ASSEMBLY AND FUNCTION OF RNA SILENCING COMPLEXES

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Abstract | In the RNA-interference pathway, double-stranded RNA induces sequence-specific mRNA degradation through the action of the RNA-induced silencing complex (RISC). Recent work has provided our first glimpses of the RISC-assembly pathway and uncovered the biochemical roles of critical RISC components. These advances have taken our mechanistic understanding of RNA interference to a new level and promise to improve our ability to exploit this biological process for use in experimental biology and medicine.

TRANSPOSON
A mobile genetic element that can relocate within a host genome. An autonomous transposon encodes a transposase protein that catalyses its excision and reintegration in the genome, and the transposon can therefore direct its own transposition.

SHORT INTERFERING RNA
(siRNA). A non-coding RNA (~22-nt long) that is processed from longer dsRNA during RNA interference. Such non-coding RNAs hybridize with mRNA targets, and confer target specificity to the silencing complexes in which they reside.

During the past seven years, our view of eukaryotic gene regulation has been transformed. The discovery that injecting double-stranded (ds)RNA into *Caenorhabditis elegans* silences the cognate genes[1] triggered a wave of reports that collectively gave birth to the new field of RNA silencing. Distinct forms of RNA silencing have been found to affect transcription, mRNA turnover, and protein synthesis or stability, and are now known to control the expression of endogenous genes as well as those expressed by TRANSPOSONS and pathogens[2]. The first hints of RNA silencing were observed in plants[3] and fungi[4], and these pathways are now known to operate in nearly all eukaryotic species. One form of RNA silencing, which is known as RNA interference (RNAi), uses dsRNA to guide the destruction of specific mRNAs and is now widely used for gene-inactivation studies in a broad range of eukaryotes[5], including humans[6].

Within two years of the discovery of RNAi in 1998, the first *in vitro* systems were developed that used extracts of *Drosophila melanogaster* cultured cells[7] and embryos[8]. Since then, robust cell-free systems have been established with extracts from human cells[9] and wheat-germ[10]. These biochemical systems have complemented the genetic analysis of RNAi (REF. 11) and have contributed greatly to our mechanistic understanding of the pathway. The biochemical identification of the RNA-induced silencing complex (RISC)[7], which assembles on the dsRNA that triggers silencing, was a crucial advance. Base-pairing interactions between mRNAs and the processed products of the dsRNA silencing trigger guide RISC to its mRNA targets, which it then destroys. Much of the subsequent work in this field has focused on characterizing the components and activities of this complex. Here, recent advances in RNAi biochemistry and structural biology are reviewed, and their impact on our view of RISC assembly and catalysis is described.

The discovery of RISC

Within three years of the explicit demonstration of RNAi in *C. elegans*[1], some of the most fundamental features of the RNAi pathway were discerned. Hamilton and Baulcombe[12] reported that RNA silencing in plants was accompanied by the appearance of ~25 nucleotide (nt) RNAs that were complementary to the target transcript. The small RNAs (later called SHORT INTERFERING RNAS, or siRNAs[13]) were excellent candidates for the specificity determinants of silencing, and evidence that supported this hypothesis quickly accumulated[7,8,13,14]. Shortly thereafter, Hannon and colleagues identified a RIBONUCLEASE-III enzyme, Dicer (Dcr), that processes long dsRNAs into siRNAs during the initiation of RNAi[15]. At about the same time, lysates from *D. melanogaster* S2 cells[7] and embryos[8] were shown to faithfully recapitulate dsRNA-directed mRNA degradation *in vitro*, and a form of the RISC target-specific nuclease complex was identified from S2 cells and was found to associate with siRNAs[7,16]. dsRNA processing and target-mRNA degradation were found to be biochemically separable activities[15,16], which prompted the view that RNAi can

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doi:10.1038/nrm1568
Published online 14 January 2005

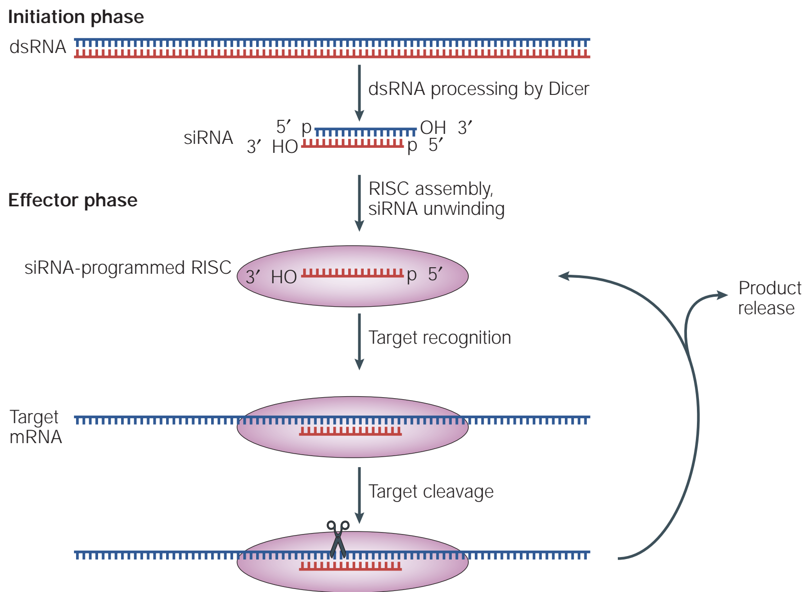


Figure 1 | **The general pathway of RNAi *in vitro*.** In the initiation phase of RNA interference (RNAi), the ribonuclease-III enzyme Dicer cleaves double-stranded (ds)RNA molecules into 21–23-nt short interfering (si)RNA duplexes[15]. siRNAs bear 5′-phosphate groups and 2-nt 3′ overhangs, both of which are important for subsequent siRNA-induced silencing complex (siRISC) assembly[17,26]. In the effector phase of RNAi, the siRNA becomes unwound[9,17], presumably by an RNA-unwindase enzyme, and assembles into RISC. The activated effector complex recognizes the target by siRNA–mRNA base pairing, and then cleaves the mRNA strand with its endoribonuclease activity[29,61,66,67]. The presence of ATP accelerates enzyme turnover, perhaps by promoting siRNA–product unwinding and product release[71].

RIBONUCLEASE III
A family of nucleases that cleave dsRNAs, generally leaving 2-nt 3′-overhangs.

ARGONAUTE (Ago) FAMILY
A family of proteins that are characterized by the presence of two homology domains, PAZ and PIWI. These proteins are essential for diverse RNA silencing pathways.

PAZ DOMAIN
A conserved nucleic-acid-binding structure that is found in members of the Dcr and Ago protein families.

PIWI DOMAIN
A conserved structure that is found in members of the Ago protein family. It is structurally similar to ribonuclease-H domains and, in at least some cases, has endoribonuclease activity.

PARALOGUE
A sequence, or gene, that originates from a common ancestral sequence, or gene, by a duplication event.

be divided into distinct 'initiator' and 'effector' phases that process the dsRNA trigger and degrade the mRNA target, respectively (FIG. 1).

