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This article is dedicated to the memory of Alan Wolffe, an inspirational leader to all of us who have pondered the mysteries of chromatin and gene regulation.

VIEWPOINT

RNA: Guiding Gene Silencing

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In diverse organisms, small RNAs derived from cleavage of double-stranded RNA can trigger epigenetic gene silencing in the cytoplasm and at the genome level. Small RNAs can guide posttranscriptional degradation of complementary messenger RNAs and, in plants, transcriptional gene silencing by methylation of homologous DNA sequences. RNA silencing is a potent means to counteract foreign sequences and could play an important role in plant and animal development.

RNA silencing is a new field of research that has coalesced during the last decade from independent studies on various organisms. Scientists who study plants and fungi have known since the late 1980s that interactions between homologous DNA and/or RNA sequences can silence genes and induce DNA methylation (*1*). The discovery of RNA interference (RNAi) in *Caenorhabditis elegans* in 1998 (*2*) focused attention on double-stranded RNA (dsRNA) as an elicitor of gene silencing, and indeed, many gene-silencing effects in plants are now known to be mediated by dsRNA (*3*). RNAi is usually described as a posttranscriptional gene-silencing phenomenon in which dsRNA triggers degradation of homologous mRNA in the cytoplasm (*4*). However, the potential for nuclear dsRNA to enter a pathway leading to epigenetic modifications of homologous DNA sequences and silencing at the transcriptional level should not be discounted. Although the nuclear aspects of RNA silencing have been studied primarily in plants, there are hints that similar RNA-directed DNA or chromatin modifications might occur in other organisms as well. Here we adopt a broad definition of RNA silencing that encompasses effects in the cytoplasm and the nucleus, and consider their possible developmental roles and evolutionary origins.

RNA Guiding Homologous RNA Degradation

Although they may differ in detail, RNAi in animals and the related phenomena of posttranscriptional gene silencing (PTGS) in plants and quelling in *Neurospora crassa* result from the same highly conserved mechanism, indicating an ancient origin (*5–10*). The basic process involves a dsRNA that is processed into shorter units that guide recognition and targeted cleavage of homologous mRNA. dsRNAs that trigger PTGS/RNAi can be made in the nucleus or cytoplasm in a number of ways, including transcription through inverted DNA repeats, simultaneous synthesis of sense and antisense RNAs, viral replication, and the activity of cellular or viral RNA–dependent RNA polymerases (RdRP) on single-stranded RNA templates (Fig. 1). In *C. elegans*, dsRNAs can be injected or introduced simply by soaking the worms in a solution containing dsRNA or feeding them bacteria expressing sense and antisense RNA (*10*).

Genetic and biochemical approaches are being used to dissect the mechanism of PTGS/RNAi. Putative RdRPs, putative helicases, and members of the PAZ/Piwi family are some of the common proteins identified in genetic screens in *N. crassa*, *C. elegans*, and *Arabidopsis* (*3*, *5*, *8*, *10*). Although these proteins provide clues about dsRNA synthesis and processing, the most detailed insight into the two-step RNA degradation process has come from biochemical experiments with cytoplasmic extracts from *Drosophila* (*11–15*) (Fig. 1). The first step involves a dsRNA endonuclease [ribonuclease III (RNase III)–like] activity that processes dsRNA into sense and antisense RNAs 21 to 25 nucleotides (nt) long. These small interfering RNAs (siRNAs), which were first described in a plant system (*16*), are generated in *Drosophila* by an RNase III–type protein termed Dicer. Orthologs of Dicer, which contains a helicase, dsRNA binding domains, and a PAZ domain, have been identified in *Arabidopsis*, *C. elegans*, mammals, and *Schizosaccharomyces pombe* (*15*). In the second step, the antisense siRNAs produced by Dicer serve as guides for a different ribonuclease complex, RISC (RNA-induced silencing complex), which cleaves the homologous single-stranded mRNAs. RISC from *Drosophila* extracts cofractionates with siRNAs that guide sequence-specific mRNA cleavage (*12*). RISC cuts the mRNA approximately in the middle of the region paired with antisense siRNA (*14*) (Fig. 1), after which the mRNA is further degraded. Although most protein components of RISC have not yet been identified, they might include an endonuclease, an exonuclease, a helicase, and a homology-searching activity (*6, 10*). A candidate for a 3′,5′-exonuclease is *C. elegans* MUT7, an RNase D–like protein recovered in a screen for RNAi mutants (*10*). Another component of RISC is a protein of the PAZ/Piwi family (*17*), which could interact with Dicer through their common PAZ domains (*18*) to incorporate the siRNA into RISC (*17*). Genes encoding members of the PAZ/Piwi family (*Arabidopsis*: AGO1; *N. crassa*: QDE2; *C. elegans*: RDE1), which are homologous to the translation factor eIF2C, have been shown to be required for PTGS/RNAi in several mutant screens (*3*, *5*, *8*, *10*).

