Mechanisms of gene silencing by double-stranded RNA

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Double-stranded RNA (dsRNA) is an important regulator of gene expression in many eukaryotes. It triggers different types of gene silencing that are collectively referred to as RNA silencing or RNA interference. A key step in known silencing pathways is the processing of dsRNAs into short RNA duplexes of characteristic size and structure. These short dsRNAs guide RNA silencing by specific and distinct mechanisms. Many components of the RNA silencing machinery still need to be identified and characterized, but a more complete understanding of the process is imminent.

First discovered in plants, where it was known as post-transcriptional gene silencing, RNA silencing or RNA interference (RNAi)[1] occurs in a wide variety of eukaryotic organisms[2,3]. It is triggered by dsRNA precursors that vary in length and origin. These dsRNAs are rapidly processed into short RNA duplexes of 21 to 28 nucleotides in length, which then guide the recognition and ultimately the cleavage or translational repression of complementary single-stranded RNAs, such as messenger RNAs or viral genomic/antigenomic RNAs. The short RNAs have also been implicated in guiding chromatin modification (see review in this issue by Lippman and Martienssen, page 364).

According to their origin or function, three types of naturally occurring small RNA have been described: short interfering RNAs (siRNAs), repeat-associated short interfering RNAs (rasiRNAs) and microRNAs (miRNAs). In nature, dsRNA can be produced by RNA-templated RNA polymerization (for example, from viruses) or by hybridization of overlapping transcripts (for example, from repetitive sequences such as transgene arrays or transposons). Such dsRNAs give rise to siRNAs or rasiRNAs, which generally guide mRNA degradation and/or chromatin modification. In addition, endogenous transcripts that contain complementary or near-complementary 20- to 50-base-pair inverted repeats fold back on themselves to form dsRNA hairpins. These dsRNAs are processed into miRNAs that mediate translational repression, although they may also guide mRNA degradation. Finally, artificial introduction of long dsRNAs or siRNAs has been adopted as a tool to inactivate gene expression, both in cultured cells and in living organisms.

RNA silencing mechanisms were first recognized as antiviral mechanisms that protect organisms from RNA viruses[4], or which prevent the random integration of transposable elements. But the general role of silencing in the regulation of gene expression only became apparent when it was realized that specific genes in plants and animals encode short forms of fold-back dsRNA[5] (the precursor molecules of miRNAs; see review in this issue by Ambros, page 350). Many of these miRNA genes are evolutionarily conserved. In plants, miRNAs mainly function as siRNAs that guide the cleavage of sequence-complementary mRNAs. In animals such as the nematode *Caenorhabditis elegans* miRNAs appear predominantly to inhibit translation by targeting partially complementary sequences located within the 3′ untranslated region (UTR) of mRNAs.

Here, we review the mechanisms of RNA gene silencing, and the roles of the proteins that make up the cellular post-transcriptional RNA silencing machinery. We discuss how dsRNAs are processed into small RNAs, how they are incorporated into effector complexes, and what the functions of these various complexes are. Finally, we discuss cellular regulatory processes and viral mechanisms that modulate RNA silencing efficiency. The picture that emerges is that RNA silencing is an evolutionarily conserved gene-regulatory mechanism with many species-specific variations, for example in the origin of the dsRNAs and in the number of homologous RNA silencing proteins expressed.

Processing dsRNA precursors

The maturation of small RNAs is a stepwise process catalysed by dsRNA-specific RNase-III-type endonucleases, termed Drosha and Dicer, which contain catalytic RNase III and dsRNA-binding domains (dsRBDs) (Figs 1 and 2). Drosha is specifically required for the processing of miRNA precursors, but not for the processing of long dsRNA. miRNAs are transcribed as long primary transcripts, which are first processed by Drosha in the nucleus[6,7]. When Drosha excises the fold-back miRNA precursor, a 5′ phosphate and a 2-nucleotide 3′ overhang remain at the base of the stem[7,8]. The miRNA precursor is then exported to the cytoplasm by means of the nuclear export receptor, exportin-5 (refs 9–11). Because exportin-5 lacks an obvious single-stranded or double-stranded RBD, it is not known whether the miRNA precursor binds directly to exportin-5 or to an RNA-binding adaptor protein.