Despite these successes, a single, coherent view of RISC has failed to materialize. siRNA-directed mRNA-cleavage activity has been reported to reside in complexes that have a wide range of apparent sizes, which include ~160 kDa[9], ~200 kDa[17], ~500 kDa[16], ~550 kDa[18,19] and ~80S[20]. The only common theme is that all effector complexes that have been characterized so far contain a member of the ARGONAUTE (Ago) FAMILY of proteins, as defined by the presence of PAZ and PIWI DOMAINS[21]. The variety of size estimates for RISC (summarized in TABLE 1) could be due to either genuine differences in complexes from different cellular sources, or could result from variations in fractionation techniques, or both. Whatever the reason, the reported differences in size, associated factors and other features have complicated efforts to understand the activities and properties of RISC. The discrepancies have also hampered attempts to delineate the pathway of RISC assembly, as there is little agreement on the endpoint of the assembly process. Nonetheless, many features of RNAi effector complexes have been clarified recently, including some unexpected mechanistic connections between the initiator and effector phases of RNAi.

dsRNA processing and RISC assembly

The protein families that are most closely associated with the two phases of RNAi are Dcr, which is associated with the initiation phase, and Ago, which is associated with the effector phase. Co-immunoprecipitation experiments in *D. melanogaster* S2 cell extracts revealed a physical interaction[16] between the RISC component Ago2 and Dcr1, which is one of two Dcr PARALOGUES in *D. melanogaster*[15]. The *C. elegans* Ago protein RDE-1 was also found to co-immunopurify with DCR-1 (the only Dcr protein in worms)[22]. These results provided the first hint of a direct mechanistic link between the dsRNA-processing machinery and RISC, despite the fact that their activities are biochemically separable. Human DCR has recently been shown to bind to both human Ago proteins that have been tested so far (AGO2 and HIWI) through direct interactions between an RNase-III domain of DCR and the PIWI domain of the Ago proteins[23].

A second, functional link between RISC and the dsRNA-processing apparatus was recognized with the characterization of R2D2, a *D. melanogaster* dsRNA-binding protein[24]. This protein heterodimerizes with Dcr2, which is the Dcr protein that generates most siRNAs in *D. melanogaster*[24,25]. In the absence of R2D2, Dcr2 can still process dsRNA efficiently, but the resulting siRNAs are not effectively channelled into RISC. siRNA binding is readily detected with the Dcr2–R2D2 heterodimer but not with Dcr2 alone, which indicates that siRNA binding by the heterodimer is important for RISC entry[24]. Therefore, Dcr2 and R2D2 seem to connect the initiator and effector phases of RNAi. The *C. elegans* protein RDE-4 has a similar domain architecture to R2D2, is required for RNAi *in vivo*, and associates with DCR-1 *in vitro*[22], which indicates that the deduced role of R2D2 is probably conserved in other organisms.

A downstream role for Dicer. The most direct indication of mechanistic coupling between the two phases of RNAi would be a requirement for Dcr downstream of dsRNA processing. Synthetic 21-nt siRNAs do not undergo dsRNA processing, but nonetheless function as potent RNAi triggers[6,9,13,14,26,27], which indicates that they might simply bypass Dcr. An initial report was consistent with this possibility: the immunodepletion of DCR from human cell extracts did not block siRNA-induced target cleavage *in vitro*[9]. However, a subsequent study reported that RNAi-mediated DCR knockdown in cultured human cells did inhibit siRNA-mediated silencing of a reporter protein[28], which indicated a possible role for DCR downstream of siRNA generation. More definitive results emerged from a genetic screen for *D. melanogaster* RNAi-defective mutants that yielded null *Dcr2* alleles[25]. The injection of siRNA into *Dcr2*-null mutant embryos triggered a far weaker RNAi response than the injection of siRNA into wild-type embryos, and extracts from *Dcr2*-null embryos failed to cleave significant amounts of target mRNA in response to an siRNA trigger[25]. Importantly, RISC activity was restored when the *Dcr2*-mutant extracts were supplemented with a partially purified Dcr2-containing complex[20] (see below), which indicated that the RISC defect was probably a direct consequence of the absence of Dcr2. The evidence therefore points towards a direct role for Dcr in RISC assembly that is triggered by double-stranded siRNAs. Results with human AGO2 immunoprecipitates

Table 1 | **Complexes implicated in RISC assembly and function**

Complex (original names)	Source	Known or apparent components	Estimated size	Apparent functions in the RNAi pathway	References
B	*D. melanogaster* embryos	NR	NR	siRNA binding, precursor to the RISC-loading complex?	32
Dcr2–R2D2	*D. melanogaster* S2 cells	Dcr2, R2D2	~250 kDa	dsRNA processing, siRNA binding	24
Dcr2–R2D2 (R1)	*D. melanogaster* embryos	Dcr2, R2D2	~250 kDa	dsRNA processing, siRNA binding, precursor to RISC	20
RISC-loading complex‡, RLC (A)	*D. melanogaster* embryos	Dcr2, R2D2	NR	dsRNA processing, siRNA binding, precursor to RISC	20,32
R2	*D. melanogaster* embryos	NR	NR	RISC-assembly intermediate?	20
Holo-RISC (R3, RISC)	*D. melanogaster* embryos	Ago2, Dcr1, Dcr2, Fmr1/Fxr, R2D2, Tsn, Vig	~80S	Target-RNA binding and cleavage	20,32
RISC	*D. melanogaster* S2 cells	Ago2, Fmr1/Fxr, Tsn, Vig	~500 kDa	Target-RNA binding and cleavage	7,16,59, 60
RISC	*D. melanogaster* S2 cells	Ago2	~140 kDa	Target-RNA binding and cleavage	58
RISC*	*D. melanogaster* embryos	NR	~200 kDa	Target-RNA binding and cleavage	17
Fmr1-associated complex	*D. melanogaster* S2 cells	L5, L11, 5S rRNA, Fmr1/Fxr, Ago2,Dmp68	NR	Target-RNA binding and cleavage?	48
Minimal RISC	HeLa cells	eIF2C1 (AGO1) or eIF2C2 (AGO2)	~160 kDa	Target-RNA binding and cleavage	9,29,61,75
miRNP	HeLa cells	eIF2C2 (AGO2), Gemin3, Gemin4	~550 kDa	miRNA association, target-RNA binding and cleavage	18,19

‡Possibly analogous to a *Caenorhabditis elegans* DCR-1–RDE-1–RDE-4–DRH1-2 complex characterized by co-immunoprecipitation[22]. Ago, Argonaute; Dcr, Dicer; Dmp68, the *Drosophila melanogaster* orthologue of the mammalian p68 RNA unwindase; dsRNA, double-stranded RNA; eIF2C1, eukaryotic translation-initiation factor 2C1; eIF2C2, eukaryotic translation-initiation factor 2C2; Fmr1/Fxr, the *D. melanogaster* orthologue of the fragile-X mental-retardation protein (FMRP); miRNA, microRNA; miRNP, miRNA–protein complex; NR, not reported; RNAi, RNA interference; RISC, RNA-induced silencing complex; rRNA, ribosomal RNA; siRNA, short interfering RNA; Tsn, tudor-staphylococcal nuclease; Vig, vasa intronic gene.

indicate that single-stranded siRNAs can enter RISC in the absence of DCR[29], although the physiological significance of this observation is not clear, as dsRNAs generally trigger RNAi *in vivo*. Recent experiments with cultured human cells have shown that 27-nt RNA duplexes and short hairpin RNAs (shRNAs) function as DCR substrates and are much more potent RNAi triggers compared with standard 21-nt siRNA duplexes, which suggests that the dsRNA-processing step can potentiate RISC entry[30,31].

Dcr2, R2D2 and the RISC-loading complex. A mechanistic basis for the involvement of Dcr beyond dsRNA processing has recently been uncovered. Native gel electrophoresis has shown that when siRNAs are added to *D. melanogaster* embryo lysates, they rapidly bind one or more Dcr2–R2D2-containing complexes that function as precursors to RISC[20,32]. Native polyacrylamide gel analysis[20] revealed a Dcr2-dependent complex known as R1 that corresponds to an siRNA–protein assembly (which was originally referred to as RISC) that has also been characterized by gel filtration chromatography[17,25]. The factors that form this complex cofractionate with dsRNA-processing activity[20] and apparently consist solely of Dcr2 and R2D2 (J. W. Pham and E.J.S., unpublished results). A commitment/chase experiment showed that the Dcr2–R2D2–siRNA complex is a precursor to active RISC[20]. The siRNA within R1 is double-stranded, and formation of the complex does not require ATP[17,20]. The 5′-phosphate groups that are present on siRNAs are important for RNAi activity[17], and this requirement can be traced (at least in part) to the Dcr2–R2D2 heterodimer, as siRNAs that lack 5′ phosphates fail to associate stably with this complex[17,20].

