siRNA and miRNA: an insight into RISCs

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Two classes of short RNA molecule, small interfering RNA (siRNA) and microRNA (miRNA), have been identified as sequence-specific posttranscriptional regulators of gene expression. siRNA and miRNA are incorporated into related RNA-induced silencing complexes (RISCs), termed siRISC and miRISC, respectively. The current model argues that siRISC and miRISC are functionally interchangeable and target specific mRNAs for cleavage or translational repression, depending on the extent of sequence complementarity between the small RNA and its target. Emerging evidence indicates, however, that siRISC and miRISC are distinct complexes that regulate mRNA stability and translation. The assembly of RISCs can be traced from the biogenesis of the small RNA molecules and the recruitment of these RNAs by the RISC loading complex (RLC) to the transition of the RLC into the active RISC. Target recognition by the RISC can then take place through different interacting modes.

Introduction

Although small interfering RNA (siRNA; see Glossary) and microRNA (miRNA) were initially discovered in unrelated studies, both types of small RNA are closely related in their biogenesis, assembly into RNA–protein complexes and ability to regulate gene transcripts negatively in diverse eukaryotes [1–4]. Both siRNAs and miRNAs are generated by Dicer (DCR), a multidomain enzyme of the RNase III family. DCR cuts long, double-stranded RNA (dsRNA) into siRNAs and chops short precursor miRNAs with imperfect stem-loop structure into miRNAs.

The nascent siRNAs and miRNAs are double-stranded duplexes. These duplexes need to be unwound before they can be assembled into an RNA-induced silencing complex (RISC). By comparing the thermodynamic stabilities at the two ends, siRNAs can be divided into two classes: symmetric siRNAs and asymmetric siRNAs. A symmetric siRNA has two equally stable ends; thus, both strands of the siRNA are assembled into the RISC with equivalent efficiency [5]. By contrast, an asymmetric siRNA has one end that is less stable than the other. Because it is easier to unwind siRNA from the less stable end, one strand of the siRNA is preferentially incorporated into the RISC complex in a process referred to as the ‘asymmetric assembly of RISCs’ [5,6]. Intriguingly, most miRNAs are highly asymmetric, ensuring efficient asymmetric assembly of a miRISC in cells [5,6].

In vitro and in vivo biochemical studies have shown that a siRISC can function as a miRISC to repress translation of the target mRNA; similarly, a miRISC can function as a siRISC to cleave the target mRNA. This functional interchangeability between a siRISC and a miRISC argues that siRISCs and miRISCs are highly similar, if not identical [7–10]. Much evidence suggests, however, that siRISCs and miRISCs are distinct types of complex.

First, the biogenesis, maturation and subsequent assembly of siRNAs and miRNAs into silencing complexes are different [1], which can result in RISCs with distinct functions. Second, Argonaute (AGO) proteins, which are principal components of RISCs, are encoded by a multigene family and can be divided into functionally distinct subgroups [11–15]. These functionally different AGOs endow their corresponding RISCs with distinct functions. Third, the complementarity between the small RNAs and their target mRNAs has been proposed to affect the functional mode of RISCs in terms of the regulation of mRNA stability and translation. Nevertheless, RISCs containing small RNAs that are extensively complementary to their target mRNAs do not always specify efficient cleavage of the targets as was previously predicted: some RISCs direct efficient target cleavage [7,8,16,17], whereas others do not [15] (G. Tang et al., unpublished). Fourth, RISCs vary markedly in size, from the smallest ‘core RISC’ of ~160 kDa to the largest ‘holo RISC’ of 80 Svedberg (80S) [18–21].

Last, siRNA and miRNA programmed RISCs have distinct targeting functions in cells. Many endogenous miRNAs and their RISCs are genetically programmed to regulate gene expression and thus are important for the growth and development of an organism [22]. By contrast, siRNAs are produced from dsRNAs that are often synthesized in vitro or in vivo from viruses or repetitive sequences introduced by genetic engineering. In addition, dsRNA can be produced from endogenously activated transposons. Thus, siRNAs have been proposed to function in: (i) antiviral defense (despite the fact that viruses develop counter-defense strategies as well [23,24]), (ii) silencing mRNAs that are overproduced or translationally aborted, and (iii) guarding the genome from disruption by transposons [4,25,26].

In this review, I focus on RISC assembly by discussing the initial synthesis of siRNA and miRNA by DCRs, the formation of a RISC loading complex (RLC), the transition from an RLC to an active RISC, and target recognition and...
interaction by the RISC. Finally, I discuss how two general types of RISC can explain the complexity of their functions in cleavage and translational repression of the target mRNA.

