# RNA interference

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**A conserved biological response to double-stranded RNA, known variously as RNA interference (RNAi) or post-transcriptional gene silencing, mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids, and regulates the expression of protein-coding genes. RNAi has been cultivated as a means to manipulate gene expression experimentally and to probe gene function on a whole-genome scale.**

The phenomenon of RNAi was first discovered in the nematode worm *Caenorhabditis elegans* as a response to double-stranded RNA (dsRNA), which resulted in sequence-specific gene silencing[1]. Following on from the studies of Guo and Kemphues, who had found that sense RNA was as effective as antisense RNA for suppressing gene expression in worms[2], Fire, Mello and colleagues[1] were attempting to use antisense RNA as an approach to inhibit gene expression. Their breakthrough was to test the synergy of sense and antisense RNAs, and they duly found that the dsRNA mixture was at least tenfold more potent as a silencing trigger than were sense or antisense RNAs alone[1]. Silencing by dsRNAs had a number of remarkable properties — RNAi could be provoked by injection of dsRNA into the *C. elegans* gonad or by introduction of dsRNA through feeding either of dsRNA itself or of bacteria engineered to express it[3]. Furthermore, exposure of a parental animal to only a few molecules of dsRNA per cell triggered gene silencing throughout the treated animal (systemic silencing) and in its $F_1$ (first generation) progeny (Fig. 1).

From this discovery emerged the notion that a number of previously characterized, homology-dependent gene-silencing mechanisms might share a common biological root. Several years previously, Richard Jorgensen had been engineering transgenic petunias with the goal of altering pigmentation. But introducing exogenous transgenes did not deepen flower colour as expected. Instead, flowers showed variegated pigmentation, with some lacking pigment altogether (refs 4, 5, and reviewed in ref. 6). This indicated that not only were the transgenes themselves inactive, but also that the added DNA sequences somehow affected expression of the endogenous loci. This phenomenon, called co-suppression, can be produced by highly expressed, single-copy transgenes[7,8] or by transgenes, expressed at a more modest level, that integrate into the genome in complex, multicopy arrays[9]. In parallel, several laboratories found that plants responded to RNA viruses by targeting viral RNAs for destruction[10–13]. Notably, silencing of endogenous genes could also be triggered by inclusion of homologous sequences in a virus replicon.

What is clear in retrospect is that both complex transgene arrays and replicating RNA viruses generate dsRNA. In plant systems, dsRNAs that are introduced from exogenous sources or that are transcribed from engineered inverted repeats are potent inducers of gene silencing (reviewed in ref. 14). But co-suppression phenomena are not restricted to plants: similar outcomes have been noted in unicellular organisms, such as *Neurospora*, and in metazoans, such as *Drosophila*, *C. elegans* and mammals[15–18]. In a few cases, silencing has been correlated with integration of transgenes as complex arrays that can produce dsRNA directly, although silencing can also be triggered by the presence of single-copy or dispersed elements[18]. What remains a mystery is how, and indeed whether, such elements produce the dsRNA silencing trigger that has become a hallmark of RNAi. It has been proposed that endogenous RNA-directed RNA polymerases (RdRPs) may recognize 'aberrant transcripts' derived from highly expressed loci and convert these into dsRNA[19]. Indeed, homologues of these enzymes have proven essential for silencing in *C. elegans*, fungi and plants, and this is discussed below.

Genetic and biochemical studies have now confirmed that RNAi, co-suppression and virus-induced gene silencing share mechanistic similarities, and that the biological pathways underlying dsRNA-induced gene silencing exist in many, if not most, eukaryotic organisms (Fig. 1). What are the mechanisms by which dsRNAs induce silencing of homologous sequences, either exogenous or endogenous? What are the biological functions of these processes? And how are they related in evolutionarily divergent fungi, plants and animals?

## Silencing machinery operates at multiple levels

In *C. elegans*, initial observations were consistent with dsRNA-induced silencing operating at the post-transcriptional level. Exposure to dsRNAs resulted in loss of corresponding messenger RNAs (mRNAs), and promoter and intronic sequences were largely ineffective as silencing triggers[1]. A post-transcriptional mode was also consistent with data from plant systems in which exposure to dsRNA[20], for example in the form of an RNA virus, triggered depletion of mRNA sequences without an apparent effect on the rate of transcription[21]. Indeed, viral transcripts themselves were targeted, despite the fact that these were synthesized cytoplasmically by transcription of RNA genomes[10]. These studies led to the notion that RNAi induced degradation of homologous mRNAs, and this hypothesis has been validated by biochemical analysis.

But the RNAi machinery affects gene expression through additional mechanisms. In plants, exposure to dsRNA induces genomic methylation of sequences homologous to the silencing trigger[22]. If the trigger shares sequence with a promoter, the targeted gene can become transcriptionally silenced[23]. Recent studies have suggested that the RNAi machinery may also affect gene expression at the level of chromatin structure in *Drosophila*, *C. elegans* and fungi (refs 18, 24–26, and R. Martienssen, T. Volpe, I. Hall and S. Grewal, unpublished data). Finally, in *C. elegans*, endogenously


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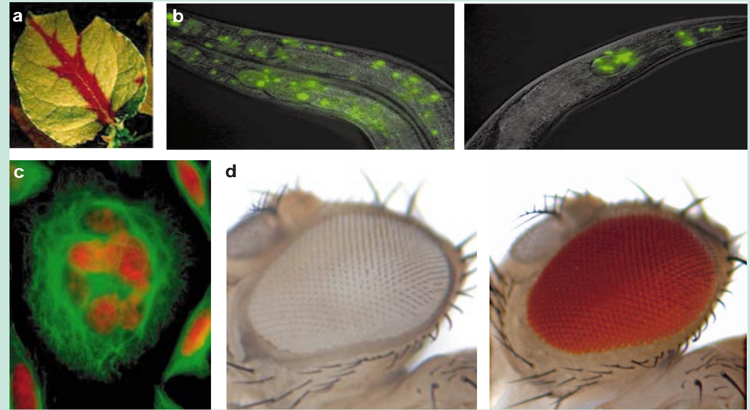


