ELSEVIER

Post-transcriptional gene silencing by siRNAs and miRNAs

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Recent years have seen a rapid increase in our understanding of how double-stranded RNA (dsRNA) and 21- to 25-nucleotide small RNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs), control gene expression in eukaryotes. This RNA-mediated regulation generally results in sequence-specific inhibition of gene expression; this can occur at levels as different as chromatin modification and silencing, translational repression and mRNA degradation. Many details of the biogenesis and function of miRNAs and siRNAs, and of the effector complexes with which they associate have been elucidated. The first structural information on protein components of the RNA interference (RNAi) and miRNA machineries is emerging, and provides some insight into the mechanism of RNA-silencing reactions.

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Introduction

Research undertaken during the past decade with plants, fungi, protozoa and metazoan animals has revealed several novel and evolutionarily conserved gene control mechanisms that use double-stranded RNA (dsRNA) and 21- to 25-nucleotide (21–25-nt) small RNAs as sequence-specific regulators. These reactions, often collectively referred to as RNA silencing, include RNA interference (RNAi), RNAi-mediated chromatin silencing and DNA rearrangements, and post-transcriptional regulation of mRNA by endogenously encoded microRNAs (miRNAs) (reviewed in [1–7]). During RNAi, the dsRNA formed in cells by DNA- or RNA-dependent synthesis of complementary strands, or introduced into cells by viral infection or artificial expression is processed to ~20-bp double-stranded small interfering RNAs (siRNAs) containing 2-nt 3′ overhangs. The siRNAs are then incorporated into an RNA-induced silencing complex (RISC), which mediates the degradation of mRNAs with sequences fully complementary to the siRNA (Figure 1). In another reaction, occurring in the nucleus, siRNAs formed from repeat element transcripts and incorporated into the RNAi-induced transcriptional silencing (RITS) complex may guide chromatin modification and silencing. The genetics and biochemistry of the latter process are best characterized for the fission yeast *Schizosaccharomyces pombe* and plants, but related reactions also operate in other organisms [5,6].

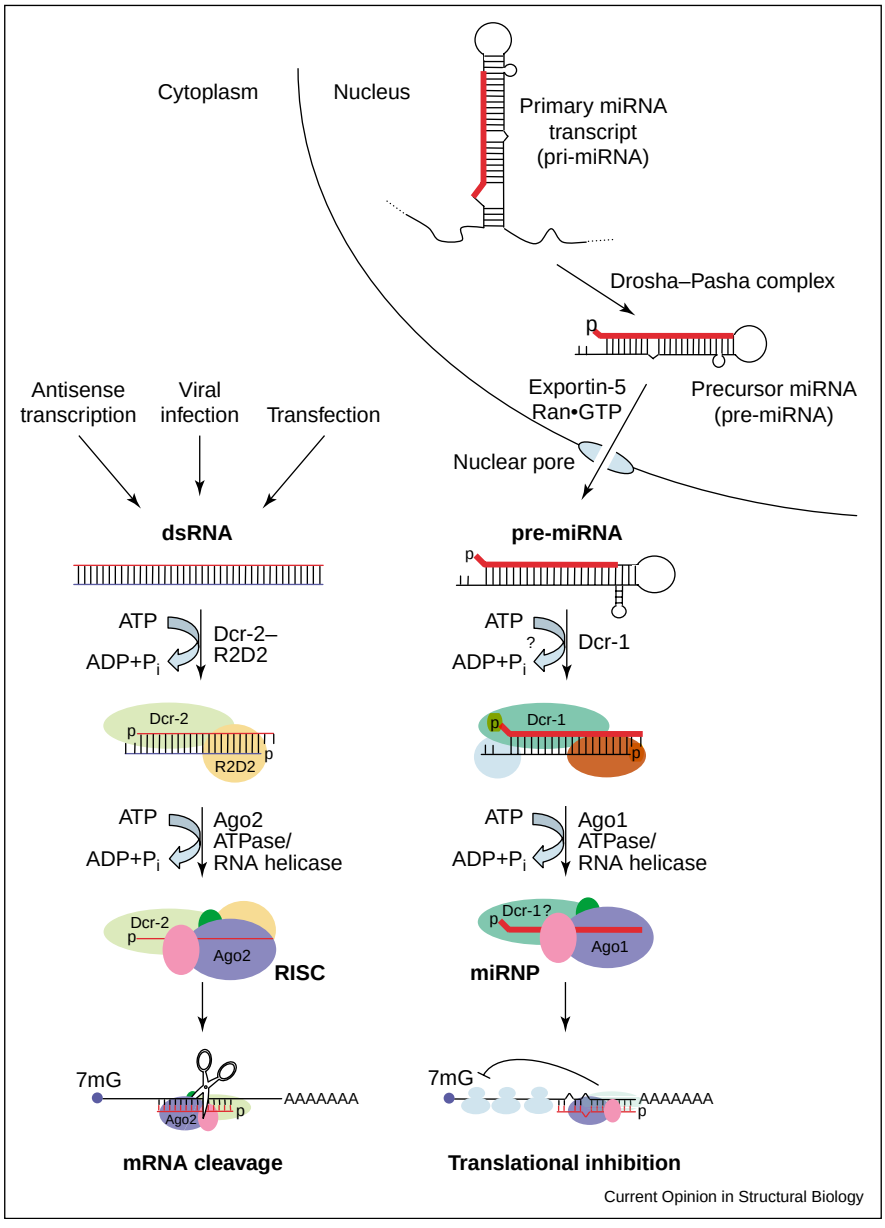
miRNAs are a large family of ~21-nt regulatory RNAs expressed in metazoan animals and plants. They are processed from endogenous precursor molecules, which fold into dsRNA-like hairpins. With very few exceptions, animal miRNAs regulate gene expression by base pairing imperfectly to the 3′-untranslated region (3′-UTR) of target mRNAs, inhibiting protein synthesis by an unknown mechanism. By contrast, plant miRNAs generally show nearly precise complementarity to target mRNAs and trigger mRNA degradation via a mechanism similar to that operating during RNAi. Like siRNAs, miRNAs form part of RISC-like ribonucleoprotein particles, miRNPs or miRISCs (Figure 1). There is partial overlap in the protein composition of RISCs and miRNPs (e.g. both complexes contain proteins of the Argonaute family), consistent with the ability of miRNAs to act, under some circumstances, as siRNAs and vice versa [1,2,4].

