# RNAi: nature abhors a double-strand

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In organisms as diverse as nematodes, trypanosomes, plants, and fungi, double-stranded RNA triggers the destruction of homologous mRNAs, a phenomenon known as RNA interference. RNA interference begins with the transformation of the double-stranded RNA into small RNAs that then guide a protein nuclease to destroy their mRNA targets.

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**Abbreviations**

| | |
|---|---|
| **dsRNA** | double-stranded RNA |
| **miRNA** | microRNA |
| **nt** | nucleotide |
| **PTGS** | post-transcriptional gene silencing |
| **RdRP** | RNA-dependent RNA polymerase |
| **RISC** | RNA-induced silencing complex |
| **RNAi** | RNA interference |
| **siRNA** | small interfering RNA |
| **stRNA** | small temporal RNA |
| ***Su[Ste]*** | *Suppressor of Stellate* |

## Introduction

Confronted with double-stranded RNA (dsRNA), eukaryotic cells respond in a rather surprising way: they destroy their own mRNAs that share sequence with the double strand. This phenomenon, termed RNA interference (RNAi), has provided biologists with a remarkable tool for reverse genetics [1]. Thus, investigators studying *Caenorhabditis elegans*, *Drosophila melanogaster*, and a host of other invertebrates, plants such as *Arabidopsis thaliana*, fungi like *Neurospora crassa* (but not *Saccharomyces cerevisiae*), and mouse embryonic stem cells, oocytes, and early embryos can disrupt expression of virtually any gene by delivering dsRNA corresponding to that gene's sequence [2–14]. Recently, the use of RNAi has been extended to differentiated cultured mammalian cells [15,16••].

Here, we first describe the two models recently proposed to explain the mechanism of RNAi. We then discuss our evolving understanding of the biological functions of the RNAi pathway.

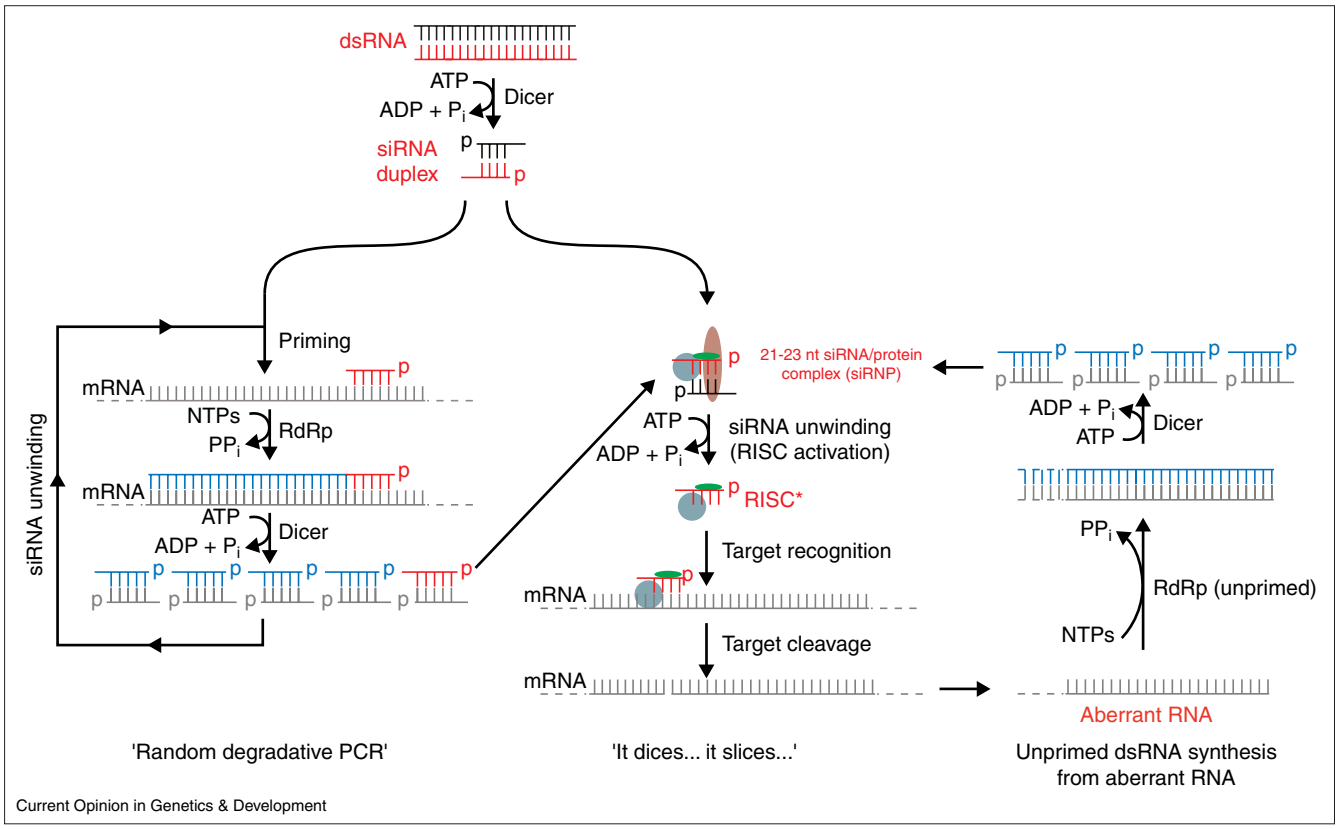
## It dices… it slices…?

