On the road to reading the RNA-interference code

Haruhiko Siomi¹ & Mikiko C. Siomi¹,²

The finding that sequence-specific gene silencing occurs in response to the presence of double-stranded RNAs has had an enormous impact on biology, uncovering an unsuspected level of regulation of gene expression. This process, known as RNA interference (RNAi) or RNA silencing, involves small non-coding RNAs, which associate with nuclease-containing regulatory complexes and then pair with complementary messenger RNA targets, thereby preventing the expression of these mRNAs. Remarkable progress has been made towards understanding the underlying mechanisms of RNAi, raising the prospect of deciphering the ‘RNAi code’ that, like transcription factors, allows the fine-tuning and networking of complex suites of gene activity, thereby specifying cellular physiology and development.

The discovery of RNA interference (RNAi)¹ heralded a revolution in RNA biology. Researchers uncovered ‘hidden’ layers of regulation of gene expression, in which many previously unidentified families of small RNAs (consisting of ~20–30 nucleotides) mediate gene silencing. A diverse set of gene-regulatory mechanisms were found to use key steps in the RNAi process, including mechanisms that silence endogenous genes and mechanisms that restrain the expression of parasitic and pathogenic invaders such as transposons and viruses²–⁵. The basic RNAi process can be divided into three steps⁶–⁷. First, a long double-stranded RNA (dsRNA) that is expressed in, or introduced into, the cell (for example, as a result of the base-pairing of sense and antisense transcripts or the formation of stem–loop structures) is processed into small RNA duplexes by a ribonuclease III (RNaseIII) enzyme known as Dicer. Second, these duplexes are unwound, and one strand is preferentially loaded into a protein complex known as the RNA-induced silencing complex (RISC). Third, this complex effectively searches the transcriptome and finds potential target RNAs. The loaded single-stranded RNA (ssRNA), called the guide strand, then directs an endonuclease that is present in the RISC (sometimes called the ‘slicer’ and now known to be an Argonaute protein) to effect their targeting function (discussed further in the section ‘Loading and sorting of small RNAs by the RISC’).

In different organisms, the RNAi pathways comprise different proteins and mechanisms, but they operate by strikingly convergent strategies. In all organisms that have been studied, RNAi involves two main components: small RNAs, which determine the specificity of the response; and Argonaute proteins, which carry out the repression. Depending on both the nature of the Argonaute in the RISC and the degree of complementarity between the small RNA and the target sequence in the mRNA, the association of the RISC with target mRNAs has been shown to have different outcomes: it can control protein synthesis and mRNA stability, maintain genome integrity or produce a variety of other effects. One emerging finding in the field is that the activity of RNAi pathways is subject to intense regulation at various levels, from the level of biogenesis of small RNAs to the silencing mode of the RISC. In this Review, we describe the biogenesis of the guide strand of small RNAs and the formation and actions of the RISC, and we discuss the current understanding of the molecular mechanisms of RNAi in the light of recent insights into how silencing pathways are specified and regulated.

Biogenesis of small RNAs

A hallmark of RNAi is that short (~20–30 nucleotide) dsRNAs — known as small RNAs — are generated by the activity of RNaseIII enzymes (either Dicer alone or Drosha and Dicer). Two main categories of small RNAs have been defined on the basis of their precursors. The cleavage of exogenous long dsRNA precursors in response to viral infection or after artificial introduction generates short interfering RNAs (siRNAs), whereas the processing of genome-encoded stem–loop structures generates microRNAs (miRNAs). Using high-throughput sequencing technology, several new classes of endogenous small RNA species have recently been uncovered, and these include PIWI-interacting RNAs (piRNAs) and endogenous siRNAs (endo-siRNAs or esiRNAs).

A common feature of all of these small RNAs is that they are loaded onto Argonaute proteins to effect their targeting function (discussed further in the section ‘Loading and sorting of small RNAs by the RISC’). An overview of the generation of small RNAs is presented in Fig. 1.

siRNA biogenesis

Dicer (Table 1) processes long RNA duplexes and generates siRNAs. These small RNAs are ~21–25-nucleotide duplexes with a phosphate group at both 5’ ends, and hydroxyl groups and two-nucleotide overhangs at both 3’ ends, all hallmarks of RNaseIII-mediated cleavage. The Dicer protein contains a PAZ domain, which binds to the 3’ end of an siRNA, and two RNaseIII domains, which have the catalytic activity. It functions as a monomer⁸–¹⁴, but the RNaseIII domains associate with each other to include built-in molecular ‘rulers’ that define the size of small RNAs, structures that determine which strand of a small RNA is selected, mechanisms that direct further rounds of small RNA amplification, or safeguards against off-target (unrestricted and unrelated) silencing.

Another emerging finding in the field is that the activity of RNAi pathways is subject to intense regulation at various levels, from the level of biogenesis of small RNAs to the silencing mode of the RISC. In this Review, we describe the biogenesis of the guide strand of small RNAs and the formation and actions of the RISC, and we discuss the current understanding of the molecular mechanisms of RNAi in the light of recent insights into how silencing pathways are specified and regulated.