This review summarizes recent (2003–2004) progress in understanding the function of dsRNA and small 21–25-nt RNAs in the post-transcriptional control of gene expression, focusing primarily on mechanistic and structural aspects of regulation in animal cells. For extensive discussions of the role of the RNAi machinery in chromatin modification and DNA rearrangements in protozoa, see the reviews by Lippman and Martienssen [5], and Matzke and Birchler [6]; RNA-silencing reactions in plants are reviewed by Baulcombe [3].

Biogenesis of miRNAs

miRNAs are processed from precursor molecules, which correspond either to transcripts of independent miRNA genes or to fragments, most frequently originating from introns, of protein-coding RNA polymerase II transcripts. Several miRNAs, often structurally or functionally related, can be co-transcribed [8]. Maturation of miRNAs occurs in two steps, both catalyzed by enzymes of the RNase III family, Drosha and Dicer. Droshas are 130–160 kDa nuclear proteins containing two RNase III

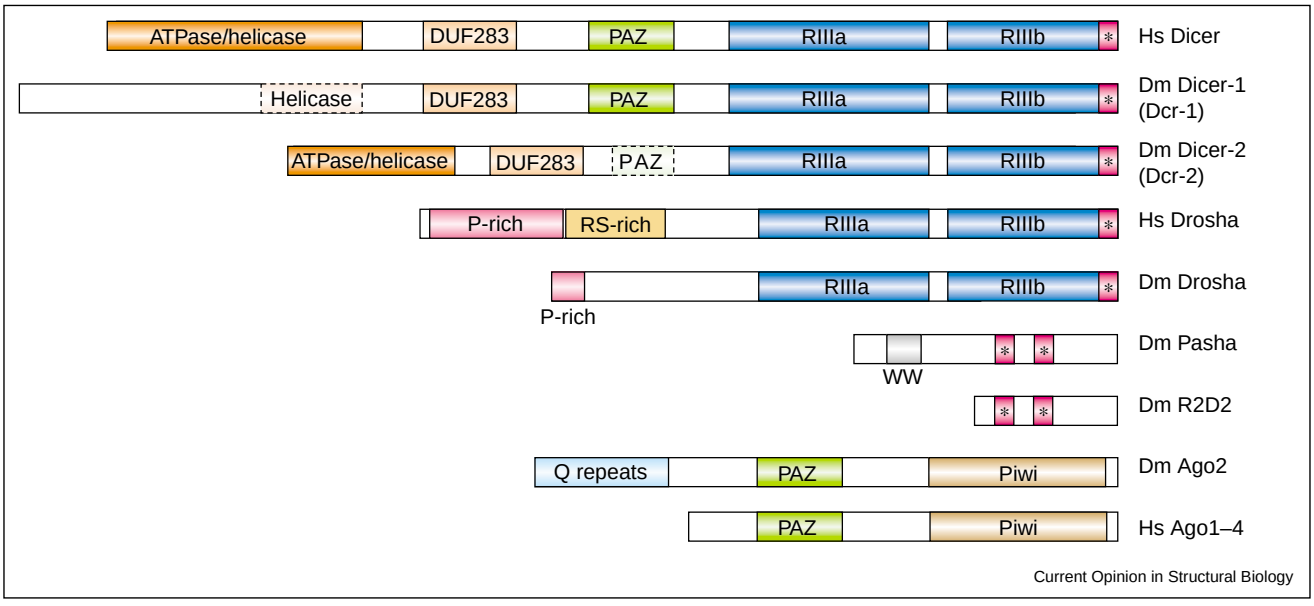
Figure 1



miRNA biogenesis and post-transcriptional gene regulation by miRNAs and siRNAs. The pathway shown is primarily based on data from *Drosophila*, although processing and assembly steps in mammalian cells are similar. Pri-miRNAs frequently contain more than one pre-miRNA hairpin. The Dcr-2–R2D2 complex recognizes the thermodynamic asymmetry of siRNA, with Dcr-2 binding to the less stable end of the duplex, near the 5′ end of the selected guide strand. Rules of miRNA strand selection are similar, but details of the proteins involved are not known. Although not absolutely required, Dcr-1 also plays a role in siRNA-dependent RNAi [20••]. In mammals, a single Dicer participates in RNAi and miRNA pathways, but whether the protein is part of the mature RISC/miRNP is not yet established. *Drosophila* Ago1 and Ago2 function in miRNA and RNAi reactions, respectively. In mammals, all four Ago proteins, Ago1–4, seem to function in translational repression, but only Ago2 is a component of the cleavage-competent RISC.

