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Minireview

Dicing and slicing
The core machinery of the RNA interference pathway

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Abstract RNA interference (RNAi) is broadly defined as a gene silencing pathway that is triggered by double-stranded RNA (dsRNA). Many variations have been described on this theme. The dsRNA trigger can be supplied exogenously, as an experimental tool, or can derive from the genome in the form of microRNAs. Gene silencing can be the result of nucleolytic degradation of the mRNA, or by translational suppression. At the heart of the pathway are two ribonuclease machines. The ribonuclease III enzyme Dicer initiates the RNAi pathway by generating the active short interfering RNA trigger. Silencing is effected by the RNA-induced silencing complex and its RNaseH core enzyme Argonaute. This review describes the discovery of these machines and discusses future lines of work on this amazing biochemical pathway.

Keywords: RNA interference; miRNA; microRNA; Dicer; Drosha; Argonaute; Slicer; RNA-induced silencing complex

1. RNAi as a ribonuclease engine

RNA interference (RNAi) was discovered as a convergence of three unrelated lines of experimentation. The most familiar work is from the labs of Andrew Fire and Craig Mello. They made the groundbreaking discovery that double-stranded RNA (dsRNA) could potently induce gene silencing in the nematode *Caenorhabditis elegans* [1]. Previous to this discovery, however, studies in plants and fungi had uncovered gene silencing pathways that were triggered by transgene expression or viral replication [2]. The famous short interfering RNA (siRNA) was first detected in plant systems, and its linkage to RNAi in animals provided the connection between gene silencing pathways across kingdoms [3,4]. The third experimental component of RNAi began 20 years ago with the study of developmental timing genes in *C. elegans*. Ruvkun and Ambros [5,6] had identified *lin-4*, a small untranslated RNA that regulated the expression of the mRNA for *lin-14*. This was the first discovered microRNA. We now know that microRNAs are naturally occurring triggers of the RNAi pathway and play an important role in gene regulation in many organisms ranging from nematodes to plants to humans [7].

A simplified model for the RNAi pathway is based on two steps, each involving a ribonuclease machine. In the first step, the trigger RNA (either dsRNA or microRNA primary transcript) is processed into an siRNA by the RNaseIII enzymes Dicer and Drosha. dsRNA binding domain proteins (dsRBD) Pasha, Loquacious, and R2D2 are cofactors for processing events. In the second step, siRNAs are loaded into the effector complex RNA-induced silencing complex (RISC). The siRNA is unwound in a strand specific manner during RISC assembly. This single-stranded siRNA locates mRNA targets by Watson–Crick base pairing. Gene silencing is a result of the nucleolytic degradation of the targeted mRNA by the RNaseH enzyme Argonaute (Slicer). If the siRNA/mRNA duplex contains mismatches at the scissile site, often the case for microRNAs, the mRNA is not cleaved. Rather, gene silencing is a result of translational inhibition.

The outline for this biochemical pathway was derived from the earliest studies by Fire and Mello [1,8]. Recent work by many labs has begun to fill in our understanding of some of the intricacies of the RNAi pathway. For example, one strand of the siRNA is preferentially incorporated into RISC. This was first observed with microRNAs, but also occurs with siRNAs and long dsRNAs. One immediate impact of this discovery was the improvement in siRNA design. Secondly, RISC assembly is a multistep process. Dicer and RISC do not function independently, but act as part of a coordinated pathway. And, this basic model does not explain translational suppression observed with microRNAs. This review will discuss the core RNAi activities of Dicer and RISC, focusing on Drosophila and mammalian systems, and present recent work that details some of the more subtle mechanistic aspects of RNA-induced post-transcriptional gene silencing.

2. Initiator machinery

The goal of the initiator step of RNAi is the generation of siRNAs from long dsRNAs, or mature microRNAs from their primary transcripts. This is achieved by the action of two families of RNase III genes, Dicer and Drosha.

