# Single Processing Center Models for Human Dicer and Bacterial RNase III

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# Summary

Dicer is a multidomain ribonuclease that processes double-stranded RNAs (dsRNAs) to 21 nt small interfering RNAs (siRNAs) during RNA interference, and excises microRNAs from precursor hairpins. Dicer contains two domains related to the bacterial dsRNAspecific endonuclease, RNase III, which is known to function as a homodimer. Based on an X-ray structure of the Aquifex aeolicus RNase III, models of the enzyme interaction with dsRNA, and its cleavage at two composite catalytic centers, have been proposed. We have generated mutations in human Dicer and Escherichia coli RNase III residues implicated in the catalysis, and studied their effect on RNA processing. Our results indicate that both enzymes have only one processing center, containing two RNA cleavage sites and generating products with 2 nt 3' overhangs. Based on these and other data, we propose that Dicer functions through intramolecular dimerization of its two RNase III domains, assisted by the flanking RNA binding domains, PAZ and dsRBD.

# Introduction

Dicer is a large endoribonuclease responsible for processing double-stranded RNAs (dsRNAs) to  $\sim$ 20 bplong small interfering RNAs (siRNAs) acting as effectors during RNA interference (RNAi), and also for excision of microRNAs (miRNAs) from the hairpin precursors. Being a key enzyme for RNAi and for miRNA function, Dicer proteins have been found in all eukaryotes studied to date, with the exception of baker's yeast. The number of genes encoding Dicer-like proteins varies from four in Arabidopsis to one in vertebrates (reviewed by Hannon and Zamore, 2003). Mutations in Dicer proteins in different organisms have developmental phenotypes (reviewed by Hannon and Zamore, 2003; Schauer et al., 2002), likely resulting from the compromised formation of miRNAs (Carrington and Ambros, 2003). In zebrafish and mouse, the Dicer-encoding gene is essential (Bernstein et al., 2003; Wienholds et al., 2003).

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Dicers are approximately 200 kDa multidomain proteins. Typically, their domains include a DExH RNA helicase/ATPase domain, the DUF283 and PAZ signatures, two neighboring RNase III-like domains (RIIIa and RIIIb), and a dsRNA binding domain (dsRBD) (Figure 1A). Although no mutagenesis has been performed to date on Dicer proteins, the dsRBD and RNase III domains are most certainly involved in dsRNA binding and cleavage. This is supported by the findings that  $\sim$ 20 bp products of Dicer processing contain 2 nt 3' overhangs and 5'-p and 3'-OH termini, characteristic features of RNase IIImediated reactions (Elbashir et al., 2001; Nicholson, 2003). Functions of the remaining Dicer domains are not known. The PAZ domain is also found in Dicerinteracting proteins involved in RNAi and miRNA function, referred to as PAZ and Piwi Domain (PPD) proteins (reviewed by Carmell et al., 2002). Structural studies of the PAZ domain of the Drosophila PPD proteins Ago1 and Ago2 revealed similarity to the oligonucleotide binding (OB) fold, consistent with the RNA binding activity of the domain (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). The presence of the helicase/ATPase domain could be related to the findings that generation of siRNAs by C. elegans and Drosophila Dicers is greatly stimulated by the addition of ATP (Bernstein et al., 2001; Ketting et al., 2001; Liu et al., 2003; Nykänen et al., 2001). However, no such effect is observed for the mammalian enzyme (Zhang et al., 2002). Moreover, Dicer of Dictyostelium is devoid of the helicase/ATPase domain (Martens et al., 2002).

Irrespective of the specific role of individual domains, Dicer emerges as a very complex and dynamic enzyme, interacting with other cellular proteins. Apart from Ago proteins, shown recently to associate with Dicer directly (Tahbaz et al., 2004), these are two related small proteins, R2D2 of Drosophila (Liu et al., 2003) and RDE-4 of C. elegans (Tabara et al., 2002), and the Drosophila ortholog of the human fragile X mental retardation protein, dFMR1 (Ishizuka et al., 2002). Recent studies with recombinant human and Drosophila Dicers revealed some additional properties of the enzyme (Liu et al., 2003; Provost et al., 2002; Zhang et al., 2002). For example, the human enzyme has a strong preference for cleaving siRNAs from the ends of dsRNA substrates. In addition, activity of both the endogenous and recombinant human Dicer is strongly stimulated by proteolysis, suggesting that access to the catalytic center of the enzyme is perhaps regulated by other domains of the protein (Zhang et al., 2002).

Sequence similarity to RNase III was crucial for identification of Dicer as a protein involved in generating siRNAs (Bass, 2000; Bernstein et al., 2001; Billy et al., 2001; Ketting et al., 2001). According to current classification (Blaszczyk et al., 2001; Nicholson, 2003), Dicer belongs to the class 3 enzymes of the RNase III superfamily. The class 1 members include RNases III superfamily. The class 1 members include RNases III from bacteria and fungi, and the class 2 encompasses metazoan RNases III, exemplified by Drosha. In contrast to Dicer and metazoan RNases III, prokaryotic and lower eukaryotic RNases contain one RNase III domain (Figure



Figure 1. Schemes of the RNase III Superfamily Proteins and Summary of RNase III and Dicer RNase III Domain Sequences and Mutants (A) Three classes of the RNase III family proteins represented by human Dicer (class 3), human Drosha (class 2), and bacterial RNase III (class

1). Individual protein domains are indicated in different colors.

(B) The postulated mechanism of dsRNA cleavage by Aa-RNase III (Blaszczyk et al., 2001). (A) and (B) represent two subunits of the homodimer enzyme, which was proposed to bind dsRNA (shown as a stack of the Watson-Crick [WC] base pairs) in the intersubunit cleft and to contain two compound catalytic centers. Residues E37 and E64, and D44 and E110 of each center, proposed to be responsible for the generation of products with 2 nt 3' overhangs, are indicated. Residues E40 and D107, involved with D44 and E110 in coordinating the metal ion, are not shown.

(C) Alignment of conserved regions of Aa-RNase III (SwissProt accession O67082), Ec-RNase (P05797), and Dicer domains RIIIa and RIIIb (Q9UPY3; Zhang et al. 2002), containing residues equivalent to the Aa-RNase III E37 and D44, E64 and E110 (in red).

(D) Summary of single amino acid mutants in RNase III domains of human Dicer and Ec-RNase III.

