44,120–122]. DCL1, assisted by its dsRBD partner HYL1, converts pri-miRNAs to miRNA–miRNA* duplexes in the nucleus, after which the miRNA–miRNA* duplex is thought to be exported to the

Box 2 | High-throughput sequencing and small RNA discovery

Much of the credit for the identification of small RNAs rests with advances in high-throughput sequencing. Currently, there are three commercial 'high depth' sequencing systems: the Roche 454 GS FLX Genome Analyzer, the Illumina Solexa Genome Analyzer and, most recently, the Applied Biosystem SOLiD System. REF. 224 describes how each method works. Whereas 454 has the advantage of sequencing >250 bp per read, compared with ~35–50 bp for Solexa and SOLiD, the Solexa and SOLiD platforms provide 70-fold to 400-fold greater sequencing depth. All three platforms have been used successfully to identify novel small RNA species and to discover new small RNA classes in plants and animals.

Using less than 10 µg of total RNA, high-throughput sequencing, together with advances in small RNA library preparation, has revealed the length distribution, sequence identity, terminal structure, sequence and strand biases, isoform prevalence, genomic origins, and mode of biogenesis for millions of small RNAs. Initial small RNA sequencing experiments sought simply to identify novel small RNA species and classes. Increasingly, high-throughput sequencing is being used to profile small RNA expression across the stages of development and in different tissues and disease states. Profiling by deep sequencing provides quantitative information about small RNA expression, as would PCR or microarray-based approaches, but can also precisely detect subtle changes in small RNA sequence or length.

Perhaps the most problematic step in small RNA sequencing is preparing the small RNA library. The most frequently used cloning protocols require the small RNAs to have a 5′ phosphate and a 3′ hydroxyl group — the hallmarks of Dicer products. This approach identifies small RNAs with the expected termini, but alternative methods must be used to find small RNAs, such as *Caenorhabditis elegans* secondary siRNAs, with other terminal structures. Additionally, finding every possible small RNA in a cell using exhaustive deep sequencing is an approach with diminishing returns. For example, although many microRNAs have been sequenced hundreds of thousands or even a million times, the *C. elegans* miRNA *lsy-6*, which is apparently expressed in less than ten cells of the adult, has so far eluded high-depth sequencing[225].

cytoplasm by HASTY, an Exportin 5 homologue (*hasty* mutants develop precociously, hence their name)[65,122–124]. Unlike animal miRNAs, plant miRNAs are 2′-O-methylated at their 3′ ends by HEN1 (REFS 34,120,125). HEN1 protects plant miRNAs from 3′ uridylation, which is thought to be a signal for degradation[36]. HEN1 probably acts before miRNAs are loaded into AGO1, because both miRNA* and miRNA strands are modified in plants[34].

Target regulation by miRNAs. The mechanism by which a miRNA regulates its mRNA target reflects both the specific Argonaute protein into which the small RNA is loaded and the extent of complementarity between the miRNA and the mRNA[126–128]. A few miRNAs in flies and mammals are nearly fully complementary to their mRNA targets; these direct endonucleolytic cleavage of the mRNA[129–133]. Such extensive complementarity is considered the norm in plants, as target cleavage was thought to be the main mode of target regulation in plants[39,62,134]. However, in flies and mammals, most miRNAs pair with their targets through only a limited region of sequence at the 5′ end of the miRNA called the 'seed region'; these miRNAs repress translation and direct degradation of their mRNA targets[135–140]. The seed region of all small silencing RNAs contributes most of the energy for target binding[141,142]. Thus, the seed is the primary specificity determinant for target selection. The small size of the seed means that a single miRNA can regulate many, even hundreds, of different genes[143,144]. Intriguingly, recent data suggest that the nuclear transcriptional history of an mRNA influences whether a miRNA represses its translation at the initiation or the elongation step[145].

As plant miRNAs are highly complementary to their mRNA targets, they can direct mRNA target cleavage. Nonetheless, AGO1-loaded plant miRNAs can also block translation, suggesting a common mechanism between plant and animal miRNAs, despite the absence of specific miRNAs shared between the two kingdoms[146].

Functions of miRNAs. Like transcription factors, miRNAs regulate diverse cellular pathways and are widely believed to regulate most biological processes in plants and animals, ranging from housekeeping functions to responses to environmental stress. Covering this vast body of work is beyond the scope of this article; the cited reviews provide valuable insight[147–149].

The study of miRNA pathway mutants provided early evidence for the influence of miRNAs on biological processes in both plants and animals. Loss of Dicer or miRNA-associated Argonaute proteins is nearly always lethal in animals, and such mutants show severe developmental defects in both plants and animals. In *Drosophila* species, *dcr-1* mutant germ-line stem-cell clones divide slowly; in *Arabidopsis* species, embryogenesis is abnormal in *dcl1* mutants; in *C. elegans*, *dcr-1* mutants display defects in germline development and embryonic morphogenesis; zebrafish lacking both maternal and zygotic Dicer are similarly defective in embryogenesis; and mice lacking Dicer die as early embryos, apparently devoid of stem cells[14,120,150–153]. Loss of Dicer in mouse embryonic fibroblasts causes increased DNA damage and, consequently, the upregulation of signalling by the tumour suppressor proteins p19ARF and p53, which induces premature senescence[154].

Many miRNAs function in specific biological processes, in specific tissues and at specific times[155]. The importance of small silencing RNAs goes far beyond the RNA silencing field: long-standing questions about the molecular basis of pluripotency, tumorigenesis, apoptosis, cell identity and so on are finding answers in small RNAs[147,156].

piRNAs: the longest small RNAs

piRNAs function in the germ line. piRNAs are the most recently discovered class of small RNAs and, as their name suggests, they bind to the Piwi clade of Argonaute proteins. (Animal Argonaute proteins can be subdivided by sequence relatedness into Argonaute and Piwi subfamilies.) The Piwi clade comprises Piwi, Aubergine (AUB) and AGO3 in flies, MILI, MIWI and MIWI2 in mice (also called PIWIL1, PIWIL2 and PIWIL4, respectively), and HILI, HIWI1, HIWI2 and HIWI3 in humans (also called PIWIL2, PWIL, PIWIL4 and PIWIL3, respectively).

Sequencing depth
The number of sequences obtained by high-throughput sequencing for a specific sample. Sequencing depth is often expressed as 'genome-matching reads'.

piRNAs were first proposed to ensure germline stability by repressing transposons when Aravin and colleagues discovered in flies a class of longer small RNAs (~25–30 nucleotides) associated with silencing of repetitive elements[157]. Later, these 'repeat associated small interfering RNAs' — subsequently renamed piR-NAs — were found to be distinct from siRNAs: they bind Piwi proteins and do not require DCR-1 or DCR-2 for their production, unlike miRNAs and siRNAs[33,158,159]. Moreover, they are 2'-O-methylated at their 3' termini, unlike miRNAs but similar to siRNAs in flies[32,158,160–162].

High-throughput sequencing of vertebrate piR-NAs revealed a class of piRNAs unrelated to repetitive sequences[87,163–169]. Mammalian piRNAs can be divided into pre-pachytene and pachytene piRNAs, according to the stage of meiosis at which they are expressed in developing spermatocytes. Like piRNAs in flies, pre-pachytene piRNAs predominantly correspond to repetitive sequences and are implicated in silencing transposons, such as L1 and intracisternal A-particle (IAP)[166]. In male mice, gametic methylation patterns are established when germ cells arrest their cell cycle 14.5 days post-coitum, resuming cell division 2–3 days after birth[170,171]. Both MILI and MIWI2 are expressed during this period, and *Miwi2*- and *Mili*-deficient mice lose DNA methylation marks on transposons[172]. The pre-pachytene piRNAs, which bind MIWI2 and MILI, might serve as guides to direct DNA methylation of transposons. In contrast to pre-pachytene piRNAs, the pachytene piRNAs mainly arise from unannotated regions of the genome, not transposons, and their function remains unknown[166].

