CMLS, Cell. Mol. Life Sci. 60 (2003) 871–882
1420-682X/03/050871-12
DOI 10.1007/s00018-003-2245-2

CMLS Cellular and Molecular Life Sciences

Review

RNA-mediated gene silencing

A. S. Pickford and C. Cogoni*

Dipartimento di Biotecnologie Cellulari e Ematologia, Università di Roma 'La Sapienza', Viale Regina Elena 324, 00161 Rome (Italy), Fax +39 06 4457731, e-mail: carlo@bce.med.uniroma1.it

Received 11 September 2002; accepted 2 October 2002

Abstract. A number of gene-silencing phenomena including co-suppression discovered in plants, quelling in fungi and RNA interference in animals have been revealed to have steps in common. All occur in the cytoplasm at a post-transcriptional level with the mRNAs of target genes degraded in a sequence-specific manner. Small non-coding RNA molecules demonstrated to be mediators of these silencing phenomena have also been shown to mediate a parallel post-transcriptional gene silencing (PTGS) mechanism that regulates the expression of developmental genes, although in this latter mechanism, rather than being degraded, the translation of target mRNAs is inhibited. Both types of small RNA appear to be processed from longer double-stranded RNAs (dsRNAs) by a common endonuclease. RNAs may also operate as regulators of gene expression at a transcriptional level in the nucleus, via chromatin remodelling or RNA-directed DNA methylation. Methylation of promoter sequences leads to transcriptional gene silencing, while methylation of coding sequences by the same homology-dependent mechanism does not block transcription, but leads to PTGS. In some organisms, the RNA silencing signal may spread to other tissues inducing systemic RNA silencing.

Key words. Post-transcriptional gene silencing; siRNA; stRNA; Dicer; RISC; RdDM; chromatin remodelling; systemic RNA silencing.

Introduction

Since the discovery of catalytic RNA molecules during the 1980s and the consequential theory of life originating in an 'RNA world' before the evolution of DNA, several types of non-protein-coding RNA molecules have been identified and demonstrated to have crucial roles in eukaryotic cells, acting either alone, or with proteins such as ribonucleic proteins (RNPs). A large class of microRNAs (miRNAs), 21–25 nucleotides (nt) in length, has been demonstrated to be involved in two separate, but overlapping pathways that regulate gene expression at a post-transcriptional level, in a sequence-specific manner. The first is a degrative mechanism that destroys mRNA corresponding to duplicated or foreign gene sequences, in a process mediated by small interfering RNAs (siRNAs), while small temporal RNAs (stRNAs) regulate the expression of developmental genes by inhibiting translation of their target mRNAs. The post-transcriptional gene silencing (PTGS) mediated by both types of miRNA involves a two-step process whereby siRNAs and stRNAs are processed by an RNA endonuclease from longer dsRNA precursor molecules and then act as 'guide' molecules to target single-stranded RNAs (ssRNAs). The siRNAs carry out their task as part of the degradation RNA-induced-silencing complex (RISC) that comprises 'adapter' proteins and probably an RNAse and RNA helicase. The stRNAs have also been shown to interact with protein components that block the translation of the mRNA of target genes. The demonstration that siRNAs can interact with DNA to induce heterochromatin formation and/or DNA methylation in a process known as

* Corresponding author.

RNA-directed DNA methylation (RdDM) extends their role in the regulation of gene expression to include transcriptional gene silencing (TGS), providing further evidence that non-coding RNA molecules are not just vestiges of an antique world, but have maintained a fundamental role in both regulating the expression and protecting the genome [1]. Moreover, the recent identification of a whole class of miRNAs of as yet unknown function in both plants and animals may indicate that siRNAs and stRNAs are just the tip of an iceberg and that RNA molecules may truly play a central role in cell regulation [2].

PTGS phenomena

The first PTGS phenomenon to be identified was termed 'co-suppression' due to the fact that a transgene, introduced into a plant with the aim of increasing the quantity of the product encoded by the transgene, determined the suppression of both the transgene itself and the expression of a homologous endogenous gene. In petunia plants, the petal pigmentation genes chalacone synthase (*cho*) or dihydroflavonol-4-reductase (*dfr*) involved in anthocyanine biosynthesis were introduced via a transgene in an attempt to increase the intensity of flower colour but, instead, total or partial suppression of these genes determining the appearance of white or variegated flowers was observed, while loss of the transgene caused re-establishment of wild-type flower colour showing that co-suppression was a reversible phenomenon [3, 4]. Co-suppression of the polygalcturonase gene responsible for fruit ripening in tomato [5] showed that the phenomenon was not peculiar to petunia and evidence that co-suppression correlated with a decrease in mRNA was obtained in tobacco plants transformed with the β-1,3-glucanase gene [6].

A similar phenomenon called 'quelling' was identified in the fungus *Neurospora crassa* as a result of transformation with a transgenic copy of the *albino-1* gene necessary for carotene biosynthesis. Instead of a more intense orange phenotype, approximately 30% of colonies were albino [7]. The 'quelling' effect was also demonstrated to be the result of a reduction in the level of steady-state mRNA and, as in plants, was shown to be transient, reversion correlating with a loss of transgenes during vegetative growth [8].

PTGS was also observed in animals, in the nematode *Caenorhabditis elegans,* as the result of the introduction into embryos of antisense or sense RNA constructs [9]. Subsequently, Mello and colleagues obtained the same silencing effect using double-stranded RNA (dsRNA) instead of transgenes [10]. The mechanism triggered by dsRNA which they termed 'RNA-mediated genetic interference' or simply RNA interference (RNAi), was subsequently shown to occur in *Drosophila* [11, 12], *Typanosoma brucei* [13], *Paramecium* [14] and *Planaria* [15]. Furthermore, RNAi was also shown to be effective in plants such as *Arabidopsis thaliana* [16] and vertebrates including mice [17, 18] and zebrafish [19, 20]. Co-suppression was hypothesized as also being mediated by dsRNA and the demonstration that the two processes are mechanistically related was provided by the use of RNA extracts of a co-suppressed tobacco plant to induce RNAi of the same reporter gene in *C. elegans* [21].

Because a cell would normally only encounter dsRNA during the replicative phase of a virus infection or as part of the replication process of a transposable element (TE), the suggestion was made that PTGS mechanisms could have evolved as a form of defence or as a genomic 'immune' system, to counter virus disease and the mutagenic potential of TEs that can be replicated and move to different locations in the genome [22]. The ability of viral genes to cause silencing was first noted in experiments designed to induce virus resistance in tobacco plants using transgenes from Potato Virus X (PVX) and Tobacco Etch Virus (TEV) [23, 24]. These experiments made apparent that a virus can both trigger a gene-silencing response and then suffer the consequences in a mechanism called 'virus-induced gene silencing' (VIGS) [25]. The increased rate of transposition of TEs in PTGS mutants in *C. elegans* further demonstrated the involvement of PTGS components in harnessing the movement of TEs in an analogous process called 'transposon silencing' [26, 27].

While the various forms of PTGS described above may all be viewed as host defence responses, in which siRNAs are the mediators of a genetic surveillance mechanism destroying the mRNAs of duplicated or anomalous genetic elements, stRNAs are active during normal development to regulate the expression of developmental genes by repressing the synthesis of and consequently, silencing target proteins. The first hint of a link between 'developmental gene silencing' and PTGS phenomena came from the fact that PTGS-defective mutants in *C. elegans* and *A. thaliana* also presented developmental defects [27, 28]. However, not until siRNAs were identified as mediators of PTGS, did their similarity to stRNAs, first identified in *C. elegans* as small antisense RNAs with sequence complementarity to heterochronic genes [29, 30], suggest that the two processes could be mediated in a similar way, explaining why mutants defective in the production of either type of small RNA could have both silencing- and developmental-defective phenotypes.