1A). However, they function as homodimers, as established by biochemical work (reviewed by Nicholson, 2003) and structural studies on the bacterium Aquifex aeolicus (Aa) RNase III (Blaszczyk et al., 2001). Based on the X-ray structure of the catalytic domain of Aa-RNase III and mutagenesis of the Escherichia coli RNase III (Ec-RNase III), Blaszczyk et al. (2001) proposed a model of the dsRNA cleavage by the enzyme. In this model, shown schematically in Figure 1B, the RNase III dimer contains two compound catalytic centers, positioned at the ends of the postulated dsRNA binding cleft, each cutting two nearby phosphodiester bonds, positioned on opposite RNA strands. Within each catalytic center, two clusters of acidic residues, one comprising Glu40, Asp44, Asp 107, and Glu110, and another residues Glu37 and Glu64, would be responsible for cleavage of individual diester bonds. The former four residues coordinate single metal ions, Mn<sup>2+</sup> or Mg<sup>2+</sup>, present in each enzyme monomer. Based on the spacing of the catalytic centers, the model predicts generation of 9 bp products with 2 nt 3' overhangs, consistent with the size of products generated by RNase III in vitro (Blaszczyk et al., 2001).

The bacterial RNase III structure and activity models raised many speculations regarding the mechanism of dsRNA cleavage by Dicer and the need to explain the size difference—approximately 10 versus 20 bp—in the products of RNase III and Dicer reactions. Is Dicer functioning as a pseudodimer, with RIIIa and RIIIb domains of one molecule interacting with each other, or as a true dimer in which RIII domains associate together intermolecularly in either homologous or heterologous combinations? Irrespective of the model, the size difference between RNase III and Dicer products was suggested to be due to inactivation of one of the two catalytic centers, with an evolutionarily conserved glutamate changed to proline in the position equivalent to the Aa-RNase III Glu64, a putative catalytic residue (Blaszczyk et al., 2001; Hannon, 2002; Nicholson, 2003; Zamore, 2001; see Figure 1C).

We generated mutations in all human Dicer and Ec-RNase III residues implicated in the catalysis and studied their effect on processing of dsRNA and hairpin substrates. Our results demonstrate that both enzymes have only one dsRNA processing center, containing two catalytic sites and generating products containing 3'-protruding ends. We also studied sedimentation properties of the human Dicer and the effect of mutations in its PAZ and dsRBD domains. Collectively, our data indicate that Dicer functions as an intramolecular dimer of RIIIa and RIIIb domains, assisted by two RNA



## Figure 2. Activity of wt and Mutant Dicer Proteins

(A) SDS-8% PAGE of purified proteins. The gel was stained with GelCode Blue Stain (Pierce). Lane M, protein size markers (in kDa).
 (B) Processing of the internally <sup>32</sup>P-labeled 50 bp dsRNA. Lane "-",dsRNA incubated without addition of the protein. Positions of oligoribonucleotide markers are indicated.

(C) Processing of the 30 bp dsRNA containing 2 nt 3' overhangs and <sup>32</sup>P-labeled at the 5' end of either upper (30 bp U\*) or lower (30 bp L\*) strands. Lanes T1 and L, RNase T1 and alkaline ladders. Positions of cleavages affected in 44a and 110a, and 44b and 110b mutants are indicated in red and green, respectively and marked in the scheme in the lower image. Thickness of arrows represents cleavage intensity. (D) Schematic representation of processing of the 50 bp dsRNA substrate <sup>32</sup>P-labeled at the 5' end of either the upper or lower strand. For the autoradiogram of the gel, see Supplemental Figure S3 available on *Cell* website. Cleavages marked with the composite red/green arrows represent secondary events occurring only when processing of both strands in the central region of the substrate has taken place (see text and Figure S3).

binding domains, dsRBD and PAZ, the latter domain being likely involved in the recognition of the 3'-overhang end.

## Results

# Processing of dsRNAs by Dicer RNase III Domain Mutants

For insight into the mechanism of Dicer cleavage we generated single amino acid mutations in its residues equivalent to the Glu37, Asp44, Glu64, and Glu110, which have been proposed to comprise the catalytic centers in Aa-RNase III. With the exception of Dicer residue Pro1731, which was substituted by glutamine, all other residues were changed to alanine. To simplify mutant description and to facilitate comparison of the Aa-RNase III, Ec-RNase III, and human Dicer data, we follow the Aquifex protein numbering and refer to the two RNase III domains of Dicer as RIIIa and RIIIb. Accordingly, mutations in Dicer residues Glu1313, Asp1320, Glu1340, and Glu1652 are referred to as 37a, 44a, 64a, and 110a, respectively, and mutations in residues Gln1702, Asp1709, Pro1729, and Glu1813, as 37b, 44b, 64b, and 110b, respectively (for summary of all RIII domain mutations, see Figure 1D). Mutant proteins were overexpressed in insect cells and purified as described previously (Zhang et al., 2002), yielding preparations of comparable purity (Figure 2A).

Activity of wild-type (wt) and mutant proteins was first tested with the 50 bp and 70 bp internally <sup>32</sup>P-labeled dsRNA substrates, which were previously shown to un-

dergo effective processing by the recombinant human enzyme. Surprisingly, four of the mutant proteins (37a, 64a, 37b, and 64b) processed the substrates into 21 nt fragments with an activity comparable to that of the wt Dicer (Figure 2B, Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/118/1/57/DC1, and data not shown). The remaining mutants were considerably less active. With the 50 bp substrate, which gives a clearer readout, mutants 44b and 110b yielded lower levels of ~21 nt RNAs and also products of ~29 nt, while mutants 44a and 110a only generated products having length of 25–27 nt. Notably, each pair of mutants appeared to generate a subset of products formed by the wt enzyme (Figure 2B, and see below).

We also generated three sets of double mutants in Dicer RIII domains. Mutants 44a110a and 44b110b combine mutations 44 and 110 in Dicer domains RIIIa and RIIIb, respectively. Mutants 44ab and 110ab combine mutations in equivalent positions in each Dicer RIII domain, and mutants 37ab and 64a37b are combinations of mutations which had no apparent effect on Dicer activity. 44ab110ab is a quadruple mutant combining mutations 44a110a and 44b110b. Mutants 44ab and 110ab, and the quadruple mutant were found to be completely inactive, while other double mutants mimicked precisely the activity of the single amino acid mutants from which they were derived; identical results were obtained for both the 70 bp (Supplemental Figure S1 available on Cell website) and the 50 bp (data not shown) substrates.

