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# Biochemical Principles of Small RNA Pathways

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## Key Words

assay, dsRNA, miRNA, piRNA, siRNA

## Abstract

The discovery of RNA interference (RNAi) is among the most significant biomedical breakthroughs in recent history. Multiple classes of small RNA, including small-interfering RNA (siRNA), micro-RNA (miRNA), and piwi-interacting RNA (piRNA), play important roles in many fundamental biological and disease processes. Collective studies in multiple organisms, including plants, *Drosophila*, *Caenorhabditis elegans*, and mammals indicate that these pathways are highly conserved throughout evolution. Thus, scientists across disciplines have found novel pathways to unravel, new insights in probing pathology, and nascent technologies to develop. The field of RNAi also provides a clear framework for understanding fundamental principles of biochemistry. The current review highlights elegant, reason-based experimentation in discovering RNA-directed biological phenomena and the importance of robust assay development in translating these observations into mechanistic understanding. This biochemical template also provides a conceptual framework for overcoming emerging challenges in the field and for understanding an expanding small RNA world.

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## Contents

INTRODUCTION .................. 296
Logical Flow of Classic Biochemistry .................. 296
HARNESSING THE PHENOMENON OF RNAi ...... 297
Molecular Events Underlying RNAi .......................... 297
Cell-Free Assay Development ...... 299
Scaling Up for Biochemical Purification ........ 299
IDENTIFICATION OF SMALL-INTERFERING RNA ... 299
A PARALLEL MICRO-RNA PATHWAY ......... 299
SMALL RNA BIOGENESIS ......... 300
siRNA EFFECTOR FUNCTION .... 301
miRNA EFFECTOR MECHANISMS................... 303
ACTIVATING RISC ................. 304
Helicase Model of RISC Activation ...................... 304
Slicer Model of RISC Activation .... 305
Overcoming Key Challenges for Understanding RISC Activation ...................... 305
DEFINING HOLO-RISC............ 306
GENOME-ENCODED SMALL RNA............................. 307
miRNA .......................... 307
Endogenous siRNA ................ 309
piRNA .......................... 310
REGULATION OF SMALL RNA PATHWAYS................. 310
Endogenous Inhibitors of RNAi .... 311
Posttranscriptional Control of miRNA Biogenesis ........... 311
Modulating an miRISC-Target mRNA Relationship ............ 311
Cellular Regulation of Small RNA Pathways ......... 312
CONCLUDING REMARKS ......... 312

## INTRODUCTION

Modern biological sciences encompass an expanding array of tools to probe the processes of life and disease. The rapid pace of discovery in the RNAi field has been the result of numerous outstanding contributions by investigators across biomedical disciplines. Although the importance of genetics, molecular biology, and cell biology cannot be overstated, advances in elucidating mechanisms of small RNA pathways have largely been achieved through traditional biochemical approaches. The purpose of the current review is to highlight the principles of classic biochemistry as applied to RNAi. Emphasis is placed on outlining the major achievements through which the field has matured, underscoring the rational basis for experimentation and key elements of assay development. For broader analyses, readers are directed to many wonderful recent reviews (1–9). In recounting the path the field has taken toward elucidating the biochemical mechanisms of RNAi, the present work may be particularly useful in providing a framework for understanding emerging regulatory RNA pathways.

### Logical Flow of Classic Biochemistry

The purpose of biochemistry is to deconstruct and reconstruct biological events in a cell-free system. The entry point for biochemistry is identification of specific molecular events underlying a biological process (**Figure 1**). This molecular insight enables the design of a cell-free system that recapitulates the biological activity. Two critical elements are required for in vitro assay development: (*a*) Engineered substrates must harbor essential features for catalytic processing to defined products, and (*b*) biological extracts must contain robust enzymatic activity to execute the reaction. Biochemistry is susceptible to in vitro artifacts that are often caused by a deficiency in assay design, i.e., the biochemical readout does not accurately reflect the biological process. Successful reproduction of a biological process in vitro is a fundamental biochemical achievement, which

enables direct and in-depth studies of a cellular event, minimizing the complexities that often confound cellular and in vivo studies.

Biochemical purification is a powerful and unbiased approach to identify the factors responsible for the biological activity (**Figure 1**). Two golden rules for biochemical purification are robust assay and unlimited material, i.e., a simple, rapid, and robust assay should be developed, often through repeated optimization of the original assay; and given the large-scale of purification, an abundant and economic source of material must be identified. Following this, a successful purification scheme can be developed, provided that the activity is resistant to salt exposure, exhibits excellent chromatographic behavior, and is sufficiently stable to withstand the arduous process of purification. A key indicator for success is the enrichment of specific activity (total activity relative to total protein) following sequential chromatography. The goal of purification is to approach homogeneity, i.e., a single factor(s) closely correlates with the activity, such that the candidate factor(s) responsible for the biochemical activity is (are) unequivocally identified by mass spectrometric analysis.

Functional validation of the candidate factor(s) typically involves three lines of experiments (**Figure 1**): (*a*) Immunodepletion of candidates should diminish, whereas immunoprecipitates concentrate, the catalytic activity from biological extract; (*b*) biochemical activity is reconstituted using recombinant proteins; and (*c*) biological function of candidates must be demonstrated in vivo. Genetic approaches are a powerful complement to biochemical studies and a gold standard for validation. However, the enormous amount of time and resources required for these studies, even in organisms accessible to genomic manipulation, often discourage such attempts. Fortunately, RNAi has significantly reduced these barriers for reverse genetic studies in most areas. Despite the high degree of difficulty, there is simply no substitute for traditional biochemistry in fundamental understanding of biological processes.

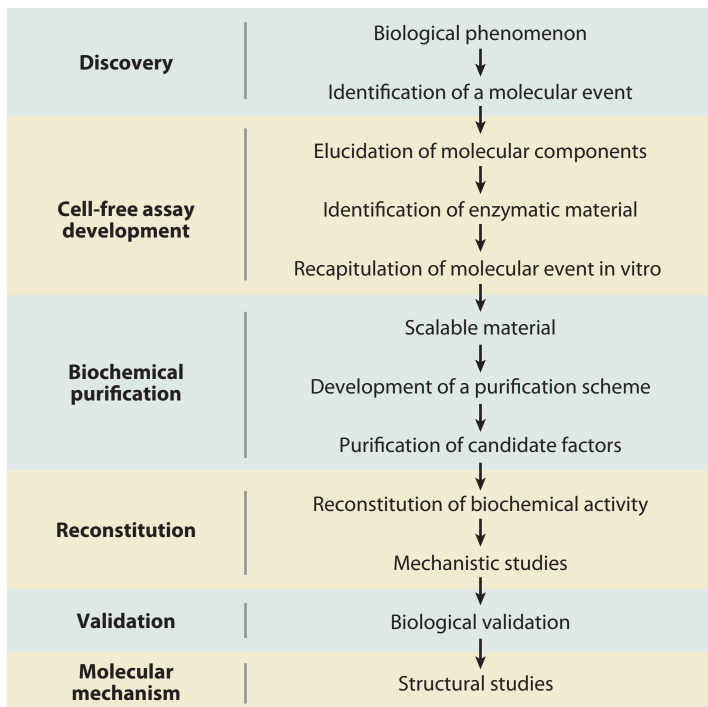


**Figure 1**

Logical flow of classic biochemistry.

## HARNESSING THE PHENOMENON OF RNAi

The first documentation of RNA silencing was derived from attempts to alter aesthetic characteristics of petunia. Aiming to overexpress chalcone synthase to enrich flower pigmentation, Jorgensen and coworkers (10) unexpectedly noted transgene-induced silencing (**Figure 2*a***). Based on the premise of nucleic acid homology, antisense RNA and oligonucleotides were used as loss-of-function perturbations. However, the mechanisms by which these interventions effected gene silencing were poorly understood.

**RNAi:** RNA interference

**Chromatography:** a method of purifying molecules in solution by taking advantage of differences in binding properties to stationary phase surfaces

**Immunodepletion:** use of antibody-coated solid phase material to remove a particular protein from solution

**Transgene:** genetic material that is expressed through engineered methods

**dsRNA:** double-stranded RNA

### Molecular Events Underlying RNAi

Andrew Fire and Craig Mello and their coworkers (11) systematically determined that injection of double-stranded RNA (dsRNA) homologous to *unc-22* messenger RNA (mRNA) was significantly more effective at mimicking



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the twitching phenotype of *unc-22* mutant worms than either sense or antisense RNA. This dsRNA-induced silencing phenomenon, termed RNA interference (RNAi), was shown to be systemic, heritable, and accompanied by reduction of target transcript. This work also established a green fluorescent protein (GFP) reporter assay such that RNAi silencing could be easily visualized in vivo (**Figure 2*b***). These seminal studies defined the molecular events underlying RNAi, i.e., dsRNA functions as a trigger for target transcript degradation.