catalytic domains and a dsRNA-binding domain (dsRBD) in the C-terminal half of the protein, and additional domains of unknown function in the N-terminal half [9••] (Figure 2). As recently shown for mammals, *Drosophila melanogaster* and *Caenorhabditis elegans*, Drosha does not work in isolation, but exists in a complex with a dsRBD protein called Pasha (in *Drosophila*) or DGCR8 (in mammals) (Figure 2) [10•–13•]. Like Drosha, Pasha/DGCR8 is essential for the processing of primary miRNA transcripts, pri-miRNAs, to ~70-nt hairpins referred to as precursor miRNAs (pre-miRNAs). The large ~650 kDa size of the Drosha–DGCR8 complex may be due to

Figure 2



Domain structures of proteins involved in the RNA-silencing pathways discussed in this review. Dicers and Droshas belong to the RNase III family of proteins, and contain two RNase III catalytic domains (RIIIa and RIIIb) and a dsRBD (denoted by the asterisk). Dicers have an N-terminal ATPase/RNA helicase domain, followed by a domain of unknown function (DUF283) and a PAZ domain, which is also present in members of the Argonaute family. The two *Drosophila* (Dm) Dicers differ in functional domain composition: Dcr-1 contains only a C-terminal part of the helicase domain, whereas the PAZ of Dcr-2 diverges from the domains found in other proteins. The Argonautes contain a C-terminal Piwi domain that structurally resembles the RNase H fold. The Droshas have N-terminal proline-rich regions and the human (Hs) enzyme has a domain rich in Arg-Ser (RS) dipeptides, similar to the protein–protein interaction domains found in many splicing factors. Drosha and Dicer are assisted in their function by two proteins containing dsRBDs, Pasha and R2D2, respectively. The WW domain is a module with two conserved tryptophan residues. Although WW modules generally interact with proline-rich sequences, the WW module of Pasha/DGCR8 is dispensable for the interaction with Drosha [11•]. *Drosophila* Ago2 is a considerably larger protein than Ago1 and human Ago1–4, because it contains glutamine-rich repeats at its N terminus.

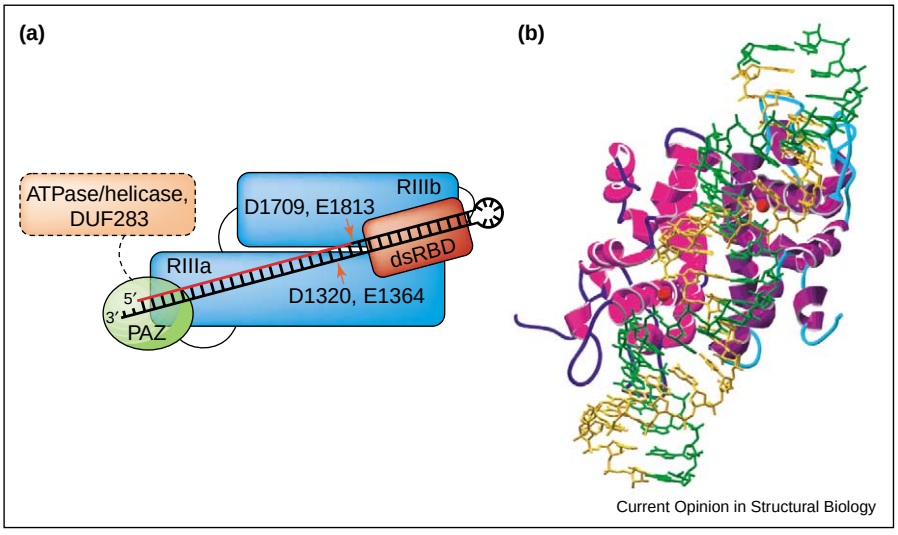
dimerization of its components [11•] or the presence of additional proteins. In human cells, Drosha also appears to form part of a much larger multiprotein complex, but whether this complex functions in the biogenesis of miRNAs or other RNAs is unclear [13•]. Conceivably, as for protein-coding transcripts, the synthesis and processing of pri-miRNAs are tightly coordinated in the nucleus. *Trans*-splicing may be important for effective processing of pri-let-7 RNA in *C. elegans* [14]. Mammalian Drosha contains a serine-arginine (SR)-rich region resembling protein–protein interaction domains of the SR family of splicing factors. However, such a domain is not present in the *Drosophila* protein. Plant genomes do not seem to encode Drosha homologs and, in *Arabidopsis*, all miRNA biogenesis steps may be carried out by one of the four Dicer-like proteins, DCL-1, localized in the nucleus [15].

Function of Dicer

Following the nuclear maturation steps, the ~70-nt pre-mRNA hairpins are transported to the cytoplasm, in a process mediated by exportin-5 (reviewed in [8]), where they are further cleaved by Dicer to yield ~20-bp miRNA duplexes. Dicers are large ~200 kDa proteins containing generally an ATPase/RNA helicase, DUF283, PAZ domains, two catalytic RNase III domains (RIIIa and RIIIb) and a C-terminal dsRBD (Figure 2). In addition to excising miRNAs from pre-miRNAs, Dicer enzymes process dsRNA to siRNAs. Vertebrates, *C. elegans* and *S. pombe* contain single Dicer genes, whereas *Drosophila*, *Arabidopsis* and some other organisms express two or more Dicers with specialized functions (reviewed by Carmell and Hannon [16]).

