CHAPTER X

RISC Biology

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1. Introduction

The study of modern gene regulation began from the groundbreaking discovery of the DNA double helix by Watson and Crick in 1953 [1]. Half a century later, the breakthrough discovery of small, non–coding RNAs (sncRNAs) and their multiple functions in gene regulation [2-28] have brought us a unique research field, the biology of small RNA-Induced Silencing Complexes (RISCs) or RISC biology [5, 29-45]. RISCs, the key negative players in gene regulation, amazingly counteract gene activation by destroying the gene transcripts, blocking the production of specific proteins from the gene transcripts, and insulating numerous regions of the genome via chromatin modification in a developmental and cell/tissue- specific manner.

The key components of RISCs are the 21-30 nucleotide (nt) RNAs that were largely identified recently, and the numerous proteins that are directly or indirectly associated with these small RNAs [5, 29-45]. The small RNAs play a key role in defining the specificity of the interaction between RISCs and their target RNAs, while the numerous protein components provide a fundamental platform for RNA-RNA, RNA-protein, or even RNA-DNA interactions. The consequences of these RISC-target interactions are, (1) mRNAs are cleaved at specific sites and eventually destroyed, (2) protein translation is blocked in a specific cellular compartment, and (3) chromatin structures are remodeled at specific regions on chromosomes. These biochemical and epigenetic changes ensure, amongst other things, that organisms develop properly, pathogens have a hard time to infect their hosts, flowers are colorful to please animals, progenies look similar to their parents, and species differ from one another. Surely, there are many more unknown functions of these small RNA-directed RISCs waiting to be discovered. In this chapter, we focus on RISC biology by summarizing the recent advances in RISC studies and discussing the origin of the small RNAs, RISC components, RISC assembly, and the mechanisms of RISC function.

2. The discovery of the small RNA world: siRNAs and miRNAs

Prior to a number of recent discoveries, the biology of sncRNA had, for the most part, been "hidden". For example, when antisense RNA technologies were first used to knock down genes in tomato and petunia plants in 1988 [46, 47], no one knew that it was small RNAs that triggered certain key events in the plants. These included the inactivation of the genes of PolyGalacturonase (PG) that controls tomato ripening and chalcone synthase that controls flower pigmentation. Two years later, when Jorgensen, Stuitje and their colleagues discovered that extra copies of the same gene in a genome suppressed one another's expression [48, 49], they did not envisage that tiny RNAs were behind the phenomenon of "co-suppression". In animals, when the antisense RNA technique became established as a tool for the study of gene function, the biology of small RNA was "obscured" once again. In 1995, when Gao and Kemphues injected worms with antisense RNA to target specific gene for down-regulation and with the sense RNA as a

control [50], they actually repeated the traditional plant experiments of antisense RNA and "co-suppression" simultaneously. However, this extraordinary experiment in worms caught the attention of Mello, Fire and their colleagues: why did both sense and antisense RNA have similar gene silencing effects in worms? The reasonable explanation is that the real trigger of the gene silencing was not the single-stranded antisense or the control sense RNA, but the traces of double-stranded RNAs (dsRNAs) that might contaminate the antisense and sense RNAs used for injection. Their attention and speculation led to the amazing discovery of RNA interference (RNAi) in 1998, a phenomenon in which small amounts of dsRNAs induce remarkable silencing of genes that share the same sequences of the dsRNAs [51, 52].

Genetic studies of RNAi in worms not only led to its application in a wide range of eukaryotes, but also triggered an intense interest of several young biochemists. One year after the discovery of RNAi, Tuschl and Zamore diverged from their previous researches and started to establish their famous *in vitro* biochemical RNAi system, using fly embryo extracts [53]. Their pioneering *in vitro* RNAi experiments opened a wide window as well as a platform for studying RNAi mechanisms using a biochemical approach. Soon, Zamore and Tuschl discovered that small RNAs of 21-23 nt, termed short/small interfering RNAs (siRNAs), are the key players in mediating specific RNA degradation in fly embryo extracts [2, 6]. By that time, Baulcombe and colleagues found that a population of small RNAs of similar size also exist in the traditional transgene silenced plants [54]. Thus, a part of the small RNA "world", siRNAs, finally came into light.

One aspect of the small RNA world actually first came to our attention much earlier than that of siRNA. In 1993, Ambros and coworkers discovered that an endogenous small RNA of 22 nt, *lin-4,* originated from a non-coding region of the genome, and controls aspects of developmental timing in worms [55]. Later another endogenous small RNA of 22 nt, termed *let-7,* also involved in developmental timing in worms [56], was discovered. Subsequently, the hunt for siRNAs, led to the discovery of hundreds small non-coding RNAs [15-17, 20-22, 25]. It turned out that these small RNAs, now known as microRNAs (miRNAs), belong to a large population of ubiquitous and endogenous small ribo-regulators that control growth and development of almost all eukaryotic organisms investigated to date. It therefore seems that miRNAs and siRNAs constitute an expanding collection of small RNAs that appears to play a big role in regulation of gene expression.

3. The biogenesis of the small RNAs: RNAi, miRNA, and RITS pathways

What could be the origins of small RNAs? The breakthrough discovery in RNAi, namely that dsRNAs induced the silencing of their complementary target genes, triggered an increasing interest in mechanistic studies of RNAi among geneticists and biochemists. Geneticists investigated the RNAi pathway by intensively screening for RNAi mutants while biochemists simply followed the conversion of the dsRNAs and their target RNAs in *in vitro* biochemical systems. These intensive and complementary studies not only revealed a great deal of detail on the RNAi machinery but also led to the discovery of two other related pathways: the miRNA pathway and the RNA-Induced Transcriptional

Silencing (RITS) pathway.

3.1. The RNAi pathway

The RNAi pathway came into light initially in worms with the breakthrough discovery of dsRNA-triggered sequence-specific gene silencing by Mello, Fire and colleagues [51, 52]. Major progress in revealing the RNAi pathway, however, came from an *in vitro* study done by Tuschl, Zamore and colleagues using *Drosophila* embryo extracts [53]. Based on their *in vitro* studies, Zamore and Tuschl proposed their first prototype model [2] of the RNAi pathway in 2000: (1) RNAi begins with the cleavage of long dsRNA into small dsRNA fragments of 21-23 nt by dsRNA-specific nuclease, perhaps in a multiprotein complex; (2) the 21-23 nt short dsRNA fragments are then subjected to strand separation by an unidentified ATP-dependent helicase; (3) RNAi-specific proteins are associated with the long dsRNA and remain on the 21-23 nt small fragments before and after the small RNA strand separation; (4) after the strand separation, the small single-stranded RNA fragments, which are coated with RNAi-specific proteins, recognize and bind their complementary mRNA targets to form specific interacting complexes on the targets; and (5) independent endonucleases recognize such complexes and cleave the target mRNAs for destruction. Zamore and Tuschl's model laid a solid framework for the subsequent dissection of the RNAi pathway. Questions on each step in this model led to a new discovery. For example, questions on how the long dsRNA are cleaved into 21-23 nt small RNAs led to the discovery of the first RNAi enzyme termed Dicer by Hannon and colleagues [5]. Similarly, questions on how small RNAs cleave their target mRNAs led to the discovery of the RNA catalytic engine termed "slicer" by Hannon and colleagues as well [40].

The RNAi pathway has subsequently been subjected to extensive exploration in animals and plants. Although there is some deviation and divergence in different organisms, the basic pathway follows the initial RNAi model proposed by Zamore and Tuschl. A unified model [57] with more defined details could be summarized as the following (Figure 1, left): (1) dsRNAs are the essential substrates to initiate RNAi [51, 52] and these dsRNAs can be generated endogenously, for example, by specific RNA polymerases that use RNA as templates, termed RNA-directed RNA polymerase (RdRP) [58-61] and exogenously, for example, by viral infection [60, 62] or *in vitro* engineering [52]; (2) the dsRNA will be first converted into 21-30 nt small RNAs termed small interfering RNAs (siRNAs) [2-4, 6] by RNase III family enzymes [5] that are tightly stabilized by their partners, dsRNA binding proteins [35]; (3) siRNAs are the direct gene silencing triggers that are assembled into siRNA-induced silencing complexes (siRISCs) [3, 5, 6], which contain a member of Argonaute (AGO) proteins with "slicer" (endonuclase) activity [40]; (4) RISCs are the "effectors" that recognize and bind the complementary target mRNAs for cleavage [5, 32]; and finally (5) RNAi is an active process that needs ATP at several specific steps [2, 10]. Thus, RNAi is a major pathway for the biogenesis of small RNAs.

3.2. The miRNA pathway

The search for endogenous siRNAs for intrinsic RNAi functions has led to the discovery of hundreds of miRNAs from diverse eukaryotes. The pathway that is responsible for the

production of these miRNAs remained unknown even after the discovery of RNAi pathway. The size similarity between *lin-4*, *let-7* and siRNAs triggered our curiosity about the potential relationship between these small RNAs. While Mello and coworkers used a series of RNAi mutants to assay for the production of *lin-4* and *let-7* in worms [7], Zamore and coworkers investigated the production of *let-7 in vitro* in fly embryo extracts and *in vivo* in human cells [63]. The outcome from these independent investigations revealed that the production of *lin-4* or *let-7* was in a Dicer-dependent manner in worms, flies and humans. This suggests that the biogenesis of *lin-4*, *let-7* and siRNAs shared common or similar processing machineries [7, 63]. Shortly after this finding, hundreds of *lin-4*- and *let-7*-like non-coding small RNAs termed miRNAs were discovered from numerous eukaryotic organisms [15-17, 20-22, 25]. Knocking down of Dicer-like enzymes similarly reduced the production of these miRNAs [21, 22].