Once it is in the cytoplasm, the miRNA precursor is further processed by Dicer[12–16]. Processing of dsRNAs by Dicer yields RNA duplexes of about 21 nucleotides in length, which — like Drosha-processing products — have 5′ phosphates and 2-nucleotide 3′ overhangs[17]. Several organisms contain more than one Dicer gene, with each Dicer preferentially processing dsRNAs that come from a specific source (Fig. 1). *Drosophila melanogaster* has two paralogues: Dicer-1 (DCR-1) preferentially processes miRNA precursors[14], and Dicer-2 (DCR-2) is required for long dsRNA processing[14,18,19]. DCR-2 is stably associated with the dsRBD-containing protein R2D2 (ref. 18), and this complex then associates with siRNA duplexes (as monitored by gel-shift analysis[19]). The processing of dsRNA to siRNAs by the recombinant DCR-2 monomer or by the DCR-2/R2D2 heterodimer is ATP-dependent and requires a functional RNA helicase domain in DCR-2 (refs 14, 18, 20). For

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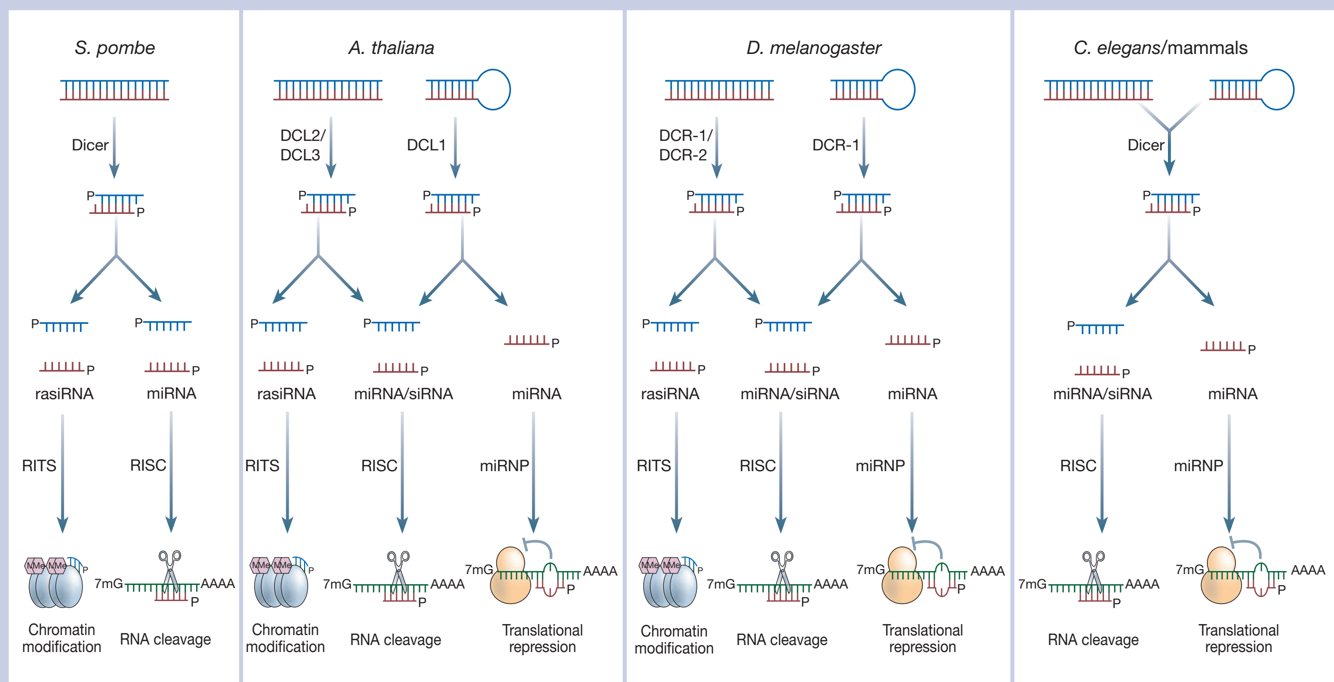

Figure 1 RNA silencing pathways in different organisms. Long dsRNA and miRNA precursors are processed to siRNA/miRNA duplexes by the RNase-III-like enzyme Dicer. The short dsRNAs are subsequently unwound and assembled into effector complexes: RISC, RITS (RNA-induced transcriptional silencing) or miRNP. RISC mediates mRNA-target degradation, miRNPs guide translational repression of target mRNAs, and the RITS complex guides the condensation of heterochromatin. In animals, siRNAs guide cleavage of complementary target RNAs, whereas miRNAs mediate translational repression of mRNA targets. rasiRNAs guide chromatin modification. *S. pombe*, *C. elegans* and mammals carry only one Dicer gene. In *D. melanogaster* and *A. thaliana,* specialized Dicer or DLC proteins preferentially process long dsRNA or miRNA precursors. 7mG, 7-methyl guanine; AAAA, poly-adenosine tail; Me, methyl group; P, 5′ phosphate.

human recombinant Dicer, however, ATP-dependence of dsRNA processing has not been observed[21,22]. The reason for this species difference in ATP-dependence is unclear.