Although the central dogma of molecular biology (DNA → RNA → protein) provided a useful framework to conceptualize gene expression, there were inherent constraints with this simplified picture. RNA was viewed primarily as a housekeeper simply transmitting genetic information from DNA to protein. Technically, RNA was traditionally studied as large polymers by agarose gel electrophoresis. Challenging these assumptions and reasoning that RNA silencing could be mediated by RNA smaller than typical transcripts, Hamilton & Baulcombe (12) employed polyacrylamide gel electrophoresis to connect RNA silencing with ∼25-nucleotide (nt) RNA in plants. The simplicity of these experiments highlighted the sophistication of

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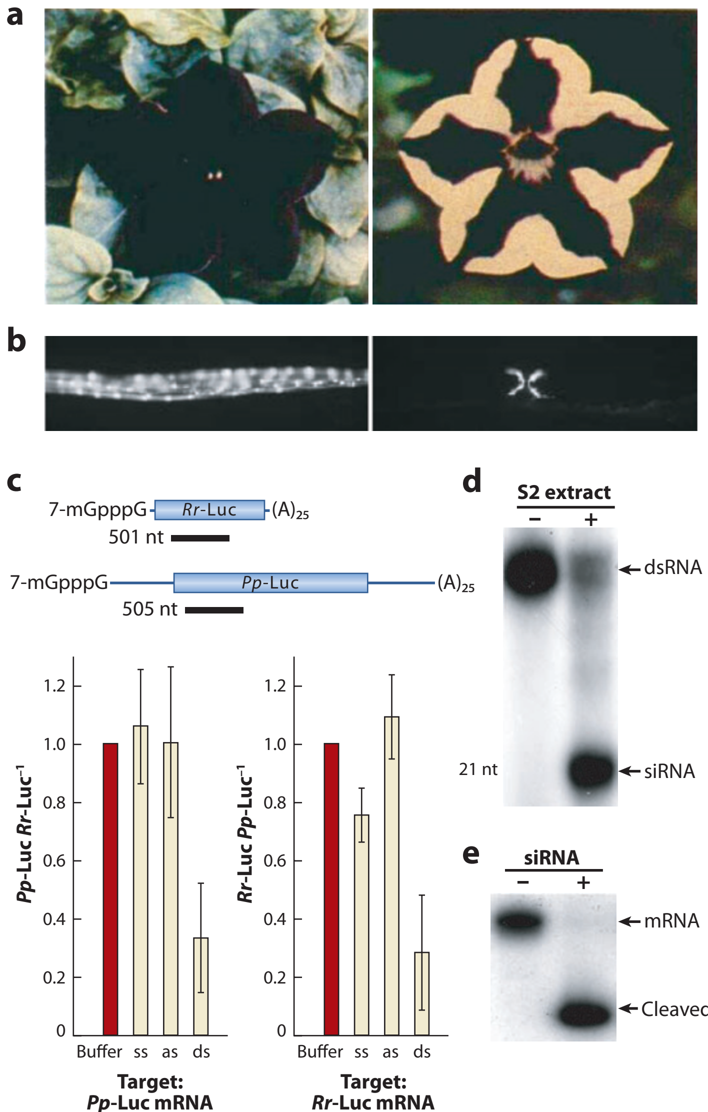


**Figure 2**

From biological phenomenon to cell-free assays. (*a*) Phenotypes of parental (*left*) and transgenic petunia expressing an ectopic chalcone synthase gene (*right*). Contrary to the enhanced pigmentation expected, Jorgensen and coworkers (10) observed reduced flower pigmentation. (*b*) Fire et al. (11) demonstrated dsRNA-induced silencing of a *gfp* transgene in adult worms. Animals were injected with control (*left*) or *gfp* dsRNA (*right*). (*c*) Tuschl et al. (13) established a cell-free system that recapitulated RNAi in *Drosophila* embryo extract. (*top*) Schematic indicates Renilla (*Rr*-Luc) and firefly luciferase (*Pp*-Luc) transcripts and homologous dsRNA. (*bottom*) Normalized reporter activity when using sense (ss), antisense (as), or dsRNA. (*d*) The Dicer assay showing that radiolabeled dsRNA is processed to 21–22-nt small-interfering RNA (siRNA) in *Drosophila* S2 cell extract. (*e*) The RNA-induced silencing complex (RISC) assay showing that duplex siRNA initiates RISC cleavage of 5′-radiolabeled target mRNA in S2 extract. Both assays were first established by Zamore (14) and Tuschl (16) and their coworkers in *Drosophila* embryo extract. **Figure 2*a*** was adapted by permission from the American Society of Plant Physiologists, *Plant Cell* © 1990 (10). **Figure 2*b*** was adapted by permission from Macmillan Publishers Ltd, *Nature* © 1998 (11). **Figure 2*c*** is reprinted by permission from Cold Spring Harbor Laboratory Press, *Genes and Development* © 1999 (13).

departing from the central dogma and facilitated the emergence of small regulatory RNAs.

## Cell-Free Assay Development

Tuschl et al. (13) developed a cell-free system that recapitulated dsRNA-induced silencing and established a foundation for biochemical analysis of RNAi. Using dsRNA against luciferase transcripts, sequence-specific silencing of reporter mRNA translation was demonstrated in extract prepared from syncitial blastoderm *Drosophila* embryos (**Figure 2*c***). Similar to what was observed in worms (11), this in vitro silencing was also accompanied by degradation of the target transcript (13). These studies provided direct evidence for dsRNA and target mRNA as the molecular players involved and for mRNA degradation as the functional outcome of RNAi. To simplify this translation-based assay, Zamore et al. (14) employed radiolabeled mRNA to directly monitor target transcript degradation and indicated that RNAi was mediated by sequence-specific cleavage of target mRNA. Similarly, radiolabeled dsRNA was used to demonstrate that long dsRNA was processed to 21–22-nt small RNA fragments (**Figure 2*d***) (14). These findings provided a critical link between the long dsRNA silencing trigger used by Fire et al. (11) and the small RNA corresponding to RNAi observed by Hamilton & Baulcombe (12).

## Scaling Up for Biochemical Purification

However, the limited quantities in which *Drosophila* embryos can be obtained presented a problem for biochemical purification. Hammond et al. (15) solved this problem by establishing RNAi activity in *Drosophila* Schneider 2 (S2) cells grown in large-scale suspension culture. In vitro target mRNA cleavage activity was achieved by "preloading" S2 cells with dsRNA prior to preparation of cell extract. Following chromatographic fractionation, an RNA-induced silencing complex (RISC) was isolated that harbored small RNA and catalyzed sequence-specific mRNA cleavage. Therefore, it was hypothesized that long dsRNA was processed to small RNA, which programmed the RISC to direct target transcript degradation (14, 15).

**RISC:** RNA-induced silencing complex

**siRNA:** small-interfering RNA

# IDENTIFICATION OF SMALL-INTERFERING RNA

Careful mapping studies indicated that target transcript cleavage corresponded to regions complementary to dsRNA and occurred at 21–22-nt intervals similar to the size of dsRNA-derived small RNAs (16). To directly test if small RNAs mediate RISC activity, 21–22-nt RNA duplexes with symmetric 2-nt 3′ overhangs were synthesized to mimic dsRNA-processing products. Indeed, these synthetic oligonucleotides induced target mRNA cleavage at sites corresponding to the middle of small RNA (**Figure 2*e***) (16–18). Therefore, these dsRNA-derived small RNAs were designated as small-interfering RNAs (siRNAs). Furthermore, a most important contribution was made by demonstration of target transcript silencing through transfection of synthetic siRNAs into mammalian cells (19). This work provided the foundation for numerous RNAi-based applications, including powerful loss-of-function tools and a new class of potential therapeutics. As such, these landmark studies provided an excellent example of the importance of basic science, including traditional biochemistry, in generating new areas for biomedical applications.

# A PARALLEL MICRO-RNA PATHWAY

These fundamental advances in the study of siRNA also provided new insight into a previously known regulatory RNA pathway. In *Caenorhabditis elegans*, LIN-14 protein is a master regulator of developmental gene expression (20). The *lin-4* locus is also required for proper timing of development, but this genomic region was not known to harbor a protein-coding sequence (21, 22). Rather, *lin-4* was found to encode ∼22- and 61-nt RNAs that were

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**UTR:** untranslated region

**miRNA:** micro-RNA

**Epitope tagged:** fusion of a protein-coding sequence with a short polypeptide marker to facilitate antibody recognition

complementary to multiple elements within the 3′-untranslated region (UTR) of *lin-14* mRNA (21, 22). Phenotypic and reporter assays indicated that both *lin-4* and 3′-UTR of *lin-14* were necessary and sufficient for developmental patterning (21, 22). Subsequent work revealed that lin-4 and the highly conserved 21-nt let-7 RNA govern a network of regulatory factors in directing temporal development (23). Similar to siRNA, these micro-RNAs (miRNAs), also known as small temporal RNAs (stRNA), were found to negatively regulate expression of target transcripts (24).