A putative RdRP was the first cellular protein shown to be required for PTGS/RNAi in genetic screens (*N. crassa*: QDE1; *C. elegans*: Ego1; *Arabidopsis*: SGS2/SDE1) (*3*, *5*, *8*, *10*), but its exact role is unclear and the predicted enzyme activity remains to be established. This protein might be dispensible when large amounts of dsRNA are produced from transgenes or when viral RdRPs are present (*5*). RdRP might be needed only when dsRNA is synthesized to initiate silencing—for example, from "aberrant" sense RNAs that are prematurely terminated or processed improperly (*19*). RISC-cleaved mRNAs may also be used as templates and converted into dsRNA, increasing the level of siRNAs and enhancing PTGS/RNAi (Fig. 1).

Putative helicases are another class of enzyme found repeatedly in mutant screens (*N. crassa*: QDE3; *C. elegans*: SMG-2; *Chlamydomas*: MUT6; *Arabidopsis*: SDE3) (*3*, *5*, *8*, *10*). Those recovered so far are not highly related and have not yet been characterized biochemically. A DNA helicase (QDE3) and members of two RNA helicase superfamilies (MUT6 and SMG2/SDE3, respectively) have

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been identified. The requirement for a DNA helicase for quelling in *N. crassa* suggests a nuclear step, which must still be clarified. The various RNA helicases might facilitate different steps in PTGS/RNAi, implying that additional helicases remain to be identified for each organism. Alternatively, the different helicases identified until now might stimulate the same steps, but their divergent evolution reflects the specific needs for optimizing RNA silencing in each species.

Different Pathways for dsRNA Processing?

Although dsRNAs from different sources can enter the processing pathway leading to PTGS/RNAi (*5*) (Fig. 1), recent work with HC-Pro (helper component proteinase), a plant viral suppressor of PTGS, suggests that there may be more than one pathway for dsRNA cleavage, producing distinct classes of short RNAs that might not be functionally equivalent. HC-Pro prevents accumulation of siRNAs required for PTGS (*20*, *21*) (Fig. 1), but not small RNAs associated with transcriptional gene silencing and RNA-directed DNA methylation (*22*). Whether these differential effects of HC-Pro on small RNA accumulation reflect alternative degradation pathways for dsRNAs produced in different ways, the activity of different RNase III–type enzymes, or compartmentalization of separate dsRNA-processing pathways in the nucleus and cytoplasm is not yet known. There is indirect evidence for the latter two possibilities. In plants infected by viroids, which are pathogens consisting solely of a rod-shaped, noncoding RNA that replicates in the nucleus by means of dsRNA, viroid-derived small RNAs can be detected (*23*), suggesting processing of nuclear dsRNAs by a Dicer-like activity. The *Arabidopsis* protein CARPEL FACTORY (CAF) (*24*), which is a candidate Dicer homolog (*15*), contains two bipartite nuclear localization signals (NLSs). However, CAF expression yields two RNA species, the shorter of which lacks the NLSs and contains only the RNase III and dsRNA-binding domain (*24*). This version of CAF could be involved in PTGS/RNAi in the cytoplasm, whereas the complete CAF protein containing the helicase activity might be required for RNA silencing in the nucleus (Fig. 1). The MUT6 RNA helicase, which is required for PTGS and transposon silencing in *Chlamydomonas* (*19*), also contains putative NLSs, further suggesting that common proteins are used for RNA silencing in the cytoplasm and nucleus.

