Ribo-gnome: The Big World of Small RNAs

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Small RNA guides—microRNAs, small interfering RNAs, and repeat-associated small interfering RNAs, 21 to 30 nucleotides in length—shape diverse cellular pathways, from chromosome architecture to stem cell maintenance. Fifteen years after the discovery of RNA silencing, we are only just beginning to understand the depth and complexity of how these RNAs regulate gene expression and to consider their role in shaping the evolutionary history of higher eukaryotes.

In 1969, Britten and Davidson proposed that RNAs specify which genes are turned on and which are turned off in eukaryotic cells (*1*). Their elegant idea was that the base-pairing rules of Watson and Crick could solve the problem of eukaryotic gene regulation. With the subsequent discovery of protein transcription factors—there are perhaps 1850 in humans—the idea that a diverse array of RNA guides sets the expression profile of each cell type in a plant or animal was abandoned.

In fact, RNAs—specifically, tiny RNAs known as "small RNAs"—do control plant and animal gene expression. Distinct classes of these small RNAs—microRNAs (miRNAs), small interfering RNAs (siRNAs), and repeat-associated small interfering RNAs (rasiRNAs)—are distinguished by their origins, not their functions [see the poster in this issue (*2*)]. One class alone, the miRNAs, is predicted to regulate at least one-third of all human genes (*3*). Small RNAs, 21 to 30 nucleotides (nt) in length, provide specificity to a remarkable range of biological pathways. Without these RNAs, transposons jump (wreaking havoc on the genome), stem cells are lost, brain and muscle fail to develop, plants succumb to viral infection, flowers take on shapes unlikely to please a bee, cells fail to divide for lack of functional centromeres, and insulin secretion is dysregulated. The production and function of small RNAs requires a common set of proteins: double-stranded RNA (dsRNA)–specific endonucleases such as Dicer (*4*), dsRNA-binding proteins, and small RNA–binding proteins called Argonaute proteins (*5*, *6*). Together, the small RNAs and their associated proteins act in distinct but related "RNA silencing" pathways that regulate transcription, chromatin structure, genome integrity, and, most commonly, mRNA stability. The RNAs may be small, but their production, maturation, and regulatory function require the action of a surprisingly large number of proteins.

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A Brief History of Small RNA

In 1990, two groups overexpressed a pigment synthesis enzyme in order to produce deep purple petunia flowers, but instead generated predominantly white flowers (Fig. 1) (*7*, *8*). This phenomenon was dubbed "cosuppression" because the transgenic and endogenous genes were coordinately repressed, and its discovery quietly ushered in the study of RNA silencing. By the end of the decade, RNA silencing phenomena were discovered in a broad spectrum of eukaryotes, from fungi to fruit flies. RNA interference (RNAi) is perhaps the best known RNA silencing pathway, in part because its discovery makes it possible to block expression of nearly any gene in a wide range of eukaryotes, knowing only part of the gene's sequence (*9*, *10*). Human clinical trials testing RNAi-based drugs are currently under way.

Building on the unexpected finding that both sense and antisense RNA could silence gene expression in *Caenorhabditis elegans* (*11*), the key breakthrough in RNA silencing was the discovery that dsRNA is the actual trigger of specific mRNA destruction, with the sequence of the dsRNA determining which mRNA is destroyed (*9*). Later, the dsRNA was found to be converted into siRNAs—fragments of the original dsRNA, 21 to 25 nt in length, that guide protein complexes to complementary mRNA targets, whose expression is then silenced (*12*–*14*). Thus, the actual mechanism of RNAi is remarkably like an early model for plant cosuppression, which postulated that small RNAs derived from the overexpressed gene might guide inactivation of cosuppressed genes (*15*).

In contrast to siRNAs, which derive from dsRNA hundreds or thousands of base pairs long, miRNAs derive from long, largely unstructured transcripts (pri-miRNA) containing stem-loop or "hairpin" structures ~70 nt in length [reviewed in (*16*)]. The hairpins are cut out of the pri-miRNA by the dsRNA-specific endonuclease Drosha, acting with its dsRNA-binding protein partner DGCR8 in humans or Pasha in flies, to yield a pre-miRNA (Fig. 2) (*2*). Each mature miRNA resides in one of the two sides of the ~30–base pair stem of the pre-miRNA. The mature miRNA is excised from the pre-miRNA by another dsRNA-specific endonuclease, Dicer, again acting with a dsRNA-binding protein partner, the *tar*-binding protein (TRBP) in humans or Loquacious (Loqs) in flies. The April 2005 release of the miRNA Registry, an online database that coordinates miRNA annotation, records 1650 distinct miRNA genes, including 227 from humans and 21 from human viruses; 1648 of these were discovered in the 21st century. Whereas siRNAs are found in eukaryotes from the base to the crown of the phylogenetic tree, miRNAs have been discovered in plants and animals and their viruses only.

