PERSPECTIVE

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RNase III enzymes and the initiation of gene silencing

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Our understanding of RNA interference has been enhanced by new data concerning RNase III molecules. The role of Dicer has previously been established in RNAi as the originator of 22-mers characteristic of silencing phenomena. Recently, a related RNAse III enzyme, Drosha, has surfaced as another component of the RNAi pathway. In addition to biochemistry, protein structures have proven to be helpful in deciphering the enzymology of RNase III molecules.

RNAi is an evolutionarily conserved process by which double-stranded RNA (dsRNA) induces highly specific gene silencing. The sources of silencing triggers include exogenously introduced dsRNA, RNA viruses, transposons and endogenous short dsRNAs (reviewed in ref. 1). In the current mechanistic model, these triggers are processed by the RNase III enzyme Dicer into small, 21–24-nucleotide (nt) short interfering RNAs (siRNAs)[2–5]. The siRNAs then serve as sequence-specific guides to an effector complex called the RNA-induced silencing complex (RISC) that carries out the destruction of homologous mRNAs[5,6]. RNAi has been linked to several seemingly disparate processes, including control of development and regulation of heterochromatin[7,8]. The Dicer enzyme has been implicated in these processes. It produces siRNAs with characteristic chemical structures. The presence of such species is a hallmark of all RNAi-related phenomena.

Over the past two years, several groups have described a populous class of endogenous substrates that enter silencing pathways[9–13]. These substrates, called microRNAs or miRNAs, are short, noncoding RNAs that form hairpin-like structures. The stems of miRNAs are largely dsRNA but contain mismatches and GU wobble pairs. They enter silencing pathways, leading to either suppression of protein synthesis or mRNA destruction[14]. MicroRNAs are transcribed from the genomes of diverse organisms. In human cells, some clustered miRNA genes are transcribed polycistronically as primary precursors for miRNAs (pri-miRNAs) that are several hundred bases long[15,16]. Nonclustered miRNA genes are also expressed as longer nascent transcripts that require further processing. Both poly- and monocistronic pri-miRNAs undergo a processing step in the nucleus that produces shorter, ~70-nt pre-miRNAs. The pre-miRNAs are then exported from the nucleus by exportin-5, a nuclear export factor that binds pre-miRNAs directly and specifically[17,18]. In the cytoplasm, pre-miRNAs are processed by Dicer into mature, ~22-nt miRNAs[15]. These RNAs were first described as molecules that control developmental timing events in *Caenorhabditis elegans*[19,20]. A defect in miRNA processing may account for the developmental phenotypes associated with disruption of RNAi pathways in other organisms from plants to mice[21,22].

This review will focus on recent developments in the biochemistry of RNAi, particularly in reference to the role of RNase III molecules. The role of Dicer has been established in RNAi, and recently a related RNase III enzyme, Drosha, has been identified as another component of the RNAi pathway[2–5]. We also discuss several models of Dicer cleavage informed by the structural analysis of the PAZ domain of *Drosophila melanogaster* Argonautes 1 and 2, and a second look at that of the *Aquifex aeolicus* RNase III domain.

RNase III molecules and the biochemistry of RNAi

RNase III (RIII) enzymes were first described in 1968 by Zinder and colleagues[23]. All RNase III molecules exhibit specificity for dsRNA. RIII enzymes are involved in RNA metabolism in organisms ranging from phage to animals (reviewed in ref. 24). Three structural classes of RIII molecules have been described. The first class is represented by *Escherichia coli* RNase III, the second by Drosha and the third by Dicer (**Fig. 1**). The first class is comprised of the simplest RIII proteins, each of which contains one catalytic endonuclease domain (RIII domain) and a dsRNA-binding domain (dsRBD). *E. coli* RNase III promotes maturation of ribosomal RNAs (rRNAs), tRNAs and mRNAs, and can also initiate mRNA degradation[24]. Highly structured, hairpin-like RNA precursors are cleaved in the stem region by RIII enzymes. As has been observed for processing of long dsRNA, structured RNA substrates such as rRNA precursors are cleaved in a staggered fashion. The products generated contain 5′ monophosphate groups and two-nucleotide 3′ overhangs.