Initiation of the RNAi and miRNA pathways: role of DCRs

It is generally accepted that the RNA interference (RNAi) and miRNA pathways are initiated by DCR members of the RNase III enzyme family. DCR was initially identified in Drosophila [27] and has been subsequently found in diverse eukaryotic organisms including humans, plants and fungi (Table 1). Some organisms have a single homolog of DCR [28–35], whereas others have more [8,36]. In species with several DCRs, different homologs are responsible for producing siRNAs and miRNAs with similar or different sizes [8,21,37,38].

In Drosophila there are two DCR homologs, DCR1 and DCR2 (Table 1). DCR1 is responsible for miRNA production, whereas DCR2 produces siRNAs [38–40]. The fly R2D2, so named because it has two dsRNA-binding domains and tightly interacts with DCR2, has a chief role in bridging the initiation step (siRNA production) and the effector step (siRNA loading onto the effector RISC complex) of RNAs by presenting siRNA to the RLC [40,41] (Figure 1a). R2D2 and DCR2 form a stable heterodimer, and the absence of one makes the other unstable [40]. Knocking out either DCR2 or R2D2 leads to loss of RNAi [40]. Intriguingly, whereas the dcr2 mutant shows no obvious change in fly development, the r2d2 mutant produces a female-sterile phenotype (Q. Liu and D. Smith, personal communication), showing that R2D2 is essential for fly development and suggesting that it has functions that are independent of DCR2.

The only DCR in C. elegans, DCR-1 (Table 1), is required for both the RNAi and the miRNA pathways. Genetic screening for RNAi-deficient worms has led to the identification of numerous RNAi-deficient (rde) genes. Biochemical studies have suggested that the dsRNA-binding protein RDE-4, an R2D2 homolog, interacts with both an AGO protein, RDE-1, and a DCR-related DExH-box RNA helicase (DRH-1) do not seem to be involved in initiating the miRNA biogenesis. In humans, Pasha is also known as DGCR8. Dicer (DCR): A multidomain enzyme of the RNase III family that was first identified in Drosophila. Dicer cleaves dsRNA or stem-loop structured RNA precursors into small RNAs (≈21–25 nucleotides), termed siRNAs and miRNAs. DCR is widely conserved in diverse organisms.

Drosophila: A nuclear RNA III enzyme discovered first in humans and subsequently in Drosophila and C. elegans that is implicated in initiation of the miRNA pathway.

Exportin: A nuclear transmembrane protein that transports precursor miRNA from the nucleus to the cytosol.

Holo RISC: The largest RNA-protein complex (80S) with RISC activity that has been found in Drosophila. The holo RISC associates with all possible RISC components, RLC components and proteins from other pathways. The existence of the holo RISC indicates that RISC assembly is an inseparable and sequential process and that the RISC-centered RNAi and miRNA pathways closely interact with many other pathways, possibly to control the growth and development of an organism.

Microprocessor: A nuclear complex composed of Drosha and Pasha that functions in miRNA biogenesis from the primary miRNA to the precursor miRNA.

Non-cleaving RISC: A RISC that lacks endonuclease activity and thus cannot target miRNA for cleavage or translational repression. The RISCs of <200 kDa identified in humans and Drosophila might represent the core RISC. AGO proteins are associated with the core RISC.

Dicer-related DExH-box RNA helicase (DRH): A protein, first identified in C. elegans, that has most homology to the helicase domain of DCR. There are two DRHs: DRH-1 and DRH-2. Both interact with the dsRNA-binding protein RDE-4 and are required for RNAi in C. elegans. Neither seems to be required for miRNA processing.

DCR (DCR): Dicer: A nuclear transmembrane protein that transports precursor miRNA from the nucleus to the cytosol.

Glossary

Argonaute (AGO): A large protein family that constitutes key components of RISCs.AGO proteins are characterized by two unique domains, PAZ and PIWI, whose functions are not fully understood. Current evidence suggests that the PAZ domain binds the 3′-end two-nucleotide overhangs of the siRNA duplex, whereas the PIWI domain of some AGO proteins confers slicer activity. PAZ and PIWI domains are both essential to guide the interaction between the siRNA and the target mRNA for cleavage or translational repression. Distinct AGO members have distinct functions. For example, human AGO2 programs RISCs to cleave the mRNA target, whereas AGO1 and AGO3 do not.

