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Ribonuclease revisited: structural insights into ribonuclease III family enzymes

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Ribonuclease III (RNase III) enzymes occur ubiquitously in biology and are responsible for processing RNA precursors into functional RNAs that participate in protein synthesis, RNA interference and a range of other cellular activities. Members of the RNase III enzyme family, including *Escherichia coli* RNase III, Rnt1, Dicer and Drosha, share the ability to recognize and cleave double-stranded RNA (dsRNA), typically at specific positions or sequences. Recent biochemical and structural data have shed new light on how RNase III enzymes catalyze dsRNA hydrolysis and how substrate specificity is achieved. A major theme emerging from these studies is that accessory domains present in different RNase III enzymes are the key determinants of substrate selectivity, which in turn dictates the specialized biological function of each type of RNase III protein.

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Introduction

An endonuclease specific for double-stranded RNA (dsRNA) substrates was first described and partially purified from *Escherichia coli* by Zinder and colleagues in 1968 [1]. Subsequent studies in bacteriophage, bacteria and other cell types showed that ribonuclease III (RNase III) enzymes produce functional ribosomal and other RNAs from precursor molecules by cleaving within helical segments. The discovery that RNase-III-type enzymes are responsible for generating microRNAs (miRNAs) and short interfering RNAs (siRNAs) during the initial steps of RNA interference (RNAi) further fueled interest in RNase III enzyme structures and mechanisms.

Over the past five years, as Dicer and Drosha were discovered to be eukaryotic RNase III family enzymes

responsible for processing precursor miRNA transcripts, structural data were emerging from studies of bacterial and yeast RNase III proteins. Structures of an *Aquifex* RNase III ribonuclease domain dimer [2] and the *Saccharomyces cerevisiae* Rnt1 dsRNA-binding domain bound to an RNA hairpin [3**] provided the first insights into RNase III active site organization and substrate recognition. Although models of RNase III substrate cleavage involving a dimeric active site had been proposed, careful enzymatic studies of RNase III active site mutants led to a model in which a single processing center catalyzes dsRNA cleavage [4**]. This model was confirmed by recent crystallographic structures of a Dicer enzyme and of *Aquifex* RNase III bound to a product dsRNA [5**,6**].

In this review, we discuss structural insights into the catalytic mechanisms, substrate recognition properties and macromolecular interaction surfaces of RNase III family enzymes. Recent findings highlight the roles of accessory domains present in different RNase III enzymes as determinants of substrate selectivity, which in turn governs the specific biological function of each type of RNase III protein.

The RNase III family

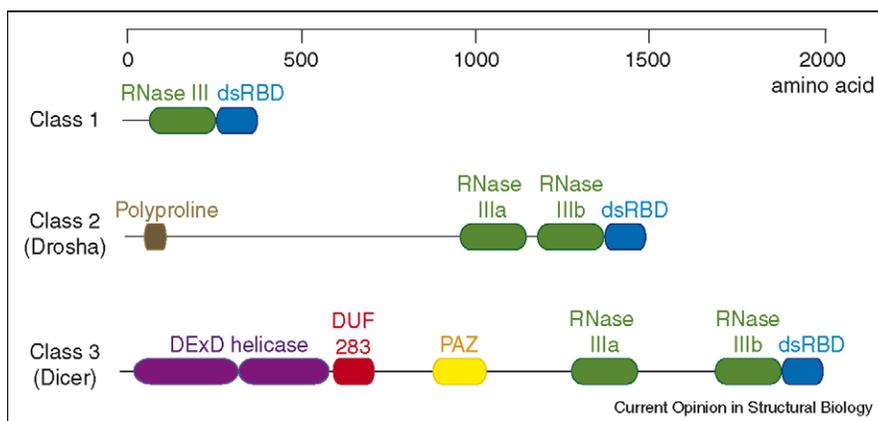
The RNase III family of enzymes is a collection of endoribonucleases that specifically cleave dsRNA. All RNase III family members contain a characteristic ribonuclease domain, which is commonly called the ‘RNase III domain’. Cleavage by RNase III produces a characteristic terminal dsRNA structure consisting of a 5′ phosphate group and a two base overhang at the 3′ end [1]. RNase III proteins vary widely in length, from ~200 to ~2000 amino acids, and have been subdivided into three classes based on domain composition [2,7] (Figure 1). Class 1 RNase III enzymes are the simplest and smallest, containing a single ribonuclease domain and a dsRNA-binding domain (dsRBD). Class 2 proteins have a dsRBD and two ribonuclease domains, which are commonly referred to as RNase IIIa and IIIb. Class 3 proteins are the largest and typically contain two ribonuclease domains, a dsRBD and an N-terminal DEXD/H-box helicase domain followed by a small domain of unknown function (DUF283) and a PAZ domain. Class 3 RNase III proteins are also known as the Dicer family of enzymes [8].