## SMALL RNA BIOGENESIS

That siRNAs and miRNAs were established as functional guides in governing silencing of target transcripts raised the question of how these small RNAs were produced. Examination of dsRNA-processing products indicated an RNaseIII-like pattern of endonucleolytic cleavage (16, 25). Bernstein et al. (26) expressed candidate RNaseIII enzymes as epitope-tagged proteins in *Drosophila* S2 cells and found that CG4792 (named Dicer-1) immunoprecipitates processed dsRNA to siRNA in vitro. This work identified Dicer as the small RNA-generating enzyme and established the first RNAi factor with a defined biochemical activity. Subsequently, a six-step chromatographic purification of siRNA-generating activity from S2 cell extract revealed CG6493 (named Dicer-2) (27). dsRNA-mediated knockdown of Dicer-2, but not Dicer-1, in S2 cells decreased siRNA production and compromised RNAi silencing. Thus, two functional Dicer proteins are encoded in the *Drosophila* genome.

Genomic analysis indicated that the let-7 miRNA might be derived from a conserved stem-loop precursor (28), suggesting a similar dicing step may be required for miRNA biogenesis. By Northern blotting, Hutvagner et al. (29) verified the existence of this ~70-nt let-7 precursor (pre-let-7) in *Drosophila* pupae and HeLa cells. Furthermore, in vitro transcribed pre-let-7 was processed to mature let-7 in *Drosophila* embryo lysate. siRNA-mediated knockdown of Dicer in HeLa cells resulted in accumulation of pre-let-7 and attenuated expression of let-7. Thus, in addition to siRNA production, Dicer is also required for miRNA biogenesis.

To dissect small RNA pathways in *Drosophila*, Lee et al. (30) isolated *dicer-1* (*dcr-1*) and *dicer-2* (*dcr-2*) mutants from an elegant RNAi genetic screen. Analyses of these mutants indicated that Dicer-1 and Dicer-2 were responsible for miRNA and siRNA biogenesis, respectively (30). Reconstitution studies confirmed that recombinant Dicer-1 preferentially processed pre-miRNA independent of ATP, whereas Dicer-2 preferentially processed long dsRNA in an ATP-dependent manner (31). In contrast, mammalian genomes encode only one Dicer that generates both miRNA and siRNA (29, 32). Recombinant human Dicer preferentially processes pre-miRNA versus dsRNA (Q. Liu, unpublished information) and does not require ATP for catalysis (33, 34). Therefore, the biochemical characteristics of human Dicer more closely resemble *Drosophila* Dicer-1 versus Dicer-2.

Two hallmarks of RNaseIII catalysis are the discrete size of dsRNA products and the 2-nt 3′ overhang. Prokaryotic RNaseIII carries a single RNaseIII domain and functions as a homodimer (35), whereas eukaryotic Dicer contains two RNaseIII domains and functions as a monomer (34). Through mutagenesis studies of putative catalytic residues of *E. coli* RNaseIII and human Dicer, Zhang and coworkers (34) proposed that RNaseIII/Dicer contains a single catalytic center for processing dsRNA (**Figure 3*a*,*b***). The tandem RNaseIII domains of Dicer form an intramolecular dimer and cleave opposing strands of dsRNA in an offset manner to produce a 2-nt 3′ overhang. Similar results were obtained with corresponding experiments for *Drosophila* Dicer-1 (36). In addition, structural and modeling studies of a primitive Dicer from *Giardia intestinalis* suggested that the PAZ domain recognizes the dsRNA terminus and that the size of small RNA produced is determined by the physical distance between the PAZ and RNaseIII domains (**Figure 3*c***) (37).

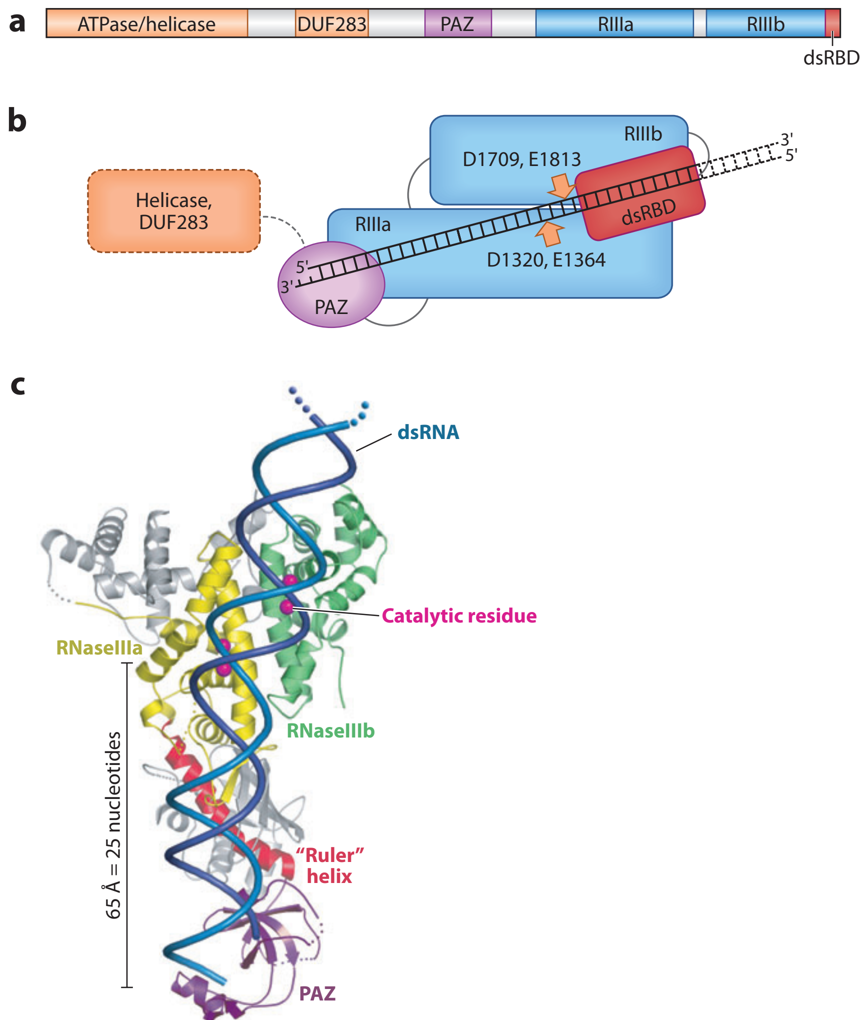


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**Figure 3**

Molecular mechanism of Dicer function. (*a*) Domain structure of human Dicer, indicating ATPase/helicase, domain of unknown function (DUF)283, PAZ, RNaseIII (RIII), and dsRNA-binding domains (dsRBDs). (*b*) Single processing center model of Dicer catalysis. The PAZ domain recognizes the terminus of the dsRNA substrate. The two RNaseIII domains approximate to form one catalytic center and cleave the opposing strands of dsRNA in an offset manner, resulting in a characteristic 2-nt 3′ overhang. (*c*) Ribbon depiction of *Giardia* Dicer structure modeled with dsRNA substrate. The axe-like arrangement of the core enzyme is formed by a PAZ-dsRNA handle and RNaseIII domains forming the blade. The distance between PAZ and the catalytic residues (*balls*) is the ruler that determines the size of small RNA products. **Figure 3*a*,*b*** is from *Cell* © 2004 (34), reprinted by permission from Elsevier. **Figure 3*c*** provided courtesy of J. Doudna, University of California, Berkeley.

## siRNA EFFECTOR FUNCTION

Identification of siRNA- and miRNA-generating enzymes intensified efforts to elucidate the mechanisms through which these small RNAs effected silencing. The work of Hammond et al. (15) indicated that the RNAi effector RISC contains a nuclease that targets mRNA through programming

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by siRNA. Chromatographic purification of preloaded RISC activity from S2 cell extract identified CG7439, named Argonaute2 (Ago2) (38). A similar independent purification scheme further suggested Ago2 involvement in *Drosophila* RISC (39). Human Argonautes were also identified as RISC constituents through affinity purification of biotinylated siRNA from HeLa cell extract (40). This family of proteins had previously been implicated in RNAi as *C. elegans*, *Neurospora*, and *Arabidopsis* mutants exhibited defects in silencing (41–43).

RISC was demonstrated as a magnesium-dependent endoribonuclease (44, 45). Affinity purification of epitope-tagged proteins indicated that all four human Agos associated with endogenous miRNA and transfected siRNA, but only Ago2-programmed RISC exhibited in vitro target mRNA cleavage activity (46, 47). Furthermore, Ago2-deficient murine embryonic fibroblast cells were defective for RNAi and could be rescued only by Ago2 but not by the Ago1 transgene (46). These findings strongly implicated Ago2 as a candidate effector nuclease for RISC.

Reasoning that catalytic residues may be surmised through sequence comparison between the cleaving Ago2 and noncleaving Ago proteins, Liu and coworkers (46) found that mutations of selected human Ago2 residues resulted in a loss of mRNA cleavage (slicer) activity. Song et al. (48) provided critical insight for Ago function by defining the crystal structure of a primitive Ago from *Pyrococcos furosus* (48). This analysis revealed that the PIWI domain of Ago has a fold similar to RNaseH, which degrades RNA in RNA-DNA hybrid. Thus, the PIWI domain of Ago2 could slice target mRNA in an mRNA-siRNA hybrid. Indeed, mutation of conserved RNaseH-based catalytic residues abolished in vitro slicer activity of immunoprecipitated Ago2 (46). A key biochemical achievement was the reconstitution of a minimal RISC enzyme using *E. coli*-produced recombinant human Ago2 and single-strand siRNA (49). Recombinant human Ago2-bearing mutations in catalytic residues D597, D669, and H807 did not exhibit slicer activity (46, 49). The lack of this "DDH" motif explains why human Ago1 and Ago4 do not exhibit slicer activity (1).