**Figure 1** Double-stranded RNA can be introduced experimentally to silence target genes of interest. In plants, silencing can be triggered, for example, by engineered RNA viruses or by inverted repeat transgenes. In worms, silencing can be triggered by injection or feeding of dsRNA. In both of these systems, silencing is systemic and spreads throughout the organism. **a**, A silencing signal moves from the veins into leaf tissue. Green is green fluorescent protein (GFP) fluorescence and red is chlorophyll fluorescence that is seen upon silencing of the GFP transgene. **b**, *C. elegans* engineered to express GFP in nuclei. Animals on the right have been treated with a control dsRNA, whereas those on the left have been exposed to GFP dsRNA. Some neuronal nuclei remain florescent, correlating with low expression of a protein required for systemic RNAi[59]. **c**, HeLa cells treated with an ORC6 siRNA and stained for tubulin (green) and DNA (red). Depletion of ORC6 results in accumulation of multinucleated cells. Stable silencing can also be induced by expression of dsRNA as hairpins or snap-back RNAs. **d**, Adult *Drosophila* express a hairpin homologous to the white gene (left), which results in unpigmented eyes compared with wild type (right).

encoded inducers of the RNAi machinery (for example, *lin-4*) operate at the level of protein synthesis[27]. Although translational control by dsRNA has not been established definitively in other systems, the conservation of *let-7* and related RNAs[28] suggests that this regulatory mode may be a further common mechanism through which RNAi pathways control the expression of cellular genes.

## Mechanism of post-transcriptional gene silencing

Our present understanding of the mechanisms underlying dsRNA-induced gene silencing is derived from genetic studies in *C. elegans* and plants and from biochemical studies of *Drosophila* extracts. In the latter case, Carthew and colleagues laid the foundations by showing that injection of dsRNA into *Drosophila* embryos induced sequence-specific silencing at the post-transcriptional level[29]. Sharp and colleagues then tested the possibility that *Drosophila* embryo extracts, previously used to study translational regulation, might be competent for RNAi[30]. Incubation of dsRNA in these cell-free lysates reduced their ability to synthesize luciferase from a synthetic mRNA. This correlated with destabilization of the mRNA and suggested that dsRNA might bring about silencing by triggering the assembly of a nuclease complex that targets homologous RNAs for degradation.

This effector nuclease, now known as RISC (RNA-induced silencing complex), was isolated from extracts of *Drosophila* S2 cells in which RNAi had been triggered by treatment with dsRNA *in vivo*[31]. A key question was how this complex might identify cognate substrates. Fire and Mello had originally proposed that some derivative of the dsRNA would guide the identification of substrates for RNAi, and the first clue in the hunt for such 'guide RNAs' came from the study of silencing in plants. Hamilton and Baulcombe[32] sought antisense RNAs that were homologous to genes being targeted by co-suppression. They found a ~25-nucleotide RNA that appeared only in plant lines containing a suppressed transgene, and found that similar species appeared during virus-induced gene silencing. Similar small RNAs were produced from dsRNAs in *Drosophila* embryo extracts[33], and partial purification of the RISC complex showed that these small RNAs co-fractionated with nuclease activity[31].

These findings forged a link between transgene co-suppression in plants and RNAi in animals. In addition, a model for RNAi and related silencing phenomenon began to emerge (Fig. 2). According to this model, initiation of silencing occurs upon recognition of dsRNA by a machinery that converts the silencing trigger to ~21–25-nucleotide RNAs. These small interfering RNAs (siRNAs) are a signature of this family of silencing pathways and, by joining an effector complex RISC, they guide that complex to homologous substrates.

This convergence of observations from diverse experimental systems suggested that a conserved biochemical mechanism would lie at the core of homology-dependent gene-silencing responses. However, the varied biology of dsRNA-induced silencing — for example, the heritable and systemic nature of silencing in *C. elegans* compared to apparently cell-autonomous, non-heritable silencing in *Drosophila* and mammals — suggested that this core machinery probably adapted to meet specific biological needs in different organisms.

## The initiation step

The model outlined in Fig. 2 implies that the dsRNA silencing trigger is cleaved to produce siRNAs. Support for this emerged first from studies of *Drosophila* embryo extracts, which contained an activity capable of processing long dsRNA substrates into ~22-nucleotide fragments[33]. Analysis of these RNAs showed that they were double stranded and contained 5′-phosphorylated termini[33,34]. The quest for the enzyme that initiates RNAi led to the RNase III ribonuclease family, which displays specificity for dsRNAs and generates such termini.

RNase III enzymes can be divided into three classes based upon domain structure: bacterial RNase III contains a single catalytic domain and a dsRNA-binding domain; Drosha family nucleases contain dual catalytic domains[35]; and a third family also contains dual catalytic domains and additional helicase and PAZ motifs[36]. Members of this third class of RNases were found to process dsRNA into siRNAs and were therefore proposed to initiate RNAi[36]. This family, now named the Dicer enzymes, are evolutionarily conserved, and proteins from *Drosophila*, *Arabidopsis*, the insect *Spodoptera frugiperda*, tobacco, *C. elegans*, mammals and *Neurospora* have all been shown to recognize and process dsRNA into siRNAs of a characteristic size for the relevant species (refs 36, 37, and A. M. Denli and G.J.H., unpublished data). Genetic evidence has also emerged from *C. elegans* and *Arabidopsis* that is consistent with Dicer acting in the RNAi pathway: Dicer is required for RNAi in the *C. elegans* germline[37–39], and a hypomorphic allele of *Carpel Factory* can intensify the phenotypes of weak *Argonaute-1* alleles in *Arabidopsis* (C. Kidner and R. Martienssen, personal communication).