Three recent studies report that the previously discovered germline '21U' RNAs in *C. elegans* are piR-NAs[114,173–175]. These small RNAs were initially identified by high-throughput sequencing[114]. They are precisely 21 nucleotides, begin with a uridine 5'-monophosphate and are 3' modified. They bind Piwi-related gene (PRG-1), a *C. elegans* Piwi protein. Each 21U-RNA might be transcribed separately, as they all are flanked by a common upstream motif. Like piRNAs in *Drosophila* species, the 21U-RNAs are required for maintenance of the germ line and fertility, and like *Drosophila* AUB and other piRNA pathway components, PRG-1 is found in specialized 'P granules', which are associated with germline function, in a perinuclear ring called nuage. Worm piRNAs resemble pachytene piRNAs in mammals: their targets and functions are largely unknown.

Biogenesis of piRNAs. piRNA sequences are stunningly diverse, with more than 1.5 million distinct piRNAs identified thus far in flies, but collectively they map to a few hundred genomic clusters[77,79,81,159,176–178]. The best studied cluster is the *flamenco* locus. *flamenco* was identified genetically as a repressor of the *gypsy*, *ZAM* and *Idefix* transposons[33,179–183]. Unlike siRNAs, *flamenco* piRNAs are mainly antisense, suggesting that piRNAs arise from long, single-stranded precursor RNAs. In fact, disruption of *flamenco* by insertion of a P-element near the 5' end of the locus blocks the

production of distal piRNAs up to 168 kb away. Thus, an enormously long, ssRNA transcript seems to be the source of the piRNAs that derive from the *flamenco* locus[177].

The current model for piRNA biogenesis was inferred from the sequences of piRNAs that are bound to Piwi, AUB and AGO3 (REFS 177,184). piRNAs bound to Piwi and AUB are typically antisense to transposon mRNAs, whereas AGO3 is loaded with piRNAs corresponding to the transposon mRNAs themselves (FIG. 1). Moreover, the first 10 nucleotides of antisense piRNAs are frequently complementary to the sense piRNAs found in AGO3. This unexpected sequence complementarity has been proposed to reflect a feed-forward amplification mechanism — 'piRNA ping-pong'— that is activated only after transcription of transposon mRNA[177,184] (FIG. 4). A similar amplification loop has been inferred from high-throughput piRNA sequencing in vertebrates, implying that it has been conserved through evolution[163,172]. Many aspects of the ping-pong model remain speculative. Why AGO3 seems to bind only sense piRNAs derived from transposon mRNAs is unknown. An untested idea is that different forms of RNA Pol II transcribe primary piRNA transcripts and transposon mRNAs, and that the specialized RNA Pol II that transcribes the primary piRNA precursor recruits Piwi and AUB, but not AGO3. How the 3' ends of piRNAs are made is also not known.

piRNA function and regulation. Piwi family proteins are indispensable for germline development in many, perhaps all, animals; but they have thus far been most extensively studied in *Drosophila* species. Piwi is restricted to the nucleoplasm of *Drosophila* germ cells and adjacent somatic cells. Piwi is required to maintain germline stem cells and to promote their division; the protein is required in both the somatic niche cells that support germline stem cells and in the stem cells themselves[185,186]. In the male germ line, AUB is required for the silencing of the repetitive *Stellate* locus, which would otherwise cause male sterility. Expression of *Stellate* is controlled by the related repetitive *Suppressor of Stellate* locus, the source of antisense piRNAs that act through AUB to repress *Stellate*[157,158,187].

aubergine was originally identified because it is required for specification of the embryonic axes[188]. The loss of anterior–posterior and dorsal–ventral patterning in embryos from mothers lacking AUB is an indirect consequence of the dsDNA breaks that occur in the oocyte in its absence[189]. The breaks seem to activate a DNA-damage checkpoint that disrupts patterning of the oocyte and, consequently, of the embryo. The defects in patterning, but not in silencing repetitive elements, are rescued by mutations that bypass the DNA damage signalling pathway, suggesting the breaks are caused by transposition. That activation of a DNA damage checkpoint should inappropriately reorganize embryonic polarity was unexpected, but further underscores the vital part piRNAs play in germline development.

Pachytene
Pachytene is the stage of meiotic prophase during which the homologous chromosomes condense and separate into two chromatids. At the pachytene stage, homologous chromosomes pair and crossing over — the exchange of DNA segments — occurs between two non-sister chromatids.
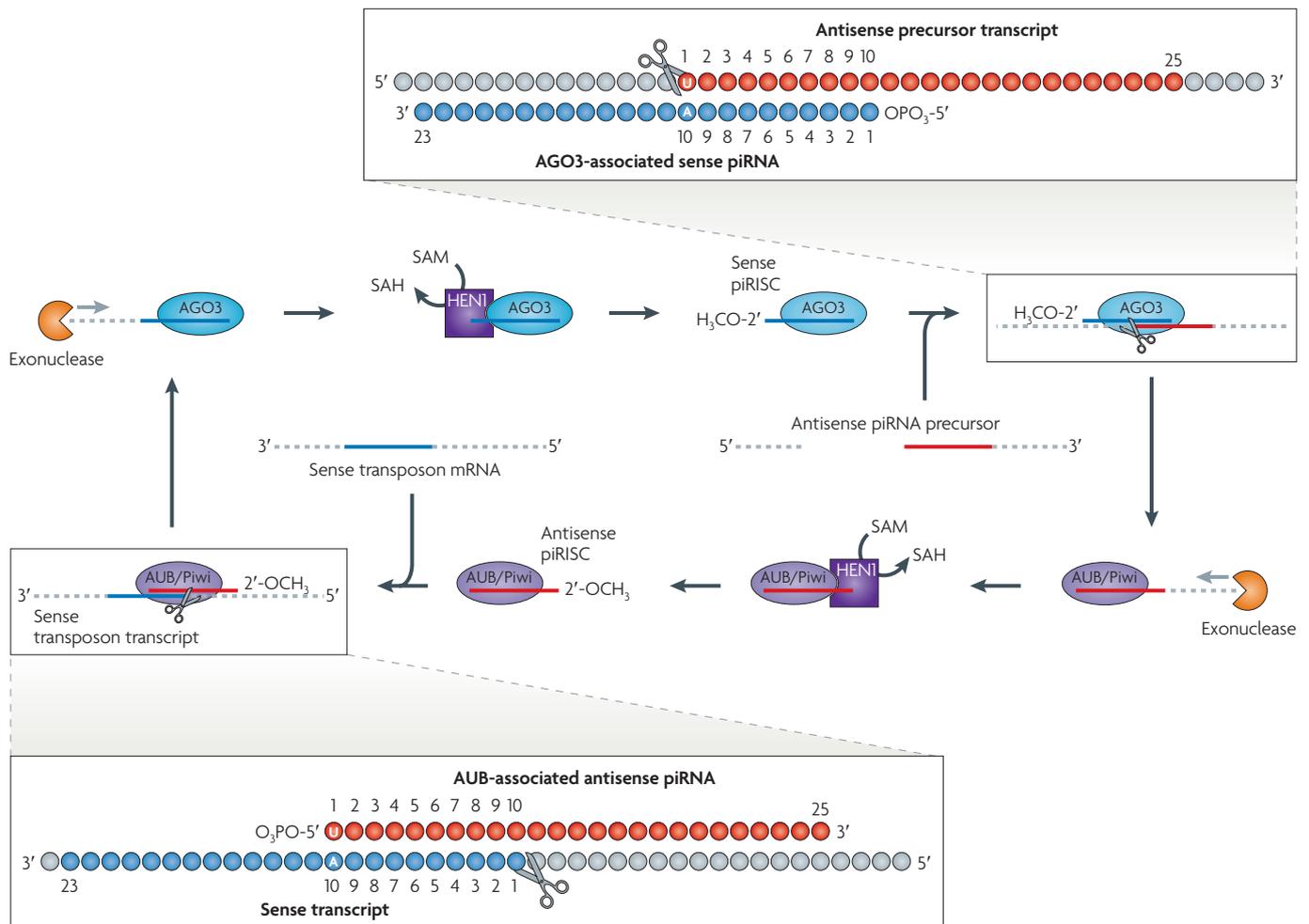
Figure 4 | **Feed-forward or 'ping-pong' model for Piwi-interacting RNA (piRNA) amplification.** According to this model, antisense piRNAs in Piwi or Aubergine (AUB) first bind transposon mRNAs and cleave them across from position 10 of the antisense piRNA guide. The 5′ end of the cleaved product is thought to then load into AGO3 and generate an AGO3-bound sense piRNA. The sense piRNA can, in turn, guide cleavage of an antisense piRNA precursor transcript, fuelling the feed-forward amplification loop. A key postulate of the model is that the intracellular concentrations of piRNA-loaded Piwi and AUB are much greater than of piRNA-loaded AGO3. The amplification loop is proposed to facilitate piRNA surveillance of transposon transcription in the germ line. The methyltransferase HEN1 adds the 2′-O-methyl modification at the 3′ end. SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine.