Much of what was learned from human Ago2 was also found for *Drosophila* Ago proteins. Immunoprecipitated siRNA-programmed dAgo2 exhibited in vitro slicer activity, whereas immunoprecipitated dAgo1 cleaved a target mRNA bearing a sequence complementary to bantam miRNA (50). Minimal RISC activity was reconstituted for recombinant dAgo1 and a truncated dAgo2 produced from *E. coli* (50). However, there appear to be key differences in how small RNAs program Argonautes in different organisms. In *Drosophila*, dAgo1 is programmed through miRNA, whereas dAgo2 is primarily programmed through siRNA (51). By contrast, human Ago1–4 were found to harbor both siRNA and miRNA (46, 47). The molecular basis for the difference between generalized functions of human Agos versus specialized roles of *Drosophila* Agos remains unknown.

Structural studies also provided great insight into the functional relationships among Ago, siRNA, and target mRNA (1). The PAZ domain of Ago contains an oligonucleotide-binding fold that interacts with the 2-nt 3′ overhang of siRNA (**Figure 4*a*,*b***) (1). In conjunction with PAZ, the PIWI, N-terminal, and middle (Mid) domains of Ago form a crescent base and a positively charged channel, wherein guide siRNA recognizes target mRNA, and the 5′ phosphate of siRNA is anchored within the binding pocket of the Mid domain (**Figure 4*b*,*c***) (1, 52, 53). Consistent with earlier biochemical studies (16, 44, 45), structural studies revealed that helical interaction between siRNA and target RNA positioned the scissile phosphate corresponding to the ninth and tenth positions of guide RNA at the catalytic center (1, 52, 53). Cleavage of target RNA results in a 5′ fragment with a 3′-hydroxyl terminus and a 3′ fragment with a 5′ phosphate (44, 45). Collectively, these biochemical and structural studies provided the foundation for understanding small RNA effector functions.

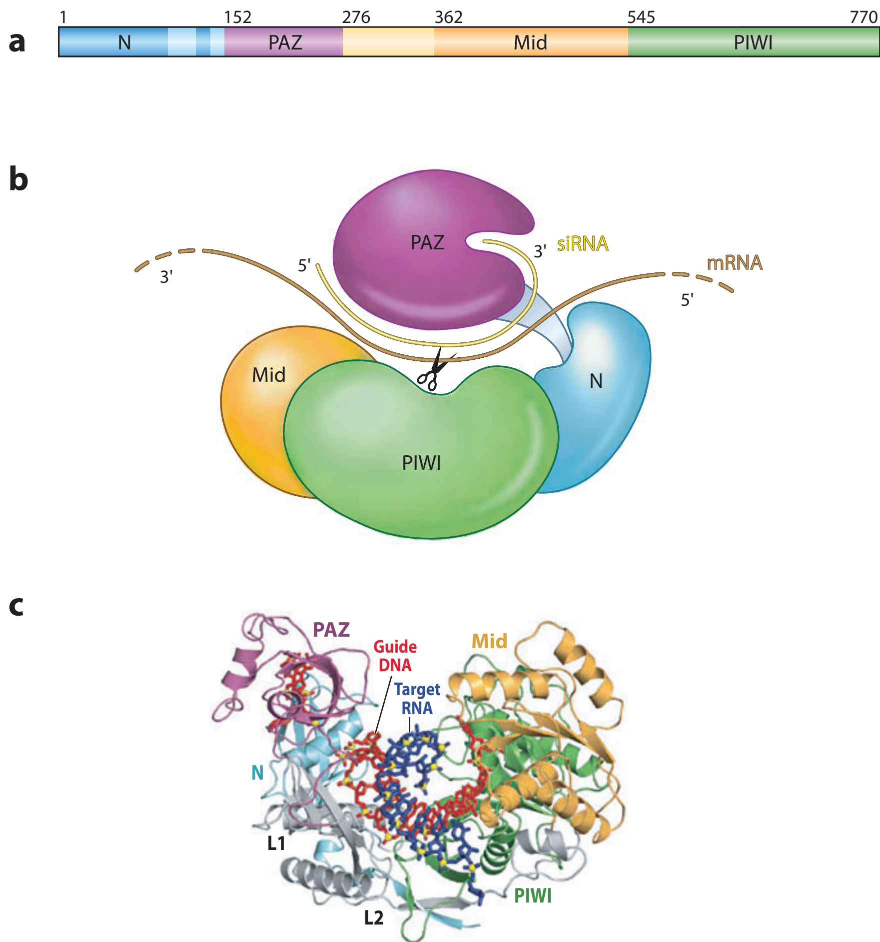


**Figure 4**

Molecular mechanism of slicer function. (*a*) Domain structure of argonaute from *Pyrococcus furiosus*. (*b*) A schematic representation of siRNA-directed mRNA cleavage. The 3′ end of siRNA is positioned in the cleft of the PAZ domain. The mRNA situates between the upper PAZ domain and the lower crescent-shaped base formed by the N-terminal, PIWI, and middle (Mid) domains. The catalytic site (*scissors*) slices mRNA at a position that corresponds to the ninth and tenth nucleotides of guide siRNA. (*c*) Crystal structure of *Thermus thermophilus* argonaute bound to 5′-phosphorylated 21-nt guide DNA and 20-nt target RNA. **Figure 4*a,b*** is from *Science* © 2004 (48), reprinted with permission from AAAS. **Figure 4*c*** was adapted by permission from Macmillan Publishers Ltd., *Nature* © 2008 (52).

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## miRNA EFFECTOR MECHANISMS

Despite the many similarities between siRNA and miRNA, where siRNA has a clearly defined effector mechanism, miRNA function is much more controversial [for a comprehensive review, see Sonenberg (53a) in the current issue]. A key reason for this disparity was the early establishment of a widely accepted in vitro assay for siRNA-induced RISC (siRISC) activity (14). The lack of a comparable, well-defined biochemical readout for miRNA-induced RISC (miRISC) activity has been a key obstacle in establishing biochemical assays to understand miRNA effector mechanisms.

**Cross-linking:** a technique used to induce covalent bond formation between proteins or between proteins and nucleic acids

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Current thinking on animal miRNA function has been heavily influenced by the earliest report that lin-4-mediated repression of Lin-14 protein expression occurred in the absence of detectable changes in *lin-14* mRNA levels (22). Thus, it was proposed that miRNAs effect target silencing through inhibition of translation. However, these studies employed RNase protection assays to monitor transcript levels. Subsequent work using Northern blotting indicated reduction of full-length target transcripts (54). Moreover, cloning of *lin-41* 3′-UTR fragments indicated cleavage products upstream of let-7 target sites (54). Similar cleavage products were detected for 3′-UTR constructs bearing let-7 sites in human cells (55). Additionally, among the first miRNA-mRNA target relationships identified was miR-196-directed cleavage of *HOXB8* mRNA (56). Indeed, perturbation of miRNA expression followed by global transcript profiling has been widely successful in identifying miRNA targets (57). Thus, miRNAs appear to modulate protein expression through (*a*) a slicer mechanism for high homology mRNA targets, (*b*) slicer-independent mRNA cleavage or degradation, and (*c*) translational inhibition without changes in mRNA levels. Distinguishing among these outcomes may help resolve the stewing tension in the field between master miRNA regulators that govern expression of a few key targets versus fine-tuning miRNAs that moderately influence expression of a large number of transcripts (58).

Cell-free systems of miRNA-mediated silencing have been instrumental in identifying the molecular requirements for target transcript silencing (59, 60). Although these assays are not suited for biochemical purification, they may be useful in testing candidate factors that modulate miRNA-mediated silencing. Identification of miRNA-mediated deadenylation is a promising lead for more in-depth biochemical investigation (60). The lack of a widely accepted molecular readout, robust assay, and unlimited material remains an outstanding challenge for in-depth biochemical investigation.