Class 1: bacterial RNase III enzymes

Class 1 is the best characterized and most extensively studied of the RNase III family [9]. These smallest

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Figure 1



Linear schematic of the RNase III classes. The top bar indicates scale in units of amino acid residues. 'RNase III domain' or simply 'RNase III' is commonly used as shorthand for the ribonuclease domain of RNase III enzymes. Functional domains are colored as follows: ribonuclease domain (RNase III), green; dsRBD, blue; proline-rich region, brown; DExD helicase domain, purple; DUF283, red; PAZ domain, yellow.

members of the RNase III family are found ubiquitously in bacteria, bacteriophage and some fungi. In *E. coli*, RNase III plays an important role in processing rRNA precursors (pre-rRNAs) [10,11] and also regulates translation by acting on target mRNAs [12–15]. Typical *E. coli* RNase III substrates are cellular or viral RNAs that have two complementary segments that are able to fold back and form dsRNA regions [16–18]. *In vitro*, bacterial RNase III will cleave any perfect-duplex dsRNA with little regard for sequence [19]. However, the RNAs targeted by RNase III in bacteria and bacteriophage contain recognition elements that direct RNase-III-mediated cleavage to precise and specific positions. Although the recognition elements in dsRNAs targeted by *E. coli* RNase III have been investigated for decades, a clear consensus sequence for RNase III recognition has not been established. However, disfavored or 'anti-determinant' sequences in RNase III substrates have been identified [20,21].

New insight into RNase III substrate recognition has come from the recent work of Pertzev and Nicholson [22^{••}], who established a minimal substrate of *E. coli* RNase III based on the bacteriophage T7 R1.1 processing signal RNA. Extensive analysis of RNase III activity using variants of this minimal substrate demonstrated that the dsRNA sequence extending 10 base pairs from the cleavage site can affect RNase III activity, with the first four base pairs (termed the proximal box) and the last two pairs (termed the distal box) being the most potent. Most bases in the proximal and distal boxes affect activity by contributing to substrate affinity, with the noted exception of the base pair on the 3' side of the scissile bond, which appears to have a direct impact on catalysis. The authors propose that RNase III recognizes the structure of the sugar–phosphate backbone in the proximal and distal boxes, and that mutating the RNA