piRNAs outside the germ line? The role of piRNAs in the fly soma is hotly debated. Piwi and AUB are required to silence tandem arrays of *white*, a gene required to produce red eye pigment[190]. It is not understood if piRNAs are produced in the soma as well as in the germ line, or if piRNAs that are present during germline development deposit long-lived chromatin marks that exert their effects days later.

Both piRNAs and endo-siRNAs repress transposons in the germ line, where mutations caused by transposition would propagate to the next generation. siRNAs, which are produced by the RNAi pathway, probably provide a rapid response to the introduction of a new transposon into the germ line, a challenge not dissimilar to a viral infection. By contrast, the piRNA system seems to provide a more robust, permanent solution to the acquisition of a transposon. In the soma, however, endo-siRNAs are the predominant transposon-derived small RNA class, and their loss in *dcr-2* and *ago2* mutants increases transposon expression[76,77,79,81]. Somatic piRNA-like small RNAs have been observed in *ago2* mutant flies[76]. Perhaps, in the absence of endo-siRNAs, piRNAs are produced somatically and resume transposon surveillance. Such a model implies significant cross-talk between the piRNA and endo-siRNA–generating machineries.

Intertwined pathways

The RNAi, miRNA and piRNA pathways were initially believed to be independent and distinct. However, the lines distinguishing them continue to fade. These pathways interact and rely on each other at several levels, competing for and sharing substrates, effector proteins and cross-regulating each other.

Competition for substrates during loading. Both the siRNA and miRNA pathways load dsRNA duplexes containing an ~19 bp double-stranded core flanked by 3′ overhangs of two nucleotides. An siRNA duplex contains guide and passenger strands and is complementary throughout its core; a miRNA–miRNA* duplex contains mismatches, bulges and GU wobble pairs. In *Drosophila* species, biogenesis of small RNA duplexes is uncoupled from its loading into AGO1 or AGO2 (REFS 191,192). Instead, loading is governed by the structure of the duplex: duplexes bearing bulges and mismatches are sorted into the miRNA pathway and hence loaded into AGO1; duplexes with greater double-stranded character partition into AGO2, the Argonaute protein that is associated with RNAi.

The partitioning of small RNAs between AGO1 and AGO2 also has implications for target regulation. AGO1 primarily represses translation, whereas AGO2 represses by target cleavage, reflecting the faster rate of target cleavage by AGO2 compared with AGO1 (REF. 192). Sorting creates competition between the two pathways for substrates[191,192]. In *Drosophila*, loading of a small RNA duplex into one pathway decreases its association with the other pathway.

Different dsRNA precursors require distinct combinations of proteins to produce small silencing RNAs. For example, *Drosophila* endo-siRNAs derived from structured loci require LOQS rather than R2D2 (REFS 77–79). We presume that under some circumstances the endo-siRNA and miRNA pathways might therefore compete for LOQS. The endo-siRNA and RNAi pathways probably also compete for shared components.

In contrast to *Drosophila*, plants load small RNAs into Argonautes according to the identity of the 5′ nucleotide of the small RNA[69,193]. AGO1 is the main effector Argonaute for miRNAs, and the majority of miRNAs begin with uridine; AGO4 is the major effector of the heterochromatic pathway and is predominantly loaded with small RNAs beginning with an adenosine[194]. AGO2 and AGO5, however, have no characterized function in plants[194]. Changing the 5′ nucleotide from adenosine to uracil shifts the loading bias of plant small RNAs from AGO2 to AGO1, and vice versa. Similarly, *Arabidopsis* AGO4 binds small RNAs that begin with adenosine, whereas AGO5 prefers cytidine.

piRNAs bound to AUB and Piwi typically begin with uracil, whereas those bound to AGO3 show no 5′ nucleotide bias. It remains to be determined if this reflects a 5′-nucleotide preference like the situation for the plant Argonautes or some feature of an as yet undiscovered piRNA-loading machinery that sorts piRNAs between Piwi proteins.

Cross talk. Small RNA pathways are often entangled. tasiRNA biogenesis in *Arabidopsis* is a classic example of such cross talk between pathways. miRNA-directed cleavage of tasiRNA-generating transcripts initiates tasiRNA production and subsequent regulation of tasiRNA targets[64–68]. In *C. elegans* at least one piRNA has been implicated in initiating endo-siRNA production[173,174], and in flies the endo-siRNA pathway might

repress expression of piRNAs in the soma[76]. Moreover, small RNA levels might be buffered by negative feedback loops in which small RNAs from one pathway alter the expression levels of RNA silencing proteins that act in the same or in other RNA silencing pathways[195–199].

Conclusions

Despite our growing understanding of the mechanism and function of small RNAs, their evolutionary origins remain obscure. siRNAs are present in all three eukaryotic kingdoms — plants, animals and fungi — and provide antiviral defence in at least plants and animals. Thus, the siRNA machinery was present in the last common ancestor of plants, animals and fungi. By contrast, miRNAs have only been found in land plants, in the unicellular green alga *Chlamydomonas reinhardtii* and in metazoan animals, but not in unicellular choanoflagellates or fungi[200–202]. Deep-sequencing experiments have found no miRNAs shared by plants and animals, suggesting that miRNA genes, unlike the miRNA protein machinery, arose independently at least twice in evolution. Finally, piRNAs seem to be the youngest major small RNA family, having been found only in metazoan animals[202]. Although Dicer proteins have been identified only in eukaryotes, Argonaute proteins can also be found in eubacteria and archaea, raising the prospect that small nucleic acids might have served as guides for proteins at the dawn of cellular life, and even though the machinery might be ancient, the small RNA guides have diversified over time to acquire specialized roles.

The history of small silencing RNAs makes predicting the future particularly daunting, as new discoveries have come at a breakneck pace, with each new small RNA mechanism or function forcing a re-evaluation of cherished models and 'facts'. Several longstanding but unanswered questions, however, are worth highlighting. First, does RNAi — in the sense of an siRNA-guided defence against external nucleic acid threats, such as viruses — exist in mammals? Second, how do miRNAs repress gene expression? Do several parallel mechanisms coexist *in vivo*, or will the current, apparently contradictory models for miRNA-directed translational repression and mRNA decay ultimately be unified in a larger mechanistic scheme? Third, can miRNA-regulated genes ever be identified by computation alone, or will computational predictions ultimately give way to high-throughput experimental methods for associating individual miRNA species with their regulatory targets? Will network analysis uncover themes in the relationships between miRNAs and their targets that reveal why miRNA regulation is so widespread in animals? Fourth, how are piRNAs made? The feed-forward amplification 'ping-pong' model is appealing, but probably underestimates the complexity of piRNA biogenesis mechanisms. We do not yet know how piRNA 3′ ends are generated. Nor do we have a coherent model for how long, antisense transcripts from piRNA clusters are fragmented into piRNAs. Finally, will the increasing number of examples of small RNAs carrying epigenetic information across generations[3,203] ultimately force us to re-examine our Mendelian view of inheritance?

1. Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811 (1998).
   The landmark discovery of dsRNA as the trigger for RNAi.
2. Voinnet, O. Non-cell autonomous RNA silencing. *FEBS Lett.* **579**, 5858–5871 (2005).
3. Grishok, A., Tabara, H. & Mello, C. C. Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* **287**, 2494–2497 (2000).
4. Hamilton, A. J. & Baulcombe, D. C. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950–952 (1999).
   This paper identified small RNAs — now known as siRNAs — as the guides for post-transcriptional gene silencing in plants.
5. Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33 (2000).
   This paper and reference 6 showed that siRNAs are the specificity determinants for endonucleolytic cleavage of RNA targets by RISC.
6. Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. J. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296 (2000).
7. Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366 (2001).
   In this work, Dicer was shown to be the dsRNA-specific ribonuclease that converts long dsRNA into siRNAs.
8. Elbashir, S. M., Lendeckel, W. & Tuschl, T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* **15**, 188–200 (2001).
   This paper and reference 9 deduced the structure of siRNAs and discovered that they direct cleavage at the phosphodiester bond in the target that lies across from bases 10 and 11 in the small RNA guide, now a general principle for Argonaute-mediated, small RNA-guided silencing.
9. Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W. & Tuschl, T. Functional anatomy of siRNAs for mediating efficient RNAi *in Drosophila melanogaster* embryo lysate. *EMBO J.* **20**, 6877–6888 (2001).
10. Liu, J. *et al.* A role for the P-body component GW182 in microRNA function. *Nature Cell Biol.* **7**, 1161–1166 (2005).
11. Hutvágner, G. *et al.* A cellular function for the RNA-interference enzyme Dicer in the maturation of the *let-7* small temporal RNA. *Science* **293**, 834–838 (2001).
    This study, together with references 12 and 13, linked the RNAi and miRNA pathways, establishing that they share proteins, such as Dicer, and mechanisms of biogenesis and function.
12. Grishok, A. *et al.* Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* **106**, 23–34 (2001).
13. Ketting, R. F. *et al.* Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev.* **15**, 2654–2659 (2001).
14. Knight, S. W. & Bass, B. L. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*. *Science* **293**, 2269–2271 (2001).
15. Lee, Y. S. *et al.* Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* **117**, 69–81 (2004).
16. Obbard, D. J., Jiggins, F. M., Halligan, D. L. & Little, T. J. Natural selection drives extremely rapid evolution in antiviral RNAi genes. *Curr. Biol.* **16**, 580–585 (2006).
17. Tabara, H., Yigit, E., Siomi, H. & Mello, C. C. The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DexH-Box helicase to direct RNAi in *C. elegans*. *Cell* **109**, 861–871 (2002).
18. Shaham, S. Worming into the cell: viral reproduction in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA* **103**, 3955–3956 (2006).
19. Williams, B. R. PKR; a sentinel kinase for cellular stress. *Oncogene* **18**, 6112–6120 (1999).
20. Kunzi, M. S. & Pitha, P. M. Interferon research: a brief history. *Methods Mol. Med.* **116**, 25–35 (2005).
21. Vilcek, J. Fifty years of interferon research: aiming at a moving target. *Immunity* **25**, 343–348 (2006).
22. Schwarz, D. S. *et al.* Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**, 199–208 (2003).
23. Khvorova, A., Reynolds, A. & Jayasena, S. D. Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**, 209–216 (2003).
24. Aza-Blanc, P. *et al.* Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. *Mol. Cell* **12**, 627–637 (2003).
25. Liu, Q. *et al.* R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. *Science* **301**, 1921–1925 (2003).
    This paper established the paradigm that the RNase III proteins that act in RNA silencing pathways have dsRNA-binding protein partners, and showed that the Dicer partner R2D2 facilitates loading of the double-stranded siRNA into an Argonaute protein.
26. Tomari, Y., Matranga, C., Haley, B., Martinez, N. & Zamore, P. D. A protein sensor for siRNA asymmetry. *Science* **306**, 1377–1380 (2004).
27. Matranga, C., Tomari, Y., Shin, C., Bartel, D. P. & Zamore, P. D. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**, 607–620 (2005).
28. Kim, K., Lee, Y. S. & Carthew, R. W. Conversion of pre-RISC to holo-RISC by Ago2 during assembly of RNAi complexes. *RNA* **13**, 22–29 (2006).
29. Leuschner, P. J., Ameres, S. L., Kueng, S. & Martinez, J. Cleavage of the siRNA passenger strand during RISC assembly in human cells. *EMBO Rep.* **7**, 314–320 (2006).
30. Miyoshi, K., Tsukumo, H., Nagami, T., Siomi, H. & Siomi, M. C. Slicer function of *Drosophila* Argonautes and its involvement in RISC formation. *Genes Dev.* **19**, 2837–2848 (2005).
31. Rand, T. A., Petersen, S., Du, F. & Wang, X. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* **123**, 621–629 (2005).
32. Horwich, M. D. *et al.* The *Drosophila* RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. *Curr. Biol.* **17**, 1265–1272 (2007).
33. Pelisson, A., Sarot, E., Payen-Groschene, G. & Bucheton, A. A novel repeat-associated small interfering RNA-mediated silencing pathway downregulates complementary sense *gypsy* transcripts in somatic cells of the *Drosophila* ovary. *J. Virol.* **81**, 1951–1960 (2007).
    The discovery that siRNAs are 2'-*O*-methylated in flies.
34. Yu, B. *et al.* Methylation as a crucial step in plant microRNA biogenesis. *Science* **307**, 932–935 (2005).
    The first report of small silencing RNA methylation. This paper alerted the field to the idea that small RNA stability and function could be controlled by post-transcriptional modification.
35. Ramachandran, V. & Chen, X. Small RNA metabolism in *Arabidopsis*. *Trends Plant Sci.* **13**, 368–374 (2008).
36. Li, J., Yang, Z., Yu, B., Liu, J. & Chen, X. Methylation protects miRNAs and siRNAs from a 3'-end uridylation activity in *Arabidopsis*. *Curr. Biol.* **15**, 1501–1507 (2005).
37. Tolia, N. H. & Joshua-Tor, L. Slicer and the Argonautes. *Nature Chem. Biol.* **3**, 36–43 (2007).
38. Hamilton, A., Voinnet, O., Chappell, L. & Baulcombe, D. Two classes of short interfering RNA in RNA silencing. *EMBO J.* **21**, 4671–4679 (2002).
39. Tang, G., Reinhart, B. J., Bartel, D. P. & Zamore, P. D. A biochemical framework for RNA silencing in plants. *Genes Dev.* **17**, 49–63 (2003).
40. Dunoyer, P., Himber, C., Ruiz-Ferrer, V., Alioua, A. & Voinnet, O. Intra- and intercellular RNA interference in *Arabidopsis thaliana* requires components of the microRNA and heterochromatic silencing pathways. *Nature Genet.* **39**, 848–856 (2007).
41. Bouche, N., Lauressergues, D., Gasciolli, V. & Vaucheret, H. An antagonistic function for *Arabidopsis* DCL2 in development and a new function for DCL4 in generating viral siRNAs. *EMBO J.* **25**, 3347–3356 (2006).
42. Deleris, A. *et al.* Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* **313**, 68–71 (2006).
43. Gasciolli, V., Mallory, A. C., Bartel, D. P. & Vaucheret, H. Partially redundant functions of *Arabidopsis* DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Curr. Biol.* **15**, 1494–1500 (2005).
44. Xie, Z. *et al.* Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol.* **2**, E104 (2004).
45. Chan, S. W. Inputs and outputs for chromatin-targeted RNAi. *Trends Plant Sci.* **13**, 383–389 (2008).
46. Qu, F. *et al.* RDR6 has a broad-spectrum but temperature-dependent antiviral defense role in *Nicotiana benthamiana*. *J. Virol.* **79**, 15209–15217 (2005).
47. Voinnet, O. Use, tolerance and avoidance of amplified RNA silencing by plants. *Trends Plant Sci.* **13**, 317–328 (2008).
48. Gazzani, S., Lawrenson, T., Woodward, C., Headon, D. & Sablowski, R. A link between mRNA turnover and RNA interference in *Arabidopsis*. *Science* **306**, 1046–1048 (2004).
49. Gy, I. *et al.* *Arabidopsis* FIERY1, XRN2, and XRN3 are endogenous RNA silencing suppressors. *Plant Cell* **19**, 3451–3461 (2007).
50. Ambros, V., Lee, R. C., Lavanway, A., Williams, P. T. & Jewell, D. MicroRNAs and other tiny endogenous RNAs in *C. elegans*. *Curr. Biol.* **13**, 807–818 (2003).
51. Zilberman, D., Cao, X. & Jacobsen, S. E. *ARGONAUTE4* control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* **299**, 716–719 (2003).
52. Chan, S. W. *et al.* RNA silencing genes control de novo DNA methylation. *Science* **303**, 1336 (2004).
53. Zheng, X., Zhu, J., Kapoor, A. & Zhu, J. K. Role of *Arabidopsis* AGO6 in siRNA accumulation, DNA methylation and transcriptional gene silencing. *EMBO J.* **26**, 1691–1701 (2007).
54. Boutet, S. *et al.* *Arabidopsis* HEN1: a genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. *Curr. Biol.* **13**, 843–848 (2003).
55. El-Shami, M. *et al.* Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. *Genes Dev.* **21**, 2539–2544 (2007).
56. Herr, A. J., Jensen, M. B., Dalmay, T. & Baulcombe, D. C. RNA polymerase IV directs silencing of endogenous DNA. *Science* **308**, 118–120 (2005).
57. Kanoh, J., Sadaie, M., Urano, T. & Ishikawa, F. Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres. *Curr. Biol.* **15**, 1808–1819 (2005).
58. Lee, S. K. *et al.* Lentiviral delivery of short hairpin RNAs protects CD4 T cells from multiple clades and primary isolates of HIV. *Blood* **106**, 818–826 (2005).
59. Onodera, Y. *et al.* Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. *Cell* **120**, 613–622 (2005).
60. Pontier, D. *et al.* Reinforcement of silencing at transposons and highly repeated sequences requires the concerted action of two distinct RNA polymerases IV in *Arabidopsis*. *Genes Dev.* **19**, 2030–2040 (2005).
61. Tran, R. K. *et al.* DNA methylation profiling identifies CG methylation clusters in *Arabidopsis* genes. *Curr. Biol.* **15**, 154–159 (2005).
62. Llave, C., Kasschau, K. D., Rector, M. A. & Carrington, J. C. Endogenous and silencing-associated small RNAs in plants. *Plant Cell* **14**, 1605–1619 (2002).
63. Mette, M. F., Aufsatz, W., van der Winden, J., Matzke, M. A. & Matzke, A. J. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J.* **19**, 5194–5201 (2000).
64. Vazquez, F. *et al.* Endogenous *trans*-acting siRNAs regulate the accumulation of *Arabidopsis* mRNAs. *Mol. Cell* **16**, 69–79 (2004).
65. Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H. L. & Poethig, R. S. *SGS3* and *SGS2/SDE1/RDR6* are required for juvenile development and the production of *trans*-acting siRNAs in *Arabidopsis*. *Genes Dev.* **18**, 2368–2379 (2004).
66. Yoshikawa, M., Peragine, A., Park, M. Y. & Poethig, R. S. A pathway for the biogenesis of *trans*-acting siRNAs in *Arabidopsis*. *Genes Dev.* **19**, 2164–2175 (2005).
67. Williams, L., Carles, C. C., Osmont, K. S. & Fletcher, J. C. A database analysis method identifies an endogenous trans-acting short-interfering RNA that targets the *Arabidopsis* ARF2, ARF3, and ARF4 genes. *Proc. Natl Acad. Sci. USA* **102**, 9703–9708 (2005).
68. Allen, E., Xie, Z., Gustafson, A. M. & Carrington, J. C. microRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. *Cell* **121**, 207–221 (2005).
69. Montgomery, T. A. *et al.* Specificity of ARGONAUTE7-miR390 interaction and dual functionality in *TAS3 trans*-acting siRNA formation. *Cell* **133**, 128–141 (2008).
70. Axtell, M. J., Jan, C., Rajagopalan, R. & Bartel, D. P. A two-hit trigger for siRNA biogenesis in plants. *Cell* **127**, 565–577 (2006).