The precursors of siRNAs were previously defined to be the dsRNAs. What then, are the precursors for miRNAs? Seeking to answer this question using bioinformatics and RNA "mfold" analysis resulted in two interesting observations [15-17, 21, 22]: (1) most, if not all, miRNAs are located in the non-coding regions of the genome, and (2) the sequences extended from both ends of the miRNAs, have the potential to be folded up into "stem-loop" structures in which the native miRNAs are located at the stem region. Thus, miRNA precursors (pre-miRNAs) for specific miRNAs may exist in the form of "stem-looped" structures. Indeed, such precursors of about 70 nt were actually detectable in worms, flies and humans [7, 63]. Interestingly, when Dicer was knocked down in these organisms, the pre-miRNA expression increased while the mature miRNAs decreased [7, 63]. These observations strongly supported the idea that Dicer produces miRNAs from their pre-miRNAs, the stem-loop structured RNAs.

What are the origins of the pre-miRNAs? Bioinformatics analysis of the miRNAs has identified many Expression Sequence Tag (EST) clones that contain the miRNAs. These EST clones originally came from long mRNA-like transcripts with poly(A) tails but lacked apparent translational open reading frames (ORFs) [64]. This observation indicates that miRNAs might initially have much longer primary RNA transcripts that are produced by RNA polymerase II (pol-II). A previous experiment done by Kim and coworkers in human cells *in vitro* and *in vivo* confirmed this hypothesis. Based on their experiment, they proposed the first prototype model of the miRNA pathway [65] as the following: (1) miRNA genes are transcribed in the nucleus by an unidentified RNA polymerase to generate the long primary miRNAs termed pri-miRNAs; (2) the pri-miRNAs are then processed in nucleus by an unknown factor(s) into shorter (~ 70 nt in humans) miRNA precursors termed pre-miRNAs; (3) the pre-miRNA are sequentially exported from the nucleus to the cytoplasm by an unidentified export receptor; and (4) pre-miRNAs are further processed by Dicer and possibly other factors to produce the mature miRNAs.

Like the prototype RNAi model [2] proposed by Zamore and Tuschl, Kim and coworkers' miRNA biogenesis model [65] provided a guideline to the detailed dissection of the miRNA pathway in plants and animals. Currently, a comprehensive miRNA pathway can be summarized as the following (Figure 1, right): (1) miRNAs are initially transcribed by RNA polymerase II (pol-II) as long primary miRNA (pri-miRNA) transcripts from the

non-coding regions of the genomes [64-66]; (2) the long pri-miRNAs are first processed into short miRNA precursors (pre-miRNA) of ~70 nt in nucleus by a microprocessor composed of a Drosha-like enzyme of RNase III family and a Pasha-like protein of dsRNA binding protein family [66-70]; and (3) pre-miRNAs are then transported from the nucleus to the cytoplasm by Exportin-5 for maturation into miRNAs by a cytoplasmic complex composed of Dicer and Loquacious-like protein of the dsRNA binding protein family [71-77].

3.3. The RITS pathway

RNAi and miRNA pathways are two related endogenous pathways that regulate gene expression post-transcriptionally. The previously discovered phenomenon of "co-suppression" in plants was also considered to be related to RNAi but could actually be divided into two kinds of gene silencing: Post-Transcriptional Gene Silencing (PTGS) and Transcriptional Gene Silencing (TGS). Only PTGS is the process that is most related to RNAi while the TGS is a different pathway that involves chromatin modification or DNA methylation. Why "co-suppression" displayed two forms of gene silencing was hard to understand. What is the relationship between PTGS and TGS? The discovery of small RNAs and the small RNA-Induced Transcriptional Silencing (RITS) in *Schizosaccharomyces pombe* provided convincing data to support the idea that TGS and PTGS intersect with each other. Bartel, Grewal, Moazed, and colleagues observed that numerous siRNA-like small RNAs generated from the centromeric repeat regions eventually initiate the silencing of these regions in *S. pombe* [23, 78, 79]. This surprising discovery indicates that an active RNA transcription and production of small RNAs happened in this heterochromatin region. The paradox that heterochromatin silencing needs an active transcription from the heterochromatin region provided the first evidence that RNA is a major player in initiation of epigenetic changes of chromatin (Figure 2).

Direct biochemical evidence that a RNAi-like mechanism links TGS and PTGS came from the identification of RITS Complex (RITSC) that contains the same family components of RISCs in *S. pombe* (Figure 2). Grewal, Moazed, and colleagues found that RITSC also contains small RNAs and AGO1 protein [78, 79] and is likely to link the chromatin remodeling proteins in nucleus. Indeed, RITSC is associated with two additional proteins, a novel protein tas3, and the chromodomain protein 1 (chp1), in addition to the general RISC components. The protein chp1 and another protein, the histone methyl transferase clr4 (cryptic loci regulator 4), are required for RITSC to co-localize with histone 3 lysine 9 (H3-K9) methylated heterochromatin [80-83].

To seemingly make the RITS pathway even more complicated, another complex, termed the RNA-Directed RNA polymerase Complex (RDRC), has also been shown to interact with the RITS complex (RITSC) [79] (Figure 2). RDRC is composed of RNA dependent RNA polymerase 1 (RdRP1 or rdp1) and two other conserved proteins, an RNA helicase that is required for RITS-directed heterochromatin assembly 1 (hrr1) and a poly(A) polymerase-like enzyme, a previously identified protein as caffeine-induced death resistant 12 (cid12) [79]. RdRP was proposed to play a role in the amplification of RNA silencing signals in both plants and *C. elegans*. The poly(A) polymerase-like enzyme may be involved in tagging specific RNAs as the RdRP templates to generate dsRNAs [79, 82].

As a new RNAi factor, the poly(A) polymerase-like enzyme homolog, RNAi deficient 3 (rde-3), was also found to be required for RNA interference in *C. elegans* by the Mello group [84]. In plants, a specific new RNA polymerase IV (pol-IV) was found to function in RITS-like complex-directed chromatin modification [85, 86]. Basically, the model of the RITS pathway describes the multiple interaction of protein complexes in the nucleus (Figure 2): (1) the RISC-like complex RITSC, RDR complex (RDRC), and chromatin remodeling proteins are interacting and associated with each other in RITS pathway; (2) the RDRC is likely to tag RNA templates, to amplify the silencing trigger and to anchor the RISC-like structures near the active transcription region; (3) the RITSC itself functions as a scaffold for chromatin remodeling proteins to modify the centromeric regions; and (4) Dicer protein should be coupled with RDRC for production of the small RNAs that are recruited in RITSC.

In summary, small RNAs, specifically siRNAs and miRNAs, appear to be mainly produced in three different, but related, processes i.e., the RNAi, miRNA, and RITS pathways. Dicer is the primary enzyme that directly converts the dsRNA, or "stem-loop" structured pre-miRNA into siRNAs or miRNAs. RdRP is an important enzyme that is coupled with Dicer to amplify the production of the small RNAs, which helps the spreading of TGS and PTGS.

4. Small RNA binding proteins of the Argonaute family: domain structures and functions

Among many proteins that are associated with the gene silencing complexes, Argonaute (AGO) family proteins are the only small RNA binding proteins identified in all RISCs and RITS complexes. Most AGO proteins are about 100 kDa and highly basic and have two unique signature domains: the PAZ domain shared by Piwi, Argonaute and Zwille/Pinhead proteins and the PIWI domain initially found in Piwi protein from *Drosophila*. The PAZ domain is about 130 amino acids and has been identified in both AGO proteins and Dicer-like enzymes. The PIWI domain is about 300 amino acids in size and is located at the highly conserved C-terminal of the AGO protein [87].

How AGO proteins recognize and bind the single-stranded small RNAs to form active RISCs has been a focus in RISC studies in recent years. Protein structural studies plus biochemical and molecular dissection of the AGO protein family have largely expanded our understanding of the interaction between the AGO proteins and the small RNAs. The crystal structures of two prokaryotic AGO-like proteins, *Pf*-AGO from an archaebacterium *Pyrococcus furiosus* [88], and *Aa*-AGO from an eubacterium *Aquifex aeolicus* [89], have revealed multiple domains in AGO proteins: the N-terminal (N) and the middle (Mid) domains in addition to the PAZ and PIWI domains. At least two domains, the PAZ and the Mid domains are directly involved in the interaction between the small RNA and the AGO protein, while the PIWI domain functions as a catalytic domain to interact with the down-stream RNA targets.

4.1. Small RNA-AGO interaction: the PAZ domain

The PAZ domain on AGO proteins, revealed by three-dimensional structures, is actually an RNA binding module, much similar to the oligonucleotide/oligosaccharide-binding (OB) fold structure, which not only binds single-stranded nucleic acids, but specifically recognizes and binds the 3' end two nucleotide overhangs of the small RNA duplexes [34, 37, 90]. The nucleic acid-binding surface of the PAZ domain is located in a hydrophobic cleft-like region between a central five-stranded β-barrel and an α-helix/α-hairpin of a ~35 conserved amino acid residue module. The aromatic and positively charged residues of this nucleic binding surface endow the ability to bind the negatively charged nucleic acids [34, 37, 90, 91].

The PAZ domain can indiscriminately bind any single-stranded small RNAs or double-stranded RNA duplexes with 3' overhangs, whereas blunt-ended double-stranded RNAs and dsRNAs with 5' overhangs are poorly recognized by the PAZ binding surface [34, 37, 90, 91]. The 3' end two nucleotide overhangs of the siRNA duplexes produced by Dicer is the optimal end structure for PAZ to bind; reducing or increasing the length of the 3' end overhanging nucleotides dramatically reduced the binding affinity of the PAZ domains. Because they can effectively recognize and bind the Dicer product siRNA duplex ends, the PAZ domains may play an important role in the initial recognition of siRNA duplexes by AGO proteins in RISC assembly. Both Dicer and AGO proteins contain a PAZ domain. It is not yet known if the PAZ domains of the Dicer and AGO proteins could play a role in handing siRNA duplexes between the two proteins.