¹Department of Molecular Biology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. ²Japan Science and Technology Agency (JST), Core Research for Evolutional Science and Technology (CREST), 4-1-8 Hon-chou, Kawaguchi, Saitama 332-0012, Japan.
form an ‘internal dimer’ (see page 405). The distance between the PAZ domain and the two RNaseIII domains is the length spanned by 25 base pairs (bp) of RNA\(^1\). Thus, Dicer itself is a molecular ruler.

**miRNA biogenesis**

Similarly, miRNAs are short (~21–25-nucleotide) RNA molecules\(^1\); however, their biogenesis differs markedly from that of siRNAs. The primary precursors of miRNAs (pri-miRNAs) are encoded in the genome, and the relevant genomic regions are mostly transcribed by RNA polymerase II (ref. 17). The pri-miRNAs contain stem–loop structures that harbour the miRNA in the 5’ or 3’ half of the stem. During miRNA production in plants, one type of RNaseIII, Dicer-like protein 1 (DCL1), generates the miRNA–miRNA\(^*\) duplex in the nucleus (miRNA\(^*\) being the sequence in the stem–loop that pairs with the miRNA, equivalent to the passenger strand of siRNA duplexes; discussed later). By contrast, in animals, miRNAs are derived in a two-step process, in which the nuclear-localized RNaseIII Drosha defines one end of the miRNA–miRNA\(^*\) duplex and releases a precursor miRNA (pre-miRNA) of ~65–70 nucleotides. The pre-miRNA hairpin is then exported to the cytoplasm, where Dicer completes the processing.

**Figure 1 | Small RNA production and RNA silencing.**

a, Natural transcripts that form dsRNAs and hairpin-shaped structures can be sources of small RNAs. These precursors are processed by an RNaseIII enzyme (such as Drosha or Dicer), yielding small RNA duplexes. Duplexes with a perfect match (left pathway) are further processed by an enzyme with slicer activity (an Argonaute protein) into single-stranded small RNAs. By contrast, small RNA duplexes with a mismatch or bulge in the centre (right pathway) are not substrates for the slicer and thus become single-stranded in a cleavage-independent manner. The identity of the protein that carries out this unwinding is unknown. Single-stranded small RNAs generated in this way can then be loaded onto Argonaute proteins and silence gene expression.

b, Some small RNAs found in Caenorhabditis elegans and plants are known to be produced in an RNA-dependent RNA polymerase (RdRP)-dependent manner. Natural transcripts (often aberrant RNAs) can be substrates for this type of small RNA synthesis. This does not occur in organisms that lack RdRP activity, such as mammals and Drosophila melanogaster. Single-stranded small RNAs generated in this way can then be loaded onto Argonaute proteins and silence gene expression. c, The PIWI subfamily of Argonaute proteins, which are germline specific, are loaded with piRNAs. These complexes function to silence transposons. Single-stranded precursors give rise to piRNAs, through a mechanism called the primary processing pathway. The proteins required for this pathway are unknown. The silencing of transposons by PIWI proteins simultaneously amplifies piRNAs in germ cells. This pathway is known as the secondary processing pathway (or the ping-pong amplification loop) and is conserved in a variety of organisms, including mice and zebrafish. In this pathway, the slicer activity of the PIWI proteins reciprocally forms the 5’ ends of piRNAs by cleaving transposon transcripts (piRNA precursors). Proteins required to form the 3’ end of piRNAs remain unidentified.
Drosha is present in a large complex, known as the microprocessor complex, which functions like a molecular ruler to determine the cleavage site in the pri-miRNAs. In this complex, Drosha interacts with its cofactor, known as DGCR8 or Pasha (depending on the species), which also binds to dsRNA (through its dsRNA-binding domain; dsRBD). A typical metazoan pri-miRNA consists of a 33-bp stem, a terminal loop and ssRNA flanking segments. The flanking segments are crucial for binding to DGC8, and the 33-bp stem is also required for efficient cleavage. Drosha can interact transiently with the stem of this ‘pre-cleavage’ complex, and the processing centre of the enzyme, located at ~11 bp from the ssRNA–dsRNA junction, makes a staggered pair of breaks in the RNA to create the ~65–70-nucleotide pre-miRNA. Thus, DGCR8 might function as the molecular anchor that measures the distance from the ssRNA–dsRNA junction, and the processing centre of the enzyme, located at ~11 bp from the terminal loop that mitigate processing from this direction.

Although many of the sequences encoding miRNAs are located within introns, clusters encoding miRNAs that are processed directly by the spliceosome, instead of Drosha, were recently identified. The 3’ end of the stem–loop precursor of these intronic miRNAs (known as mirtrons) coincides with the 3′ splice site of a small annotated intron and is cleaved in the same splicing pathway as pre-mRNA in the nucleus instead of by Drosha. Subsequently, the mirtron precursors, which are released by the spliceosome in the shape of a lasso, are linearized by a de-branching enzyme. They then enter the miRNA-processing pathway directly (by mimicking the structural features of pre-miRNA hairpins) and are therefore exported to the cytoplasm and processed by a Dicer protein, bypassing Drosha-mediated cleavage.