Cleaving RISC: A cleaving or cleavage-competent RISC that contains an endonuclease or slicer activity that binds a target for cleavage. The endonuclease or slicer identity has not been clearly identified in most organisms. Much evidence suggests that some types of AGO protein or their tightly associated identities are candidates for such an endonuclease. A cleaving RISC can direct a specific target mRNA for cleavage or translational repression, depending on the base-pairing conditions between the small RNA and the mRNA target.

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Drosophila and humans, an RNase III enzyme termed Drosha interacts with Pasha (a partner of Drosha with a dsRNA-binding domain similar to RDE-4 and R2D2) to form a nuclear protein complex of ~500–650 kDa termed Microprocessor. Drosha cuts the long primary miRNA transcripts into short (~70 nucleotides or longer) miRNA precursors [44–46]. These precursor miRNAs are then exported from the nucleus to the cytoplasm by Exportin-5 for maturation by cytosolic DCR [47,48]. Notably, although Pasha and DCR-1 in worms seem to be dominant in different cellular compartments, knocking down the expression of either one leads to an accumulation of primary miRNA transcripts. This suggests that there is close communication between the nuclear Microprocessor complex and cytosolic DCR-1.

Drosha and Pasha, the key components of Microprocessor, are conserved in C. elegans, Drosophila and humans but not in plants, which suggests that miRNA biogenesis diverges in different types of organism. In plants, three factors have been implicated to have a role in the biogenesis of miRNAs: DCL1, a DCR-like protein; HEN1, an uncharacterized protein; and HYL1, a nuclear dsRNA-binding protein [49,50] (Table 1). Knockout of the genes encoding these proteins leads to a reduction in the mature miRNAs in Arabidopsis and an accumulation of the corresponding target mRNAs [49–51]. DCL1, HEN1 and HYL1 might interact with each other to initiate the plant miRNA pathway [49,50].