Four Dicer-like (DCL) proteins (DCL1 to DCL4) have been identified in *Arabidopsis thaliana*, three of which are involved in processing dsRNAs that come from different sources[15]. DCL1 processes miRNA precursors[15], requiring two more proteins to do so: HEN1 (ref. 23) and the dsRBD protein HYL1 (refs 24, 25). DCL2 is required for the production of siRNAs from plant viruses, and DCL3, which also cooperates with HEN1 (ref. 15), is involved in the production of rasiRNAs. In *C. elegans*, only one Dicer (DCR-1) has been identified. This cooperates with the dsRBD protein RDE-4 during RNAi, although RDE-4 is not required for miRNA function[26].

It is conceivable that in *C. elegans* and mammals, which possess only a single Dicer gene, as-yet-unidentified Dicer-interacting dsRBD-containing proteins allow Dicer to recognize different sources of dsRNA.

Assembly into RNA silencing effector complexes

The siRNA- and miRNA-duplex-containing ribonucleoprotein particles (RNPs) are subsequently rearranged into the RNA-induced silencing complex (RISC)[27]. The functional RNPs contain only single-stranded siRNAs or miRNAs. Although it is difficult to assign distinct functional labels at this point, an siRNA-containing effector complex is commonly referred to as a RISC, whereas a miRNA-containing effector complex is referred to as a miRNP[28]. Every RISC or miRNP contains a member of the Argonaute (Ago) protein family; the Ago protein probably binds directly to the RNA in these complexes, although formal evidence for this is lacking[28–31]. Several forms of RISC or miRNPs that differ in size and composition have been reported, and these presumably differ in activity or even in function. The estimated apparent molecular mass ranges from between 130 to 160 kDa in the case of high-salt purified RISC from human cells[30,32] to 500 kDa[20,29] or up to the 80S range in the case of RISC isolated from *D. melanogaster* cell lysates[19]. The differences in mass are unlikely to be caused by oligomerization of smaller RISC units (minimal RISC). Instead, the gain in molecular mass appears to be due to the weak and/or transient association of proteins involved in the initial processing of dsRNA (Dicer, R2D2)[19,29,33], and of other factors of unknown function.

The assembly of RISC and presumably also of miRNPs is ATP-dependent[19,20], which probably reflects the requirement for energy-driven unwinding of the siRNA- or miRNA-duplex and/or other conformational or compositional changes of the pre-assembled RNA-duplex-containing RNP. Likely candidates for factors that promote these ATP-dependent conformational changes are DEAD-box RNA helicases (Fig. 3). Several ATPases have been implicated in RNA silencing (Tables 1 and 2), but only one has been characterized in detail. In *D. melanogaster*, the putative DEAD-box RNA helicase Armitage is required after Dicer processing for RISC assembly[34] and presumably also for miRNP assembly[35].

In the course of identifying more active and more specific siRNA duplexes to guide mRNA cleavage, it was noticed that the sequence composition of the siRNA duplex has an impact on the ratio of 'sense' (same sequence as the target gene) and 'antisense' (complementary to the target gene) siRNAs entering the RISC complex[36,37]. Naturally occurring miRNAs also show a strong bias for accumulating only one strand into a miRNP[5]. Effective, or more potently silencing, siRNAs or miRNA duplexes show reduced thermodynamic stability at the 5′ end of the antisense siRNA or miRNA relative to the 3′ end within the duplex. This strand bias is presumably caused by a rate-limiting unwinding step that occurs during the transition from the duplex-containing RNP to the larger RISC/miRNP complex, which allows the 5′ end of the strand positioned at the weakly paired end of the dsRNA to enter RISC/miRNP first.

The single-stranded siRNAs/miRNAs residing in RISC/miRNP are extremely tightly bound to an Ago protein: salt concentrations as high as 2.5 M KCl do not affect the association of the small RNA with

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the Ago protein during affinity purification of RISC[32]. Ago proteins have a molecular mass of about 100 kDa and contain two conserved domains: PAZ (for piwi–argonaute–zwille) and PIWI (Fig. 3)[38]. The PIWI domain has been implicated in an interaction with Dicer[33], although the analysis was carried out without using the full-length proteins. Recently, the crystal structure of an archaebacterial Ago protein revealed striking similarity of the PIWI domain with members of the RNase H family[39]. As RNase H cleaves the RNA strand of RNA/DNA duplexes, it was proposed that Ago proteins act by cleaving target RNA in target RNA/siRNA hybrids. Initially, the PAZ domain was suggested to function as a protein-interaction domain between Ago and Dicer, because a PAZ domain is also present in many Dicer-RNase-III enzymes and Ago proteins co-immunoprecipitate with Dicer[29]. Recent biochemical and structural studies, however, converged on the view that PAZ is an RBD that specifically recognizes the terminus of the base-paired helix of siRNA and miRNA duplexes, including the characteristic 2-nucleotide 3′ overhangs[40,41]. This siRNA/miRNA-duplex-specific interaction with PAZ ensures the safe transitioning of small RNAs into RISC/miRNP by minimizing the possibility of unrelated RNA-processing or RNA-turnover products entering the RNA silencing pathway. The preferred recognition of the termini of dsRNA[22] or miRNA precursors[8] by PAZ-domain-containing Dicer suggests that the processing reaction is guided by the PAZ domain docking at the terminus of long dsRNAs.