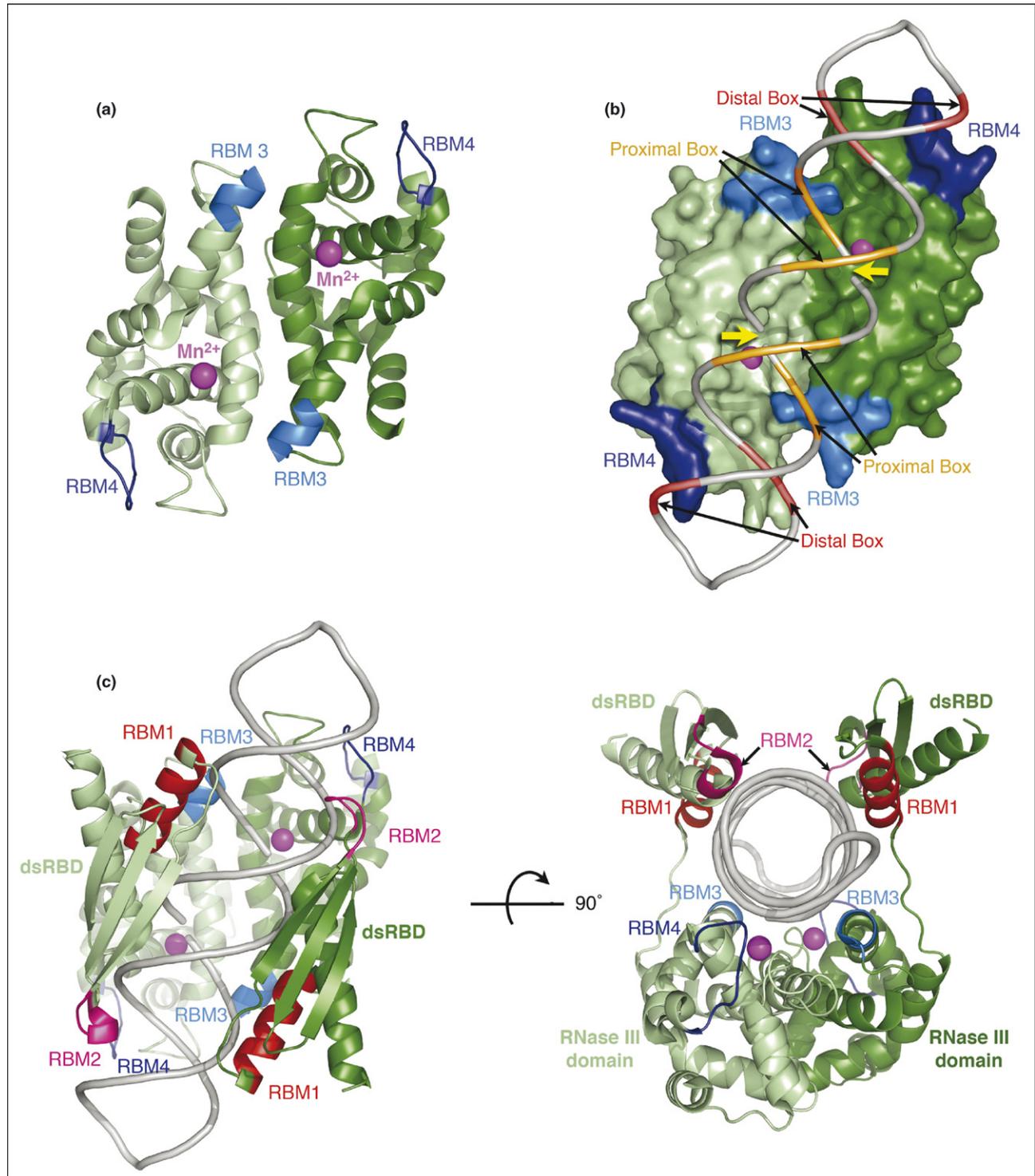
sequence in these regions leads to subtle changes in the duplex structure, which in turn reduces the affinity of the enzyme for the dsRNA.

Class 1 RNase III proteins function as homodimers [23,24], with dimerization occurring through the ribonuclease domains (Figure 2a). Recent biochemical work has shown that the two ribonuclease domains combine to form a single processing center, with each domain contributing to the hydrolysis of one RNA strand of the duplex substrate [4^{••}]. The two active sites lie at opposite ends of a long surface cleft (50 Å long and 20 Å wide) that has been termed the 'catalytic valley' [2] (Figure 2b).

The recent structure of *Aquifex* RNase III in complex with a cleaved product dsRNA revealed that the catalytic valley contains two discrete dsRNA-binding motifs (RBMs) that are conserved throughout the RNase III family (termed RBM3 and RBM4) [6^{••}] (Figure 2b). Amino acid sidechains from RBM3 and RBM4 form hydrogen bonds with non-bridging phosphate oxygen atoms and 2'-hydroxyl ribose oxygen atoms in the dsRNA backbone. In the product-bound structure, there is a single magnesium ion present in each active site. However, it is currently believed that RNase III uses a two-metal mechanism of catalysis, with each active site containing two magnesium cations during substrate hydrolysis [5^{••},25^{••},26^{••}]. The additional metal ions are thought to be only loosely associated with the protein until a substrate dsRNA is bound or are perhaps only present in the transition state.

In bacterial RNase III, the dsRBDs are connected to the ribonuclease domains by flexible linkers [27,28,29[•]]. In the structure of the protein bound to a product dsRNA, the dsRBDs sit on almost the opposite side of the RNA duplex — putting the dsRNA in a molecular 'bear hug'

Figure 2



Structural insight into bacterial RNase III. **(a)** Crystal structure of the ribonuclease domain dimer of *Aquifex* RNase III (PDB code 1RC5). The two polypeptide chains are identical in sequence and colored different shades of green for clarity. Conserved RBMs are colored blue. Purple spheres represent catalytic divalent metal ions. **(b)** Surface representation of the *Aquifex* RNase III ribonuclease domains bound to cleaved dsRNA, with dsRBDs omitted for clarity (PDB code 2EZ6). Yellow arrows point to the cleavage sites in the RNA. Proximal and distal boxes of the RNA are colored red and orange, respectively. **(c)** Structure of the complete *Aquifex* RNase III bound to cleaved dsRNA. RBM1 and RBM2 are colored red, and RBM3 and RBM4 are colored blue.