# ACTIVATING RISC

The establishment of robust enzymatic assays (**Figure 2*d*,*e***) enables biochemical identification of principal factors responsible for siRNA biogenesis and RISC function. However, understanding how these two processes connect has been a major challenge for the field. While newly synthesized siRNA is double stranded, only single-strand siRNA could directly program recombinant Ago2 into a minimal RISC (46, 49), suggesting that additional factors are required for incorporating nascent siRNA into RISC. Liu and coworkers (27) identified a biochemical link between dsRNA processing and RISC activation through biochemical purification of *Drosophila* siRNA-generating activity from S2 cell extract. Identified was a heterodimeric complex of Dicer-2 and CG7138, named R2D2 for its two dsRNA-binding domains (R2) and association with Dicer-2 (D2) (27). As recombinant R2D2 had no effect on dicing activity, it was hypothesized that R2D2 functions downstream of siRNA production. A partial reconstitution system was developed to determine that the Dicer-2-R2D2 complex coordinately recruits duplex siRNA to Ago2 to facilitate RISC activation (27). Genetic studies indicated that both Dicer-2 and R2D2 were required for efficient RISC activity and RNAi silencing (27, 30, 61). Native gel-shift analyses indicated that Dicer-2 and R2D2 interacted with duplex siRNA to assemble a RISC-loading complex that preceded formation of an active RISC (27, 62, 63). Moreover, cross-linking studies suggested that the Dicer-2-R2D2 complex senses thermodynamic asymmetry of siRNA with R2D2 preferentially binding the more stable end and Dicer-2 binding the less stable end (64–66). This asymmetric binding of Dicer-2-R2D2 appears to facilitate selection of the guide strand for incorporation into RISC.

## Helicase Model of RISC Activation

The central step of RISC activation is the separation of two siRNA strands and loading of one

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strand, the guide strand, onto Ago2. Using native polyacrylamide gel electrophoresis to resolve double-strand and single-strand siRNA, Nykanen et al. (67) demonstrated that ATP was required for single-stranded siRNA production. Moreover, this siRNA-unwinding activity correlated well with the RISC activity following gel filtration chromatography (67). Thus, RNA helicases were implicated in RISC activation.

Multiple factors with annotated helicase domains have been implicated in RNAi. For example, both Dicer-1 and Dicer-2, known to influence RNAi activity, contain a DExH helicase motif (27, 30, 62). Ovary extracts deficient in the ATP-dependent helicase Armitage were defective for RISC activity (63). Human homologs of Armitage, MOV10, coimmunoprecipitated with human Ago1 and Ago2, and knockdown of MOV10 resulted in attenuated gene silencing (68). Affinity purification of biotinylated siRNA transfected in human cells indicated an association between RNA helicase A (RHA) and active RISC complex (69). Knockdown of RHA resulted in reduced association of siRNA with Ago2, decreased RISC activity, and attenuated gene silencing (69). In addition to duplex siRNA unwinding, RNA helicases may serve other important roles in small RNA pathways, including dsRNA processing, target mRNA recognition, and release of cleavage products. Given the importance of helicases in viral biology and many reports of cross talk between virology and RNAi, understanding the roles of helicases in regulatory RNA pathways may be of direct clinical significance.

## Slicer Model of RISC Activation

An alternative model for RISC activation involves the same mechanism by which Ago2 and guide siRNA cleave target mRNA. Here, duplex siRNA is recruited to Ago2, and the (passenger) strand to be excluded from RISC is the target of slicer activity. Ago2 cleaves the passenger strand into 9-nt and 12-nt fragments, leaving the guide strand behind with Ago2 to form an active RISC. In support of this model, passenger strand cleavage was detected in wild-type, but not in *dcr-2*, *r2d2*, or *ago2* mutant *Drosophila* embryo lysates (50, 69, 70). Phosphorothioate and 2′-O-methyl siRNA modifications blocked passenger strand cleavage and attenuated RISC activity (69, 70). In S2 extract deficient in Ago2 activity, the production of single-strand siRNA was rescued with recombinant wild-type, but not catalytic mutant, Ago2 (71). A similar in vivo requirement for the slicer activity of Ago2 was also observed in flies (72). In *Neurospora*, the catalytic mutant *qde-2* (Ago2) strains were defective for production of single-strand, but not double-strand, siRNA (73). These studies indicated that the slicer mechanism plays a prominent role in *Drosophila* and *Neurospora* RISC activation.

Although various factors and models have been proposed, there is a lack of consensus in defining the biochemical mechanisms of RISC activation. One reason for this is the possibility of multiple mechanisms for RISC activation, each playing more or less significant roles depending on the species, tissues, and cell types under study. As not all Ago proteins exhibit slicer activity, passenger strand cleavage cannot explain all effector complex programming. Additionally, miRNAs have central mismatches that preclude Ago-mediated passenger strand cleavage. Thus, understanding the mechanisms by which small RNA effector complexes are activated remains a clear challenge for the field.

## Overcoming Key Challenges for Understanding RISC Activation

Although an immensely powerful approach, biochemical fractionation and reconstitution impose many significant challenges. A critical obstacle in the setting of RNAi has been the salt sensitivity of in vitro RISC activity. Specifically, exposure to salt or chromatographic fractionation could irreversibly damage the ability to form RISC in *Drosophila* embryo or S2 extract (39, 74). Hammond et al. (15, 38) circumvented this problem by treating S2 cells with dsRNA to preload RISC prior to making cell extract. Unlike *de novo* RISC activity, preloaded

RISC activity was resistant to salt treatment and could withstand chromatographic fractionation. This disparity may be the result of stabilization of Ago2 conformation upon siRNA binding, conferring resistance to salt-induced structural changes. However, a solution to the salt-sensitivity problem was needed to purify additional factors involved in RISC activation.

It was determined that *Drosophila* Ago2 was a key salt-sensitive factor, and hence, the problem of RISC reconstitution was solved by using recombinant Ago2 purified at a low-salt condition (71). The production of full-length *Drosophila* Ago2 had been confounded by its extensive N-terminal polyglutamine repeats. Thus, an active, truncated Ago2 recombinant protein was generated that could fully restore RISC activity to *ago2* mutant extract (71). Recombinant Dicer-2-R2D2 and Ago2 proteins successfully reconstituted long dsRNA- and duplex siRNA-initiated RISC activity (71), demonstrating that these factors compose the catalytic core of RNAi. Such experiments may provide important guidance for solving key problems in reconstituting human RISC.

Liu and coworkers (71) employed this core reconstitution system to biochemically purify a new RISC activator, named C3PO (component 3 promoter of RISC), which consists of two evolutionarily conserved subunits: Translin/TB-RBP and Translin-associated factor X (Trax). Recombinant C3PO enhanced core RISC activity of Dicer-2/R2D2/Ago2, and genetic depletion of C3PO attenuated RISC activity in vitro and in vivo (71). Biochemical studies indicated that C3PO is a $Mg^{2+}$-dependent endoribonuclease (71). Mutagenesis of putative catalytic residues on the Trax subunit resulted in loss of C3PO's nuclease activity and RISC-enhancing activity, suggesting a functional link between these processes (71). Specifically, C3PO was found to promote RISC activation by removing siRNA passenger strand fragments (71). A similar mechanism had also been observed in *Neurospora*, wherein the exonuclease QIP degraded QDE-2-nicked duplex siRNA to facilitate RISC activation (73). These studies collectively illustrated a three-step process for slicer-mediated RISC activation: (*a*) Duplex siRNA is recruited to Ago2 (e.g., by Dicer-2-R2D2), (*b*) Ago2 cleaves the passenger strand, and (*c*) passenger strand fragments are actively removed by C3PO or QIP.

## DEFINING HOLO-RISC

A three-component model of human RISC was proposed through studies of immunopreciptiated complexes containing Dicer, Ago2, and the HIV transactivating response element-binding protein (TRBP, a homolog of R2D2) from human cells (75, 76). Direct support for this model was provided by reconstitution studies employing recombinant Dicer, TRBP, and Ago2 proteins (77). Following incubation of individually purified components, gel filtration chromatography revealed the formation of a trimeric complex exhibiting pre-miRNA-processing and pre-miRNA-initiated RISC activity. However, recent reports have shown that recombinant human Ago2 devoid of pre-miRNA-processing activity could be programmed by pre-miRNA encoding 5′-, but not 3′-, derived miRNA to cleave target mRNA (77a; J.A. Doudna & Q. Liu, unpublished information). Thus, an alternative interpretation of the data is that the intact pre-miRNA may serve as a guide for human Ago2 to direct target mRNA cleavage. Importantly, long dsRNA- and duplex siRNA-initiated RISC activities have yet to be reconstituted in the human system. The lack of this reconstitution system has hindered further mechanistic understanding of human RISC.

The formation of an 80S holo-RISC was detected by sedimentation analysis in *Drosophila* embryo extract (62). Recombinant Dicer-2-R2D2 and Ago2 could reconstitute core RISC activity, and addition of C3PO markedly enhanced this baseline activity (71), suggesting that the trimeric model may be an underrepresentation of holo-RISC function. Development of a robust reconstitution system should greatly facilitate in-depth studies of the assembly, function, and regulation of holo-RISC. A key objective of biochemical reconstitution is



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to identify all factors that are necessary and sufficient for holo-RISC activity. Other objectives are to assign a specific role to each factor and to define the functional relationships among different components. Moreover, the reconstitution system can be employed to purify regulators of holo-RISC activity and to study posttranslational regulation of the RNAi machinery.

## GENOME-ENCODED SMALL RNA

Perhaps the biggest surprise of the Human Genome Project was the relatively small number of protein-coding genes relative to genome size (78, 79). Only five percent of the genome is believed to encode proteins. miRNA, endogenous siRNA (endo-siRNA), and piwi-interacting RNA (piRNA) are constituents of the remaining noncoding genome. There is growing understanding of the importance of these and other noncoding RNAs in regulating genome function.