Components of RNA silencing

The similarities between co-suppression, quelling, RNAi, VIGS and transposon silencing indicate that they are re-

Table 1. Genes involved in PTGS.

Gene	Organism	Function	References
RNA-dependent RNA polymerase (RdRP)			
qde-1	*N. crassa*	quelling	31
ego-1	*C. elegans*	RNAi	39
sgs-2/sde-1	*A. thaliana*	co-suppression	40, 41
rrf-1/rrf-2/rrf-3	*C. elegans*	RNAi	42
piwi-PAZ domain (PPD) proteins			
qde-2	*N. crassa*	quelling	65
rde-1	*C. elegans*	RNAi/transposon silencing	27
rde-4	*C. elegans*	RNAi	27
alg-1/alg-2	*C. elegans*	development	53
ago-1	*A. thaliana*	co-supression	28, 60
ago-1 (homologue)	*D. melanogaster*	development	62
ago-2	*D. melanogaster*	RNAi	59
piwi	*D. melanogaster*	development	63
sting	*D. melanogaster*	development	64
pinhead/zwille	*A. thaliana*	development	107
dsRNA-dependent RNA endonuclease (RNAse III)			
dcr-1	*C. elegans*	RNAi/development	52, 53
Dicer	*D. melanogaster*	RNAi/development	33
sin-1/caf-1	*A. thaliana*	development	108
Helicase-MOI	*H. sapiens*	RNAi/development	109
RNAseD			
mut-7	*C. elegans*	RNAi/transposon silencing	26
DNA helicase			
qde-3	*N. crassa*	quelling	110
RNA helicase			
mut-6	*C. reinhardtii*	RNAi/transposon silencing	75
sde-3	*A. thaliana*	RNAi	73
smg-2, mut-14, drh-1/drh-2,	*C. elegans*	RNAi	47, 74, 77
Unknown function			
rde-2/rde-3	*C. elegans*	RNAi	27
mut-2/mut-6/mut-8/mut-9	*C. elegans*	RNAi	27
sgs-1	*A. thaliana*	co-suppression	111
sgs-3	*A. thaliana*	RNAi	40

lated phenomena with an underlying evolutionarily conserved mechanism and, as such, are likely to be driven by genes of homologous function. Analysis of both plant and animal RNA-silencing mutants has, in fact, led to the identification in these organisms of homologous genes involved in silencing, confirming that PTGS is indeed a conserved mechanism (table 1).

Several key discoveries have been of fundamental importance in understanding the complexities of RNA silencing. The first was an RNA-dependent RNA polymerase (RdRP) shown to be a determinant of quelling in *Neurospora* by Cogoni and Macino in 1999 [31]. This enzyme, homologous to tomato RdRP [32], was hypothesized to be responsible for recognizing and transforming aberrant transgenic mRNAs into dsRNAs shown to be triggers of PTGS. The identification of a bidentate RNA endonuclease called Dicer in *Drosophila melanogaster* by Bernstein et al. [33] in 2001, capable of 'dicing' dsRNA into 21 to 25-nt pieces, provided an explanation for the presence of siRNAs of the same length, homologous to silenced transgenes or viral genes, found by Hamilton and Baulcombe [34] in 1999 in co-suppressed plants and by Hammond and colleagues [35] in 2000 in animals silenced by RNAi. Furthermore, in *Drosophila*, siRNAs homologous to the silencing trigger were shown to be associated with a RISC [35]. This discovery, together with evidence that siRNAs originating from the cleavage of dsRNA were required for the subsequent degradation of cognate mRNAs from silenced *Drosophila* embryo extracts and that these mRNAs were also cleaved at 21- to 25-nt intervals [36, 37], led to the formulation of a unified model for all PTGS phenomena, hypothesizing a two-step process in which dsRNA, recognized as alien to the cell, is targeted for degradation by Dicer into 21-nt RNA fragments that are incorporated into the RISC multicomponent nuclease as 'guide' molecules to direct target mRNA degradation in a sequence-specific manner [38]. In this model, events downstream from the formation or introduction of dsRNA are common to all RNA-silencing mechanisms (fig. 1). Refine-

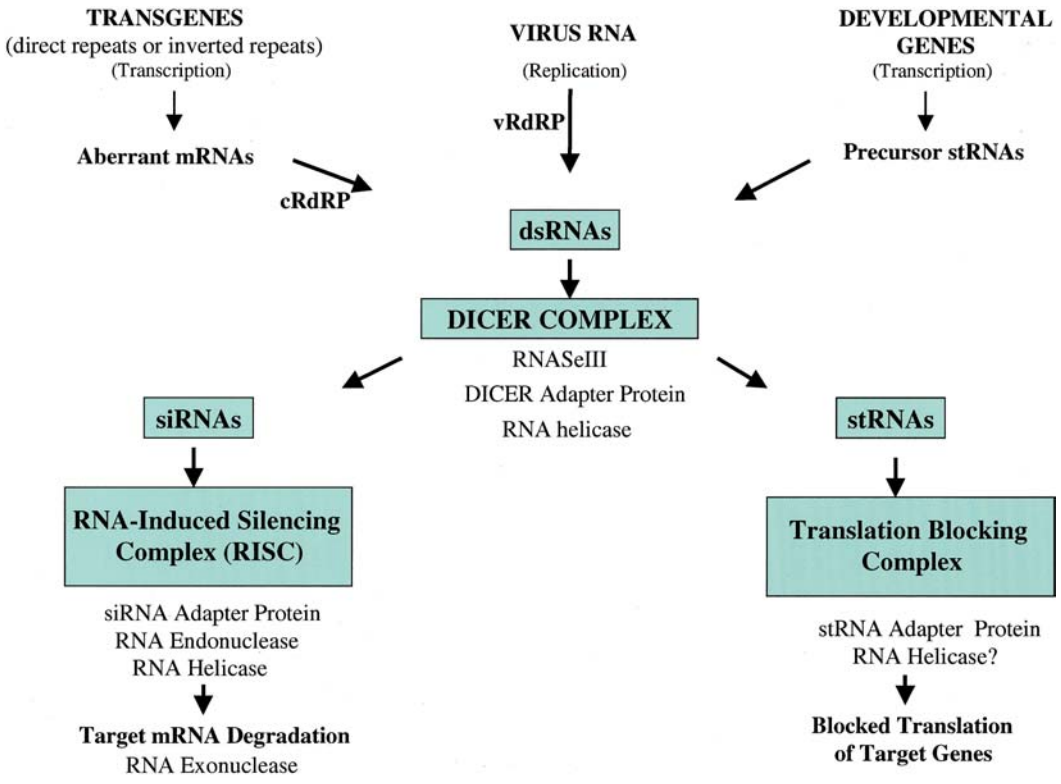

Figure 1. Model of PTGS. Double-stranded RNA (dsRNA), as well as triggering PTGS directly, may originate in the cell as an intermediate in three different pathways (i) Transgenes integrated into the genome as either direct or inverted repeats are transcribed into aberrant mRNAs that are recognized by cellular RNA-dependent RNA polymerase (cRdRP) and converted to dsRNA. (ii) RNA virus genomes are converted into a dsRNA replication intermediate by viral RdRP (vRdRP). (iii) Developmental genes are transcribed into mRNAs that fold into double-stranded intermediates, as precursor small temporal RNAs (stRNAs). DsRNA is then processed into small 21- to 25-nt RNA fragments by the Dicer complex comprising the Dicer RNAseIII enzyme itself and probably adapter proteins and an RNA helicase. Small interfering RNAs (siRNAs) with phosphorylated 5′ termini are recognized and incorporated into the RNA-induced silencing complex (RISC), probably comprising an adapter protein and an RNA helicase that unwinds the double-stranded siRNAs to enable the antisense strand to pair with target mRNAs which are then also cut into 21- to 25-nt fragments by an RNA endonuclease. An RNA exonuclease may be responsible for complete degradation of target mRNA fragments. Small temporal RNAs (stRNAs) are possibly incorporated into a translation-blocking complex comprising stRNA adapter proteins and an RNA helicase, to enable pairing with target mRNA of developmental genes to inhibit translation.