The imprecision of Drosha or Dicer cleavage could result in the production of a set of miRNA–miRNA* duplexes with a variety of 3′ and 3′ ends. Most miRNAs in animals form imperfect hybrids with sequences in the target mRNA, with most of the pairing specificity being provided by the 5′-proximal region of the miRNA (that is, positions 2–8; also known as the seed region) . Imprecise cleavage either alters the seed sequence or inverts the relative stabilities of the 5′ and 3′ ends of the duplex (see the section ‘Loading and sorting of small RNAs by the RISC’). The results of recent deep-sequencing studies of small RNAs, however, indicate that human cells might take advantage of such imprecise cleavage, because the generation of a diverse set of miRNAs from a single precursor could be a way of broadening the network of factors and processes that are regulated by miRNAs.

**RNAseIII-independent pathways of small RNA biogenesis**

In some systems, small RNAs do not seem to be produced in response to dsRNA, but silencing signals are still amplified. Because these small RNAs do not arise from dsRNA precursors, RNaseIII enzymes cannot be involved in their generation. These findings therefore call into question the definition of RNAi. In this subsection, we describe the known RNAseIII-independent pathways of small RNA production, including those that generate piRNAs, 21U-RNAs, and secondary sRNAs in *Caenorhabditis elegans*.

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**Table 1: Key proteins in RNA silencing in various organisms**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Yeast (Schizosaccharomyces pombe)</th>
<th>Plant (Arabidopsis thaliana)</th>
<th>Nematode (Caenorhabditis elegans)</th>
<th>Fruitfly (Drosophila melanogaster)</th>
<th>Mouse</th>
<th>Mammal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rnaselli</strong></td>
<td>Dcr1</td>
<td>DCL1</td>
<td>DCR-1</td>
<td>DCR1</td>
<td>DICER</td>
<td>DICER</td>
</tr>
<tr>
<td><strong>Argonaute: AGO subfamily</strong></td>
<td>Ago1</td>
<td>AGO1</td>
<td>AGO1</td>
<td>AGO1</td>
<td>AGO1</td>
<td>AGO1</td>
</tr>
<tr>
<td><strong>Argonaute: PIWI subfamily</strong></td>
<td>None</td>
<td>None</td>
<td>ERGO-1</td>
<td>AGO3</td>
<td>MIU</td>
<td>HIWI</td>
</tr>
<tr>
<td><strong>Argonaute: WAGO subfamily</strong></td>
<td>None</td>
<td>None</td>
<td>RDE-1</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>Double-stranded-RNA-binding domain (dsRBD)-containing cofactor of Rnaselli</strong></td>
<td>None</td>
<td>HYL1</td>
<td>PASH-1</td>
<td>DGC8</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>RNA-dependent RNA polymerase (RdRP)</strong></td>
<td>Rdp1</td>
<td>RDR1</td>
<td>EGO-1</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Molecules that belong to these categories but have unknown functions are not listed but are indicated as ‘others’. Common synonyms are indicated in parentheses. Data were taken from refs 8, 12, 29, 50 and 98.
The small RNAs known as piRNAs were named for their ability to bind to a group of Argonaute proteins known as PIWI proteins. As noted earlier, members of the Argonaute family bind directly to small guide RNAs and lie at the core of all known RISCs. Argonaute proteins consist of a variable amino-terminal domain and three conserved domains (the PAZ, middle (MID) and PIWI domains)\(^\text{30,39}\). The 5′ end of a small RNA interacts with the PAZ domain, whereas the phosphate group at the 5′ end of small RNAs binds to a cleft bridging the MID domain and the PIWI domain\(^\text{29,30}\) (see page 405). The PIWI domain has an RNaseH-like folded structure\(^\text{19}\) and slicer activity (although some Argonaute proteins seem to have no slicer activity). There are three phylogenetic groups of Argonaute proteins\(^\text{31}\): the AGO subfamily (or AGO clade), named after the founding member Arribidopsis thaliana ARGOAUTA 1 (AGO1); the PIWI subfamily, named after D. melanogaster PIWI (P-element-induced wimpy testis); and the WAGO (worm-specific Argonaute) subfamily of C. elegans-specific proteins. PIWI-subfamily proteins bind to piRNAs\(^\text{31,37}\) (Table 1). These small RNAs have been found only in germ cells, and they are important for germline development and suppress transposon activity in the germline cells of mammals, fish and D. melanogaster. They are ~24–31 nucleotides (slightly longer than miRNAs), usually have a uridine at the 5′ end and carry a 5′ monophosphate. Unlike mammalian miRNAs, but similarly to plant miRNAs, piRNAs have a 2′-O-methyl (2′-O-Me) modification on the nucleotide at the 3′ end, a modification that is carried out by a HEN1-like methyltransferase\(^\text{38,39}\). If Dicer is mutated, the production of piRNAs is not affected, indicating that their biogenesis is distinct from that of miRNAs and siRNAs and does not involve dRNA precursors\(^\text{40,42}\).