The discovery of the siRNA began with the study of co-suppression. This is a related gene silencing pathway, best exemplified in plants, that is triggered by 'aberrant' RNAs. Highly effective triggers of co-suppression include transcripts derived

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from transgene repeats or viruses. The transgene (or virus) is suppressed, and homologous genes are also suppressed, thus the term 'co-suppression'. The active species of the silencing pathway was assumed to be RNA, however none had been identified by Northern-blot analysis. The team of Hamilton and Baulcombe surmised that the active RNA was too small to be detected by conventional Northern blot. Using polyacrylamide gel-based Northern blot analysis they identified 25 nucleotide RNAs that were complementary to co-suppressed sequences [3]. These were present in plants undergoing transgene or virus induced co-suppression. Subsequently, similar small RNAs were identified in a Drosophila RNAi model system [4]. The discovery of these RNAs, later termed siRNAs, provided the first evidence for a universal biochemical pathway of silencing phenomena in plants and animals, whether triggered by transgenes or dsRNA. Subsequent genetic and biochemical studies have reinforced this connection.

The existence of these small RNAs suggested a biochemical activity that would generate them from dsRNA. This activity was first characterized in a Drosophila embryo extract, and the precise molecular nature of the small RNAs was defined [9]. They were shown to contain a 5′ phosphate and a 3′ hydroxyl terminus [10]. These properties are features of RNaseIII cleavage products. So began the search for the initiator enzyme for RNAi.

3. Dicer

RNaseIII enzymes fall into three classes (see Fig. 1, [11] for a review). Class I enzymes, found in bacteria and yeast, contain a single RNaseIII domain joined to a dsRBD. Class II and III enzymes contain two RNaseIII catalytic domains. Class III enzymes are further characterized by a helicase domain and a PAZ (Piwi/Argonaute/Zwille) domain. This last domain is also present in Argonaute family proteins, already known to be essential for RNAi, which led to the proposal that Class III enzymes are the initiator of RNAi [12,13]. This was experimentally proven in a Drosophila model system. The Drosophila genome has two Class III genes, CG4792 and CG6493, and one Class II gene, Drosha (see Fig. 1). The suspected role of Drosha in rRNA processing was a further suggestion that a Class III enzyme was the RNAi initiator. Using dsRNA processing assays of immunopurified RNaseIII proteins, Hannon and colleagues [14] conclusively demonstrated that a Class III gene (CG4792) housed the RNAi initiator activity. This gene was named Dicer-1 to reflect its biochemical function.

The generation of an siRNA from dsRNA potentially requires four endonucleolytic reactions. How does Dicer achieve this? Early models were based on the prediction that Dicer forms a dimer on the substrate and performs four cleavage reactions [15]. Recent data, however, favors a model whereby Dicer acts as a monomer, using two endonucleolytic reactions to generate one new terminus [16,17]. This would occur if Dicer bound to an existing terminus and made a cut ~21 nucleotides from the end. This was first suggested by studies using dsRNA substrates with blocked termini [16]. If the enzyme could not initiate processing from the end and was forced to process internally, the reaction was significantly delayed. The authors' interpretation was that internal binding was less efficient and caused a lag in processing. Once binding occurred and a single new terminus was created, further processing occurred at normal rates, since the enzyme now had terminal ends from which to process. In the same study, glycerol sedimentation indicated the enzyme existed principally as a monomer. How does this compare to single RNase III domain enzymes from prokaryotes? *E. coli* RNase III exists as a stable dimer, thus brings two catalytic domains together on the substrate [18]. With dsRNA as a substrate, this enzyme produces double stranded products in the 11 to 15 nucleotide size range. But does this enzyme cut internally, or process from the terminal end?

Work on the enzymatic model of Class I enzymes has culminated with the crystal structure of RNaseIII from *Aquifex aeolicus* [19]. The structure was obtained in the absence of a dsRNA substrate, but the positioning of the substrate was inferred based on the location of essential catalytic residues. The structural model predicted two active centers per monomer, with residues from each monomer contributing to form compound active sites. The dimer therefore could bind internally on a dsRNA substrate, and generate two new termini. The canonical 2 nucleotide 3′ overhang, as well as the length of the dsRNA products, was a result of spacing between residues on individual peptide chains. This model fit the existing data on prokaryotic enzymes, but was difficult to reconcile with Dicer. For example, several 'essential' catalytic residues were missing in Dicer. More surprising, mutation of additional catalytic residues did not impair cleavage activity [17]. These data