A possible variation in the general mechanism of siRNA transfer from the dsRNA-processing complex into RISC has been observed for Dicer variants that presumably lack the PAZ domain[18]. For example, *D. melanogaster* DCR-2, which predominantly processes siRNAs, requires R2D2 — not for dsRNA processing but for the incorporation of siRNA into RISC[18]. R2D2 contains two dsRBDs. The binding mode of DCR-2 and R2D2 may expose the 2-nucleotide staggered terminus of the processed siRNA for docking of the Ago protein and its PAZ domain. The 2-nucleotide 3′ overhanging structure of siRNA duplexes is essential for effective assembly of RISC in *D. melanogaster* lysate[42]. This implies that a step exists during RISC assembly that involves specific recognition of the 2-nucleotide overhang, presumably by the incoming Ago protein and its PAZ domain. The *C. elegans* RDE-4 protein, which is structurally related to R2D2, was shown to bind to the RDE-1 Ago protein[26], and therefore may have a similar function to R2D2 in forming active RISC.

The way in which single-stranded siRNA/miRNA binds to Ago after unwinding of the small RNA duplex is not understood because recombinant full-length Ago proteins are difficult to express and therefore the reconstitution of a functional complex containing a defined RNA sequence has not yet been accomplished.

Different organisms have different numbers of Ago proteins, ranging from one in *Schizosaccharomyces pombe* to more than 20 in *C. elegans*[38]. In *A. thaliana* ten members have been identified[43], compared with five in *D. melanogaster*[44] and eight in humans[45]. So far, only a small subset of this family has been functionally characterized; the specific functions of these Ago proteins are summarized in Tables 1 and 2. The evidence suggests that the different Ago proteins are not redundant. In species expressing more than one Dicer protein, the specificity of small-RNA loading into RISC/miRNP is probably controlled by the individual Dicer and Ago interactions. In species with a single Dicer, it is unclear whether and how the loading of the different Ago protein members is controlled for the different sources of dsRNA. Ago proteins may have evolved an intrinsic sequence-specificity that allows them to bind preferentially to small RNAs of specific sequence, or they may use specific adaptor proteins that are associated with the different cellular sources of dsRNA. A recent study in *D. melanogaster* revealed that AGO1 is required for miRNA accumulation whereas AGO2 is required for siRNA-triggered mRNA degradation[46]. Moreover, two recent studies in human systems showed that AGO1–AGO4 all associate with miRNAs and siRNAs, but that only the AGO2-containing RNPs exhibit RISC activity[47,48]. Finally, different expression patterns and levels of the various Ago proteins may control

