A brief history of RNAi: the silence of the genes

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ABSTRACT **The use of the RNA interference (RNAi) pathway to eliminate gene products has greatly facilitated the understanding of gene function. Behind this remarkable pathway is an intricate network of proteins that ensures the degradation of the target mRNA. In this review, we explore the history of RNAi as well as highlighting recent discoveries.—Sen, G. L., Blau, H. M. A brief history of RNAi: the silence of the genes. *FASEB J.* 20, 1293–1299 (2006)**

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ORIGINS OF RNA INTERFERENCE

RNA interference (RNAi) has revolutionized studies to determine the role of a gene. The advent of massive genome sequencing projects has highlighted the marked need for a means of elucidating gene function. Loss of function studies using antisense and homologous recombination are cumbersome and variably successful. RNAi now provides a rapid means of depleting mRNAs by introducing double-stranded RNA homologous to a particular message leading to its sequence-specific degradation. As with many great discoveries, the history of RNAi is a tale of scientists able to interpret unexpected results in a novel and imaginative way.

Napoli and Jorgensen were the first to report an RNAi type of phenomenon in 1990 (1). The goal of their studies was to determine whether chalcone synthase (CHS), a key enzyme in flavonoid biosynthesis, was the rate-limiting enzyme in anthocyanin biosynthesis. The anthocyanin biosynthesis pathway is responsible for the deep violet coloration in petunias. In an attempt to generate violet petunias, Napoli and Jorgensen overexpressed chalcone synthase in petunias, which unexpectedly resulted in white petunias. The levels of endogenous as well as introduced CHS were 50-fold lower than in wild-type petunias, which led them to hypothesize that the introduced transgene was "cosuppressing" the endogenous CHS gene. In 1992, Romano and Macino reported a similar phenomenon in *Neurospora crassa* (2), noting that introduction of homologous RNA sequences caused "quelling" of the endogenous gene. RNA silencing was first documented in animals by Guo and Kemphues, who observed that the introduction of sense or antisense RNA to par-1 mRNA resulted in degradation of the par-1 message in *Caenorhabditis elegans* (3). At that time, antisense was one of the most attractive means of eliminating gene expression. Antisense was thought to function by hybridization with endogenous mRNAs resulting in double-stranded RNA (dsRNA), which either inhibited translation or was targeted for destruction by cellular ribonucleases. Surprisingly, when Guo and colleagues performed control experiments using only the sense par-1 RNA, which would not hybridize with the endogenous par-1 transcript, the par-1 message was still targeted for degradation. This finding caused investigators to rethink the current dogma.

ELUCIDATION OF THE SILENCING TRIGGER

In 1998, Fire and Mello published a seminal paper that provided an explanation for the previously reported silencing of endogenous genes by "cosuppression, quelling and sense mRNA" (4). Working with *C. elegans*, they tested the hypothesis that the trigger for gene silencing was not single-stranded RNA (ssRNA) but double-stranded RNA (dsRNA). They reasoned that the seemingly paradoxical finding of Guo and Kemphues showing that introduction of sense RNA leads to gene silencing could have been due to the contamination of preparations of ssRNA by dsRNA resulting from the activity of bacteriophage RNA polymerases (4). To address this possibility, Fire and Mello extensively purified the sense and antisense ssRNA preparations, then directly compared their effects to dsRNA on the unc-22 gene. The purified ssRNAs (sense or antisense) were consistently found to be 10- to 100-fold less effective than dsRNA targeting the same mRNA. Indeed, ssRNA was found to be effective only if the sense strand was injected into the animals, followed by the antisense strand or *vice versa*, suggesting that hybridization of the ssRNA to form dsRNA occurred *in vivo*. Thus, Fire and Mello provided the first explanation for previous observations, implicating integrated transgenes in the production of dsRNA in plants and fungi, and contamina-

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tion of sense RNA by dsRNA in worms. While this work established an entirely new conceptual framework for the effects of RNA on gene silencing by highlighting a role for dsRNA, a plethora of questions remained regarding the mechanism by which dsRNA could cause the degradation of endogenous mRNA.