Ambros and co-workers discovered the first miRNA, *lin-4*, in 1993. They identified two RNA transcripts—one small and one smaller—derived from the *lin-4* locus of *C. elegans* (*17*). Earlier experiments showed that loss-of-function mutations in *lin-4* disrupted the developmental timing of worms, much as did gain-of-function mutations in the protein-coding gene *lin-14*. Noting that *lin-4* could form base pairs, albeit imperfectly, with sites in *lin-14*, Ambros and colleagues proposed that the 22-nt *lin-4* regulates the much longer *lin-14* mRNA by multiple RNA-RNA interactions between the miRNA and the 3′ untranslated region of its mRNA target. This remarkable paper predicted the contemporary miRNA pathway, suggesting that the longer 61-nt transcript corresponds to a precursor RNA that folds into a hairpin structure from which the 22-nt mature *lin-4* miRNA is excised. Eight years later, the prescient observation that "*lin-4* may represent a class of developmental regulatory genes that encode small antisense RNA products" (*17*) was amply validated by the discovery that miRNAs compose a large class of riboregulators (*18*–*23*).

The *lin-4* miRNA was discovered 3 years after the first reports of RNA silencing in plants (*7*, *8*) and 2 years before the first hint of RNAi in nematodes (*11*). However, no formal connection between miRNAs and siRNAs was made until 2001, when Dicer, the enzyme that converts long dsRNA into siRNAs (*4*, *24*), was shown to convert pre-miRNAs, such as the longer 61-nt transcript from *lin-4*, into mature miRNAs, like *lin-4* itself (*25*–*27*).

The human genome may contain ~1000 miRNAs, a few of which may not only be unique to humans, but may also contribute to making us uniquely human. Recent efforts to define the entirety of this small RNA class have uncovered 53 miRNAs unique to primates (*28*). Because miRNAs are small, they may evolve

rapidly, with new miRNA genes arising by duplication and mutation of the 21-nt miRNA sequence.

Small on Specificity

From the standpoint of binding specificity, small RNAs are truly diminutive. A mere six or seven of the 21 nucleotides within a miRNA or siRNA provide the bulk of binding specificity for the small RNA–protein complexes they guide. As first proposed by Lai (*29*), and subsequently confirmed computationally (*3*, *30*) and experimentally for miRNAs (*31*–*34*) and siRNAs (*35*, *36*), the 5′ end of a miRNA or an siRNA contributes disproportionately to target RNA binding. Kinetic and structural studies suggest that the first nucleotide of a small RNA guide is unpaired during small RNA function (*36*–*38*). The small region of the small RNA that mediates target binding has been called the "seed sequence," a term intended to suggest that the region nucleates binding between the small RNA guide and its target, and that the more 3′ regions of the small RNA subsequently zipper-up—if they can—with the 5′ regions of the binding site on the target RNA (*39*).

In truth, current experimental evidence cannot discern the order in which distinct regions of the small RNA interact with its binding site on the target RNA. Both computational and experimental approaches detect only the binding contributions of specific small RNA regions at equilibrium. But the finding that stable binding between the small RNA and its target derives from such a small region of an already puny RNA oligonucleotide implies that the manner in which the small RNA interacts with its target is very different from antisense oligonucleotide–target RNA pairing. This radical and unexpected mode of nucleic acid interaction is almost certainly a consequence of the way the small RNA—both alone and paired to its RNA target—is bound by a member of the Argonaute family of proteins. These multidomain proteins are specialized for binding the small RNAs that mediate RNA silencing; understanding the relationship of Argonaute protein structure to their functions in controlling gene expression is now the key to understanding the deeper physical meaning of the small RNA "seed sequence."

The small RNAs that act in RNA silencing pathways are like fancy restriction enzymes whose recognition sites occur at random once every ~4000 to ~65,000 nt of sequence. But unlike restriction enzymes, which cut DNA wherever they bind, small RNAs can act in two distinct ways, each of which dramatically extends their functional specificity (Fig. 3) (*2*). When a small RNA pairs extensively with its RNA target, it directs cleavage of a single phosphodiester bond in the target RNA, across from nucleotides 10 and 11 of the small RNA guide (*40*). Thus, small RNA–directed cleavage is much more specific than small RNA binding itself, as it occurs only when most of the 21 nt of the siRNA or miRNA can base pair to form at least one turn of an A-form helix with the RNA target (*36*, *41*, *42*). Even when the small RNA is fully complementary to its target RNA, cleavage only occurs when the RNA is bound to the right Argonaute protein (*43*, *44*). In humans, only one of the four Argonaute proteins examined in detail retains all the amino acids required to catalyze target RNA cleavage (*45*). Argonaute proteins contain two RNA-binding domains: the Piwi domain, which binds the small RNA guide at its 5′ end, and the PAZ domain, which binds the single-stranded 3′ end of small RNA. The endonuclease that cleaves target RNAs resides in the Piwi domain, and this domain is a structural homolog of the DNA-guided RNA endonuclease RNase H (*46*). Target RNA cleavage is commonly viewed as the siRNA or RNAi mode, but is actually the dominant mechanism by which plant miRNAs regulate their targets (*47*, *48*) and is found for at least a small number of animal (*49*, *50*) and viral miRNAs (*51*).