Table 1 **Factors involved in RNAi or transcriptional gene silencing**

Protein	Domains and motifs	Function	Species	References
Dicer and Dicer-like proteins				
Dicer	RNase III, DEAD, PAZ,dsRBD	Long dsRNA processing	Hs	16
DCR-1	RNase III, DEAD, PAZ,dsRBD	Long dsRNA processing	Ce	13
DCR-1	RNase III, HELICc, PAZ,dsRBD	Long dsRNA processing, RISC assembly	Dm	14, 19
DCR-2	RNase III, DEAD, dsRBD	Long dsRNA processing, RISC assembly	Dm	14, 18, 19
DCL2	RNase III, DEAD, PAZ,dsRBD	Long dsRNA processing	At	15
DCL3	RNase III, DEAD, PAZ	Long dsRNA processing	At	15
Ago protein family				
AGO1	PAZ, PIWI	Short RNA binding	Hs, Dm	30, 44
AGO1	PAZ, PIWI		At, Sp	80, 81
AGO2	PAZ, PIWI	Short RNA binding	Hs, Dm	29, 30
AGO3	PAZ, PIWI	Short RNA binding	Hs	48
AGO4	PAZ, PIWI		At	82
Piwi	PAZ, PIWI		Dm	83
Aubergine	PAZ, PIWI		Dm	84
RDE-1	PAZ, PIWI	Initiation of RNAi	Ce	26
PPW-1	PAZ, PIWI		Ce	85
QDE-2	PAZ, PIWI		Nc	86
Putative RNA helicases				
Armitage	DEAD	RISC assembly	Dm	34
Spindle E	DEAD		Dm	84
Rm62	DEAD		Dm	51
SDE3	DEAD		At	87
DRH-1/2	DEAD		Ce	26
MUT-14	DEAD		Ce	88
SMG-2	DEAD		Ce	89
RNA-dependent RNA polymerases				
SGS2/ SDE1	RNA polymerase	RNA amplification	At	90
RDR2	RNA polymerase	RNA amplification	At	91
EGO-1	RNA polymerase	RNA amplification	Ce	92
RRF-1	RNA polymerase	RNA amplification	Ce	67
RRF-3	RNA polymerase		Ce	74
QDE-1	RNA polymerase	RNA amplification	Nc	93
Other factors				
R2D2	dsRBD	RISC assembly	Dm	18
FMRp	KH, RGG	RNA binding, translational regulation	Dm	49
Vasa intronic gene (VIG)	RGG		Dm	49
TudorSN (p100)	Tudor, nuclease		Dm, Hs	50
WEX-1	Exonuclease		At	94
SGS3			At	90
SDE4			At	82
RDE-4	dsRBD	Initiation of RNAi	Ce	26
MUT-7	Exonuclease		Ce	95
ADAR-1/2	Deaminase	Adenosine deamination	Ce	71
SID-1		dsRNA transport	Ce	96
ERI-1	Exonuclease	siRNA degradation	Ce	73
QDE-3	DNA helicase		Nc	97
CHP1	Chromo	Heterochromatin association	Sp	81
TAS3			Sp	81

Hs, *Homo sapiens*; Dm, *D. melanogaster*; At, *A. thaliana*; Ce, *C. elegans*; Nc, *Neurospora crassa*; Sp, *S. pombe*.

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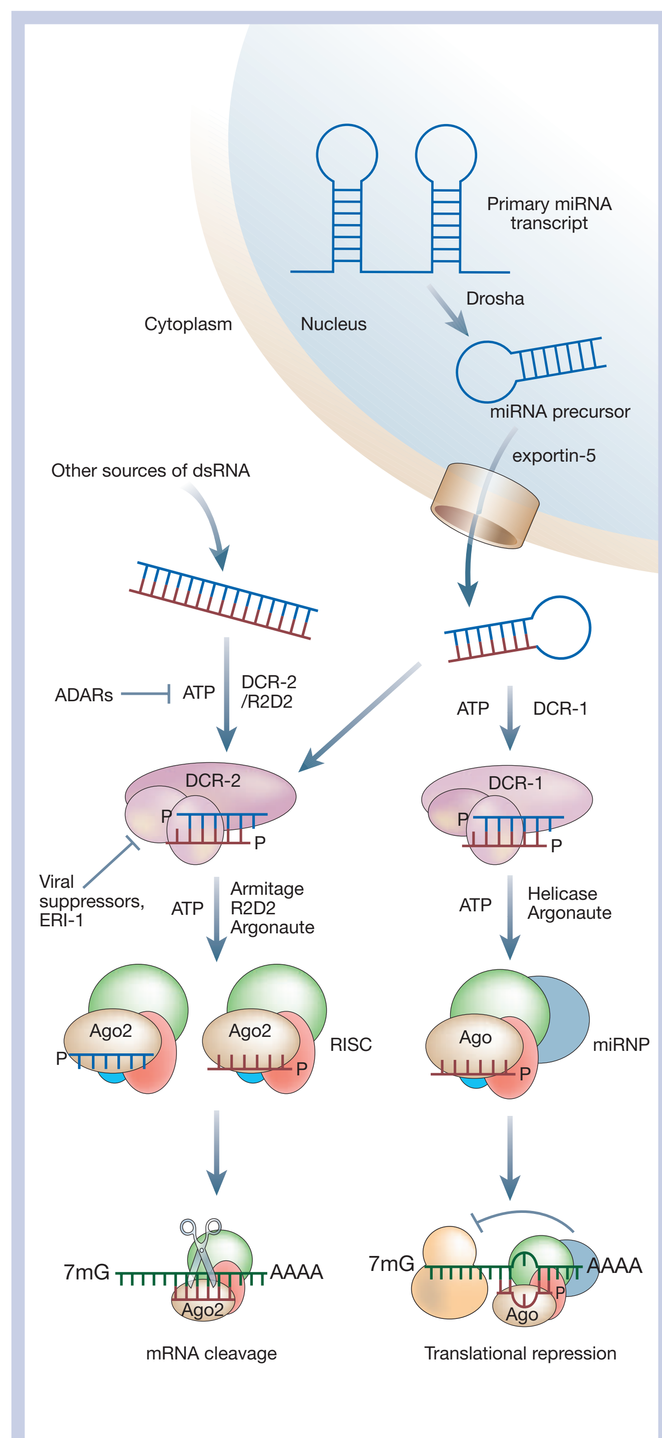