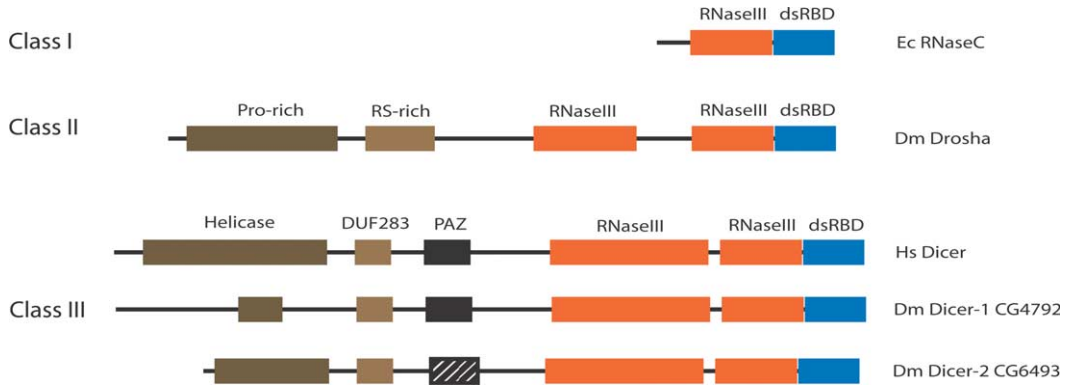

Fig. 1. Domain organization of RNaseIII gene family. Three classes of RNaseIII genes are shown. The PAZ domain in Dm-Dicer-2 contains mutations in several residues required for RNA binding and may not be functional. Abbreviations are: Ec: *E. coli*; Dm: *Drosophila melanogaster*; Hs: *Homo sapiens*; DUF283: Domain of unknown function 283.

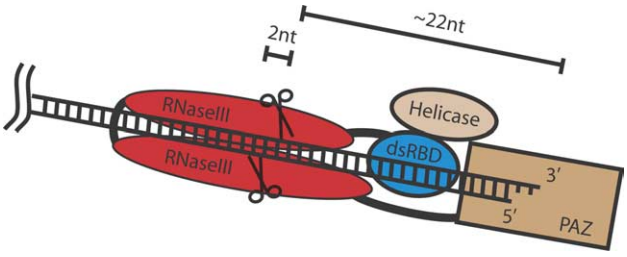

Fig. 2. Model for Dicer catalysis. This model is essentially as described in [17]. The PAZ domain binds the 2 nt 3′ overhang of a dsRNA terminus. The RNaseIII domains form a pseudo-dimer. Each domain hydrolyzes one strand of the substrate. The binding site of the dsRBD is not defined. The function of the helicase domain is not known.

ruled out the existence of two active centers per RNaseIII domain. The most congruous model is shown in Fig. 2. The two RNaseIII domains in Dicer associate in an intramolecular pseudo-dimer, creating an active site similar to *Escherichia coli* RNaseIII. Each domain cuts a single strand of the duplex, thus generating one new terminus. The 2 nucleotide overhang is measured by the dimer alignment, rather than the distance between active residues on one peptide chain. The 21 nucleotide product length is measured by the distance between the terminal binding PAZ domain and the active site. Further data suggested a similar model for Class I enzymes [17]. Since these enzymes lack PAZ domains, or any appreciable sequence N terminal to the active core, it is not clear what binds the substrate terminus, and thus measures the length of the product. Since the products for this enzyme are more heterogenous in size (for dsRNA substrates), one might predict a less effective terminal binding domain. A more complete model awaits the structure determination in complex with a substrate [20].

In addition to processing dsRNAs into siRNAs, this gene family also participates in maturation of microRNAs. The biology of these small RNAs had been studied for years, but little was known about the proteins involved in their biochemical mechanism of action. In hindsight the connection between microRNAs and siRNAs is clear: they are both non-translated RNAs of comparable size that reduce expression of genes with complementary sequences. The formal demonstration that these related RNAs share a mechanism of action was made possible by the identification of components of the RNAi machinery. Shortly after the discovery of Dicer four groups demonstrated that this enzyme is also a part of the microRNA machinery [21–24]. This linkage was groundbreaking, for it provided an endogenous role for the RNAi pathway. It explained the developmental phenotypes that were associated with mutations in the RNAi pathway in flies and plants, i.e., *piwi*, *carpal factory*, *zwille*. The connection with development was extended to mammals. Targeted deletion of Dicer in the mouse leads to early embryonic lethality [25].