### Table 1. Principal protein components of the RLC and RISC and their putative functions

<table>
<thead>
<tr>
<th>Organism</th>
<th>Component</th>
<th>Putative functions</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. elegans</td>
<td>DCR-1</td>
<td>Produces siRNA and miRNA</td>
<td>[34,75]</td>
</tr>
<tr>
<td>Drosophila</td>
<td>Drosha</td>
<td>RNase III enzyme with a predominant nuclear location; interacts with Pasha to initiate the miRNA pathway</td>
<td>[44]</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>RDE-1</td>
<td>A major AGO component of the RISC involved in target mRNA cleavage; interacts with DCR-1 and RDE-4 and seems to be involved in siRNA production, probably by keeping DCR-1 and RDE-4 in proper tertiary structure</td>
<td>[34]</td>
</tr>
<tr>
<td>S. pombe</td>
<td>RDE-4</td>
<td>dsRNA-binding protein proposed to channel dsRNA to DCR1; interacts with RDE-1</td>
<td>[34]</td>
</tr>
<tr>
<td>Human</td>
<td>Pasha</td>
<td>dsRNA-binding protein (RDE-4 and R2D2 homolog) that interacts with Drosha to initiate the miRNA pathway in the nucleus</td>
<td>[44]</td>
</tr>
<tr>
<td>Human</td>
<td>DRH-1</td>
<td>RNA helicase that interacts with RDE-4 and DCR-1; is probably involved in dsRNA handling and siRNA unwinding</td>
<td>[34]</td>
</tr>
<tr>
<td>Drosophila</td>
<td>DRH-2</td>
<td>Similar to DRH-1</td>
<td>[34]</td>
</tr>
<tr>
<td>Drosophila</td>
<td>DCR1</td>
<td>Produces miRNAs and probably interacts with AGO1</td>
<td>[21,27,38,40,76]</td>
</tr>
<tr>
<td>Drosophila</td>
<td>DCR2</td>
<td>Produces siRNAs, interacts with R2D2 and probably interacts with AGO2</td>
<td>[21,38,40]</td>
</tr>
<tr>
<td>Drosophila</td>
<td>Drosha</td>
<td>See entry for C. elegans Drosha</td>
<td>[44]</td>
</tr>
<tr>
<td>Drosophila</td>
<td>R2D2</td>
<td>dsRNA-binding protein that binds and stabilizes DCR2, senses and binds siRNA duplexes, and probably bridges RLC to RISC</td>
<td>[21,40]</td>
</tr>
<tr>
<td>Drosophila</td>
<td>Pasha</td>
<td>See entry for C. elegans Pasha</td>
<td>[44]</td>
</tr>
<tr>
<td>Drosophila</td>
<td>AGO1</td>
<td>Major AGO component in miRISCs that probably interacts with DCR1; a potential weak slicer that forms a weak cleaving RISC</td>
<td>[39]</td>
</tr>
<tr>
<td>Drosophila</td>
<td>AGO2</td>
<td>Major AGO component in siRISCs that probably interacts with DCR2; a potential slicer that forms a cleaving RISC</td>
<td>[39,59]</td>
</tr>
<tr>
<td>Human</td>
<td>DCR1</td>
<td>Produces siRNAs and miRNAs</td>
<td>[30,32,76]</td>
</tr>
<tr>
<td>Human</td>
<td>Drosha</td>
<td>See entry for C. elegans Drosha</td>
<td>[43,44]</td>
</tr>
<tr>
<td>Human</td>
<td>Pasha</td>
<td>See entry for C. elegans Pasha; also named DGCGR8</td>
<td>[46,77]</td>
</tr>
<tr>
<td>Human</td>
<td>AGO1</td>
<td>An AGO component that forms a non-cleaving RISC and probably interacts with DCR1</td>
<td>[13]</td>
</tr>
<tr>
<td>Human</td>
<td>AGO2</td>
<td>Major AGO component in siRISCs or miRISCs that probably interacts with DCR1 and functions as a slicer to form a cleaving RISC</td>
<td>[13,15]</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>AGO3</td>
<td>An AGO component that forms a non-cleaving RISC and probably interacts with DCR1</td>
<td>[13]</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>DCL1</td>
<td>DCR-like 1 enzyme that produces miRNAs that target endogenous mRNAs for regulation; might interact with HYL1 and HEN1 to initiate the plant miRNA pathway</td>
<td>[37,49]</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>DCL2</td>
<td>DCR-like 2 enzyme; produces siRNAs that target viral RNAs</td>
<td>[37]</td>
</tr>
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<td>Arabidopsis</td>
<td>DCL3</td>
<td>DCR-like 3 enzyme; produces siRNAs that direct the formation of heterochromatin</td>
<td>[37]</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>AGO1</td>
<td>Major AGO component in siRISCs; a potential slicer on the cleaving RISC and component of another kind of RNA–protein complex that directs transgene methylation; probably interacts with DCL1</td>
<td>[62,63]</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>HYL1</td>
<td>A nuclear dsRNA-binding protein implicated in the biogenesis of plant miRNAs</td>
<td>[49]</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>HEN1</td>
<td>A novel protein implicated in the biogenesis of plant miRNAs</td>
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**siRNA– and miRNA–protein interactions: the RLC**

The DCR-cleaved siRNAs and miRNAs are initially double stranded. The transition from double-stranded to single-stranded RNAs during RISC assembly is achieved via RNA–protein and protein–protein interactions. The RLC is the initial RNA–protein complex formed in cells after the production of small RNAs. The small RNAs in the RLC are probably double stranded and ready to be unwound for functional RISC assembly. The RLC has been characterized to some extent in Drosophila and C. elegans, but its exact composition is unknown.

In Drosophila, formation of the RLC is initiated via interactions between the siRNA duplex and DCR2–R2D2 heterodimers [40]. A UV cross-linking study has shown that R2D2 is important in sensing the asymmetry of a siRNA duplex and selects which strand of the siRNA duplex to incorporate into the RISC (while the other
This finding indicates that R2D2 is not only the DCR2 stabilizer, as mentioned above, but also is involved in functional assembly of the RISC [40,41]. How R2D2 functions in the RLC and guides asymmetric assembly of the RISC is poorly understood. R2D2 tends to bind the more stable end of the siRNA duplex and enables the siRNA duplex to unwind from the less stable end, leading to asymmetric RISC assembly [41].

Whether R2D2 or an R2D2-like sensor is required for miRISC assembly has not been fully determined. R2D2 is required for the normal development of a female fly, suggesting that it might be involved in miRNA biogenesis (Q. Liu and D. Smith, personal communication). Most mature miRNAs are detected in cells in a single-stranded form, and the hypothetical miRNA antisense strand (miRNA*) is often undetectable [50,51]. The hypothetical miRNA duplexes produced from precursor miRNA are often characterized by unequal base-pairing stability at the small duplex ends. These findings suggest that endogenous miRNAs might also use an asymmetric RISC assembly mechanism [5,6].