ments of this model are being made constantly with the identification of additional factors involved in PTGS. For example, the identification of protein components required for both steps raises the possibility that DICER and the RISC may form a large multiriboprotein complex. Moreover, the recently acquired evidence of an amplification step in which the siRNAs not only 'guide' cognate mRNA degradation but can also act as primers to amplify the silencing signal may provide clues to many outstanding questions such as the nature of the factors involved in systemic RNA silencing and RdDM, discussed in the following paragraphs (outlined in fig. 2). Lastly, the identification of proteins of as yet unknown function that are required for RNAi indicates that there may be further additions to the basic model, and protein components of RNAi that have been demonstrated to be involved in parallel silencing processes indicate that areas of overlap exist between these mechanisms.

RNA-dependent RNA polymerase

The isolation of the *qde-1* gene in *N. crassa* was the first experimental evidence for the involvement of an RdRP in PTGS, as previously suggested by Lindbo and co-workers [24] in 1993, and was confirmed by the identification of the *qde-1* homologue *ego-1,* in silencing-defective *C. elegans* strains [39] and of *sde-1/sgs-2* mutants in *A. thaliana* impaired both in transgene-induced gene silencing and virus-induced PTGS [40, 41]. Although *sde-1/sgs-2* is necessary for transgene-induced PTGS, not all viruses are silenced by cellular RdRPs [41]. Six other RdRP-related genes have been identified in *A. thaliana*; however, a specific role for these homologues has not yet been demonstrated. In *C. elegans, ego-1* has also three homologues, called the RdRP family (*rrf*) genes. Ego-1 is expressed in germ cell lines only and RNAi defectiveness is limited to maternal genes [39]. This finding indicates possible common steps in silencing and developmental

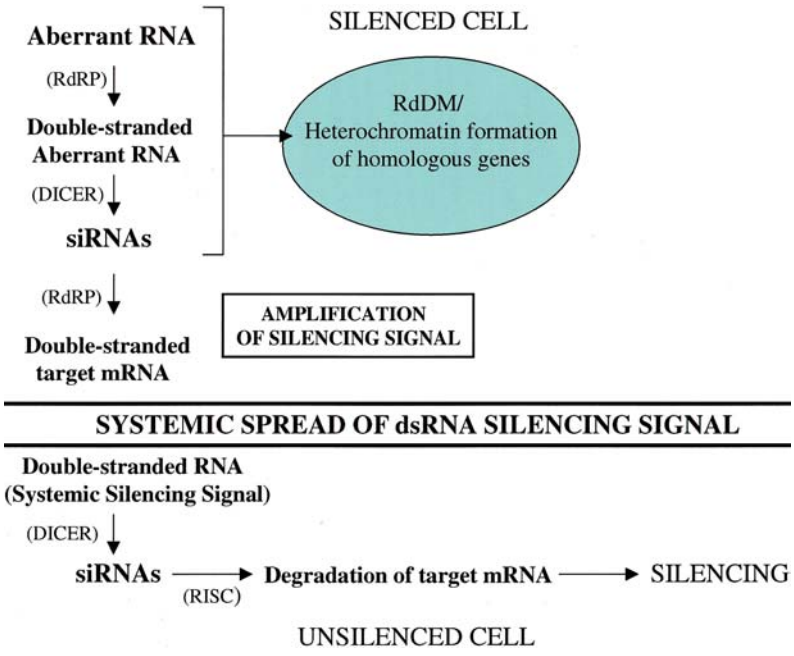

Figure 2. PTGS may be reinforced in a silenced cell by the RNA-silencing signal returning to the nucleus to induce RNA-directed DNA methylation (RdDM) or chromatin changes. The silencing signal may either be the aberrant RNA itself, the double-stranded aberrant RNA created by RNA-dependent RNA polymerase (RdRP), or the small interfering RNAs (siRNAs) produced by Dicer. An amplification step may involve the action of RdRP and siRNAs on target mRNAs that are made double-stranded, thus becoming themselves targets for the Dicer complex. The dsRNA-silencing signal may induce silencing by degradation of target mRNAs in an unsilenced cell distant from the initial site in a process known as systemic RNA silencing. The exact nature of the systemic silencing signal has not yet been identified.

pathways (discussed below). The *rrf-1* gene, although closely linked to *ego-1*, does not appear to be involved in development, but instead is involved in RNAi in somatic cells [42].

While dsRNA can induce RNAi directly, transgene-induced gene silencing in co-suppression and quelling may involve single-stranded transgenic transcripts that would need to be transformed into a dsRNA by an RdRP to then enter the RNAi pathway. Several recognition criteria have been proposed for RdRP 'sensing' of transgenic RNA [43]. These include 'aberrancy' of transgenic mRNAs due to pre-termination, incorrect maturation or secondary structures. On the other hand, transgenes present in tandem or inverted repeats may form dsRNA as hairpin or cruciform structures as a result of intramolecular pairing, in which case they could trigger PTGS directly without the need for RdRP conversion. In fact, short hairpin RNA (shRNA) has been shown to be as efficient as dsRNA in triggering RNAi in *Trypanosoma brucei* [44] and in mammalian cells [45]. The role of RdRP in PTGS triggered directly by dsRNA (as in RNAi) presented an enigma, but may now be explained by the recent evidence indicating that RdRP may be involved in an amplification step of the silencing signal that reinforces the silencing effects of PTGS [46]. Such an amplification step would also explain the extreme efficiency of RNAi that can be triggered with just a few molecules of dsRNA [10]. In vitro studies on *Drosophila* embryos have demonstrated that 'primary' siRNAs produced by the action of Dicer on dsRNA trigger molecules act as primers for RdRP to convert cognate mRNA into dsRNA which is subsequently degraded into 'secondary' siRNAs. Consistent with this, short single-stranded antisense RNAs have been found to work as efficiently as dsRNA in RNAi in *C. elegans* [47]. In this amplification reaction, new dsRNAs are created that may be immediately degraded into new siRNAs. This process has been termed 'degradative PCR' [46].

The 3′ hydroxyl group of siRNAs has been shown to enable elongation by RdRP [48]; however, evidence in human cells that siRNAs with blocked 3′ hydroxyl termini may still trigger efficient, but transient silencing in vivo indicates that the use of siRNAs as primers for RdRP synthesis in an amplification step may not be crucial in all organisms [49].