4. Drosha

The link between the RNAi and the microRNA pathways provided an exciting role for RNAi in the regulation of gene expression. An overview of the microRNA processing pathway is shown in Fig. 3. MicroRNAs are transcribed from RNA polymerase II as long primary transcripts (see [26] for a review). The active microRNA species, termed the mature RNA, is present in a stem–loop structure within the primary transcript. The stem–loop can be located in an exon or an intron. For example, the microRNAs miR-106b, miR-93, and miR-25 are located within an intron of the protein coding gene *mcm-7*. After transcription, the microRNAs are processed out of the primary transcript, and the spliced mRNA is exported and translated. Whether the microRNA is processed before, during, or after splicing is not known. Sequential processing of the primary transcript by the RNaseIII enzymes Drosha and Dicer liberates the mature RNA. Drosha cleavage releases the stem–loop, termed the precursor, which is exported from the nucleus in an Exportin-5/RAN-GTPase-dependent manner. In the cytoplasm, the precursor is processed into a siRNA-like structure by Dicer. Drosha generates a 2 nt 3′ overhang terminus on the precursor which is recognized by the PAZ domain of Dicer, analogous to the recognition of dsRNA termini. The double-stranded microRNA is incorporated into RISC in a similar manner as siRNAs.

Drosha is a Class II enzyme as shown in Fig. 1. This enzyme assumes a pseudo-dimer catalytic core similar to Dicer [27]. The substrate of Drosha, microRNA primary transcripts, is structurally distinct from Dicer substrates. Drosha does not process from a dsRNA terminus. Rather, data suggests that the stem–loop structure is recognized. In particular, the loop size appears to be important for recognition [28]. In addition, unstructured sequences flanking the stem–loop are essential for processing [29,30]. It is not evident how Drosha would recognize these sequences, as they are outside of the dsRNA stem. Possibly other, unidentified cofactors play a role. Conserved sequence elements have been found in flanking regions of *C. elegans* microRNAs [31].

Evidence to date suggests that microRNA expression is regulated at the level of transcription. Several microRNA promoters have been studied. The polycistronic cluster of miR-17-18-19a-20-19b-92 is positively regulated by the oncogenic transcription factor *c-myc*, and the muscle specific miR-1 is positively regulated by Serum Response Factor (SRF), MyoD, and Mef2 [32,33]. Regulation of microRNA expression at the level of Drosha or Dicer processing has not been reported, though this has not been systematically tested.

5. dsRBD cofactors

While Dicer and Drosha proteins contain the required RNaseIII domains for activity, recent data has shown these proteins function as components of larger complexes. Minimally they are associated with dsRBD cofactors. The first dsRBD that was identified, *rde-4* (RNAi deficient-4), arose from a genetic screen in *C. elegans* [12,34]. In Drosophila, Dicer-1, Dicer-2 and Drosha are associated with Loquacious, R2D2, and Pasha, respectively [35–40]. The role of R2D2 in directing strand specific incorporation of the siRNA is well established (see below). Loquacious may perform a similar role with microRNA loading into RISC. The function of Pasha is less clear, since strand specificity appears to occur downstream of Dicer action. One possibility is that Pasha confers regulation of microRNA expression at the level of Drosha processing. The limited data does not suggest such a role, since knockdown of Pasha reduced processing of all microRNAs tested [38,40]. This possibility has not been fully explored, however.