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(Figure 2c). The dsRBDs contain two conserved RBMs (termed RBM1 and RBM2), which make extensive contacts with the sugar–phosphate backbone of the distal box of the bound dsRNA [22**].

Class 1: Rnt1

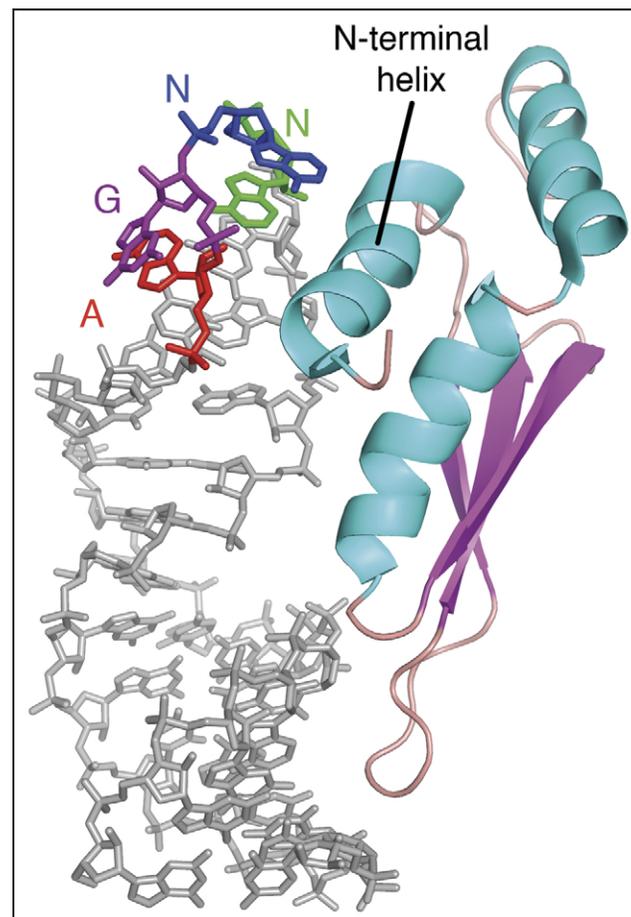
The best-characterized eukaryotic class 1 RNase III is the *S. cerevisiae* enzyme Rnt1. Rnt1 plays important roles in the processing of yeast pre-rRNAs [30,31], and many small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) [7,32–34]. Rnt1 also participates in the regulation of specific mRNAs [35,36]. The common feature of Rnt1 substrates is a dsRNA hairpin with a tetraloop structure containing the consensus sequence AGNN [24,37]. Recognition of the hairpin structure is mediated by the Rnt1 dsRBD [38*]. The solution structure of the Rnt1 dsRBD in complex with an AGNN tetraloop hairpin revealed that the dsRBD contacts the RNA at successive minor, major and tetraloop minor grooves on one face of the dsRNA helix [3**] (Figure 3). Surprisingly, the structure also showed that the conserved guanine and adenine bases do not participate in specific hydrogen bonds with the protein, but instead are important for forming a distinct tetraloop minor groove [39], which accommodates the N-terminal helix of the dsRBD. This finding highlights the importance of subtle RNA structures in mediating interactions with RNase III proteins. Recognition of the tetraloop by the dsRBD positions the ribonuclease domains on the cleavage site, which is 13–16 base pairs from the tetraloop.

Class 2: Drosha

The founding member of the class 2 RNase III proteins is Drosha. There is currently no published structure of Drosha or any closely related protein, but biochemical experiments indicate that Drosha functions as a monomer, with its ribonuclease domains forming an internal ‘dimer’ structure [40*], similar to that seen in Dicer (see below).