We have demonstrated previously that Dicer preferen-

tially cleaves off siRNAs from the termini of dsRNA substrates (Zhang et al., 2002). We took advantage of this observation to map cleavage sites introduced by the wt and mutant proteins in the 30 bp substrate. The 30 bp dsRNA can be cleaved by Dicer only once yielding the  $\sim$ 21 nt siRNA-like products and the cut-off fragments (Zhang et al., 2002; and our unpublished results). The 30 bp RNA can be accessed by Dicer from either end but the resulting "end-specific" cleavages can be monitored independently from each other when single strands of the substrate are labeled at the terminus. As shown in Figure 2C, incubation of the wt Dicer with the 30 bp substrate containing either the upper or lower strand labeled at the 5' terminus yielded series of processing products diagnostic of the enzyme approaching the substrate either from the label-containing (20-23 nt fragments) or opposite (9-12 nt fragments) end. Consistent with the analysis of the internally labeled substrates (see Figure 2B), processing of the 5'-end-labeled 30 bp RNA was unaffected by mutations 37a, 64a, 37b, and 64b (Figure 2C). In contrast, mutations 44a and 110a prevented processing at the labeled-end proximal but not distal sites, while the reverse was true for mutations 44b and 110b. This was irrespective of whether the 5' label was on the upper or the lower strand of the substrate (Figure 2C). The 30 bp substrates used in this experiment contained 2 nt 3' overhangs at both ends but similar results were obtained with the blunt-ended 30 bp dsRNA (data not shown). Likewise, processing of the terminally labeled 30 bp dsRNA substrate with a different sequence and base composition yielded a very similar cleavage pattern (Supplemental Figure S2 available on Cell website).

The most straightforward interpretation of the data presented above is that residues equivalent to the Aa-RNase III Asp44 and Glu110 of both Dicer RNase III domains are part of one processing center responsible for the dsRNA cleavage, with residues of the domain RIIIa being required for the cleavage of one RNA strand and residues of RIIIb for the nearby cleavage of the second strand, resulting in the formation of products with 3' overhangs. This conclusion is further supported by analysis of terminally labeled 50 bp substrate processing by Dicer mutants (Figure 2D; for autoradiogram of the gel, see Supplemental Figure S3 available on Cell website). This analysis also provides an explanation for the differences in the length of processing products of the internally labeled 50 bp dsRNA generated by the RIIIa and RIIIb domain mutants (see Figure 2B). Since inactivating mutations in domain RIIIa, 44a and 110a, prevent cleavage at sites separated by  $\sim$ 21 nt from RNA 3'-hydroxyl ends (sites shown in red in Figure 2D), the only products formed are those resulting from processing at the nearby sites (shown in green) on complementary strands; for the substrate containing blunt ends, as with the 50 bp dsRNA, these products are expected to be approximately 23 to 27 nt in length, consistent with the data shown in Figure 2B. Conversely, the inactivating mutations in the domain RIIIb, 44b and 110b, prevent cleavage at sites close to the center of the dsRNA (sites shown in green in Figure 2D), and only the products of processing at sites on the complementary RNA strands (shown in red in Figure 2D) accumulate; these products are expected to have lengths of approximately 21 and 29 nt, also in agreement with the data in Figure 2B.

Analysis of terminally labeled 50 bp substrates revealed additional sets of cleavages, marked with the composite red/green arrows in Figure 2D. Positions of these cleavages and the fact that they are observed with the wt protein and its mutants 37 and 64, but not mutants 44 and 110, in both RIII domains (see Supplemental Figure S3 available on *Cell* website), indicate that they represent secondary events occurring only when processing of both strands in the central region of the substrate has taken place. The requirement for the primary processing event explains why no labeled products diagnostic of the cleavage at complementary sites on opposite RNA strands could be identified (Supplemental Figure S3 available on *Cell* website).

# Processing of Pre-let-7 RNA by Dicer Mutants

We extended the analysis of Dicer RIII domain mutants to miRNA precursors, which represent another class of cellular substrates of Dicer (Hannon and Zamore, 2003). Long primary transcripts containing miRNA sequences are first processed in the nucleus by Drosha into  $\sim$ 70 nt long hairpins referred to as pre-miRNAs. The premiRNAs are then matured by Dicer to  $\sim$ 21 nt miRNAs in the cytoplasm (Lee et al., 2003). We generated the precursor of let-7 miRNA, expected to correspond to the Drosha cleavage product (Basyuk et al., 2003; Lee et al., 2003; see Figure 3C), by the self-processing of the in vitro hybrid transcript, which contains the hammerhead ribozyme upstream of the pre-let-7 sequence. Processing of the internally labeled pre-let-7 RNA by the wt protein yielded the double-stranded siRNA-like product, as established by gel electrophoresis under nondenaturing conditions (Figure 3A). Analysis of processing of pre-let-7 RNA, terminally labeled at either the 5' or 3' end, by different Dicer mutants revealed that the cleavage is unaffected by mutations 37a, 64a, 37b, and 64b, and that mutations 44a and 110a, and 44b and 110b strongly inhibit processing at adjacent sites on the descending and ascending hairpin arms, respectively (Figures 3B and 3C). These data corroborate conclusions derived from the dsRNA substrate experiments. Collectively, they demonstrate that residues equivalent to the Aa-RNase III Asp44 and Glu110 of both Dicer RIII domains are part of one processing center containing two RNA cleavage sites functioning independently of each other, and that amino acids equivalent to Aa-RNase III residues Glu37 and Glu64 are not essential for the catalysis. The data also indicate that during both the pre-let-7 RNA (Figure 4) and dsRNA (e.g., see Figure 2D) processing, Dicer accesses the substrate in a polar fashion, with the RIIIa domain always cutting the RNA strand bearing a 3'-hydroxyl, at approximately 21 nt from the end.

## Activity of the E. coli RNase III Mutants

The Dicer mutagenesis and dsRNA and pre-miRNA processing data are inconsistent with the proposed model of dsRNA cleavage by the bacterial RNase III. They are also in conflict with some previous experimental data indicating that the Ec-RNase III residues equivalent to



Figure 3. Processing of Pre-let-7 RNA by Recombinant Dicer Enzymes

(A) Processing of internally <sup>32</sup>P-labeled pre-*let-7* RNA by the wt Dicer monitored by nondenaturing PAGE. The substrate (3 fmol) was incubated in the absence (lane 1) or presence (lanes 2 and 3, containing 10 and 30 ng of Dicer, respectively). Lanes 4 and 5, 21 nt single-stranded and double-stranded siRNA markers, respectively. The band marked with the asterisk corresponds the top region of the hairpin.
(B) Mapping of cleavage sites in the 5'-end (left image) or 3'-end (right image) <sup>32</sup>P-labeled pre-*let-7* RNA. The RNA was incubated in the absence (lanes "-") or presence of indicated Dicer preparations. Dicer-induced cleavage sites on ascending and descending hairpin arms are indicated by green and red bars, respectively. Unspecific, nonenzymatic cleavages at UA and UG phosphodiesters in the bulge are marked with asterisks.