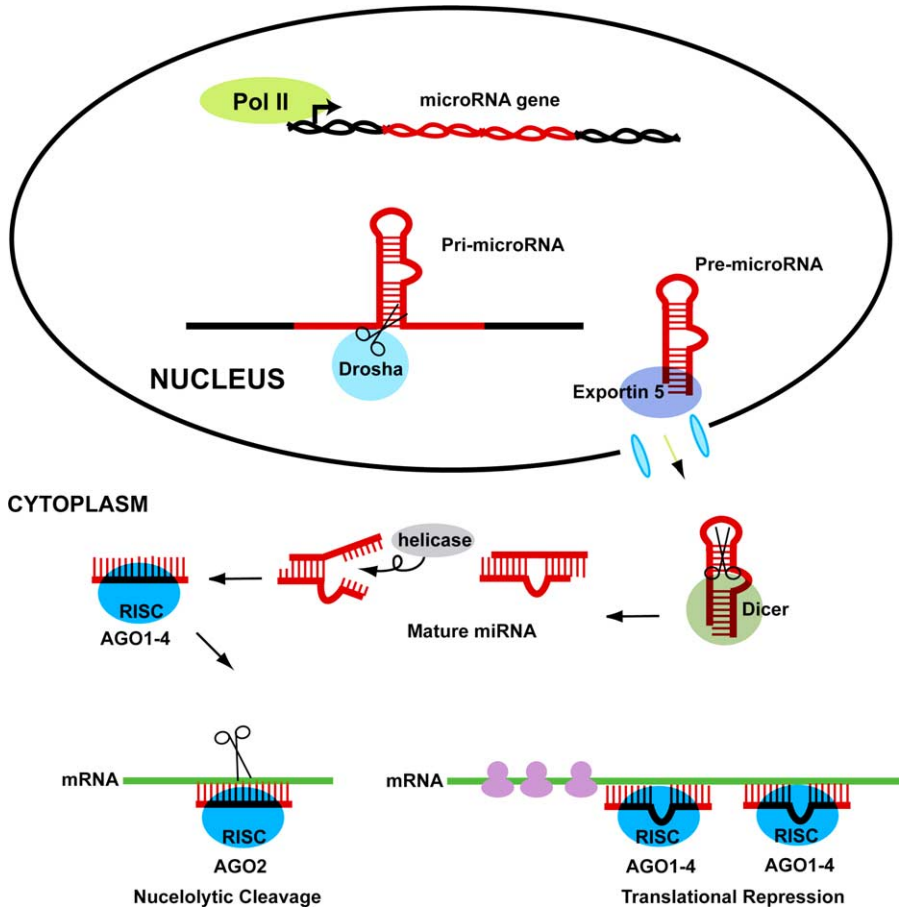

Fig. 3. Biogenesis pathway of microRNAs. MicroRNA genes are transcribed by RNA polymerase II. The primary transcript is referred to as "pri-microRNA". Drosha processing occurs in the nucleus. The resulting precursor, "pre-microRNA", is exported to the cytoplasm for Dicer processing. In a coordinated manner, the mature microRNA is transferred to RISC and unwound by a helicase. mRNA targets that duplex in the Slicer scissile site are cleaved and degraded, if the microRNA is loaded into an Ago2 RISC. Mismatched targets are translationally suppressed. All Ago family members are believed to function in translational suppression.

6. Effector machinery

The endgame of the RNAi pathway is the nucleolytic destruction of the targeted mRNA. This is achieved by the multiprotein complex RISC. Central to this complex is a member of the Argonaute family. Selected members of this family have a nuclease activity which is responsible for mRNA target cleavage (Slicer activity). An alternate mode for RISC activity is suppression of translation without mRNA cleavage, which is the more typical mode for microRNA-mediated silencing.

7. RISC

The existence of a sequence-specific nuclease complex was first predicted by Fire [8] after initial studies on the RNAi pathway in *C. elegans*. The formal demonstration of this activity was reported by two independent groups working in Drosophila cell-free model systems. The first report, from a collaboration among Zamore, Sharp, Bartel, and Tuschl [41], demonstrated that dsRNA could induce gene silencing in a Drosophila embryo extract. This was accompanied by destruction of the target mRNA. In a second report, using cell-free extracts from Drosophila cultured cells, Hannon's [4] group characterized this nuclease activity. They showed that it existed as a preformed, fractionable entity. Importantly, this report also showed the nuclease activity contained an siRNA as an integral component. These reports solidified the hypotheses that RNAi was effected by a sequence-specific nuclease complex. Size-exclusion chromatography suggested several possible sizes for the RISC, ranging from 500 to 360 kD to 140 kD, depending on the model system [42–44]. This discrepancy may reflect the absence or presence of non-essential cofactors, or remnants of RISC assembly factors. If this was the case, 140 kD would represent the minimal RISC nuclease. We now know this is likely the case, since Argonaute and an siRNA are sufficient for minimal target cleavage activity [45].