Recently, the structure of an RNase III catalytic domain has led to a model for the generation of ~22-nucleotide RNAs by Dicer cleavage[40] (Fig. 2). It is thought that bacterial RNase III functions as a dimeric enzyme and, in the structural model, antiparallel RNase III domains produce two compound catalytic centres, each of which is formed by contributions from both monomers. The sequences of Dicer and Drosha RNase III domains reveal deviations from the consensus in both enzymes. Introduction of these alterations into bacterial RNase III permitted a genetic test for domain function: defects were noted upon introduction of residues that form part of the catalytic centre from the second RNase III domain of Dicer family members. Antiparallel alignment of Dicer's RNase III motifs on a dsRNA substrate could produce four compound active sites, but the central two of these would be inactive. In this way, cleavage would


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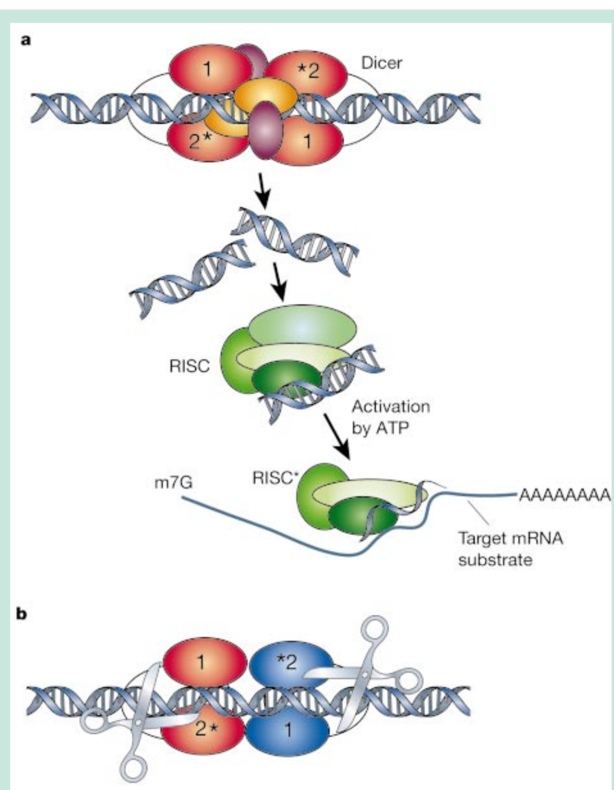


**Figure 2** Dicer and RISC (RNA-induced silencing complex). **a**, RNAi is initiated by the Dicer enzyme (two Dicer molecules with five domains each are shown), which processes double-stranded RNA into ~22-nucleotide small interfering RNAs[36]. Based upon the known mechanisms for the RNase III family of enzymes, Dicer is thought to work as a dimeric enzyme. Cleavage into precisely sized fragments is determined by the fact that one of the active sites in each Dicer protein is defective (indicated by an asterisk), shifting the periodicity of cleavage from ~9–11 nucleotides for bacterial RNase III to ~22 nucleotides for Dicer family members[40]. The siRNAs are incorporated into a multicomponent nuclease, RISC (green). Recent reports suggest that RISC must be activated from a latent form, containing a double-stranded siRNA to an active form, RISC*, by unwinding of siRNAs[41]. RISC* then uses the unwound siRNA as a guide to substrate selection[31]. **b**, Diagrammatic representation of Dicer binding and cleaving dsRNA (for clarity, not all the Dicer domains are shown, and the two separate Dicer molecules are coloured differently). Deviations from the consensus RNase III active site in the second RNase III domain inactivate the central catalytic sites, resulting in cleavage at 22-nucleotide intervals.

occur at ~22-base intervals, and subtle alterations in Dicer structure could alter the spacing of these catalytic centres and explain the species-specific variation in siRNA length (A. Denli and G.J.H, unpublished results).

## The effector step

In the *Drosophila* system, RNAi is enforced by RISC, a protein–RNA effector nuclease complex that recognizes and destroys target mRNAs. The first subunit of RISC to be identified was the siRNA, which presumably identifies substrates through Watson–Crick base-pairing[31]. Zamore and colleagues have recently shown that RISC is formed in embryo extracts as a precursor complex of ~250K; this becomes activated upon addition of ATP to form a ~100K complex that can cleave substrate mRNAs[41]. Cleavage is apparently endonucleolytic, and occurs only in the region homologous to the siRNA. siRNAs are double-stranded duplexes with two-nucleotide 3′ overhangs and 5′-phosphate termini[33,34], and this configuration is functionally important for incorporation into RISC complexes[34,41]. However, single-stranded siRNAs should be most effective at seeking homologous targets, and one intriguing correlation with the transition of RISC zymogens to active enzymes is siRNA unwinding[41].

My laboratory has purified RISC from *Drosophila* S2 cells as a ~500K ribonucleoprotein with slightly different characteristics[31,42]. In embryo extracts, RISC* (the 100K active RISC species) cleaves its substrates endonucleolytically[41]. Intermediate cleavage products are never observed in even the most highly purified RISC preparations from S2 cells, suggesting the presence of an exonuclease in this enzyme complex. Therefore, the complex formed *in vivo* probably contains additional factors that account for observed differences in size and activity. Alternatively, RISC purified from S2 cells may become activated — perhaps changing size and subunit composition — upon incubation with ATP.

RISC from S2 cells co-purifies with AGO2, a member of the *Argonaute* gene family[42]. Argonaute proteins were first identified in *Arabidopsis* mutants that produced altered leaf morphology[43], and form a large, evolutionarily conserved gene family with representatives in most eukaryotic genomes, with the possible exception of *Saccharomyces cerevisiae* (reviewed in ref. 44). These proteins are characterized by the presence of two homology regions, the PAZ domain and the Piwi domain, the latter being unique to this group of proteins. The PAZ domain also appears in Dicer proteins, and may be important in the assembly of silencing complexes[36].