The Dicers of vertebrates and *Drosophila* are the best characterized biochemically and genetically [7,16]. Recent analysis of mutants of purified recombinant human Dicer led to a model of dsRNA and pre-miRNA processing [17••]. The enzyme functions as a monomer containing a single processing center formed through intramolecular dimerization of the two RNase III domains of the same Dicer molecule. The center contains two independent catalytic sites, each cutting one RNA strand of the duplex and generating products with 2-nt 3′ overhangs (Figure 3). Based on the crystal structure of the catalytic domain of bacterial RNase III [18] and the predicted substrate-docking site, each catalytic site

Figure 3



Model of Dicer function. **(a)** Scheme summarizing a possible mechanism of pre-miRNA processing by human Dicer. The helicase/ATPase and DUF283 domains, with no assigned function, are delineated with a broken line. The enzyme contains a single dsRNA cleavage center with two independent catalytic sites. The center is formed by the RIIIa and RIIIb domains of the same Dicer molecule, and processes the dsRNA ~20 bp from its terminus. The PAZ domain recognizes the 3′ overhang end created by Drosha processing. The placement of the RIII domains illustrates the asymmetry of the catalytic region, with RIIIa cleaving the descending arm and RIIIb the ascending arm of the pre-miRNA hairpin [17••]. **(b)** Model of bacterial (*Aquifex aeolicus*) RNase III interaction with dsRNA. The model is based on a crystal structure of the catalytic domain dimer [18] and the docking of the 30-bp dsRNA A helix, permitting cleavage of each RNA strand at phosphodiesters separated by 2 bp [17••]. Two RNase III monomers are in different shades of purple. Mn^{2+} ions present in each catalytic center are shown as red spheres. The interaction of dsRNA with the intramolecular pseudo-dimer of the Dicer RIIIa and RIIIb domains is very probably similar.

contains a single Mg^{2+} ion coordinated by four conserved acidic amino acid residues. The Dicer mechanism is similar to that proposed for bacterial RNase III [17••] (Figure 3). Dicer preferentially cuts dsRNAs at their termini [16,19] and also excises miRNAs from the end of pre-miRNA hairpins produced by Drosha [9••,17••]. The PAZ domain of Dicer is responsible for the recognition of termini with 3′ overhangs (Figure 3), consistent with similar RNA-binding properties of the PAZ domain of Argonaute proteins (see below). Properties of *Drosophila* Dicer-2 (Dcr-2) mutants, assayed both *in vivo* and *in vitro*, support the catalytic center model as outlined above [20••]. This work also demonstrated that *Drosophila* Dcr-2 requires a functional helicase for dsRNA processing *in vivo*, consistent with the strong effect of ATP on the activity of both Dcr-2 and *C. elegans* Dicer *in vitro* [21,22••]. By contrast, ATP has no effect on dsRNA cleavage by either endogenous or recombinant human Dicer [19]. Drosha may process its substrates using a mechanism similar to that of Dicer. Like Dicer, Drosha contains two RNase III catalytic domains (Figure 2), which form an intramolecular dimer containing two catalytic sites functioning independently of each other [11•].

Drosophila expresses two Dicers, Dcr-1 and Dcr-2, which have subtle differences in the composition of their functional domains (Figure 2), but play very distinct roles in RNA silencing. Dcr-1 mainly functions in the processing of miRNA precursors, whereas Dcr-2 is required for RNAi, both for the cleavage of dsRNA and for the assembly of the RISC [20••,23••,24••]. To perform the latter function, Dcr-2 heterodimerizes with R2D2, a small protein containing two dsRBDs [22••]. Because vertebrates and *C. elegans* express only one Dicer protein, interactions with additional proteins must modulate the specificity of these enzymes. Indeed, an R2D2-like protein, RDE-4, which functions in a complex with Dicer, the Argonaute protein RDE-1 and other proteins, and is essential for RNAi but not miRNA function, has been characterized in *C. elegans* [25]. Dicer proteins also interact with Argonaute proteins [7,16], and regions responsible for the direct interaction between human proteins have been mapped to RNase III and Piwi domains [26,27].

Components of RNA-induced silencing complexes

siRNAs and miRNAs function as components of ribonucleoprotein complexes, RISCs and miRNPs, respectively. These complexes have been isolated from human and *Drosophila* cells, and have also been identified in *C. elegans* (reviewed in [7,28]). Apart from Argonautes, the only proteins consistently found in all RISC and miRNP forms, identified RISC components include the Vasa intronic gene (VIG) protein, Tudor-SN (a protein