In *Drosophila* or human cell lysates, small RNA–programmed Argonaute2 (Ago2) acts as a multiple-turnover enzyme, with each small RNA directing the cleavage of hundreds of target molecules (*36*, *52*). Small RNA–directed mRNA cleavage cuts an mRNA into two pieces, and efficient release of these fragments requires adenosine triphosphate (ATP) (*36*). Proteins besides Ago2 may be required for release of the products of small RNA–directed target cleavage. In fact, Ago2 alone can direct a single round of target cleavage but cannot efficiently catalyze additional cycles, likely because the cleavage products remain bound to the small RNA within the enzyme (*45*). After the cleaved pieces of the target are released, the 3′ fragment is destroyed in the cytoplasm by the exonuclease Xrn1 while the 5′ fragment is degraded by the exosome, a collection of exonucleases dedicated to 3′-to-5′ RNA degradation (*53*). In plants and animals, when miRNAs direct mRNA cleavage, a short polyuridine [poly(U)] tail is subsequently added to the 3′ end of the 5′ cleavage fragment (*54*). Addition of poly(U) correlates with decapping and 5′-to-3′ destruction of the target RNA cleavage fragment, at least in plants, suggesting an alternative route to the exosome for degradation of the 5′ cleavage product.

When siRNAs or miRNAs pair only partially with their targets, they cannot direct mRNA cleavage. Instead, they block translation of the mRNA into protein (*55*, *56*). However, binding of a single miRNA alone is usually insufficient to measurably block translation; instead, several miRNAs bind to the same target—opening the door to combinatorial control of gene expression by sets of coordinately expressed miRNAs (*39*). Initially, miRNAs were proposed to repress translation at a step after ribosomes have bound the mRNA, i.e., after translational initiation (*55*). One idea was that they direct degradation of the nascent polypeptide as it emerges from the ribosome. Alternatively, they might "freeze" ribosomes in place on the mRNA, stalling elongation of the growing protein chain. Recent findings, however, call these ideas into question. For example, translational repression by miRNAs was thought to affect

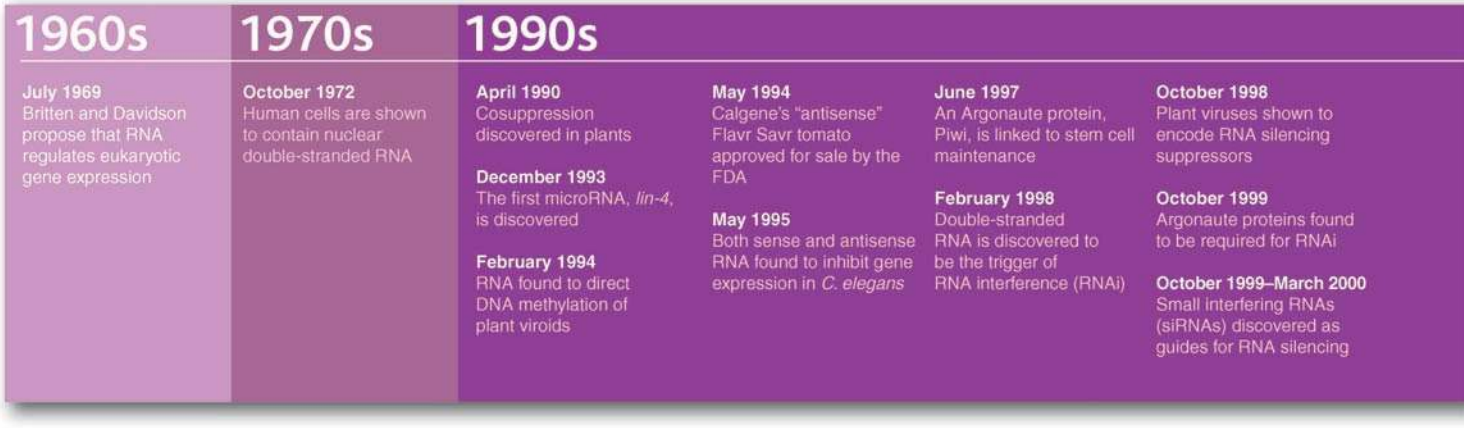

Fig. 1. A chronology of some of the major discoveries and events in RNA silencing.

only protein synthesis, not mRNA stability. Yet Lim and co-workers found that miRNAs can alter the stability of hundreds of mRNAs (*57*). And Pasquinelli and co-workers have now shown that even the founding miRNAs, worm *lin-4* and *let-7*, trigger destruction of their mRNA targets (*58*). These changes in steady-state mRNA levels are unlikely to reflect cleavage of the miRNA targets, because the complementarity between the miRNAs and their mRNA targets is restricted mainly to the seed sequence. How, then, could miRNAs make mRNA less stable? New studies offer a potential explanation.