The mechanism of asymmetric miRISC assembly is unknown and might differ from that of asymmetric siRISC assembly. First, miRNA maturation requires two steps: the production of precursor miRNA from the primary miRNA transcript in the nucleus by Microprocessor (the Drosha–Pasha complex), followed by the maturation of miRNA in the cytosol by a specific DCR (Figure 1b). It is not known how cytosolic DCR recognizes the precursor miRNA that is transported from the nucleus or how it produces a mature miRNA in the cytosol. Second, most miRNAs have a 5' uridine that forms a U•G wobble or mismatch base pair at the hypothetical miRNA duplex end [5,6,8]. These 5' U•G wobble or mismatch pairs might have a role in facilitating miRISC assembly.

The RLC has not been specifically studied in *C. elegans*; however, DCR-1, RDE-1, RDE-4 and DRH-1 interact with each other during RNAi initiation, which suggests that a similar complex might function as an RLC in this organism [34].

**RLC structure and assembly: orientation of the small RNA duplex**

In *Drosophila*, asymmetric RISC assembly requires an asymmetric RLC, which is primarily determined by the structure of the siRNA duplex [41]. The composition of the RLC has not been determined in full. The minimal components of the RLC are thought to be the siRNA–DCR2–R2D2 complex and the following RISC components to form an RLC-like complex that is rapidly converted into active RISC. In *C. elegans*, the DCR-1–RDE-1–RDE-4–DRH-1 complex seems to function in both siRNA production and subsequent assembly of the RISC. By contrast, miRISC assembly is initiated in the nucleus, where the gene encoding the miRNA is transcribed into a primary miRNA (Pri-miRNA) transcript. The primary miRNA is processed into a miRNA precursor (Pre-miRNA) by Drosha (green) and Pasha (red) in the Microrn processor complex, and is then exported into cytoplasm by Exportin-5 (purple) for maturation into miRNA by a cytosolic DCR (DCRx; green). miRNA in the miRNA–DCRx complex is subsequently assembled into the miRISC. Panel (b) is modified from Ref. [74].
duplex end [41]. These results suggest that the assembly of an active RLC favors proper orientation of the siRNA.

In *Drosophila*, siRNA duplexes are produced from long dsRNA, probably in a random direction, by DCR2–R2D2. Each duplex binds one DCR2–R2D2 heterodimer, most likely via the R2D2 moiety. If the R2D2 happens to bind the more stable base-pairing duplex end, thereby positioning the DCR2 moiety at the opposite, less stable end, a highly asymmetric RLC will form [41]. How the siRNA duplex is dissociated from and rebound to DCR2–R2D2 in its proper orientation for subsequent active assembly of the RLC is unknown.

**The transition from RLC to RISC: RNA helicase and AGO proteins**

Handing siRNAs from the RLC to the RISC requires an AGO protein and possibly also an RNA helicase (Table 1). Even though *Drosophila* DCR2 has a DEAH helicase domain, it does not unwind siRNA duplexes *in vitro* [41], which suggests that a specific RNA helicase or other factors might be required during the transition from RLC to RISC. Such a hypothetical RNA helicase is thought to unwind the siRNA duplex, after which the RISC is formed by the recruitment of an AGO protein as the core component. Indeed, purified AGO proteins from transfected cells are associated with single-stranded siRNAs or miRNAs [12,13,15,18,19], which suggests that the siRNAs or miRNAs are unwound before their stable association with AGO proteins. The helicase responsible for this unwinding has not been identified. Alternatively, other types of protein that lower the base-paring energy might exist. These proteins might bind small RNA duplexes and help to recruit AGO proteins to one or the other single-stranded siRNA without the need of any helicase.

A common feature of RISCs from diverse organisms is that the AGO proteins are always associated with RISCs. AGO proteins constitute a large gene family in diverse organisms [11]. Most eukaryotic organisms have several AGO homologs: human has eight [52], Arabidopsis has ten [11], *Drosophila* has five [11] and *C. elegans* has twenty-seven [53]; however, fission yeast has only one AGO homolog [28,29,53]. It has been proposed that different resident AGO proteins might specify distinct RISC functions. This possibility is clearly demonstrated in *Drosophila*, in which AGO2 cofractionates and functionally associates with DCR2, whereas AGO1 cofractionates and functionally associates with DCR1 [39,54,55]. These results correlate with the fact that DCR1 is implicated in the maturation of miRNAs, whereas DCR2 has been shown to initiate RNAi in *Drosophila* [38,39] (Table 1). It seems that the fly AGO proteins functionally interact with different DCRs for the downstream assembly of distinct RISCs.