The sequencing of small RNAs associated with D. melanogaster PIWI-subfamily proteins (PIWI, Aubergine (AUB) and AGO3)\(^\text{32–37}\) showed that piRNAs associated with AUB and PIWI are derived mainly from the antisense strand of retrotransposons, whereas AGO3-associated piRNAs arise mainly from the sense strand. AUB- and PIWI-associated piRNAs show a strong preference for uridine at their 5′ ends, whereas AGO3-associated piRNAs show a preference for adenosine at nucleotide 10. The first ten nucleotides of AUB-associated piRNAs can be complementary to the first ten nucleotides of AGO3-associated piRNAs. In addition, PIWI-subfamily proteins have slicer activity that allows them to cleave an RNA substrate opposite position 10 of their bound piRNA\(^\text{32–37}\). These observations suggest that piRNAs have a self-amplifying loop (Fig. 1), in which sense piRNAs associated with AGO3 cleave long antisense transcripts and guide the formation of the 5′ end of antisense piRNAs bound to AUB or PIWI, and vice versa. Thus, in this amplification loop, which is called the ping-pong cycle\(^\text{43}\), transposons are both a source of piRNAs and a target of piRNA-mediated silencing. After the resultant cleavage products have been loaded onto another member of the PIWI subfamily, further (as yet unidentified) nuclease activity generates the 3′ end of the piRNA, with the specific size of the piRNA determined by the footprint of the PIWI-subfamily protein on the RNA, a step that seems to precede 2′-O-Me modification\(^\text{44}\). In each PIWI-subfamily protein, the PAZ domain might be positioned at a distance from the MID domain that corresponds to the length of each piRNA. Thus, the PAZ domain might function as part of a molecular ruler for processing piRNAs of a defined size. Signatures of this amplification cycle are also apparent in zebrafish (Danio rerio) germ cells and in mammalian germ cells before the pachytene stage of meiosis during spermatogenesis\(^\text{42,45}\).

PIWI-subfamily proteins and, presumably, their associated piRNAs are loaded into embryos from the ova\(^2\), implying that the piRNAs that initiate an amplification cycle of piRNA biogenesis (which generates secondary piRNAs) could be supplied by germline transmission. But several findings indicate that there must be mechanisms of piRNA biogenesis other than amplification induced by maternal piRNAs. First, the amplification cycle in D. melanogaster engages mainly AGO3 and AUB\(^\text{32,44}\) but piRNAs are still loaded onto PIWI, which is spatially separated from these proteins at the subcellular and cell-type levels\(^\text{32,44}\). Second, piRNAs derived from a particular piRNA cluster in the genome (the flamenco locus) associate almost exclusively with PIWI\(^\text{43}\). These findings indicate that flamenco-derived piRNAs are produced by a pathway independent of the amplification loop. Whether such a piRNA-biogenesis pathway exists remains to be determined.

What at first seemed to be another type of small RNA, 21U-RNA, is found in C. elegans. These small RNAs are precisely 21 nucleotides and have a bias towards uridine at the 3′ end (but not in the remaining 20 nucleotides), and the genetic regions that encode them contain a characteristic sequence motif ~42 bp upstream of the first nucleotide of the small RNA\(^\text{46}\). It is possible that these RNAs are derived from thousands of separate, autonomously expressed, loci that are broadly scattered in two large regions of one chromosome. They are expressed solely in the germ line and interact with the PIWI-subfamily protein PRG-1 (refs 47, 48); therefore, 21U-RNAs are the C. elegans equivalent of piRNAs by definition. Like piRNAs, they depend on PRG-1 activity for their accumulation and are independent of DCR-1 (the C. elegans Dicer protein) for their production. C. elegans with mutations in prg-1 have a smaller brood and a temperature-sensitive sterile phenotype, which is consistent with the idea that PIWI-subfamily proteins are involved in germline maintenance. Like the piRNAs found in mammalian germ cells in pachytene\(^\text{33,34}\), 21U-RNAs have remarkable sequence diversity but lack obvious targets.

Small RNAs with a similar role to piRNAs have also been found in the ciliate Tetrahymena thermophila. These scan RNAs (scnRNAs) direct the elimination of transposon-like DNA sequences and associate with a PIWI-subfamily protein, TW11 (ref. 8) but, in contrast to piRNAs and 21U-RNAs, are produced by a Dicer-dependent pathway\(^\text{49}\).

These three examples (piRNAs, 21U-RNAs and scnRNAs) indicate that the core PIWI and piRNA machinery might have evolved to produce small RNAs and silence targets by different strategies.