Another Dcr2–R2D2-containing assembly, which is known as the A complex, was identified by native agarose gel electrophoresis[32]. This complex (which is now known as the RISC-loading complex, or RLC[33]) differs from the R1 complex in that its assembly requires ATP[32], and it seems to contain other factors in addition to Dcr2 and R2D2 (Y. Tomari and P. D. Zamore, personal communication). The relationship between R1 and the RLC is not yet understood, but both seem to be RISC precursors[20,32]. The RLC contains predominantly double-stranded siRNA, although low levels of unwound siRNA have also been observed[33]. Both Dcr2 and R2D2 directly contact siRNAs within the RLC[20,32,33]. A complex from *C. elegans* that consists of DCR-1, RDE-4, the Ago protein RDE-1, and one or both of a pair of related RNA UNWINDASES (DRH-1 and -2) is a good candidate for an RLC-like assembly in *C. elegans*[22].

RNA UNWINDASE
An enzyme that catalyses the dissociation of RNA base pairs.

The B and R2 complexes. The same *D. melanogaster in vitro* systems that revealed the existence of R1 and the RLC have indicated that those complexes are only part of a more extensive small interfering (si)RISC-assembly

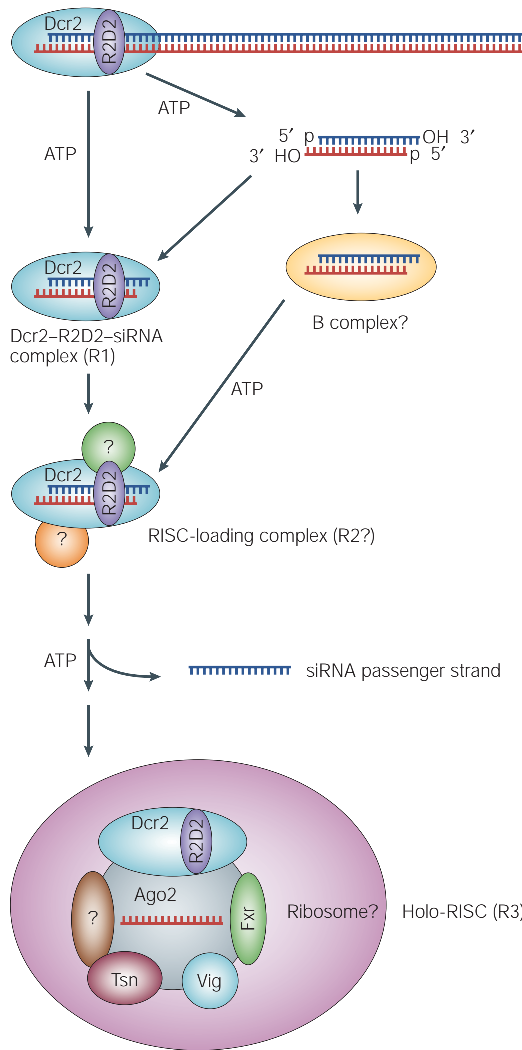


Figure 2 | **RISC assembly in *Drosophila melanogaster*.** The short interfering (si)RNA silencing trigger can be chemically synthesized, or it can be processed from longer double-stranded (ds)RNA by the heterodimer that is composed of Dicer-2 (Dcr2) and the dsRNA-binding protein R2D2 (REFS 24,25). After dsRNA processing, the siRNA might remain bound to the Dcr2–R2D2 heterodimer that generated it, or it might dissociate and then be taken up by a different Dcr2–R2D2 heterodimer. The Dcr2–R2D2–siRNA complex is identical to a complex that was originally known as R1 (REF. 20). The siRNA then enters the RNA-induced silencing complex (RISC)-loading complex (RLC)[33], which was originally known as complex A (REF. 32) and is probably similar to another complex that is known as R2 (REF. 20). The RLC might receive the siRNA from a smaller complex (B) of unknown composition[32]. The RLC associates with other factors to form RISC in an ATP-dependent process that is accompanied by siRNA unwinding[17,20,32], presumably by an ATP-dependent RNA unwindase (not shown). One siRNA strand (the 'passenger' strand) is discarded[50], and the other (the 'guide' strand) probably associates with the PAZ domain of an Ago protein[53,54,66]. At this point, RISC assembly is complete, and the complex is ready to recognize and cleave target mRNAs. In *D. melanogaster* embryo lysates, RISC activity resides in an ~80S holo-RISC assembly that might include a ribosome[20]. Ago2, Argonaute-2; Fxr, the *D. melanogaster* orthologue of the fragile-X mental-retardation protein; Tsn, tudor-staphylococcal nuclease; Vig, vasa intronic gene.

MICRORNA
(miRNA). An ~21–22-nt RNA silencing trigger that is processed from short stem–loop precursors that are encoded in the genomes of metazoans and certain viruses.

pathway[17,20,32] (FIG. 2). The protein complexes that are implicated in RISC assembly are listed in TABLE 1. It has been suggested that two complexes, B and R2, are located immediately upstream of the RLC and downstream of R1, respectively, during siRISC assembly[20,32]. However, much less is known about these two complexes, and their positions along the productive assembly pathway are less firmly established than those of R1 and the RLC. They are also not detected as consistently as R1 and the RLC[20,32].

The proposed role of the B complex downstream of dsRNA processing but upstream of the RLC indicates that it accepts newly generated siRNAs from Dcr2–R2D2, only to transfer them back to the same proteins. A functional rationale for such a role is not yet clear, especially as a purified recombinant Dcr2–R2D2 heterodimer tightly binds siRNAs without assistance from any other factors[24]. The B complex is ATP independent and contains double-stranded siRNA[32]. The R2 complex can also assemble in ATP-depleted extracts, and is derived from the Dcr2–R2D2–siRNA complex in a commitment/chase assay[20]. However, R2 has not been definitively shown to give rise to functional siRISC. In the native agarose gel electrophoresis assay, the assembly pathway fails to progress beyond the RLC in mutant extracts that lack the presumptive RNA unwindase Armitage[32], the PAZ/PIWI-domain protein Aubergine[32] or Ago2 (REF. 34). The ability to form the R2 complex in these extracts has not yet been reported.

RISC and other RNA silencing pathways. R1 and the RLC have been shown to assemble on siRNAs, but siRNAs are not the only RNAi triggers to inhabit RISC. Metazoan genomes encode large numbers of ~22-nt RNAs called MICRORNAS (miRNAs) that enter RNA silencing complexes and help to control the expression of endogenous genes by affecting either mRNA stability or protein synthesis[35–37] (BOX 1). Although both siRNAs and miRNAs are present in forms of RISC (siRISC and micro (mi)RISC, respectively) that can cleave mRNA targets[19,38], far less is known about miRISC assembly, including how distinct it might be from siRISC assembly, and whether it proceeds through complexes that resemble R1 and the RLC. Some organisms encode multiple Dcr paralogues (for example, Dcr1 and Dcr2 in *D. melanogaster*, and DCL1, DCL2, DCL3 and DCL4 in *Arabidopsis thaliana*) that function differentially in siRNA and miRNA biogenesis[25,34,39], providing a potential route toward distinct assembly pathways that involve multiple R1-like and RLC-like intermediates. However, other organisms (for example, *C. elegans* and humans) use only a single Dcr protein to process both categories of silencing triggers, and it is less clear how a single Dcr protein might control entry into multiple assembly pathways.

In addition to the RNA silencing pathways that affect RNA stability and protein synthesis, dsRNA can also direct sequence-specific gene silencing at the transcriptional level[40–43], and a complex that executes this process has recently been identified in fungi[44] (BOX 2). Its assembly pathway is completely uncharacterized.