Both PTGS and RNAi produce a mobile signal that induces silencing at distant sites (*5*, *6*). Systemic silencing in plants can be impeded by the viral suppressor of PTGS, the p25 protein (*25*). The signal has not yet been characterized, but it presumably contains a nucleic acid because the sequence specificity of silencing is retained. HC-Pro prevents production of the siRNAs required for PTGS but does not interfere with systemic silencing (*20*), suggesting either that the mobile signal is produced upstream of the siRNAs or that a separate class of small RNAs that are unaffected by HC-Pro is involved (Fig. 1).

RNA Guiding Homologous DNA Modification

A role for RNA in guiding de novo cytosine (C) methylation of homologous DNA se-

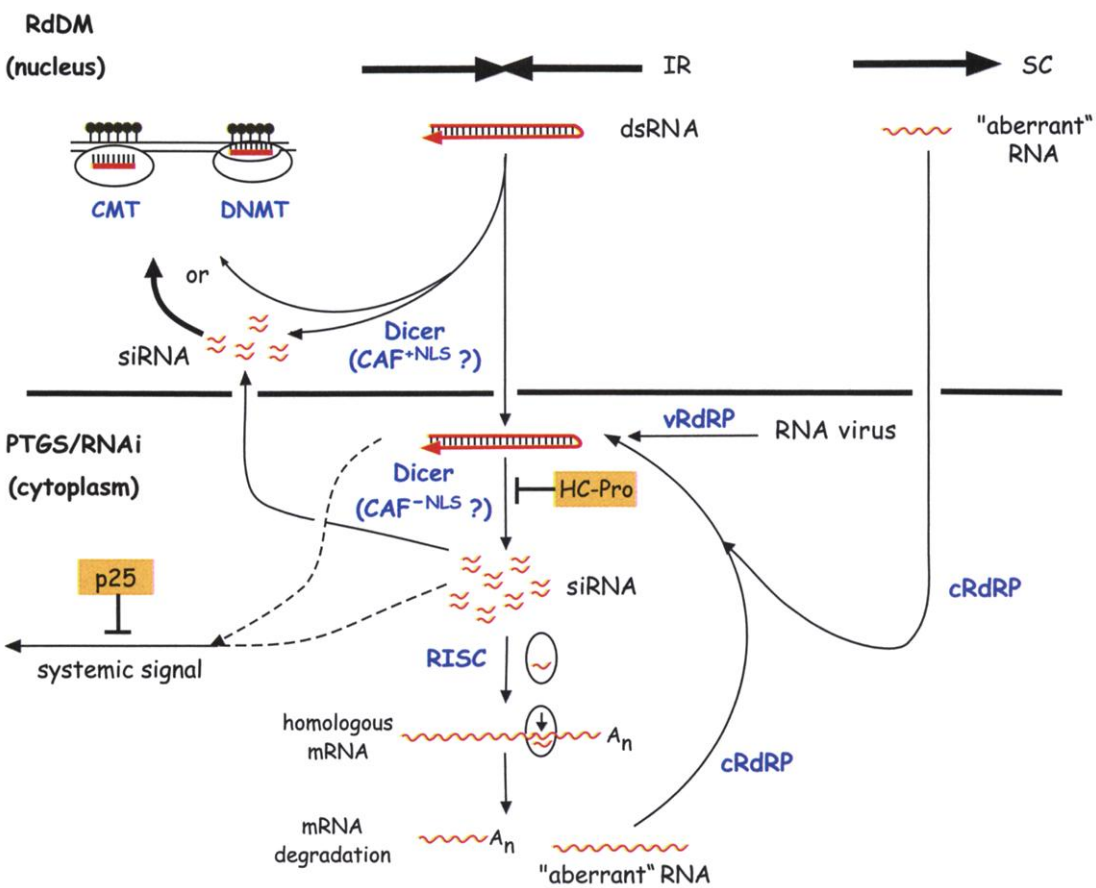

Fig. 1. Model for RNA silencing. RNA-directed DNA methylation (RdDM) and posttranscriptional gene silencing/RNA interference (PTGS/RNAi) are both triggered by double-stranded RNAs (dsRNAs) that are cleaved by RNAse III–type enzymes (e.g., Dicer, CAF) into small interfering RNAs (siRNAs), probably in both the nucleus (+ nuclear localization signal, NLS) and cytoplasm (−NLS). In the cytoplasm, the siRNAs serve as guides for endonucleolytic cleavage of homologous mRNA degradation in association with the RNA-induced silencing complex (RISC). In the nucleus, short RNAs possibly guide methylation of homologous DNA (thick arrow), although it cannot be ruled out that dsRNA triggers RdDM directly. RNA triggering of RdDM might either interact with the chromodomain of chromomethylase (CMT) and guide it to the homologous DNA sequence, or unwound dsRNA might base pair with homologous single-stranded DNA, producing an RNA-DNA duplex and single-stranded DNA bulge, an unusual structure that might attract a de novo DNA methyltranferase (DNMT). DNA methylation is indicated by filled circles. dsRNAs can be made by transcribing through inverted DNA repeats (IR), or by the activity of cellular RNA-dependent RNA polymerase (cRdRP) acting on "aberrant" (prematurely terminated and/or lacking polyadenylation: A_n) RNA templates synthesized from single-copy (SC) genes in the nucleus or generated in the cytoplasm by RISC cleavage of mRNA. Replicating RNA viruses produce dsRNA by means of a viral RdRP (vRdRP). Plant viral suppressors of PTGS block accumulation of siRNAs (HC-Pro) and systemic silencing (p25). The exact nature of the systemic silencing signal is unknown, but it involves either dsRNA or a special class of siRNAs (dotted lines).