IDENTIFICATION OF A SILENCING INTERMEDIATE

When dsRNA was injected into one region of a worm or plant, it caused systemic silencing, which led to the hypothesis that the RNAi effect was mediated by a stable silencing intermediate. This hypothesis was further supported by the observation that gene silencing could be passed from parent to progeny in *C. elegans* (5, 6). The existence of stable intermediates was first demonstrated by plant virologists Hamilton and Baulcombe (7). Although it was generally thought that the dsRNA had to unwind in order for the antisense strand to bind to the mRNA, the full-length antisense strand was never detected. This led Hamilton and Baulcombe to search for shorter forms of the antisense RNA derived from the dsRNA. They hypothesized that antisense RNA could serve as a guide, binding to the mRNA and causing its degradation. When Hamilton and Baulcombe detected antisense RNA that had an estimated length of 25 nucleotides (nt), they suggested that this length was necessary for RNAi specificity. The following year, two independent teams of biochemists used extracts from *Drosophila* cells to identify the silencing intermediate (8, 9). Upon fractionation, both groups found that 21–23 nt RNA always copurified with RNAi, suggesting that dsRNA was converted to shorter intermediates, small interfering RNAs (siRNAs) capable of binding to their homologous target mRNAs, leading to cleavage of the transcript (8, 9).

To determine definitively that the 21–23 nt dsRNAs are the effector molecules of the RNAi pathway, Tuschl and colleagues incubated Drosophila cell extracts with chemically synthesized 21–22 nt dsRNAs targeting a firefly luciferase transcript (10). The siRNAs were able to act as guides to mediate cleavage of the target mRNA. siRNAs with 2–3 nt overhangs on their 3′ ends were more efficient in reducing the amount of target mRNA than siRNAs with blunt ends. The target mRNA was found to be cleaved near the center of the region encompassed by the 21–22 nt RNAs 11 or 12 nt downstream of the first base pair between the siRNA and target mRNA (10). These short chemically synthesized 21–22 nt siRNAs were capable of silencing not only heterologous but also endogenous genes in mammalian cells (11). Up to this point, the use of RNAi was limited to flies, worms, and plants, as the introduction of long dsRNA into mammalian cells elicits an interferon response that causes a general inhibition of translation abrogating the specificity of RNAi. The finding that short dsRNA could silence genes heralded the use of RNAi in mammalian cells.

DISCOVERY OF THE dsRNA ENDONUCLEASE, DICER

In an attempt to determine whether the enzymes responsible for the cleavage of the dsRNA into siRNAs were different from those involved in the cleavage of the target mRNA, Bernstein et al. turned to *Drosophila* cell extracts to test whether the two enzymatic activities were separable (12). Remarkably, they found that the two activities could be resolved simply by high-speed centrifugation. The activity that cleaved the target mRNA, which they coined RNA-induced silencing complex (RISC), was cleared by centrifugation, whereas the activity that cleaved the dsRNA into siRNAs remained in the supernatant (8, 12). These two distinct phases of the RNAi pathway were designated the initiator (dsRNA converted to siRNAs) and effector (RISC-mediated cleavage of target mRNA) phases.

Hannon and colleagues took a candidate gene approach to the isolation of the initiator enzyme. Since they knew that the enzyme had to be a dsRNA ribonuclease, they tested representatives from the three types of RNase III family members. Type I of the RNase III enzymes is similar to bacterial ribonucleases, which contain a single RNase III motif and a dsRNA binding domain. Type II enzymes, which include Drosha, are characterized by two RNase III motifs and a dsRNA binding domain, whereas type III enzymes contain two RNase III motifs but also have an amino-terminal helicase domain (12). The T7 epitope was used to tag a representative from each type of RNase III enzyme. These constructs were then transfected into Drosophila S2 cells and the proteins were immunoprecipitated using the T7 tag. Each immunoprecipitate was tested for dsRNA cleavage activity capable of yielding the characteristic 21–23 nt siRNAs. Only one enzyme had the requisite activity, a type III enzyme encoded by gene number CG4792, later given the more memorable name of Dicer (12). Depletion of Dicer using dsRNAs resulted in loss of dsRNA-induced gene silencing of other genes, indicating that Dicer was essential for the initiator phase of the RNAi pathway. Dicer homologues have now been found in all organisms in which RNAi activity has been reported (13). Following the identification of Dicer, research on RNAi shifted to an elucidation of the components of RISC, in particular the enzyme that mediated the cleavage of the target mRNA.

IDENTIFICATION OF "SLICER"

To purify RISC, Tuschl and colleagues used cell extracts derived from human HeLa cells. They devised a clever means of partially purifying RISC by conjugating the 3′ termini of siRNAs to biotin, which enabled coimmunoprecipation of the siRNA with associated protein complexes. Precipitated complexes were further purified based on size and molecular weight (14). Two proteins of ~100 kDa were identified that corre-

sponded to Argonaute 1 and Argonaute 2 (Ago1 and Ago2) (14).