In contrast to *Drosophila*, humans and *C. elegans* contain only one DCR that initiates the assembly of both siRISCs and miRISCs. Nevertheless, different human AGO proteins (AGO1–AGO3) can be recruited by siRNA or miRNA to form RISCs, although only AGO2-associated RISC can direct the target mRNA for cleavage [13,15]. How one DCR transfers the small RNAs to different AGO proteins for the assembly of distinct RISCs remains unknown.

**RISC assembly from RLC: a sequential process**

Recent biochemical studies have shown that RISCs vary markedly in size from complexes as small as 160 kDa in humans to ones as large as 80S in *Drosophila* [12,18–21,56]. *In vitro* RISC assembly in *Drosophila* indicates that assembly starts with the formation of an RLC that contains DCR2 and R2D2, and ends with a large protein complex the size of 80S ribosome [21]. Furthermore, a series of intermediate complexes has been observed during the transition from RLC to RISC. Notably, the RLC, the intermediates and the 80S active RISC all contain DCR2 and R2D2 [21]. The complexes that form during the transition are dependent on ATP and can be chased quantitatively on a native gel, which suggests that RISC assembly is a sequential process and that the active core RISC might be tightly associated with the RLC [21,56]. Whether such a phenomenon can be observed in other organisms by a similar experimental approach has not been tested.

Why do the RISCs seem to be so large and heterogeneous? One explanation is that these complexes might associate with ribosomes [55,57,58]. Indeed, active RISC cofractionates with ribosomes on a gel filtration column [21]. In *Drosophila*, the RISCs could be heterogeneous if the RLC and the RISC can interact with each other. For example, R2D2 in an RLC might interact with AGO proteins on a RISC. Such an interaction occurs in *C. elegans*, in which the R2D2-like protein RDE-4 physically interacts with the AGO homolog RDE-1 [34]. Intriguingly, *Drosophila* DCR1, which is implicated in the production of miRNA, also seems to associate with siRNA-programmed RLCs and RISCs [21]. This observation suggests that *Drosophila* DCR1 might share structural regions with DCR2 that enable it to interact with these complexes.

**RISC functions: target mRNA cleavage and the ‘slicer’ activity**

In RNAi, cleavage of an RNA target by a RISC is defined mainly by the endonuclease or ‘slicer’ activity of the RISC. Much recent evidence has demonstrated that slicer is a subtype of the AGO protein family [13,15,59]. AGO proteins have been previously shown to be in tight association with siRNAs and miRNAs by affinity purification studies using either tagged AGO protein or tagged siRNA [13,15,18,19]. Notably, among the tagged AGO proteins that have been expressed in human cells, only mammalian AGO2 has been found to program a cleavage-competent RISC *in vivo* [13,15]. Moreover, purified tagged human AGO2, together with 5’ phosphorylated single-stranded siRNA, can be forced into a core RISC-like complex that can direct target mRNA for cleavage *in vitro* [13]. This experiment indicates that human AGO2 has slicer activity. This finding is not limited to mammals. In *Drosophila*, purification of the RISC coupled with protein mass analysis has also shown that *Drosophila* AGO2 is the only protein associated with an active RISC activity [59]. Slicer activity has been mapped to the PIWI domain in AGO proteins through mutagenesis [13], a finding that is supported by the crystal structure study of an
archae bacterium protein, *Pyrococcus furiosus* AGO (Pf-AGO), and bioinformatic data [13,14,59]. The PIWI domains of Pf-AGO and *Drosophila* AGO2 are similar to RNase H or endonuclease V in secondary structure [14,59] and share conserved amino acid residues in the active site. These findings suggest that the PIWI domain of these AGO proteins confers the endonuclease activity of slicer. Several biochemical and crystal structure studies suggest that the PAZ domain of AGO proteins binds the 3' end of the single-stranded siRNA [60,61] that interacts with the target mRNA. The crystal structure of Pf-AGO suggests that the PAZ and PIWI domains define a substrate-binding groove that supports interactions between the siRNA and the target mRNA and subsequent cleavage of the target mRNA [14].