Argonaute proteins were linked to RNAi by genetic studies in *C. elegans*, whose genome contains >20 related genes. The *rde-1* gene was isolated by Mello and colleagues[25] from a mutant worm that was unable to sustain RNAi in germline or soma. Using genetic methods, Grishok and colleagues[45] found a requirement for RDE-1 and RDE-4 for initiation of silencing in a parental animal; however, neither function was required for systemic silencing in $F_1$ progeny. In contrast MUT-7 (ref. 46) and RDE-2 were both dispensable in the parent, but were required in their progeny.

Rationalizing these results with the simple model proposed above is difficult. Indeed, RDE-4 is a small dsRNA-binding protein, and both RDE-1 and RDE-4 can interact with *C. elegans* Dicer (H. Tabara *et al.*, unpublished data). Perhaps RDE-4 initially recognizes dsRNA and delivers it to the Dicer enzyme. This would be consistent with the observation that siRNA levels are greatly reduced in worms that lack RDE-4 function, but are abundant in worms that lack RDE-1 (ref. 47). Similarly, in *Neurospora*, mutations in the Argonaute family member *qde-2* eliminate quelling (transgene co-suppression), but do not alter accumulation of siRNAs[48]. Thus RDE-1, and perhaps other Argonaute proteins as well, might shuttle siRNAs to appropriate effector complexes (RISCs). Consistent with this notion, we have detected transient interactions in S2 cell extracts between Dicer and Argonaute family members (ref. 42, and A. Caudy, unpublished data). This model has implications for signal amplification and systemic silencing.

## Amplification and spreading of silencing

One of the most provocative aspects of RNAi in *C. elegans* is its ability to spread throughout the organism, even when triggered by minute quantities of dsRNA[1]. Similar systemic silencing phenomena have been observed in plants, in which silencing could pervade a plant or even be transferred to a naive grafted scion[49]. Accounting for these phenomena requires firstly a system to pass a signal from cell to cell, and secondly a strategy for amplifying the signal.

Recently, a phenomenon termed 'transitive RNAi' has provided some useful clues. Transitive RNAi refers to the movement of the silencing signal along a particular gene (Fig. 3). For example, in *C. elegans*, targeting the 3′ portion of a transcript results in suppression of that mRNA and in the production of siRNAs homologous to the targeted region. In addition, siRNAs complementary to regions of the transcript upstream from the area targeted directly by the silencing trigger also appear and accumulate[50]. If these siRNAs are complementary to other RNAs, those are also targeted (hence, 'transitive' RNAi).

In both plants and *C. elegans*, dsRNA-induced silencing requires proteins similar in sequence to a tomato RNA-directed RNA


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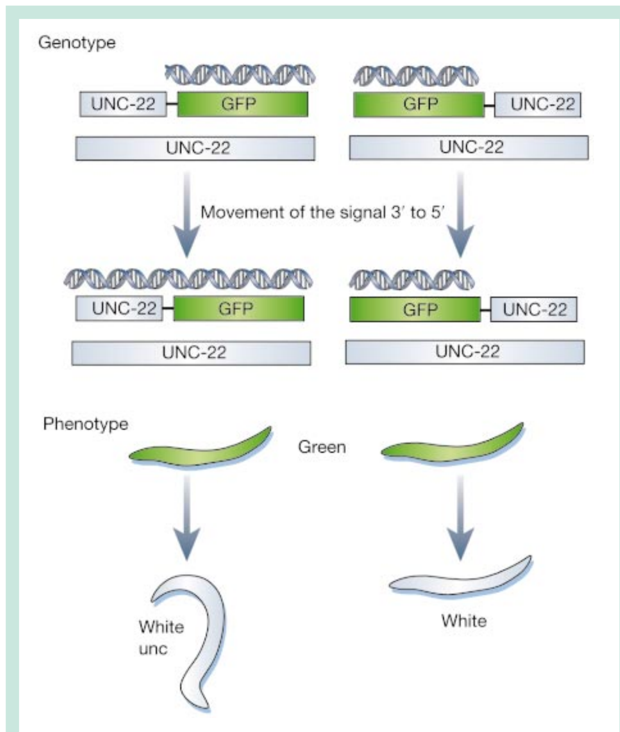


**Figure 3** Transitive RNAi. In transitive RNAi in *C. elegans*, silencing can travel in a 3′ to 5′ direction on a specific mRNA target[50]. The simplest demonstration comes from the creation of fusion transcripts. Consider a fragment of green fluorescent protein (GFP) fused 3′ to a segment of UNC-22 (left). Targeting GFP abolishes fluorescence but also creates an unexpected, uncoordinated phenotype. This occurs because of the production of double-stranded RNA and consequently small interfering RNAs homologous to the endogenous UNC-22 gene. In a case in which GFP is fused 5′ to the UNC-22 fragment (right), GFP dsRNA still ablates fluorescence but does not produce an uncoordinated phenotype.

polymerase (RdRP)[51], which could be involved in amplifying the RNAi signal. However, only the tomato enzyme has been shown to possess polymerase activity, and biochemical studies will be required to establish definitively the role these proteins play in RNAi. In *Arabidopsis*, SDE1/SGS2 is required for transgene silencing, but not for virally induced gene silencing (VIGS)[19,52]. This suggests that SDE1/SGS2 may act as an RdRP, as viral replicases could substitute for this function in VIGS. In *Neurospora*, QDE-1 is required for efficient quelling[53]. EGO-1 is essential for RNAi in the germline of *C. elegans*[54], and another RdRP homologue, RRF-1/RDE-9, is required for silencing in the soma[50] (D. Conte and C. Mello, unpublished data).