Decisive evidence that short RNAs can act as primers in the production of dsRNAs has been provided by the fact that action of RdRP can extend beyond the initial sequence complementary to dsRNA and the siRNAs formed as a result of the degradation of the extended dsRNA (secondary siRNAs) have the ability to induce secondary RNAi termed 'transitive RNAi'; however, the effects of transitive RNAi diminish with distance from the primary target sequence. The RdRP encoded by *ego-1* in *C. ele-*

gans has been shown also to be involved in the production of secondary siRNAs in germline cells, while its homologue *rrf-1* appears to be necessary for transitive RNAi in somatic cells only [42]. Thus although RdRPs homologous to tomato RdRP can sustain both primed and unprimed RNA synthesis, two different RdRP enzymes may possibly carry out the 'initiation' and 'maintenance' steps.

Dicer

The bidentate dsRNA-specific endonuclease isolated by Hammond and co-workers [33] from extracts of *Drosophila* S2 cells transfected with a dsRNA to induce RNAi was shown to be a class II RNAseIII with two ribonuclease domains, an RNA-binding domain as well as an ATP-dependent RNA helicase domain and PAZ motif (see below). The enzyme was named Dicer due to its ability to digest dsRNA into uniformly sized small RNAs. A characteristic of class II RNAseIII is that it acts as a dimer and cleaves dsRNA into 21-nt fragments with 2-nt 3′ overhangs [37, 50]. The Dicer cleavage product has been shown to be a double-stranded siRNA [51].

The Dicer orthologue *dcr-1* identified in *C. elegans* [52, 53] has been shown to be important both for the production of siRNAs in RNAi and in the processing of stRNAs, providing concrete evidence of a common step in the two processes, both of which produce small RNAs from longer dsRNA substrates. Further evidence that RNAi and stRNA pathways intersect has been obtained both in vitro in *Drosophila* and in vivo with human cultured cells in which down-regulation of the human Dicer-like enzyme (Helicase MOI) resulted in an accumulation of stRNA precursors [54].

In *C. elegans,* the non-coding heterochronic regulatory stRNAs *lin-4* and *let-7* are complementary to the 3′ untranslated regions (3′ UTRs) of the mRNAs of their target genes *lin-14, lin-28* and *lin-41*, involved in larval development. They are expressed as approximately 70-nt precursor RNAs that are predicted to fold into stem-loop structures forming dsRNAs analogous to those that trigger RNAi. Whereas *lin-4* that regulates an early larval transition stage appears to be unique to *C. elegans*, *let-7* that regulates the transition from the late larval to the adult stage of development is highly conserved in a number of higher organisms, including humans. The temporal conservation is also conserved in *Drosophila*, zebrafish, annelids and molluscs, indicating that *let-7* may control development across animal phylogeny, but not in yeast, bacteria or plants where no homologue has been detected [55]. The recent identification of 15 new miRNAs in *C. elegans* of which several are expressed during larval development, and the identification of 3 homologues in mammals and/or insects suggest that small non-coding RNAs may have an even more extensive role in the regulation of gene expression than seen to date [56]. Moreover, 25 miRNA sequences identified in *Arabidopsis* indicate that regulation of gene expression by miRNAs may also occur in plants [57].

Despite the fact that stRNAs and siRNAs may both be processed by Dicer-like enzymes from longer dsRNA molecules, differences exist between the two types of small RNA: apart from the fact that siRNAs lead to RNA destruction while stRNAs determine the inhibition of translation, stRNAs are sense ssRNAs, whereas siRNAs are double-stranded [58]. SiRNAs can target sequences anywhere in homologous mRNAs, although a report that different siRNAs have varying silencing efficiency in RNAi directed to the human tissue factor, important for blood coagulation, may indicate positional effects for siRNAs [49]. In contrast, stRNAs bind to the 3′ UTRs of target genes by complementarity with sequences contained in the UTRs. The nature of the RNA hybrid may be decisive for the destiny of target mRNAs. Imperfect homology between the antisense strand of stRNAs and their target RNAs cause bulges at unpaired nucleotide sites which may provide recognition sequences for RNA-binding proteins that control translation, while the perfect sequence homology between siRNAs and mRNAs determines the formation of a continuous base pair hybrid which may be recognized by proteins involved in mRNA degradation [53].

RNA-induced silencing complex

The role of siRNAs as guide molecules for target mRNA degradation in PTGS was first proposed by Hammond and co-workers who found that small 21- to 25-nt RNAs, with sequence homology to target mRNAs, co-fractionated with an RNA nuclease that specifically degraded exogenous mRNAs homologous to a dsRNA used to transfect *Drosophila* S2 cells to induce RNAi. They called the nuclease the RNA-induced silencing complex (RISC) [35]. Subsequently, a protein constituent Argonaute-2 (Ago-2) was found to be part of a 500-kDa active RISC [59] and proposed to have an 'adapter' function to bind siRNAs, committing them to the RNAi pathway. The isolation of Ago-2 provided a biochemical link to the genetic evidence previously obtained from mutants of a family of homologous proteins identified in developmental and RNAi-defective mutants unable to carry out mRNA degradation. The Argonaute family of proteins, due to homology with Ago-1 in *A. thaliana* [28, 60] includes RNAi-defective-1 (Rde-1) and Rde-4 in *C. elegans* [27, 61], Ago-1, Piwi and Sting in *Drosophila* [62–64] and Qde-2 in *Neurospora crassa* [65]. In *N. crassa,* siRNAs have been co-purified with Qde-2, indicating that siRNAs may be part of a complex equivalent to the RISC in quelling also [66].

These proteins are characterized by a Piwi domain and a PAZ (Piwi/Argonaute/ Zwille) domain and are therefore referred to as the PPD (PAZ and Piwi domain) family of proteins [67]. An interaction between PPD proteins and Dicer via their PAZ domains has been proposed to facilitate incorporation of siRNAs in the RISC [68]; however, although Ago-2 and Dicer can be co-immunoprecipitated, Dicer activity has not been demonstrated in RISC fractions isolated from *Drosophila* extracts [59].

The indisputable involvement of double-stranded siRNAs as the true intermediates of RNAi has been demonstrated in vitro in *Drosophila* and in cultured human cells using synthetic 22-nt siRNAs to mediate cleavage of target mRNA which is shown to occur at a single site near the centre of the region spanned by the guiding siRNA [69, 70]. PTGS induced by synthetic siRNAs has now also been demonstrated in vivo in *Xenopus* embryos [71].

The double-stranded siRNAs produced by Dicer are proposed to first assemble into a 360-kDa RNP complex. Although this step is ATP independent, both native siRNAs and synthetic siRNAs must be phosphorylated at their 5′ termini, a step that requires ATP. The 5′ phosphate that may be generated by a specific kinase could be a distinguishing factor for siRNA with respect to other small RNA species. As this 360-kDa complex is not competent to cleave target mRNA, an ATP-dependent siRNA unwinding step which separates the sense and antisense strand of the siRNA in the complex has been proposed, which would be in accordance with the antisense strand of siRNA guiding the RISC to target mRNA. In fact, modifications of the antisense strand can compromise PTGS efficiency [72]. The identification of a <232-kDa active RISC* comprising single-stranded siRNAs may result from the action of an RNA helicase on the 360-kDa inactive RISC. Still not known, however, is whether only one or both siRNA strands are retained in this <232-kDa active complex [51]. The 500-kDa complex identified by Hammond et al. [59] may, in fact, represent a complex in which a larger precursor and smaller active species remain associated. In support of the notion that an ATP-dependent RNA helicase may be part of the RISC as a dissociable co-factor is the fact that several PTGS mutants are defective in RNA helicase genes. RNA helicases implicated in PTGS are represented by Sde-3 in *A. thaliana* [73], Smg-2 in *C. elegans* [74] and Mut-6 in the green alga *Chlamydomonas reinhardtii* [75]. The recent identification of a second RNA helicase Mut-14 involved in RNAi, but specifically in a step downstream from siRNA processing [47], supports this hypothesis.