The possibility of reconstituting *in vitro* core RISC activity that can cleave a mRNA target provides a practical approach to studying the structure and function of AGO proteins in terms of slicer activity. Why do some AGOs have slicer activity and others not? Mutagenesis and bioinformatics analyses of the PIWI domain of human AGO2 and *Drosophila* AGO2 have identified key amino acid residues that are essential for slicer activity [13]. A loss of these residues during the evolution of AGO protein family might be the reason for the lack of slicer activity in some AGO proteins. Most eukaryotes have conserved RNAi and miRNA pathways and also contain several AGO proteins. These organisms are expected to have at least one member of the AGO family with slicer activity for RNAi to function. In *Drosophila*, both AGO1 and AGO2 are good candidates for this activity [39,59]. In *Schizosaccharomyces pombe*, only one AGO1 protein, which functions in both the RNAi and the chromatin-remodeling pathways, has been identified [28,53]. In *Arabidopsis*, AGO1 is a good candidate for slicer activity [62,63]. Experiments should be designed to test whether these candidate 'slicers', like human AGO2, can be reconstituted into core RISCs that are competent for target mRNA cleavage *in vitro*. More extensive mutagenesis and domain-swapping experiments on the PIWI domain of different AGO family members will be essential to expand our understanding of the slicer activity.

**Two types of RISC: multiple functions**

RISC assembly is most complex in the RNAi and miRNA pathways. It involves small RNA producers (DCRs), small RNA duplex structures and RLC assemblies, and it requires the unwinding of a symmetric–asymmetric RNA duplex and the recruitment of distinct AGO proteins. Different AGO proteins on RISCs have distinct functions that are most probably determined by the PIWI domain of the AGO protein. Moreover, some PIWI domains confer slicer activity, whereas others do not. On the basis of the type of AGO protein that is recruited to a RISC, these complexes can be tentatively divided into two general types: a cleaving RISC and a non-cleaving RISC (Figure 2).

A cleaving RISC has dual functions that can direct the target mRNA either for cleavage or for translational repression, depending on the rate of target cleavage. When the small RNA (siRNA or miRNA) is extensively complementary to its target mRNA, most cleaving RISCs direct cleavage of the target mRNA at a high rate and efficiently destroy it. These cleaving RISCs can become non-cleaving if the base-pairing conditions between the small RNA and the target mRNA are not met. An extensively complementary cleaving RISC can also result in translational repression when the target cleave rate is below a certain threshold. By contrast, when bound to the target mRNA a non-cleaving RISC can direct the target mRNA only for translational repression, regardless of complementarity between the small RNA and the target.

![Figure 2. Model of RISC formation and function. Distinct subtypes of Argonaute protein (AGOx and AGOy) are proposed to interact with siRNAs or miRNAs to form two types of RISC: a cleaving and a non-cleaving RISC. A cleaving RISC can direct the target mRNA for either cleavage or translational repression, depending on the rate of target cleavage. When the small RNA (siRNA or miRNA) is extensively complementary to its target mRNA, most cleaving RISCs direct cleavage of the target mRNA at a high rate and efficiently destroy it. These cleaving RISCs can become non-cleaving if the base-pairing conditions between the small RNA and the target mRNA are not met. An extensively complementary cleaving RISC can also result in translational repression when the target cleave rate is below a certain threshold. By contrast, when bound to the target mRNA a non-cleaving RISC can direct the target mRNA only for translational repression, regardless of complementarity between the small RNA and the target.](http://www.sciencedirect.com)
the base-pairing complementarity between the siRNA or miRNA and its mRNA target is less extensive, the target mRNA might be physically unreachable by the active center of the endonuclease (slicer) in the cleaving RISC, because of the distorted helix that forms between the siRNA or miRNA and the target [7,64,65]. This will result in translational repression instead of efficient cleavage of the target mRNA. By contrast, non-cleaving RISCs lack endonuclease (slicer) activity in the PIWI domain of AGO proteins and can direct the target mRNA only for translational repression (Figure 2).

In addition to these two types of RISC, other kinds of RISC-like identity, such as the RNA-induced initiation of transcriptional gene silencing (RITS) complex that directs chromatin remodeling, also exist [28,66]. The RITS complex also contains DCR-generated siRNA and AGO protein, and functions in heterochromatic silencing by binding to heterochromatic loci [67]. It is not yet known whether the AGO protein on the RITS complex confers the slicer activity.

Both cleaving and non-cleaving RISCs need to recognize and to bind their targets to function in cleavage or translational regulation. Several observations indicate that target recognition and binding by a RISC are determined mainly by the base-pairing between the 5' portion of the siRNA or miRNA and its target mRNA. For example, a siRISC- or miRISC-directed target is cleaved between positions 10 and 11 from the 5' end of a siRNA or miRNA guide strand, regardless of the size (e.g., 21–25 nt) of the siRNA or miRNA [7,68]. This suggests that the 5' end (~9 nt) of a siRNA or miRNA is crucial for target recognition, binding and cleavage. In addition, bioinformatics analysis of miRNA target regions and in vitro target cleavage analysis of the targets with mutations in the siRNA- or miRNA-binding regions also strongly support the importance of base-pairing between the 5' portion of the siRNA or miRNA and its target [16,64,69].