Box 1 | **miRNA control of protein synthesis or stability**

Most metazoan genomes encode numerous small stem–loop transcripts that are known as pre-micro (pre-mi)RNAs[35–37]. These are processed by Dicer (Dcr)[77–79] to give rise to ~22-nt miRNAs, which assemble into Argonaute (Ago)-containing complexes (similar to small interfering (si)RNAs). In both plants and animals, endogenous miRNAs are present in cleavage-competent RNA-induced silencing complexes (RISCs) and direct the degradation of mRNAs that bear sites of perfect or near-perfect complementarity[19,38]. In animals, however, most miRNAs recognize sites of imperfect complementarity in the 3′ untranslated regions (UTRs) of their target mRNAs. With only one known exception[80], the binding of miRNA to natural target mRNAs in animals does not induce transcript degradation, but nonetheless reduces protein production[70]. An *in vitro* system has not been reported for this mode of regulation, and the mechanism is not understood. The translation of target mRNA could be directly inhibited by miRNAs, and the observed polysome association of miRNA-regulated mRNAs[70] could possibly indicate an effect on elongation or termination. Alternatively, miRNA binding could somehow induce the cotranslational degradation of the nascent polypeptide. Whatever the mechanism, the ability of miRNAs to silence target genes at two distinct levels (mRNA stability and protein synthesis) raises the question of whether a single miRNA–protein effector complex has both activities, or whether miRNAs are channelled into two separate complexes that function differently. In both flies and worms, the siRNA and miRNA pathways seem to require distinct Ago paralogues[34,78,81], which is consistent with the possibility that the two categories of silencing triggers can exist in distinguishable complexes. There is some biochemical support for this interpretation[59]. Nonetheless, human AGO2-containing complexes associate with siRNAs and miRNAs[29,75] and show both target-cleavage[29,75] and translational-inhibition[82] activities, which indicates that a single type of complex could mediate both functions.

siRNA unwinding and RISC entry

siRISC contains single-stranded siRNA[9,45], and therefore siRNA unwinding must occur at some point along the siRISC-assembly pathway. The thermal stability of 19–21-nt duplexes indicates that siRNA unwinding is likely to be an active, energy-consuming process, which is probably catalysed by one or more members of the DEXD/H FAMILY of RNA-unwindase enzymes[46]. Several *D. melanogaster* proteins with putative ATPase/RNA-helicase domains have been implicated in RNAi (including Dcr2 (REFS 20,24,25,34), Spindle-E (REF. 47), Dmp68 (REF. 48) and Armitage[32]), but none has been unambiguously linked to a discrete siRNA-unwinding step. Mutations in conserved residues in the putative helicase domain of Dcr2 abolish dsRNA-processing activity, but have no effect on siRNA-mediated silencing, which indicates that Dcr2 does not directly catalyse siRNA unwinding[25]. Extracts from mutant *D. melanogaster* ovaries that lack functional Armitage protein fail to support the siRISC-assembly pathway beyond RLC formation, which indicates that Armitage might be involved in unwinding siRNAs[32].

DEXD/H FAMILY
A family of enzymes that uses the energy from ATP hydrolysis to drive RNA unwinding.

OFF-TARGET EFFECT
In the context of RNA silencing, this refers to the decreased expression of genes other than the intended target gene.

Synthetic siRNA strand selection. The control of RNA unwinding seems to be an important step in the specificity of siRNA-triggered RNAi. siRNAs generally enter the siRISC-assembly pathway in a double-stranded form, but only one strand is present in activated siRISC[9], and strand selection for functional siRISC uptake is not random. The rules for RISC entry for synthetic siRNAs that do not undergo dsRNA processing seem to be largely dictated by the relative thermal stability of base pairing at the two ends of the siRNA duplex[49,50]. If one end of the siRNA duplex is less stably base-paired than the other, then the strand that has its 5′ terminus at that end is favoured for RISC incorporation and becomes the 'guide' strand, and the other strand (the 'passenger' strand) is discarded (FIG. 3a). When designing siRNAs for gene-silencing experiments, these 'thermodynamic asymmetry' rules are among the features of siRNA that can be exploited to bias RISC entry in favour of the intended antisense strand, thereby minimizing OFF-TARGET EFFECTS due to sense-strand-programmed siRISC[51,52].

How does the siRISC-assembly machinery assess the relative stabilities of the two siRNA ends? The answer probably lies in the nature of the interactions of siRNAs with Dcr2 and R2D2 within the complexes that initiate RISC assembly. Most Dcr-family members contain a PAZ domain, and the structures of two distinct dsRNA-bound PAZ domains have revealed that each has a binding pocket for 2-nt 3′-overhanging RNA ends[53,54]. *In vitro* experiments indicate that recombinant human DCR processes dsRNA from the ends[55], with the PAZ and dsRNA-binding domains (dsRBDs) interacting with terminal and internal regions of the dsRNA substrate, respectively[56] (FIG. 3b). If DCR binds to synthetic siRNAs in a similar asymmetrical manner, then differential base-pairing stability could bias PAZ-domain binding toward one or other end. Similarly, the dsRBDs of *D. melanogaster* R2D2 might bind more rapidly or avidly to the more stably base-paired end of the siRNA, and results from recent crosslinking studies are consistent with this possibility[33]. Such asymmetrical Dcr–R2D2–siRNA interactions could then direct the asymmetrical recruitment of an siRNA unwindase to one end of the siRNA, which would lead to biased RISC incorporation[33].

Processed siRNA strand selection. When long dsRNAs, rather than synthetic siRNAs, are used as a silencing trigger, other factors might be important in the specification of the entry of the siRNA strand into RISC. Tuschl and co-workers have shown that when Dcr generates an siRNA from one specific end of a longer dsRNA, only the strand with its 3′ terminus at the processed end enters siRISC[13], at least for the dsRNA that was used in that experiment. One possible interpretation of the result is that when the silencing trigger undergoes the dsRNA-processing step, the direction of Dcr processing could influence strand selection.

A relationship between siRNA strand selection and the polarity of Dcr processing might result from the fact that Dcr both generates siRNAs[15] and escorts them into siRISC[20,25,32]. These dual roles of Dcr indicate that the enzyme might remain associated with newly generated siRNAs as they are processed from the end of a dsRNA (FIG. 3b). Recombinant human DCR 'turns over' very poorly during dsRNA processing[55,56], which is consistent with the possibility of sustained product interactions. If Dcr establishes asymmetrical interactions with the exposed, pre-existing end of the siRNA while it is still embedded within a longer precursor, then, during dsRNA processing, Dcr might not be free to 'choose' to

Box 2 | **Control of chromatin structure by the RITS complex**

The effects of RNA silencing pathways are not limited to cytoplasmic processes such as mRNA turnover and protein synthesis. The initiation of HETEROCHROMATIN **formation at centromeres and at other chromosomal locations has been shown to rely on small RNAs and the RNA silencing apparatus[40]. In addition to its roles in controlling chromosome structure during normal cellular growth and division, dsRNA-induced heterochromatin formation also seems to underlie** TRANSCRIPTIONAL GENE SILENCING **that can occur in response to transgene or transposon expression[40–43]. Depending on the organism and the specific cellular context, small RNAs that are derived from transgenes or repetitive sequences can direct either DNA methylation or histone methylation. These phenomena have been described in protists[83], plants[84], insects[85,86] and vertebrates[87–89], but are most well understood in the fission yeast *Schizosaccharomyces pombe*[90–92]. Recently, Grewal, Moazed and co-workers demonstrated the existence of an RNA-induced transcriptional silencing (RITS) complex that contains small repeat-associated RNAs, the Argonaute (Ago) protein Ago1, the** CHROMODOMAIN **protein Chp1, and a novel protein, Tas3** (REFS 44,93)**. The presence of small RNAs in the RITS complex requires functional Dicer (Dcr1) protein. Chromatin immunoprecipitation (ChIP) experiments showed that the RITS complex associates directly with heterochromatic regions at centromeres, as long as the small RNAs are present (*dcr1* null mutants still contain the complex, but association with heterochromatin is lost). The RITS complex apparently recruits heterochromatin-specific histone modification factors to the bound loci. It is not known whether the RITS-complex-associated RNAs recognize DNA directly, or whether they hybridize to nascent transcripts. Whatever the targeting mechanism, the identification of an Ago-containing effector complex provides a direct parallel with miRISC and siRISC (see above) and portends rapid progress in understanding the mechanism of dsRNA-induced heterochromatin formation and transcriptional gene silencing.**

bind to a specific siRNA end on the basis of relative base-pairing stability, as it can when it binds to a synthetic siRNA[33]. Accordingly, any asymmetrical Dcr–siRNA interactions that are established during the dsRNA-processing step could persist during siRISC assembly and direct the asymmetrical recruitment of an siRNA unwindase, leading to biased siRISC uptake.

