RNAi: an ever-growing puzzle

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In recent years, sequence-specific gene silencing has been an area of increasing focus, both because of its interesting biology and because of its power as an experimental tool. A growing understanding of one such phenomenon, RNA interference (RNAi), has provided clues that many homology-dependent gene-silencing mechanisms share a common trigger, double-stranded RNA. Recent findings that RNAi and related pathways are involved not only in the response to exogenous pathogenic and endogenous parasitic nucleic acids but also in basic cellular processes, such as gene regulation and heterochromatin formation, have further fueled interest in this rapidly expanding field.

The phenomenology of double-stranded RNA (dsRNA)-induced silencing has been known for some years in plant systems, as at least one form of CO-SUPPRESSION and as virus-induced gene silencing (VIGS) (reviewed in [1]), before dsRNA itself was known to be the trigger or before similar responses were noted in animals. However, it was the latter observation and the evolution of RNA INTERFERENCE (RNAi; see glossary) as an experimental tool that really drew broad attention to this burgeoning field. In one sense, the story started with a test of the efficacy of antisense RNA in *Caenorhabditis elegans*. Sense RNA was a logical control but, interestingly, antisense and sense RNAs caused specific silencing of the targeted gene to similar degrees [2]. What might have been considered a failed experiment, in retrospect, built the foundation for coalescence in the field of gene silencing. Fire and colleagues reasoned that the *in vitro* transcribed RNA preparations used by Guo and Kemphues in their antisense studies were not purely single-stranded RNA and that dsRNA in these preparations might be the key trigger of silencing. Indeed, dsRNA was a much more potent silencing trigger than either strand alone. This phenomenon was named RNA interference (RNAi), distinguishing it mechanistically from classical antisense-mediated suppression [3].

The mechanism of RNAi

An examination of the phenomenology of RNAi in *C. elegans* and in plants required that any proposed mechanism explain certain unusual characteristics of the process. First, the process had to be flexible and instructed as to its targets by both exogenous and endogenous nucleic acids. These triggers had to serve as a sequence-based template for recognition by machinery that then degraded the target. In some circumstances, mechanisms for signal amplification and for spread of silencing throughout the organism needed to be proposed, although the latter properties are not universal characteristics of RNAi, and will be discussed separately.

In the past several years, the field has come a substantial way towards understanding the mechanism of RNAi through a combination of biochemical and genetic studies in several distinct experimental systems. Many proteins have been linked to RNAi and related phenomena (Table 1).

Dicer

One of the first mysteries to be unraveled, at least to a superficial level of understanding, was how the dsRNA-silencing trigger provided specificity to the silencing machinery. A key breakthrough came from the Baulcombe group. In work on transgene- and virus-induced post-transcriptional gene silencing (PTGS), this group observed the formation of discrete, small RNAs that were complementary to the target of silencing [4]. The discovery of these small RNAs prompted biochemical studies on the activity that formed these RNAs. Could these small RNAs be products of the silencing trigger, and thus possibly key components of the silencing pathway? The answer came when Zamore and colleagues showed that *Drosophila* embryo extracts could produce small dsRNAs [named SMALL-INTERFERING RNAS (siRNAS)] directly from the dsRNA silencing trigger [5]. In addition, the Hannon group simultaneously found these small RNAs in association with the effector complex of RNAi, RNA-induced silencing complex (RISC; see below), thus implicating them as direct participants in the silencing pathway [6].

RNaseIII family ribonucleases specifically recognize dsRNAs and were thus candidates for the activity that

Glossary

Co-suppression: A term that refers to silencing of endogenous loci following the introduction of transgenes. It can also refer to silencing of transgenic loci in a copy-number-dependent fashion.
Dicer: An RNase III family member that produces siRNAs.
PTGS: Post-transcriptional gene silencing, a term used for RNAi in plants where expression is lost without effects on mRNA transcription.
RISC: RNA-induced silencing complex. The multicomponent machine that enforces silencing.
RNA interference: RNAi. Becoming an umbrella term to describe homology-dependent gene silencing events triggered by double-stranded RNA.
Small-interfering RNAs: siRNA. The specificity component of the RNAi machinery.
TGS: Transcriptional gene silencing. As is becoming increasingly clear, dsRNA can also direct changes at the genome level. TGS refers to suppression of transcription in plants following exposure of cells to dsRNAs that are homologous to promoter regions.