As a final step in the degradation process, an RNA exonuclease has been proposed as a further component of the RISC. Mut-7, homologous to the RNAseD of the Werner syndrome DNA helicase, has been identified in *C. elegans* [26]. *Mut-7* mutants resistant to RNAi are also characterized by an increase in transposition of TEs. The 3′-5′ exonuclease activity of Mut-7 may be responsible for the degradation of transposon-specific mRNAs and therefore a component of transposon silencing in this organism. (26). An exonuclease such as Mut-7, proposed to function together with the Rde-2 protein in a downstream step common to both RNAi and transposon silencing [61] may also act in a rate-determining step subsequent to RISC cleavage of human tissue factor in RNAi in human cells [49].

Accessory proteins in PTGS

Recent evidence from *C. elegans* indicates that Rde-1, previously shown to act downstream from siRNA production, forms a complex with a homologous protein Rde4, implicated in the production of siRNAs [76] and may, therefore, also be important in the initiation step of RNAi. The two dsRNA-binding domains (dsRBMs) of Rde-4 have been demonstrated to be necessary for cleavage of trigger dsRNA by Dicer, confirming an upstream role for Rde-4 [42]. In fact, Rde-4 forms a complex with Dcr-1 and has been proposed to act as an adapter protein that presents trigger dsRNA to Dcr-1 for processing [77]. Furthermore, the interaction of Rde-4 with Dicer-related helicases encoded by *drh-1* and *drh-2* in *C. elegans* indicates a role for RNA helicases in an initiation step also, to unwind long dsRNA trigger molecules to enable processing by Dcr-1. The interaction of both the Dcr-1/Rde-4 complex and the RISC with Rde-1 could indicate that Rde-1 has a role to transfer siRNAs from one complex to the other, or that both steps of RNAi occur in a single multiprotein dsRNA-processing complex.

Although Dcr-1 has also been shown to be involved in stRNA processing [52, 53], *rde-4* and *drh-1* mutants do not exhibit developmental defects [27, 77], indicating that other proteins may be specific to the RNA-regulated expression of developmental genes and that the shorter stem-loop stRNA precursors do not need the action of an RNA helicase. In fact, two other Rde-1 homologues, Alg-1 and Alg-2, have been proposed to be involved as co-factors in the action of stRNAs and their subsequent regulation of heterochronic genes, maybe to prevent the single-stranded stRNAs from being degraded [53]. Likewise, the *ago-1* homologue in *Drosophila*, together with *piwi* and *sting*, and *ago-1* in *Arabidopsis* together with *pinhead/zwille*, may also be specific to the stRNA pathway.

RNA-mediated epigenetic DNA modifications

Epigenetic DNA modifications that silence genes at a transcriptional level without altering their structure include chromatin remodelling and DNA methylation. Once thought to be the result of DNA-DNA interactions,

it is now becoming clear that dsRNA may also be involved in such modifications, linking PTGS with transcriptional silencing phenomena. For the economy of the cell it would be reasonable that once the mRNA of a gene has been targeted for degradation in the cytoplasm, the same trigger or guide molecule would be able to return to the nucleus to induce DNA methylation or chromatin changes to silence homologous genes at the transcriptional level. The ability of RNA to induce methylation of DNA was first discovered in plants where the presence of viroids determined sequence-specific de novo methylation of nuclear DNA [78]. Similarly, RNA viruses may also become methylated when integrated into the nucleus [79]. Interestingly, targets as short as 30 nt can be modified [80], which suggests a link with siRNAs. Whether trigger dsRNA or siRNAs guide RdDM in the nucleus has still not been firmly established, however, both promoter region hairpin RNAs and viral dsRNAs processed into small dsRNAs have been shown to direct DNA methylation [81–83]. Such dsRNAs conceivably act as guide molecules for DNA methyltransferases to methylate unmodified homologous DNA sequences, or for chromatin remodelling proteins to induce chromatin modifications, in a manner similar to the role of siRNAs as guide molecules in mRNA degradation. Evidence in support of this hypothesis is provided by the fact that RNA molecules have been shown to interact with the chromodomain of a chromomethylase protein in plants [84] and with the *mof* gene in *Drosophila* that encodes a histone deacetylase [85]. RNA-mediated methylation or heterochromatin formation may have evolved to sequester TEs and repetitive elements in parallel to PTGS mechanisms that lead to the degradation, for example, of transposase mRNA. In *C. elegans,* silencing of tandem arrays of endogenous genes such as those of the Polycomb complex [86, 87] and tandem repeats of the *Su* (*Ste*) gene involved in the maintenance of male fertility in *Drosophila* [88], have both been shown to involve mechanisms analogous to co-suppression. In *C. elegans, mes-3, -4* and *-6* that have been identified as additional genes involved in RNAi are homologues to chromatin-binding Polycomb group proteins, indicating that chromatin remodelling and RNAi may be linked [89].

In *Arabidopsis*, PTGS fails to become established in *ddm-1* (decrease in DNA methylation) mutants lacking the SNF2/SW12 chromatin-remodelling protein [90], indicating a role for chromatin remodelling in the establishment of PTGS. RdDM may represent a maintenance step for PTGS, as the coding regions of silenced transgenes are often methylated [91]. In *Arabidopsis,* although PTGS can be established in DNA-methylation-deficient (*met-1*) plants, silenced genes become reactivated with growth, indicating that the maintenance of DNA methyltransferase activity encoded by *met-1* is important for maintaining silencing during plant development and through meiosis in future generations [90]. Confirmation of the role of methylation in PTGS in plants has been provided by a partial release from silencing, as a result of drug-induced hypomethylation, of a tobacco transgene [92]. On the other hand, efficient quelling in *N. crassa dim-2* mutants indicates that DNA methylation is dispensable for quelling in fungi [8] and also for RNAi in invertebrates, as these organisms do not methylate their DNA.

Viral suppressors of PTGS

Viruses have found ways to evade cellular defence, and identification of viral proteins involved in counteracting gene silencing, has shed light on various aspects of the PTGS mechanism itself. The helper component proteinase (Hc-Pro) from potyviruses inhibits the accumulation of siRNAS [93, 94], via its interaction with the regulator of gene silencing-calmodulin-like (rgs-CaM) protein, a protein which itself may suppress gene silencing when overexpressed [95]. This is the first example of an endogenous PTGS gene which may be expressed by the cell to temporarily disable PTGS when this would otherwise be detrimental. How Hc-Pro ultimately suppresses PTGS is still not known, as the target gene of rgs-CaM has not yet been identified.

While Hc-Pro blocks the maintenance of PTGS in tissues where silencing has already been established, the Cucumber Mosaic Virus (CMV) 2b protein blocks the spread of silencing to new tissues [96], probably by blocking a nuclear step of PTGS [97]. The B2 protein of Flock House Virus (like the 2b protein of CMV) can suppress RNA silencing both in plants and animals, demonstrating for the first time that this response is conserved across kingdoms [98]. A further strategy is adopted by the p25 cell-to-cell movement protein of PVX, which suppresses PTGS by targeting the mobile silencing signal [99], and the p19 protein of Tomato Bushy Stunt Tombusvirus (TBSV) which suppresses both transgene-induced local and systemic silencing, but not virus-induced local silencing, by binding to PTGS-generated siRNAs [100].