One question that remains to be resolved is whether the two parameters that have been reported to affect strand entry — the relative stabilities of the ends of the siRNA duplex and the direction of Dcr processing — ever come into conflict. If the polarity of Dcr processing can help to determine which strand of a nascent siRNA enters siRISC[13], then, for a given set of processed siRNAs, the identity of the strand that is taken up by RISC will be consistent with the thermodynamic asymmetry rules[49–51] only half of the time, on average (FIG. 3c). It is possible that the newly generated siRNA does not remain associated with Dcr, but is instead released, therefore allowing re-binding to Dcr in the favoured orientation in preparation for RISC assembly. Alternatively, the thermodynamic asymmetry rules might exert less effect with processed siRNAs compared with synthetic siRNAs. Analysis of steady-state populations of dsRNA-derived siRNAs in plants suggests that dsRNA processing might not affect adherence to thermodynamic asymmetry rules[49,57], but siRISC assembly has not yet been directly assessed with processed siRNAs.

From minimal RISC to holo-RISC

The siRISC-assembly pathway culminates in a complex that harbours siRNA-directed target-mRNA-cleavage activity, and several active forms of this complex have been reported (TABLE 1). During fractionation, RISC activity from *D. melanogaster* S2 cells pellets with POLYSOMES and other large complexes, and can be released from the pellet by a high salt wash[7]. The resulting active material behaves as a ~500-kDa complex during gel filtration chromatography[16]. More extensive purification from S2 cell extracts has yielded an active fraction that apparently consists of only a single ~130-kDa protein, Ago2 (REF. 58). By contrast, size fractionation of a *D. melanogaster* embryo lysate was reported to yield RISC activity in a broad range of fractions, with peak RISC activity occurring in the ~200-kDa fractions and almost no activity in the ~500-kDa fractions[17]. A form of RISC (which is known as 'minimal RISC') that was purified from cultured human cells under high salt conditions is even smaller (~160 kDa)[9]. Target-mRNA-cleavage activity is also associated with human proteins that reside within a ~550-kDa complex[18,19]. Most recently, *D. melanogaster* embryo lysates that were fractionated under mild conditions showed RISC activity predominantly in an ~80S holo-RISC complex (originally known as the R3 complex)[20].

Some forms of active RISC have been sufficiently purified to allow direct subunit identification by mass spectrometry[9,16,58–60]. In other cases, resident components have been identified by western blotting[20,32]. Proteins that have been found to be in physical contact with active RISC complexes are listed in TABLE 2. The one family of proteins that is common to all forms of RISC that have been analysed so far is the Ago family, and therefore the presence of an Ago protein seems to be a defining feature of RISC. In human minimal RISC, AGO1 and AGO2 are the only proteins identified so far. Ago proteins are very tightly bound to single-stranded siRNAs within RISC, as the RNA–protein interaction persists even under high salt conditions[9,29,58,61,75]. The PAZ domain of Ago has been implicated in RNA binding[53,54,63–65], and the PIWI domain seems to furnish RISC with effector-nuclease function[29,66] (see below).

Other proteins have been found to associate with functional silencing complexes in *D. melanogaster*. Dcr2 and R2D2 cofractionate with holo-RISC[20,32], which indicates that these proteins assemble into active siRISC together with the siRNAs that they bind in R1 and the RLC. Dcr1 was also found to cofractionate

HETEROCHROMATIN
A highly condensed and transcriptionally less active form of chromatin that occurs at defined sites, such as centromeres, silencer DNA elements or telomeres.

TRANSCRIPTIONAL GENE SILENCING
A form of gene silencing that inhibits RNA synthesis in response to dsRNA.

CHROMODOMAIN
A conserved protein structure that is common to some chromosomal proteins. It interacts with chromatin by binding to methylated lysine residues in histone proteins.

POLYSOME
(or polyribosome). Two or more ribosomes that are bound to different sites on the same mRNA.