These genetic studies have led to a model for transitive RNAi in which siRNAs might prime the synthesis of additional dsRNA by RdRPs. RdRP activity has been reported recently from *Drosophila* embryo extracts[55], although transitive RNAi has yet to be observed in flies. While numerous experiments suggest that an RdRP is not required for RNAi in *Drosophila* extracts, the possibility remains that such an enzyme might act, for example, in triggering RNAi by the production of dsRNA from dispersed, multicopy transgenes.

The fact that RDE-1 and RDE-4 are required only for initiation of RNAi in parental *C. elegans* adds an additional layer of complexity to the model. Perhaps exogenous dsRNAs are recognized initially in manner that is distinct from recognition of secondary dsRNA, which may be produced by RdRPs. For example, the proposed function of RDE-4 in delivering dsRNA to Dicer could be substituted for secondary dsRNAs by another hypothetical protein. Alternatively, Dicer could exist in a stable complex with an RdRP, making dsRNA delivery unnecessary. The requirement for RRF-1/RDE-9 throughout the *C. elegans* soma — and the similar requirement for SDE1/SGS2 in plants — also suggests that most RNAi in these systems is driven by secondary siRNAs produced through the action of RdRPs.

However, other possibilities also exist. Indeed, in plants, transitive RNAi travels in both 3′→5′ and 5′→3′ directions[56], which is inconsistent with the simple notion of siRNAs priming dsRNA synthesis. Instead, one can imagine that genomic loci may serve as a reservoir for silencing. In some systems, it is known that exposure to dsRNA can produce alterations in chromatin structure, which could lead to the production of 'aberrant' mRNAs that are substrates for conversion to dsRNA by RdRPs. This model would permit bi-directional spread, as such an expansion of altered chromatin structure is an established phenomenon. Moreover, a similar model could explain co-suppression that is occasionally triggered by single-copy, dispersed transgenes. Finally, this model would be consistent with transitive effects that have been observed for both transcriptional and post-transcriptional silencing in *Drosophila*, which operate in the absence of any homology in the transcribed RNA, and thus differ from 'transitive RNAi' in *C. elegans*[18,24]. But support for a genome-based amplification model remains elusive, as does the nature of the 'aberrant' RNAs that trigger siRNA formation and an explanation for how chromatin modifications could induce their production.

Although these models suggest mechanisms for cell-autonomous amplification of the silencing signal, the character of the signal that transmits systemic silencing in plants and animals is unknown. Two candidates are siRNAs themselves or long dsRNAs, perhaps formed via RdRP-dependent amplification.

Note that, in plants, two types of transmission must be considered. The first is short-range, cell-to-cell transmission. Plant cells are intimately connected through cytoplasmic bridges known as plasmodesmata. Movement of RNA and proteins via these cell–cell junctions is well known, and it is likely that either long dsRNA or siRNAs could be passed through these connections. But the silencing signal must also be passed over a longer range through the plant vasculature[57]. In this regard, studies of a viral silencing inhibitor have provided evidence against siRNAs being critical for systemic silencing in plants. Hc-Pro suppresses silencing and also interferes with the production of siRNAs from dsRNA triggers[58]. Expression of Hc-Pro does not interfere with transgene methylation, which results in transcriptional gene silencing (TGS) if present in the promoter and which may contribute to post-transcriptional gene silencing (PTGS) if present in the transcribed sequence. Hc-Pro expression in a silenced rootstock relieves silencing and inhibits siRNA production, but a systemic signal can still be passed from this rootstock to an engrafted scion lacking Hc-Pro expression.

Recently, Hunter and colleagues identified a protein in *C. elegans* that is required for systemic silencing[59]. The ***sid-1*** gene encodes a transmembrane protein that may act as a channel for import of the silencing signal. Expression of ***sid-1*** is largely lacking from neuronal cells, perhaps explaining initial observations that *C. elegans* neurons were resistant to systemic RNAi. SID-1 homologues are absent from *Drosophila*, consistent with a lack of systemic transmission of silencing in flies, but are present in mammals, raising the possibility that some aspects of RNAi may act non-cell autonomously in mammals.

## Other components of the RNAi machinery

A combination of genetics and biochemistry has led to much progress towards understanding the mechanism of PTGS, but many questions remain. In *Drosophila* embryo extracts, pre-RISC becomes activated upon unwinding of siRNAs in an ATP-dependent process. A number of different helicases have been identified in searches for RNAi-deficient mutants (for example, QDE-3, MUT6 and MUT-14), and any of these might be candidates for a RISC activator[60–62]. Additionally, the identities of RISC-associated nucleases that cleave targeted mRNAs remain elusive. Studies of RISC formed in embryo extracts suggest an endonuclease that cleaves the siRNA–mRNA hybrid near the middle of the duplex, while RISC


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formed *in vivo* may have additional exonuclease activities. The MUT-7 protein, which is essential for RNAi in the *C. elegans* germ line, has nuclease homology, but a *Drosophila* relative of this protein has not yet been found in RISC (ref. 46, and S. Hammond, unpublished data). The efficiency of RNAi suggests an active mechanism for searching the transcriptome for homologous substrates. Most *Drosophila* RISC might be associated with the ribosome[31], and recent studies have extended this observation to trypanosomes (E. Ullu, unpublished data). Finally, relationships between the RNAi machinery and other aspects of RNA metabolism in the cell must be explored. For example, genetic evidence[63] suggests a link between RNAi and nonsense-mediated decay, raising the possibility that the RNAi machinery may be important in destruction of improperly processed mRNAs or in the general regulation of mRNA stability.

## RNAi and the genome

In plants, dsRNA induces genomic methylation at sites of sequence homology (ref. 22, reviewed in ref. 64). Methylation is asymmetric and is not restricted to CpG or CpXpG sequences. If methylation occurs in the coding sequence, it has no apparent effect on the transcription of the locus, although silencing still occurs at the post-transcriptional level. Methylation of the promoter sequence induces TGS[23], which unlike PTGS is stable and heritable[21]. Thus, dsRNA can clearly trigger alterations at the genomic level, but the degree to which these alterations are relevant to PTGS remains uncertain.