(C) Secondary structure of pre-let-7 RNA and positions of cleavage sites as determined in B. Cleavages affected by mutations 44a and 110a, and 44b and 110b, are in red and green, respectively.

the Aa-RNase III Glu37 and Glu64 are essential for the catalysis. Since the latter conclusion was based on a rather indirect assessment of effects of mutations on enzyme activity, namely determining the Ec-RNase IIIdependent expression of the  $\lambda N$ -lacZ reporter in vivo (Blaszczyk et al., 2001), we reinvestigated activity of Ec-RNase III mutants by more direct methods. Four single amino acid mutants of Ec-RNase III (Glu38Ala, Asp45Ala, Glu65Ala, and Glu117Ala, referred to as 37ec, 44ec, 64ec, and 110ec, respectively; see Figure 1D), in positions corresponding to Aa-RNase III residues Glu37, Asp44, Glu64, and Glu110, were constructed and proteins overexpressed and purified (Figure 4A). Their activity was compared with that of the wt protein, using dsRNA, R1.1 RNA, and R1.1[WC] RNA as substrates. R1.1 and R1.1 [WC] RNAs, shown in Figure 4E, represent well-characterized Ec-RNase III substrates. R1.1 RNA corresponds to the phage T7 RNA fragment processed only at the descending arm of the hairpin, and R1.1[WC] RNA is modified such that it is processed at both hairpin arms (Li and Nicholson, 1996; Nicholson, 2003).

Reactions with the internally labeled 70 bp (Figure 4B) and 50 bp (data not shown) dsRNAs revealed that mutations 44ec and 110ec very strongly compromise activity of the enzyme; low levels of products and intermediates were only observed when a large excess of mutant proteins was used. This is consistent with the previous findings that mutation of Glu117 (residue substituted in the 110ec mutant) strongly inhibits activity of the *E. coli* enzyme (Nicholson, 2003; and references therein). In contrast, mutants 37ec and 64ec showed activity comparable with that of the wt protein (Figure 4B).

Terminally labeled 30 bp dsRNA, and R1.1 and R1.1[WC] RNAs were used to map cleavage sites by wt and mutant E. coli proteins (Figures 4C-4E). The wt Ec-RNase III cleaved 30 bp dsRNA in the central region and, as expected, processed R1.1 and R1.1 [WC] RNAs at the descending arm and at both the ascending and descending arms, respectively. Mutants 37ec and 64ec cleaved all substrates in a manner similar to the wt protein, while mutants 44ec and 110ec were inactive. These findings are consistent with the results of Dicer mutagenesis and indicate that the bacterial RNase III, which functions as a homodimer, also has only one processing center, with the Ec-RNase III residues Asp45 and Glu117 (equivalent to Aa-RNase III residues Asp44 and Glu110) of each monomer contributing to the cleavage of two diester bonds separated by two base pairs and present on opposite RNA strands.

We used the R1.1 single cleavage substrate to compare kinetic parameters of the wt Ec-RNase III with that of its mutants 37ec and 64ec.  $V_{max}$  values for all three proteins were similar but  $K_m$  values for mutants 37ec and 64ec were, respectively, 5 and 3 times higher than the 137 nM value determined for the wt enzyme (Supplemental Table S1 available on *Cell* website), suggesting a small deficiency in the substrate binding. It is possible that this deficit of mutant proteins is an underlying factor explaining the inability of similar mutants to process the  $\lambda N$ -*lacZ* reporter in vivo as reported by Blaszczyk et al. (2001). (We note that in this work only one of the Glu residues was mutated to alanine; the other was mutated to valine.) Since the  $\lambda N$ -*lacZ* reporter processing was measured following the shift of bacteria to 42°C (Blasz-



Figure 4. Activity of Recombinant wt and Mutant Ec-RNases III

(A) The purified wt or mutant proteins (0.5  $\mu$ g) were analyzed by SDS-15% PAGE. Lane M, size markers.

(B) Processing of the 70 bp internally labeled dsRNA (3 fmol). Assays contained either 10 ng (left image) or 70 ng (right image) of the indicated protein.

(C) Processing of the 30 bp dsRNA containing 2 nt 3' overhangs and terminally labeled on either the upper (30 bp U\*) or lower (30 bp L\*) strand. Cleavage positions are shown in the bottom image.

(D) Processing of the 5'-<sup>32</sup>P-labeled R1.1 (left image) and R1.1[WC] (right image) RNAs. RNase T1 and alkaline ladders in the right image represent longer exposure of the same gel. Cleavage positions at  $U_{47}$  (R1.1) and  $U_{20}$  and  $U_{48}$  (R1.1[WC]) are indicated; assignment of cleavages to  $U_{47}$  and  $U_{48}$  was confirmed by long migration gels. Longer exposure of the  $U_{48}$  region is shown at the bottom.

(E) Structure of R1.1 and R1.1[WC] RNAs with marked cleavage sites.

czyk et al., 2001), we have tested whether mutant proteins retain activity at this temperature. The 37ec and 64ec mutants processed dsRNA at 37°C and 42°C with comparable activity (data not shown).

## **Gradient Sedimentation Analysis of Dicer**

As shown above, each of the two RNase III domains of Dicer, RIIIa and RIIIb, contributes amino acids to one processing center generating products with a short 3' overhang. Thus, Dicer likely operates as a monomer, which functionally represents an intramolecular pseudo-dimer. We used glycerol gradients to determine whether Dicer sediments as a monomer or a larger complex. Analysis of purified Dicer indicated that the enzyme fractionates at the position corresponding to a molecular mass of ~180 kDa (Figure 5A). The enzyme in a complex with the 30 bp dsRNA substrate having 3' overhangs sedimented at similar position (Supplemental Figure S4 available on *Cell* website), as did Dicer present in the extract prepared from insect cells overproducing the protein (data not shown). We also analyzed properties of

endogenous Dicer in extracts of mouse teratocarcinoma P19 cells (Billy et al., 2001). The protein was found to sediment at  $\sim$ 230 kDa, suggesting that it may be associated with other cellular component(s) of a relatively small size (Figure 5B). Shorter centrifugation of the gradient revealed that an appreciable amount of Dicer also sediments in a region of 80S or larger, suggesting that a fraction of Dicer in P19 cells is associated with ribosomes or another large complex (Figure 5C). The data support a model in which domains RIIIa and RIIIb of a single Dicer molecule interact together to form an intramolecular pseudodimer.

## Activity of Mutants in Dicer PAZ and dsRBD Domains

Human Dicer and most of its orthologs in other species contain a PAZ domain positioned N-terminally to RIIIa (Hannon and Zamore, 2003; Schauer et al., 2002). This approximately 150 amino acid long domain is also found in PPD proteins (Carmell et al., 2002), and structural studies of the PAZ domain of *Drosophila* PPD proteins



Figure 5. Sedimentation of Purified Dicer (A) and P19 Cell Extract (B and C) on 10%–30% Glycerol Gradients

Position of protein markers (aldolase, 158 kDa; catalase, 232 kDa; ferritin, 440 kDa), and ribosomal subunit and 80S ribosome markers, run in parallel gradients, are indicated. Lanes C, fraction of the input extract. Dicer was visualized by Western blotting. Centrifugation was for 20 hr in (A) and (B), and for 4.5 hr in (C).