Chromatographic purification of RISC nuclease activity from Drosophila cells revealed several RISC components. The first identified component was Argonaute2 (Ago2) [43]. This protein is a member of a gene family conserved in most eukaryotic and several prokaryotic genomes. The *C. elegans* homolog, *rde-1*, was previously identified in a genetic screen for RNAi-deficient mutants, reinforcing its connection with RNAi [12]. Structurally, this protein family is characterized by two domains, the PAZ domain and the PIWI domain. Structures for both domains have been solved (see below). Additional RISC components with unknown roles in RNAi

have also been identified. These include the RNA binding protein VIG, the Drosophila homolog of the Fragile X protein, dFXR, helicase proteins, and Tudor-SN [46–48]. This last protein has five staphylococcal nuclease (SNase) domains and a Tudor domain. The presence of SNase domains made it an obvious candidate for Slicer. Several lines of evidence, however, are inconsistent with this. Many essential catalytic residues are absent in the SNase domains of Tudor-SN [47]. While the protein still exhibits some nuclease activity, the chemistry of the cleavage reaction differs from that observed with Slicer. Specifically, products of the Slicer reaction have 5′ phosphate and 3′ OH moieties. The scissile bond has been mapped to the center of the siRNA, indicating an endonucleolytic reaction. SNase, however, is an exonuclease that produces 5′ OH and 3′ phosphate products [49,50]. While Tudor-SN may have a role in degrading Slicer products, it is not Slicer itself.

8. Slicer

Several lines of experimentation were pointing to Argonaute itself as Slicer. Purification of a Drosophila RISC activity to homogeneity revealed Ago2 as the only remaining protein as determined by mass spectrometry [51]. This does not rule out the possibility, while remote, that Slicer is very small and is not represented in tryptic fragments. Further evidence was obtained in mammalian model systems. In humans, there are four closely related Argonaute family members, named Ago1-4. All four bind siRNAs and microRNAs at similar levels, and are widely expressed. Only Ago2, however, is present in a cleavage-competent RISC [52,53]. Similarly, siRNA-mediated knockdown, or targeted knockout, of Ago2 impairs RNAi of a reporter, while knockdown of Ago1, 3, 4 had no effect. These data can be interpreted in two ways: Ago2 alone is capable of interacting with Slicer, or Ago2 itself is Slicer. The answer was provided by the crystal structure of an Argonaute family member from *Pyrococcus furiosus* [54]. The structure revealed an RNaseH fold for the signature PIWI domain. The crystal structure of a second archaean Argonaute, *Archaeoglobus fulgidus* Piwi (AfPiwi), confirmed the RNaseH fold [55]. The final demonstration that Slicer activity was contained within Ago2 was the reconstitution of minimal RISC with bacterially expressed, purified Ago2 and a single-stranded siRNA [45].

Mechanistic studies on RISC have recently reached an apex with the crystal structure of AfPiwi complexed with a dsRNA [56,57]. The AfPiwi protein is not a perfect model, since it lacks the PAZ domain, and its cellular role is a mystery. Nevertheless, this structure provided molecular details to a number of experimental observations. For example, microRNA/target pairs have demonstrated the importance of nucleotides 2–8 in the microRNA, termed the 'seed' region, for target recognition [7]. The first nucleotide does not contribute to target recognition, and nucleotides 9 to the 3′ terminus have reduced importance. The AfPiwi structure shows that the first nucleotide of the siRNA does not pair with the target, but is sequestered in a binding pocket. Not only is base pairing unnecessary, but a strong pairing may distort RNA binding and reduce Slicer activity. Interestingly, a strong base pair at the 5′ terminus of the siRNA should not occur in any case. Rules that govern strand incorporation into RISC are based on low pairing energy at the 5′ end of the incorporated (guide) strand, compared to the discarded (passenger) strand [58,59]. This means that an effective siRNA (or microRNA) will begin with an A or U, thus will not prevent proper binding to Argonaute. Of course, another way to achieve specific strand loading is to have a mismatched G or C at the 5′ end, which would strongly base pair to a matched target. This did not reduce Slicer catalytic rate, however [58].