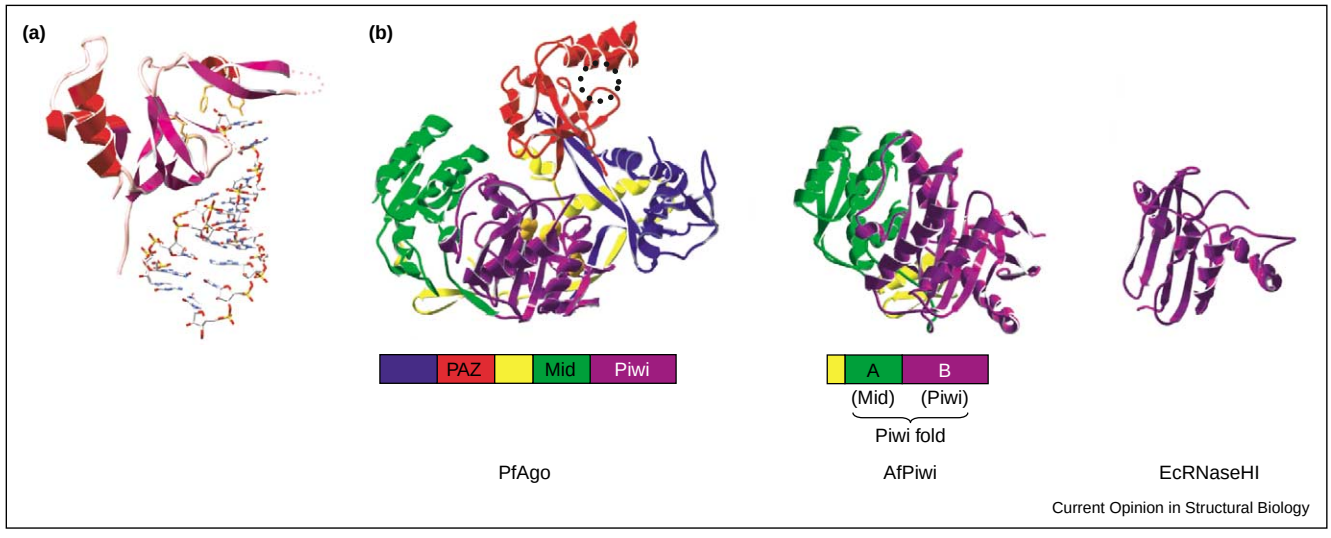
containing five staphylococcal/micrococcal nuclease [SN] domains and a Tudor domain) and dFXR, the *Drosophila* ortholog of human fragile X mental retardation protein (FMRP) [7,28–30]. These proteins or their orthologs also seem to form part of miRNPs from other organisms. Additional associated proteins have also been identified. In human cells, miRNAs were found in an ~15S complex containing Gemin 3 and Gemin 4 [31]; in *Drosophila*, dFXR–miRNA complexes included ribosomal proteins RPL5 and RPL11, and the RNA helicase Dmp68 [32]. The significance of the association with Gemins 3 and 4, proteins also found in the SMN (survival of motor neurons) complex, which functions in the assembly of different cellular RNPs, is not understood, but interaction with FXR1 or FMRP, RNA-binding proteins known to act as modulators of translation, particularly in neurons, may be related to the role of miRNAs in translational repression (reviewed in [33]). The latter argument also applies to the association with RPL5 and RPL11.

Structure and function of Argonaute proteins

A central role for Argonaute proteins in RNA silencing in different organisms first emerged upon isolation of mutants deficient in RNAi and related phenomena. Argonautes were then identified as components of RISCs and miRNPs that associate with siRNAs or miRNAs, and as proteins that interact with Dicer (reviewed in [7,34]). The number of Argonaute paralogs in different organisms ranges from 1 in *S. pombe* to 27 in *C. elegans*. The proteins can be divided into two subfamilies, referred to as Ago and Piwi. Humans, as with other mammals, contain four Ago proteins, hAgo1–4, expressed ubiquitously, and four Piwi family members of ill-defined function, expressed only in testis and hematopoetic stem cells. Five Argonaute proteins are present in *Drosophila*, two of which, Ago1 and Ago2, are best characterized in the context of RNAi and miRNA pathways [7,34].

Recent structural studies of Ago proteins have greatly advanced our understanding of RNA-silencing reactions. Characteristic signatures of all eukaryotic Argonautes, ~100 kDa highly basic proteins, are the PAZ and Piwi domains, the former also found in Dicer proteins and the latter unique to the Ago/Piwi family. Three-dimensional structures of the ~130 amino acid PAZ domains of *Drosophila* Ago1 and Ago2 revealed that they contain an oligonucleotide-binding (OB)-like nucleic-acid-binding fold [35–37]. These findings were soon followed by structures of hAgo1 PAZ in complex with a 7-bp siRNA-like duplex with a 2-nt 3′ overhang [38••] and *Drosophila* Ago2 PAZ complexed with single-stranded RNA or DNA pentamers [39•]. In the hAgo1 PAZ–RNA structure, the 2-nt overhang is inserted into a cleft positioned between a β barrel and α-β modules (previously identified in protein-only structures). In the 7-bp A-form duplex, the strand with an anchored 3′ end is in contact with the positively charged top of the β barrel and the C-terminal tail of the PAZ domain [38••] (Figure 4a). Notably, all important protein contacts are with the phosphodiester backbone, consistent with the largely sequence-

Figure 4



Structure of Argonaute proteins. **(a)** Structure of the hAgo2 PAZ domain in complex with a 7-bp siRNA-like duplex [38••]. The PAZ domain resembles an OB-fold. Amino acid residues that recognize 3′-terminal overhang nucleotides, projecting from the cleft between a β barrel and α-β modules, are shown as orange sticks. The anchored strand is bound along the phosphate backbone. The projection of the PAZ domain is similar to that of PfAgo PAZ in (b). **(b)** Structures of PfAgo [40••], AfPiwi [41•] and *Escherichia coli* RNase HI (EcRNaseHI; PDB code 1RNH), shown in a similar view. Structurally conserved domains are traced in the same color and a schematic is shown below. In the PfAgo structure, the DDE triad amino acids are represented as orange balls; a pocket containing conserved amino acids involved in the binding of the 3′-protruding nucleotides is marked as a dotted circle.

independent recognition of RNA during the reaction. Solution structures of the *Drosophila* Ago2 PAZ complexes confirm the docking of two 3′-terminal nucleotides in the cleft, which is lined with aromatic and hydrophobic residues [39•].