Processing of primary precursors of miRNAs by Drosha

Members of the second class of RIII proteins, comprised of Drosha and homologs, contain two RIII domains, a dsRBD, and a long N-terminal segment[25]. The N termini of human and mouse Drosha contain two domains thought to be involved in protein-protein interactions, namely a proline-rich region (PRR) and a serine-arginine-rich (RS) domain[26,27]. RS domains are commonly found in RNA metabolism and splicing factors[28]. Drosha homologs have been identified in flies, worms, humans and mice, but not yet in plants or *Schizosaccharomyces pombe* (although they do have other RIII proteins)[25–27]. The human Drosha functions in processing of highly structured ribosomal RNA precursors, much like *E. coli* RNase III[26]. The degree to which a panel of antisense RNAs against human Drosha

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decreased its expression correlated very well with an increase in the 32S and 12S ribosomal precursors. Recently, Drosha has emerged on the RNAi scene as an important player in the maturation of miRNAs[29]. These RNAs are initially transcribed as long pri-miRNAs[15,16]. Drosha clips pri-miRNAs in the nucleus into shorter ~70-nt pre-miRNAs. The pre-miRNAs are then exported from the nucleus and processed by Dicer into mature ~22-nt miRNAs[15]. Thus, Drosha processes two distinct types of substrates with hairpin-like secondary structures.

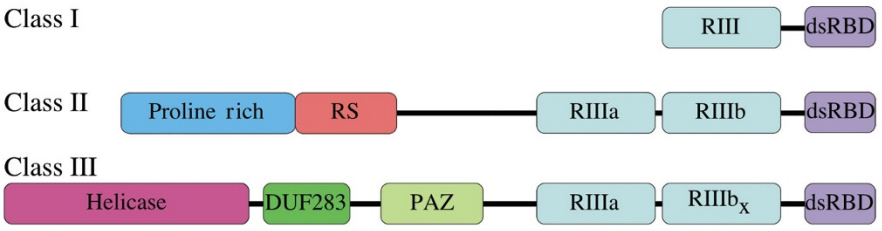

Figure 1 Domain structures of class I–III RNase III proteins. Class I is represented by *E. coli* RNase III, class II by mammalian Drosha and class III by Dicer. The nonfunctional catalytic site in the second RIII domain of Dicer is denoted by an X. Domain sizes are approximate.

Generation of siRNAs and mature miRNAs by Dicer

The third class of RIII enzymes, comprised of Dicer-like proteins, are ~200-kDa multidomain proteins[2]. Typically, Dicer enzymes contain an N-terminal DEXH-box RNA helicase domain, a domain of unknown function (DUF283), a PAZ domain, two RIII domains and a dsRBD. The PAZ domain is also found in the Argonaute protein family involved in RNAi effector complexes[6], and is in fact named after three founding Argonaute proteins, Piwi, Argonaute and Zwille. The potential functions of the PAZ domain will be discussed in the next section.

Despite progress toward clarifying the biochemical mechanism of RNAi, many key questions have yet to be answered. For example, what characteristics distinguish molecules able to elicit an RNAi response from other segments of dsRNA that might exist within coding and noncoding cellular RNAs? Also, how do *bona fide* silencing triggers exclusively enter and remain in the RNAi pathway? Certainly, one clue is the characteristic chemical structure of intermediates in the pathway. For example, biochemical evidence indicates that siRNAs with two-nucleotide 3′ overhangs are optimal for inducing silencing[5]. Likewise, siRNAs lacking 5′ phosphate groups characteristic of RIII nuclease activity cannot become incorporated into RISC complexes[30,31]. MicroRNAs share these terminal structures after cleavage by Drosha[29,32,33]. The presence of these specific chemical groups may be important for quality control in the RNAi pathway. For example, they may ensure that pre-miRNAs created by Drosha in the nucleus are readily recognized by Dicer in the cytoplasm. The overall picture can be completed in several ways. One or more proteins in the pathway may be able to distinguish intermediaries from other RNAs in the cell. This recognition could occur through either direct or indirect recognition of one or more of the chemical structures. Notably, exportin-5, which mediates nuclear export of pre-miRNAs, shows a marked preference for 3′ overhangs[17,34].