Drosha is involved in the processing of human pre-rRNA [41] and also plays a major role in the maturation of small regulatory miRNAs [42]. miRNAs arise from long RNA polymerase II transcripts that fold back on themselves to form stable internal RNA hairpins called pri-miRNAs [43–46]. Drosha excises pre-miRNA hairpins from the long primary transcripts [42]. Pre-miRNA hairpins are subsequently cleaved by Dicer into mature miRNA duplexes [47–49]. Interestingly, purified Drosha alone cannot excise pre-miRNA hairpins accurately, but instead acts as a general RNase III that cleaves pri-miRNAs indiscriminately. The specificity of substrate recognition comes from a Drosha-associated protein called DiGeorge syndrome critical region gene 8 (DGCR8) in humans [50**]. The *Caenorhabditis elegans* ortholog of DGCR8 is named Pasha (partner of Drosha) [51,52] and the Drosha–Pasha complex has been aptly

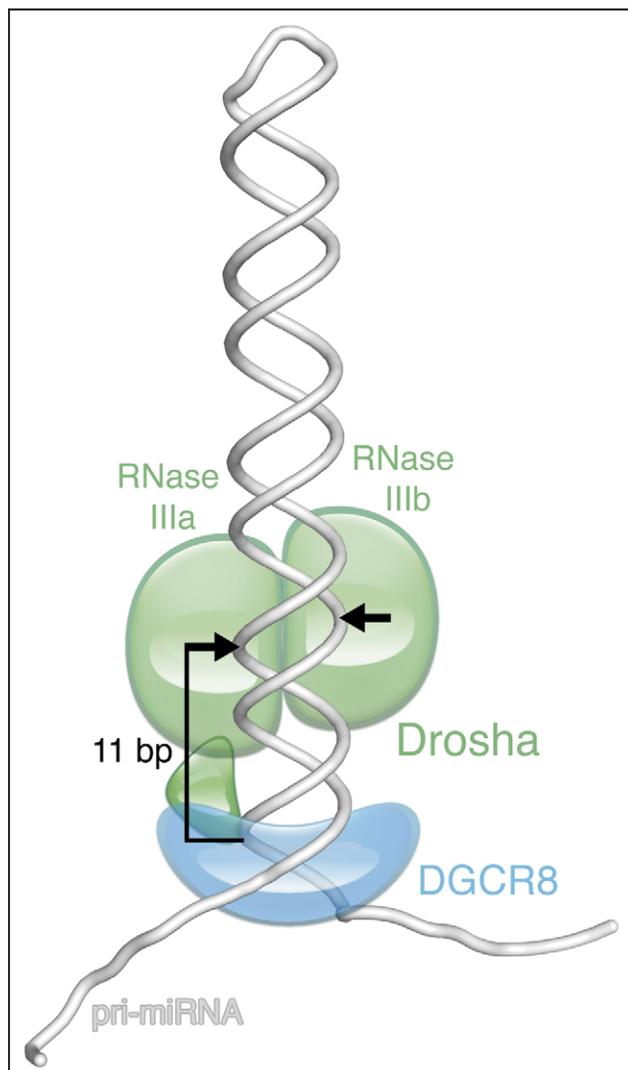
Figure 3



The dsRBD of Rnt1 specifically recognizes dsRNAs containing AGNN tetraloops. The NMR structure of the dsRBD bound to a target RNA is shown (PDB code 1T4L). The AGNN bases of the tetraloop are labeled and colored red, purple, blue and green, respectively.

named the ‘Microprocessor’ to signify its role in the processing of pri-miRNAs. DGCR8 is an RNA-binding protein that recognizes dsRNA–ssRNA junctions and positions the Drosha ribonuclease domains eleven nucleotides, or one turn of the dsRNA helix, away [50**] (Figure 4). Thus, the function of DGCR8 in the Microprocessor is analogous to the function of the dsRBD of Rnt1 (and the PAZ domain of Dicer, discussed below); DGCR8 confers substrate specificity and proper positioning of the Drosha ribonuclease center. However, in the case of Drosha–DGCR8, the specificity domain is located on a polypeptide chain separate from the RNase III domains. Interestingly, DGCR8 contains a proline-binding WW domain that has been proposed to mediate interactions with the proline-rich N terminus of Drosha [51]. It is therefore possible that Drosha may have other WW-domain-containing binding partners that engender it with alternative substrate specificities and additional biological functions.

Figure 4



Schematic cartoon of the Microprocessor complex bound to a pri-miRNA. Drosha and DGCR8 subunits are colored green and blue, respectively. Black arrows indicate the cleavage sites in the pri-miRNA.