4.2. Small RNA-AGO interaction: the Mid domain

The interaction between the small RNA duplex ends and the PAZ domains is not enough for the small RNAs to stably set on the AGO proteins to form functional complexes. The overall crystal structures of two prokaryotic AGO proteins, the *Pf*-AGO and the *Aa*-AGO, revealed more detailed domain structures [88, 89]. Both *Pf*-AGO and *Aa*-AGO are very similar in their global architectures and composed of four major domains in a similar order and orientation: the N-terminal (N), the PAZ, the middle (Mid) and the PIWI domains. While the PAZ domain interacts with the 3' end nucleotide overhangs of the small RNA duplexes, the C-terminal half of the Mid domain forms a conserved deep basic pocket surface adjacent to the interface with the PIWI domain [89]. This deep basic pocket interacts with and binds the 5' end monophosphate group of the small guide RNA [44, 89, 92, 93]. The separated binding features of the PAZ and the Mid domains to the 3' and 5' end of small RNAs result in assembling active and stable RISCs that place the small RNAs in an appropriate position on AGO proteins for subsequent target recognition.

4.3. The catalytic engine of RISCs: the PIWI domain of the AGO proteins

The overall crystal structures of the *Pf*-AGO and the *Aa*-AGO indicate that the PAZ domain is held by a 'stalk' structure between N and PAZ regions whereas the N, Mid and PIWI domains form a crescent-shaped base to support the "stalk" structure. This places

the PAZ domain right on top of the PIWI domain at the center. Interestingly, the overall structure of the PIWI domain is rather similar to the RNase H family enzymes, a class of enzymes known to cleave the single-stranded RNA of RNA/DNA hybrid duplexes [44, 88, 89, 92, 94, 95]. Like the RNase H fold structure, the PIWI domain fold structure also contains a conserved catalytic motif Asp-Asp (DD), indicating that the PIWI domain has RNA cleaving activity similar to RNase H. Indeed, biochemical studies showed that human and *Drosophila* AGO2 and *Arabidopsis* AGO1 proteins have endonuclease or "slicer" activity that cleaves the target mRNA. The key catalytic motif on the PIWI domain has been identified as Asp-Asp-His (DDH) in human AGO2, which is similar to the Asp-Asp-Glu (DDE) triad in RNase H [40, 93]. Comparison of the PIWI domains among different AGO proteins showed that not all the AGO family members have such conserved catalytic motif on their PIWI domains. This indicates that some AGO proteins may lose their "slicer" activity during evolution.

4.4. The AGO protein family: multiple members and diverse functions

Most eukaryotic organisms have multiple members of the AGO protein family, and only a small subset has been functionally characterized. It is not yet known how many of them are involved in the assembly of the gene silencing complexes. Increasing evidence, however, indicates that different subsets of the AGO family members are involved in different kinds of gene silencing and have diverse functions in cells.

Humans appear to have eight different AGO members that can be classified into two subfamilies: the PIWI subfamily (PIWIL1/HIWI, PIWIL2/HILI, PIWIL3, and PIWIL4/HIWI2) and the eIF2C/AGO subfamily (EIF2C1/hAgo1, EIF2C2/hAgo2, EIF2C3/hAgo3, and EIF2C4/hAgo4) [96]. The four members of eIF2C/AGO subfamily, hAGO1-4, have similar affinity to bind miRNAs or siRNAs, but only hAGO2 has the 'slicer' activity to cleave the target mRNA [40, 42, 93]. Furthermore, hAGO2 with "slicer" activity may also mediate the miRNA-guided translational repression when the miRNAs are imperfectly paired to the target mRNAs, as do the hAGO3 and hAGO4. When tethered to the mRNA, the hAGO2 can mimic the miRNA-mediated repression of protein synthesis, independent of miRNAs [97]. This indicates that "slicer" containing AGO protein may have dual functions of either cleaving the target mRNA or blocking the mRNA translation, while the AGOs with no "slicer" activity have no ability to cleave the target mRNAs.

Drosophila has five AGO homologs, dAGO1, dAGO2, dAGO3, piwi and aubergine [87, 98]. Both dAGO1 and dAGO2 have 'slicer' activity, but dAGO1 is involved in miRNA-directed RISC assembly in miRNA pathway, and dAGO2 in siRNAs-directed RISCs in RNAi pathway [43]. The contributions of dAGO3, piwi and aubergine in RISC assembly have not yet been clarified although both piwi and aubergine are required in the female germline for oogenesis and indicated for maintaining PTGS [87].

The *C. elegans* genome encodes more than 20 Argonaute-like genes [7], but only four of them (RED-1, PPW-1, ALG-1, and ALG-2) have been explored so far. Among them, RDE-1 and PPW-1 were required for exogenous double-stranded RNA induced gene

silencing but show no function in the miRNA pathway [99, 100]. In contrast, ALG-1 and ALG-2 bind miRNAs and have functions in miRNA-directed translational repression and/or cleavage of the target mRNAs, but have no role in the RNAi pathway [7, 101].

Among the ten members of the *Arabidopsis* AGO family [87, 102, 103], AGO1 is the only one so far confirmed to have the "slicer" activity to cleave the target mRNAs [102, 103]. Sequence comparisons indicate that Arabidopsis AGO1, 5, 7 and 10 all have a conserved 'slicer' catalytic motif, the DDH triad that was identified in hAGO2 [93]. The exact "slicer" activity of each individual AGO protein, however, needs further experimental testing [102]. Data have shown that the *Arabidopsis* AGO1 is required for both miRNA regulated plant development and DNA methylation triggered by sense transgene (S-PTGS) but not inverted-repeat transgenes (IR-PTGS) [102, 104-106].

In *Aradidopsis*, different AGO family members have also been shown to have different functions in RISC assembly or in different gene silencing pathways. Affinity-purified AGO1 proteins from transgenic AGO1 plants were not only physically associated with miRNAs, but also with miRNA-induced trans-acting siRNAs (tasiRNA), a subtype of siRNAs that come from the products of the miRNA-directed target cleavage via RdRP and Dicer [107], and transgene-derived siRNAs. However, the *Arabidopsis* AGO1 protein seemed not associated with virus-specific siRNAs or the 24-nt siRNAs that are involved in chromatin modification [102]. Intriguingly, both 21- and 24-nt siRNAs could be loaded on to *Arabidopsis* AGO1 proteins in *in vitro* RISC assembly [103]. The difference between the *in vivo* and the *in vitro* RISC assemblies indicate that cellular compartmentalization or other factor(s) are involved in loading different size of small RNAs on to different gene silencing complexes in cells [102, 103]. *Arabidopsis* AGO4 is required for DNA methylation and more likely plays a role in the RITS pathway [108, 109]. Genetic studies indicated that the *Arabidopsis* AGO7/ZIP and AGO10/ZLL/PNH have functions in control of plant development, but their related RISCs need to be determined [110-112].

Interestingly, the budding yeast *Schizosaccharomyces cerevisiae* is missing in AGO proteins and shows no RNAi pathway in this organism. In fission yeast *S. pombe*, however, a single set of RNAi-related proteins with a single copy of AGO1 protein, Dicer-like enzyme, and the RNA-dependent RNA polymerase (RdRP/RdR1) are involved in both transcriptional (RITS pathway) and posttranscriptional gene silencing (RNAi pathway) [78, 113].

5. Small RNA-protein interaction: the RISC associated proteins or cofactors

What could be the other protein components in gene silencing complexes besides the AGO proteins? Both *in vitro* and *in vivo* studies showed that an AGO protein and a small RNA alone can form a functional "core" RISC to cleave the target mRNA. For example, the smallest active RISC purified biochemically might contain only an AGO protein and a guide small RNA [32, 40, 102, 114]. The recombinant human AGO2 protein and synthetic siRNA can reconstitute an active RISC *in vitro* [93]. Similarly in *Arabidopsis*, the

immunoaffinity-purified AGO1 proteins can interact with single-stranded siRNAs to form active RISCs [103]. These recombinant "core" RISCs shared similar properties with the RISCs purified from human or *Drosophila* cells. For example, only single-stranded siRNAs were found to be specifically cross-linked to either the recombinant or the endogenous hAGO2. Both recombinant and endogenous RISC-directed target cleavages are in a divalent cation (Mg^{2+} or Mn^{2+})-dependent manner [93].

Other examples, however, indicate that active RISCs need other protein components or cofactors. For example, the RISC-directed multiple turnover target cleavage needs ATP. Such a cofactor that binds and hydrolyses ATP has not yet been identified [93]. Another example is that RISC assembly needs the unwinding of the small RNA duplexes and the protein factors that help the small RNA unwinding have not been identified [115, 116]. In addition, RISCs may also need other associated proteins for functioning in specific pathways or compartments. Thus, it is not surprising that purification for RISCs using cell extracts or lysates has resulted in complexes of variable sizes, from ~150 kDa "core" RISCs to 80 S "holo" RISCs [14, 20, 29, 45, 114, 115, 117, 118]. The "holo" RISCs were found to be associated with many proteins in addition to the AGO protein and the small RNA.

For example, Dicer-like enzymes, the small RNA producers, interact with AGO proteins during the gene silencing effector phase in *Drosophila in vitro* lysates [35, 39, 45, 71, 103, 115, 116, 119, 120]. Genetic studies in *C. elegans* also indicated such interaction between the AGO (RDE-1) and Dicer [99]. Much evidence indicates that Dicer is important for siRNA or miRNA to be loaded onto RISCs. Dicer-like enzymes and their partners, the double-stranded RNA binding proteins (dsRBDP), are considered to be important factors that link the initiation and effecting phases of RNAi [35, 45, 71, 115-117, 121, 122].