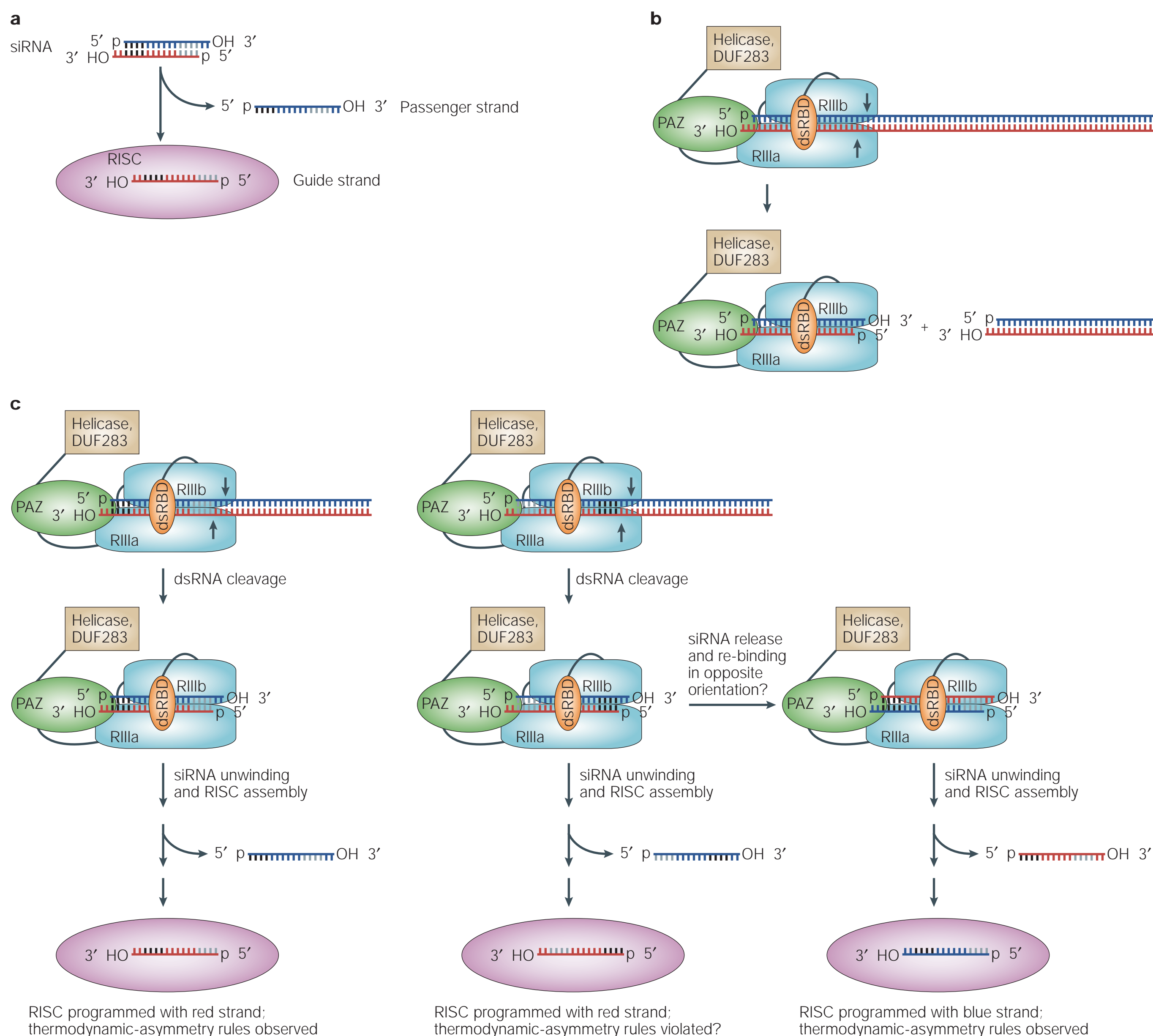


Figure 3 | **siRNA asymmetry, dsRNA processing and the implications for RISC assembly. a** | When 21-nt short interfering (si)RNAs are used to initiate RNA interference (RNAi), the strand with its 5′ terminus at the thermodynamically less stable end of the duplex is preferentially incorporated into the RNA-induced silencing complex (RISC)[49,50]. The guide strand is shown in red and the passenger strand in blue. Less stably base-paired regions of the siRNA are depicted in grey and the more stable regions are shown in black. **b** | Dicer (Dcr) generates siRNAs from the ends of double-stranded (ds)RNAs[55,56]. The multiple domains that are found in most Dcrs (the RNA helicase, DUF283, PAZ, RNase IIIa (RIIIa), RNase IIIb (RIIIb), and dsRNA-binding (dsRBD) domains) are shown. Current models indicate that Dcr interacts with nascent siRNAs asymmetrically, with its PAZ domain bound to the pre-existing dsRNA end and its dsRBD bound closer to the dsRNA-cleavage sites[56,94] (arrows). The dual roles of Dcr in dsRNA cleavage and RISC assembly indicate that Dcr might remain bound to the siRNA after it is processed from the dsRNA precursor. Combined dsRNA-processing and target-cleavage assays suggest that the strand that has its 3′ end bound to the PAZ domain preferentially assembles into RISC[13]. **c** | Potential pathways that might specify siRNA strand entry when the siRNA is processed from a longer dsRNA. The relative base-pairing stabilities of the siRNA ends are as shown in part **a**, and the Dcr domains are as shown in part **b**. If an asymmetrical siRNA is orientated within the dsRNA precursor as shown on the left, with the more stably paired end located at the dsRNA terminus, then both the orientation of Dcr and thermodynamic asymmetry could favour RISC entry by the red strand. By contrast, if the siRNA is embedded within the dsRNA precursor as shown in the middle, with the less stably paired end located at the dsRNA terminus, then Dcr interactions could favour RISC entry by the red strand (middle), whereas thermodynamic asymmetry would favour RISC entry by the blue strand (right). This implies that the bound siRNAs might be released to allow rebinding in the 'correct' orientation, as shown on the right. Alternatively, the functional asymmetry rules that are observed with chemically synthesized siRNAs (which bypass the Dcr processing step) might be less dominant when the siRNAs are generated from longer dsRNAs by Dcr, as shown in the middle. DUF283, domain of undefined function 283; PAZ, piwi/argonaute/zwille; RNase, ribonuclease.

Table 2 | **Biochemically documented RISC-assembly factors and RISC-associated proteins***

Protein	Species	Domains	References
Dcr1	*D. melanogaster*	DUF283, PAZ, RNase III, dsRBD	20
Dcr2	*D. melanogaster*	Helicase, DUF283, RNase III, dsRBD	20,32
R2D2	*D. melanogaster*	dsRBD	20,24,32
Ago2	*D. melanogaster*	PAZ, PIWI	16,20,48,58
Fmr1/Fxr	*D. melanogaster*	RGG, KH	20,48,59
Vig	*D. melanogaster*	RGG	20,59
Tsn	*D. melanogaster*	Tudor, SN	20,60
Dmp68	*D. melanogaster*	Helicase	48
Polyribosomes, ribosome components	*D. melanogaster, T. brucei*	Numerous	7,16,20,48,69
eIF2C1(AGO1)	*H. sapiens*	PAZ/PIWI	9
eIF2C2(AGO2)	*H. sapiens*	PAZ/PIWI	9,19,29,75
Gemin3	*H. sapiens*	Helicase	18,19
Gemin4	*H. sapiens*	None known	18,19

*Numerous other proteins involved in RNAi in a range of species have been discovered by genetic or bioinformatic means[95]. In a few cases, for example, Ago1, Aubergine and Armitage in *Drosophila melanogaster*, the biochemical roles of the proteins have been investigated by examining the activities of extracts from mutant embryos[32,34,96]. However, these proteins have not been physically detected in association with active RISC. Ago, Argonaute; Dcr, Dicer; Dmp68, the *D. melanogaster* orthologue of the mammalian p68 RNA unwindase; dsRBD, double-stranded-RNA-binding domain; DUF283, domain of undefined function 283; Fmr1/Fxr, the *D. melanogaster* orthologue of the fragile-X mental-retardation protein (FMRP); KH, heterogeneous nuclear ribonucleoprotein (hnRNP) K homology; PAZ, piwi/argonaute/zwille; RGG, arginine–glycine–glycine RNA binding; RNase, ribonuclease; SN, staphylococcal nuclease; Vig, vasa intronic gene.

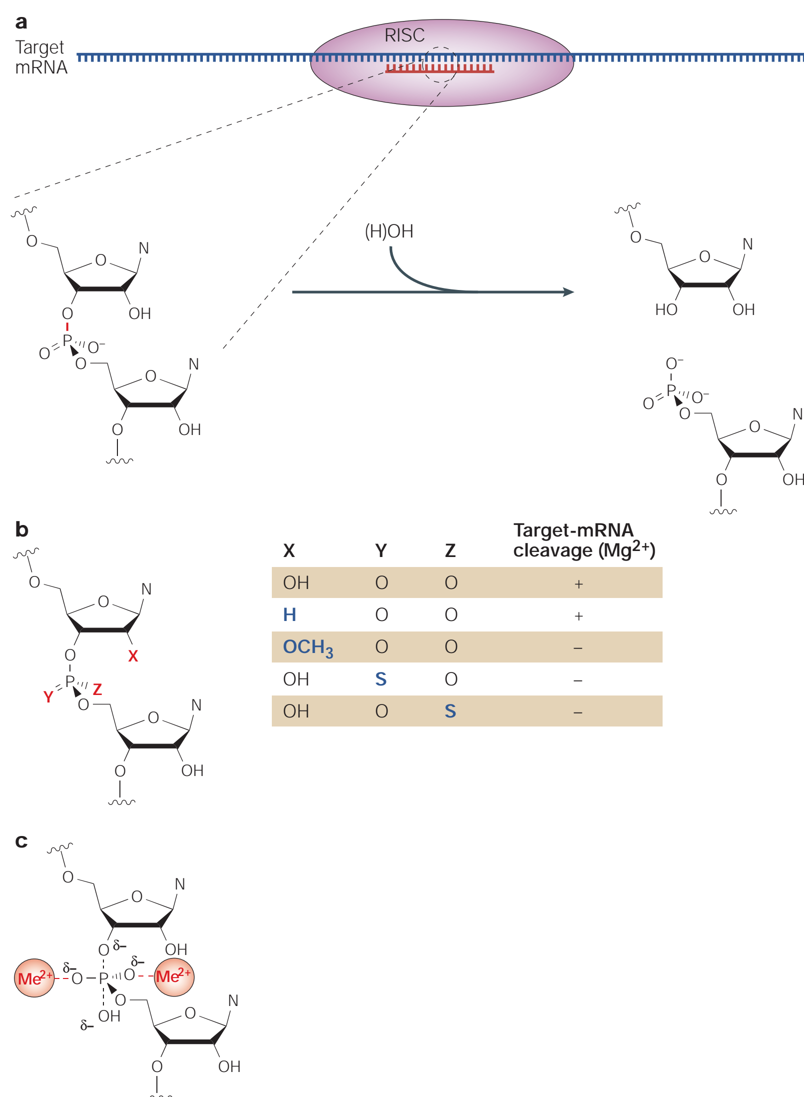
with holo-RISC[20], and its association might be related to miRNA function within RISC[25,34]. Two *D. melanogaster* proteins with RNA-binding domains — the vasa intronic gene (Vig) protein and the orthologue of the human fragile-X mental-retardation protein (FMRP), which is known as Fxr or Fmr1 — have also been found to associate with RISC, although their functions in RISC are not clear[48,59]. Purified *D. melanogaster* RISC contains Tsn, a protein with multiple repeats of the staphylococcal nuclease domain[60]. Chemical considerations as well as the results of enzyme-inhibitor experiments seem to exclude a role for Tsn in siRNA-directed target cleavage[61,67], although its proposed nuclease activity could still be involved in the more extensive degradation of the initial cleavage products of RISC. Vig, Fmr1/Fxr and Tsn co-fractionate with holo-RISC[20]. Affinity-tagged Fmr1/Fxr co-purifies with Ago2, components of the large ribosomal subunit and the presumptive RNA helicase Dmp68 (REF. 48). The presence of Ago2 in this complex indicates that it might have RISC activity, but this has not been directly shown. Knockdown of Dmp68 expression inhibited the RNAi response in *D. melanogaster* S2 cells, which is consistent with a possible role in RISC[48].