Class 3: Dicer

Class 3 RNases III, or Dicer enzymes, process dsRNA substrates into small RNA fragments of discrete size, typically 21–27 nucleotides in length [8]. The small RNAs produced by Dicer act as guides for sequence-specific silencing of cognate genes through RNAi and related pathways. The recent crystal structure of Dicer from the human parasite *Giardia intestinalis* revealed that the ability of Dicer to generate dsRNA products of discrete length stems from its accessory domains [5^{••}]. Unlike Dicer proteins found in higher eukaryotes, *Giardia* Dicer does not possess a DExD/H-box helicase domain, a DUF283 domain or a dsRBD (Figure 5a). Containing only the PAZ and tandem ribonuclease domains, the *Giardial* protein may be considered a

‘minimal’ Dicer, which probably represents the core structure of all Dicer proteins.

Giardia Dicer is an elongated molecule measuring 100 Å long and 30–50 Å wide. In contrast to class 1 RNase III proteins, Dicer functions as a monomer. The two ribonuclease domains of Dicer associate with each other, forming an internal ‘dimer’ that resembles the ribonuclease homodimer of class 1 enzymes [4^{••},5^{••}] (Figure 5b). When viewed from the front, the molecule resembles the shape of a hatchet, with the RNase III ribonuclease domains making up the blade and the PAZ domain at the base of the handle (Figure 5a). The PAZ and ribonuclease domains are connected by a long helix that runs the length of the handle. The N-terminal residues of the protein encompass the connector helix, forming a flat platform on the face of the molecule.

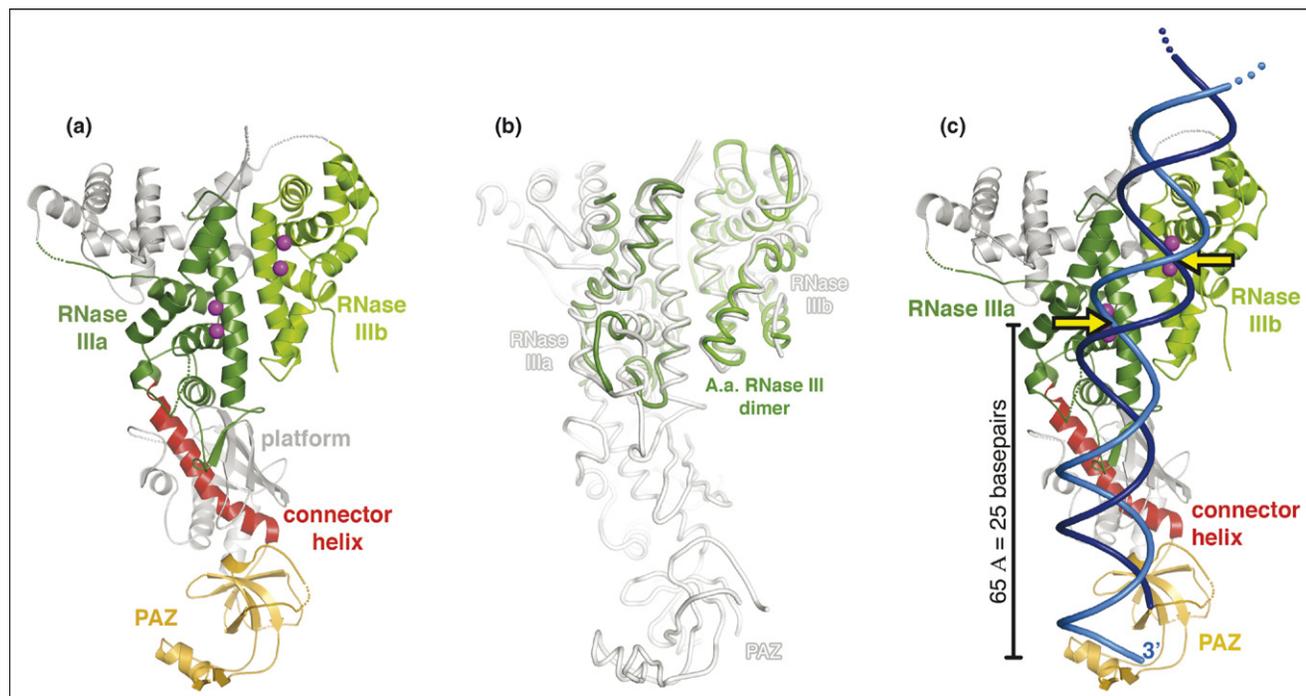
Dicer is thought to function as a molecular ruler that generates dsRNA fragments of discrete size by recognizing the end of a dsRNA substrate and cleaving a set distance away (Figure 5c). The PAZ domain is responsible for binding to the end of the dsRNA [53[•],54–56] (see [57] for a review) and the connector helix is the structural element that sets the measuring distance from the dsRNA end to the cleavage site. In the case of *Giardia* Dicer, which generates dsRNA products 25–27 nucleotides in length, the distance from the region of the PAZ domain that binds the RNA 3' end to the catalytic center in the first ribonuclease domain is 65 Å. This distance closely matches the length spanned by 25 dsRNA base pairs. In human Dicer, which produces dsRNAs 21–23 nucleotides long, this distance is likely to be slightly shorter, suggesting that human Dicer may have a shorter connector helix. Thus, as in the case of Rnt1 and Drosha-DGCR8, it is the accessory domains that endow Dicer with its specific function by directing the ribonuclease domains to the proper dsRNA target site.