Systemic RNA silencing

In plants, PTGS was shown to spread from an initial site to the rest of the plant [101, 102]. Short-range transmission from cell to cell occurs via plasmodesmata, while silencing signals can travel long distances via phloem. Systemic silencing probably evolved as a means of defence to halt the spread of viral infections via the vascular tissue of a plant. The PTGS counter-defence strategies of viruses have provided elucidation of the steps involved in systemic gene silencing. SiRNAs were initially

proposed as the silencing signal [34]; however, although Hc-Pro suppresses the accumulation of siRNAs, it does not eliminate the mobile silencing signal or DNA methylation but, rather, prevents the plant from responding to the mobile signal [94], suggesting that RdDM may also not be mediated by siRNAs, but a different dsRNA. DsRNA has been hypothesized to be the diffusible signal in plants. Bound to an RNA movement protein, dsRNA may move both through plasmodesmata and through nuclear pores. In the nucleus, together with an RNA helicase, dsRNA can mediate RdDM, while in new cells, as in the case of grafted tissue, the action of Dicer would produce siRNAs that can then act as primers on target mRNA and with RdRP to produce dsRNAs amplifying the mobile silencing signal [103]. The proposal that p25, the viral inhibitor of PTGS of PVX, targets host RdRP to block the production of the mobile silencing signal [104] substantiates this model in which dsRNA is the diffusible factor. Moreover, the involvement of RdRP in an amplification step has been identified both in *C. elegans* [42] and in *Drosophila* [46], although systemic silencing has not been revealed in flies. The p19 protein of TBSV shown in vitro to bind to siRNAs probably inhibits the production of an active signal complex by depleting the cell of siRNAs that could act as primers to generate dsRNAs [100].

The systemic spread of RNAi also occurs in *C. elegans*. In this organism, RNAi not only spreads from one tissue to another, but can also be transmitted to progeny [10], indicating that the diffusible factor must be a nucleic acid. Systemic RNAi can be induced by simply injecting dsRNA into the body cavity, or by feeding worms with bacteria expressing dsRNA [105]. Evidence against siRNAs as the diffusible signal in *C. elegans* is provided by the fact that even in homozygous *rde-1* and *rde-4* mutant strains deficient in the production of siRNAs, RNAi can be transmitted to heterozygous progeny [76].

To identify components of systemic silencing in *C. elegans*, systemic RNA interference defective *(sid*) mutants were isolated and found to fall into three complementation groups (*sid-1, sid-2* and *sid-3*) [106]. In *sid-1* mutants, RNAi cannot spread from one tissue to another and silencing cannot be transmitted to progeny. Sid-1 has been shown to be a trans-membrane protein and is probably required for the import/export of the systemic RNAi signal. The absence of a Sid-1 homologue in *Drosophila* explains the lack of systemic silencing in this organism.

Perspectives

The practical implications of RNA silencing, once viewed as an impediment to genetic engineering of plants, may instead be of fundamental importance both for controlling gene expression and for use as a tool for functional genomics. Specific genes can now be targeted by RNAi, while dsRNA-induced TGS can be used to control gene expression. The theraputic potential of synthetic siRNAs and shRNAs has become apparent as these RNAi intermediates alleviate the non-specific responses previously found with dsRNA in mammalian cells. Uncovering the still unknown function of many miRNAs that may have important roles in gene regulation is the next step in deciphering the complexities of RNA silencing.

1 Storz G. (2002) An expanding universe of non-coding RNAs. Science **296:** 1260–1262
2 Mattick J. S. (2001) Non-coding RNAs: the architects of eukaryotic complexity. EMBO Rep. **2:** 989–991
3 Napoli C., Lemieux C. and Jorgensen R. (1990) Introduction of a chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in trans. Plant Cell **2:** 279–289
4 Krol A. R. van der, Mur L. A., Beld M., Mol J. N. and Stuitje A. R. (1990) Flavonoid genes in *Petuni*a: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2:** 291–299
5 Smith C. J. S., Watson C. F., Bird C. R., Ray J., Schoch W. and Grierson D. (1990) Expression of a truncated tomato polygalacturonase gene inhibits expression of endogenous gene in transgenic plants. Mol. Gen. Genet. **224:** 477–481
6 Carvalho F. de, Niebel F., Frendo P., Van Montagu M. and Cornelissen M. (1995) Post-transcriptional co-suppression of β-1,3-glucanase genes does not affect accumulation of transgene nuclear mRNA. Plant Cell **7:** 347–358
7 Romano N. and Macino G. (1992) Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. Mol. Microbiol. **6:** 3343–3353
8 Cogoni C., Irelan J. T., Schumacher M., Schmidhauser T. J., Selker E. U. and Macino G. (1996) Transgene silencing of the al-1 gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. EMBO J. **15:** 3153–3163
9 Guo S. and Kemphues K. J. (1995) *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell **81:** 611–620
10 Fire A., Xu S., Montgomery M. K., Kostas S. A., Driver S. E. and Mello C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:** 806–811
11 Kennerdell J. R. and Carthew R. W. (2000) Heritable gene silencing in *Drosophila* using double stranded RNA. Nat. Biotechnol. **17:** 896–898
12 Misquitta L. and Paterson B. M. (1999) Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. Proc. Natl. Acad. Sci. USA **96:** 1451–1456
13 Ngo H., Tschudi C., Gull K. and Ullu E. (1998) Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. Proc. Natl. Acad. Sci. USA **95:** 14687–14692
14 Ruiz F., Vayssie L., Klotz C., Sperling L. and Madeddu L. (1998) Homology-dependent gene silencing in *Paramecium*. Mol. Biol. Cell **9:** 931–943
15 Alvarado A. S. and Newmark P. A. (1999) Double stranded RNA specifically disrupts gene expression during planarian regeneration. Proc. Natl. Acad. Sci. USA **96:** 5049–5054