REVIEWS

71. Katiyar-Agarwal, S. *et al.* A pathogen-inducible endogenous siRNA in plant immunity. *Proc. Natl Acad. Sci. USA* **103**, 18002–18007 (2006).

72. Borsani, O., Zhu, J., Verslues, P. E., Sunkar, R. & Zhu, J. K. Endogenous siRNAs derived from a pair of natural *cis*-antisense transcripts regulate salt tolerance in *Arabidopsis*. *Cell* **123**, 1279–1291 (2005).

73. Zhang, D. & Trudeau, V. L. The XS domain of a plant specific SGS3 protein adopts a unique RNA recognition motif (RRM) fold. *Cell Cycle* **7**, 2268–2270 (2008).

74. Katiyar-Agarwal, S., Gao, S., Vivian-Smith, A. & Jin, H. A novel class of bacteria-induced small RNAs in *Arabidopsis*. *Genes Dev.* **21**, 3123–3134 (2007).

75. Yang, N. & Kazazian, H. H. J. L1 retrotransposition is suppressed by endogenously encoded small interfering RNAs in human cultured cells. *Nature Struct. Mol. Biol.* **13**, 763–771 (2006).

76. Ghildiyal, M. *et al.* Endogenous siRNAs derived from transposons and mRNAs in *Drosophila* somatic cells. *Science* **320**, 1077–1081 (2008).

77. Czech, B. *et al.* An endogenous small interfering RNA pathway in *Drosophila*. *Nature* **453**, 798–802 (2008).

78. Okamura, K. *et al.* The *Drosophila* hairpin RNA pathway generates endogenous short interfering RNAs. *Nature* **453**, 803–806 (2008).

79. Kawamura, Y. *et al. Drosophila* endogenous small RNAs bind to Argonaute 2 in somatic cells. *Nature* **453**, 793–797 (2008).

80. Okamura, K., Balla, S., Martin, R., Liu, N. & Lai, E. C. Two distinct mechanisms generate endogenous siRNAs from bidirectional transcription in *Drosophila melanogaster*. *Nature Struct. Mol. Biol.* **15**, 581–590 (2008).

81. Chung, W. J., Okamura, K., Martin, R. & Lai, E. C. Endogenous RNA interference provides a somatic defense against *Drosophila* transposons. *Curr. Biol.* **18**, 795–802 (2008).

82. Tabara, H. *et al.* The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* **99**, 123–132 (1999).
 The first study to link an Argonaute protein to an RNA silencing pathway. The authors showed that the Argonaute protein RDE-1 is required for RNAi in worms.

83. Ketting, R. F., Haverkamp, T. H., van Luenen, H. G. & Plasterk, R. H. *mut-7* of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* **99**, 133–141 (1999).

84. Zhou, R. *et al.* Comparative analysis of Argonaute-dependent small RNA pathways in *Drosophila*. *Mol. Cell* **32**, 592–599 (2008).

85. Förstemann, K. *et al.* Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. *PLoS Biol.* **3**, e236 (2005).

86. Tam, O. H. *et al.* Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. *Nature* **453**, 534–538 (2008).

87. Watanabe, T. *et al.* Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. *Nature* **453**, 539–543 (2008).

88. Sasidharan, R. & Gerstein, M. Genomics: protein fossils live on as RNA. *Nature* **453**, 729–731 (2008).

89. Neilson, J. R. & Sharp, P. A. Small RNA regulators of gene expression. *Cell* **134**, 899–902 (2008).

90. Lee, R. C., Feinbaum, R. L. & Ambros, V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* **75**, 843–854 (1993).
 This paper reported the discovery of the first miRNA, *lin-4*, and, together with reference 92, proposed that it regulated an mRNA, *lin-14*, by partially base-pairing to sites in the 3′ UTR, a relationship now considered as the hallmark of miRNA regulation.