Conclusions and future directions

The dimeric catalytic domain structure shared by all RNase III family enzymes explains the unique ability of these endoribonucleases to catalyze dsRNA cleavage, with each ribonuclease domain acting on one strand of the duplex RNA substrate. Building on this core structure, different classes of RNase-III-type enzymes have evolved additional domains or interaction partners that recognize distinct kinds of dsRNA substrates. The yeast protein Rnt1, a class 1 RNase III, uses its specialized dsRBD to recognize AGNN tetraloops, whereas the class 3 Dicer enzymes use their PAZ domain to bind free dsRNA ends. The class 2 Drosha proteins associate with the accessory protein DGCR8, which recognizes ssRNA-dsRNA junctions. These differences in substrate specificity enable the RNase III family to process a wide variety of RNA precursors into mature RNAs with diverse functions.

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Figure 5



Crystal structure of Dicer from *G. intestinalis*. **(a)** Ribbon representation of *Giardia* Dicer with structural domains colored individually (PDB code 2FFL). Purple spheres represent the proposed binding sites for the catalytic metal ions. **(b)** Superposition of the *Aquifex* ribonuclease domain homodimer (green) onto the ribonuclease domains of *Giardia* Dicer (white) reveals the same core ribonuclease structure. **(c)** Modeling dsRNA onto the structure of Dicer demonstrates that the distance from the PAZ domain to the ribonuclease domains determines the length of the RNA produced by Dicer. Yellow arrows indicate sites of RNA cleavage.

Despite recent structural advances, numerous intriguing questions remain. Mechanistic details of RNase III catalysis await future high-resolution structural studies of substrate-bound enzymes, using either RNA containing a non-hydrolyzable phosphodiester linkage or a protein active site mutant. Studies with *E. coli* RNase III have shown that substrate secondary structure and sequence influence binding and cleavage rate, suggesting possible similar effects of pre-miRNA secondary structure on Dicer activity that need to be explored. A model of Dicer bound to a dsRNA substrate compares favorably with the crystal structure of a bacterial RNase III–dsRNA product complex, although this model predicts that Dicer substrates are bent. Whether Dicer induces dsRNA bending, or whether the protein flexes to accommodate dsRNA, will be important to determine, as this impacts the resulting size of dsRNA products. Finally, the roles of accessory domains of RNase III family members remain to be fully elucidated. For example, what is the function of the second dsRBD of the Rnt1 dimer? Only one is necessary to bind the RNA substrate AGNN tetraloop, but two are present in the protein dimer. In Dicer, the functions of the DExD/H-box helicase and DUF domains are currently not known. Future structural and biochemical research will be necessary to address these questions. And the continued discovery of new

small-RNA families, such as the PIWI-interacting RNAs (piRNAs) associated with spermatogenesis [58–61] and the repeat-associated small interfering RNAs (rasiRNAs) implicated in germline stability [62], raises the possibility that RNase-III-type enzymes, perhaps assisted by different accessory domains or interaction partners, may produce ssRNA products. Or perhaps other, as yet unidentified, ribonucleases are responsible for piRNA and rasiRNA production. Stay tuned.

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