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Table 1. Proteins and domains involved in RNAi and related phenomena

Protein(s) or protein family	Domains	Domain function	Refs
Dicer family	RNA helicase	RNA unwinding	
	PAZ	Putative protein–protein interaction	
	RNaseIII	Ribonuclease	
	dsRNA binding	dsRNA binding	
Argonaute family	PAZ	Putative protein–protein interaction	
	PIWI	Unknown	
RNA-dependent RNA polymerases	RdRP	RNA-dependent RNA polymerization	
RNA helicases	Putative RNA helicase	RNA unwinding	
QDE-3	DNA helicase	DNA unwinding	[52]
RDE-4	dsRNA binding	dsRNA binding	[53]
MUT-7	Rnase D	RNA degradation	[42]
Fragile X related protein (dFXR)	KH	Putative RNA binding	[24]
	RGG	Putative RNA binding	
Vasa intronic gene (VIG)	RGG	Putative RNA binding	[24]
SGS3	Coiled-coil	Protein–protein interaction	[54]
Polycomb proteins	SET (MES-4)	Histone lysine methyltransferase	[55]
	WD-40 (MES-6)	Unknown	

produced siRNAs. In *Drosophila*, Bernstein *et al.* showed that a class of RNaseIII enzymes produces siRNAs from long dsRNAs in an ATP-dependent manner [7]. These enzymes – named DICERS – are evolutionarily conserved in organisms competent for RNAi, and, in many cases, have been shown to exhibit characteristic biochemical activity. Structural information on archaebacterial RNaseIII [8] has led to a model in which Dicer functions as an anti-parallel dimer to form ~22mer siRNAs [9]. In this model, only two of four possible active sites (those on the ends) are competent for cleavage, yielding 22-nucleotide (nt) RNAs rather than the ~11-nt RNAs that could be produced by similarly arranged bacterial RNase III enzymes. A recent study on recombinant human Dicer shows that dsRNAs are cleaved preferentially at their termini and, in this study, ATP was not required for activity [10]. This raises questions about possible differences between the *in vivo* dicer complex and recombinant Dicer protein.

When siRNAs from silenced plants are analyzed, two distinct species (~21 nt and ~25 nt) are observed. Recently, it has been suggested that these two kinds of siRNA species are involved in different aspects of silencing. Based on the effects of different viral suppressors of gene silencing and RNAi mutants, the Baulcombe group propose that short siRNAs drive mRNA degradation whereas long siRNAs are involved in systemic silencing and DNA methylation (see below) [11]. Considering the number of Dicer homologs in *Arabidopsis*, and the differential effects of viral suppressors on these siRNA species, it is plausible that long and short siRNAs might be produced by different Dicer enzymes.

siRNAs are not the only products of Dicer. Dicer mutants in *C. elegans* have phenotypes that resemble those seen in *let-7* and *lin-4* mutants, both of which have developmental defects [12–14]. *let-7* and *lin-4* are non-coding small RNAs [now known generically as microRNAs (miRNAs)] that bind to 3′ untranslated regions (UTRs) of target mRNAs and regulate gene expression at the level of protein synthesis. It is important to note that the complementarity between 3′ UTR sequences and these small RNAs is not perfect. These single-stranded miRNAs are produced by post-transcriptional processing of ~70 base precursors that form stem–loop structures. Recent data suggest that, in some instances, several clustered miRNAs might be transcribed polycistronically and processed to generate the ~70 base precursor form in the nucleus [15]. Dicer then processes these ~70 bp hairpins to the mature ~21-nt form [12–14,16]. miRNAs now form a broad class of small RNAs that have been identified in *C. elegans*, *Drosophila*, plants and mammals [17–21] The ever-growing number of these developmentally regulated miRNAs suggests an important role for RNAi in endogenous gene regulation.

Dicer proteins appear to be a common denominator in both siRNA and miRNA pathways. However, a key question raised by the discovery of the link between miRNAs and the RNAi machinery, is the degree to which silencing at the level of mRNA degradation might be related to silencing via suppression of protein synthesis. Although no definitive answer has emerged, clues are beginning to emerge from the analysis of RNAi effector complexes.

RNA-induced silencing complex

At present, the only effector function of RNAi that has been amenable to *in vitro* analysis is the siRNA-directed degradation of mRNAs. This began with analyses of activities in extracts of *Drosophila* embryos and cultured S2 cells, but is now being extended to other organisms, including *C. elegans* and mammals. To date, four RISC components have been identified in *Drosophila* and, more recently, four components have been found as part of the mammalian enzyme. However, these do not completely overlap, suggesting either species-, cell type- or developmental stage-specific differences, or the presence of multiple independent effector complexes.

The first protein component to be identified as part of the RISC complex was Argonaute-2, a homolog of *C. elegans* RDE-1. RDE-1 had previously been shown to be required for RNAi in *C. elegans*, and belongs to the Argonaute family of proteins [22]. Argonaute proteins are highly basic ~100 kDa proteins that have been linked to RNAi through mutant screens in plants, *Neurospora* and *C. elegans*.