Argonaute proteins are a highly conserved family that have been implicated in RNAi across several species (15). Argonaute proteins were named for Ago1 mutants in *Arabidopsis*, which resulted in various phenotypic abnormalities, including radialized leaves, and abnormal infertile flowers that resembled small squid (15). Family members have two characteristic domains: PAZ and PIWI (14). Argonaute proteins have been found to be associated with RNAi in various organisms in screens for RNAi-deficient mutants. These include *C. elegans* rde-1, *Arabidopsis* AGO1, and *Neurospora* QDE2 (16–18). There are eight human Argonaute proteins, of which four belong to the Argonaute subcategory (Ago1–4) and four to the Piwi subcategory (hPiwi 1–4).

Whether the Ago1 and 2 isolated by purifying RISC had "Slicer" activity responsible for cleaving target mRNAs was unclear (14). Although Argonaute proteins had been identified as essential for RNAi, as discussed above, conclusive evidence that any of the Argonaute proteins had endonuclease activity has been lacking. Moreover, the amino acid sequence of Ago1 and Ago2 revealed no canonical ribonuclease domains. Over the next few years various laboratories reported other proteins that were part of RISC, but none of these was "Slicer."

In 2004, two groups reported the identification of "Slicer." A group led by Joshua-Tor crystallized an Argonaute protein from the archebacterium *Pyrococcus furiosus* (PfAgo) (19). The Piwi domain of PfAgo showed a remarkable similarity to the conserved secondary structure of RNase H enzymes. The active site of the RNase H domain is comprised of the DDE motif, which consists of three amino acids with the side chain carboxylates positioned to catalyze the cleavage reaction. The reaction is dependent on divalent cations such as Mg^{2+} or Mn^{2+}. The RNase H enzymes are unlike most ribonucleases and resemble deoxyribonucleases, which leave 3′ OH and 5′ phosphate termini. Slicer activity, like RNase H enzyme activity, is dependent on divalent cations and leaves 3′ OH and 5′ phosphate termini (20). Two of the three DDE (aspartate-aspartate-glutamate) motif catalytic carboxylates are in the same secondary structure positions in the PfAgo as in the RNase H enzymes (19). Using the structure of PfAgo, Joshua-Tor and colleagues modeled the structure of human Argonautes. Accordingly, the N-terminal, PIWI, and middle domains of Argonaute lie in a crescent to support the fourth domain, PAZ. The 3′ end of the single-stranded siRNA sits in the groove of the PAZ domain, which allows the rest of the siRNA to bind to target mRNA. The target mRNA then sits on top of the PIWI domain, which causes cleavage of the message.

Collaborating with the group of Joshua-Tor, Hannon, and co-workers Myc tagged Ago1–4 and immunoprecipitated each complex from human 293-T cells and showed that only Ago2 immunoprecipitates retained cleavage activity, even though all Argonaute proteins could bind to siRNAs (21). This finding indicated that Ago2 might have "Slicer" activity. Since Ago2 was the only Argonaute capable of forming cleavage-competent RISC, Ago2 null mice were generated. These mice were embryonic lethal due to several developmental abnormalities, including failure of the neural tube to close and swelling inside the pericardial membrane (21). It was unclear why Ago1, 3, and 4 exhibited no cleavage. To address this question, Hannon's group mutated key amino acids corresponding to those in the cleavage-incompetent Argonautes. A series of eight mutants was generated that retained siRNA binding activity, but two Q633R and H634P exhibited a loss of cleavage activity. In addition, analysis of the PIWI domain revealed that two of the three amino acids were conserved between the catalytic triad of RNase H enzymes and Ago2, suggesting that mutation of these amino acids could lead to a loss of cleavage activity. Indeed, when the two conserved aspartates of the catalytic triad were converted to alanine, D597A and D669A, the resulting mutants no longer had Ago 2 cleavage capacity but retained siRNA binding activity (21). Once "Slicer" was found, attention focused on how siRNAs were unwound and became incorporated into RISC.

ENTRY OF siRNAs INTO RISC

It has long been assumed that the double-stranded siRNAs must be unwound by an RNA helicase in order to be incorporated into RISC, as RISC contains only a single-stranded siRNA (14). This putative "unwindase" enzyme remains to be identified.