Eukaryotic Ago proteins and their isolated Piwi domains are difficult to prepare in a recombinant purified form. Help, rather unexpectedly, came from prokaryotes. The genomes of a few archaea and eubacteria encode proteins of unknown function but with identifiable Piwi domains. Song *et al*. [40••] and, shortly thereafter, Parker *et al*. [41•] reported the structures of two such proteins, PfAgo and AfPiwi, originating from the archaeons *Pyrococcus furiosus* and *Achaeoglobus fulgidus*, respectively. Three domains of PfAgo, N-terminal, middle and Piwi, form a crescent-shaped base, with a PAZ domain sitting in the middle of the crescent above the Piwi domain (Figure 4b). The sequence of PfAgo PAZ is poorly conserved and the domain could only be recognized at the three-dimensional structure level. However, PfAgo PAZ contains aromatic amino acids that are the equivalent of those in eukaryotic proteins that bind the 3′ overhang nucleotides of siRNA [40••]. The second characterized protein, 427 amino acid AfPiwi, is smaller than PfAgo, and structurally corresponds to the PfAgo middle and Piwi domains. In the AfPiwi structure, they are referred to as domains A and B, and jointly as the Piwi fold [41•] (Figure 4b).

Most interestingly, the Piwi domains of PfAgo and AfPiwi turned out to have a fold similar to that of RNase H, an enzyme that cleaves the RNA strand of DNA–RNA hybrids, arguing that the Piwi domain may represent an elusive 'Slicer' that is responsible for mRNA cleavage in RISCs [40••,41•]. RNase H and structurally related enzymes contain a triad of acidic amino acids (DDE) involved in catalysis. Equivalent amino acids appear to be conserved in PfAgo and most eukaryotic Argonaute proteins. Mutagenesis of human Ago2, the only one of the four mammalian Ago proteins able to support mRNA degradation upon incorporation into the RISC, demonstrated that the two aspartates of the DDE triad are indeed essential for mRNA cleavage ([42••]; see also Update). The similar metal ion requirements and chemistry of RNA cleavage of RNase H and the RISC (both enzymes generate 5′-phosphate and 3′-OH termini) [43•,44•], and the demonstration that Ago2 is the only polypeptide present in the purified RISC of *Drosophila* [45], further support a role for Argonaute as Slicer (see also Update).

siRNA strand selection and RNA-induced silencing complex assembly

The products of dsRNA and pre-miRNA processing by Dicer are ~20-bp duplexes with 3′ overhangs. However, miRNAs and siRNAs present in functional RISCs have to be single stranded for pairing with the target RNA. How are the duplexes converted to single-chain forms and how is a correct (i.e. antisense or 'guide') strand selected for loading onto the RISC? The latter question is of practical importance because artificial siRNAs can be directly used to trigger RNAi in order to knock-down genes. Measurements of the potency of different double- and single-stranded siRNAs (single-stranded siRNAs can induce RNAi, although with much lower efficiency than duplex forms), and sequence analysis of the duplexes formed by pre-miRNA processing by Dicer have indicated that the strand incorporated into the RISC or selected as an miRNA is generally the one whose 5′ terminus is the thermodynamically less stable end of the duplex [46••,47••]. Investigating the mechanism of RISC assembly on synthetic siRNAs, Tomari *et al*. [48••] found that, in *Drosophila*, the Dcr-2–R2D2 heterodimer senses the differential stability of the duplex ends and determines which siRNA strand gets selected. Photocross-linking to siRNAs containing 5-iodouracils at different positions demonstrated that Dicer binds to a less stable and R2D2 to a more stable siRNA end.

Identification of the Dcr-2–R2D2–siRNA complex [22••] and use of *Drosophila* strains with mutations in RNAi genes helped considerably in dissecting RISC assembly (reviewed in [28]). ATP-independent formation of the Dcr-2–R2D2–siRNA complex clearly represents an early step in the pathway. The complex probably positioned immediately downstream is the RISC loading complex, RLC. This contains additional unidentified proteins and its formation requires ATP. siRNA unwinding is initiated within the RLC and the process requires functional Ago2, which gradually displaces the Dcr-2–R2D2 heterodimer, as monitored by photocross-linking of proteins to RNA. The Dcr-2–R2D2–siRNA complex and the RLC are true assembly intermediates; both can be 'chased' into the active RISC [23••,24••,48••].

Functional RISCs able to cleave mRNA targets have been isolated in distinct forms with varying size and protein composition [7,28]. This may reflect their functional heterogeneity, but may also be due to differences in preparatory procedures. 'Minimal' active RISCs of 100–160 kDa may contain only Ago proteins associated with siRNAs [45,49], consistent with the notion that Argonautes catalyze mRNA cleavage. The largest complex, a holo-RISC identified in *Drosophila*, sediments on gradients at ~80S [23••] and probably corresponds to the ribosome-associated form of previously characterized 300–500 kDa RISCs [29,30,50]. Proteins cofractionating with the holo-RISC, in addition to VIG, Tudor-SN, dFXR and Ago2, also include Dcr-2 and R2D2, indicating that these early siRNA-loading factors may have additional, still unidentified, functions in the RISC [23••,24••]. Within the RISC, mRNA cleavage occurs between the residues that are base paired to nucleotides

10 and 11 of the siRNA, counting from its 5′ end [51], and cleavage itself does not require ATP [7,24••,50]. The guide siRNA remains associated with the complex, allowing it to carry out multiple rounds of RNA cleavage. The turnover of the enzyme is dependent on ATP, suggesting that release of the cleaved mRNA halves may involve an RNA helicase [28,52•,53••]. Several proteins implicated in RNAi in *Drosophila* and other organisms contain ATPase/RNA helicase domains, consistent with a requirement for ATP at different steps of the reaction [50]. However, which proteins participate in specific steps of RNAi is not yet established. Armitage, identified in *Drosophila*, is perhaps the best candidate for unwinding siRNAs, as extracts from *armitage* mutants are defective in steps downstream of Dcr-2–R2D2–siRNA complex assembly [24••].