In addition to Dicer-like enzymes and their partners, many other proteins or cofactors were identified to be associated with RISCs. A *Drosophila* homolog of human fragile-X mental-retardation protein (FMRP, dFXR), a Vasa Intronic Gene protein (VIG) and a Tudor Staphylococcal Nuclease (TSN) were also found to be associated with both a ~500-kDa RISC from *Drosophila* S2 cells and the 'holo' RISC from *Drosophila* embryos [29, 30, 45, 118]. FMRP is an RNA binding protein that is associated with ribosome and negatively regulates the translation [31]. In *Drosophila*, dFMR was found to be associated with a complex that contained Dicer, dAGO2, a p68 RNA helicase homolog (Dmp68), and two ribosome proteins L5 and L11 along with 5S ribosomal RNA in a dFMR-tandem affinity purification experiment [31]. FMRP was proposed to be part of miRISC apparatus to mediate translational repression [31].

VIG is an evolutionarily conserved putative RNA-binding protein in RNA metabolism pathways with homologs found in *C. elegans*, *Arabidopsis*, mammals and *S. pombe* [30]. TSN is a protein with five staphylococcal/micrococcal nuclease domains and a tudor domain [33]. Although the TSN purified from *Drosophila* has nuclease activity, it is excluded from "slicer" activity that is assigned to the AGO protein [40, 44, 88, 89, 92, 93, 95]. TSN was suggested to be involved in the more extensive degradation of the initial cleavage products of RISC [123]. But recent data indicates that TSN regulates miRNA

biogenesis by cleaving the ADAR modified inosine containing pri-miRNAs in mouse, indicating that TSN is not only a cofactor of RISCs but also a ribonuclease specific to inosine-containing dsRNAs to control the miRNA biogenesis [124].

Gemin3 and Gemin4, two additional proteins that were originally found in the Survival of Motor Neurons (SMN) protein complex in biogenesis of diverse ribonucleoproteins [125, 126], were found to be associated with many human miRNAs [20]. Gemin3 is a DEAD-box RNA helicase that potentially works together with its co-factor Gemin-4 [125, 126] to mediate RNA unwinding or RNP reconstruction during miRNA maturation and/or downstream target RNA recognition [20]. Several other putative RNA helicases were also implied in gene silencing pathways in different organisms. These helicases include the Spindle-E [127], Dmp68 [31], DCR2 [39] and Armitage [115, 128] in *Drosophila*, the putative DEAx-box RNA helicases in *C. elegans* [99, 100], and the *Arabidopsis* RNA mutant SDE3 [129, 130].

The ribosome has been found to be associated with 'holo' RISC from *Drosophila* embryos [45] and S2 cells [29, 31, 118]. In *Trypanosoma brucei*, siRNAs were reported to associate with polyribosomes [131]. miRNAs and miRISCs/miRNPs have also been found to be associated with polyribosomes from a human neuronal cell line [132, 133]. The exact relationship between the ribosome and silencing effectors is not clear. *In vitro* experiments indicate that the target mRNA translation was not required for siRNA- or miRNA-directed target mRNA recognition and cleavage [2, 118, 133]. Speculatively, the ribosome might play a role in stabilizing small RNAs and their target mRNAs in control of mRNA translation [133].

In *C. elegans*, an AGO interacting protein AIN-1, ALG-1 Interacting protein-1, was found to associate with ALG-1 on the miRISCs to regulate the worm developmental timing [134]. AIN-1 functions in translocating its associated miRISCs intracellularly to a special mRNA decay center termed processing bodies (P-bodies) [134-136]. It is possible that AIN1 and its associated miRISCs control the target mRNA translation by binding the target mRNAs and sequestering them into P-body for storage or degradation.

In summary, RISC associated proteins or cofactors may or may not be the essential components of the RISCs for activity, but potentially play diverse roles in guiding RISC-target interaction and downstream events for functions in a spatial or temporal specific manner. These RISC interacting partners involve multiple pathways including the protein translation pathway, RNA metabolism pathways, and some disease development pathways. This suggests that protein translation, RNA metabolism, some disease-related processes, and most likely, other unknown pathways intersect with endogenous RNAi or miRNA regulation machinery. Potentially, this kind of interaction might serve as one of the endogenous strategies in the control of RNAi- and miRNA- regulated pathways.

6. RISC assembly

RISC assembly is a process in which the small RNAs (siRNAs and miRNAs) recognize and interact with a series of cellular proteins to form active RNA-induced silencing complexes for down-stream target regulation. How miRNAs and siRNAs are assembled with specific AGO proteins to form specific RISCs is the principal mechanistic question for us in the study of RNA induced gene silencing. Recent studies indicate that RISC assembly is a sequential process that starts from the biogenesis of the small RNAs and requires multiple steps of RNA-protein and protein-protein interactions. According to the nature of small RNA biogenesis, RISC assembly can be divided into two sub-types: the siRNA-induced RISC (siRISC) assembly and the miRNA-induced RISC (miRISC) assembly [39, 74, 137, 138]. While the siRISC assembly has been more extensively dissected, the miRISC assembly is still not well understood. Currently, the RISC assembly is thought to have the following biochemical steps: small RNA duplex-protein interaction to form RISC-Loading Complex (RLC) and RISC maturation. Because RISC assembly is a sequential process, the distinct structures of RLCs or RISCs are still not fully defined.

6.1. Small RNA duplex-protein interaction: 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 is characterized or implied to some extent in *Drosophila* and *C. elegans*, but the exact composition is unknown.

siRNA- directed RISC assembly in *Drosophila* is the most experimentally dissected and understood system *in vitro*. It is now rather clear that *Drosophila* has two Dicers (DCRs) [5, 35, 39, 43, 45]: DCR1 and DCR2. Only DCR2 is involved in siRNA production and subsequently siRISC assembly while DCR1 is involved in miRNA biogenesis [43]. DCR2 is tightly associated with its partner, a dsRNA binding protein termed R2D2, to form a stable heterodimer (DCR2-R2D2), without which both are unstable in cells [35]. Increasing evidence indicates that DCR2-R2D2 heterodimer is not only essential for siRNA biogenesis but also important for assisting the siRNAs to be loaded onto RISCs [45, 116]. The formation of RLC starts from the interaction between the siRNA duplex and the DCR2-R2D2 heterodimer [45, 116].

Specifically, it is proposed that siRNA duplexes can be released from DCR2-R2D2 after their production from long dsRNA precursors and recruited back by DCR2-R2D2 heterodimers [57]. It is not yet known how the siRNA duplexes are released from DCR2-R2D2 but, for example, synthetic, naked siRNA duplexes, can be easily recruited by DCR2-R2D2 in an ATP independent manner [57]. The ATP seems required for the RLC assembly, which makes the RLC different from the simple complex of siRNA-DCR2-R2D2 trimer [57].

There are two models for RLC. One, proposed by Zamore and colleagues, is that RLC contains DCR2-R2D2 heterodimer, siRNA, and other unidentified protein(s) [45, 57, 116]. The RLC might be associated with an unidentified ATPase or RNA helicase because the RLC assembly and the initiation of siRNA unwinding on RLC require ATP and helicase [115]. The key RISC component AGO proteins seem not to be required for the formation of RLC. The other model is that RLC is simply a tri-mer of siRNA-DCR2-R2D2 [117]. This model is supported by a recent study that confirmed the recombinant siRNA-DCR2-R2D2 trimer has the exact size of RLC previously identified on a native gel [117]. However, it is not excluded that small protein cofactors may be indistinguishable in a large complex on a native gel. Both models agree on the fact that siRNA duplexes must first bind to DCR2-R2D2 heterodimer and then the subsequent RISC assembly can be initiated.

The small dsRNA binding protein R2D2, so named because it has two dsRNA binding domains (dsRBDs) tightly associated with DCR2 [35], play a big role in formation of a functional RLC. Recent studies indicate that R2D2 is not simply a partner of DCR2 for their stabilization, but also essential in binding the small RNA duplexes and sensing the structures of the duplexes [116]. Neither recombinant DCR2 alone, nor DCR2-R2D2^{M} heterodimer with point mutations within the two dsRBDs of R2D2 can bind siRNAs effectively [35]. Further *in vitro* studies showed that R2D2 is a sensor protein that tends to bind the thermodynamically more stable siRNA duplex end and places the siRNA duplex onto DCR2-R2D2 in a right direction [116]. This directional positioning of the siRNAs on RLC during RISC assembly is referred to as "asymmetric assembly of RISCs" [116] or "asymmetric assembly of RLC". Thus, the asymmetric complex assembly is mainly defined by R2D2 and the structures of the small RNA duplexes. In the case of a symmetric siRNA duplex that has two equally stable ends, R2D2 tends to bind both ends with equivalent efficiency to form two different RLCs with opposite orientation of siRNA. In contrast, in the case of an asymmetric siRNA duplex that has one end less stable than the other, the R2D2 tends to bind the more stable end to form a highly asymmetric RLC [116].

Whether an R2D2-like sensor is required for miRISC loading complex (miRLC) assembly has not yet been fully explored. But R2D2 homologs or orthologs have been found to associate with the miRNA production-specific Dicers in several organisms. In *Drosophila*, R3D1, so named because it has three dsRDBs and tightly associated with DCR1 [74], or termed Loquacious (Loqs) because it is talkative to many pathways and has diverse functions [73, 139], is a R2D2 homolog and forms a stable complex with DCR1 *in vitro* and *in vivo*. Genetic deletion of either DCR1 or R3D1/Loqs resulted in the accumulation of pre-miRNA in *Drosophila* S2 cells [73, 74, 139]. *In vitro* recombinant DCR1 and R3D1-L, a larger form of R3D1/Loqs, form a complex that can effectively bind a synthetic miRNA/miR* duplex [74]. This indicates that DCR1 and R3D1/Loqs may play a similar role in miRNA biogenesis and the subsequent RLC assembly [74]. In humans, the single Dicer also has a dsRBDP partner previously termed TRBP/DGCR8 that contains three dsRBDs [71]. Dicer and TRBP/ DGCR8 form a stable complex *in vivo* and show importance for both miRNA biogenesis and RISC assembly [68, 69, 139]. Similar to

Drosophila DCR1 and Loqs, knockdown of either human Dicer or TRBP/DGCR8 results in the destabilization of the partner protein and loss of miRNA biogenesis. However, TRBD/DGCR8 is rather a Dicer stabilizer than a Dicer activity enhancer in miRNA production because there was no difference between Dicer alone and Dicer-TRBD complex in producing miRNAs in an *in vitro* assay [71].