Extracts from human cells have yielded an apparent RNAi effector complex in addition to minimal RISC. Immunoaffinity purification and proteomic analysis identified a ~15S miRNA–protein complex that includes AGO2, a putative RNA helicase (Gemin3) and another protein with no known domains or motifs (Gemin4)[18]. Although the initial report did not provide evidence that the complex had RISC activity, anti-Gemin3 and anti-Gemin4 immunoprecipitates were later shown to cleave miRNA-targeted mRNA[19], which indicates that the complex may function as a form of RISC.

The ~80S size of *D. melanogaster* holo-RISC indicates possible RISC–ribosome association[20] and, indeed, large ribosomal subunit components have been found in Ago2-containing complexes[48]. Translationally dormant mRNAs are refractory to RNAi in *D. melanogaster* oocytes[47], which indicates possible functional connections between translation and RNAi (although it should be noted that translationally quiescent transcripts are susceptible to RNAi in mouse oocytes[68]). Interactions between ribosomes and RISC probably exist in other species besides *D. melanogaster*: a fraction of human DCR sediments at ~80S, which indicates that ribosome-sized RNAi complexes might exist in humans[56], and siRNAs have been shown to associate with polyribosomes in *Trypanosoma brucei*[69]. Ribosomes are clearly dispensable for target cleavage, as smaller forms of RISC are active *in vitro*. Nonetheless, ribosome association might facilitate mRNA-target scanning by RISC *in vivo*, possibly by exploiting the ability of the ribosome to disrupt secondary structures. Furthermore, miRISC in animals might function by inhibiting post-initiation phases of protein synthesis[70], and this inhibitory activity (which has not yet been observed *in vitro*) could easily depend on direct ribosome association. In *D. melanogaster* embryo lysates, some RISC components are present in ~80S fractions even in the absence of exogenous siRNA[20], which indicates that endogenous silencing complexes (such as those assembled on miRNAs) might be of similar size.

Mechanism of target-mRNA cleavage by RISC

Once the functional siRISC is assembled and activated, it contains a single siRNA strand[9] that is available for base pairing with its mRNA target. If the mRNA–siRNA duplex is sufficiently complementary (see below), RISC



X	Y	Z	Target-mRNA cleavage (Mg^{2+})
OH	O	O	+
H	O	O	+
OCH_3	O	O	–
OH	S	O	–
OH	O	S	–

Figure 4 | **Chemical mechanism of RISC-catalysed target-mRNA cleavage. a** | The RNA-induced silencing complex (RISC) catalyses a phosphodiester hydrolysis reaction that generates 3′-hydroxyl and 5′-phosphate termini[61,67]. The nucleotides of the target mRNA that are paired to the short interfering RNA (siRNA) nucleotides 10 and 11 (counting from the siRNA 5′ end) are depicted on the left. The scissile bond is shown in red; N represents any nucleobase. **b** | Target cleavage by RISC is not affected by a 2′-deoxyribose (H) substitution at the cleavage site (scissile bond), but 2′-*O*-methyl ribose (OCH_3) and phosphorothioate (S) substitutions at the same phosphodiester linkage inhibit cleavage[61,67]. These effects begin to provide insight into the active-site environment within RISC. X, Y and Z represent atoms or functional groups. **c** | RISC activity requires divalent metal ions[61,67], and a sulphur-substitution experiment indicates that one or more metal ions (Me^{2+}) might directly coordinate one or both of the non-bridging oxygen atoms of the scissile phosphate, perhaps in the transition state[67].

A-FORM RNA
A helical structural form that is commonly adopted by dsRNA and RNA–DNA hybrid duplexes.

severs the mRNA phosphodiester backbone. When nonspecific exonucleases are removed or inhibited, both the 5′- and 3′-cleavage products can be isolated and analysed, which shows that RISC functions as an endonuclease[61,67]. The 5′- and 3′-terminal cleavage products carry 3′-hydroxyl and 5′-phosphomonoester termini, respectively, and this observation reveals the cleavage chemistry of RISC[61,67] (FIG. 4a). The target cleavage step itself is ATP independent and leaves the siRNA intact, allowing RISC to function as a multiple-turnover enzyme[9,19,61,71]. Although ATP is not essential for cleavage, unpurified *D. melanogaster* RISC 'turns over' more rapidly in the presence of hydrolysable ATP, which indicates that ATP can drive product release, promote a conformational step that restores RISC to a productive ground state, or both[71].

Cleavage-site selection and specificity. For a given siRNA, the site of mRNA-backbone cleavage is highly specific: only the phosphodiester linkage that joins the residues paired to siRNA nucleotides 10 and 11 (counting from the siRNA 5′ end) is severed[26]. Surprisingly, this accuracy is not compromised when the pairing partners of the siRNA 5′-terminal 4–5 nts are mismatched or absent[61,71]. Because the cleavage site is selected in the same manner even when the structure of the intervening RNA changes, target-site selection apparently involves more than a simple measurement along an A-FORM RNA duplex, and the target site might in fact be specified before siRNA–mRNA hybridization. Although limited numbers of siRNA–mRNA mismatches generally do not affect cleavage accuracy, they can have significant effects on cleavage efficiency[61,71].

The target-cleavage reaction. The effects of specific chemical modifications on reactivity can provide useful information about reaction mechanisms and enzyme–substrate interactions. Target molecules that consist exclusively of 2′-deoxy or 2′-*O*-methyl ribonucleotides are not cleaved by human or *D. melanogaster* siRISC[61,62,72]. However, limited substitutions in an otherwise all-RNA background are generally well tolerated by human minimal RISC, including a 2′-deoxyribonucleotide substitution at the target cleavage site itself[61] (FIG. 4b). If the chemical step were rate-limiting, the 2′-deoxyribonucleotide substitution at the cleavage site would be expected to decrease the reaction rate by ~10^3- to 10^4-fold[73], which indicates that a binding or conformational step might limit the overall rate of siRISC-catalysed mRNA hydrolysis. A single 2′-*O*-methylribose substitution at the cleavage site slowed the reaction by almost 100-fold, which indicates possible steric interference by the added methyl group[61]. The two non-bridging phosphate oxygen atoms at the scissile phosphate are important for reactivity, as a target mRNA with a racemic mixture of R_p- and S_p-phosphorothioate substitutions at the cleavage site is not significantly hydrolysed under standard reaction conditions (that is, with Mg^{2+} as the sole divalent cation)[67]. When Mn^{2+} was used instead, however, phosphorothioate inhibition could be partially alleviated. Because Mg^{2+} resists inner-sphere coordination with sulphur to a much greater degree than Mn^{2+} (REF. 74), the observed 'metal-specificity switch' suggests that one or both of the non-bridging phosphate oxygen atoms might establish an inner-sphere contact with one or more divalent metal ions during the hydrolysis reaction[67] (FIG. 4c).