quences was first discovered in viroid-infected transgenic plants (*26*) and subsequently in nonpathogenic plant systems (*3*). RNA-directed DNA methylation requires dsRNAs that are cleaved into small RNAs similar to those guiding homologous RNA degradation in PTGS/RNAi (*27*). Only DNA sequences complementary to the guide RNA become modified, suggesting direct RNA-DNA interactions. DNA sequences as short as 30 base pairs can be targets for methylation, which occurs at all Cs, including those not present in symmetrical CpG (and in plants, CpNpG) nucleotide groups (*26*), which are the conventional substrates for methylation. Any DNA sequence can apparently become modified by RNA-directed DNA methylation, even ones that are not usually thought to be transcribed, such as promoters. dsRNAs that contain promoter sequences are thus able to direct methylation and transcriptional silencing of homologous promoters in trans (*27*, *28*). Moreover, RNAs produced in the cytoplasm as a consequence of PTGS can enter the nucleus and trigger homologous DNA methylation (*29*) (Fig. 1). In some instances of PTGS, RNA-directed DNA methylation might be required for initiation or maintenance of silencing, as indicated by the alleviation of PTGS in *Arabidopsis* mutants deficient in DNA methylation (*ddm1* and *ddm2/met1*) (*30*).

The protein machinery involved in RNA-directed DNA methylation has not yet been determined, but the minimal enzymatic activities presumably include a de novo DNA methyltransferase (MTase) (*31*) and an RNA helicase to unwind dsRNA. Whether the dsRNA or the small RNA degradation products are required for RNA-directed DNA methylation is not yet known. Several studies suggest the involvement of small RNAs (*21*, *22*, *32*), which might have ready access to partially unwound DNA to form an RNA-DNA duplex and single-stranded DNA loop, or an RNA-DNA triple helix. These unusual structures might attract MTase (*33*) (Fig. 1). Alternatively, small RNAs could interact with MTase and guide the enzyme to a homologous DNA sequence. A possible candidate MTase is the so-called chromomethylase, a special chromodomain-containing MTase that has been found so far only in plants (*34*). Chromodomains are believed to mediate interactions between chromatin regulatory proteins. Intriguingly, the chromodomain of the histone acetylase MOF in *Drosophila* has been shown to act as an RNA interaction module (*35*). Conceivably, small RNAs might interact with a chromomethylase through the chromodomain to direct methylation of homologous DNA sequences (Fig. 1) or to maintain methylation at non-CpGs (*34*). Whether RNA-binding ability is a general property of chromodomains remains to be seen. The MOF example raises the fascinating possibility that not only RNA-directed DNA methylation but also certain chromatin modifications might be targeted to specific regions of the genome by guide RNAs, even in organisms that do not methylate their DNA. Several special cases of epigenetic silencing, including X-chromosome dosage compensation in mammals (*36*) and *Drosophila* (*37*) and some cases of genomic imprinting in mammals (*38*), involve noncoding RNAs or overlapping sense and antisense RNAs, which might be involved in triggering the chromatin modifications or methylation associated with these phenomena.