In *C. elegans,* RDE-4, an ortholog of R2D2, is required for RNAi pathway but not required for miRNAs-mediated cell development [99]. *In vivo*, RDE-4 seems to form a complex with DCR1 and a DExH-box RNA helicase-related protein F15B10.2 (also named DRH1 because of its most related to DCR-1 in worms), and interacts with RDE-1, one of the *C. elegans* AGO proteins that involves in exogenous dsRNA induced gene silencing [99].

That the AGO protein interacts with Dicer and RNA helicase proteins in worms strongly support the idea that RISC assembly is a sequential process and it is hard to divide this process into specific steps. Similarly, both *Drosophila* DCR1 and R3D1/Loqs interact with dAGO1 and are associated in a complex capable of Pre-miRNA processing [73, 74, 139]. The human Dicer-TRBD complex is also associated with hAGO2 in cells. An *in vitro* reconstitution experiment demonstrated that Dicer-TRBD and siRNA form a Dicer-TRBP-siRNA complex, and that TRBP is required for the recruitment of hAGO2 to the Dicer-TRBP-siRNA complex [71].

It is not clear how siRNAs and miRNAs are loaded into RISCs in plants. In *Arabidopsis*, the nuclear dsRBDP HYL1 is required to interact with *Arabidopsis* Dicer-1 for miRNA generation and plant development but not involved in the loading of miRNA into RISC [140]. Plant miRNAs and siRNAs have several distinct characteristics: (1) plant miRNAs seem to mature in the nucleus and the unwinding of the miRNA duplexes seems also to be occurring in the nucleus by an unknown mechanism [141]; (2) plant miRNAs are methylated at the 3' end [142]; and (3) an siRNA duplex is unable to be assembled into a RISC, but rather, a single-stranded siRNA can effectively direct a RISC assembly in *in vitro* studies [103, 143]. This indicates that RISC assembly could have alternative pathways. Indeed, single-stranded siRNA can direct RNA silencing also in *Drosophila* and human cell extracts [32, 144]. In these cases, siRNAs directly interact with AGO proteins without the need of DCR, dsRDBP, or helicase protein.

In summary, Dicer and dsRBD proteins form complexes that initiate the RLC or RISC assemblies in *Drosophila*, *C. elegans* and human cells. Most, if not all, of these Dicer-dsRBDP complexes interact with AGO proteins and are important in both the biogenesis of small RNAs and RLC or RISC assembly [68, 69, 73, 74, 99, 139]. There could exist alternative pathways to this general process. A single-stranded small RNA could directly interact with AGO protein to form active RISC [32, 140, 144], thereby bypassing the so-called RLC stage .

6.2. RISC maturation

RISC maturation starts from the strand separation of the small RNA duplex on RLC and ends with the recruitment of AGO proteins by the single-stranded small RNA [57, 137]. The exact process is still not fully understood. The structure of the small RNA duplexes is important in selection of which strand of the duplex could potentially be assembled into the RISCs, which has been defined during the formation of RLC [116]. What could be the mechanisms for small RNA duplex unwinding/separation and the destruction of one of the two strands that is not associated with the RISC during RISC maturation?

In vitro siRNA duplex unwinding experiments in *Drosophila* showed a detectable single-stranded siRNA portion of the less RISC-assembly competent strands in the absence of the target mRNA [36], indicating that strand separation and the strand elimination (degradation) are two separated steps. Several pieces of evidence indicate that RNA helicase could be employed to unwind the small RNA duplex. First, DEAD-box RNA helicases can open RNA duplex in an ATP-dependent manner and the siRNA duplex unwinding *in vitro* needs ATP [10]. Second, several identified DEAD-box RNA helicases are involved in the RNAi process in different organisms. In *C. elegans,* two DCR-1 closely related DEAD-box helicases, (DRH-1/DRH-2) were required for efficient RNAi activity [99]. DRH-1/DRH-2 interact with DCR-1, RDE-4, a paralog of the fly R2D2, and the AGO protein RDE-1 to form a complex that seems required for both long dsRNA processing and subsequent RISC assembly. DRH-1/DRH-2 homologues are also present in other organisms including flies and humans. Three additional DEAD-box helicases in worms were found to be required for gene silencing in an RNAi-based screen and also to have their homologues in *Drosophila* and vertebrates [145]. Gemin-3, a RISC associated protein [146], is an ATP-dependent DEAD-box helicase. In addition, non-canonical RNA helicases, armitage (armi) in *Drosophila* and SDE3 in *Arabidopsis*, are required for RNAi *in vivo* and *in vitro* in fly and *in vivo* in plants, respectively [115, 128, 129].

On the other hand, several pieces of recent evidence indicate that small RNA duplex separation and elimination may not require RNA helicase. Based on their independent, experimental results, Zamore, Wang, and their colleagues recently proposed a mechanism for siRNA passenger strand elimination via a *cis* cleavage by the *Drosophila* AGO2 protein during RISC assembly [147, 148]. These experiments showed that the passenger strand of the siRNA duplex is initially cleaved by the AGO protein before the complete separation of the two strands (in *cis*) rather than that the passenger strand is first separated from the guide strand-directed RISC and then binds back to the RISC leading to the cleavage of the passenger strand (in *trans*). Since not all small RNA duplexes are perfectly base paired, which might affect the cleavage of the passenger strand by the AGO protein, Zamore and colleagues also proposed a bypass mechanism for miRNA duplex strand separation that does not require a cleavage of the passenger strand by the AGO proteins [147]. In the bypass model, the mismatches and bulges on the miRNA duplexes may play an important role in strand separation. It is not yet known how the two strands are separated during non-cleaving RISC assembly in which the AGO protein has no “slicer” activity. In plants, only single-stranded siRNA rather than an siRNA duplex

can be assembled into a cleaving RISC in *in vitro* experiments, suggesting that strand separation and elimination may occur in more than one way during RISC maturation.

In summary, the formation of RNA-protein complex, the RISC, is a hallmark of the small RNA-directed gene silencing. Currently, the siRNA-directed RISC (siRISC) assembly is better understood to be a sequential process in *Drosophila*. It starts from the siRNA duplex production by Dicer complex to the interaction between the duplex and the Dicer complex to form RLC, and ends with RISC maturation by recruiting the AGO and other associated proteins and elimination of one strand of the siRNA duplex. It is not yet known whether RISC assemblies in other organisms follow a similar pathway. In contrast, miRNA-directed RISC (miRISC) assembly seems more complicated. It starts in the nucleus and ends in the cytoplasm. Different organisms may take different RISC assembly approaches. The exact RISC assembly pathways need further dissection.

7. RISC-target interaction: the mechanism

As we have seen above, RISC assembly is a complex process in both RNAi and miRNA pathways. It involves small RNA producers (DCRs), small RNA duplex structures, RLC assemblies, unwinding of a symmetric/asymmetric duplex, and recruitment of distinct AGO proteins. Distinct AGO proteins on RISCs have distinct functions that are determined most likely by the PIWI domain of AGO proteins. Moreover, certain PIWI domains confer "slicer" activity; others do not. Based on what kind of AGO protein is recruited on a RISC, RISCs can be tentatively divided into two general types: cleaving and non-cleaving RISCs (Fig. 3). A cleaving RISC has dual functions that can direct the target mRNA for either cleavage or translational repression, depending on the pairing features between the small RNA and the target mRNA [14, 143, 149]. If the pairing complementarity between the siRNA or miRNA and their targets is less extensive, the target mRNA may be physically unreachable by the active center of the endonuclease ("slicer") in the cleaving RISC because of the distorted helix formed between siRNA or miRNA and the target [14, 38, 150]. This will result in translational repression instead of efficient cleavage of the target mRNA. In contrast, non-cleaving RISCs lack endonuclease ("slicer") activity on the PIWI domain of AGO proteins and generally direct the target mRNA for translational repression (Fig. 3). However, non-cleaving RISCs may serve as the target mRNA translocators to sequester the target mRNA into specific loci for degradation. In addition to cleaving and non-cleaving RISCs, RITS complex (RITSC) may represent a nucleus RISC to modify the chromatin [78].

For function, cleaving, non-cleaving RISCs and RITSC need first to recognize and bind the target RNAs. The interaction between these RNA-protein complexes and the target RNAs will result in target RNA cleavage, translational repression, or chromatin modification. Specifically, the mechanisms for the interaction between different kinds of RISCs or RITSC and the target RNAs or loci could be divided into three main types: (1) RISC-mediated mRNA degradation; (2) RISC-mediated mRNA translational repression; and (3) RITS-mediated chromatin remodeling.

7.1. Mechanism of RISC-mediated mRNA degradation

A 'minimal' or "core" RISC is comprised of AGO protein and its associated single-stranded small guide RNA, while a 'complete" or "holo" RISC could be huge in size. What other components are needed to constitute a "holo" RISC? A few studies confirmed that a "holo" RISC is a large complex that almost contains all the proteins so far identified in RNAi and miRNA pathways [45]. Both "core" and "holo" RISCs are functional in cleaving the target RNAs *in vitro* when the targets are designed to be perfectly complementary to the small guide RNAs on RISCs. So far, both siRISCs and miRISCs have been found to lead either to the destruction of the target mRNAs or to the suppression of the target mRNA translation. Three conditions are essential for target mRNA to be site-specifically cleaved by a cleaving RISC: (1) the sequence complementarity between the guide RNA and the mRNA target [14, 38, 143, 150, 151]; (2) an AGO protein with RNase H-like endonulease 'slicer' activity for forming a cleaving RISC [40, 44, 88, 89, 95, 102]; and (3) the scissile phosphodiester bond on the mRNA target accessible to the endonuclease active motif located in the PIWI-domain of the AGO protein [40, 44, 88, 89, 95, 102].