91. Wightman, B., Burglin, T. R., Gatto, J., Arasu, P. & Ruvkun, G. Negative regulatory sequences in the lin-14 3′-untranslated region are necessary to generate a temporal switch during *Caenorhabditis elegans* development. *Genes Dev.* **5**, 1813–1124 (1991).

92. Wightman, B., Ha, I. & Ruvkun, G. Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell* **75**, 855–862 (1993).

93. Lagos-Quintana, M., Rauhut, R., Lendeckel, W. & Tuschl, T. Identification of novel genes coding for small expressed RNAs. *Science* **294**, 853–858 (2001).

94. Lee, R. C. & Ambros, V. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* **294**, 862–864 (2001).

95. Lau, N. C., Lim, L. P., Weinstein, E. G. & Bartel, D. P. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* **294**, 858–862 (2001).
 References 93–95 ushered in the age of miRNA discovery, increasing the number of known miRNAs from 2 to 76.

96. Griffiths-Jones, S., Saini, H. K., van Dongen, S. & Enright, A. J. miRBase: tools for microRNA genomics. *Nucleic Acids Res.* **36**, D154–D158 (2008).

97. Lee, Y., Jeon, K., Lee, J. T., Kim, S. & Kim, V. N. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J.* **21**, 4663–4670 (2002).

98. Lee, Y. *et al.* MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* **23**, 4051–4060 (2004).

99. Cai, X., Hagedorn, C. H. & Cullen, B. R. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA* **10**, 1957–1966 (2004).

100. Birney, E. *et al.* Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* **447**, 799–816 (2007).

101. Lee, Y. *et al.* The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415–419 (2003).
 The discovery that Drosha generates pre-miRNAs in the nucleus from longer, primary transcripts.

102. Denli, A. M., Tops, B. B., Plasterk, R. H., Ketting, R. F. & Hannon, G. J. Processing of primary microRNAs by the Microprocessor complex. *Nature* **432**, 231–235 (2004).

103. Gregory, R. I. *et al.* The Microprocessor complex mediates the genesis of microRNAs. *Nature* **432**, 235–240 (2004).

104. Han, J. *et al.* The Drosha–DGCR8 complex in primary microRNA processing. *Genes Dev.* **18**, 3016–3027 (2004).

105. Landthaler, M., Yalcin, A. & Tuschl, T. The human DiGeorge syndrome critical region gene 8 and its *D. melanogaster* homolog are required for miRNA biogenesis. *Curr. Biol.* **14**, 2162–2167 (2004).

106. Yi, R., Qin, Y., Macara, I. G. & Cullen, B. R. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev.* **17**, 3011–3016 (2003).

107. Bohnsack, M. T., Czaplinski, K. & Gorlich, D. Exportin 5 is a Ran GTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* **10**, 185–191 (2004).

108. Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E. & Kutay, U. Nuclear export of microRNA precursors. *Science* **303**, 95–98 (2004).

109. Zeng, Y. & Cullen, B. R. Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. *Nucleic Acids Res.* **32**, 4776–4785 (2004).

110. Chendrimada, T. P. *et al.* TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436**, 740–744 (2005).

111. Jiang, F. *et al.* Dicer-1 and R3D1-L catalyze microRNA maturation in *Drosophila*. *Genes Dev.* **19**, 1674–1679 (2005).

112. Lee, Y. *et al.* The role of PACT in the RNA silencing pathway. *EMBO J.* **25**, 522–532 (2006).

113. Saito, K., Ishizuka, A., Siomi, H. & Siomi, M. C. Processing of pre-microRNAs by the Dicer-1–Loquacious complex in *Drosophila* cells. *PLoS Biol.* **3**, e235 (2005).

114. Ruby, J. G. *et al.* Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in *C. elegans*. *Cell* **127**, 1193–1207 (2006).

115. Okamura, K., Hagen, J. W., Duan, H., Tyler, D. M. & Lai, E. C. The mirtron pathway generates microRNA-class regulatory RNAs in *Drosophila*. *Cell* **130**, 89–100 (2007).

116. Ruby, J. G., Jan, C. H. & Bartel, D. P. Intronic microRNA precursors that bypass Drosha processing. *Nature* **448**, 83–86 (2007).

117. Glazov, E. A. *et al.* A microRNA catalog of the developing chicken embryo identified by a deep sequencing approach. *Genome Res.* **18**, 957–964 (2008).

118. Berezikov, E., Chung, W. J., Willis, J., Cuppen, E. & Lai, E. C. Mammalian mirtron genes. *Mol. Cell* **28**, 328–336 (2007).

119. Babiarz, J. E., Ruby, J. G., Wang, Y., Bartel, D. P. & Blelloch, R. Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs. *Genes Dev.* **22**, 2773–2785 (2008).

120. Park, W., Li, J., Song, R., Messing, J. & Chen, X. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Curr. Biol.* **12**, 1484–1495 (2002).

121. Reinhart, B. J., Weinstein, E. G., Rhoades, M. W., Bartel, B. & Bartel, D. P. MicroRNAs in plants. *Genes Dev.* **16**, 1616–1626 (2002).

122. Papp, I. *et al.* Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors. *Plant Physiol.* **132**, 1382–1390 (2003).

123. Vazquez, F., Gasciolli, V., Crete, P. & Vaucheret, H. The nuclear dsRNA binding protein HYL1 is required for miRNA accumulation and plant development, but not posttranscriptional transgene silencing. *Curr. Biol.* **14**, 346–351 (2004).

124. Park, M. Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H. & Poethig, R. S. Nuclear processing and export of microRNAs in *Arabidopsis*. *Proc. Natl Acad. Sci. USA* **102**, 3691–3696 (2005).

125. Yang, Z., Ebright, Y. W., Yu, B. & Chen, X. HEN1 recognizes 21–24 nt small RNA duplexes and deposits a methyl group onto the 2′ OH of the 3′ terminal nucleotide. *Nucleic Acids Res.* **34**, 667–675 (2006).

126. Hutvágner, G. & Zamore, P. D. A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297**, 2056–2060 (2002).

127. Meister, G. *et al.* Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol. Cell* **15**, 185–197 (2004).

128. Liu, J. *et al.* Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**, 1437–1441 (2004).

129. Mansfield, J. H. *et al.* MicroRNA-responsive 'sensor' transgenes uncover Hox-like and other developmentally regulated patterns of vertebrate microRNA expression. *Nature Genet.* **36**, 1079–1083 (2004).

130. Pfeffer, S. *et al.* Identification of virus-encoded microRNAs. *Science* **304**, 734–736 (2004).

131. Yekta, S., Shih, I. H. & Bartel, D. P. MicroRNA-directed cleavage of *HOXB8* mRNA. *Science* **304**, 594–596 (2004).

132. Davis, E. *et al.* RNAi-mediated allelic *trans*-interaction at the imprinted *Rtl1/Peg11* locus. *Curr. Biol.* **15**, 743–749 (2005).

133. Sullivan, C. S., Grundhoff, A. T., Tevethia, S., Pipas, J. M. & Ganem, D. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature* **435**, 682–686 (2005).

134. Rhoades, M. W. *et al.* Prediction of plant microRNA targets. *Cell* **110**, 513–520 (2002).

135. Brennecke, J., Stark, A., Russell, R. B. & Cohen, S. M. Principles of microRNA-target recognition. *PLoS Biol.* **3**, e85 (2005).

136. Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P. & Burge, C. B. Prediction of mammalian microRNA targets. *Cell* **115**, 787–798 (2003).
 This paper reports the first computational evidence for a seed sequence and uses seed pairing to identify miRNA targets.