The Slicer catalytic model is shown in Fig. 4. The 5′ end of the siRNA guide is bound to the Piwi domain. The 5′ phosphate, which is important for high affinity binding, is coordinated by four conserved residues, and torsioned away from the mRNA target strand. The 3′ end of the siRNA extends beyond the Piwi domain. Structural studies on the isolated PAZ domain suggest it binds 3′ OH terminal ends of RNA, or duplexes with a 3′ overhang [60–64]. Since AfPiwi lacks a PAZ domain, one can only predict that this domain binds to the 3′ end of the siRNA guide. The target mRNA duplexes primarily with the 5′ seed region of the siRNA, in the context of the Piwi domain. The affinity of mRNA target binding is largely based on this interaction, though efficient Slicer catalytic rate depends on duplex formation with the 3′ region of the siRNA [49]. The catalytic engine is the RNaseH fold in the Piwi domain. Typical RNase H endonucleases cleave the RNA strand of a RNA/DNA duplex, in a cation dependent manner, generating 5′ phosphate 3′ OH products. When presented with a long RNA substrate duplexed with a short DNA oligonucleotide, however, the enzyme cleaves the RNA in the center of the oligonucleotide. This is essentially a Slicer activity, though the siRNA guide takes the place of the DNA oligonucleotide.

This model also explains binding of mRNA targets to microRNA RISC. The 5′ seed region of the microRNA is essential for binding affinity, as has been observed for bona-fide, and artificial mRNA targets [7]. Since Slicer activity is not required, the 3′ region of the microRNA is relatively unimportant. What is not clear is the mechanism of translational suppression. Target degradation does occur, but this does not appear to be the primary cause of gene silencing [65]. Loss of function of 5′–3′ exonuclease activity in *C. elegans* caused

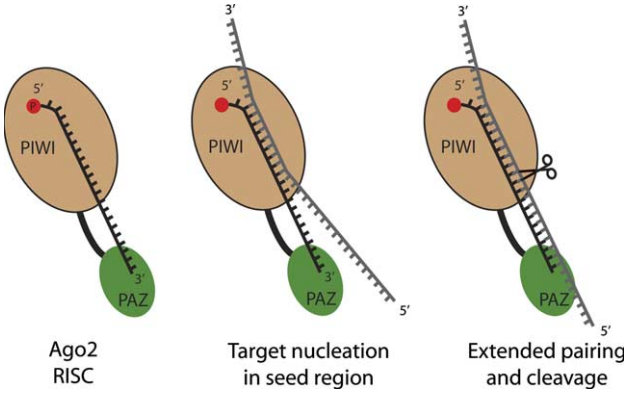

Fig. 4. Model for Slicer catalysis. The siRNA guide strand is bound at the 5′ end by the PIWI domain and at the 3′ end by the PAZ domain. The 5′ phosphate is coordinated by conserved basic residues. mRNA targets are initially bound by the seed region of the siRNA and pairing is extended to the 3′ end. The RNaseH fold hydrolyzes the target in a cation dependent manner. Slicer cleavage is measured from the 5′ end of the siRNA. Product is released by an unknown mechanism and the enzyme recycles.

an increase in the *let-7* target *lin-41*, without an increase in *lin-41* protein (based on phenotype). The most compelling model for microRNA function has recently been published by the Filipowicz group [66]. They presented evidence that microRNA/RISC blocks cap-dependent initiation of translation. Cap-independent reporters that initiate from an internal ribosome entry site (IRES) are not targetable by microRNAs. Targeted mRNAs are localized to cytoplasmic mRNA processing bodies (P-bodies) [66–68]. These are loosely defined structures that contain populations of mRNAs and nucleolytic processing and degradation machinery. The Filipowicz model predicts that this is a consequence of translational inhibition. This is in harmony with 5′–3′ exonucleolytic degradation of mRNA targets, since this activity is located in P-bodies [69].