Small RNAs, bound to Ago2, can move the mRNAs they bind from the cytosol to sites of mRNA destruction called "P-bodies" (*59*, *60*). Ago2 concentrates in P-bodies only when it binds small RNAs like miRNAs and siRNAs; Ago2 mutants that cannot bind small RNAs remain in the cytosol (*59*). Moreover, Ago2 associates with the enzymes that remove the 5′ 7-methylguanosine cap characteristic of mRNAs, a prerequisite for their destruction in the P-body (*59*, *60*). It is tempting to imagine that this new role for small RNAs, moving an mRNA to P-bodies, explains the mystery of small RNA–directed translational repression: By sequestering mRNA in the P-body, small RNAs would block translation. Subsequent destruction of the mRNA would then be a secondary consequence of relocating the mRNA from the cytosol to the P-body, which contains no ribosomal components (Fig. 4). Binding of a miRNA to the mRNA would not alter its inherent decay rate. The steady-state abundance of mRNAs that intrinsically turn over rapidly would therefore be reduced more than that of intrinsically more stable mRNAs when each is targeted by small RNA, but the translational rate of the two mRNAs would be reduced equally.

Is repression of mRNA translation by miRNAs just a consequence of the relocalization of the mRNA to the P-body? Filipowicz and colleagues argue in this issue of *Science* that translational repression comes first (*61*). They show that when bound to an mRNA target, human *let-7* miRNA blocks translational initiation. They propose that the consequence of miRNA-directed inhibition of translational initiation is relocalization of the mRNA target to the P-body. Once in the P-body, the mRNA may then be degraded, releasing the miRNA-programmed protein complex so it can return to the cytosol to begin a new round of target mRNA repression (Fig. 4). This pathway is presumed to be distinct from the small RNA–directed cleavage pathway, in which Ago2 in flies or mammals first cleaves a single phosphodiester bond in the mRNA target, and then the 5′ cleavage product is degraded by the exosome without obligate decapping.

Do all miRNAs repress gene expression? At least one human miRNA appears to act positively. Replication of hepatitis C virus (HCV) requires binding of human miR-122 to the 5′ noncoding region of the virus (*62*). Thus, for HCV, miR-122 acts as an enhancer of replication, and only cells expressing miR-122 support efficient HCV replication. Whether the positive effect of miR-122 on HCV is unique or represents an undiscovered mode of miRNA action remains unknown.

Aberrant, Unwanted

RNAi has been implicated in silencing parasitic DNA sequences, such as transposons and repetitive sequences. In many organisms, a specialized RNA silencing pathway senses the "aberrant RNA" transcribed from such sequences, and then initiates silencing posttranscriptionally and even transcriptionally. A candidate for an aberrant RNA sensor is a class of RNA silencing proteins that can copy single-stranded RNA into dsRNA. These RNA-dependent RNA polymerases (RdRPs) are found in nearly every eukaryote with a functioning RNA silencing pathway—except insects and mammals. In addition to initiating silencing responses from single-stranded trigger RNAs, RdRPs have been proposed to amplify and sustain silencing triggered by dsRNA. How RdRP enzymes distinguish between normal and aberrant transcription remains a key mystery of RNA silencing.

Meanwhile, Back in the Nucleus

RNA-directed transcriptional silencing was first identified in plants, where dsRNA corresponding to nontranscribed sequences can direct DNA methylation and transcriptional repression (*63*, *64*). Genetic studies in worms, plants, and *Schizosaccharomyces pombe* implicate small RNAs and the canonical components of the RNA silencing machinery—RdRP, Dicer, and Argonaute—in transcriptional silencing (*65*–*70*). Components of the RNAi machinery are also required for transcriptional silencing in flies (*71*, *72*). Transcriptional silencing directed by small RNAs is typically associated with the formation of heterochromatin, a transcriptionally repressed, compact form of chromatin in which the amino terminus of histone H3 is modified by methylation at lysine 9 ("H3K9"). In some organisms, such as plants and mammals, heterochromatic DNA is also hypermethylated. In *Tetrahymena*, small RNA–directed heterochromatin formation drives the deletion of specific regions of chromosomal DNA in the macronucleus (*73*, *74*).

A well-studied example of siRNA-directed assembly of heterochromatin is the outer regions of the centromere in *S. pombe*. Without this heterochromatin, *S. pombe* centromeres cannot reliably mediate chromosome segregation during cell division. Such a role for the RNA silencing machinery in assembling centromeric heterochromatin may be quite common, as chicken and mouse cells lacking Dicer also fail to assemble silent heterochromatin at their centromeres (*75*, *76*).