Ago1 and Ago2 revealed similarity to the OB fold, consistent with the RNA binding activity of the domain (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). Three Dicer mutant proteins were overexpressed and purified to test the importance of the PAZ domain for RNA processing. In mutants F960A and YY971/2AA, conserved aromatic amino acids, Phe960, and Tyr971 and Tyr972, respectively, are replaced by alanine. Equivalents of the mutated residues were demonstrated to be important for optimal RNA binding of the Ago protein PAZ domains (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003) (for an alignment of PAZ domains of Dicer and PPD proteins, see Supplemental Figure S5 available on Cell website). The third mutant is E1036A. Equivalent mutation in Drosophila Ago1 had no effect on RNA binding activity (Yan et al., 2003), but in Ago2, the mutation of the corresponding Glu residue to Lys rendered the PAZ protein insoluble (Lingel et al., 2003). In addition, Dicer protein with the C-terminal dsRBD domain deleted,  $\Delta$ dsRBD, was prepared. When assayed with the 70 bp blunt-ended dsRNA as a substrate, all four mutations markedly decreased Dicer activity. Mutations of the two adjacent tyrosines in the PAZ domain and deletion of the dsRBD had the strongest effect, lowering activity of the enzyme approximately 10-fold (Supplemental Figure S6 available on Cell website).

Binding studies with the PAZ domain of Ago proteins indicated that it may recognize the 3'-protruding ends of siRNAs (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). Thus, we compared activity of the wt and mutant Dicer proteins in processing of 30 bp substrates containing either blunt (30 bp) or 2-nt 3'-overhang (30 bp-UU) ends. The wt protein processed the 30 bp-UU RNA with nearly 2-fold higher efficiency than the 30 bp RNA, and each of the three PAZ mutations had a 1.4to 1.9-fold stronger effect on processing of the 30 bp-UU than on the 30 bp substrate (Figure 6A; for quantification, see the legend). A stronger discrimination between 30 bp and 30 bp-UU substrates was displayed by the  $\Delta$ dsRBD mutant. While this mutation decreased the cleavage of the 30 bp-UU RNA only 1.9-fold, it decreased 4-fold the cleavage of the 30 bp RNA (Figure 6B). We also compared ability of the wt Dicer and the ∆dsRBD mutant to process the let-7 RNA precursor containing either 2 nt 3'-protruding (pre-let-7) or blunt (pre-let-7<sub>BL</sub>) ends. As with the dsRNA substrates, the wt protein showed an ~2.5-fold preference for the pre-*let-7* RNA with the 3' overhang, and the processing of this substrate was less strongly affected by the dsRBD deletion than the processing of pre-*let-7*<sub>BL</sub> (Figure 6C). Of note, we found that processing of pre-let-7 RNA by Dicer is not affected by the absence of a phosphate on the recessed 5'-end (data not shown). Taken together, these results indicate that Dicer has a preference for processing of substrates bearing 3' overhangs; this preference gets accentuated by the deletion of dsRBD. Moreover, the results support a role for the Dicer PAZ domain in the recognition of the substrate 3'-protruding end (see Discussion).

## Discussion

Our data indicate that both the human Dicer and the bacterial RNase III contain only a single dsRNA processing center responsible for cleavage of two nearby phosphodiester bonds on opposite RNA strands, generating products containing 2 nt 3'-protruding ends. Residues equivalent to the Aa-RNase III Asp44 and Glu110 of two RNase III domains contribute to this active center, while residues equivalent to Glu37 and Glu64, previously proposed to be required for the RNA cleavage (Blaszczyk et al., 2001), were found to be nonessential. Dispensability of Glu37 and Glu64 equivalents for the catalysis is further supported by the recent finding that mutation of these two residues in the RIIIa domain of the Drosophila Dicer does not inactivate the enzyme (Lee et al., 2004). Since in the case of Dicer, two different RNase III domains, RIIIa and RIIIb, contribute to the processing center, analysis of Dicer proteins bearing mutations in individual RIII domains was particularly revealing. This analysis, performed with both dsRNA and pre-let-7 substrates, demonstrated that mutations of Asp44 and Glu110 equivalents in the RIIIa domain prevent cleavage of one RNA strand but have no effect on cutting the other, while the reverse is true for mutations in the RIIIb domain. Hence, the two catalytic sites present in the RIIIa/RIIIb dimer function independently of each other.

Simultaneous mutations in both Dicer RIII domains (mutants 44a44b or 110a110b) abolished cleavage of each RNA strand. These Dicer double mutants are functionally equivalent to the Ec-RNase III single amino acid



Figure 6. Activity of Dicer PAZ and dsRBD Domain Mutants

(A) Kinetics of processing of the internally labeled 30 bp dsRNA containing either blunt ends (30 bp) or 2 nt 3' overhangs (30 bp-UU) by the wt Dicer and the PAZ domain mutants. Proteins added to the reaction and times of incubation are indicated at the top. PhosphorImager quantification revealed that the wt Dicer cleaves 1.92-fold and 1.54-fold more of the input 30 bp-UU then 30 bp RNA at time points 3 min and 10 min, respectively (ratios based on averages of three independent experiments similar to those shown in (A) and (B), The PAZ mutations E1036A, YY971/2AA, F960A had 1.4-, 1.4-, and 1.9-fold stronger effect on processing of 30 bp-UU then 30 bp RNA, respectively (values represent averages of 3 and 10 min time points from two independent experiments). Note that the 30 bp-UU RNA contains four additional U residues (see Figure 2C), which are labeled with <sup>32</sup>P. Appropriate corrections were made to calculate moles the substrate and the reaction products. (B and C) Comparison of activities of wt and ∆dsRBD Dicers to cleave 30 bp and 30 bp-UU substrates (B) and pre-let-7 RNA containing either blunt (pre-let-7<sub>BL</sub>) or 3'-protruding (prelet-7) ends (C). In (B), the AdsRBD mutation decreased cleavage of the 30 bp-UU RNA 1.9-fold, and cleavage of 30 bp RNA 4.0-fold (averages from three independent experiments). Reactions in (A-C) contained 40 ng of Dicer proteins and 3 fmol of RNA.

mutants 44ec and 110ec. Since bacterial RNase III functions as a homodimer (Nicholson, 2003), the mutations affect processing of both RNA strands. Notably, mutation 44ec inhibited RNA cleavage more severely than mutation 110ec. It is possible that Asp44 (Asp45 in Ec-RNase III; changed to alanine in the 44ec mutant), which is engaged in metal ion binding via the water molecule in Aa-RNase III (Blaszczyk et al., 2001), acts as a general base that deprotonates the metal bound water (see also Nicholson, 2003).