Strategies for Methylating Nucleic Acids

When considering the origins of RNA-directed DNA methylation, it is interesting to compare the different strategies used by bacteria and eukaryotes to methylate nucleic acids. To methylate ribosomal RNA (rRNA), bacteria use different protein enzymes, each dedicated to modifying a specific site. In contrast, eukaryotes and Archaea use small nucleolar RNAs (snoRNAs) that guide methylases and other modifying enzymes to complementary sequences on rRNA (*39*). This elaborate system of RNA-guided rRNA modification allows more sites of modification than are possible in bacteria. Although the original idea of an RNA world proposed that functions once performed by RNAs were gradually taken over by proteins, snoRNA-guided rRNA modification might provide an example of RNAs usurping the role of proteins (*39*). A similar principle might apply to DNA methylation. Bacteria have many different DNA MTases, each of which recognizes a specific short sequence. In contrast, eukaryotes that methylate their DNA possess only a handful of DNA MTases yet can modify an infinite variety of sequences (*40*, *41*), a capability that could potentially be accounted for by the use of guide RNAs.

Evolution of RNA Silencing

RNA silencing, which is active at different levels of gene expression in the cytoplasm and the nucleus, appears to have evolved to counter the proliferation of foreign sequences, such as transposable elements (TEs) and viruses, many of which produce dsRNAs during replication. This conclusion is substantiated by the mobilization of some TEs in several RNAi mutants in *C. elegans* (*10*) and the heightened sensitivity of some PTGS-defective mutants to certain RNA viruses in plants (*5*). Moreover, some TEs are unleashed in methylation-defective *Arabidopsis* (*34*, *42*–*44*), although it is not known whether these TEs are methylated by RNA-directed DNA methylation. Despite the existence of host defenses to parasitic sequences, higher eukaryotic genomes are laden with TEs and endogenous viral sequences. A possible beneficial outcome of faulty host defenses has been the evolution of epigenetic regulatory mechanisms that are required for proper development of plants and animals. TE insertions into host genes might have rendered them conspicuous to the defense machinery, thus imposing on host genes the type of epigenetic control used to silence foreign sequences (*45*).

The extent to which RNA silencing contributes to normal development and not just to host defense is uncertain. Only a few RNAi/PTGS mutants identified in genetic screens display developmental defects (*Arabidopsis*: *ago1*; *C. elegans*: *ego-1*) (*5*, *10*), and no mutants defective in RNA-directed DNA methylation are yet available for inspection. In plants, dramatic effects on flower development are seen in mutants of CAF, an *Arabidopsis* ortholog of Dicer. Although not initially recovered in screens for plants impaired in RNA silencing, *caf* mutants display unregulated cell division in floral meristems, indicating a role for dsRNA processing in plant morphogenesis (*24*). It is expected that additional mutant screens and improved understanding of RNA silencing mechanisms through biochemical approaches will reveal a larger contribution of these processes to development. Another type of small RNA regulates developmental timing in *C. elegans* and perhaps other bilaterally symmetric animals. The *lin-4* and *let-7* small temporal RNAs (stRNAs) presumably act by base pairing to the 3′-untranslated region of target mRNAs and interfering with translation, in contrast to siRNAs, which affect mRNA stability. However, the length of stRNAs (21 nt) and the fact that they are processed from larger duplex RNAs provide striking similarities to siRNAs (*46*). Indeed, recent work has demonstrated that Dicer is involved in the maturation of stRNAs (*47*, *48*), providing evidence that the RNAi and stRNA pathways intersect at the step where small RNAs that repress gene expression are produced.