Repetitive, transposon-like sequences compose the outer regions of the *S. pombe* centromere. Mammalian centromeres likewise comprise repetitive sequences. Thus, how the RNA silencing machinery silences centromeric repeats may be just an example of the broader question of understanding the mechanism by which the RNA silencing machinery detects

2000s

October 2000
Double-stranded RNA shown to direct DNA methylation

January 2001
Dicer shown to make siRNAs

May 2001
RNAi discovered in human cells

July 2001
Dicer found to make microRNAs (miRNAs)

October 2001
miRNAs are established as a large class of gene regulators

July 2002
Plant miRNAs are discovered

July 2002
siRNAs are revealed as triggers of RNAi in mice

September 2002
Small RNAs guide the production of heterochromatin at centromeres

November 2002
miRNAs implicated in cancer

September 2003
It is clear that miRNA maturation begins in the nucleus

November 2003
Dicer shown to be required for mouse embryogenesis, and perhaps for stem cell production

March 2004
Human genome-wide RNAi libraries become available

April 2004
Animal viruses found to encode miRNAs

August 2004
First "investigational new drug" application filed for a therapeutic siRNA

September 2004
Argonaute is revealed as the RNAi endonuclease, "Slicer"

June 2005
miRNAs shown to act as oncogenes

July 2005
Primate-specific miRNAs identified

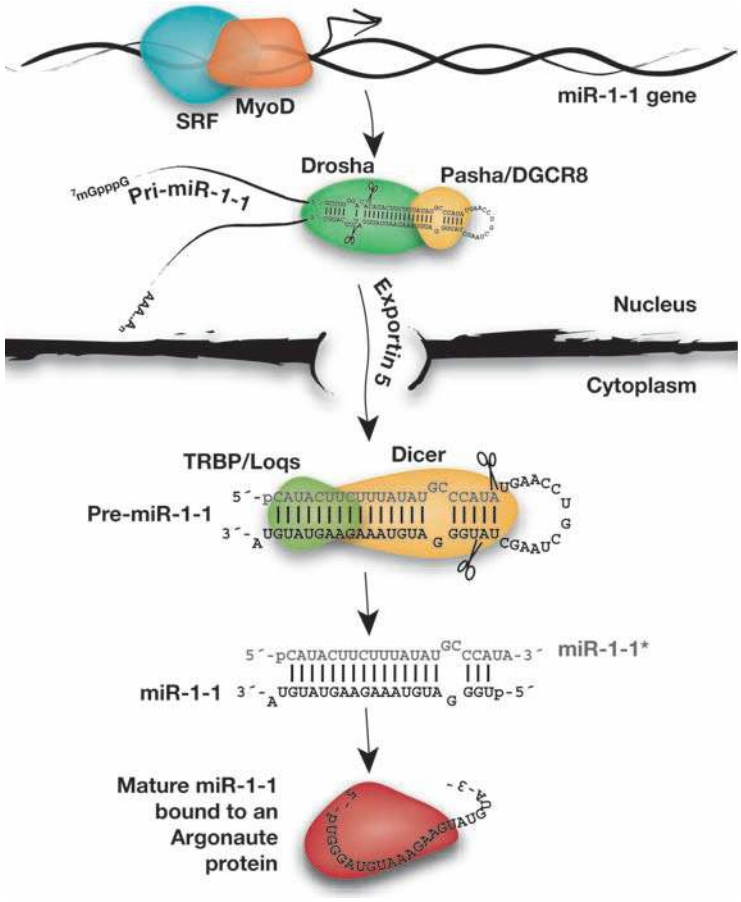

Fig. 2. A day in the life of the miRNA miR-1. In developing cardiac tissue, the transcription factors SRF (serum response factor) and MyoD promote RNA Pol II–directed transcription of pri-miR-1. In the nucleus, the RNase III endonuclease Drosha, together with its dsRNA-binding partner, Pasha/DGCR8, excises pre-miR-1 from pri-miR-1, breaking the RNA chain on both the 5′ and 3′ sides of the pre-miR-1 stem, leaving a ~2-nt, single-stranded 3′ overhang end. Exportin 5 recognizes this characteristic pre-miRNA end structure, transporting pre-miR-1 from the nucleus to the cytoplasm. In the cytoplasm, a second RNase III endonuclease, Dicer, together with its dsRNA-binding partner protein, Loqs/TRBP, makes a second pair of cuts, liberating miR-1 as a "miRNA/miRNA*" duplex. Mature, 21-nt long miR-1 is then loaded from the duplex into an Argonaute family member and miR-1* is destroyed. miR-1 guides the Argonaute protein to its target RNAs, such as the 3′ untranslated region of the *hand2* mRNA. Binding of the miR-1–programmed Argonaute protein represses production of Hand2 protein, halting cardiac cell proliferation.