Figure 2 Model of small-RNA-guided post-transcriptional regulation of gene expression. Primary miRNA transcripts are processed to miRNA precursors in the nucleus by the RNase-III-like enzyme Drosha. The miRNA precursor is subsequently exported to the cytoplasm by means of the export receptor exportin-5. The miRNA precursor is further processed by Dicer to siRNA-duplex-like intermediates. The duplex is unwound while assembling into miRNP/RISC. Mature miRNAs bind to Ago proteins, which mediate translational repression or cleavage of target mRNAs. Other sources of long dsRNA in the cytoplasm of a cell are viral RNAs, artificially introduced dsRNA, dsRNAs generated by RdRPs, and genomic sense and antisense transcripts. Like miRNA precursors, long dsRNA is processed by the RNase III enzyme Dicer into 21–23 nucleotide dsRNA intermediates. Assisted by the RNA helicase Armitage and R2D2, the single-stranded siRNA-containing RISC is formed. The stability of the dsRNA and its recognition by Dicer can be regulated by specific ADARs and the exonuclease ERI-1.

the extent to which the different RNA silencing processes operate. The functional characterization of the individual Ago proteins remains a considerable challenge in the field.

Besides the core Ago protein and the small RNA component of the various RNA silencing complexes, other proteins have been identified in the larger forms of purified complexes. Three more components were identified in purified *D. melanogaster* RISC: the Vasa intronic gene product (VIG), fragile-X-related protein (dFXR), and the tudor staphylococcal-nuclease-domain-containing protein (Tudor-SN)[49,50]. In a preparation of *D. melanogaster* miRNPs, AGO2, dFXR, the DEAD-box helicase RM62, and the ribosomal proteins RPL5 and RPL11 were identified, indicating a role of miRNPs in the regulation of mRNA translation[51]. In human biochemical systems, interactions of AGO2/eIF2C2 (eukaryotic translation initiation factor 2C2) with FMRP (fragile X mental retardation protein) — the human homologue of dFXR — has also been observed[52]. Furthermore, human miRNAs, which reside in a defined 15S complex, were shown to contain AGO2 and the RNA helicases Gemin3 and Gemin4 (ref. 28). The precise function of these different proteins in the RNA silencing process remains unclear.

mRNA cleavage and translational repression

The single-stranded siRNA in RISC guides sequence-specific degradation of complementary or near-complementary target mRNAs[30,32]. RISC cleaves the target mRNA in the middle of the complementary region, ten nucleotides upstream of the nucleotide paired with the 5′ end of the guide siRNA[17]. The cleavage reaction guided by RISC/miRNP does not require ATP[20,31]. However, multiple rounds of mRNA cleavage — which requires the release of cleaved mRNA products — are more efficient in the presence of ATP[31]. ATP-dependent enhancement of multiple rounds of mRNA cleavage may reflect the assistance of an RNA helicase. This may facilitate the release of mRNA products from the RISC/miRNP complex, or reorganize the endonuclease complex for another round of target-RNA recognition.

RISC/miRNP complexes catalyse hydrolysis of the target-RNA phosphodiester linkage, yielding 5′ phosphate and 3′ hydroxyl termini[32,53]. This hydrolysis reaction is different from classic pancreatic-RNase endonucleolytic cleavage, which occurs by nucleophilic attack of the adjacent 2′ hydroxyl group and yields a 2′,3′-cyclic phosphate intermediate. The target-RNA hydrolysis reaction requires magnesium ions and resembles mechanistically the hydrolysis reaction that occurs when Dicer generates siRNA duplexes from dsRNA precursors — a reaction that also requires magnesium ions[21,22]. However, the small molecular mass of minimal RISC argues against a role for Dicer in directly catalysing target-RNA cleavage[30,32]. The Tudor-SN nuclease, which has been characterized as a component of RISC[50], can also be eliminated as a candidate for the unidentified endonuclease activity in RISC because, as a member of the micrococcal nuclease protein family, it should produce 3′ phosphate rather than 5′ phosphate termini. A smaller double-strand-specific endonuclease may be part of RISC, or as has recently been proposed on the basis of the similarity of the PIWI domain with RNase H[40], Ago proteins themselves may act as nucleases. However, it remains unresolved, why nuclease activity has been detected with Ago2 but not Ago1 or Ago3 in human cells, even though the proposed catalytic DDE motif is intact in all three proteins. It has been speculated that the RNA subunit of RISC may assist catalysis, but this is unlikely given that base pairing during target recognition positions the small RNA sugar-phosphate backbone far away from the active site. In animals, the binding sites for miRNAs in target mRNAs are generally considered to be of insufficient complementarity to allow the target to be cleaved. Recently, however, it was found that RISC/miRNP can cleave significantly mismatched targets, albeit at a reduced rate, although this depends on the type, position and structure of the pairing interaction[32].