The two *Drosophila* Dicers, Dcr-1 and Dcr-2, are not the only proteins with roles dedicated to either miRNA or RNAi pathways. In *Drosophila*, the primary function of Ago1 is in the miRNA pathway, whereas that of Ago2 is mainly confined to RNAi [29,54•]; Ago proteins also have specialized functions in *C. elegans* [55]. Of the four human Ago proteins, hAgo1–4, only complexes incorporating either endogenous or recombinant hAgo2 are able to cleave mRNA targets [42••,53••]. Consistently, mouse Ago2 knockout cells are resistant to induction of the RNAi response [42••]. On the other hand, all mammalian Ago proteins associate with endogenous miRNAs and appear to function in translational repression [42••, 53••,56•]. The inability of mammalian Ago1 and Ago3 to act as a Slicer is puzzling, because, like hAgo2, these proteins contain the conserved DDE triad. Subtle differences in amino acids other than DDE are probably responsible for the inactivity of hAgo1 and hAgo3 ([42••]; see also Update).

Function of miRNAs

In contrast to the assembly of the mRNA-cleaving RISCs, relatively little is known about the assembly of functional miRNPs. However, the disposition of miRNA within the mature complex must be similar to that of siRNA within RISCs, because endogenous miRNPs can cleave RNA when confronted with targets perfectly complementary to the miRNA. Moreover, vertebrate miRNA miR-196 [57] and most known plant miRNAs [2,3] function by cleaving target mRNAs. Initial studies in *C. elegans* indicated that miRNAs downregulate protein accumulation without affecting the mRNA level and mRNA association with polysomes, suggesting that translation is either repressed at the step downstream of initiation or that proteins actually undergo synthesis but are rapidly degraded [1,2,58]. In *Drosophila* and mammalian cells also, the decrease in protein accumulation occurs without mRNA degradation [59–62]. The repressive effect of miRNAs can be mimicked in HeLa cells by the miRNA-independent tethering of human Argonautes hAgo2–4 to the mRNA reporter, showing that a major function of miRNAs is to guide their associated proteins to the mRNA [56•]. These data also indicate that a default effect of depositing Ago proteins, including hAgo2 Slicer, on mRNA is translational repression, with cleavage requiring proper base pairing of guide and target RNAs.

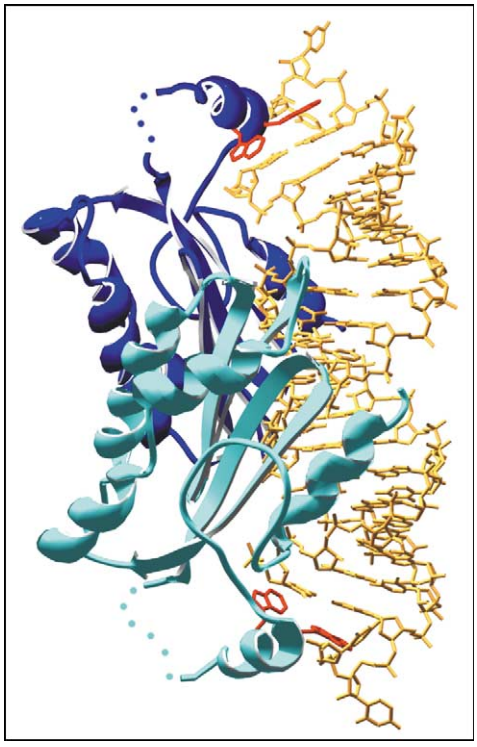
Two major characteristics of animal miRNA–mRNA interactions, established by experimental and bioinformatics approaches, are contiguous Watson–Crick base pairing in the miRNA 5′-proximal seed region and a lack of complementarity in a central part of the miRNA [2,63•,64,65•]. Whereas one perfectly complementary site is sufficient for siRNA- (or miRNA-) induced target RNA cleavage, effective translational repression of mRNA usually requires multiple imperfect sites recognized by the same or several different miRNAs [60,63•] (for a discussion of exceptions to this generalization, see [65•]). The molecular basis of this apparent miRNP cooperativity remains unknown.

Although none of the known signatures of Argonaute proteins provides any clue as to their possible role in effecting translational repression, one of the many proteins that associates with Argonautes and miRNAs, FMRP/dFXR [29,32], is a possible candidate for mediating at least some translational repression functions. FMRP is known to act as a regulator of mRNA translation, and is strongly implicated in promoting synaptic maturation and function. Notably, FMRP is most abundant in brain and a large number of miRNAs are also specifically expressed in this tissue [4,33].