While the microRNA and RNAi pathway share the same core machinery, some specialization may exist. For example, in Drosophila Ago1 preferentially binds microRNAs and Ago2 siRNAs [46,70]. Similarly, Dicer-1 is essential for microRNA processing [71]. Flies lacking Dicer-1 have minimal mature microRNAs and exhibit developmental phenotypes that would be expected from this deficiency. These flies can process long dsRNAs at normal levels. Dicer-2 mutant flies, conversely, have normal levels of mature microRNAs but cannot process long dsRNAs. Interestingly, both mutants have reduced levels of gene silencing triggered by siRNAs. This is not surprising for Dicer-2, due to its role in RISC assembly (see below). Why Dicer-1 would be required for siRNA function is a mystery. It is possible that some overlap in function exists, since partitioning of siRNAs and microRNAs into Ago2 and Ago1 complexes, respectively, is not absolute. An alternative explanation is that Dicer-1 flies, lacking microRNA function, have a host of cellular defects, and this leads to reduced siRNA effectiveness.

It is interesting that humans have only one Dicer gene, which is more related to the Drosophila Dicer-1 gene. Similarly, human Ago1-4 are related to Drosophila Ago1. There is no ortholog of Drosophila Ago2 in the human genome. This suggests that the human genome has preferentially retained the microRNA sub-pathway. Since long dsRNAs are toxic to mammalian cells, the processing pathway for this type of RNAi trigger would be unnecessary. Slicer function, however, has been retained in one human family member, Ago2. The experimental use of RNAi in mammalian cells, therefore, is based on co-opting the microRNA pathway's one remaining Slicer Argonaute.

9. RISC assembly

Recent work has begun to refine the roles of Dicer proteins in RNAi. For example, based on the simple two-step model for RNAi, direct introduction of siRNAs should not require Dicer function. However, depletion of Dicer does reduce effectiveness of siRNA silencing [72]. There is also evidence that synthetic hairpin RNAs that act as Dicer substrates are more effective RNAi triggers than siRNAs [73,74]. These observations point to an interaction between Dicer and RISC. Such an interaction was first suggested by co-immunoprecipitation studies, but the nature was unknown [43]. Recent data from two labs has outlined a multistep assembly process for RISC that requires Dicer [75,76]. At an early step, Dicer and a dsRBD partner (i.e., Drosophila R2D2 and Dicer-2) bind to the siRNA. Assembly of this RISC loading complex (RLC) may be a single step or may include multiple steps with different, uncharacterized accessory proteins. The orientation of R2D2 binding is asymmetric, favoring the loading of the guide strand of the siRNA into RISC [77]. In a concerted manner, the siRNA is unwound and the guide strand is transferred from the RLC into RISC. Evidence suggests this assembly occurs on an ~80S complex [75]. Since RISC has been reported to be bound to ribosomes, this large complex may be the ribosome [78]. This 'holo-RISC' is now active, and may target mRNAs while ribosome bound, or may dissociate as free RISC.

10. Outlook

The field of RNAi has progressed at an amazing rate in the seven years since its discovery. What began as an oddity in *C. elegans* has revolutionized cell biology in many model systems. A poorly understood gene silencing mechanism has reached detailed understanding including crystal structures for RNase III and Argonaute family proteins. While the Dicer/Slicer pathway for RNAi is becoming well understood, two major arms of the pathway still require much work. MicroRNA-mediated gene silencing, while sharing the same pathway, remains enigmatic. Even less understood is the transcriptional arm of the RNAi pathway. This has been well established in *Schizsaccharomyces pombe* and plants, and evidence for its existence has been reported in Drosophila and mammals. The extent of its role in biology is still a mystery and its mechanism is poorly understood. Another unexplored facet of RNAi is its potential connection with mRNA localization. Mutations in several RNAi components disrupt mRNA localization in Drosophila oocytes. It is not known whether this localization machinery is based on RISC and small RNAs, or whether there is duplicated function of some RNAi components. Either way, it is clear that the global pathway of RNAi will extend into many areas of cell biology.

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