The base-pairing condition between the guide RNA and the mRNA target is not only important for target recognition but also essential for the RISC-directed cleavage event. The more complementarity there is, the more efficiently the target-cleavage occurs [14, 38, 143, 150, 151]. However, expression profiling and biochemical analyses indicate that mismatches between the guide and the target are tolerated to some extent and certain partial complementarities will lead to a cleavage of the mRNA target by RISCs [41, 152-154].

The common rules for the mismatch-tolerance and partial complementarity have been revealed by several studies [14, 38, 41, 106, 143, 150-152]. Generally, the mismatches would reduce the target-cleavage efficiency [41, 154], with the exception of the first base of the 5' terminal of the guide RNA [92, 149, 154-156]. Mismatch of this base with the target mRNA will increase the target cleavage, most likely facilitating the release of the cleavage product [92, 154, 155]. The complementary proximity to the 5' end of the guide RNA is more essential than that to 3'end of the guide. *In vitro* data indicate that the mismatches at the 5' end could be reached up to first 4-5 nt of the guide RNA, compared to the mismatches up to the last 9-nt at the 3'end [41, 154]. However, the perfect complementarity close to the 3' end could have a compensatory effect on the 5' end mismatches in terms of target cleavage. This is confirmed by the observation that certain mismatches near both ends of the guide RNA will lead to a complete loss of target cleavage while the target cleavage is still observable if the guide RNA has mismatches to the target near one end, either the 5' end or 3' end [154].

In general, target recognition, binding and cleavage by a RISC are mainly determined by the base pairing between the 5′ portion (~9 nt) of an siRNA or miRNA and their target mRNA. First, the target cleavage site is measured from the 5' end of the small guide RNAs. An siRISC- or miRISC-directed target is cleaved between position 10 and 11 from the 5′ end of the siRNA or miRNA guide-strand [4, 14] (regardless of the size of siRNA or miRNA , e.g. 21-25 nt). Second, 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 their target [38, 153, 157]. It has been proposed that the formation of an A-form RNA helix between the guide RNA and the target mRNA is required for RISC cleavage [150, 151]. The ~9 base-pares at the 5' end portion might be enough to initiate an A-form RNA helix (11 base-pairs per turn) between the guide RNA and the target mRNA with one or two mismatches.

Only a few AGO proteins, such as, hAGO2 in human [40, 42, 93, 158], dAGO1 and dAGO2 in *Drosophila* [43], RDE1, PPW-1, ALG1 and ALG2 in *C. elegans* [99, 100], and *Arabidopsis* AGO1 [102, 103] have RNase H-like motifs at the PIWI domains that potentially have been identified to have endonuclease ('slicer') activity so far. These AGO proteins potentially form cleaving RISCs with the small guide RNAs to cleave the target mRNAs.

The cleaving activity of the AGO 'slicers' is divalent cation- (Mg^{2+}, etc.) dependent [93, 159]. It catalyses the hydrolysis of the phosphodiester linkage of mRNA target at specific site and yields 3' hydroxyl and 5' phosphate termini on the cleavage products [4, 41]. This specific cleavage site is pre-determined on RISCs and located precisely at the phosphodiester bond in the mRNA target between the nucleotides paired to base 10 and 11 of the guide RNA from the 5'end [4, 6, 93, 154]. The scissile phosphate on the target mRNA remains the same without being affected by the base-pairing mismatches between the guide RNA and its target RNA at either 5'end or 3'end [154].

Biochemical and structural studies found that the phosphate group at the 5' end of the guide RNA has an impact on the cleavage site fidelity by a RISC 'slicer' active site [44, 92, 93]. An altered or repositioned cleavage site will occur to the target cleavage directed by a minimal RISC that contains a guide RNA without the 5' end monophosphate [93]. This altered or repositioned cleavage site may result from the slippage of the guide RNA on the AGO protein that cannot accurately anchor the 5' end of the small guide RNA into the phosphate binding pocket of the AGO Mid-/Piwi-domain without the phosphate at the 5' end of the guide RNA [44, 89, 92, 95].

The guide RNA normally positions on the AGO protein in a RISC effector with the 5' end anchored into the phosphate-binding basic pocket in Mid-/Piwi-domain, the 3' end bound with the PAZ-domain, and the middle sequence channelled with the 'slicer' active site between the Mid-/Piwi-domain and the PAZ-domain [44, 89, 92]. The interaction between the guide RNA and the AGO protein probably makes the hydrophilic side face the solution [92]. The conformation would be changed during the interaction between the guide RNA and the target. Indeed, it was observed that the 3' end of the guide RNA loosed off binding with the PAZ-domain, whereas the guide and the target RNA-duplex merged into the solvent RISC environment once guide RNA in the RISC forms a duplex with its target mRNA in a crystal structure formed by *Af*-Piwi-siRNA guide-RNA target [95]. To make the cleavage event occur, the conformational changes induced by the interaction among the AGO protein, the guide RNA and the target mRNA has to position the scissile phosphodiester bond of the target accessible by the 'slicer' active region [92, 95].

The RISC cleavage reaction itself does not requires ATP, but ATP is required for the releasing of the cleaving products from RISC so that the RISC enzyme could function in a multiple turnover manner [10, 93, 101, 154]. The ATP-assisted cleavage product release likely requires an ATPase/RNA helicase. An ATPase/RNA helicase was also suggested to play a role in the upstream loading of siRNA or miRNA duplexes onto RISCs [35, 115]. It is not clear whether the candidate ATPase/RNA helicase remains the same or different at the two different stages.

7.2. Mechanism of RISC-mediated translational repression

RISC can initiate gene silencing by repressing the target mRNA translation in addition to directing the site-specific target cleavage. RISCs with no "slicer" activity or with small guide RNAs that are imperfectly paired with the target mRNAs often induce the translational repression of the target mRNAs. In most cases, the target mRNAs appear at a relatively stable level, but their protein syntheses are blocked [56, 160-163].

Several miRNAs were found to form a functional imperfect duplex with their target mRNAs whose protein translations were blocked [157, 164-166]. Most metazoan miRNAs lack their perfect target mRNAs and are thus proposed to predominantly function in suppression of the target mRNA translation [167]. There are some requirements for formation of such functional miRNA-target duplexes. Generally, extensive base pairing to 5' end of the miRNA is more important than that to 3'end of the miRNA. The position 2-8-nt from 5' end proximity is the most conserved regions of metazoan miRNAs and specifically termed the "seed sequence" [168]. Changes in the base-pairing between miRNA and mRNA target at this region would dramatically reduce the regulation efficiency or result in loss of the target recognition completely [165, 166]. A functional "seed" region requires a continuous helix of at least 4 or 5 nucleotides [165]. However, a strong base-pairing condition at the 3' end could provide a compensation to a weak complementarity at the 5' end [165]. Fulfilling these requirements, a single miRNA is anticipated to have on average approximately 100 target genes or targeting sites [165, 166]. For more efficient translational repression, a target mRNA needs to have several miRNA targeting sites or one miRNA needs to target an mRNA with multiple targeting sites [169].

It is still not clear how these imperfect small RNA binding sites on the target mRNA inhibit the mRNA translation. There are three possibilities for RISC-directed target translational repression: (1) RISC might repress translation itself at the stage of either protein translation initiation, elongation or termination; (2) protein translation might not be affected but the nascent protein is degraded rapidly; (3) RISC might sequester the target mRNA away from the translation machinery [135, 167]. Recent findings support the idea that miRISCs repress the translation by sequestering mRNA targets away from the translation apparatus, the ribosome [134-136, 169-173].

It has not been clear until recently what specific protein translation stage is blocked by the miRISCs. Filipowicz and colleagues investigated the mechanism of the miRISC-directed translational repression in two approaches. One was tethering the human AGO protein to the 3'-UTR of a reporter gene to mimic the miRNA-mediated translational

repression in HeLa cells. The other was to assay the endogenous *let-7* miRISCs for their inhibition of protein translation of a reporter mRNA that contains the *let-7* miRISC targeting sites. The results demonstrated that miRNA guided/associated miRNP/miRISC blocked the protein translation initiation in a M(7)G-cap-dependent manner, suggesting that miRNPs interfere with recognition of the cap [169].

Intriguingly, hAGO1-4 and *let-7* target mRNA were co-localized within cytoplasmic processing bodies (P-bodies) in a *let-7*-depedent manner [135, 169]. The cytoplasmic P-bodies, also known as Dcp-bodies or GW-bodies in human cells, are discrete cytoplasmic foci enriched with mRNA decapping and degradation proteins such as the two decapping proteins Dcp1a and Dcp2, 5'-to-3' exonuclease Xrn1, a putative RNA helicase Dhh1, and others [174]. P-bodies were observed in both yeast and human cells and defined as an mRNA decay center. But recent observation indicates that P-bodies could also serve as a site to temporarily store mRNAs for regulation [174-176].