Biochemical experiments conducted in *Drosophila* embryo lysates and cultured S2 cells support a four-step model for the RNAi pathway (Figure 1). The model envisions that RNAi is initiated by the ATP-dependent, processive cleavage of long dsRNA into 21–25 nucleotide (nt) double-stranded fragments, termed small interfering RNAs (siRNAs) [16••,17,18•,19•,20••]. These siRNA duplexes are then incorporated into a protein complex that is not yet competent to mediate RNAi [21•]. ATP-dependent unwinding of the siRNA duplex remodels the complex to generate an active RNA-induced silencing complex (RISC*, where the asterisk indicates the active conformation of the complex) [18•,21•]. Finally, in a step that requires little or no ATP, the RISC* can recognize and cleave a target RNA complementary to the guide strand of the siRNA [21•,22].

siRNAs are produced by the enzyme Dicer, a member of the RNase III family of dsRNA-specific endonucleases [20••]. Like the products of other RNase III enzymes, siRNA duplexes contain 5′ phosphate and 3′ hydroxyl termini, and two single-stranded nucleotides on their 3′ ends [23••]. These structural features are important for the entry of siRNAs into the RNAi pathway; blunt-ended siRNAs or siRNAs lacking a 5′ phosphate group are inefficient triggers of RNAi both *in vitro* and *in vivo* [15,21•,24–26]. The length of an siRNA is species-specific and may reflect differences in the spacing or structure of the RNase III domains of Dicer homologs. Among RNase III enzymes, Dicer is unique in that cleavage of dsRNA requires ATP, a finding that has been attributed to the presence of an ATP-dependent RNA 'helicase' domain at its amino terminus [19•,20••,21•,27]. The domain may serve to unwind dsRNA locally, allowing its subsequent cleavage, but this seems unlikely as other RNase III enzymes require no such helicase function. More likely, the helicase domain may be an RNA translocase, using ATP energy to drag Dicer down the dsRNA. Other evidence suggests that Dicer binding to RNA or catalytic activity might be ATP-regulated [28].

*In vitro*, both synthetic siRNAs and purified, native siRNAs generated by Dicer cleavage of dsRNA directly mediate RNAi [21•,23••]. Synthetic siRNAs mediate RNAi *in vivo* in Drosophila, and in both fly and mammalian cells *ex vivo*, but are less effective in *C. elegans* [15,16••,29]. Although the mechanism of target RNA destruction has not been demonstrated *in vivo*, biochemical studies support the view that each RISC contains a single siRNA and a protein ribonuclease [19•,21•,23••]. This nuclease has not yet been identified, but several lines of evidence suggest that it is distinct from Dicer and that it might aptly be named 'Slicer'. First, Dicer activity can be chromatographically separated from the active RISC [20••]. Second, an active, siRNA-containing complex (RISC*) that is assembled in the presence of ATP can subsequently mediate robust, sequence-specific cleavage of its target in the absence of ATP [21•]; Dicer function requires ATP. Similarly, the RISC has been purified extensively, contains both siRNA and protein, and is active in the absence of exogenous nucleotide cofactors [22]. *In vivo*, the RNA