Argonaute proteins are comprehensively reviewed in [23]. There are five Argonaute proteins in *Drosophila*: dAgo1, dAgo2, Piwi, Aubergine and a predicted protein,

dAgo3. The first four of these proteins have been implicated in RNAi or in potentially related silencing phenomena. Several Argonaute mutants also have developmental defects. *Drosophila piwi* and *aubergine/sting* exhibit developmental phenotypes manifested in the gonads, and dAgo1 appears to be essential for viability and normal development of the nervous system. *Arabidopsis ago1* and *zwille* are associated with defects in the shoot apical meristem and exhibit severe phenotypes affecting general plant structure. In *C. elegans*, two Argonaute homologs, *alg-1* and *alg-2*, exhibit developmental phenotypes in the gonad also. Interestingly, this phenotype is consistent with their role in the metabolism of *let-7* and *lin-4* miRNAs. How intertwined these developmental and RNAi-related functions are remains to be determined. The answer might lie in further examination of the components of discrete Argonaute-containing RISC complexes, and the identification of their preferential substrates.

Various Argonaute proteins have been isolated in RISC complexes that exhibit a tendency towards either siRNA or miRNA substrates. For example, dAgo1 and dAgo2 are present in separate complexes that preferentially contain siRNAs or miRNAs, respectively [24], and mammalian dAgo1 homolog eIF2C2/hAgo2 resides in a complex associated with miRNAs [21]. Table 2 summarizes the set of component proteins and interactions revealed to date.

The *Drosophila* homolog (dFXR) of the human fragile X mental retardation protein (FMRP) is another component of the RISC complex. Interestingly, human FMRP has long been known to be a component of ribonucleoprotein complexes, and is believed to regulate a set of genes by affecting protein synthesis. Solidifying the validity of dFXR as a *bona fide* RISC component, miRNAs, siRNAs and nuclease activity co-immunoprecipitate with dFXR. In addition, transfection of cells with dsRNA against dFXR impairs their RNAi response [24,25]. dAgo2 associates with dFXR in an RNaseA-resistant manner, suggesting a direct interaction between the two rather than one mediated by mutual binding to target mRNAs [25]. The precise role that dFXR plays in RNAi, and the role that RNAi might play in fragile X syndrome, remains to be seen.

Recently, purification from mammalian cells yielded a RISC-like complex containing an Argonaute family member and two novel components whose involvement has not yet been demonstrated in other systems. In a study aiming to identify gemin3- [a DEAD-box helicase associated with the survival of motor neurons (SMN) complex] associated proteins, Mourelatos and colleagues identified the human Argonaute member, eIF2C2/hAgo2, as an interacting protein. Using immunoprecipitation and partial purification, they showed that gemin3, eIF2C2/hAgo2, gemin4 (a protein with no known motifs) and miRNAs are present in a 15S ribonucleoprotein complex. Like dAgo2, mammalian eIF2C2/hAgo2 is also present in complexes with siRNAs [26], and eIF2C–Gemin complexes are competent for miRNA-directed cleavage of homologous substrates, suggesting that this is a mammalian RISC [27].

In summary, RISC-like complexes are involved in pathways that enforce both siRNA- and miRNA-mediated silencing. Perhaps the most interesting property of these complexes is an apparent flexibility that enables their involvement in these seemingly disparate processes. siRNAs are thought to function through mRNA degradation (Fig. 1). Although miRNAs can function at the level of protein synthesis, a recent finding suggests that miRNAs can also direct mRNA cleavage [20]. One hypothesis is that small RNAs with perfect homology can induce mRNA degradation or mRNA cleavage, and imperfectly paired small RNAs act mainly at the level of protein synthesis. Thus far, the nuclease involved in these complexes remains the most elusive component; however, it is equally unclear as to how miRNA-containing complexes mediate translational suppression.

Table 2. Dicer- and Argonaute-associated proteins

Dicer/Argonaute family members	Organism	Interacting protein(s)	Refs
DCR-1	*C. elegans*	RDE-4	[53]
DCR-1	*C. elegans*	RDE-1	[53]
Dicer	*Drosophila*	dAgo1	[24]
Dicer	*Drosophila*	dAgo2	[24]
Dicer	*Drosophila*	dFXR	[25]
dAgo2	*Drosophila*	dFXR, VIG	[24]
dAgo2	*Drosophila*	dFXR, Dmp68	[25]
eIF2C2/hAgo2	Mammalian	Gemin3, Gemin4	[21]
eIF2C2/hAgo2	Mammalian	eIF2C1/hAgo1	[26]