137. Lewis, B. P., Burge, C. B. & Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**, 15–20 (2005).

138. Stark, A., Brennecke, J., Bushati, N., Russell, R. B. & Cohen, S. M. Animal microRNAs confer robustness to gene expression and have a significant impact on 3′UTR evolution. *Cell* **123**, 1133–1146 (2005).

139. Xie, X. *et al.* Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. *Nature* **434**, 338–345 (2005).

140. Lai, E. C. Predicting and validating microRNA targets. *Genome Biol.* **5**, 115 (2004).

141. Ameres, S. L., Martinez, J. & Schroeder, R. Molecular basis for target RNA recognition and cleavage by human RISC. *Cell* **130**, 101–112 (2007).

142. Haley, B. & Zamore, P. D. Kinetic analysis of the RNAi enzyme complex. *Nature Struct. Mol. Biol.* **11**, 599–606 (2004).

143. Baek, D. *et al.* The impact of microRNAs on protein output. *Nature* **455**, 64–71 (2008).

144. Selbach, M. *et al.* Widespread changes in protein synthesis induced by microRNAs. *Nature* **455**, 58–63 (2008).

145. Kong, Y. W. *et al.* The mechanism of micro-RNA-mediated translation repression is determined by the promoter of the target gene. *Proc. Natl Acad. Sci. USA* **105**, 8866–8871 (2008).

146. Brodersen, P. *et al.* Widespread translational inhibition by plant miRNAs and siRNAs. *Science* **320**, 1185–1190 (2008).

147. Stefani, G. & Slack, F. J. Small non-coding RNAs in animal development. *Nature Rev. Mol. Cell Biol.* **9**, 219–230 (2008).
148. Yekta, S., Tabin, C. J. & Bartel, D. P. MicroRNAs in the Hox network: an apparent link to posterior prevalence. *Nature Rev. Genet.* **9**, 789–796 (2008).
149. Bartel, D. P. & Chen, C. Z. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nature Rev. Genet.* **5**, 396–400 (2004).
150. Bernstein, E. *et al.* Dicer is essential for mouse development. *Nature Genet.* **35**, 215–217 (2003).
151. Wienholds, E. *et al.* MicroRNA expression in zebrafish embryonic development. *Science* **309**, 310–311 (2005).
152. Giraldez, A. J. *et al.* MicroRNAs regulate brain morphogenesis in zebrafish. *Science* **308**, 833–838 (2005).
153. Hatfield, S. D. *et al.* Stem cell division is regulated by the microRNA pathway. *Nature* **435**, 974–978 (2005).
154. Mudhasani, R. *et al.* Loss of miRNA biogenesis induces p19^Arf-p53 signaling and senescence in primary cells. *J. Cell Biol.* **181**, 1055–1063 (2008).
155. Landgraf, P. *et al.* A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* **129**, 1401–1414 (2007).
156. Wienholds, E. & Plasterk, R. H. MicroRNA function in animal development. *FEBS Lett.* **579**, 5911–5922 (2005).
157. Aravin, A. A. *et al.* Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster* germline. *Curr. Biol.* **11**, 1017–1027 (2001).
    This paper was the first to identify piRNAs, then called repeat-associated siRNAs.
158. Vagin, V. V. *et al.* A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **313**, 320–324 (2006).
    The first report that piRNAs are distinct from siRNAs. This paper proposed that piRNAs are made from single-stranded precursors without the involvement of Dicer, are 3′ modified and are bound to Piwi proteins, and that they are largely antisense to selfish genetic elements.
159. Saito, K. *et al.* Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the *Drosophila* genome. *Genes Dev.* **20**, 2214–2222 (2006).
160. Saito, K. *et al.* Pimet, the *Drosophila* homolog of HEN1, mediates 2′-*O*-methylation of Piwi- interacting RNAs at their 3′ ends. *Genes Dev.* **21**, 1603–1608 (2007).
161. Kirino, Y. & Mourelatos, Z. Mouse Piwi-interacting RNAs are 2′-O-methylated at their 3′ termini. *Nature Struct. Mol. Biol.* **14**, 347–348 (2007).
162. Ohara, T. *et al.* The 3′ termini of mouse Piwi-interacting RNAs are 2′-O-methylated. *Nature Struct. Mol. Biol.* **14**, 349–350 (2007).
163. Houwing, S. *et al.* A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. *Cell* **129**, 69–82 (2007).
164. Aravin, A. *et al.* A novel class of small RNAs bind to MILI protein in mouse testes. *Nature* **442**, 203–207 (2006).
165. Lau, N. C. *et al.* Characterization of the piRNA complex from rat testes. *Science* **313**, 363–367 (2006).
166. Aravin, A. A., Sachidanandam, R., Girard, A., Fejes-Toth, K. & Hannon, G. J. Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science* **316**, 744–747 (2007).
167. Girard, A., Sachidanandam, R., Hannon, G. J. & Carmell, M. A. A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature* **442**, 199–202 (2006).
168. Grivna, S. T., Beyret, E., Wang, Z. & Lin, H. A novel class of small RNAs in mouse spermatogenic cells. *Genes Dev.* **20**, 1709–1714 (2006).
169. Grivna, S. T., Pyhtila, B. & Lin, H. MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis. *Proc. Natl Acad. Sci. USA* **103**, 13415–13420 (2006).
170. Kato, Y. *et al.* Role of the Dnmt3 family in *de novo* methylation of imprinted and repetitive sequences during male germ cell development in the mouse. *Hum. Mol. Genet.* **16**, 2272–2280 (2007).
171. Lees-Murdock, D. J., De Felici, M. & Walsh, C. P. Methylation dynamics of repetitive DNA elements in the mouse germ cell lineage. *Genomics* **82**, 230–237 (2003).
172. Aravin, A. A. *et al.* A piRNA pathway primed by individual transposons is linked to *de novo* DNA methylation in mice. *Mol. Cell* **31**, 785–799 (2008).