Dicer seems to preferentially recognize the ends of its substrates. For example, when Dicer is presented with a 40-bp dsRNA, discrete products of ~22 and ~18 bp are produced[35]. If Dicer were not cleaving precisely from the termini, the result would be a product of ~22 bp and heterogenous products ranging from 1 to 18 bp. It is unclear how Dicer recognizes the ends of a substrate, and how stringent the requirement for end recognition is. One study suggests that although there is a clear preference for an end, the exact nature of the end may not matter[35]. For example, chemically altering the ends of the dsRNA substrate with tetraloops or DNA bases slows, but does not prevent, initial cleavage by Dicer[35].

The function of another RIII enzyme upstream of Dicer may contribute to specific entry of miRNAs into the RNAi pathway. This may be due to the fact that Drosha creates ends with a chemical structure that is readily recognized by Dicer. Dicer binds to, and indeed can be inhibited by, its own reaction product, making it no surprise that Dicer binds to Drosha products that share the same termini[36]. Biochemical evidence indicates that preprocessing of substrates by Drosha enhances the ability of Dicer to produce mature miRNAs[29]. In fact, Drosha processing improves both the efficiency and accuracy of subsequent Dicer cleavage[29]. Specifically, Drosha activity leads to a greater buildup of 22-mers than with twice the amount of Dicer alone. Drosha preprocessing also leads to a more homogeneous population of 22-mers, supporting the idea that Drosha defines the end from which Dicer makes its cut. The same principle is also true with long dsRNA substrates. Treating long dsRNA with limiting amounts of *E. coli* RNase III increases the rate of subsequent Dicer processing to create siRNAs (J. Silva and G.J.H., unpublished data). Similarly, Dicer's apparent processivity on long dsRNA substrates may be achieved by recognition of the ends created by the previous Dicer cleavage.

The PAZ domain as molecular sensor?

PAZ domains are highly conserved domains of 130 amino acids that are found in Dicer and members of the Argonaute family. Argonaute proteins are core components of RISC and also interact with Dicer, either directly or indirectly[6]. To gain insight into the function of the PAZ domain, several groups have recently solved the structures of the PAZ domains of *D. melanogaster* Argonautes 1 (ref. 37) and 2 (refs. 38,39). The structure reveals a high degree of similarity to an oligo-binding (OB) fold, a previously characterized single-stranded nucleic acid–binding domain[40]. To investigate whether the PAZ domain might bind nucleic acid directly, all three groups test PAZ binding to single- and double-stranded siRNAs and DNA. Although there is some discrepancy about the species of nucleic acid that preferentially binds to PAZ domains, it is clear that all of the observed binding is of low affinity, with dissociation constants in the micromolar range. The study of Ago1 reports that the PAZ domain interacts with RNA preferentially over DNA[37]. One Ago2 study agrees with this finding[38], whereas the other finds that both single- and double-stranded DNA bind with equal affinity to the Ago2 PAZ[39]. Although none of the solved structures contained RNA, the binding site is predicted to involve highly conserved residues in an intersubdomain cleft. Mutational studies confirm that this cleft contains aromatic residues critical for RNA binding[37–39]. All three studies demonstrate that binding is independent of nucleic acid sequence.