A role for dsRNA in controlling gene expression was anticipated in 1979 by Britten and Davidson in their proposal that RNA-RNA duplexes regulate gene expression at the posttranscriptional level (*49*). Most of the tissue-specific nuclear RNAs they identified were derived from repetitive sequences and probably consisted primarily of TEs and related sequences. RNA silencing by means of transcripts produced from TE-derived sequences could conceivably contribute to developmentally regulated gene expression in plants and animals and would be compatible with the steady accumulation of TEs in the genomes of these groups during evolution (*45*).

RNA silencing is proving to be useful for the study of functional genomics in invertebrates (*50*) and plants (*5*). Routine RNAi might be possible in mammalian cells, where dsRNA normally elicits a global shutdown of protein synthesis, by injecting directly siRNAs (*51*). Continued efforts in ribonomics, the RNA analog of proteomics (*52*), should identify the full complement of natural noncod-

ing RNAs that act as riboregulators (*53*) and determine the degree to which they contribute to epigenetic regulation of gene expression in diverse organisms.

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VIEWPOINT

Epigenetic Aspects of X-Chromosome Dosage Compensation

Yongkyu Park and Mitzi I. Kuroda

The X chromosomes of mammals and fruit flies exhibit unusual properties that have evolved to deal with the different dosages of X-linked genes in males (XY) and females (XX). The X chromosome dosage-compensation mechanisms discovered in these species are evolutionarily unrelated, but exhibit surprising parallels in their regulatory strategies. These features include the importance of noncoding RNAs, and epigenetic spreading of chromatin-modifying activities.

Sex chromosomes have posed a fascinating puzzle for biologists. The dissimilar organization, gene content, and regulation of the X and Y chromosomes are thought to reflect selective forces acting on original pairs of identical chromosomes (*1–3*). The result in many organisms is a male-specific Y chromosome that has lost most of its original genetic content, and a difference in dosage of the X chromosome in males (XY) and females (XX).

The processes that animals use to respond to differences in X-chromosome dosage have been most intensively studied in three very different groups of organisms: (i) mammals, including marsupials; (ii) the nematode *Caenorhabditis elegans*; and (iii) the fruit fly, *Drosophila melanogaster*. The dosage-compensation mechanisms in these species differ dramatically: eutherian (placental) mammals randomly inactivate one of their two X chromosomes in females to form a Barr body (*4*); female marsupials display nonrandom X inactivation (*5*); XX hermaphrodite nematodes halve the expression from each X (*6*); and male *Drosophila* increase the transcription of their single X approximately twofold (*7*, *8*). In each case however, the regulation of chromatin composition and structure in one sex is central to the dosage-compensation mechanism. The striking result is sex- and chromosome-specific localization of activities that acetylate and deacetylate histones, methylate DNA, condense chromosomes, or otherwise modify chromatin architecture. The chromosome-specific mechanisms that have been investigated are either very unusual, or are simply dramatic examples of regulation that might be much more common than currently recognized.

What are these unusual aspects of X-chromosome regulation? We focus on mammalian and fruit fly dosage compensation to illustrate surprising parallels in their distinct regulatory strategies. The first common aspect is that in each case, chromatin-modifying activities spread long distances in cis from specific initiation sites on the X chromosome (*9–11*). The second parallel is that these initiation sites produce a new class of untranslated, chromosome-associated RNAs (*12–15*).

In mammals, the *Xist* RNA originates from the X inactivation center (*Xic*), "coats" one chromosome in cis, and is required for X inactivation (*12*, *13*, *16*, *17*). The *Tsix* gene is transcribed in an antisense orientation to *Xist* and regulates the choice of which X chromosome remains active (*18*). *Xist* transgenes inserted on autosomes can cause DNA methylation, histone hypoacetylation, late replication, accumulation of a variant histone (macroH2A), and transcriptional inactivation of distantly linked genes, all characteristics of the endogenous inactive X chromosome (*9*, *10*, *19*) (Fig. 1).

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