Target recognition and binding by RISC do not necessarily result in target cleavage. Several factors determine whether a target gets cleaved or not. First, only cleaving RISCs have the potential to direct the targets for cleavage; a non-cleaving RISC can direct a target mRNA only for translational repression. Second, the base-pairing condition between the small RNA and its target determines whether the target mRNA is cleaved or not; in other words, a cleaving RISC might not cleave its target if the base-pairing requirement is not met. This idea is strongly supported by the fact that miRNAs and siRNAs are functionally interchangeable, depending on complementarity between the small RNAs and their targets [7,9,10]. Only extensive complementarity in specific regions results in cleavage of the target mRNA; otherwise, the RISC seems to target the mRNA for translational repression [70–72].

Third, the modes of regulating target mRNA (cleavage or translational repression) by siRNA or miRNA sometimes intersect with each other. In this case, both modes of regulation are involved. Which mode of regulation occurs in vivo depends on which mode is dominant. For example, miR172, an Arabidopsis miRNA, shows slow cleavage of its target AP2 mRNA both in vitro (G. Tang et al., unpublished) and in vivo (the 3' cleavage product can be cloned by a PCR-based approach) [71]. Generally, however, AP2 mRNA does not appear to be cleaved in vivo; instead, it seems to be translationally repressed by miR172 [70,71], indicating that miR172-directed AP2 mRNA translational repression is predominant in Arabidopsis. It seems that some threshold of the rate of RISC-directed cleavage determines whether a target mRNA is destroyed or suppressed against translation.

The sequence context of a small RNA might also have a role in determining whether a cleaving or non-cleaving RISC is assembled. For example, chemically synthesized Drosophila miR2b, although perfectly complementary to its target mRNA, does not direct apparent cleavage of the target in vitro, even under conditions that are favorable for a cleaving RISC (G. Tang et al., unpublished). Furthermore, the characteristics of a target mRNA (e.g., abundance, turnover, structure and translatability) might also affect the efficiency of target clearance or translational repression by specific RISCs.

**Interactions between the RISC and other pathways**

RISCs function in the control of specific pathways primarily by negatively regulating gene expression. Sometimes, the RISC regulates its targeting pathways by interacting with proteins from these pathways. For example, immunoprecipitation and sucrose gradient fractionation of RISCs have identified not only the common components of an active holo siRISC [21], but also proteins from other pathways [54,58,73]. These RISC interacting partners include ribosome proteins from the protein translation pathway, vasa intronic gene (VIG) and tudor staphylococcal nuclease (TSN) from RNA metabolism pathways, and fragile X-related protein (FXR) and the survival of motor neurons (SMN) complex from pathways involved in disease.

This type of interaction suggests that protein translation, RNA metabolism, some disease-related processes and most probably other unknown pathways intersect with the endogenous RNAi or miRNA regulation machinery. Potentially, this kind of interaction might be an endogenous strategy in the control of RNAi- and miRNA-regulated pathways.

**Concluding remarks**

In summary, RISC assembly is central to the RNAi and miRNA pathways. Biochemical and genetic studies have identified many putative RISC components, RISC interacting proteins and also the structural features of the siRNAs and miRNAs. The various components of RISCs belong to families of proteins, and each family member specifies a RISC with distinct functions. The discovery of distinct AGO proteins and the identification of slicer identity on these proteins have established two general types of RISC: a cleaving and a non-cleaving RISC (Figure 2). Whereas non-cleaving RISCs can direct only translational repression on their target mRNAs, cleaving RISCs can direct their target mRNAs for either cleavage or translational repression, depending on the base-pairing conditions between the small RNA and its target (Figure 2). The sequence context of a siRNA or a miRNA might also contribute to functional assembly of the RISC.

The variation in size and heterogeneity of RISCs reflects either complexes formed during sequential RISC
assembly or interactions between the RNAi or miRNA pathways and their regulatory components. Future directions should focus on dissecting the core RISCs to elucidate how these complexes regulate target gene expression, while also exploring the holoholo RISCs to understand how the RNAi and miRNA pathways interact with other cellular mechanisms.

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