173. Batista, P. J. *et al.* PRG-1 and 21U-RNAs interact to form the piRNA complex required for fertility in *C. elegans*. *Mol. Cell* **31**, 67–78 (2008).
174. Das, P. P. *et al.* Piwi and piRNAs act upstream of an endogenous siRNA pathway to suppress Tc3 transposon mobility in the *Caenorhabditis elegans* germline. *Mol. Cell* **31**,79–90 (2008).
175. Wang, G. & Reinke, V. A *C. elegans* Piwi, PRG-1, regulates 21U-RNAs during spermatogenesis. *Curr. Biol.* **18**, 861–867 (2008).
176. Ruby, J. G. *et al.* Evolution, biogenesis, expression, and target predictions of a substantially expanded set of *Drosophila* microRNAs. *Genome Res.* **17**, 1850–1864 (2007).
177. Brennecke, J. *et al.* Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* **128**, 1089–1103 (2007).
    This paper, along with reference 184, proposed the 'ping-pong' model for piRNA biogenesis. The authors were the first to use high-throughput sequencing data to deduce the biogenesis mechanism of a small RNA pathway.
178. Nishida, K. M. *et al.* Gene silencing mechanisms mediated by Aubergine piRNA complexes in *Drosophila* male gonad. *RNA* **13**, 1911–1922 (2007).
179. Prud'homme, N., Gans, M., Masson, M., Terzian, C. & Bucheton, A. *flamenco*, a gene controlling the *gypsy* retrovirus of *Drosophila melanogaster*. *Genetics* **139**, 697–711 (1995).
180. Pelisson, A. *et al.* Gypsy transposition correlates with the production of a retroviral envelope-like protein under the tissue-specific control of the *Drosophila flamenco* gene. *EMBO J.* **13**, 4401–4411 (1994).
181. Desset, S., Buchon, N., Meignin, C., Coiffet, M. & Vaury, C. In *Drosophila melanogaster* the COM locus directs the somatic silencing of two retrotransposons through both Piwi-dependent and -independent pathways. *PLoS ONE* **3**, e1526 (2008).
182. Desset, S., Meignin, C., Dastugue, B. & Vaury, C. COM, a heterochromatic locus governing the control of independent endogenous retroviruses from *Drosophila melanogaster*. *Genetics* **164**, 501–509 (2003).
183. Mevel-Ninio, M. T., Pelisson, A., Kinder, J., Campos, A. R. & Bucheton, A. The *flamenco* locus controls the *gypsy* and *ZAM* retroviruses and is required for *Drosophila* oogenesis. *Genetics* **175**, 1615–24 (2007).
184. Gunawardane, L. S. *et al.* A Slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in *Drosophila*. *Science* **315**, 1587–1590 (2007).
185. Cox, D. N. *et al.* A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes Dev.* **12**, 3715–3727 (1998).
186. Cox, D. N., Chao, A. & Lin, H. *piwi* encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* **127**, 503–514 (2000).
187. Aravin, A. A. *et al.* Dissection of a natural RNA silencing process in the *Drosophila melanogaster* germ line. *Mol. Cell Biol.* **24**, 6742–6750 (2004).
188. Schupbach, T. & Wieschaus, E. Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. *Genetics* **129**, 1119–1136 (1991).
189. Klattenhoff, C. *et al.* *Drosophila* rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. *Dev. Cell* **12**, 45–55 (2007).
190. Pal-Bhadra, M. *et al.* Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. *Science* **303**, 669–672 (2004).
191. Tomari, Y., Du, T. & Zamore, P. D. Sorting of *Drosophila* small silencing RNAs. *Cell* **130**, 299–308 (2007).
192. Förstemann, K., Horwich, M. D., Wee, L.-M., Tomari, Y. & Zamore, P. D. *Drosophila* microRNAs are sorted into functionally distinct Argonaute protein complexes after their production by Dicer-1. *Cell* **130**, 287–297 (2007).
193. Mi, S. *et al.* Sorting of small RNAs into *Arabidopsis* Argonaute complexes is directed by the 5′ terminal nucleotide. *Cell* **133**, 116–127 (2008).
194. Vaucheret, H. Plant ARGONAUTES. *Trends Plant Sci.* **13**, 350–358 (2008).
195. Tokumaru, S., Suzuki, M., Yamada, H., Nagino, M. & Takahashi, T. *let-7* regulates Dicer expression and constitutes a negative feedback loop. *Carcinogenesis* **29**, 2073–2077 (2008).

196. Forman, J. J., Legesse-Miller, A. & Coller, H. A. A search for conserved sequences in coding regions reveals that the *let-7* microRNA targets Dicer within its coding sequence. *Proc. Natl Acad. Sci. USA* **105**, 14879–14884 (2008).
197. Xie, Z., Kasschau, K. D. & Carrington, J. C. Negative feedback regulation of Dicer-like1 in *Arabidopsis* by microRNA-guided mRNA degradation. *Curr. Biol.* **13**, 784–789 (2003).
198. Vaucheret, H., Vazquez, F., Crete, P. & Bartel, D. P. The action of *ARGONAUTE1* in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev.* **18**, 1187–1197 (2004).
199. Vaucheret, H., Mallory, A. C. & Bartel, D. P. AGO1 homeostasis entails coexpression of *MIR168* and *AGO1* and preferential stabilization of miR168 by AGO1. *Mol. Cell* **22**, 129–136 (2006).
200. Zhao, T. *et al.* A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*. *Genes Dev.* **21**, 1190–1203 (2007).
201. Molnar, A., Schwach, F., Studholme, D. J., Thuenemann, E. C. & Baulcombe, D. C. miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii*. *Nature* **447**, 1126–1129 (2007).
202. Grimson, A. *et al.* Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. *Nature* **455**, 1193–1197 (2008).
    This paper established that miRNAs and piRNAs existed before the divergence of bilaterian animals from the animal kingdom.
203. Brennecke, J. *et al.* An epigenetic role for maternally inherited piRNAs in transposon silencing. *Science* **322**, 1387–1392 (2008).
204. Sijen, T. *et al.* On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**, 465–476 (2001).
205. Voinnet, O., Vain, P., Angell, S. & Baulcombe, D. C. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* **95**, 177–187 (1998).
206. Vaistij, F. E., Jones, L. & Baulcombe, D. C. Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell* **14**, 857–867 (2002).
207. Fusaro, A. F. *et al.* RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. *EMBO Rep.* **7**, 1168–1175 (2006).
208. Moissiard, G., Parizotto, E. A., Himber, C. & Voinnet, O. Transitivity in *Arabidopsis* can be primed, requires the redundant action of the antiviral Dicer-like 4 and Dicer-like 2, and is compromised by viral-encoded suppressor proteins. *RNA* **13**, 1268–1278 (2007).
209. Sijen, T., Steiner, F. A., Thijssen, K. L. & Plasterk, R. H. Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. *Science* **315**, 244–247 (2007).
210. Pak, J. & Fire, A. Distinct populations of primary and secondary effectors during RNAi in *C. elegans*. *Science* **315**, 241–244 (2007).
211. Smardon, A. *et al.* EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. *Curr. Biol.* **10**, 169–178 (2000).
212. Aoki, K., Moriguchi, H., Yoshioka, T., Okawa, K. & Tabara, H. *In vitro* analyses of the production and activity of secondary small interfering RNAs in *C. elegans*. *EMBO J.* **26**, 5007–5019 (2007).
213. Makeyev, E. V. & Bamford, D. H. Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. *Mol. Cell* **10**, 1417–1427 (2002).
214. Tijsterman, M., Ketting, R. F., Okihara, K. L., Sijen, T. & Plasterk, R. H. RNA helicase MUT-14-dependent gene silencing triggered in *C. elegans* by short antisense RNAs. *Science* **295**, 694–697 (2002).
215. Caplen, N. J. *et al.* Rescue of polyglutamine-mediated cytotoxicity by double-stranded RNA-mediated RNA interference. *Hum. Mol. Genet.* **11**, 175–184 (2002).
216. Xia, H., Mao, Q., Paulson, H. L. & Davidson, B. L. siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nature Biotechnol.* **20**, 1006–1010 (2002).
217. Ding, H. *et al.* Selective silencing by RNAi of a dominant allele that causes amyotrophic lateral sclerosis. *Aging Cell* **2**, 209–217 (2003).
218. Miller, V. M. *et al.* Allele-specific silencing of dominant disease genes. *Proc. Natl Acad. Sci. USA* **100**, 7195–7200 (2003).

219. Martinez, L. A. *et al.* Synthetic small inhibiting RNAs: efficient tools to inactivate oncogenic mutations and restore p53 pathways. *Proc. Natl Acad. Sci. USA* **99**, 14849–14854 (2002).
220. Schwarz, D. S., Hutvagner, G., Haley, B. & Zamore, P. D. Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. *Mol. Cell* **10**, 537–548 (2002).
221. Nykanen, A., Haley, B. & Zamore, P. D. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309–321 (2001).
222. Martinez, J., Patkaniowska, A., Urlaub, H., Lührmann, R. & Tuschl, T. Single stranded antisense siRNA guide target RNA cleavage in RNAi. *Cell* **110**, 563–574 (2002).
223. Roignant, J. Y. *et al.* Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*. *RNA* **9**, 299–308 (2003).
224. Rusk, N. & Kiermer, V. Primer: Sequencing — the next generation. *Nature Methods* **5**, 15 (2008).
225. Johnston, R. J. & Hobert, O. A microRNA controlling left/right neuronal asymmetry in *Caenorhabditis elegans*. *Nature* **426**, 845–849 (2003).
226. Jones-Rhoades, M. W. & Bartel, D. P. Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell* **14**, 787–799 (2004).
227. Elbashir, S. M. *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498 (2001).
The breakthrough paper that showed siRNAs could be used in cultured somatic mammalian cells to phenocopy loss-of-function genetic mutations.
228. Sijen, T. & Plasterk, R. H. Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi. *Nature* **426**, 310–314 (2003).