The mechanism of miRNA-guided translational regulation is not as well understood as that of target-RNA cleavage. The first evidence for

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Table 2 **Factors involved in miRNA guided translational regulation**

Protein	Motifs	Function	Species	References
Dicer and Dicer-like proteins				
Dicer	RNase III, DEAD, PAZ, dsRBD	miRNA precursor processing	Hs	12
DCR-1	RNase III, DEAD, PAZ, dsRBD	miRNA precursor processing	Ce	13
DCR-1	RNase III, HELICc, PAZ, dsRBD	miRNA precursor processing	Dm	14
DCL1	RNase III, DEAD, PAZ, dsRBD	miRNA precursor processing	At	15
Ago protein family				
AGO1	PAZ, PIWI	Short RNA binding	Dm, Hs	46
AGO2	PAZ, PIWI	Short RNA binding	Dm, Hs	28, 51, 52
AGO3	PAZ, PIWI	Short RNA binding	Hs	47
AGO4	PAZ, PIWI	Short RNA binding	Hs	47
AGO1	PAZ, PIWI		At	98
ALG-1/2	PAZ, PIWI		Ce	13
Putative RNA helicases				
Gemin3	DEAD	RNA helicase	Hs	28
Armitage	DEAD	RISC assembly	Dm	35
Other factors				
Drosha	RNase III	Processing of primary miRNA transcripts	Hs	7, 8
Exportin-5		Nuclear export of miRNA precursors	Hs	9–11
Gemin4			Hs	28
FMRP, FXR	KH, RGG	RNA binding	Dm, Hs	51, 52
Ribosomal proteins L8,L11		60S ribosomal subunit	Dm	51
HYL1	dsRBD		At	24, 25
HEN1			At	23

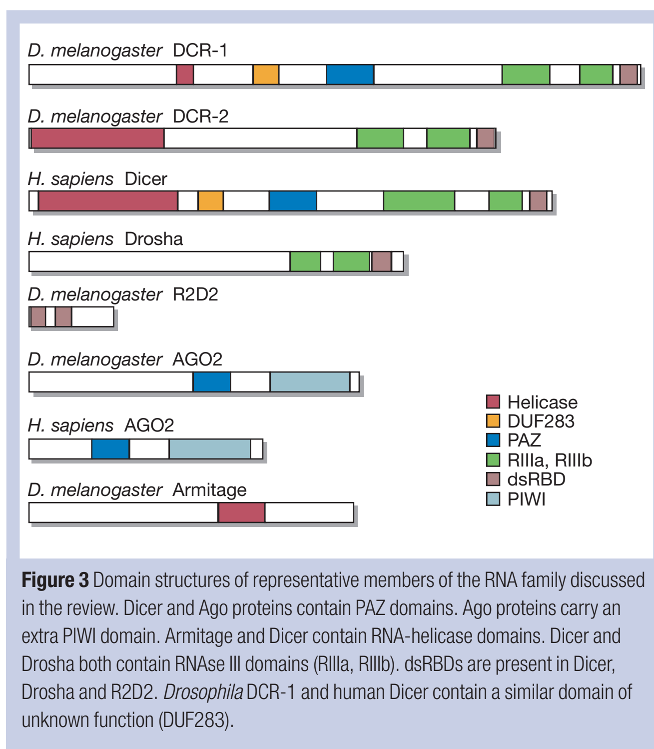

Figure 3 Domain structures of representative members of the RNA family discussed in the review. Dicer and Ago proteins contain PAZ domains. Ago proteins carry an extra PIWI domain. Armitage and Dicer contain RNA-helicase domains. Dicer and Drosha both contain RNAse III domains (RIIIa, RIIIb). dsRBDs are present in Dicer, Drosha and R2D2. *Drosophila* DCR-1 and human Dicer contain a similar domain of unknown function (DUF283).

translational repression by miRNAs was obtained from studies of mutant or transgenic *C. elegans*, where it was shown that miRNAs targeted to a specific gene reduced protein synthesis without affecting mRNA levels[5]. The target mRNA contained in its 3′ UTR several binding sites for the miRNA, and both target and miRNA were found to be associated with polyribosomes. This suggested that miRNAs block translation elongation or termination rather than translational initiation[54,55]. Ribosome profiles obtained from mammalian cell extracts also indicate an association of a small fraction of miRNAs with polysomes[56].