Proteins regulating RNA silencing

Studies of RNA silencing in different organisms have revealed its important role in taming transposon movement, in antiviral defense and in control of gene expression at different levels. Not surprisingly then, RNAi and miRNA reactions are frequently subject to elaborate regulation. For example, many plant [3] and some animal [66,67] viruses encode suppressors of post-transcriptional RNA silencing that interfere with the accumulation or function of siRNAs. Recent crystallographic studies have revealed how the p19 suppressor protein of Tombusviridae elegantly and effectively sequesters siRNAs aimed at destroying viral RNA [68••,69••]. In the structures, two pairs of tryptophan residues of the p19 dimer are positioned on both sides of the siRNA duplex, stacking on the top of terminal base pairs and measuring the distance between the ends of the RNA duplex (Figure 5). As a consequence, only 19–21-bp siRNAs are efficiently bound, independent of the presence of 3′ overhangs. Most of the protein contacts occur with the backbone phosphates and sugar 2′-OH groups of the RNA, thereby explaining the absence of sequence specificity.

Figure 5



Structure of the viral p19 suppressor in complex with 19-bp siRNA ([69••]; see also [68••]). The p19 dimer binds siRNA with the β-sheet surface interacting with the shallow minor groove of the RNA helix. Two pairs of tryptophan residues, shown as red sticks, are positioned on both sides of the siRNA duplex, stacking on the top of terminal base pairs. p19 monomers are colored light and dark blue.

Genetic screens in *C. elegans* identified two proteins, ERI-1 and RRF-3, mutations in which strongly enhance RNAi. ERI-1 contains a conserved DEDDh-like motif also found in $3' \rightarrow 5'$ exonucleases, and the *C. elegans* protein and its human ortholog degrade siRNAs *in vitro* in a reaction dependent on $3'$ overhangs [70•]. ERI-1 is highly expressed in neurons and may be responsible for the relative inefficiency of RNAi in these cells. *rrf-3* encodes one of the four putative RNA-dependent RNA polymerases (RdRPs) of *C. elegans* [71]. RdRPs are essential for many forms of RNA silencing in plants, worms and *Neurospora crassa*, and are also implicated in amplification and spreading of the RNAi response [3]. The inhibitory effect of RRF-3 on RNAi is most readily explained by the protein being a defective enzyme, acting as a dominant negative factor [71].

Conclusions and prospects

The discovery of RNA-guided silencing mechanisms revolutionized our understanding of the control of gene expression and emphasized once more the crucial regulatory role played by RNA. During the past 5–6 years, intensive genetic, biochemical and computational approaches have cataloged hundreds of different players, both proteins and RNAs, that take part in the small RNA games. First high-resolution structures have also appeared, providing some mechanistic understanding of the reactions. The breadth of small-RNA-mediated regulation was not anticipated. Current predictions put the number of different mammalian miRNAs at 500 or more [72] and the percentage of genes regulated by them is placed at 25–30% [73]. miRNAs encoded in viral genomes, probably regulating both viral and host gene expression, have also been reported [74]. Moreover, recent experiments indicate that mammalian miRNAs can reduce the levels of many of their target mRNAs that do not have fully complementary sites [75].

New modes of biogenesis of small RNA regulators are emerging, as exemplified by the endogenous *trans*-acting siRNAs processed from double-stranded forms of non-coding RNAs expressed in plants [76,77]. Single dsRNA of this kind, probably formed by the action of RdRPs, can give rise to multiple siRNAs targeting many different genes. Equally intriguing is the identification in *C. elegans* of hundreds of endogenous siRNAs precisely complementary to protein-coding regions of ~500 different genes, arguing that regulation of endogenous genes by RNAi is also common in animals [78]. New connections between RNA silencing and epigenetic mechanisms, a topic only briefly mentioned in this review, are also emerging. Identification of the plant miRNA that guides gene-specific DNA methylation in *Arabidopsis* by associating with the nascent mRNA transcript in the nucleus, and of an additional RNA polymerase (Pol IV) involved in siRNA-mediated silencing of transposons and repetitive DNA in plants are just two examples of the progress in this area [79,80]. Without doubt, small RNAs will continue to surprise us for many years to come.

Update

Three recent papers provide important additional information about the structure and function of Argonaute proteins. Parker *et al.* [81••] and Ma *et al.* [82••] solved the structure of AfPiwi complexed with siRNA mimics. In the structure, the phosphorylated $5'$-terminal nucleotide of the guide strand is non-base-paired to the complementary strand and is anchored, in the 'flipped' conformation, within the highly conserved basic pocket of the protein. The additional identified siRNA–Piwi contacts involve the sugar-phosphate backbone of four $5'$-proximal nucleotides (positions 2–5) of the guide strand. Presentation of these nucleotides on the Piwi surface in a quasi-helical form suitable for base pairing is consistent with the $5'$ end of both miRNAs and siRNAs representing a nucleation region for pairing with target mRNAs. Modeling longer A-form helices into the AfPiwi structures placed the mRNA target scissile phosphate in proximity to the proposed catalytic region. Hence, the data reinforce the idea that

the mRNA cleavage site is determined by measuring the fixed distance from the anchored siRNA 5′ end. This conclusion is also supported by the work of Rivas *et al.* [83••]. These authors found that an active RISC can be reconstituted from bacterially expressed human Ago2 and an siRNA. When siRNAs shorter than 21 nt were used for RISC assembly, the cleavage site of the substrate was shifted by precisely the number of bases removed from the siRNA 5′ end. Reconstitution of the active RISC with a recombinant hAgo2 represents a final proof that Argonaute is indeed a catalytic engine of the RISC. However, mutagenesis indicates that a DDH rather than DDE triad of amino acids is required for catalysis by the recombinant hAgo2.

The assembly and activity of RISC and RISC-like complexes are also reviewed in a recent article by Tomari and Zamore [84].

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