Based on the results of RNase III and Dicer mutagenesis, and other findings presented in this work, we propose new models for substrate cleavage by both enzymes. In the bacterial Aa-RNase III model (Figures 7A-7C, and Supplemental Figure S7 available on Cell website), the dsRNA substrate is rotated by approximately 30° with regard to its position in the model of Blaszczyk et al. (2001). The following arguments support the new placement of the substrate and its availability for the cleavage by the enzyme. (1) In the new position, each of the two scissile phosphodiester bonds is in the proximity of four acidic residues (Glu40, Asp44, Asp107, and Glu110), which coordinate the metal ion (Mn<sup>2+</sup> or Mg<sup>2+</sup>) and constitute the catalytic site for the cleavage of one strand. (2) In the model, each catalytic site of the dimer contains a metal ion required for the catalysis. The distance to the scissile bonds (see legend to Figure 7) is consistent with both metal ions participating in the catalysis, each involved in cleaving one of the two RNA strands. In the reported crystal structure of Aa-RNase III, the two metal ions coordinated by four acidic residues in each monomer are the only metal ions identified. No metal ions were found coordinated by residues Glu37 and Glu64, generating speculations about possible repositioning of the substrate in order for the second diester bond cleavage to take place (Nicholson, 2003). (3) The enzyme surface along the new dsRNA placement contains many conserved amino acid residues, likely contributing to substrate binding (Figure 7C; for positions of conserved residues in the alignment of RNase III domains of different proteins, see Supplemental Figure S8 available on *Cell* website).

In the new model, no catalytic role is assigned to residues equivalent to Aa-RNase III Glu37 and Glu64. However, these amino acids are not far from the RNA helix and could, in the presented model, contact the RNA substrate either directly or indirectly via an ion. Such a proximity might explain an increase of  $K_m$  values for Ec-RNase III mutants 37ec and 64ec. Notably, Glu37 and Glu64 residues are not 100% conserved in RNase III domains, in contrast to residues equivalent to Asp44 and Glu110. In different members of the RNase III superfamily, Glu, Gln, and Asp residues are present at the Glu37 position, and Glu, Gln, Pro, and Val residues are found at the Glu64 position. For example, in the *Thermotoga maritima* RNase III, the crystal structure of



Figure 7. Models of RNase III and Dicer Interactions with dsRNA

(A and B) Frontal view of a complete (A) and an enlarged central region (B) of the Aa-RNase III catalytic domain dimer (Blaszczyk et al., 2001), interacting with the dsRNA helix representing the 30 bp dsRNA shown in Figure 2C. Proteins are represented as ribbons; the two monomers are in different shades of purple.  $Mn^{2+}$  ions are as red spheres. Residues coordinating the  $Mn^{2+}$  ion in each monomer are shown as yellow sticks.  $Mn^{2+}$  ions present in each proposed catalytic site are 21.5 Å apart, while the distance between the two scissile phosphodiester bonds (marked with arrows), across the minor groove, is 17.2 Å;  $Mn^{2+}$  ions and phosphorus atoms of cleavable bonds in the model are approximately 5 Å apart. These distances are likely to change upon dsRNA binding to the enzyme. In the same vein, we cannot exclude that additional ions are present in the activated complex. In our model, the dsRNA substrate is rotated by approximately 30° clockwise as compared to its position, parallel to the subunit interface, in the model of (Blaszczyk et al., 2001).

(C) Distribution of conserved amino acids in 75 bacterial and fungal RNase III proteins. Residues conserved in more than 70% of proteins are in green. The modeled-in dsRNA is shown as ribbons. Two monomers are shown in different colors.

(D) A model of dsRNA processing by Dicer. Individual domains of Dicer are in different colors. Helicase/ATPase and DUF283 domains, with no assigned function, are delineated with a broken line. The RIIIa domain is drawn as a larger rectangle than RIIIb to illustrate its larger size. Its placement also illustrates the fact that this domain cleaves the 3'-OH-bearing RNA strand.

which has been recently determined (Protein Data Bank ID 100W), Glu64 is replaced by valine. Importantly, a model of the dsRNA-protein complex similar to that for the Aa-RNase III could also be built for the *T. maritima* enzyme (our unpublished results).

Mutagenesis has demonstrated that Dicer domains RIIIa and RIIIb contribute together to one "dimeric" catalytic center, analogous to that of the bacterial RNase III homodimer. Since during gradient sedimentation, Dicer behaves as a monomer rather than a dimer, also when associated with the substrate, we propose that the two interacting RIII domains originate from a single Dicer molecule. Bacterial and fungal RNases III assemble into dimers in the absence of substrate and X-ray analysis has revealed the important contribution of hydrophobic interactions to this process (Blaszczyk et al., 2001). Sequence alignments and modeling of the conserved core regions of Dicer RIIIa and RIIIb domains indicated that interactions similar to the hydrophobic "ball-socket" contacts identified in bacterial enzymes (Blaszczyk et al., 2001; Protein Data Bank ID 100W) may also occur between Dicer RIIIa and RIIIb domains (our unpublished observations). Additional support for the intramolecular dimer, containing a single processing center, is offered by the observation that long dsRNA substrates are always shortened successively by  $\sim$ 20 bp siRNA-like units and never by longer segments of dsRNA (for example, see Figure 2 in Zhang et al., 2002). The dependence of secondary processing events on prior cleavages in the center of the 50 bp substrate (Figure 2D and Supplemental Figure S3 available on *Cell* website), also argues for the stepwise excision of the  $\sim$ 20 bp segments by an enzyme with a single processing center.

In most Dicer proteins, the two RIII domains are flanked by the PAZ and dsRBD domains, positioned N- and C-terminally, respectively (see Figure 1A). The PAZ domains of Dicer and PPD proteins were originally thought to mediate interaction between these proteins, but recent work has shown that RIII and Piwi domains of Dicer and PPD proteins are responsible for the interaction (Tahbaz et al., 2004). Recent structural studies of the PAZ domain of *Drosophila* PPD proteins Ago1 and Ago2 revealed its RNA binding potential (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003), raising a possibility that the PAZ domain of Dicer contributes to substrate recognition (Song et al., 2003). Indeed, we found that mutations of four conserved amino acids, either singly (mutants F960A and E1036A) or in combination (mutant YY971/2AA), have marked inhibitory effect on the activity of the protein. A role for the PAZ domain in dsRNA processing is further supported by the finding that the purified C-terminal fragment of Dicer, encompassing RIIIa, RIIIb, and dsRBD domains, shows no dsRNA cleavage activity (our unpublished results).