### miRNA

Public miRNA databases indicate hundreds of miRNAs from many species with hundreds or thousands more estimated (80). These miRNAs have been identified through three main approaches (81): (*a*) forward genetics by isolating miRNA mutants, (*b*) bioinformatic predictions based on a stem-loop pre-miRNA and phylogenetic conservation, and (*c*) direct cloning and sequencing of small cellular RNA, which has been greatly expanded by new pyrosequencing technologies.

Hypothesizing that ~70-nt pre-miRNA was derived from longer transcripts, Lee et al. (82) performed RT-PCR using primers of increasing distance from the genomic region of pre-miRNA. This approach indicated the existence of several hundred bases long primary-miRNA (pri-miRNA) transcripts, which were confirmed by Northern blotting (82). These findings were particularly important as small RNAs, such as transfer RNA and small nucleolar RNA, are derived from short RNA polymerase III transcripts (83). That pri-miRNA transcripts were larger than expected suggested a different mechanism of transcription.

To elucidate features of pri-miRNA transcripts, total RNA was affinity purified using the guanosine cap-binding protein eIF4E (84, 85). All pri-miRNAs examined exhibited eIF4E binding, indicating 5′-guanosine capping of pri-miRNA transcripts. Affinity purification of total RNA using oligo-dT indicated that pri-miRNA transcripts were polyadenylated (84, 85). These characteristics suggested an RNA polymerase II (Pol II)-mediated transcription. Pharmacological inhibition of Pol II reduced pri-miRNA expression, and chromatin immunoprecipitation indicated Pol II occupancy of a pri-miRNA promoter (85). These findings demonstrate that miRNAs are derived from Pol II transcripts bearing a 5′-guanosine cap and 3′-poly(A) tail.

Although there was good understanding of Dicer's processing of ~70-nt pre-miRNA to mature miRNA, it was unclear how pri-miRNA transcripts, of hundreds or thousands of bases in length, were converted to pre-miRNA. Subcellular fractionation studies indicated that pri-miRNA and pre-miRNA processing occurred separately in the nucleus and cytoplasm, respectively (82). Through a series of mutagenesis studies, Lee et al. (86) deduced that the dsRNA feature of a pri-miRNA is required for its processing into pre-miRNA. Similar to the rationale employed in the discovery of Dicer (26), the specificity of RNaseIII for dsRNA processing proved useful here. The nuclear localization of the RNaseIII Drosha (87) suggested an excellent candidate for the pri-miRNA-processing enzyme. Indeed, pri-miRNA-processing activity immunoprecipitated with epitope-tagged Drosha, but not Dicer (86). Furthermore, siRNA-mediated knockdown of Drosha resulted in accumulation of pri-miRNA and reduced levels of pre-miRNA and miRNA (86). These findings indicate that miRNA biogenesis requires two spatially regulated catalytic steps. One exception is a small number of "mirtrons," whose pre-miRNAs are embedded in the introns of other transcripts, which

**piRNA:** piwi-interacting RNA

**Immunoprecipitation:** use of antibody-coated solid phase material to concentrate a particular protein out of solution

are converted to pre-miRNA by the splicing machinery and processed by Dicer into miRNA (88–90).

**dsRBP:** double-stranded RNA-binding protein

To determine how pre-miRNA produced in the nucleus connect with the pre-miRNA-processing enzyme in the cytoplasm, a search for candidate transporters was conducted. The structural similarities between pre-miRNA and adenovirus VA1 RNA suggested that the VA1 transporter Exportin-5 (91) might play a role in pre-miRNA export (92). In human cells, siRNA-mediated knockdown of Exportin-5 decreased cytoplasmic pre-miRNA levels and reduced miRNA expression (93). In addition, the transport cofactor RanGTP was required for interaction between Exportin-5 and pre-miRNA in vitro (92). Export of radiolabeled pre-miRNA following nuclear injection of *Xenopus* oocyte was specifically enhanced by coinjection of recombinant Exportin-5 (94). Depletion of RanGTP or coinjection of Exportin-5 antibodies decreased pre-miRNA export and miRNA expression (94, 95). These results provide an important spatial link between the two distinct RNaseIII-mediated events required for miRNA biogenesis.

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**Dicer partners.** Identification of Dicer and Drosha only partially represented the factors required for small RNA maturation. The discovery of RDE-4 and R2D2 (27, 96) suggested that Dicer and Drosha might function in tandem with dsRNA-binding proteins (dsRBPs). Bioinformatic analysis of the *Drosophila* genome revealed an R2D2-like CG6866, named R3D1/Loquacious (hereafter Loqs) for its three dsRNA-binding domains (R3) and association with Dicer-1 (D1) (31, 97, 98). Chromatographic fractionation of miRNA-generating activity showed excellent correlation with Dicer-1 and Loqs-PB, one of multiple Loqs isoforms (31). Unlike R2D2 that was required for siRISC assembly but not for siRNA production, Loqs is required for miRNA production (31, 97, 98) but not for miRISC assembly (99). Two roles for Loqs on miRNA production have been reported (31, 97, 98): (*a*) Loqs-PB facilitated Dicer-1-pre-miRNA interaction to enable efficient processing; and (*b*) expression of Loqs and Dicer-1 were interdependent, wherein loss of one attenuates expression of the other.

Computational analysis also indicated potential dsRBPs encoded in the human genome (97, 98). Human Dicer coimmunoprecipitated with TRBP and chromatographic fractionation of miRNA-generating activity showed close correlation with Dicer and TRBP (100–101a). Immunoprecipitation of epitope-tagged Dicer also revealed association with another dsRBP, protein activator of protein kinase R (PACT) (102). Knockdown of TRBP or PACT resulted in attenuated miRNA production and miRNA-mediated silencing (100–102). Similar to Loqs, TRBP was required for miRNA production through stabilization of Dicer expression and by facilitating Dicer-pre-miRNA interaction (100–102).

**Drosha partner.** A dsRBP partner (CG1800/Pasha) for *Drosophila* Drosha was identified by analyzing a yeast two-hybrid interaction map (103). Pasha coimmunoprecipitated with Drosha, and immunoprecipitates of both proteins exhibited pri-miRNA-processing activity in vitro (104). Moreover, chromatographic fractionation of pri-miRNA-processing activity exhibited good correlation with Drosha and Pasha (104). Immunoprecipitation of epitope-tagged Drosha from human cells revealed a putative dsRBP DGCR8 (105–107). The pri-miRNA-processing activity was observed for immunoprecipitates of Drosha and DGCR8 (105–107). Both recombinant Drosha and DGCR8 were required for in vitro reconstitution of pri-miRNA-processing activity (105, 108). Knockdown of Drosha or Pasha/DGCR8 resulted in accumulation of pri-miRNA and decreased miRNA expression (104–107). Thus, as a general rule, RNaseIII enzymes function in tandem with dsRBPs in small RNA pathways.

An interesting observation was made involving the autoregulation of Drosha and DGCR8. Following knockdown of Drosha

in cultured cells, the levels of DGCR8/Pasha mRNA and protein were unexpectedly increased (109). Overexpression of Drosha reduced, whereas expression of a dominant-negative Drosha increased DGCR8 expression (109). Predicted stem-loop structures in the 3′-UTR of *dgcr8* mRNA were processed to ~70-nt products by Drosha, thereby negatively regulating DGCR8 expression (109). Thus, Drosha also governs mRNA expression through miRNA-independent mechanisms.

dsRBPs may also modulate the conformation of partner RNaseIII enzymes. Enhanced catalytic activity was observed for human Dicer in the absence of the N-terminal helicase domain (110). Because Dicer interacts with dsRBP partners through this region (36, 102), it has been proposed that TRBP contributes to Dicer function by relieving autoinhibition of Dicer (110). Another example of dsRBP function was provided through clinical studies that found two frameshift mutations in TRBP gene in cancer cell lines (110a). Both mutations introduced premature stop codons, resulting in reduced TRBP expression. Consistent with the role of TRBP in maintaining Dicer stability, these mutations resulted in reduced Dicer expression and lower miRNA production, and were associated with accelerated cell proliferation. These findings offer a possible genetic explanation for the reduced global miRNA expression in cancer cells (111, 112).

## Endogenous siRNA

Endo-siRNAs have been shown to play important roles in regulating genome functions in diverse species. In *C. elegans*, exogenous dsRNAs are processed to rare primary siRNAs, which are amplified to more abundant secondary siRNAs (113). Specifically, primary siRNAs are loaded onto RDE-1 (homolog of Ago2) to cleave target mRNA, and RNA-dependent RNA polymerases (Rdrp) use cleaved mRNA as a template to prime synthesis of secondary siRNAs (114). Unlike primary siRNAs with 5′ monophosphate, secondary siRNAs have 5′ triphosphate (113) and are produced through a Dicer-independent mechanism (115). Secondary siRNAs are loaded onto multiple non-slicing Agos, which further contribute to target silencing (114). The production of secondary siRNAs corresponds to 5′ spreading of RNAi along the target mRNA and is linked to systemic and heritable silencing in worms (113, 114).