and silences repetitive sequences. A coherent but speculative model of small RNA–directed transcriptional silencing emerges from recent studies in both *S. pombe* and plants. Transcripts from genomic regions to be targeted for silencing must first be converted to dsRNA. RdRPs have been assigned this role. Mutation of catalytically essential amino acids demonstrates that the polymerase activity of Rdp1, the sole *S. pombe* RdRP enzyme, is required for centromeric silencing (*77*), but what template RNA is copied by the RdRP has not been directly established in any organism. The dsRNA envisioned to be generated by the RdRP must next be converted to siRNAs, presumably by Dicer. In plants, distinct RdRP and Dicer paralogs are devoted to separate RNA silencing pathways, with RDR2, the RdRP, collaborating with DCL3, the Dicer, to generate siRNAs that target repetitive sequences for both cytosine and H3K9 methylation (*68*). Presumably the double-stranded siRNAs thus generated are unwound and the resulting single strands loaded into a member of the Argonaute family of proteins: Ago1 in *S. pombe* and AGO4, among others, in plants (*65–67*, *78*). The siRNAs, bound to the Argonaute protein within a larger complex of DNA and chromatin-modifying enzymes, guide the assembly of heterochromatin. How insects and mammals derive chromatin-silencing triggers in the absence of an RdRP is unknown.

What does it mean when we propose that siRNAs guide modifying enzymes to DNA, converting it to heterochromatin? Do we imagine that the siRNAs pair directly with single-stranded DNA, somehow separating the two strands of the chromosomal DNA, as proposed by Britten and Davidson (*1*)? Or rather, do the siRNAs bind RNA, as has been proposed for centromeric silencing in *S. pombe* (*79*)? This second model is comforting because it imagines that siRNAs interact with RNA in both

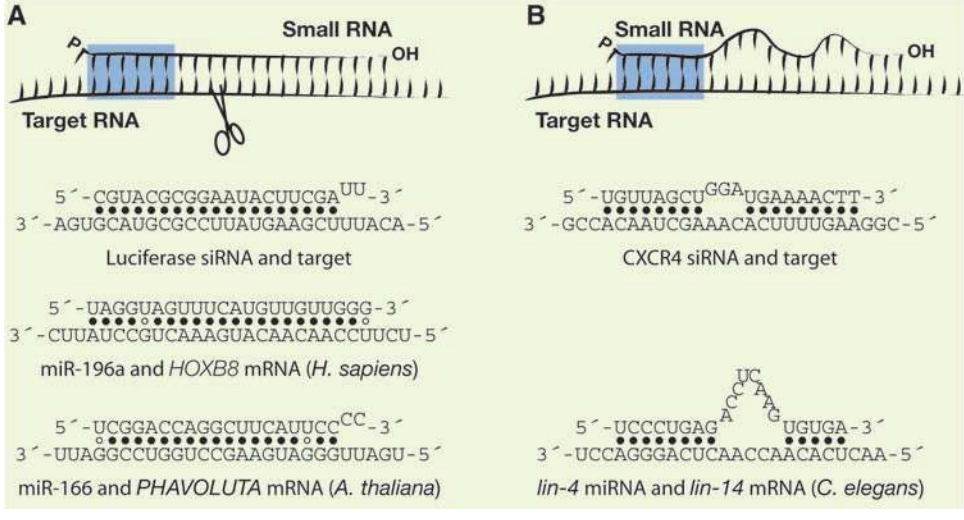

Fig. 3. Small RNA binding modes. (**A**) Extensive pairing of a small RNA to an mRNA allows the Piwi domain of a catalytically active Argonaute protein (e.g., Ago2 in humans or flies) to cut a single phosphodiester bond in the mRNA, triggering its destruction. Synthetic siRNAs typically exploit this mechanism, but some mammalian miRNAs (such as miR-196a) and most, if not all, plant miRNAs direct an Argonaute protein to cut their mRNA targets. (**B**) Partial pairing between the target RNA and the small RNA, especially through the "seed" sequence—roughly nucleotides 2 to 7 of the small RNA—tethers an Argonaute protein to its mRNA target. Binding of the miRNA and Argonaute protein prevents translation of the mRNA into protein. siRNAs can be designed to trigger such "translational repression" by including central mismatches with their target mRNAs; animal miRNAs such as *lin-4*, the first miRNA discovered, typically act by this mode because they are only partially complementary to their mRNA targets. The seed sequence of the small RNA guide is highlighted in blue.

transcriptional and post-transcriptional silencing, but it requires transcription across regions of DNA that were thought to lie untranscribed, such as promoters or intragenic regions. In plants, ''transcriptionally silenced'' DNA appears to be transcribed by a specialized type of DNA-dependent RNA polymerase, RNA polymerase IV (Pol IV) (*80*–*82*). RNA Pol IV may be specially adapted to transcribe silent heterochromatin, thereby providing a constant source of primary transcript to act as template for the RdRP and hence generating the dsRNA substrate required for Dicer to manufacture siRNAs (*80*). The model is appealing because it explains why siRNAs persist even after they have silenced the gene from which they arise.