RNA silencing pathways include mechanisms that downregulate endogenous genes and restrain the expression of selfish or exogenous genetic material, and these pathways often share common components such as Dicer. Therefore, there should be competition between different silencing pathways for particular components. Ways to overcome such competition should also exist; for example, by amplifying a weak silencing signal. In C. elegans, distinct Argonaute proteins operate at different stages of RNAi, directing gene silencing in a sequential manner\(^\text{50}\) — the second stage of which involves RNAseIII-independent biogenesis of small RNAs. First, a primary Argonaute protein (such as RDE-1 for exogenous siRNAs (exo-siRNAs) and ERGO-1 for endo-siRNAs) is guided by ‘primary’ siRNAs (that is, a first round of siRNAs), which have been generated from long dsRNAs by DCR-1. Second, the silencing signal is amplified by the production of ‘secondary’ siRNAs by the action of RNA-dependent RNA polymerases (RdRPs) (Fig. 1). These secondary siRNAs then bind differentially to secondary Argonaute proteins (SAGO, members of the WAGO subfamily), which mediate downstream silencing. In plants, RNAs with aberrant features, including lack of a poly(A) tail and lack of a 5′ cap, are copied into double-stranded forms by RdRPs and become substrates for Dicer, which converts them into siRNA duplexes\(^\text{52}\). By contrast, the C. elegans somatic RdRP mostly produces 21-nucleotide, single-stranded, 5′-triposphorylated small RNAs directly from the target mRNA in a primer-independent manner without the need for Dicer-mediated cleavage of dsRNA\(^\text{51–53}\). Such recruitment of an RdRP directly to the target mRNA allows dsRNA synthesis without consuming the siRNAs generated in response to the original trigger, although it is unclear how the 3′ end of these secondary siRNAs is formed and what the molecular ruler is that determines their size.

**Blurring of the boundaries between small RNA types**

As described above, the three main classes of small RNA — siRNAs, miRNAs and piRNAs — are distinct in their biogenesis and cellular roles. However, recent findings blur these distinctions and show that there are even more-complex interactions between factors involved in small RNA biogenesis. Deep sequencing of small RNAs from somatic tissues and cultured somatic cells in D. melanogaster has uncovered another class of small RNA, consisting of 3′-methylated, 21-nucleotide RNAs derived from the D. melanogaster genome. These endogenous RNAs are derived from transposons and from several loci, including...
locti that encode cis-natural antisense transcript pairs, and long stem–
loop structures containing many mismatched pairs in their stems. In D. melano-
gaster, distinct Dicer-containing complexes process exo-siRNAs and miRNAs. DCR-1 generates miRNAs, acting with its dsRNA-binding protein partner, Loquacious (LOQS), and the miRNAs are loaded onto AGO1. By contrast, DCR-2, together with its dsRNA-binding protein partner, R2D2 (ref. 62), generates exo-
siRNAs, which are loaded onto AGO2. Like exo-siRNAs, the recently
discovered endogenous small RNAs are produced by the DCR-2-
dependent pathway and are loaded onto AGO2, and they are therefore
called endo-siRNAs. However, the generation of many endo-siRNAs requires LOQS, the dsRBD-containing partner of DCR-1 in the
miRNA pathway, but not R2D2, the partner of DCR-2 (ref. 62).
In D. melanogaster, the dsRBD-containing partner of DCR-1 in the
miRNA pathway, but not R2D2, the partner of DCR-2 (ref. 62). DCR-1
deficient in DCR-2 or AGO2, the expression of transposons increases, so endo-siRNAs might be the main mechanism for
silencing selfish genetic elements in somatic cells, which lack the
piRNA pathway. Therefore, endo-siRNAs and piRNAs are fundamentally similar in that they defend organisms against nucleic-acid-based ‘parasites’. This finding also shows that D. melanogaster has two RNA pathways that repress transposon expression. Mouse
oocytes have also been shown to contain endo-siRNAs. These RNA are
derived from various sources, including transposons, however, some are processed from overlapping regions of functional genes and
their cognate pseudogenes. This finding suggests that pseudogenes,
which have been thought to be non-functional protein ‘fossils’, might regulate the expression of their founder genes.

Although siRNAs and miRNAs are categorized in terms of their origin rather than their size or function, the discovery of endo-siRNAs makes it difficult to distinguish between siRNAs and miRNAs. This blurring of the boundaries between the different types of small RNA has interesting evolutionary implications. The long stem–loop structures that are
processed to form endo-siRNAs are reminiscent of the pre-miRNAs in
plants. One hypothesis for the evolutionary origin of plant miRNAs is
that new plant miRNA loci might evolve from the inverted duplication
of founder loci, which when transcribed would result in hairpin RNAs.
These hairpin RNAs would have almost perfect self-complementarity and
might be processed by Dicer-like enzymes other than DCL1, the
main miRNA-processing enzyme in plants, because DCL1 has limited
activity against such substrates. Subsequent acquisition of mutations as a result of genetic drift would produce a hairpin with imperfect
complementarity, which could then be processed by DCL1. Thus, the
stem–loop structures from which endo-siRNAs are derived could be
evolutionary intermediates that are gradually transformed into miRNA
precursors. It is possible that such an adaptive switch could also occur
during the evolution of miRNA-encoding genes in D. melanogaster,
in which DCR-1 would then generate miRNAs instead of endo-siRNAs
being generated by DCR-2.

Loading and sorting of small RNAs by the RISC
In gene silencing pathways initiated by dsRNA precursors, Dicer-mediated
cleavage yields small dsRNA intermediates (small RNA duplexes). These small RNA duplexes must be dissociated into ‘competent’ single
strands in order to function as guides for RISCs. For each small RNA
duplex, only one strand, the guide strand, is loaded onto a specific Argonaute protein and assembled into the active RISC; the other strand, the
passenger strand, is destroyed. Many eukaryotes express more than one
Argonaute protein, and these proteins bind to small RNAs in a
sequence-independent manner. So how are small RNAs sorted and
loaded onto a specific Argonaute protein?