One possible explanation for the lack of high-affinity binding is an interaction of the PAZ domain with only part of an siRNA. The apparent end-sensing ability of the PAZ-containing Dicer led to speculation that the PAZ domain could be functioning in end recognition through a direct interaction with one or more characteristic terminal structures[35]. This would not be the first demonstration of OB-fold-containing proteins exhibiting end-binding properties. For example,

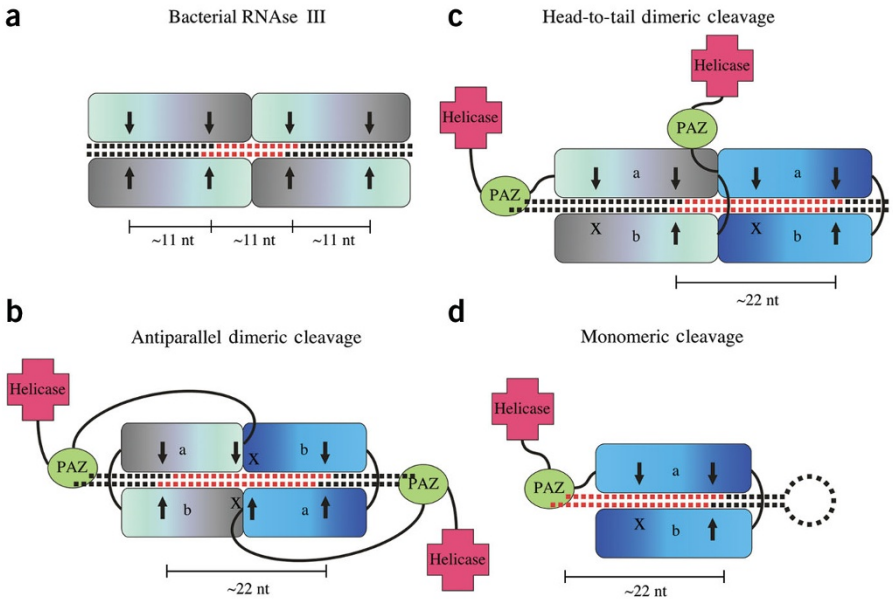

Figure 2 Models for Dicer cleavage. (**a**) Two adjacent *E. coli* RNase III dimers cleave dsRNA to generate 11-mers (adapted from ref. 45). (**b**) Antiparallel dimeric model showing close packing of RIII domains required to generate 22-mers. (**c**) Head-to-tail dimeric model. (**d**) Monomeric model shown on a hairpin. In all panels, the footprint of the RIII domain on dsRNA is to scale. Arrows indicate active catalytic sites, and X indicates a nonfunctional catalytic site. Because it is unknown whether dual cleavage chemistries are utilized to create the 3′ overhang, only one arrow is shown per compound active site. Putative cleavage products are indicated in red. For clarity, DUF283 and the dsRBD are omitted from the drawings.

two proteins containing OB folds, *S. cerevisiae* Cdc13 and *Oxytricha nova* telomere end-binding protein, bind specifically to the 3′ overhangs of telomeric DNA[41,42].

Several lines of evidence support the end-recognition hypothesis. Both studies of the Ago2 PAZ provided data demonstrating a reduced affinity of the Ago2 PAZ domain for blunt-ended dsRNA as compared with siRNAs with single-stranded 3′ ends[38,39]. Although the Ago1 study did not address the issue of an overhang, the Ago1 PAZ does have a preference for single-stranded RNA binding[37]. In addition, UV crosslinking experiments revealed that siRNAs with thymidines (which are particularly photoreactive) in the overhangs can be crosslinked to the PAZ domain efficiently[38]. These data indicate that the bases in the overhang, and not in the interior of the siRNA, have the potential to interact with the PAZ domain. It should also be noted that the cleft formed by the PAZ domain is large enough to recognize the terminus of a single-stranded RNA, but not the entire siRNA or dsRNA[38]. Notably, slight modifications of the chemical groups on the overhang of siRNAs are tolerated (such as changing the 3′ OH to a 2′,3′ dideoxy), although some decrease silencing activity[43]. In addition, certain larger adducts are tolerated, although some abolish silencing[31,44].