Identifying 'Slicer'. Despite the accumulating mechanistic insights into RISC-catalysed mRNA cleavage, a central question — which RISC component actually catalyses the hydrolytic reaction — remained unanswered for

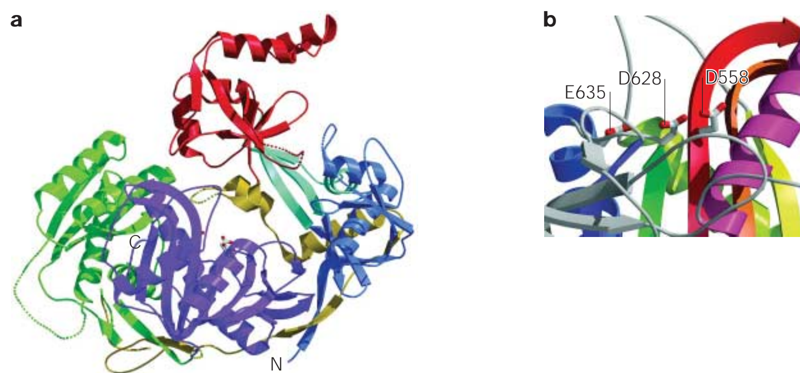


Figure 5 | **The PIWI domains of Ago proteins harbour the endonuclease activity of RISC.** **a** | The crystal structure of an Argonaute (Ago) protein from the archaebacterium *Pyrococcus furiosus*[66]. The protein consists primarily of four domains (the N-terminal (blue), PAZ (red), middle (green) and PIWI (purple) domains). The PAZ domain, which has been implicated in RNA binding, is located above a crescent-like arrangement of the three other domains. The PIWI domain is in the centre of the crescent and lies underneath the PAZ domain. A cleft with multiple positively charged residues begins at the 3′-terminal RNA-binding site of the PAZ domain and continues into the PIWI domain, and probably comprises an extended RNA-binding site. **b** | The PIWI domain has structural similarity to ribonuclease-H enzymes and other proteins with nuclease or polynucleotidyl-transferase activity. At the edge of the putative RNA-binding cleft, three carboxylate residues (E635, D628, and D558) are positioned in a manner that is reminiscent of the divalent-metal-ion-binding DDE catalytic motif of ribonuclease-H enzymes. This indicates that the PIWI domain probably contains the active site for the endonuclease activity of the RNA-induced silencing complex (RISC)[29]. Reproduced with permission from REF. 66 © (2004) The American Association for the Advancement of Science.

RIBONUCLEASE H
An enzyme that cleaves the RNA strand of a DNA–RNA hybrid duplex.

several years. Recently, however, advances in structural biology have provided strong indications that certain Ago proteins can provide RISC with RNA-hydrolysis activity. Full-length Ago proteins have generally proved difficult to produce in recombinant form, but Joshua-Tor and colleagues have successfully expressed, purified and crystallized an Ago protein from the hyperthermophilic archaebacterium *Pyrococcus furiosus*[66].

The X-ray crystal structure revealed three domains (the N-terminal, PIWI and middle domains) that lie in a crescent and together support the fourth domain, PAZ (FIG. 5a). The structure of the PAZ domain of the *P. furiosus* protein is similar to those of other Ago PAZ domains that have been solved in isolation[63–65]. Unexpectedly, the PIWI domain (which is located directly underneath the PAZ domain) adopts a fold that is strikingly similar to that of RIBONUCLEASE H (RNase H) and related polynucleotidyl-hydrolase/transferase enzymes (FIG. 5b). A positively charged cleft that extends from the 3′-terminal RNA-binding site of the PAZ domain[53,54] into the PIWI domain probably provides the siRNA–mRNA binding surface. At the edge of this cleft, the PIWI domain contains three carboxylate side chains that are positioned analogously to the DDE-motif residues, which are thought to bind a catalytically important divalent metal ion in RNase-H enzymes.

Three well-characterized attributes of RNase H — its dependence on divalent metal ions, its ability to cleave one specific strand of a duplex nucleic acid, and its reaction pathway (which involves 3′-hydroxyl and 5′-phosphate termini in the products) — are shared by RISC, which indicates that the PIWI domain could function similarly during RNAi. The Ago protein family is unique in having a representative present in every form of active RISC that has been examined so far[16,19,20] (including highly purified human minimal RISC[9]), and is regarded as the signature RISC component. Ago proteins are therefore 'in the right place, at the right time' to account for the nuclease activity of RISC. Recently, Ago2 was found to be the only detectable protein in active RISC fractions following extensive purification from *D. melanogaster* S2 cell extracts, which further indicates that Ago proteins alone are sufficient for siRISC activity[58].

Functional specificity of Argonaute proteins. In extracts from human cells, target-mRNA-cleavage activity is associated with AGO2, but not with its paralogues AGO1, AGO3 or AGO4 (REFS 29,75). Several missense mutations in human AGO2 were shown to abolish target-cleavage activity without affecting AGO2 expression or siRNA binding[29]. The most provocative of the mutated positions were two of the carboxylate residues that (by alignment) are proposed to contribute to the DDE-motif-like, metal-ion-binding site. By itself, the loss of catalytic activity in response to mutation does not prove that the residues in question comprise part of the active site of the enzyme, as there are many ways in which a mutation can inhibit activity besides direct active-site ablation. Nonetheless, the mutational and structural results together very strongly imply that the PIWI domains of at least some Ago proteins function as 'Slicer', the name given earlier[60] to the long-sought endonuclease subunit of RISC. Ago proteins are important for other RNA silencing phenomena that do not appear to involve RNA degradation[21], and some Ago proteins that (as far as we know) do not have nuclease activity, nonetheless carry PIWI domains with the presumptive active-site residues[29]. So, although the identification of Slicer as the Ago PIWI domain has answered a critical question about RNAi, it has also deepened the mysteries that are associated with other aspects of Ago protein function.

Concluding remarks

Seven years after the discovery of RNAi (REF. 1), our understanding of the pathway is now beginning to approach a satisfying level of mechanistic detail. The outlines of an siRISC-assembly pathway have emerged, and have revealed physical and functional connections between the initiator and effector phases of RNAi. The principles that control siRNA strand selection are also coming into focus. Many of the constituents of RNAi effector complexes have been identified, and members of one family of RISC components (the Ago proteins) have been shown to be the core RISC factors that catalyse target-mRNA cleavage. Finally, the mechanism of RISC-catalysed target cleavage is beginning to be revealed by rigorous chemical, kinetic and thermodynamic characterization.

Nonetheless, these advances have left many unanswered questions in their wake. Given the variety of intermediate and effector complexes that have been observed so far, what does RISC really look like *in vivo*?

What unwinds siRNAs, and how does the guide strand make its way from Dcr to Ago? What other RNAi factors remain to be discovered? What are the similarities and differences between siRISC and miRISC? Why do metazoans encode so many different Ago proteins, and what are the physical and functional distinctions between them? These and other fundamental questions will continue to provide considerable opportunities for discovery in the area of RNAi.

The widespread importance of RNA silencing in biology provides ample reason to work towards a deeper understanding of the underlying mechanisms and, as described in this review, mechanistic advances continue to be made with breathtaking speed. However, the ability and desire to harness the RNA silencing machinery for experimental and (perhaps someday) therapeutic purposes[76] adds even greater urgency to the effort to explain how it works. We have already seen several instances where basic mechanistic insights (for example, dsRNA processing, siRNA asymmetry, and the role of Dcr in potentiating RISC entry, to name just a few) have quickly translated into an improved ability to apply RNAi in the laboratory and develop it for the clinic. We can anticipate that the mechanistic and applied aspects of RNA silencing will continue to advance in parallel for years to come.

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Acknowledgements
I would like to thank R. W. Carthew, P. Bellare, J. Gorra, Z. He, J. L. Pellino, J. W. Pham and J. Preall for helpful discussions and comments on the manuscript. E.J.S. is supported by grants from the National Institutes of Health, National Science Foundation, March of Dimes and American Cancer Society.

Competing interests statement
The author declares no competing financial interests.

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