Loading
A small RNA generated from dsRNA precursors is converted from a
duplex into a single-stranded form as it is loaded into the RISC. The key
steps in converting the RISC from its precursor form (the pre-RISC),
which contains the small RNA duplex, to its mature form (the holo-
RISC), which contains the guide strand, are small RNA strand unwinding and preferential strand selection. The prevalent view of RISC loading is
that thermodynamic asymmetry along small RNA duplex determines
which RNA strand is retained and which is discarded. More specifically,
the strand that has its 5’ end at the thermodynamically less stable end of
the small RNA duplex is preferentially loaded into the RISC as the guide
strand, a phenomenon referred to as the asymmetry rule.

For siRNAs, the known interactions between Dicer and the Argonaute proteins indicate that the production of the small RNA and the assembly of the RISC might be physically coupled. For example, in D. melanogaster, DCR-2 does not simply transfer siRNAs to a distinct
RISC but, instead, forms part of the RISC together with the siRNAs,
indicating that the role of DCR-2 extends beyond the initiation phase.
The loading of siRNA duplexes onto AGO2 is facilitated by the RISC-
loading complex, which contains DCR-2 and its dsRBD-containing
partner, R2D2 (refs 62, 67). The particular strand of the siRNA duplex
that is loaded onto AGO2 seems to be determined by the orientation of
the DCR-2–R2D2 heterodimer on the siRNA duplex. R2D2 is thought to sense the thermodynamic stability of the siRNA duplexes and
bind to the more stable end of the siRNA, whereas DCR-2 is recruited to
the less stable end. The heterodimer probably recruits AGO2 through an
interaction between DCR-2 and AGO2. Previous models have proposed that the transition from a double-stranded silencing trigger to a single-stranded one is mediated by an unidentified ATP-dependent
RNA helicase. However, the unwinding of the siRNA duplex and the
loading of a single strand into the RISC are facilitated by the slicing of
the unincorporated (passenger) strand by AGO2, a process that does not
require ATP (26–27) (Fig. 1). Cleavage in the middle of the passenger
strand, as though the passenger strand were a microRNA target, would be
expected to reduce the annealing temperature and the free energy of
duplex formation, which in turn facilitates the separation of the siRNA
strands. These data support a model in which siRNAs are initially loaded
duplexes onto an AGO2-containing pre-RISC (Fig. 2).

By contrast, in humans, pre-miRNAs are known to bind to a preformed trimERIC complex of AGO2, DICER1 and DICER1’s dsRBD-
containing partner, TRBP. This complex can cleave target RNAs using
pre-miRNA and can distinguish miRNA from miRNA*, in the absence of
ATP hydrolysis, suggesting that DICER1-mediated cleavage and sensing of thermodynamic stability occur in series in the AGO2–DICER1–TRBP complex.

This process by which a pre-RISC is converted to a holo-RISC can also occur by a slicer-independent mechanism. Three of the four
Argonaute proteins in humans (AGO1, AGO3 and AGO4) lack slicer activity but are nonetheless loaded with single-stranded guide
miRNAs. Similarly, single-stranded miRNAs are found associated with AGO2 in humans, despite the expectation that mismatches in the unwound pre-miRNA should block the passenger-strand cleavage activity of AGO2. Thus, a cleavage-independent (bypass) mechanism for RISC assembly must exist. RNA helicase A has been identified as a candidate for unwinding the duplex in this process.

Sorting
Once assembled, RISCs mediate a range of the effector steps in all RNA
silencing mechanisms, from repressing translation to maintaining
genome stability. The specialized functions of RISCs are likely to
result from the particular proteins that associate with each Argonaute
protein. In other words, the different RISC variants are distinguished by their constituent Argonaute protein. Thus, it is crucial that a specific set of small guide RNAs is directed to a specific Argonaute protein.

Analyses of how different types of small RNA are channeled to different
Argonaute proteins show that there are multiple mechanisms: the
determinants for small RNA sorting vary from the structure of the small
RNA duplex to the identity of the 5’ nucleotide and the presence and
extent of modifications to this nucleotide.

In D. melanogaster, pre-miRNAs are processed by DCR-1, whereas exo-siRNA duplexes are produced by DCR-2 from long dsRNAs (Fig. 2a). Small RNAs then seem to be loaded onto either AGO1 or AGO2, depending on the structure of a small intermediate RNA duplex. If the duplex has a bulge in the middle (frequently observed in miRNA
precursors), the small RNA is routed to AGO1. If the duplex is perfectly matched, the small RNA is channelled to AGO2. This is because the DCR-2–R2D2 heterodimer, which recruits AGO2 to form the pre-RISC, binds well to highly paired small RNA duplexes but poorly to duplexes with central mismatches. Thus, the DCR-2–R2D2 heterodimer not only determines the polarity of siRNA loading on the basis of thermodynamic stability rules but also functions as a gatekeeper for AGO2-containing RISCs. If the duplex has a mismatch or a bulge in the centre (as miRNAs do), then the RNA is routed to AGO1. If the duplex is perfectly matched (as siRNAs are), then the small RNA is routed to AGO2. This selectivity occurs because the small RNAs are loaded onto Argonaute proteins from a Dicer-containing complex, and the two forms of Dicer, DCR-1 and DCR-2, associate with different RNA structures. DCR-2 pairs with R2D2, and this heterodimer binds to highly paired small RNA duplexes but recognizes small RNA duplexes with a central mismatch only poorly. AGO2 favours binding to DCR-2–R2D2 over binding to the other Dicer-containing complex, DCR-1–LOQS, which binds to small RNAs with bulges. Further processing into single-stranded small RNAs is described in Fig. 1.