We have shown previously that human Dicer preferably cleaves off siRNA products from dsRNA ends and that blocking the ends with RNA tetraloops or RNA-DNA duplexes significantly delays the cleavage reaction (Zhang et al., 2002). Moreover, studies of miRNA precursor processing in vertebrate cells have revealed that Dicer preferentially excises miRNAs from pre-miRNA hairpin intermediates produced by Drosha, possibly due to the availability of proper free ends generated by this enzyme (Lee et al., 2003). The results presented in this work directly demonstrate that Dicer not only requires free dsRNA ends but that it also cleaves substrates with 2 nt 3'-overhangs more effectively than those containing blunt ends. Although for the wt Dicer this preference was found to be relatively small, deletion of the dsRBD domain made the enzyme more dependent on the presence of the 3'-overhang during the processing of both dsRNA and pre-let-7 RNA substrates. Most probably, the deletion of dsRBD, a domain generally known to mediate unspecific interactions with dsRNA, makes Dicer's interaction with the substrate more dependent on its specific structural features and on protein domains other than its dsRBD. Our findings that mutations in the PAZ domain strongly inhibit Dicer activity, and that this inhibition is greater for the dsRNA containing 3' overhangs than that with blunt ends, strongly suggest that the PAZ domain of Dicer participates in the recognition of the terminal 3' overhangs. This conclusion is in agreement with the demonstrated preference of the PAZ domain of Drosophila Ago1 and Ago2 for single-stranded RNAs or double-stranded siRNAs with 2 nt 3' overhangs, as opposed to blunt-ended siRNAs (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). It is likely that the recognition of staggered RNA ends is a major physiological function of the PAZ domain in both Dicer and PPD proteins.

One of the features distinguishing reactions catalyzed by Dicer and the bacterial RNA III is the length of the products, approximately 20 bp and 10 bp, respectively. In previous models, based on the data of Blaszczyk et al. (2001), this size difference was suggested to be due to inactivation of one of the two processing centers, postulated for the RNase III class enzymes by these authors (see Introduction). Our demonstration that both Dicer and bacterial RNase III contain only one active processing center makes this explanation unlikely. Which Dicer domain(s) could be responsible for measuring the distance between the end and the cleavage site? Since mutations in the Dicer PAZ domain were found to have an effect on the processing efficiency but not on the length of the products (Figure 6A, Supplemental Figure S6 available on Cell website), we consider it improbable that this domain is a major size-determining factor. It is more likely that the atypical N-proximal RIII domain of Dicer, RIIIa, perhaps assisted by PAZ, measures the distance between the substrate terminus and the cleavage site. RIIIa of all Dicer proteins is much longer than the RIIIb domain and all other typical RNase III catalytic domains, including the N-proximal RNase III domain of Drosha proteins (see Figure 1A).

A model of the dsRNA cleavage by Dicer, incorporating the results of mutagenesis and gradient analyses, and all considerations discussed above, is presented in Figure 7D. The most important feature of the model is the presence of a single dsRNA cleavage center, which is formed by the RIIIa and RIIIb domains of the same Dicer molecule, and which processes the dsRNA approximately 20 bp from its terminus. The PAZ domain recognizes the substrate terminus with the 3' overhang, and the RIIIa domain, possibly in conjunction with PAZ, measures the distance to the cleavage site. The placement of the RIII domains illustrates the asymmetry of the catalytic region, with RIIIa cleaving the 3'-hydroxyland RIIIb cleaving the 5'-phosphate-bearing RNA strand.

The recombinant Dicer utilized in this study is catalytically inefficient. When assayed at excessive (30-100 nM) substrate concentrations, it generates 0.5-1.0 mole of the siRNA product per mole of enzyme in a 30 min incubation (Zhang et al., 2002; our unpublished results). The low activity of purified Dicer is at least partially due to the reaction product, which remains associated with the enzyme (Zhang et al., 2002). Recent studies of the RNA-induced silencing complex (RISC) in Drosophila demonstrated that Dicer accompanies siRNA along the RISC assembly pathway and is an essential component of mRNA-cleaving "holo-RISC", sedimenting at  $\sim$ 80S (Lee et al., 2004; Pham et al., 2004; Tomari et al., 2004). The  $\sim$ 80S Dicer-containing complex identified in this study (Figure 5C) may represent the mammalian equivalent of Drosophila holo-RISC. The finding that Dicer assembles into RISC along with the siRNA offers an explanation for the low activity of the purified enzyme. In the absence of other RISC components, Dicer would remain associated with its siRNA cargo and be incapable of catalyzing multiple cleavage reactions. The finding that Dicer forms part of RISC (Lee et al., 2004; Pham et al., 2004; Tomari et al., 2004) also provides a rationale for the observation that binding of the PPD protein hAgo2 to Dicer inhibits the RNase activity of the enzyme (Tahbaz et al., 2004). Possibly, one function of Ago proteins, established components of RISC (reviewed by Hannon and Zamore, 2003), is to prevent cleavage of dsRNA by Dicer present in the complex to avoid that a single RISC is associated with more than one siRNA.

## **Experimental Procedures**

## Preparation of RNA Substrates

Unless indicated otherwise, all substrates contained 5'-p and 3'-OH ends. dsRNAs, containing either blunt or 2 nt 3'-protruding ends, were obtained by annealing of appropriate RNAs obtained by the T7 polymerase in vitro transcription. Nonradioactive RNAs were synthesized using the Ambion T7 MegaShortScript transcription kit. After transcription, samples were treated with DNase I, extracted with phenol, and purified by denaturating 10% polyacrylamide gel electrophoresis (PAGE). Following dephosphorylation by calf intestine phosphatase (CIP), RNAs were 5'-end-labeled, using T4 polynucleotide kinase and [ $\gamma^{-32}$ P]ATP. The internally <sup>32</sup>P-labeled dsRNAs were prepared similarly, except that transcription was performed in the presence of [ $\alpha^{-32}$ P]UTP, and [ $\gamma^{-32}$ P]ATP was replaced by 10 mM cold ATP during the 5'-phosphorylation. Complementary RNA strands were annealed at 95°C for 3 min in 20 mM NaCl, transferred to 75°C, and then slowly cooled down to 20°C. The 3'-end-labeling of pre-*let-7* RNA was performed with T4 RNA ligase and [5'-<sup>32</sup>P]pCp; following ligation, the 3'-phosphate was removed by treatment with CIP, and RNA purified by PAGE. Prior to use, pre-*let-7* RNA was dissolved in water and renaturated by incubation at 90°C for 1 min, followed by incubation at 25°C for 15 min in 30 mM Tris-HCl, [pH 6.8] containing 50 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.1% Triton X-100, and 10% glycerol. The more AU-rich 30 bp dsRNA used in the experiment shown in Figure S2 was assembled from synthetic oligoribonucleotides (Dharmacon), following their 5'-end labeling.