**Figure 1**




An integrated model for RNAi and PTGS. In this model, the sequential action of Dicer (to generate siRNAs) and 'Slicer' (to cleave the target RNA) are considered the primary route for target destruction. Amplification of the siRNAs is postulated to occur by either (or both) 'random degradative PCR' or production of siRNAs from aberrant RNA – that is, the copying of the target RNA or a cleavage product of the target RNA by an RNA-dependent RNA polymerase to generate a dsRNA substrate for Dicer, thereby creating new siRNAs. In the random degradative PCR scheme, the polymerase is envisioned to be primed by an siRNA guide strand. Conversion of aberrant RNA to dsRNA is drawn here unprimed.

polymerase inhibitor, cordycepin (3′ deoxyadenosine triphosphate), inhibits embryogenesis, but not RNAi [30]. These data suggest that not only is Dicer different from 'Slicer', but also that neither RNA-synthesis nor an RNA ligase activity [31••] is required for target cleavage. (Both DNA and RNA ligases require high energy.) Finally, experiments using 5′ radiolabeled target RNAs to map the sites of cleavage triggered by dsRNA and by synthetic siRNAs demonstrate that each siRNA directs the endonucleolytic cleavage of the target RNA at a nucleotide across from the center, not the ends, of the guide siRNA strand, the strand complementary to the target sequence [19•,23••,25]. Although, these data do not exclude a model in which Dicer contains two distinct enzymatic activities, they suggest that dsRNA processing into siRNAs and destruction of the target RNA occur by two distinct mechanisms, catalyzed by Dicer and 'Slicer', respectively. Formal proof that Dicer does not act twice in the pathway awaits the demonstration *in vivo* that siRNA, but not dsRNA, mediates RNAi in the absence of its function.

## Random degradative PCR?

Screens for genes required for gene silencing in plants, fungi, and worms have identified a family of proteins whose sequences suggest they are RNA-dependent RNA polymerases (RdRPs) ([32–34,35•]; Table 1). The discovery of such RdRP proteins in the RNAi and post-transcriptional gene-silencing pathways provides a possible explanation for the remarkable efficacy of dsRNA in gene silencing. It has been estimated that in *Drosophila* embryos, ~35 molecules of dsRNA can silence a target mRNA thought to be present at >1000 copies per cell [4]. An RdRP protein might amplify either the dsRNA or the siRNA itself, providing a molecular explanation for the remarkable potency of dsRNA in triggering interference. An equally plausible explanation for the potency of interference, however, is that the RISC* is a classic multiple-turnover enzyme, albeit one carrying an RNA-encoded specificity determinant — the siRNA guide strand. In this more prosaic model, the excitement of the RNAi mechanism lies in its restricting 'Slicer' action to targets complementary to the guide siRNA, not in the finding that a single ribonuclease can degrade >30 RNA substrates. Furthermore, the production of multiple siRNAs from a single long dsRNA (perhaps 45 per kilobase of dsRNA) would, itself, provide enormous amplification in the absence of new dsRNA synthesis. As endonucleolytic cleavage of an mRNA likely creates two unstable products *in vivo* — one

capped but deadenylated and one adenylated but uncapped — a single cleavage by the RISC* might suffice to eliminate the target mRNA.

Nonetheless, it has been suggested that in *Drosophila* an RdRP activity plays a central role in the amplification of the dsRNA, and, consequently, in the amplification of the siRNAs [31••]. The 'random degradative PCR model' (Figure 1) proposes that the RdRP uses the guide siRNA strand as a primer to synthesize new RNA using the target RNA as a template and thereby converting it into dsRNA that can then be destroyed by Dicer. This, in turn, would release a new crop of siRNAs to prime additional rounds of synthesis and target destruction. In this view, Dicer not only functions in siRNA production, but also in target destruction.