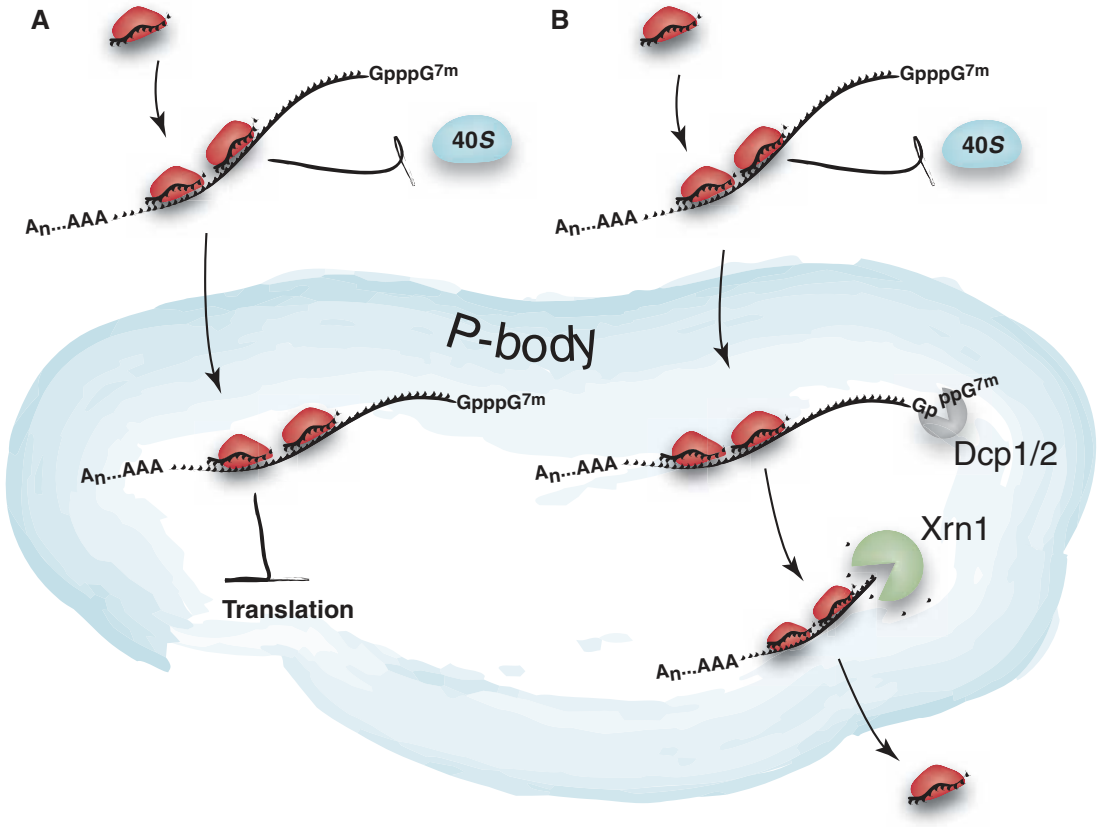

Fig. 4. A speculative model for translational repression by small RNAs: sequestration of a highly stable mRNA in the P-body. Binding of a small RNA–programmed Argonaute protein (red) to an mRNA blocks translational initiation, driving the mRNA into a P-body, the cytoplasmic site of mRNA decapping and degradation. Sequestering an mRNA in a P-body further excludes it from ribosomes, so it cannot be translated into protein. In (**A**), the mRNA is imagined to be degraded slowly in the P-body, so the miRNA appears only to repress translation. It is unknown whether mRNAs, once moved to the P-body by the binding of a small RNA, can ever return to the cytoplasm and resume translation. In (**B**), the mRNA is envisioned to be inherently prone to rapid degradation. Binding of an Argonaute-bound small RNA to an mRNA moves the mRNA to the P-body, where the decapping enzyme, Dcp1/2, is envisioned to remove its 7-methyl guanosine cap, triggering its destruction by the 5′-to-3′ exonuclease Xrn1. For the mRNA in (A), small RNAs appear to repress its translation without appreciably altering its steady-state abundance, whereas in (B), small RNAs appear to target the mRNA for exonucleolytic destruction, yet in both cases small RNAs change the cellular compartment in which the mRNA resides.

Pol IV might also supply the transcripts that provide a scaffold for siRNA-guided chromatin modification complexes to act on the adjacent DNA. Pol IV enzymes, however, occur only in plants; in *S. pombe*, formation of silent centromeric heterochromatin requires the classical RNA Pol II (*83*, *84*). This finding suggests that RNA Pol II may supply transcripts required for the production of the siRNAs themselves. For some *S. pombe* loci, perhaps even RNA Pol I provides these transcripts (*85*). Yet Pol II is required when the initial trigger of silencing is provided in trans—for example, by initiating silencing with a double-stranded hairpin—which suggests that Pol II transcription creates the target for the small RNAs as well as the trigger for small RNA production (*84*). Transcription per se is not sufficient; instead, a direct interaction between the carboxyl-terminal domain of Pol II and the RNA silencing machinery appears to help recruit the Argonaute protein, but only when loaded with siRNA, to the DNA (*84*).