16 Waterhouse P., Graham M. W. and Wang M. B. (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. USA **95:** 13959–13964
17 Svoboda P., Stein P., Hayashi H. and Schultz R. M. (2000) Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. Development **127:** 4147–4156
18 Wianny F. and Zernicka-Goetz M. (2000) Specific interference with gene function by double-stranded RNA in early mouse development. Nat. Cell Biol. **2:** 70–75
19 Wargelius A., Ellingsen S. and Fjose A. (1999) Double-stranded RNA induces specific developmental defects in zebrafish embryos. Biochem. Biophys. Res. Commun. **263:** 156–161
20 Li Y. X., Farrell M. J., Liu R., Mohanty N. and Kirby M. L. (2000) Double-stranded RNA injection produces null phenotypes in zebrafish. Dev. Biol. **217:** 394–405; (erratum **220:** 432)
21 Boutla A., Kalantidis K., Tavernarakis N., Tsagris M. and Tabler M. (2002) Induction of RNA interference in *Caenorhabditis elegans* by RNAs derived from plants exhibiting post-transcritional gene silencing. Nucleic Acids Res. **30:** 1688–1694
22 Wassenegger M. and Pélissier T. (1998) A model for RNA-mediated gene silencing in higher plants. Plant Mol. Biol. **37:** 349–362
23 Angell S. M. and Baulcombe D. C. (1997) Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. EMBO J. **16:** 3675–3684
24 Lindbo J. L., Silva-Rosales L., Proebsting W. M. and Dougherty W. G. (1993) Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus expression. Plant Cell **5:** 1749–1759
25 Ruiz M. T., Voinnet O. and Baulcombe D. C. (1998) Initiation and maintenance of virus-induced gene silencing. Plant Cell **10:** 937–946
26 Ketting R. F., Haverkamp T. H., Luenen H. G. van and Plasterk R. H. (1999) Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell **99:** 133–141
27 Tabara H., Sarkissian M., Kelly W. G., Fleenor J., Grishok A., Timmons L. et al. (1999) The rde-1 gene, RNA interference, and transposon silencing in *C. elegans.* Cell **99:** 123–132
28 Fagard M., Boutet S., Morel J.-B., Bellini C. and Vaucheret H. (2000) AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proc. Natl. Acad. Sci. USA **97:** 11650–11654
29 Lee R. C., Feinbaum R. I. and Ambros V. (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. Cell **75:** 846–854
30 Reinhart B. J., Slack F. J., Basson M., Pasquinelli A. E., Bettinger A. C., Rougvie A. E. et al. (2000) The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. Nature **403:** 901–906
31 Cogoni C. and Macino G. (1999) Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature **399:** 166–169
32 Schiebel W., Haas B., Marinkovic S., Klanner A. and Sänger H. L. (1993). RNA-directed RNA polymerase from tomato leaves. I. Purification and physical properties. II. Catalytic in vitro properties. J. Biol. Chem. **268:** 11851–11867
33 Bernstein E., Caudy A. C., Hammond S. M. and Hannon G. J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409:** 363–366
34 Hamilton A. J. and Baulcombe D. C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286:** 950–952
35 Hammond S. M., Bernstein E., Beach D. and Hannon G. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cell extracts. Nature **404:** 293–296
36 Tuschl T., Zamore P. D., Lehmann R., Bartel D. P. and Sharp P. A. (1999) Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev. **13:** 3191–3197
37 Zamore P., Tuschl T., Sharp P. and Bartel D. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101:** 25–33
38 Bass B. L. (2000) Double-stranded RNA as a template for gene silencing. Cell **101:** 235–238
39 Smardon A., Spoerke J., Stacey S., Klein M., Mackin N. and Maine E. (2000) EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans.* Curr. Biol. **10:** 169–178
40 Mourrain P., Béclin C., Elmayan T., Feuerbach F., Godon C., Morel J.-B. et al. (2000) Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell **101:** 533–542
41 Dalmay T., Hamilton A., Rudd S., Angell S. and Baulcombe D. C. (2000) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell **101:** 543–553
42 Sijen T., Fleenor J., Simmer F., Thijssen K. L., Parrish S., Timmons L. et al. (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107:** 465–476
43 Cogoni C. and Macino G. (2000) Post-transcriptional gene silencing across kingdoms. Curr. Opin. Genet. Dev. **10:** 638– 643
44 Djikeng A., Shi H., Tschudi C. and Ullu E. (2001) RNA interference in *Trypanosoma brucei*: cloning of small interfering RNAs provides evidence for retroposon-derived 24–26 nt RNAs. RNA **7:** 1–9
45 Paddison P. J., Caudy A. A., Bernstein E., Hannon G. J. and Conklin D. S. (2002) Short hairpin RNAs (shRNASs) induce sequence-specific silencing in mammalian cells. Genes Dev. **16:** 948–958
46 Lipardi C., Wei Q. and Paterson B. M. (2001) RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell **107:** 297–307
47 Tijsterman M., Ketting R. F., Okihara K. L., Sijen T. and Plasterk R. H. A. (2002) RNA helicase MUT-14-dependent gene silencing triggered in *C. elegans* by short antisense RNAs. Science **295:** 694–696
48 Elbashir S. M., Lendeckel W. and Tuschl T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev**. 15:** 188–200
49 Holen T., Amarzguioui M., Wilger M. T., Babaie E. and Prydz H. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. Nucleic Acids Res. **30:** 1757–1766
50 Zamore P. D. (2001) Thirty-three years later, a glimpse at the ribonuclease III active site. Mol. Cell **8:** 1158–1160
51 Nykänen A., Haley B. and Zamore P. D. (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell **107:** 309–321
52 Ketting R. F,. Fischer S. E., Bernstein E., Sijen T., Hannon G. J. and Plasterk R. H. (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. Genes Dev. **15:** 2654–2659
53 Grishok A., Pasquinelli A. E., Conte D., Li N., Parrish S., Ha I. et al. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. Cell **106:** 23–34
54 Hutvágner G., McLachlan J., Pasquinelli A. E., Bálint É., Tuschl T. and Zamore P. D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science **293:** 834–838

55 Pasquinelli A. E., Reinhart Stack F., Martindale M. Q., Kurodall M. I., Mallor B. et al. (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature **408:** 86–89

56 Lee R. C. and Ambros V. (2001) An extensive class of small RNAs in *Caenorhabditis elegans*. Science **294:** 862–864

57 Llave C., Kasschau K. D., Rector M. A. and Carrington J. C. (2002) Endogenous and silencing-associated small RNAs in plants. Plant Cell **14:** 1605–1619

58 Parrish S., Fleenor J., Xu S., Mello C. and Fire A. (2000) Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. Mol. Cell **6:** 1077–1087

59 Hammond S., Boettcher S., Caudy A., Kobayashi R. and Hannon G. J. (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. Science **293:** 1146–1150

60 Bohmert K., Camus I., Bellini,C., Bouchez D., Caboche M. and Benning C. (1998) AGO1 defines a novel locus of *Arabidopsis* controlling leaf development. EMBO J. **17:** 170–180

61 Grishok A., Tabara H. and Mello C. (2000) Genetic requirements for inheritance of RNAi in *C. elegans* Science **287:** 2494–2497

62 Kataoka Y., Takeichi M. and Uemura T. (2001) Developmental roles and molecular characterisation of a *Drosophila* homologue of *Arabidopsis* argonaute 1, the founder of a novel superfamily. Genes Cells **6:** 313–141

63 Cox D. N., Chao A., Baker J., Chang L., Qiao D. and Lin H. (1998) A novel class of evolutionarily conserved genes defined by *piwi* are essential for stem cell self-renewal. Genes Dev. **12:** 3715–3727

64 Schmidt A., Palumbo G., Bozzetti M. P., Tritto P., Pimpinelli S. and Schäfer U. (1998) Genetic and molecular characterization of sting, a gene involved in crystal formation and meiotic drive in the male germ line of *Drosophila melanogaster*. Genetics **151:** 749–760

65 Catalanotto C., Azzalin G., Macino G. and Cogoni C. (2000) Transcription: gene silencing in worms and fungi. Nature **404:** 245

66 Catalanotto C., Azzalin G., Macino G. and Cogoni C. (2002) Involvement of small RNAs and role of the *qde* genes in the gene silencing pathway in *Neurospora*. Genes Dev. **16:** 790–795

67 Cerutti L., Mian N. and Bateman A. (2000) Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. Trends Biochem. Sci.. **10:** 481–482

68 Baulcombe D. (2001) Diced defence. Nature **409:** 295–296

69 Caplen N. J., Parrish S., Imani F., Fire A. and Morgan R. A. (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc. Natl. Acad. Sci. USA **98:** 9742–9747

70 Elbashir S. M., Martinez J., Patkaniowska A., Lendeckel W. and Tuschl T. (2001) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J. **3:** 6877–6888