To complicate matters, miRNAs can act as siRNAs[31], and siRNAs can act as miRNAs[57,58]. This observation has occasionally been overinterpreted as suggesting that the function of an siRNA or a miRNA is solely determined by the degree of complementarity between the small RNA and its target RNA. An alternative, and probably more accurate, interpretation is that small dsRNAs are assembled into characteristic complexes that carry out distinct functions, depending on the protein factors that they recruit. This model could partly explain the apparent redundancy of the Ago protein family. Presumably, miRNA-guided translational regulation and targeted mRNA degradation are used simultaneously as natural regulatory mechanisms.

Regulators of RNAi

RNA silencing controls downstream-target gene expression but is itself under regulation. For example, systemic silencing, caused by the spread of a silencing signal between cells, occurs in plants and nematodes, and requires a group of enzymes termed RNA-dependent RNA polymerases (RdRPs; also known as RDRs). RdRPs are thought to amplify the silencing effect by dsRNA synthesis of the target mRNAs and/or its cleavage products[59,60]. In *D. melanogaster* and vertebrates, there are no equivalent RdRP-like proteins, and evidence exists against there being any amplification mechanism for silencing triggers[61–63].

In many organisms, RNA silencing mediated by dsRNA is part of an innate immune response against RNA viruses and transposable elements[64,65]. Defence strategies to thwart the host response have been found in plant viruses[66,67] and the insect flock house virus[68]. These viruses express inhibitors, such as dsRNA-binding proteins, that interfere with the host cell's RNA silencing machinery. One of the viral suppressor proteins, p19, from the plant tombusvirus, has been co-crystallized with a duplex of 21-nucleotide siRNAs[69,70]. A homodimer of p19 binds to the RNA sugar-phosphate backbone, and positions two α-helices on top of the terminal base pairs of the RNA duplex. This binding allows the p19 protein to discriminate siRNA duplexes from shorter or longer dsRNAs. Other inhibitors of RNA silencing in plants target RISC or restrict the spreading of a systemic silencing signal from infected cells[66,67]. The precise chemical nature of this spreading silencing signal is still debated: dsRNA, siRNA, RISC, or as-yet-unidentified RNAs or RNPs are possible candidates.

dsRNA acts as a substrate from which Dicer can produce siRNAs, but it also competes with the 'RNA-editing' process catalysed by dsRNA-specific adenosine deaminases (ADARs), which convert adenosine to inosine. This editing reaction not only eliminates the complementarity between the dsRNA and the target mRNA, but also destabilizes the edited dsRNA, making it a poor substrate for Dicer[71,72]. Another negative regulator of RNA silencing, the exonuclease-domain-containing protein ERI-1, was discovered in genetic screens in *C. elegans*[73]. ERI-1 is specifically expressed in neurons, and loss-of-function *eri-1* mutants show increased RNAi in neurons. Biochemical analysis suggests that siRNA is a preferred substrate for ERI-1. The presumably inactive RdRP-family protein RRF-3 (ref. 74) is also a negative regulator of RNAi in *C. elegans*. RRF-3 presumably competes for templates or primers of RNA amplification, inhibiting the production of additional short RNAs.

Outlook

Understanding the mechanisms of RNA silencing may shed light on human disease. Recently, several links between RNA-silencing factors and inherited or acquired genetic disorders have been recognized. For example, the loss or mutation of FMRP that causes the neurological disorder, fragile X syndrome, indirectly affects the translational regulation of miRNA-targeted mRNAs that are also targeted by FMRP[51,52]. Other studies have linked diseases to the loss of miRNA expression that may result from the deletion or the translocation of a

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chromosomal region. The miRNAs miR-15 and miR-16 were identified in the tumour suppressor locus for B-cell chronic lymphocytic leukaemia (*B-CLL*)[75], and consistent down-regulation of miR-143 and miR-145 has been observed in advancing colorectal neoplasia[76]. Translocation of the proto-oncogene, *c-myc*, downstream of the very strong promoter of miR-122 (ref. 77) and miR-142 (ref. 78) was also found in two cancer instances. Furthermore, it was recently discovered that some viruses express miRNAs, which presumably regulate viral as well as host gene expression[79]. To understand the underlying specificity of these processes, we need to identify the regulated targets of miRNAs, using computational or experimental methods. The development of robust biochemical and other methods for the dissection of the function and mechanism of RNA silencing are likely to provide many more insights into RNA silencing machineries. □

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