The question is how miRNPs/miRISCs are translocated to cytoplasmic P-bodies? What is the biological function of this translocation? Is the translocation the cause or the consequence of miRISC-induced translational repression *per se*? Hannon, Parker and colleagues have shown that endogenous or exogenous RISCs could transport their specific target mRNAs to P-bodies and become concentrated there. Moreover the AGO proteins of the RISCs can physically interact with P-body components Dcp1a and Dcp2 and form complexes that are not sensitive to nuclease digestion [135]. It seems that the P-bodies and the cellular translation machinery coordinate with each other to control the mRNAs for translocation or translation. Indeed, inhibition of protein translation elongation prevents mRNAs from entering P-bodies, whereas inhibition of protein translation initiation promotes mRNAs relocating in P-bodies [135]. Thus, the direct cause of miRISC-directed translocation of the target mRNAs to P-bodies is the very inhibition of translation initiation by *let7*-associated miRNP/miRISC [169]. In summary, miRNP/miRISC-directed translational repression of the target mRNAs could occur in the translational machine or the P-bodies. In either way, the protein translation will be effectively blocked, while the target mRNAs will associate with protein translation machinery or locate in P-bodies for temporary storage or simply go to the degradation pathway via the P-body [134-136, 169, 176]. In *C. elegans*, a specific protein, AIN-1, has been identified to associate with miRISCs and to help miRISCs and most likely the target mRNAs as well translocate to cytoplasmic P-bodies for the translational regulation of the target mRNAs, which appears in the change of the developmental timing of worms [134].

The intercommunication between the protein translation machine and the P-bodies via miRNP/miRISC may well explain the contradictory results of *lin-4*- and *let-7*-directed regulation of their target mRNAs. Previous studies indicate that the miRNA *lin-4* and *let-7* generally direct their target mRNAs for translational repression without affecting the target mRNA metabolism [134-136, 169-171]. However, recent findings suggested that both *lin-4* and *let-7* also guide their respective target mRNAs for degradation [173]. The report that *lin-4*- and *let-7*- directed mRNA degradation rather than translation repression suggested a new mRNA degradation pathway directed by miRISCs potentially at the P-bodies. Specifically, miRISCs sequester the target mRNAs to the P-bodies, in which the

target mRNAs are either temporarily stored or simply degraded. How exactly the P-bodies decide when the target mRNAs should be retained or degraded remains to be explored. In addition to RISC-directed target mRNA degradation in P-bodies, other protein factors, such as AU-Rich Element (ARE) binding protein TrisTetraProlin (TTP) [172], may also interact with RISCs to target specific target mRNAs for degradation.

7.3. Mechanism of RITS complex-directed chromatin remodelling

Unlike RISC-mediated target mRNA cleavage, translational repression, or general degradation that happen solely in the cytoplasm, RITS complex (RITSC)-directed chromatin remodelling is restricted to the nucleus. RITSC itself is a RISC-like complex or a nucleus RISC, but this complex interacts with many nuclear proteins or protein complexes. For example, the RITSC interacts with the RDRC and RITSC/RDRC interacts with chromodomain proteins (Figure 3).

The key question about the mechanism of RITSC-directed chromatin remodelling is how RITSCs recognize and target specific chromosome regions for remodelling. Once the RITSCs target the chromatin at specific regions, the chromatin modification will occur by the RITSC-associated chromatin remodelling proteins.

There have been two models proposed to explain how the RITS complexes target specific chromatin regions [80, 177, 178]. The first proposed that the RITS complex-associated small RNAs directly interact with DNA simply by base pairing. The chromatin needs to be open enough for unwinding of the chromosomal DNA helix to allow the direct pairing interaction between the small RNAs and the chromosomal DNA. The second model proposed that the RITS complex targeting regions are initially undergoing active RNA transcription that generates the small RNAs and the RITS complexes (Figure 3). The RITS complexes, in turn, bind the nascent RNA transcripts that are still associated with the specific chromosomal regions, so as to fulfill the recognition and targeting of the specific regions for modification.

The second model is well in agreement with the general RNAi mechanism and receives increasing supportive evidence. RNA immunoprecipitation (RIP) experiments confirmed that RITS complexes are specifically associated with non-coding centromeric RNAs from the RITS complex-targeting regions [79]. Chromatin immunoprecipitation (ChIP) experiments further confirmed that Dicer is also associated with the non-coding centromeric RNAs, indicating that the small RNAs of the RITS complexes come from the non-coding centromeric RNAs by Dicer. RITS localization to chromatin requires methylation of the Histone3-Lysine9 (H3-K9) by the histone methyltransferase Clr4 [78]. Subsequent experiments confirmed this requirement by the fact that the association of RITS complexes with centromeric RNAs is in Clr4-dependent manner [79]. In summary, RNA, rather than the DNA, is the direct mediator between the RITS complex and the chromatin, which is the hallmark of RITS complex-directed chromatin modification.

8. Summary and perspectives

The RNA-Induced Silencing Complex (RISC) is central to the processes of small RNA-directed gene regulation in RNAi, miRNA, and RITS pathways. Biochemical and genetic studies have identified many putative RISC components, RISC-interacting proteins, and also the structural features of the key components. The various components of these complexes 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 RISCs: a cleaving and a non-cleaving RISC (Figure 3). The target mRNA destruction and translational repression directed by these two types of RISCs are complex. Generally in a close association with the protein translation machine, a non-cleaving RISC directs only translational repression on its target mRNA and a cleaving RISC directs its target mRNA for either cleavage or translational repression, depending on the base-pairing condition between the small RNA and its target (Figure 3). However, either type of RISC has the potential to sequester the target mRNAs from the translation machine to P-bodies for either temporary storage or permanent destruction that is independent of the small RNA-directed target cleavage (Figure 3).

Studies of the RITS pathway in assembly of heterochromatin at specific chromosomal regions have firmly established the fundamental roles of RNA during the small RNA-induced transcriptional gene silencing. The paradox that RNA transcripts at the target locus are the initial triggers for maintaining the transcriptional silencing at this locus indicates a genome-wide dynamic regulation of the chromatic structures via RNA transcription. The finding that the nascent RNA transcripts are physically associated with the RITS complexes lead a direction to solve the puzzle of how RITS complexes recognize and bind specific chromatin regions for modification. It remains to be determined whether small RNA production by Dicer occurs in the vicinity of chromatin.

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 regulating components. Future directions should focus on dissecting the core RISCs to elucidate how these complexes regulate the target gene expression, while also exploring the holo RISCs to understand how RNAi and miRNA pathways interact with other cellular mechanisms.

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Figure legends

Figure 1. **Models for RISC assembly and RISC-target interaction.** siRNA is generated by a complex of DCR2 (green) and R2D2 (red) in *Drosophila*, or DCR-1 (green), RDE-1 (light blue), RDE-4 (red) and DRH-1 (dark green) in *C. elegans*, and remains within the complex. In *Drosophila*, the siRNA–DCR2–R2D2 complex is thought to interact with RNA helicase, AGO protein and other RISC components to form an RLC- ('RISC loading complex') 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 siRISC. By contrast, the 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 Microprocessor complex, and is then exported into cytoplasm by Exportin-5 (purple) for maturation into miRNA by a complex of cytoplasmic DCR (DCRx; green) and its partner Loqs/R3D1/TRBP (red). miRNA in the miRNA–DCR complex is subsequently assembled into the miRISC. Both siRISC and miRISC can either cleave the target mRNAs or block the target mRNA translation. The question mark represents unknown structures or unknown protein complexes. This figure was modified from Tang [137].

Figure 2. A model for RITS complex in chromatin remodelling. The chromatin of centrimeric repeats or heterochromatin is initially actively transcribed by RNA polymerase II or RNA polymerase IV (specifically in plants) into RNAs that are either complementary to each other or serve as an RdRP template to produce dsRNAs. DsRNAs are then cleaved by Dicer-like enzymes to form siRNAs. In *S. pombe*, the siRNA then recruits AGO proteins to form a RISC-like structure that further recruits the cellular chromatin remodelling enzyme Tas 3 and Chp1 to form RITS complex (RITSC). RdRP interacts with Cid12 and Hrr1 to form RdRP complex (RDRC). RITSC and RDRC interact with each other and further bind to the nascent chromatin transcript. The associated chromatin-remodelling enzymes finally use the RITSC and RDRC as a platform to change the chromatin structures. The remodelled, silenced chromatin can be reset back to active chromatin through unknown mechanisms.

Figure 3. A model for RISC working mechanisms. Distinct subtypes of Argonaute proteins (AGO*x* and AGO*y*) are proposed to interact with siRNAs or miRNAs to form two types of RISCs: cleaving and non-cleaving. A cleaving RISC can direct the target mRNA for cleavage or for translational repression. When the small RNAs (siRNAs or miRNAs) are extensively complementary to their target mRNAs, most cleaving RISCs direct efficient cleavage of the target mRNA and destroy the targets. These cleaving RISCs could become non-cleaving when the base pairing conditions between the small RNA and the target mRNA are not met. An extensively complementary cleaving RISC could also result in translational repression under certain conditions. On the contrary, a non-cleaving RISC directs the target mRNA only for translational repression when bound to the target, regardless of the complementarity between the small RNA and its target. Either type of the RISCs has the potential to sequester the target mRNAs from the translation machine ribosome to P-bodies for either temporary storage or permanent

destruction that is independent of the small RNA-directed target cleavage. It is not yet known that the target mRNA could be retro-transported back to the ribosome for protein translation.

Fig 1.