b, Arabidopsis thaliana miRNAs and trans-acting siRNAs (ta-siRNAs) have a 5′ uridine and preferentially associate with AGO1. By contrast, AGO2 and AGO5 show preferences for small RNAs containing 5′ adenosines and 5′ cytidines, respectively. However, it is unlikely that the 5′ nucleotide is the sole determinant of selective loading in A. thaliana. c, Secondary endo-siRNAs in Caenorhabditis elegans, as well as Schizosaccharomyces pombe, have a striking strand bias in which only the antisense siRNA is loaded onto Argonaute proteins. These siRNAs correspond to the RNA strand synthesized by RdRP. In C. elegans, RdRP produces small RNAs directly from the target mRNA in a primer-independent manner. Thus, these secondary small RNAs show negative polarity, and this mechanism reinforces the silencing carried out by the primary small RNAs.
miR-390, which has a 5’ adenosine. Therefore, the 5’ nucleotide does not seem to be the sole determinant of Argonaute association.

Another mechanism might operate for secondary siRNAs in C. elegans. These small RNAs are specifically loaded onto SAGOs. Secondary siRNAs carry a 5’-triphosphate modification, the hallmark of RdRP products, which might function as a recognition element for SAGO binding while excluding binding by a primary Argonaute, such as RDE-1.

Endo-siRNAs in C. elegans (including the secondary siRNAs just mentioned) and Schizosaccharomyces pombe (fission yeast) have a striking strand bias in which only the antisense siRNA strand, corresponding to the RNA strand synthesized by RdRP, is loaded into Argonaute-containing complexes. Because C. elegans RdRPs produce small RNAs directly from the target mRNA, in a primer-independent manner (Fig. 2c), all secondary siRNAs have a negative polarity and function to reinforce the silencing of the target mRNA. In S. pombe, the strand bias is probably the result of a different mechanism. The physical association of Dicer with an RdRP-containing complex known as RDRC and an Argonaute-containing complex known as the RNA-induced transcriptional silencing complex (RITS) (see page 415) may facilitate the loading of siRNAs onto Argonaute proteins in a directional manner as Dicer moves along and cleaves the dsRNA products of RdRP, giving rise to an antisense strand bias. This suggests that the polarity of Dicer processing defines the polarity of the siRNA strand loaded onto the Argonaute protein.

Argonaute proteins have diversified over evolutionary timescales, evolving a range of functions. These findings about small RNA sorting imply that the diversification of the Argonaute proteins is a consequence of which small RNA they recruit. It is possible that the conformation of the Argonaute protein dictates which small RNAs it partners, but the structures of eukaryotic Argonaute proteins will need to be determined before this can be assessed.

Safeguards in silencing pathways

During RNA silencing, a single non-sequence-specific RNA-binding protein (Argonaute) is loaded with small guide RNAs with a variety of sequences, resulting in effector complexes (RISCs). Thus, this system requires gatekeepers to ensure that Argonaute can bind to small guide RNAs but not to degraded small RNAs, thereby avoiding off-target silencing. Such gatekeeper systems seem to depend mainly on structural features specific for small guide RNAs.

As described earlier, Dicer helps to load siRNAs into the RISC, preventing siRNAs from diffusing freely in the cytoplasm after their production. This function of Dicer probably also aids in the discrimination of genuine siRNAs from various RNA-degradation products in the cell. Processing by RNaseIII enzymes (such as Dicer) characteristically yields small RNAs with 5’ monophosphates and 3’ two-nucleotide overhangs. The PAZ domain of Argonaute proteins might, as a first step, distinguish degraded RNAs (derived from unrelated pathways) from these small RNAs by binding to the characteristic 3’ overhangs of the small RNAs. In addition, to become incorporated into the RISC and mediate cleavage of the target mRNA, the guide strand of an siRNA must have a phosphate group at the 5’ end. In humans, the 5’ end of siRNAs is phosphorylated by the enzyme CLP1 (ref. 79), which also has roles in splicing transfer RNAs and forming the 5’ ends of mRNAs. Interestingly, both tRNA splicing and mRNA 3’-end formation occur in the nucleus, suggesting that siRNA duplexes with a 5’ hydroxyl group are transported to, or diffuse into, the nucleus and, after phosphorylation by CLP1, are exported to the cytoplasm and assembled into the RISC.