71 Zhou Y., Ching Y. P., Kok K. H., Kung H. F. and Jin D. Y. (2002) Post-transcriptional suppression of gene expression in *Xenopus* embryos by small interfering RNA. Nucleic Acids Res. **30:** 1664–1669

72 Yang D., Lu H. and Erickson J. W. (2000) Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. Curr. Biol. **10:** 1191–1200

73 Dalmay T., Horsefield R., Braunstein T. H. and Baulcombe D. C. (2001) SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in *Arabidopsis*. EMBO J. **20:** 2069–2078

74 Domeier M. E., Morse D. P., Knight S. W., Portereiko M., Bass B. L. and Mango S. E. (2000) A link between RNA interference and nonsense-mediated decay in *Caenorhabditis elegans*. Science **289:** 1928–1931

75 Wu-Scharf D., Jeong B., Zhang C. and Cerutti H. (2000) Transgene and transposon silencing in *Chlamydomonas reinhardtii* by a DEAH-Box RNA helicase. Science **290:** 1159–1163

76 Parrish S. and Fire A. (2001) Distinct roles for RDE1 and RDE4 during RNA interference in *Caenorhabditis elegans*. RNA **7:** 1397–1402

77 Tabara H., Yigit E., Siomi H. and Mello C. C. (2002) The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-Box helicase to direct RNAi in *C. elegans*. Cell **109:** 861–871

78 Wassenegger M., Heimes S., Riedel L. and Sanger H. L. (1994). RNA-directed de novo methylation of genomic sequences in plants. Cell **76:** 567–576

79 Jones L., Hamilton A. J., Voinnet O., Thomas C. L., Maule A. J. and Baulcombe D. C. (1999) RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. Plant Cell **11:** 2291–2302

80 Pélissier T. and Wassenegger M. (2000) A target of 30 bp is sufficient for RNA-directed DNA methylation. RNA **6**: 55-65

81 Mette M. F., Aufsatz W., Winden J. van der, Matzke M. A. and Matzke A. J. M. (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J. **19:** 5194–5201

82 Jones L., Ratcliff F. and Baulcombe D. C. (2001) RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. Curr. Biol. **11:** 747–757

83 Wang M. B., Wesley S. V., Finnegan E. J., Smith N. A. and Waterhouse P. M. (2001) Replicating satellite RNA induces sequence-specific DNA methylation and truncated transcripts in plants. RNA **7:** 16–28

84 Lindroth A. M., Cao X., Jackson J. P., Zilberman D., McCallum C. M., Henikoff S., Jacobsen S. E. et al. (2001) Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. Science **292:** 2077–2080

85 Akhtar A., Zink D. and Becker, P. B. (2000) Chromodomains are protein-RNA interaction modules. Nature **407:** 405

86 Pal-Bhadra M., Bhadra U. and Birchler J. A. (1997). Cosuppression in *Drosophila*: gene silencing of alcohol dehydrogenase by white-Adh transgenes is policomb dependent. Cell **90:** 479–490

87 Pal-Bhadra M., Bhadra U. and Birchler J. A. (1999). Cosuppression of non-homologous transgenes in *Drosophila* involves mutually related endogenous sequences. Cell **99:** 35–46

88 Aravin A. A., Naumova N. M., Tulin A. V., Vagin V. V., Rozovsky Y. M. and Gvozdev V. A. (2001) Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster* germline. Curr. Biol. **11:** 1017–1027

89 Dudley N. R., Labbe J. C. and Goldstein B. (2002) Using RNA interference to identify genes required for RNA interference. Proc. Natl. Acad. Sci. USA **99:** 4191–4196

90 Morel J-B., Mourrain P, Béclin C. and Vaucheret H. (2001) DNA methylation and chromatin structure mutants affect both post-transcriptional and transcriptional gene silencing in *Arabidopsis*. Curr. Biol. **10:** 1591–1594

91 Fagard M. and Vaucheret H. (2000) (Trans)gene silencing in plants: how many mechanisms? Annu. Rev. Plant Physiol. Plant Mol. Biol. **51:** 167–174

92 Kovarik A., Van Houdt H., Holy A. and Depicker A. (2000) Drug-induced hypomethylation of a posttranscriptionally silenced transgene locus of tobacco leads to partial release of silencing. FEBS Lett. **467:** 47–51

93 Anandalakshmi R., Pruss G. J., Ge X., Marathe R., Mallory A. C., Smith T. H. et al. (1998) A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. USA **95:** 13079– 13084.

94 Mallory A., Ely L., Smith T. H., Marathe R., Anandalakshmi R., Fagard M. et al. (2001) HC-pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. Plant Cell **13:** 571–583
95 Anandalakshmi R., Marathe R., Ge X., Herr J. M. Jr, Mau C., Mallory A. et al. (2000) A calmodulin related protein that suppresses post-transcriptional gene silencing in plants. Science **290:** 142–144
96 Brigneti G, Voinnet O., Li W. X., Ji L. H., Ding S. W. and Baulcombe D. C. (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. EMBO J. **17**: 6739-6746
97 Lucy A. P., Guo H. S., Li W. X. and Ding S. W. (2000) Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. EMBO J. **19:** 1672–1680
98 Li H., Li W. X. and Ding S. W. (2002) Induction and suppression of RNA silencing by an animal virus. Science **296:** 1319– 1321
99 Voinnet O., Lederer C. and Baulcombe D. C. (2000) A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. Cell **103:** 157–167
100 Silhavy D., Molnar A., Lucioli A., Szittya G., Hornyik C., Tavazza M. et al. (2002) A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. EMBO J. **21:** 3070–3080
101 Palauqui J. C., Elmayan T., Pollien J. M. and Vaucheret, H. (1998) Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J. **16:** 4738–4745 (erratum **17:** 2137)
102 Voinnet O., Vain P., Angell S. and Baulcombe D. C. (1998) Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell **95:** 177–187
103 Waterhouse P. M. Wang, M.-B. and Finnegan E. J. (2001) Role of short RNAs in gene silencing. Trends Plant Sci. **6:** 297–301
104 Chicas A. and Macino G. (2001) Characteristics of post-transcriptional gene silencing. EMBO Rep. **2:** 992–996
105 Timmons L. and Fire A. (2000) Specific interference by ingested dsRNA. Nature **395:** 854
106 Winston W. M., Molodowitch and Hunter C. P. (2002) Systemic RNAi *in C. elegans* requires the putative transmembrane protein SID-1. Science **295:** 2456–2459
107 Lynn K., Fernandez A., Aida M., Sedbrook J., Tasaka M., Masson P. et al. (1999) The PINHEAD/ZWILLE gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the ARGONAUTE1 gene. Development **126:** 469–481
108 Jacobsen S. E., Running M. P. and Meyerowitz E. M. (1999) Disruption of an RNA helicase/RNase III gene in Arabidopsis causes unregulated cell division in floral meristems. Development **126:** 5231–5243
109 Matsuda S., Ichigotani Y., Okuda T., Irimura T., Nakatsugawa S. and Hamaguchi M. (2000) Molecular cloning and characterization of a novel human gene (HERNA) which encodes a putative RNA-helicase. Biochim. Biophys. Acta **1490:** 163–169
110 Cogoni C. and Macino G. (1999) Posttranscriptional gene silencing in *Neurospora* by a RecQ DNA helicase. Science **286:** 2342–2344
111 Elmayan T., Balzergue S., Beon F., Bourdon V., Daubremet J., Guenet Y. et al. (1998) *Arabidopsis* mutants impaired in cosuppression. Plant Cell **10:** 1747–1758