Recent studies have begun to generalize the notion of an intimate connection between the RNAi machinery and the genome, and to draw mechanistic links between PTGS and TGS. For example, in *C. elegans*, *mut-7* and *rde-2* mutations de-repress transgenes that are silenced at the level of transcription by a polycomb-dependent mechanism[25]. Polycomb-group proteins function by organizing chromatin into 'open' or 'closed' conformations, creating stable and heritable patterns of gene expression. Recently, Goldstein and colleagues found that the polycomb proteins MES-3, MES-4 and MES-6 are required for RNAi, at least under some experimental conditions[26]. Mutant worms were deficient in the RNAi response if high levels of dsRNA were injected, but were not deficient in the presence of limiting dsRNA. Of course, the effects of these mutants could be indirect, altering the expression of other elements or regulators of the RNAi pathway. However, links between altered chromatin structures and dsRNA-induced gene silencing have also emerged from plant and *Drosophila* systems. In particular, alterations of either methyltransferases (*MET1*) or chromatin remodelling complexes (for example, *DDM1*) can affect both the degree and persistence of silencing in *Arabidopsis*[21,65]. Conversely, mutations in genes required for PTGS (for example, *AGO1* and *SGS2*) decrease both co-suppression and transgene methylation[66]. Furthermore, mutation of *piwi*, a relative of the RISC component *Argonaute-2*, compromises co-suppression of dispersed transgenes in *Drosophila* at both the post-transcriptional and transcriptional levels[24].

Thus, one of the most fascinating and least-explored responses to dsRNA involves a possible recognition of genomic DNA by derivatives of the silencing trigger, possibly siRNAs. One model suggests that a variant, nuclear RISC carries a chromatin remodelling complex rather than a ribonuclease to its cognate target. Indeed, Martienssen, Grewal and colleagues have recently noted a requirement for relatives of Dicer and RISC components in the silencing of centromeric repeats in *Schizosaccharomyces pombe* (T. Volpe, C. Kidner, I. Hall, S. Grewal and R. Martienssen, personal communication). It seems therefore that a principal biological function of the RNAi machinery may be to form heterochromatic domains in the nucleus that are critical for genome organization and stability.

## Biological functions of RNAi

Because target identification depends upon Watson–Crick base-pairing interactions, the RNAi machinery can be both flexible and exquisitely specific. Thus, this regulatory paradigm may have been adapted and adopted for numerous cellular functions. For example, in plants, RNAi forms the basis of VIGS, suggesting an important role in pathogen resistance. An elegant proof of this hypothesis comes from the genetic links between virulence and RNAi pathways (refs 52, 67, and reviewed in ref. 68). Many plant viruses encode suppressors of PTGS that are essential for pathogenesis, and these virulence determinants can be masked by host mutations in silencing pathways. RNAi has also been linked to the control of endogenous parasitic nucleic acids. In *C. elegans*, some RNAi-deficient strains are also 'mutators' owing to increased mobility of endogenous transposons[25,46]. In many systems, transposons are silenced by their packaging into heterochromatin (reviewed in ref. 64). Therefore, it is tempting to speculate that RNAi may stabilize the genome by sequestering repetitive sequences such as mobile genetic elements, preventing transposition and making repetitive elements unavailable for recombination events that would lead to chromosomal translocations. However, it remains to be determined whether RNAi regulates transposons through effects at the genomic level or by post-transcriptionally targeting mRNAs (for example, those encoding transposases) that are required for transposition.

A role for RNAi pathways in the normal regulation of endogenous protein-coding genes was originally suggested through the analysis of plants and animals containing dysfunctional RNAi components.

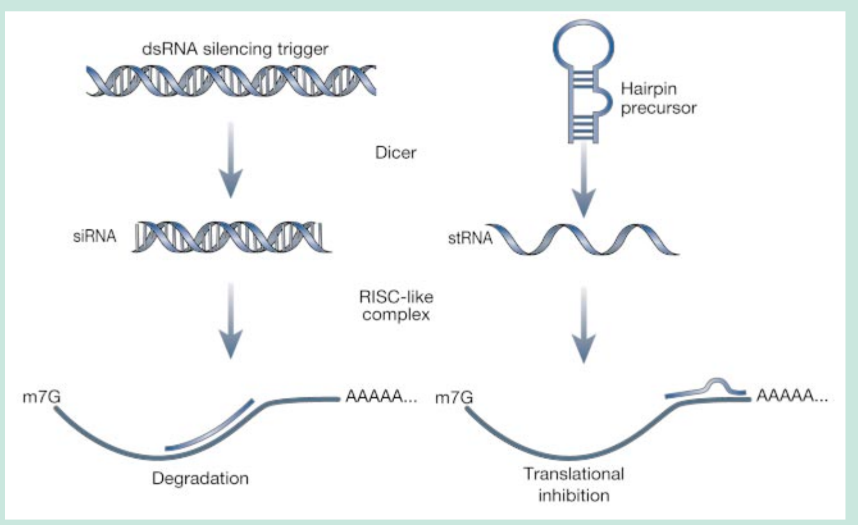


**Figure 4** Small interfering RNAs versus small temporal RNAs. Double-stranded siRNAs of length ~21–23 nucleotides are produced by Dicer from dsRNA silencing triggers. Characteristic of RNase III products, these have two-nucleotide 3′ overhangs and 5′-phosphorylated termini. To trigger target degradation with maximum efficiency, siRNAs must have perfect complementarity to their mRNA target (with the exception of the two terminal nucleotides, which contribute only marginally to recognition). stRNAs, such as *lin-4* and *let-7*, are transcribed from the genome as hairpin precursors. These are also processed by Dicer, but in this case, only one strand accumulates. Notably, neither *lin-4* nor *let-7* show perfect complementarity to their targets. In addition, stRNAs regulate targets at the level of translation rather than RNA degradation. It remains unclear whether the difference in regulatory mode results from a difference in substrate recognition or from incorporation of siRNAs and stRNAs into distinct regulatory complexes.