The report of an RdRP activity in *Drosophila* embryo lysates [31••] is at once exciting and surprising. The fungus *Neurospora crassa* contains at least one RdRP homolog; the worm genome contains four such genes; *Arabidopsis* has six. Genetic experiments clearly demonstrate an essential role for RdRP proteins in RNAi in each of these organisms [32–34,35•], but no RdRP has been identified by homology in the genomes of either flies or humans. In the *Drosophila* embryo lysate experiments, RNA-templated RNA synthesis is primed by siRNA fragments isolated from micrococcal nuclease treated siRNA–protein complexes [31••]. (Because micrococcal nuclease leaves 3′ phosphates, these siRNA fragments must be further treated with a phosphatase to generate the 3′ hydroxyl group required for priming.) Remarkably, a synthetic siRNA containing a 3′ nucleotide that cannot pair with the template RNA sequence can act as a primer [31••].

An elegant genetic test for the role of RdRP proteins in copying the target RNA into dsRNA has been described recently by Fire, Plasterk and co-workers [35•]. In their *in vivo* assay, termed 'transitive RNAi', worms bearing two target RNAs, one encoding X and the other a fusion of the sequence of X to the 5′ end of Y (XY) are challenged with dsRNA corresponding to Y sequence. If siRNAs prime the RdRP-dependent conversion of the target XY into dsRNA, followed by the cleavage of the XY dsRNA into siRNAs, then siRNAs corresponding to the X sequence should be generated that can destroy target X. This is very much what happens in worms, and an RdRP protein, RRF-1, is required in worms not only for RNAi but also for the production of 'secondary siRNAs' thought to derive from newly synthesized RNA rather than the initial dsRNA trigger. Such an experiment remains to be carried out in either flies or human cells. However, a comparable experiment has been performed in *Drosophila* embryo lysates [19•]. Three overlapping ~500 nt dsRNAs corresponding to the *Renilla reniformis* luciferase (*Rr*-luc) mRNA, each displaced along the mRNA sequence by ~100 nt, were used to trigger RNAi against a 5′ radiolabeled *Rr*-luc target mRNA. The sites of cleavage on the target mRNA were mapped for each dsRNA. For each dsRNA, the positions at which the target sequence was cleaved were precisely confined to the region spanned by the sequence of the dsRNA. In no case were cleavages observed upstream from the sequence present in the dsRNA, as would have been predicted from the 'random degradative PCR model'.

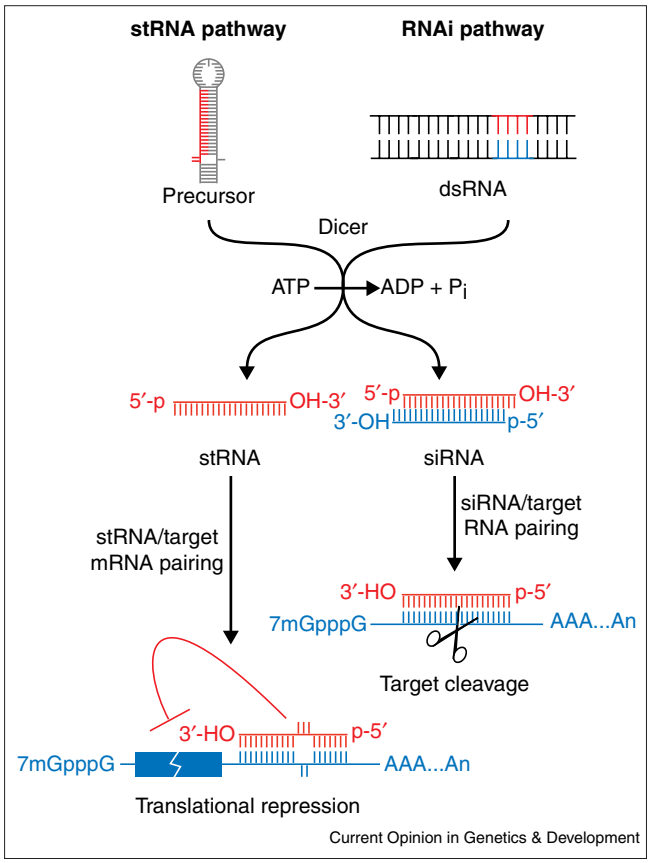
Although the 'random degradative PCR' model of target destruction is at odds with the 'it dices… it slices' mechanism, RdRP-mediated amplification of the dsRNA trigger almost certainly plays an important role in RNAi, especially in worms, where RNAi not only spreads throughout the entire animal, but also can be inherited through multiple generations [36]. The existence of transitive RNAi *in vivo* in *C. elegans* bolsters the view that Dicer might act twice in RNAi in this organism [35•], although it remains to be determined if Dicer or 'Slicer' is the primary executioner of worm mRNA targets. siRNAs can be detected in worms from sequences 5′ to the trigger RNA, indicating conversion of the target RNA into dsRNA from which come new siRNAs (secondary siRNAs) [35•]. But siRNA priming has not been demonstrated, and one might also imagine that the 5′ endonucleolytic product resulting from 'Slicer' cleavage of an mRNA could be a template for unprimed RdRP activity. In this view, one of the two mRNA cleavage products generated by 'Slicer' would correspond to the aberrant RNA thought to trigger silencing in plants and perhaps cosuppression in animals (Figure 1) [37,38]. It is worth noting that many RNA polymerases, including RdRP proteins, can synthesize RNA without an oligonucleotide primer. Unprimed synthesis of dsRNA from aberrant RNA could also account for the 3′ spreading of silencing in plants if the 3′ cleavage product is the preferred RdRP template in that organism.