These findings suggest that siRNAs interact directly with DNA and that siRNA-guided complexes can find their cognate DNA-binding sites only in the short interval during transcription of a sequence when the DNA is unpaired; when the DNA pairs again behind the polymerase, the site becomes inaccessible to the siRNA. However, most current data are also consistent with siRNAs binding nascent transcripts themselves, but in a manner that requires their being loaded on these sites by virtue of their association, through proteins, with RNA Pol II. Perhaps for genes transcribed by RNA Pol I (*85*), a similar protein-protein interaction allows the Argonaute-bound siRNA to follow closely behind the polymerase as it traverses the ''silenced'' gene.

Yet More RNA Polymerization, Just to Destroy RNA?

Template-dependent RNA polymerases are not the only RNA polymerases implicated in RNA silencing. Members of the polymerase β nucleotidyltransferase superfamily of proteins are required for RNA silencing in worms and fission yeast. This protein superfamily encompasses enzymes that either add polyadenosine to the 3′ end of RNA [poly(A) polymerases] or use ATP to make 2′-5′ polymers of adenosine monophosphate (2′-5′ oligoadenylate synthases). In worms, mutations in conserved residues in the nucleotidyltransferase domain of RDE-3 disrupt RNAi, suggesting that adenosine polymers may play a direct role in RNA silencing (*86*). In fission yeast, the putative nucleotidyl transferase Cid12 is required for heterochromatin assembly by the RNA silencing pathway (*79*). Cid12, a putative helicase protein, and Rdp1, the *S. pombe* RdRP, form a complex implicated in siRNA production. This complex also contains noncoding RNAs transcribed from centromeric DNA, the primary target of heterochromatin assembly in fission yeast, consistent with the idea that these noncoding transcripts act as templates for Rdp1, which may convert them into dsRNA, which in turn could be converted to siRNA by Dcr1, thereby triggering RNA silencing. But what do Cid12 and RDE-3 do? These enzymes may play a direct role in RNA silencing; a poly(A) tail synthesized by Cid12 may recruit enzymes that mediate heterochromatin-specific modifications to transcripts under surveillance by the RNA

silencing machinery (*79*). Alternatively, Cid12 and Rdp1 may be components of a common surveillance complex—and hence dependent on each other for their stability. This complex would contain components of two separate pathways that protect cells against "aberrant RNA"—transcripts that are misfolded, incorrectly spliced, or damaged such that they encode truncated proteins. Favoring this view, the budding yeast protein Trf4p, another polymerase β nucleotidyltransferase, adds poly(A) tails to misfolded tRNAs and to aberrant mRNAs, targeting them for destruction by the nuclear exosome, a complex of RNA-degrading enzymes (*87*–*89*). Use of a poly(A) tail as a degradation signal, rather than as a stabilizing feature that promotes mRNA translation, may be quite ancient, as bacteria use poly(A) tails to target RNA for destruction.

Stem Cells

Epigenetic marks play an important role in stem cells, which must divide to yield a daughter cell that differentiates and another that regenerates the original stem cell. RNA silencing has emerged as a vital regulatory mechanism for maintaining normal stem cell pools. Mice lacking Dicer die at embryonic day 7.5, devoid of Oct-4–expressing cells (*90*); in mammals, Oct-4 marks stem cell lineages. At least four genes in the RNA silencing pathway are required for germline stem cell function in *Drosophila melanogaster*. Piwi, an Argonaute protein, is required both to maintain female germline stem cells and to promote their proliferation (*91*). Dicer-1, which makes miRNAs and perhaps other types of small RNAs, and its dsRNA-binding protein partner, Loqs, are both required for normal germline stem cell function. In the fly ovary, germline stem cells lacking Dicer divide slowly, dramatically reducing the number of eggs generated (*92*). In contrast, in females mutant for Loqs in both the soma and the germ line, germline stem cells are lost, either because they die or because they differentiate into oocytes without replenishing the stem cell pool (*93*). It remains to be established whether these defects reflect loss of miRNAs (which require the coordinate action of Dicer-1 and Loqs for their maturation), loss of silent heterochromatin, or both.

Flies lacking Ago2 contain fewer pole cells, the germline stem cell progenitors, than do wild-type flies (*94*). The case of *ago2* mutants is particularly instructive, because loss of Ago2—like loss of the very first Argonaute protein implicated in RNA silencing, worm RDE-1 (*5*)—was originally reported to cause no cellular defects except loss of an RNAi response to exogenous dsRNA (*95*). Closer examination revealed that many aspects of early embryogenesis are defective, yet the flies somehow compensate and survive (*94*). In particular, *ago2* mutants show defects in chromosome condensation, nuclear division, spindle assembly, and nuclear timing, all perhaps caused by a loss of heterochromatin assembly normally guided by an RNA silencing pathway. It remains to be shown if Ago2 acts directly in the assembly of heterochromatin by the RNA silencing pathway, or if components common to the RNAi and transcriptional silencing pathways become unstable in the absence of Ago2 protein. But these results underscore the guiding principle of small RNA function: Small RNAs play a very big role in nearly every cellular process.

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