In *Schizosaccharomyces pombe*, a positive feedback loop of centromeric silencing is effected through the RNA-induced transcriptional silencing (RITS) complex (116). Centromeric repeats-derived dsRNAs are amplified by an Rdrp complex and processed by Dicer into siRNAs (116). Similar to RISC, these endo-siRNAs program RITS via Ago1, which recruits histone modifiers to establish and maintain heterochromatin and genomic silencing (116). In *Neurospora*, DNA damage induces the production of another class of QDE-2-interacting siRNAs, called qiRNAs (117). Biogenesis of qiRNAs requires Dicers, the DNA helicase QDE-3, and a DNA- and RNA-dependent RNA polymerase QDE-1 (117). Most qiRNAs are derived from ribosomal DNA repeats and are believed to contribute to the DNA damage response by inhibiting protein translation (117).

Recent studies have found diverse sources of endo-siRNAs in *Drosophila* and mammals (9). In general, dsRNA precursors are produced through (*a*) duplex formation of pseudogene and protein coding transcripts, (*b*) inverted repeat transcripts, (*c*) self-complementary mRNA, (*d*) retrotransposons, and (*e*) bi-directional sense and antisense transcripts (9). Although some endo-siRNAs are mapped to mRNA, others correspond to transposons and may contribute to transposon silencing in somatic cells (9). In *Drosophila*, though Dicer-1-Loqs-PB and Dicer-2-R2D2 complexes generate miRNAs and exo-siRNAs (2), respectively, the production of endo-siRNAs requires a noncanonical partnering of Dicer-2 and Loqs-PD (118, 119). These findings indicate functional cross talk among factors previously assigned to distinct small RNA pathways.

**Rdrp:** RNA-dependent RNA polymerase

**Transposon:** noncoding regions of DNA that exhibit chromosome mobility

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## piRNA

The recent history of small RNA pathways has yielded a powerful template by which to dissect other noncoding RNA pathways. Considering the possibility of small RNA independent of miRNA and siRNA, Aravin and colleagues (120) cloned 16–29-nt RNA from *Drosophila*. Among the RNAs identified were ∼24-nt repeat-associated siRNAs (rasiRNAs), which correspond to transposable and repetitive elements within the genome. To identify the mammalian equivalent of rasiRNAs, multiple labs independently cloned a class of 26–30-nt RNAs that were highly abundant in mammalian testes (3). Like *Drosophila* rasiRNAs, these small RNAs coimmunoprecipitated with Piwi proteins and were designated as piRNAs. Both *Drosophila* and mammalian piRNAs contain 3′-terminal 2′-O-methyl modification that is catalyzed by a HEN1-like methyltransferase (3). Unlike *Drosophila* piRNA, most mammalian piRNAs are mapped uniquely in the genome and cluster to a small number of loci ranging from 10 to 83 kb (3). Genomic mapping of piRNAs does not indicate potential dsRNA precursors, and piRNA biogenesis appears to involve a Dicer-independent mechanism (3).

**piRNA amplification.** Sequencing of *Drosophila* piRNAs associated with different Piwi proteins revealed interesting characteristics (121, 122). Both Aubergine- and Piwi-associated piRNAs were mainly derived from the antisense strand of retrotransposons and showed a preference for uridine (U) at the 5′ end. In contrast, Ago3-associated piRNAs were primarily derived from the sense strand and exhibited a preference for adenosine (A) at the tenth nucleotide position. Furthermore, frequent complementarity was detected between the first ten bases of Aubergine- and Ago3-associated piRNAs. These findings, together with known slicer activity of piwi proteins (122), led to a "Ping-Pong" model for piRNA amplification: Sense piRNA guides Ago3 to cleave an antisense transcript to form the 5′ end of antisense piRNA bound to Aubergine, with each successive round of cleavage generating a new piRNA (121, 122). Although details of this model remain vague, this piRNA amplification loop appeared to be disrupted in *ago3* mutant flies (123). These studies indicated that different Piwi proteins conduct piRNA functions both cooperatively and independently of one another (123).

**piRNA function.** Studies in flies, fish, and mammals suggest that piRNAs play important roles in germ line development and maintenance of genomic integrity (3). *Drosophila* piRNAs are also derived from discrete genomic loci; several of these correspond to master regulatory regions of transposons (121). Mutations of the *flamenco* locus, a regulator of *gypsy*, *ZAM*, and *Idfix*, negated silencing of these retrotransposons and diminished production of piRNAs mapped to this region (121). In mammals, a significant portion of MILI-associated prepachytene piRNAs correspond to repeat elements and play a similar role in transposon silencing (3). piRNAs also direct silencing of specific genes. In *Drosophila*, expression of the *stellate* gene is suppressed by piRNAs derived from the *Suppressor of Stellate* locus, and loss of silencing results in overexpression of Stellate protein and male sterility (3). How piRNAs effect silencing remains a subject of debate. However, recent studies suggested that piRNAs may silence transcription by regulating DNA methylation (3).

# REGULATION OF SMALL RNA PATHWAYS

Because of the importance of small RNA pathways in governing cellular activities, it has become increasingly important to understand how these pathways themselves are regulated. Spatiotemporal regulation of small RNA production and pathway components suggests tight coordination of gene silencing with wider biological processes. Defining the relationships between small RNA pathways and other cell regulatory systems represents an important and emerging area of investigation.

## Endogenous Inhibitors of RNAi

The endogenous inhibitors of RNAi were first characterized in *C. elegans*. Noting that the worm nervous system was resistant to RNAi, several groups conducted genetic screens to isolate mutants exhibiting enhanced dsRNA-induced silencing in neurons. Disruption of *rrf-3*, encoding an Rdrp-like protein, enhanced RNAi phenotypes for neuronal and nonneuronal gene targets (124). Mutants of *eri-1*, encoding a protein carrying a 3′–5′ exonuclease domain, accumulated more siRNA following dsRNA feeding or injection. Recombinant ERI-1 specifically degraded siRNA, but not other RNA substrates in vitro (125). Trans-splicing has been shown to produce the helicase Eri-6/7, which inhibits exogenous, but is required for endogenous, RNAi (126). By contrast, little is known about the regulators and regulatory mechanisms of RNAi in *Drosophila* and humans.

## Posttranscriptional Control of miRNA Biogenesis

Transcriptional control is a general mechanism for regulating miRNA expression. However, there were widespread reports of differential expression of miRNAs in the absence of apparent changes in pri- or pre-miRNA levels, suggesting that miRNA biogenesis is also regulated posttranscriptionally (127–130). For example, despite expression of pri-let-7, mature let-7 is not detected in mouse embryonic stem cells (131). Lin28 was identified as a candidate inhibitor of pri-let-7 processing through nucleic acid affinity purification (131, 132). Recombinant Lin28 inhibited in vitro pri-let-7 processing through recognition of the conserved loop region (132, 133). Overexpression of Lin28 resulted in accumulation of pri-let-7 and reduced expression of mature let-7 (131).

Lin28 has also been shown to govern pre-let-7 processing (134). Overexpression of Lin28 in human cells resulted in reduction of mature let-7 and accumulation of slower-migrating pre-let-7 species that were the result of 3′ polyuridylation (135). In vitro polyuridylation of pre-let-7 required recombinant Lin28 and the uridylyl transferase TUT4 (135, 135a). Collectively, these studies indicate that Lin28 negatively regulates let-7 biogenesis by steric inhibition of pri- or pre-let-7 processing and by recruiting uridyl transferases to mark pre-let-7 for degradation. The importance of this posttranscriptional regulatory mechanism is reflected through studies showing that knockdown of Lin28 unleashes tumor suppressor activity of let-7 and that Lin28 is a potential negative prognostic indicator for hepatocellular carcinoma (131, 136).

A number of other factors have also been identified as modulators of miRNA biogenesis. Photocross-linking and immunoprecipitation of the RNA-binding protein hnRNP A1 indicated association with the stem-loop region of pri-miR-18 (137). Unlike Lin28, which inhibits let-7 biogenesis, hnRNP A1 promoted in vitro processing of pri-miR-18 and was required for cellular expression of miR-18 (137). Following growth factor stimulation in human smooth muscle cells, Drosha processing of pre-miR-21 was enhanced by association of Smad and the RNA helicase p68 (138). The KH-type splicing regulatory protein (KSRP) coimmunoprecipitated with human Dicer and Drosha (139). KSRP binding to pri- and pre-miRNA loops facilitated maturation of a subset of miRNAs, including let-7 (139). Other factors, particularly those with known functions in RNA metabolism, are likely to emerge as regulators of small RNA biogenesis.

## Modulating an miRISC-Target mRNA Relationship

Reasoning that 3′-UTR-binding factors may influence miRISC-target mRNA interactions, experiments were conducted that showed the AU-rich element-binding protein HuR reversed miR-122 suppression of cationic amino acid transporter-1 mRNA during nutrient stress (140). Noting that target transcripts often exhibit a conserved sequence surrounding miRNA target sites, Kedde et al. (141)

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conducted immunoprecipitation experiments that indicated dead end 1 (Dnd1)–bound uridine-rich elements surrounding miRNA target sites, thereby precluding miRISC binding and inhibition of target transcripts. Similarly, Importin8 was shown to modulate interaction between miRISC and target mRNA (142). These findings suggest that miRNA-mediated silencing of target transcripts occurs in combination or in competition with other RNA-binding factors.