## Dicer, development, and small temporal RNAs

Mutations in some genes required for RNAi and in orthologs of these genes have dramatic developmental defects, especially in the germline or in proliferative tissues, suggesting a link between the RNAi pathway and development [39–46]. Mutations in the worm RdRP, *ego-1*, block RNAi in the germline and disrupt oogenesis. Deletion of the worm *dcr-1* gene, the *C. elegans* homolog of Dicer, not only abrogates RNAi but also leads to misregulation of developmental timing [27,47••] and to defects in oogenesis [48]. Similarly, disruption of *Carpel Factory* (also named *Short Integuments-1*), the *Arabidopsis* Dicer homolog, disrupts embryo development, delays flowering time, and causes unregulated cell division in floral meristems [49–51].

The dramatic consequences of disrupting Dicer function derive, at least in part, from the role of Dicer in generating small temporal RNAs (stRNAs) — 21–23 nt RNAs thought to act as sequence-specific translational repressors [27,47••]. In worms, DCR-1 is required to excise the stRNAs *lin-4* and *let-7* from their ~70 nt stem-loop precursor RNAs [27,47••]. Mature *lin-4* and *let-7* regulate the timing of worm development, ensuring the orderly progression from larva to adult [52]. Dicer is implicated in *let-7* maturation not only

**Figure 2**




The RNAi and stRNA pathways intersect. In both pathways, Dicer acts to generate the active small RNA regulator: siRNAs from dsRNA and stRNAs from ~70 nt stRNA precursors. siRNAs trigger destruction of a perfectly complementary target RNA; stRNAs are thought to repress the translation of targets with which they pair imperfectly.

in worms but also in flies and human cells [28]. Thus, the RNAi and stRNA pathways intersect, but there are striking differences between the two (Figure 2). In the RNAi pathway, Dicer cleaves dsRNA into 21–23 nt siRNAs that are duplexes, whereas it acts on stRNA precursors to generate single-stranded stRNAs. stRNA precursors contain bulges and mismatches in the stem region that encodes the stRNA, unlike the perfect dsRNA used to trigger RNAi. The pairing of stRNAs with regulatory sequences in the 3′-untranslated regions of the mRNA they regulate is also incomplete and contains bulged nucleotides and G/U mismatches. stRNAs appear to regulate gene expression by blocking translation; siRNAs cleave their targets.

## miRNAs, your RNAs

Recently, >60 potential small regulatory RNAs (microRNAs or miRNAs) were identified in worms, fly embryos, and cultured human cells [53–55]. These RNAs are encoded in regions of the genome predicted to form ~70 nt stem-loop RNAs remarkably like stRNA precursors, and two in worms have been shown to require Dicer for their production [55]. Although many of the miRNAs are constitutively expressed, others are restricted in expression to specific times in development. Some appear to be coordinately expressed from a common primary transcript encoding multiple precursor stem-loops. miRNA-sized RNAs are abundant in plants, suggesting that they may function throughout eukaryotes [56].