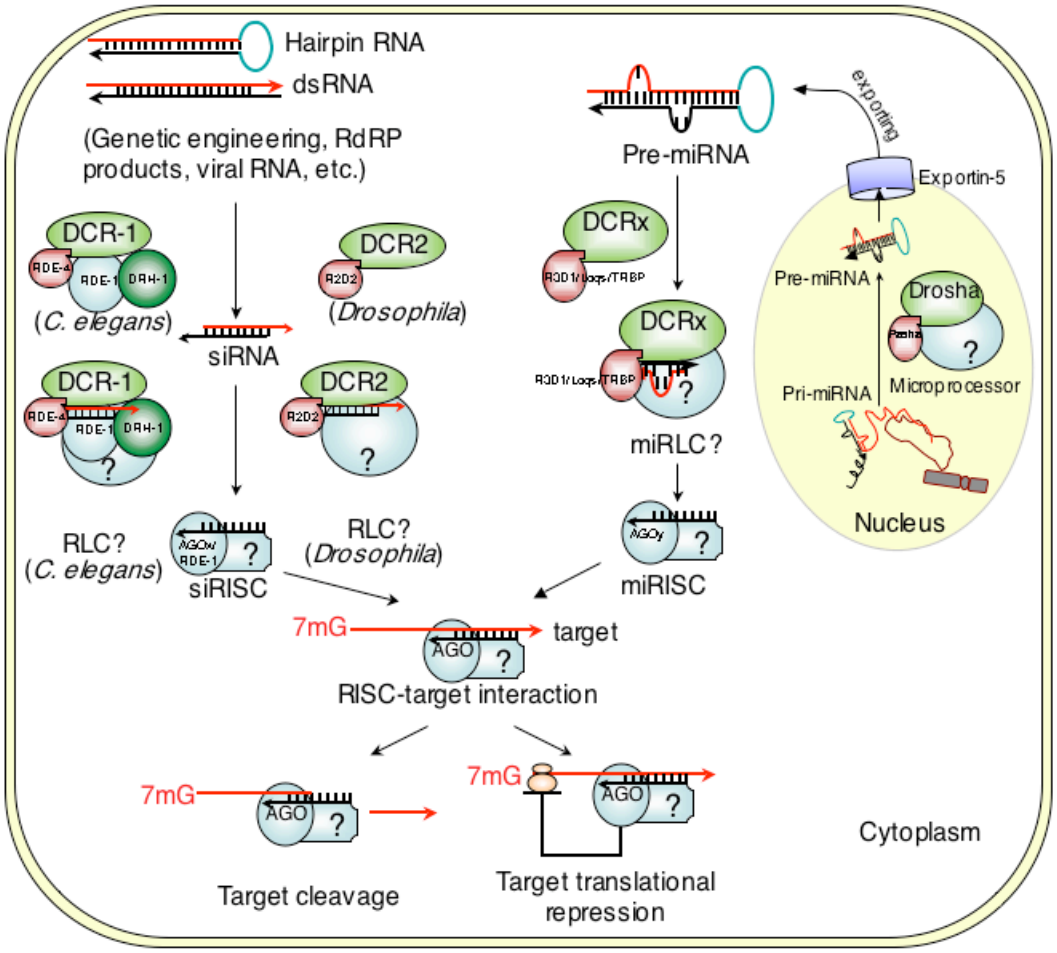

Hairpin RNA
dsRNA
(Genetic engineering, RdRP products, viral RNA, etc.)
DCR-1
RDE-4
RDE-1
DRH-1
(C. elegans)
DCR2
R2D2
(Drosophila)
siRNA
DCR-1
RDE-4
RDE-1
DRH-1
?
DCR2
R2D2
?
RLC?
(C. elegans)
AGOw
RDE-1
?
siRISC
RLC?
(Drosophila)
Pre-miRNA
exporting
Exportin-5
DCRx
R3D1/Loqs/TRBP
Pre-miRNA
Drosha
Drosha
?
DCRx
R3D1/Loqs/TRBP
?
Pri-miRNA
Microprocessor
miRLC?
Nucleus
AGOy
?
miRISC
7mG
target
AGO
?
RISC-target interaction
7mG
AGO
?
Target cleavage
7mG
AGO
?
Target translational repression
Cytoplasm

Fig 2.

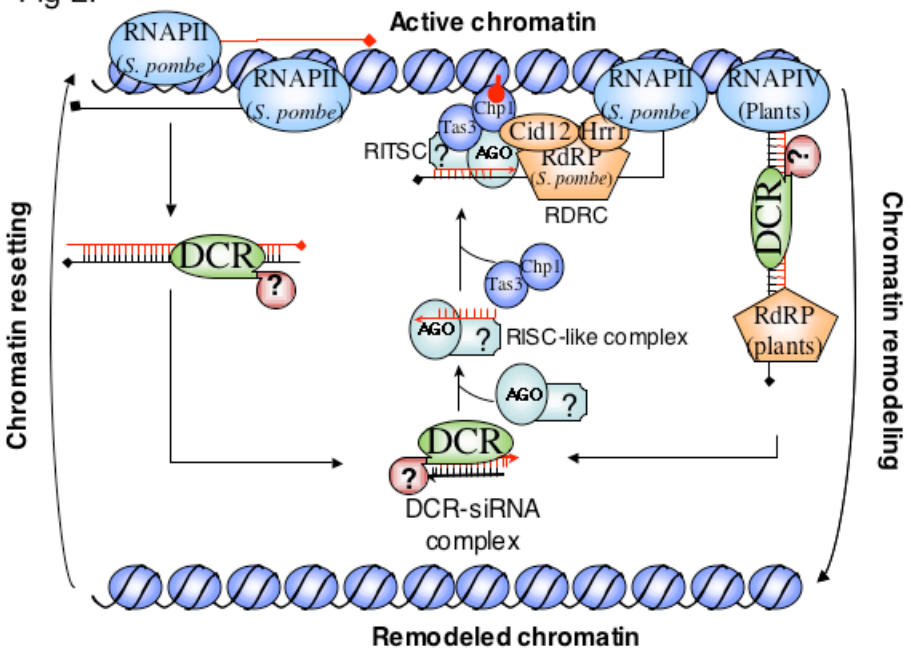
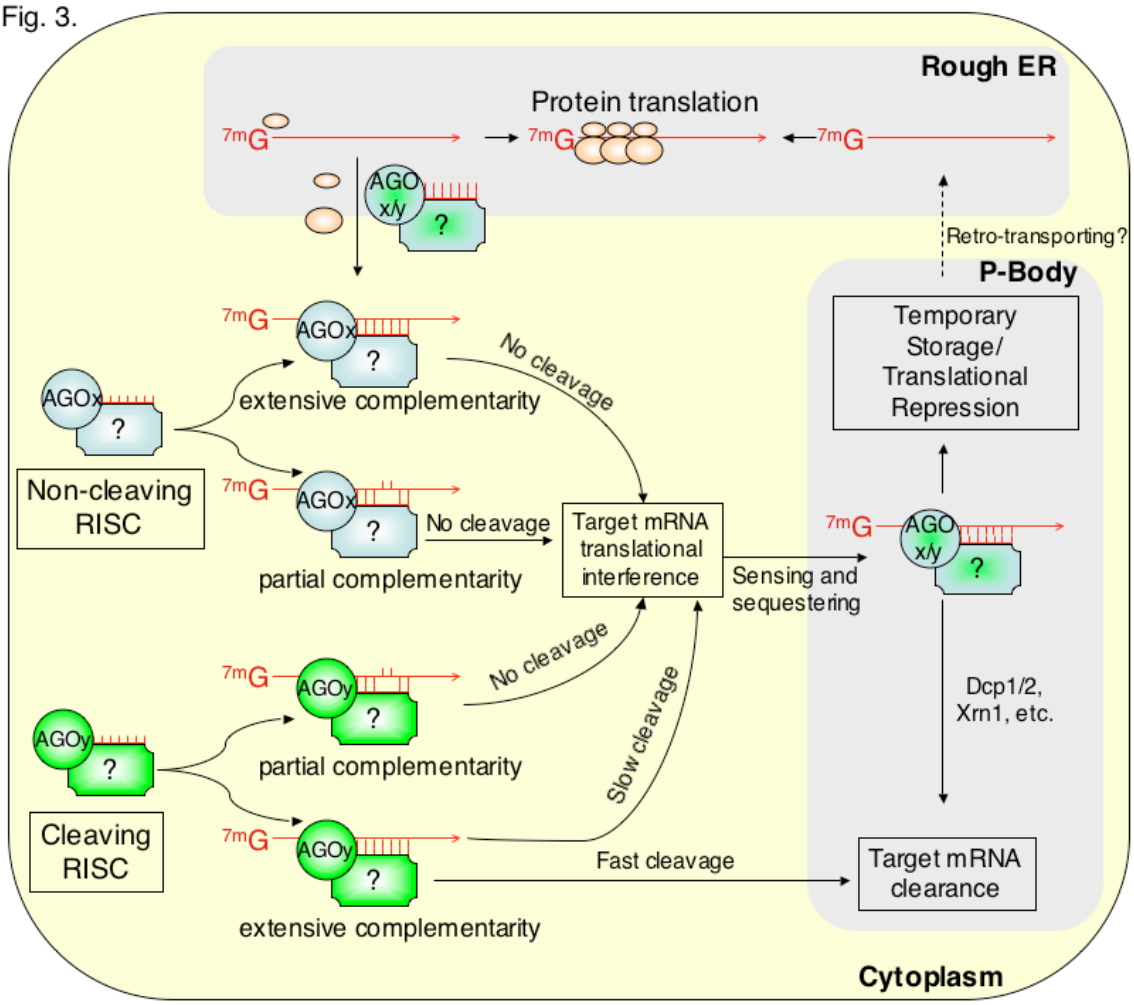

Active chromatin
RNAPII
(S. pombe)
RNAPII
(S. pombe)
RNAPII
(S. pombe)
RNAPIV
(Plants)
Chp1
Tas3
Cid12
Hrr1
RITSC
?
AGO
RdRP
(S. pombe)
RDRC
DCR
?
DCR
?
Chp1
Tas3
AGO
?
RISC-like complex
RdRP
(plants)
AGO
?
DCR
?
DCR-siRNA
complex
Chromatin resetting
Chromatin remodeling
Remodeled chromatin

Fig. 3.


Rough ER
Protein translation
7mG
AGO x/y
?
Retro-transporting?
P-Body
Temporary Storage/ Translational Repression
AGOx
extensive complementarity
No cleavage
Non-cleaving RISC
No cleavage
Target mRNA translational interference
Sensing and sequestering
partial complementarity
AGOy
Cleaving RISC
Slow cleavage
Dcp1/2, Xrn1, etc.
Fast cleavage
Target mRNA clearance
Cytoplasm