Although there is evidence that the PAZ domain recognizes a single-stranded 3′ end, it may or may not recognize the 5′ phosphate group. The two studies of Ago2 PAZ found that the presence of the 5′ phosphate is inconsequential to binding[38,39], but the Ago1 study indicates that the phosphate leads to higher-affinity binding[37]. This may represent a difference between Ago1 and Ago2 that could be reflected in their biology.

The PAZ domain is found only in Dicer and Argonautes. These molecules function at two steps that must distinguish genuine siRNAs from other RNAs in the cell. Although RISC contains single-stranded RNAs, it has not yet been determined whether RISC also transiently contains double-stranded siRNAs[30]. In either case, some component of RISC must be able to detect the chemical structure of *bona fide* intermediaries in the pathway. PAZ-containing Argonautes, shown to be core components of RISC complexes, are suitable candidates for this function.

Models for Dicer cleavage

Dicer, unlike other RNase III enzymes, produces 22-nt siRNAs that are characteristic of an RNAi response[2]. Insight from the crystal structure of *A. aeolicus* RNase III provides a platform for speculation about the mechanism of Dicer cleavage[45]. RIII domains are functional only when dimerized. This is because two compound active sites are formed across the interface of the dimer. Each RIII domain provides half of each compound active site.

One could imagine two scenarios of Dicer function. The first is that Dicer functions as a monomer with its two RIII domains forming an intramolecular dimer. Alternatively, Dicer molecules could function as dimers (with a total of four RIII domains). In either case, it must be considered that Dicer contains one nonfunctional catalytic site. The first RIII domain of Dicer has canonical active sites, but crucial catalytic residues of the second active site of the second RIII domain are not conserved (**Fig. 1**)[45]. An *E. coli* RIII domain with the same mutation lacks the ability to cleave RNA[45].

One model suggests that Dicer operates as an antiparallel dimer. If all four RIII domains are intact, a dimer of Dicer molecules should be able to cleave dsRNA at four sites, with a spacing of ~11 nt (9 bp of dsRNA with two-nucleotide 3′ overhangs) like two adjacent dimers of the bacterial RNase III (**Fig. 2a**). This model proposes that the nonfunctional active site would cause Dicer to produce 22-mers instead of 11-mers like other RIII enzymes. However, for this model to hold true, the two dimers of RIII domains would have to pack more closely to each other than do the *E. coli* RIII domains. If the steric relationship is the same as in *E. coli*, a dimer of Dicer molecules would actually produce a 31-bp helix with two-nucleotide overhangs. **Figure 2b** illustrates the close packing that would be required to bring the active sites near enough to produce 22-mers.

Another possibility for a dimeric model would be for the two Dicer molecules to be aligned in the same orientation relative to each other (head to tail) on the dsRNA (**Fig. 2c**). The production of 22-mers in this model depends on the close placement of adjacent Dicer RIII domains. It is unknown whether the domains in the N terminus of the protein would either mediate or interfere with such close packing.

A third model proposes that Dicer functions as a monomer, with its two RIII domains binding to create an intramolecular dimer (**Fig. 2d**). This model by definition cannot rely on the juxtaposition of four RIII domains to account for the production of 22-mers. Instead, another property of the molecule must define the distance of cleavage. For example, the architecture between the PAZ and the RIII domains may determine this distance. In this scenario, the PAZ, recognizing the end of a substrate, would position the site of RIII cleavage as ~22-nt away.