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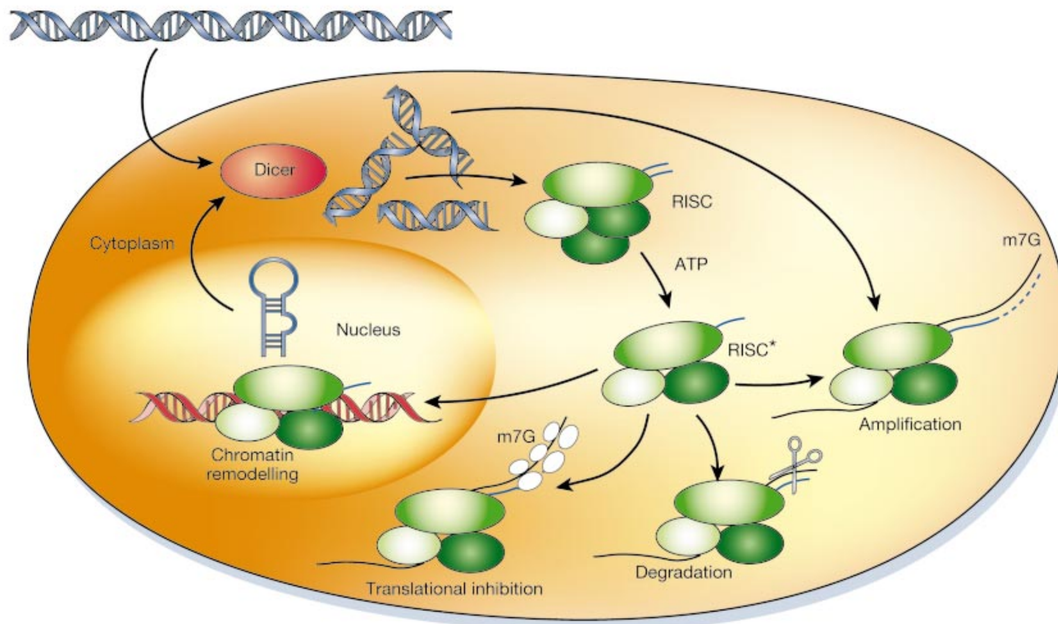


**Figure 5** A model for the mechanism of RNAi. Silencing triggers in the form of double-stranded RNA may be presented in the cell as synthetic RNAs, replicating viruses or may be transcribed from nuclear genes. These are recognized and processed into small interfering RNAs by Dicer. The duplex siRNAs are passed to RISC (RNA-induced silencing complex), and the complex becomes activated by unwinding of the duplex. Activated RISC complexes can regulate gene expression at many levels. Almost certainly, such complexes act by promoting RNA degradation and translational inhibition. However, similar complexes probably also target chromatin remodelling. Amplification of the silencing signal in plants may be accomplished by siRNAs priming RNA-directed RNA polymerase (RdRP)-dependent synthesis of new dsRNA. This could be accomplished by RISC-mediated delivery of an RdRP or by incorporation of the siRNA into a distinct, RdRP-containing complex.

Mutations in the *Argonaute-1* gene of *Arabidopsis*, for example, cause pleiotropic developmental abnormalities that are consistent with alterations in stem-cell fate determination[43]. A hypomorphic mutation in *Carpel Factory*, an *Arabidopsis* Dicer homologue, causes defects in leaf development and overproliferation of floral meristems[69]. Mutations in *Argonaute* family members in *Drosophila* also impact normal development. In particular, mutations in *Argonaute-1* have drastic effects on neuronal development[70], and *piwi* mutants have defects in both germline stem-cell proliferation and maintenance[71].

This should not be interpreted as a demonstration that PTGS pathways regulate endogenous gene expression *per se*. In fact, separation-of-function *ago1* mutants have recently been isolated that preferentially affect PTGS[72] without affecting development. Mutations in *Zwille*, another *Argonaute* family member, also alter stem-cell maintenance[73], and this occurs without perceptible impact on dsRNA-mediated silencing[72]. Thus, components of the RNAi machinery, and related gene products, may function in related but separable pathways of gene regulation.

A possible mechanism underlying the regulation of endogenous genes by the RNAi machinery emerged from the study of *C. elegans* containing mutations in their single Dicer gene, *DCR-1*. Unlike most other RNAi-deficient worm mutants, *dcr-1* animals were neither normal nor fertile: the mutation induced a number of phenotypic alterations in addition to its effect on RNAi[37–39,74]. Intriguingly, Dicer mutants showed alterations in developmental timing similar to those observed in *let-7* and *lin-4* mutants. The *lin-4* gene was originally identified as a mutant that affects larval transitions[75], and *let-7* was subsequently isolated as a similar heterochronic mutant[28]. These loci encode small RNAs, which are synthesized as ~70-nucleotide precursors and post-transcriptionally processed to a ~21-nucleotide mature form. Genetic and biochemical studies have indicated that these RNAs are processed by Dicer[37–39,74].

The small temporal RNAs (stRNAs) encoded by *let-7* and *lin-4* are negative regulators of specific protein-coding genes, as might be expected if stRNAs trigger RNAi. However, stRNAs do not trigger mRNA degradation, but regulate expression at the translational level[76,77]. This raised the possibility that stRNAs and RNAi might be linked only by the processing enzyme Dicer. However, Mello and colleagues demonstrated a requirement for Argonaute family proteins (that is, Alg-1 and Alg-2) in both stRNA biogenesis and stRNA-mediated suppression[39], which led to a model in which the effector complexes containing siRNAs and stRNAs are closely related, but regulate expression by distinct mechanisms (Fig. 4). Neither LIN-4 nor LET-7 forms a perfect duplex with its cognate target[78]. Thus, in one possible model an analogous RISC complex is formed containing either siRNAs or stRNAs. In the former case, cleavage is dependent upon perfect complementarity, while in the latter, cleavage does not occur, but the complex blocks ribosomal elongation. Alternatively, siRNAs and stRNAs may be discriminated and enter related but distinct complexes that target substrates for degradation or translational regulation, respectively. Consistent with this latter model is the observation that siRNAs or exogenously supplied hairpin RNAs that contain single mismatches with their substrates fail to repress, rather than simply shifting their regulatory mode to translational inhibition[34,79,80].