Dicer makes both siRNAs and stRNAs, so why are stRNAs (and all but one of the newly identified miRNAs) single-stranded, whereas siRNAs are double-stranded? One possibility is that the structure of the precursor, with its single-stranded loop and bulges and mismatches in the stem, guides Dicer to cut only one strand. Such strand-specific cleavage has been observed for *Escherichia coli* RNase III. Another possibility is that Dicer initially produces a double-stranded, siRNA-like RNA, but that only one strand survives when the mature stRNA enters the stRNA pathway. A third possibility is that an siRNA-like duplex is initially generated but that it dissociates rapidly because of a lack of adequate complementarity between the two strands. The stRNA would then bind its target genes, and accumulate, while the other strand is degraded. This third idea suggests that the stability of the initial double-stranded Dicer product determines which pathway a small RNA enters.

The distinctions between siRNAs and stRNAs (and the miRNAs) suggest that specific proteins, perhaps a pair of related RNA-binding proteins, control entry into the RNAi and stRNA pathways. The *C. elegans* orthologs RDE-1 and ALG-1/ALG-2 might form such a pair [47••]. Both are members of the Paz and Piwi Domain (PPD) family of proteins, of which there are 24 in worms, 5 in flies, and 4 in humans. Mutants lacking Rde-1 cannot initiate RNAi, but are otherwise normal [57]. Worms lacking ALG-1/ALG-2 function can carry out RNAi, but suffer developmental defects because they fail to generate sufficient functional *lin-4* and *let-7* [47••].

Does the RNAi pathway have a biological function beyond its role in generating stRNAs? Three lines of evidence suggest it does. First, RNAi appears to be an endogenous mechanism for thwarting transposon mobilization, presumably by degrading transposon-encoded mRNAs. In worms and in *Chlamydomonas reinhardtii*, some RNAi-defective mutants show mobilization of their transposons [57–59]. However, mutations in two worm genes, *rde-1* and *rde-4*, abrogate RNAi but transposons remain silent [57]. Endogenous siRNAs cloned from *Trypanosoma brucei*, one of the earliest representatives of the eukaryotic lineage, derive mostly from the two classes of trypanosome retrotransposons [60]. Sequencing of fly siRNAs has also identified siRNAs from transposon sequences [23••]. Second, RNAi-like mechanisms are a potent anti-viral defense in plants. Not only do plant viruses encode specific protein suppressors of post-transcriptional silencing, plants have evolved countermeasures to the silencing suppressor proteins [61–66]. Finally, *Drosophila* males use an RNAi-like