At the end of 2005, two independent groups, Gregory et al. and Matranga et al., overturned the long-held belief that helicases played a major role in unwinding ds siRNAs by demonstrating that Ago2 is responsible for cleaving the nonincorporated (passenger) strand of the siRNA duplex, allowing the other strand to be incorporated into RISC (22, 23). Evidence that the passenger strand of the siRNA serves as the first target of RISC is derived from detection of the predicted 9-nucleotide 5′ cleavage and 12-nucleotide 3′ cleavage products. How the RISC/Ago2 determines which strand of the double-stranded siRNA to cleave and which strand to incorporate into the RISC complex was a mystery. In 2003, a group led by Jayasena addressed this question by comparing the thermodynamic properties of functional and nonfunctional siRNAs (24). Their results indicated that the 5′ antisense region of the functional siRNAs were less thermodynamically stable than the 5′ sense regions, providing a basis for entry into RISC.

IDENTIFICATION OF MINIMAL RISC

Various groups have reported that RISC activity is associated with complex sizes ranging from 160 kDa, 200 kDa, 500 kDa, 550 kDa, to 80S (14, 25–27). One

thing these effector complexes have in common is the presence of an Argonaute protein. Although Ago2 is the enzyme responsible for cleaving the passenger strand siRNA as well as target mRNA, purified Ago2 cannot use double-stranded siRNAs as a substrate for target mRNA cleavage (21). This observation suggests that additional cofactors are needed for RISC activity. Recent evidence has suggested that Dicer may not only play a role in the initiator phases of the RNAi pathway, but also at later phases (27, 28).

In 2005, Gregory et al. identified a 500 kDa minimal RISC by characterizing proteins that copurified with human Dicer (29). Two proteins were found to be associated with Dicer, Ago2, and TRBP (the HIV transactivating response RNA-binding protein). The same group had shown earlier that TRBP is required for recruiting Ago2 to siRNA bound Dicer (28). Subsequently, this ternary complex was shown to be sufficient for RNAi activity when programmed with either double-stranded siRNA or unprocessed dsRNA (29).

RNAi ACTIVITY AND mRNA TRANSLATION

In *Drosophila,* RISC activity is associated with the 80S complex, suggesting a link between RISC and mRNA translation (27). The hypothesis that RISC activity was coupled to translation was attractive as it provided an expedient means for RISC to scan every translated mRNA for hybridization with siRNAs. In support of this hypothesis was the finding that translationally dormant mRNAs in *D. melanogaster* oocytes were immune to cleavage by RNAi (30). Alternatively, proteins could be bound to untranslated mRNAs in oocytes, precluding access of the translational machinery as well as RNA interference components, resulting in a block of both translation and RNAi. It remained to be determined whether inhibition of translation and RNAi was causal or coincidental. To determine definitively whether RNAi activity requires active mRNA translation, RNAi activity was assayed in the presence or absence of translation of an iron-regulated reporter mRNA. In the absence of iron, Iron regulatory proteins 1 and 2(IRP 1 and 2) can bind to iron response elements (IREs) found in iron-regulated genes preventing translation by blocking the initiation factors from loading onto the message. In the presence of iron, IRPs 1 and 2 bind to iron instead of iron response elements, allowing translation to occur. Using this system, we and others demonstrated that RISC activity does not require active mRNA translation, as siRNAs directed against a reporter gene were functional in the presence or absence of translation of the targeted mRNA (31, 32).

LOCALIZATION OF AGO2 TO mRNA DECAY CENTERS

To shed light on function, attention continued to be directed to the localization of RNAi and RISC components within the cell. Eukaryotic cells possess two major means for regulating the turnover of mRNAs (33). One pathway utilizes the 3′-5′ exonuclease, exosome, to degrade the message (34). This process is thought to occur in the cytoplasm. The other pathway is thought to occur in specialized centers known as processing (P) bodies or cytoplasmic bodies/mRNA decay centers (35,36). These centers have been identified microscopically by immunofluorescence as discrete foci in which decapping enzymes Dcp1 and 2 and the 5′-3′ exonucleases Xrn1 are concentrated (35, 37). It is thought that transcripts are transported to these centers to be degraded by the 5′-3′ exonuclease, Xrn1. In yeast, experimental evidence supports the hypothesis that unstable messages with a high turnover rate are transported to these centers to be degraded, as a genetic knockout of Xrn1 results in the accumulation of those messages in decay centers (36).