In this scenario, RISC may be viewed as a flexible platform upon which different regulatory modules may be superimposed (Fig. 5). The core complex would be responsible for receiving the small RNA from Dicer and using this as a guide to identify its homologous substrate. Depending upon the signal (for example, its structure and localization), different effector functions could join the core: in RNAi, nucleases would be incorporated into RISC, whereas in stRNA-mediated regulation, translational repressors would join the complex. Transcriptional silencing could be accomplished by the inclusion of chromatin remodelling factors, and one could imagine other adaptations might exist.


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Whether or not RISC is a flexible regulator becomes particularly important in light of recent findings that *let-7* and *lin-4* are archetypes of a large class of endogenously encoded small RNAs. Over 100 of these microRNAs or miRNAs have now been identified in *Drosophila*, *C. elegans* and mammals[81–84], and although their functions are unknown, their prevalence hints that RNAi-related mechanisms may have pervasive roles in controlling gene expression. In this regard, a number of miRNAs from *Drosophila* are partially complementary to two sequences, the K box and the Brd box, that mediate post-transcriptional regulation of numerous mRNAs[85].

## RNAi and genomics

RNAi has evolved into a powerful tool for probing gene function. In *C. elegans*, testing the functions of individual genes by RNAi has now extended to analysis of nearly all of the worm's predicted ~19,000 genes (J. Ahringer, unpublished data). Similar strategies are being pursued in other organisms, including plants (D. Baulcombe and P. Waterhouse, personal communication). Although it seemed for some time that deploying RNAi in mammalian systems would not be feasible, the first hint that the technology might work came when RNAi was demonstrated in early mouse embryos[86,87]. But this appeared to be of limited utility, as mammalian somatic cells, but not some embryonic cells, exhibit nonspecific responses to dsRNA which would obscure sequence-specific silencing. One of these is the RNA-dependent protein kinase (PKR) pathway, which responds to dsRNA by phosphorylating EIF-2$\alpha$ and nonspecifically arresting translation[88]. Tuschl and colleagues then showed that siRNAs themselves could be used to induce effective silencing in many mammalian cells[79]. These small RNAs, which are chemically synthesized mimics of Dicer products, are presumably incorporated into RISC and target cognate substrates for degradation. The siRNAs are too small to induce nonspecific dsRNA responses such as PKR[89].

One drawback that siRNAs have is that their effects are transient, as mammals apparently lack the mechanisms that amplify silencing in worms and plants. In several systems, including plants, *Drosophila*, *C. elegans* and trypanosomes, RNAi has been made stable and heritable by enforced expression of the silencing trigger, usually as an inverted repeat sequence forming a hairpin structure *in vivo*[90–95]. We have reported mammalian cell lines in which genes are stably suppressed by RNAi through the expression of a 500-base-pair dsRNA[96]. However, this approach was limited to cell types that lacked generic responses to dsRNA such as the PKR pathway. Recently, we and others have shown that short hairpin RNAs (shRNAs) modelled on miRNAs can be used to manipulate gene expression experimentally[80,97,98]. These may be expressed *in vivo* from RNA polymerase III (Pol III) promoters to induce stable suppression in mammalian cells.

The availability of stable triggers of RNAi builds upon the utility of siRNAs in several ways. Induced phenotypes can now be observed over long time spans. Stably engineered cells can be assayed either *in vitro* or *in vivo*, perhaps testing the angiogenic or metastatic potential of tumour cells in xenograft models. RNAi may potentially be used to create hypomorphic alleles rapidly in transgenic mice. If inducible Pol III promoters were used[99,100], this could permit a powerful approach akin to the use of tissue-specific Gal4-drivers in *Drosophila*. Finally, shRNAs could be combined with existing high-efficiency gene delivery vehicles to create *bona fide* RNAi-based therapeutics. In this regard, we have successfully delivered shRNAs from replication-deficient retroviruses, and foresee numerous applications for *ex vivo* manipulation of stem cells based upon this paradigm. For example, a patient's own bone marrow stem cells could be engineered to resist HIV infection by targeting either the HIV RNA itself or receptors necessary for HIV infection (for example, CCR5). Furthermore, we see no conceptual barrier to incorporating this strategy for targeted suppression into adenovirus or herpesvirus-based delivery vehicles. Ultimately, the exquisite specificity of RNAi may make it possible to silence a disease-causing mutant allele specifically, such as an activated oncogene, without affecting the normal allele.

## Perspective

Over the past few years, the way in which cells respond to dsRNA by silencing homologous genes has revealed a new regulatory paradigm in biology. This response can be triggered in many different ways, ranging from experimental introduction of synthetic silencing triggers to the transcription of endogenous RNAs that regulate gene expression. We are only beginning to appreciate the mechanistic complexity of this process and its biological ramifications. RNAi has already begun to revolutionize experimental biology in organisms ranging from unicellular protozoans to mammals. RNAi has been applied on the whole-genome scale in *C. elegans* and this goal is being pursued in plant systems. My laboratory, as part of the larger cancer genomics effort, has undertaken to target, individually, every gene in the human genome using expressed shRNAs. This will permit large-scale loss-of function genetic screens and rapid tests for genetic interactions to be performed for the first time in mammalian cells. Such approaches hold tremendous promise for unleashing the dormant potential of sequenced genomes. □


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