Observations about the RNAi pathway may yield clues pointing toward the correct model. For example, one RIII domain occupies

~23 nucleotides of an RNA helix[45]. Micro RNAs, which are cleaved by Dicer, often do not have stems >25 bp[9]. This constraint may suggest that Dicer binds miRNAs as a monomer. Notably, some organisms, including *Arabidopsis thaliana*, have two distinct sizes of siRNAs[36]. Biochemical data suggest that these distinct classes of siRNAs are produced by different Dicer-like enzymes[36]. The correct model of Dicer cleavage should be able to account for the apparent specialization of Dicer-like enzymes (where these differences cannot be accounted for by subcellular localization).

PAZ-less Dicers

Bioinformatic studies have revealed that some Dicer molecules have extremely divergent, or nonexistent, PAZ domains. These include *A. thaliana* DCL4 and *D. melanogaster* Dicer-2 (ref. 46). In light of the working model of PAZ function discussed above, this could have implications for the biochemistry and fidelity of the pathway. At least in some contexts, the absence of the PAZ domain may be compensated for by the existence of adaptor molecules for Dicer. Two such molecules have been proposed. Both proteins, Rde-4 in *C. elegans*[47], and R2D2 in *D. melanogaster*[48], contain two dsRBD domains, pointing toward a function in dsRNA binding. Rde-4 interacts with the single, PAZ-containing Dicer1 in *C. elegans*. R2D2, on the other hand, interacts only with the PAZ-less Dicer-2 in *D. melanogaster*, and not with PAZ-containing Dicer1. One could imagine that adaptor molecules such as Rde-4 and R2D2 might serve an alternative means to recognize RNA species in the pathway. These proteins may be especially important in the absence of the PAZ. Such a system would ensure that RNAi-related pathways could respond to a variety of types of triggers.

Rde-4 is found in a complex with Dicer1, Argonaute protein Rde-1 and an additional DEXH-box helicase[47]. The proposed function of Rde-4 is recognizing and presenting exogenous dsRNA triggers to Dicer. Rde-4 seems to interact only with foreign trigger dsRNA, and not with endogenous miRNAs or siRNAs. Mutations in Rde-4 substantially reduce the amount of siRNAs derived from an injected dsRNA trigger. This defect in RNAi could be partially bypassed by direct injection of siRNAs.

Although R2D2 is also proposed to function as a Dicer adaptor molecule, its role may be slightly different than that of Rde-4. The proposed role for R2D2 is facilitating siRNA transfer from Dicer2 to RISC[48]. In the absence of R2D2, Dicer2 has no defect in recognition or cleavage of dsRNA. Notably, an R2D2–Dicer2 complex, but not the PAZ-less Dicer2 alone, binds to siRNAs. This is consistent with the hypothesis that, in the absence of the recognition function supplied by the PAZ domain, Dicer may utilize adaptor molecules to recognize intermediaries in the pathway.

The PAZ domain may be particularly important for recognition of miRNA precursors. The single Dicer in *S. pombe*, whose genome does not contain miRNAs, does not contain a PAZ. In contrast, higher eukaryotes known to have miRNAs all have at least one PAZ-containing Dicer. The PAZ might be especially crucial for consistently determining the length of a mature miRNA by measuring from the end marked by Drosha cleavage. At least one adaptor molecule, Rde-4, does not seem to be involved in miRNA pathways, as Rde-4 mutants show global RNAi resistance without developmental phenotypes[47,49].

Conclusion

Our understanding of the biochemistry of RNAi has been enhanced by new data concerning the roles of RNase III molecules. The role of Dicer has been established in RNAi as the originator of 22-mers characteristic of silencing phenomena. A recent paper implicates a related RNase III enzyme, Drosha, in a processing step upstream of Dicer in miRNA biogenesis[29]. In addition to biochemistry, three-dimensional structures have proven to be helpful in deciphering the enzymology of RNase III molecules. In particular, the structures of the PAZ domains of *D. melanogaster* Argonautes 1 and 2 promise new insight into the function of this domain in Argonaute proteins as well as in Dicer. We must now anxiously await structures of relevant Dicer and RISC components in complex with their substrates to either confirm or refute these hypotheses.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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