The 5′ and 3′ cleavage products resulting from the siRNA targeted mRNAs utilize different cellular exonucleases for degradation. The 5′ cleavage product is degraded by the exosome, as RNAi-mediated depletion of the enzyme resulted in accumulation of the 5′ fragment in *Drosophila* cells (38). In contrast, the 3′ cleavage product is degraded by the 5′-3′ exonuclease, Xrn1, as RNAi-mediated depletion or genetic knockout of the enzyme results in an accumulation of the 3′ fragment (38–40). However, it is unknown whether this enzymatic activity in mammalian cells was diffusely distributed or concentrated in subcellular domains.

In 2005, we and others established the intracellular location of Ago2/RISC. Ago2 was found to be localized to the cytoplasm, with most of the Ago2 concentrated in discrete cytoplasmic bodies, the mammalian equivalent of yeast P-bodies (41–43). Ago2 localization to mRNA decay centers was demonstrated by colocalization with other cytoplasmic body proteins, including Dcp1and 2 and GW182. SiRNAs conjugated to fluorophores were also found in these mRNA decay centers (44). Recently, two groups set out to determine whether localization of Ago2 to decay centers had functional significance. To address this question, decay center integrity was disrupted by depleting cells of GW182, a protein necessary for cytoplasmic body formation, and RNAi activity was assayed (44, 45). Loss of decay centers resulted in loss of mRNA degradation via the RNAi pathway.

The localization of Ago2 to P bodies suggests two as yet unresolved hypotheses with respect to its role in RNAi function. *1) Ago2 transits between the cytoplasm and decay centers.* According to this model, the localization pattern of Ago2 is dependent on its association with siRNA and the targeted mRNA. The RNAi response would entail the conversion of dsRNA to siRNAs by Dicer, allowing TRBP to recruit Ago2. This complex would likely form in the cytoplasm, since Dicer is localized in the cytoplasm (46). The ternary complex could then associate with target mRNA, resulting in cleavage of the mRNA by Ago2. According to this hypothesis, the 5′ cleavage product would be released

and destroyed by the exosome while the remaining 3′ fragment would be transported by Ago2 to P bodies to be degraded by Xrn1. The degradation of the 3′ fragment could serve as the trigger for Ago2 to recycle back to the cytoplasm and associate with Dicer and TRBP (**Fig. 1**, Model 1).

2) Ago2 is permanently localized in decay centers. According to this model, Ago2 is associated with Dicer and TRBP in the decay centers during an RNAi response. If Ago2 is permanently localized in the P bodies, another factor must recruit the mRNAs to these decay centers. This would suggest that mRNAs are constantly directed to decay centers. Evidence in support of this hypothesis derives from the finding in yeast that there is competition between translation of messages and direction of messages to P-bodies (47). Perturbation in the levels of Dhh1p and Pat1p, two proteins responsible for translational repression and P body formation, has been shown to alter the ratio of messages translated to those sent to decay centers (47). RCK, the human homologue of Dhh1p, has been shown to have a similar function in mammalian cells (47). Thus, in this model mRNAs would be constantly sent to P bodies to allow active sampling by Ago2/RISC (Fig. 1, Model 2).