### Cellular Regulation of Small RNA Pathways

siRNA, miRNA and piRNA have emerged as important regulators of biological processes. However, regulation of these pathways and their relationships with other cellular systems is only beginning to emerge. Human Ago2 coimmunoprecipitated with the prolyl-4-hydroxylase, C-P4H(I), and was hydroxylated at proline 700 (143). This modification was required for the stability of Ago2 proteins and siRNA-induced silencing. The Piwi family of proteins contain symmetric dimethyl arginine modifications that are catalyzed by the methyltransferase PRMT5 and are subsequently recognized by Tudor proteins (144–146). Both Piwi methylation and Tudor binding were required for proper stability, localization, and function of Piwi proteins in germ line development and transposon silencing (144–146).

The human miRNA-generating complex comprises Dicer and phospho-TRBP isoforms (101a). Mitogen activated protein kinase (MAPK)/Erk-mediated phosphorylation of TRBP stabilized the miRNA-generating complex and enhanced the capacity for miRNA production. Phosphorylation of TRBP resulted in a progrowth miRNA response, including upregulation of growth-promoting miRNAs and downregulation of the let-7 tumor suppressor miRNA, and was required for mitogenic signaling. MAPK/Erk also mediated oncogenic effects of Raf through another miRNA regulatory mechanism. By attenuating Myc-mediated transcription of Lin28, the Raf kinase inhibitory protein increased let-7 expression and reduced tumor cell invasion and metastasis (147). In addition, MAPK/Erk-mediated phosphorylation of serine-387 on human Ago2 has been shown to localize RISC to processing bodies (148). Thus, the MAPK/Erk pathway appears to target multiple RNAi factors to effect cell signaling. Collectively, these early studies indicate that small RNA pathways are governed by layers of regulatory mechanisms and are intimately connected with broader cellular systems.

## CONCLUDING REMARKS

The early years following the birth of RNAi were marked by outstanding biochemical achievements providing a strong foundation for understanding small RNA function. The seminal work of Andrew Fire and Craig Mello (11) outlined dsRNA-induced target transcript silencing. Identification of these molecular events enabled development of a cell-free RNAi system using *Drosophila* embryo extract (13). Demonstration of dsRNA-induced mRNA cleavage activity in *Drosophila* S2 cells established scalable source material for biochemical purification of RISC (15). These milestone achievements provided the framework for subsequent studies elucidating biochemical mechanisms of regulatory RNA pathways.

In recent years, these advances have significantly slowed. Many of the current voids in understanding small RNA pathways stem from poorly defined molecular events. The controversy as to the mechanisms by which miRNAs exert target silencing is a clear example of this. Moreover, the lack of information on piRNA precursors and piRNA targets is a major limitation that precludes development of cell-free assays, which, in turn, severely limits understanding of the biochemical mechanisms of piRNA biogenesis and function. As genome analysis indicates expansive RNA networks far beyond current understanding, the biochemical principles of regulatory RNA functions outlined herein provide a framework for projected discoveries. Because of the importance of small RNAs in directing biological



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and pathological events, it has become increasingly important to understand the relationships between noncoding RNA pathways and wider cellular systems. A tantalizing prospect for small RNA function is the possibility of RNA-induced gene expression (149). Robust demonstration of de facto induction of gene expression would be a landmark achievement. Understanding the biochemical principles of known small RNA pathways will facilitate discovery of the many other regulatory RNA systems on the horizon.

### SUMMARY POINTS

1. Demonstration of dsRNA-induced silencing represented a monumental shift away from the central dogma of molecular biology, revealing regulatory roles for RNA.
2. Identification of dsRNA-induced mRNA degradation indicated the molecular players of RNAi, enabling development of cell-free assays.
3. Demonstration of dsRNA-induced mRNA cleavage activity in *Drosophila* S2 cells provided scalable source material for biochemical purification of RISC.
4. These fundamental advances provided a conceptual framework for elucidating the biochemical mechanisms of an expanding number of small RNA pathways.

### FUTURE ISSUES

1. What are the biochemical functions of nonslicing Agos?
2. How are small RNA effector complexes programmed?
3. What are the biochemical mechanisms by which miRNAs effect target silencing?
4. What defines a piRNA precursor, and how are primary piRNA generated?
5. What are the biochemcial mechanisms by which piRNAs effect target silencing?
6. What are the relationships between small RNA pathways and wider cellular systems?

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## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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# Contents

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**Preface**

The Power of One
*James E. Rothman* ........ v

**Prefatory Article**

Frontispiece
*Aaron Klug* ........ xiv

From Virus Structure to Chromatin: X-ray Diffraction to Three-Dimensional Electron Microscopy
*Aaron Klug* ........ 1

**Recent Advances in Biochemistry**

Genomic Screening with RNAi: Results and Challenges
*Stephanie Mohr, Chris Bakal, and Norbert Perrimon* ........ 37

Nanomaterials Based on DNA
*Nadrian C. Seeman* ........ 65

Eukaryotic Chromosome DNA Replication: Where, When, and How?
*Hisao Masai, Seiji Matsumoto, Zhiying You, Naoko Yoshizawa-Sugata, and Masako Oda* ........ 89

Regulators of the Cohesin Network
*Bo Xiong and Jennifer L. Gerton* ........ 131

Reversal of Histone Methylation: Biochemical and Molecular Mechanisms of Histone Demethylases
*Nima Mosammaparast and Yang Shi* ........ 155

The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End-Joining Pathway
*Michael R. Lieber* ........ 181

The Discovery of Zinc Fingers and Their Applications in Gene Regulation and Genome Manipulation
*Aaron Klug* ........ 213

Origins of Specificity in Protein-DNA Recognition
*Remo Rohs, Xiangshu Jin, Sean M. West, Rohit Joshi, Barry Honig, and Richard S. Mann* ........ 233

Transcript Elongation by RNA Polymerase II
*Luke A. Selth, Stefan Sigurdsson, and Jesper Q. Svejstrup* ........ 271

Biochemical Principles of Small RNA Pathways
*Qinghua Liu and Zain Paroo* ........ 295

Functions and Regulation of RNA Editing by ADAR Deaminases
*Kazuko Nishikura* ........ 321

Regulation of mRNA Translation and Stability by microRNAs
*Marc Robert Fabian, Nahum Sonenberg, and Witold Filipowicz* ........ 351

Structure and Dynamics of a Processive Brownian Motor: The Translating Ribosome
*Joachim Frank and Ruben L. Gonzalez, Jr.* ........ 381

Adding New Chemistries to the Genetic Code
*Chang C. Liu and Peter G. Schultz* ........ 413

Bacterial Nitric Oxide Synthases
*Brian R. Crane, Jawahar Sudhamsu, and Bhumit A. Patel* ........ 445

Enzyme Promiscuity: A Mechanistic and Evolutionary Perspective
*Olga Khersonsky and Dan S. Tawfik* ........ 471

Hydrogenases from Methanogenic Archaea, Nickel, a Novel Cofactor, and $H_2$ Storage
*Rudolf K. Thauer, Anne-Kristin Kaster, Meike Goenrich, Michael Schick, Takeshi Hiromoto, and Seigo Shima* ........ 507

Copper Metallochaperones
*Nigel J. Robinson and Dennis R. Winge* ........ 537

High-Throughput Metabolic Engineering: Advances in Small-Molecule Screening and Selection
*Jeffrey A. Dietrich, Adrienne E. McKee, and Jay D. Keasling* ........ 563

Botulinum Neurotoxin: A Marvel of Protein Design
*Mauricio Montal* ........ 591

Chemical Approaches to Glycobiology
*Laura L. Kiessling and Rebecca A. Splain* ........ 619

Cellulosomes: Highly Efficient Nanomachines Designed to Deconstruct Plant Cell Wall Complex Carbohydrates
*Carlos M.G.A. Fontes and Harry J. Gilbert* ........ 655

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Somatic Mitochondrial DNA Mutations in Mammalian Aging
*Nils-Göran Larsson* .......... 683

Physical Mechanisms of Signal Integration by WASP Family Proteins
*Shae B. Padrick and Michael K. Rosen* .......... 707

Amphipols, Nanodiscs, and Fluorinated Surfactants: Three Nonconventional Approaches to Studying Membrane Proteins in Aqueous Solutions
*Jean-Luc Popot* .......... 737

Protein Sorting Receptors in the Early Secretory Pathway
*Julia Dancourt and Charles Barlowe* .......... 777

Virus Entry by Endocytosis
*Jason Mercer, Mario Schelhaas, and Ari Helenius* .......... 803

**Indexes**

Cumulative Index of Contributing Authors, Volumes 75–79 .......... 835

Cumulative Index of Chapter Titles, Volumes 75–79 .......... 839

**Errata**

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