## **RNA Processing Assays**

Unless indicated otherwise, processing of terminally labeled RNAs was performed as described (Zhang et al., 2002), and incubations were for 15 min at 37°C and contained 0.1 pmol of RNA and either 100 ng of Dicer preparation (0.23 pmol; assuming 50% purity of enzyme preparations) or 0.2 ng (3.8 fmol of dimer) of Ec-RNase III. Unless indicated otherwise, cleavage reactions with internally labeled dsRNAs contained 100 ng of Dicer protein and 125 fmol of the substrate, and were incubated for 30 min. Products were analyzed by denaturing 15% PAGE. RNase T1 ladders of the 5'-end-labeled RNAs were 3'-dephosphorylated by treatment with T4 polynucleotide kinase. Native 4% PAGE was performed as described previously (Zhang et al., 2002). Quantification was done using the Storm 860 PhosphorImager (Molecular Dynamics).

#### **Other Procedures**

These are described in the Supplemental Material (available on *Cell* website).

#### Acknowledgments

We thank N. Hynes and J. Hofsteenge for valuable discussions and V. Brondani for his contribution to the gradient sedimentation analysis. F.A.K. is the recipient of a fellowship from the Human Frontier Science Program. The Friedrich Miescher Institute is a part of the Novartis Research Foundation.

Received: February 9, 2004 Revised: April 28, 2004 Accepted: May 3, 2004 Published: July 8, 2004

#### References

Bass, B.L. (2000). Double-stranded RNA as a template for gene silencing. Cell 101, 235–238.

Basyuk, E., Suavet, F., Doglio, A., Bordonne, R., and Bertrand, E. (2003). Human let-7 stem-loop precursors harbor features of RNase III cleavage products. Nucleic Acids Res. *31*, 6593–6597.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363–366.

Bernstein, E., Kim, S.Y., Carmell, M.A., Murchison, E.P., Alcorn, H., Li, M.Z., Mills, A.A., Elledge, S.J., Anderson, K.V., and Hannon, G.J. (2003). Dicer is essential for mouse development. Nat. Genet. *35*, 215–217.

Billy, E., Brondani, V., Zhang, H., Muller, U., and Filipowicz, W. (2001). Specific interference with gene expression induced by long, doublestranded RNA in mouse embryonal teratocarcinoma cell lines. Proc. Natl. Acad. Sci. USA *98*, 14428–14433.

Blaszczyk, J., Tropea, J.E., Bubunenko, M., Routzahn, K.M., Waugh, D.S., Court, D.L., and Ji, X. (2001). Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. Structure 9, 1225–1236.

Carmell, M.A., Xuan, Z., Zhang, M.Q., and Hannon, G.J. (2002). The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev. *16*, 2733–2742.

Carrington, J.C., and Ambros, V. (2003). Role of microRNAs in plant and animal development. Science *301*, 336–338.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. *15*, 188–200.

Hannon, G.J. (2002). RNA interference. Nature 418, 244-251.

Hannon, G.J., and Zamore, P.D. (2003). Small RNAs, big biology: biochemical studies of RNA interference. In RNAi A Guide to Gene Silencing, G.J. Hannon, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 87–108.

Ishizuka, A., Siomi, M.C., and Siomi, H. (2002). A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. Genes Dev. *16*, 2497–2508.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. *15*, 2654–2659.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., and Kim, V.N. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425*, 415–419.

Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J., and Carthew, R.W. (2004). Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell *117*, 69–81.

Li, H., and Nicholson, A.W. (1996). Defining the enzyme binding domain of a ribonuclease III processing signal. Ethylation interference and hydroxyl radical footprinting using catalytically inactive RNase III mutants. EMBO J. *15*, 1421–1433.

Lingel, A., Simon, B., Izaurralde, E., and Sattler, M. (2003). Structure and nucleic-acid binding of the Drosophila Argonaute 2 PAZ domain. Nature 426, 465–469.

Liu, Q., Rand, T.A., Kalidas, S., Du, F., Kim, H.E., Smith, D.P., and Wang, X. (2003). R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. Science *301*, 1921–1925.

Martens, H., Novotny, J., Oberstrass, J., Steck, T.L., Postlethwait, P., and Nellen, W. (2002). RNAi in Dictyostelium: the role of RNAdirected RNA polymerases and double-stranded RNase. Mol. Biol. Cell *13*, 445–453.

Nicholson, A.W. (2003). The ribonuclease III superfamily: forms and functions in RNA, maturation, decay, and gene silencing. In RNAi A Guide to Gene Silencing, G.J. Hannon, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 149–174.

Nykänen, A., Haley, B., and Zamore, P.D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell *107*, 309–321.

Pham, J.W., Pellino, J.L., Lee, Y.S., Carthew, R.W., and Sontheimer, E.J. (2004). A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in Drosophila. Cell *117*, 83–94.

Provost, P., Dishart, D., Doucet, J., Frendewey, D., Samuelsson, B., and Radmark, O. (2002). Ribonuclease activity and RNA binding of recombinant human Dicer. EMBO J. 21, 5864–5874.

Schauer, S.E., Jacobsen, S.E., Meinke, D.W., and Ray, A. (2002). DICER-LIKE1: blind men and elephants in Arabidopsis development. Trends Plant Sci. 7, 487–491.

Song, J.J., Liu, J., Tolia, N.H., Schneiderman, J., Smith, S.K., Martienssen, R.A., Hannon, G.J., and Joshua-Tor, L. (2003). The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nat. Struct. Biol. *10*, 1026–1032.

Tabara, H., Yigit, E., Siomi, H., and Mello, C.C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DEXHbox helicase to direct RNAi in C. elegans. Cell *109*, 861–871.

Tahbaz, N., Kolb, F.A., Zhang, H., Jaronczyk, K., Filipowicz, W., and Hobman, T.C. (2004). Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer. EMBO Rep. *5*, 189–194.

Tomari, Y., Du, T., Haley, B., Schwarz, D.S., Bennett, R., Cook, H.A., Koppetsch, B.S., Theurkauf, W.E., and Zamore, P.D. (2004). RISC assembly defects in the Drosophila RNAi mutant armitage. Cell *116*, 831–841. Yan, K.S., Yan, S., Farooq, A., Han, A., Zeng, L., and Zhou, M.M. (2003). Structure and conserved RNA binding of the PAZ domain. Nature *426*, 468–474.

Zamore, P.D. (2001). Thirty-three years later, a glimpse at the ribonuclease III active site. Mol. Cell 8, 1158–1160.

Zhang, H., Kolb, F.A., Brondani, V., Billy, E., and Filipowicz, W. (2002). Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. EMBO J. *21*, 5875–5885.