**Table 1**

**Protein domains implicated in RNA silencing phenomena.**

| Domain structure | Protein | Organism (silencing phenomenon) | Proposed function | Phenotype of mutant |
|---|---|---|---|---|
| ATP-dependent RNA helicase + PAZ domain + two RNase III domains + dsRNA-binding domain | Dicer | *Drosophila melanogaster* (RNAi) | Processing long dsRNA into siRNAs and stRNA precursors into mature stRNAs | Not analyzed |
| | Dicer | *Homo sapiens* | Production of siRNAs, stRNAs, and miRNAs | Not analyzed |
| | CARPEL FACTORY (CAF/SIN-1) | *Arabidopsis thaliana* | Not yet tested for PTGS | Over-proliferation of floral meristem; null allele is embryo lethal; delayed timing of flowering |
| | DCR-1 | *Caenorhabditis elegans* (RNAi) | Processing long dsRNA into siRNAs and stRNA precursors into mature stRNAs | Defects in developmental timing and oogenesis |
| RNA-dependent RNA polymerase (RdRpP) | EGO-1 | *Caenorhabditis elegans* (germline RNAi) | dsRNA amplification | Defects in gametogenesis, proliferation and meiosis |
| | RRF-1 | *Caenorhabditis elegans* (somatic RNAi) | Secondary siRNA production | RNAi defective |
| | Qde-1 | *Neurospora crassa* (quelling) | dsRNA generation and/or amplification | Quelling defective |
| | SDE1/SGS2 | *Arabidopsis thaliana* (PTGS) | dsRNA generation; dsRNA amplification | No transgene-induced PTGS, abnormal leaf development |
| PAZ domain + C-terminal PIWI domain (PPD proteins) | RDE-1 | *Caenorhabditis elegans* (RNAi) | Initiation of silencing subsequent to siRNA accumulation | RNAi-resistant, but still supports PTGS by cosuppression |
| | Qde-2 | *Neurospora crassa* (quelling) | Initiation of silencing | Quelling defective |
| | Ago-1 | *Arabidopsis thaliana* (PTGS) | Initiation of silencing | No transgene-induced PTGS; pleiotropic developmental defects; sterile |
| | Ago-2 | *Drosophila melanogaster* (RNAi) | Component of RISC | Not analyzed |
| | Aubergine | *Drosophila melanogaster* (*Stellate* silencing) | Translational regulator | Defects in oogenesis and embryo patterning; fail to silence *Stellate* locus in testes |
| | eIF2c | Rabbit reticulocyte lysate | Translational initiation | Not analyzed |
| | Piwi | *Drosophila melanogaster, Caenorhabditis elegans* | Cell–cell signaling | Fail to maintain germline stem cells in males and females |
| RNase D domain | MUT-7 | *Caenorhabditis elegans* (RNAi) | Target RNA degradation | RNAi-resistant; mobilizes transposons; defective for cosuppression by PTGS |
| Double-stranded RNA-binding domain | RDE-4 | *Caenorhabditis elegans* (RNAi) | Initiation of silencing prior to siRNA production | RNAi-resistant |
| ATP-dependent nucleic acid helicases | | | | |
| RecQ DNA helicase | Qde-3 | *Neurospora crassa* (quelling) | Initiation of silencing | Quelling defective |
| DEAH-motif RNA helicase | Mut-6 | *Chlamydomonas reinhardtii* (cosuppression) | RNA unwinding | No PTGS; fails to degrade aberrant RNAs; mobilizes transposons |
| Upf1p- and SMG-2-like helicase | SMG-2 | *Caenorhabditis elegans* (RNAi) | RNA unwinding | Initiate RNAi, but recover more rapidly than wild types |
| Upf1p- and SMG-2-like helicase | SDE3 | *Arabidopsis thaliana* (PTGS) | RNA unwinding | No transgene-induced PTGS |
| DEAD-motif RNA helicase | Spindle E | *Drosophila melanogaster* (*Stellate* silencing) | RNA unwinding | Defects in oogenesis and embryo patterning; failure to silence *Stellate* locus in testes; retrotransposon derepression |
| Novel | SGS3 | *Arabidopsis thaliana* (PTGS) | Not known | No PTGS; enhanced virus susceptibility |

mechanism to degrade *Stellate* transcripts [67•]. When highly expressed in fly testes, the X-encoded Stellate protein renders males sterile. The *Suppressor of Stellate* (*Su[Ste]*) locus on the Y chromosome expresses dsRNA containing *Stellate* sequences, triggering degradation of *Stellate* mRNA. *Su(Ste)*-derived small RNAs accumulate in wild-type males, but not in *Su(Ste)* deletion mutants that fail to silence *Stellate* expression. Furthermore, mutations in the *spindle E* gene lead to inappropriate *Stellate* expression — presumably because *Su(Ste)*-mediated silencing fails to occur — and derepress retrotransposons in the germline [67•].

## Conclusions and future challenges

RNAi has been a boon to biologists, bringing reverse genetics ('functional genomics') to organisms lacking established genetic tools, and quickening the pace of genetic analysis in traditional genetic models such as *C. elegans* and *Drosophila*. Large-scale RNAi analysis of all the genes in *C. elegans* is well underway [68–70], and the discovery that synthetic siRNAs trigger RNAi in mammalian cells will surely lead to similar screens for human genes. The outlines of the RNAi pathway are beginning to emerge, although many of the details are still obscure. Perhaps the biggest challenge to biologists is understanding what RNAi does for the world's eukaryotes. The answers to this question are likely to lead to a new appreciation for the role small regulatory RNAs play in regulating cell growth and development, in protecting the eukaryotic genome from parasitic DNA, and in defending against viral infection.

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