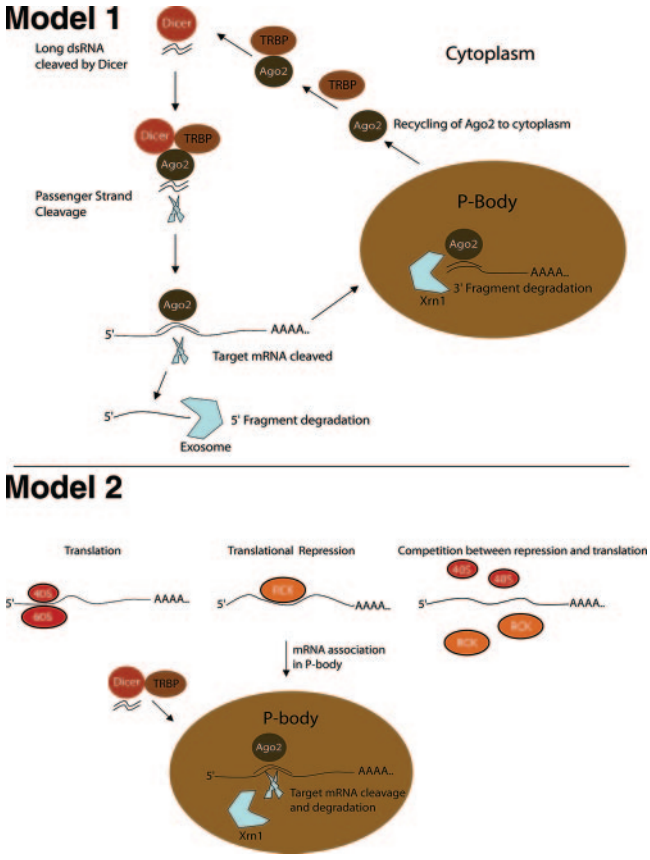

Figure 1. Two models for RNAi-mediated mRNA degradation. Model 1: Long double-stranded RNA is cleaved by Dicer, which results in the recruitment of Ago2 by TRBP. Ago2 cleaves the passenger strand siRNA, then associates with target mRNA. On cleavage of target mRNA, the 5′ fragment is destroyed by the exosome while the 3′ fragment is transported to P-bodies to be degraded by Xrn1. Degradation of the 3′ fragment releases Ago2 to the cytoplasm to once again associate with target mRNA. Model 2: mRNA associates in P-bodies due to RCK, which allows Ago2 and RISC to actively sample targets for hybridization with the siRNA. Upon hybridization, the target mRNA is cleaved and fragments are degraded all within P-bodies.

FUTURE DIRECTIONS AND REMAINING CHALLENGES

Many outstanding questions remain with respect to the silencing of the genes and the roles of components of the RNAi pathway. Double-stranded RNA that is introduced into cells results in recognition by the RNase III enzyme, Dicer, which cleaves the RNA into 21–23 nt siRNAs. Dicer forms a ternary complex with Ago2 and TRBP. Upon formation of the complex, Ago2 cleaves the passenger strand of the siRNA duplex, which then allows the complex to associate with the target mRNA. Whether this association occurs in the cytoplasm or decay centers remains to be determined. Regardless, the end result is the formation of a complex of Ago2/siRNA/mRNA in P-bodies where the cleaved message is degraded by cellular exonucleases, highlighting the importance of decay centers.

The finding that Ago2 localizes to decay centers suggests that its function may not be restricted to the RNAi pathway but may also participate more broadly in mRNA degradation pathways. For example, Ago2 may have a role in the NMD pathway, as NMD-containing transcripts are thought to be degraded in mRNA decay centers (48). Ago2 may also have a role in regulating endogenous gene expression. The degradation of certain transcripts such as alpha-globin, insulin-like growth factor II, and albumin is known to be dependent on an endonucleolytic cleavage within the 3′UTR (49–51). Could this endonuclease be Ago2? Further experiments are required to determine whether Ago2 has a role in the degradation of such endogenous mRNAs. Ago2 may not only regulate endogenous RNAs that are translated, but also untranslated RNAs, including microRNAs that have been shown to localize to cytoplasmic bodies (43). Even those messages that are translated may be regulated through untranslated regions, for example, within 3′UTRs, known to have a role in growth, differentiation, and tumorigenicity.

Recent data suggest that subsets of transcripts for example, those that have AU rich elements (ARE) in their 3′UTRs and are inherently unstable messages may be regulated via the Ago2 and Dicer pathway. In support of this hypothesis, the RNAi-mediated depletion of Ago2 or Dicer resulted in the stabilization of mRNA containing AREs of tumor necrosis factor (52). This finding raises the question of whether all ARE transcripts are subjected to Ago2 and Dicer regulation or only a specific subset. The role of other Ago proteins remains to be elucidated. Possibly the four Ago proteins have specificity for different subsets of transcripts, for example, those with roles in growth or differentiation, regulatory pathways that are generally mutually

exclusive. The role each different Argonaute protein plays in regulating gene expression needs to be explored; although they all localize to decay centers, the transcripts each controls may differ. The RNAi pathway was once thought to be evolutionarily conserved to protect cells from viral infection, but this view was clearly too limited. RNAi is likely to strongly impact all areas of cell physiology.

CONCLUDING REMARKS

In the past few years the RNAi pathway has become the predominant means of assessing loss of gene function in most organisms. The ease with which genes can be silenced via the RNAi pathway has led to the generation and screening of genome-wide siRNA libraries in many organisms (53–59). The remarkable utility of RNAi in modulating gene expression has resulted in an explosion of interest in deciphering the molecular mechanisms that control this pathway and imaginative ideas of ways to apply it to research and clinically. The pace of progress has been staggering, yet many mysteries remain to be solved as we search for ways to silence the genes in this world of small RNAs with enormous effects.

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