Amplification of the silencing signal needs to be balanced against the dangers of amplifying off-target silencing. For example, the slicer-mediated ping-pong mechanism for piRNA production does not lead to ‘transitive’ RNA silencing (in which RdRPs synthesize siRNAs complementary to sequences upstream or downstream of the initial trigger region in the target mRNA). Instead, it leads to conservative amplification of functional primary piRNA sequences (those inherited by germline transmission). However, it is conceivable that any off-target events mediated by RdRPs could lead to a chain reaction or transitive effect of silencing with deleterious consequences. Thus, there must be safeguards to prevent the pervasive use of RdRPs. A striking aspect of RdRP-based trigger amplification is that amplification occurs only when a target has been engaged, so amplification of the silencing signal is limited to cases in which there is a real target. In C. elegans, the processing of the trigger dsRNA and the loading of primary siRNAs into the RDE-1-containing complex seem to be inherently inefficient, limiting the first round of target recognition by RDE-1-containing complexes and minimizing the risk of amplifying off-target silencing reactions. In addition, each secondary siRNA seems to be generated by non-processive self-termination by RdRP, thereby restricting transitive effects. Furthermore, secondary siRNAs associate with SAGOs, which lack catalytic residues for cleaving mRNAs, suggesting that these complexes cannot generate cleaved substrates for further amplification, which in turn would prevent them from inducing the exponential generation of secondary siRNAs (but see also ref. 53 for a conflicting viewpoint). SAGOs are also present in limited supply and thus have a restricted capacity to support multiple simultaneous silencing reactions.

Another factor is that in C. elegans and S. pombe the RNAi machinery is negatively regulated by a conserved siRNA nuclease called enhanced RNAi (ERI-1 and Eri1, respectively). In S. pombe, transgene silencing is linked to a protein complex resembling the TRAMP complex of Saccharomyces cerevisiae (budding yeast), which carries out surveillance in the nucleus, targeting aberrant transcripts for degradation by the exosome. Thus, RNAi in S. pombe is actively restricted from exerting its effects throughout the genome and seems to be subject to competition from RNA quality-control machinery.

Target-sensing modes and effector modes of the RISC

When the RISC is loaded with the guide strand of a small RNA, how does it find its target mRNA? Most of the binding energy that tethers a RISC to a target mRNA is from nucleotides in the seed region of the small RNA. It seems that the accessibility of the target site can be sensed by the intrinsic, nonspecific affinity of RISC for siRNA, which follows the initial specific association between the RISC and the target (through the 5’ seed region of the small RNA). But the accessibility of the target site correlates directly with the efficiency of cleavage, indicating that the RISC cannot unfold structured RNA.

Target mRNAs are present in the cell in complex with ribonucleoproteins (RNPs), so target accessibility is also controlled by several RNA-binding proteins that either mask the target binding site or facilitate unfolding of the target. Therefore, the function of a RISC seems to be context-dependent, with its effector mode influenced not only by the structures of the small-RNA-binding sites on the target but also by the particular proteins associated with each Argonaute protein. For example, animal miRNAs silence gene expression by at least three independent mechanisms through binding sites that are mostly in the 3’ untranslated region of target mRNAs but not seem to be the sole determinant of Argonaute association.

Regulation of silencing pathways

So far, the pictures of RNA silencing pathways that we have built up (shown in Figs 1 and 2) are static. To gain further insight into silencing processes, it is important to incorporate information about how these pathways are regulated. It is already clear that competition between different silencing pathways (for example, competition between endo-siRNAs and miRNAs for LOQs in D. melanogaster) is a key step in how each stage of the RNAi mechanism is regulated. Many plant and animal viruses are known to encode suppressor proteins that block...
In the first stage of evolution, it is not clear whether changes in the activity of specific RNA-binding proteins affect the final outcome of gene regulation by small RNAs, given that RNAs in a cell are usually associated with multiple proteins that regulate many aspects of gene expression. For example, genome-wide in vivo approaches using a combination of immunoprecipitation and high-throughput sequencing will be required to establish protein–mRNA interactions or RNP complex occupancy at certain regions of mRNA, where expression is suppressed.

Finally, changes in the activity and specificity of silencing pathways could create quantitative and qualitative genetic variation in gene expression, thereby generating new gene-expression networks. Such changes might have contributed to many processes, including human evolution. Given that all vertebrates have almost exactly the same number of protein-coding genes and therefore cannot readily be distinguished in this way, it might be prophetic that the first small guide RNA to be identified, the C. elegans miRNA lin-4, has been found to regulate a gene involved in the timing of development. In humans, unlike other mammals, the brain tissue of newborns continues to grow at a similar rate to that of the fetus. This is a good example of a change in developmental timing, and there is much speculation about whether changes in this rate contributed to the evolution of humans as a new species.

References 76 and 77 show that the sorting of plant miRNAs onto Argonaute proteins depends mainly on the nucleotide at the 5′ end.


This paper shows that the final outcome of miRNA regulation is affected by the interaction of proteins other than Argonaute with the target mRNA.


This paper describes how the activity of miRNAs can be regulated by transcripts that mimic the target transcript.


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