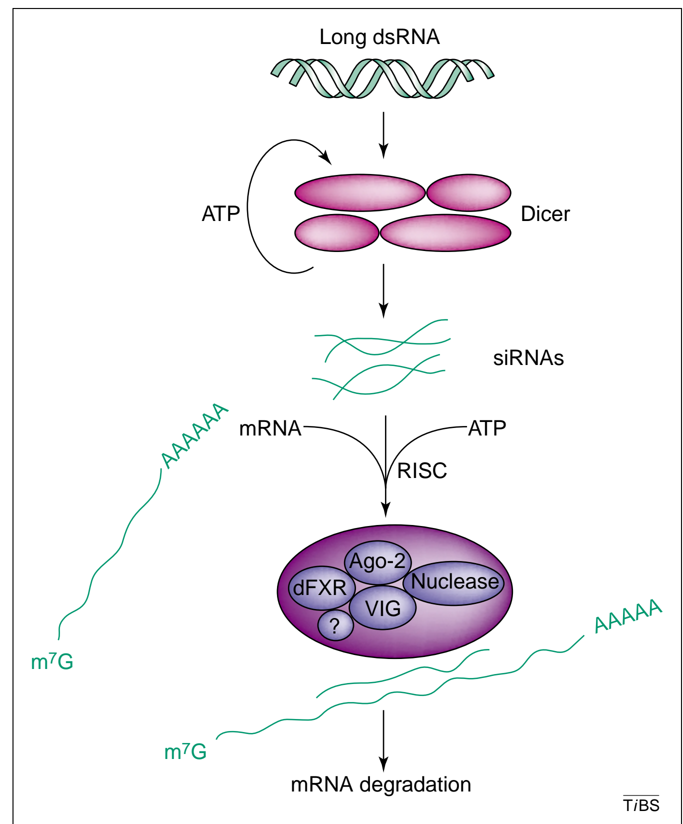

Fig. 1. Present model for the mRNA degradation pathway of RNAi. Anti-parallel Dicer dimers cleave long dsRNAs to form small-interfering RNAs (siRNAs) in an ATP-dependent manner. Recent data from studies on recombinant human Dicer suggest that ATP might be required for release of dsRNA after cleavage, and that mRNAs are cut preferentially at their termini [10]. siRNAs are incorporated in the RNA-induced silencing complex (RISC) and ATP-dependent unwinding of siRNAs activates RISC [56]. Active RISC is thus guided to degrade the specific target mRNAs.

Amplification, transitive RNAi and systemic silencing

That very small amounts of dsRNA per cell caused silencing with nearly 100% penetrance was one of the most striking observations made during early studies of RNAi in *C. elegans* [3]. This raised the possibility that some aspect of the silencing signal might be amplified. Genetic studies in plants and *C. elegans* strongly implicate the RNA-dependent RNA polymerases (RdRPs) as key elements of such an amplification loop.

sgs2/sde1 mutant plants are defective in the suppression of transgenes [28]. The *SGS2/SDE1* locus encodes a putative RNA-dependent RNA polymerase, which might function either to convert aberrant RNAs into the dsRNA silencing trigger, or to amplify silencing initiated by very small amounts of dsRNA that could originate from the transgene locus. Similarly, in *Neurospora*, co-suppression is not active in mutants with lesions in its RdRP homolog, *qde-1* [29]. By contrast, virus-induced gene silencing is unaffected in *sgs2/sde1* mutants [28]. Viruses encode their own RdRPs that are essential for producing dsRNA during replication, and thus targeting of viral mRNAs might not be dependent on the function of a host RdRP.

Putative RdRPs have also been implicated in conventional RNAi, in which the dsRNA is supplied exogenously, raising the possibility that RdRPs might participate in more than just the generation of the dsRNA silencing trigger. For example, in *C. elegans* EGO-1 is required for silencing in the germ-line [30], whereas RRF-1 is necessary for silencing in soma [31].

When a restricted portion of a particular mRNA is targeted by RNAi, siRNAs corresponding to other portions of the mRNA can also be detected. This phenomenon is called transitive RNAi and has been observed in *C. elegans* and plants. In *C. elegans*, these secondary siRNAs are homologous to sequences upstream of those targeted by the primary trigger. This $5' \rightarrow 3'$ directionality (with respect to the antisense strand) is in agreement with a model where secondary siRNAs are produced following a polymerization event [31]. Similarly, secondary siRNAs can also be observed in plants, but with a twist. In plants, spread is in both the $5' \rightarrow 3'$ and $3' \rightarrow 5'$ directions. This spread requires a putative RdRP SGS2/SDE1 and transcription of the transgene [32]. However, the precise mechanisms by which bi-directional spread is achieved remains to be identified.

Evidence for possible involvement of an RdRP activity in *Drosophila* has also been reported by Lipardi and colleagues. In their model, siRNAs act as primers for secondary dsRNA synthesis, and the majority of RNAi-mediated degradation is thought to be accomplished by Dicer [33]. However, Zamore's group has shown that replacing 3′ OH of siRNAs with groups that would not support RNA polymerization has no adverse effect on RNAi [34]. In addition, available (albeit incomplete) human and *Drosophila* genome sequences lack a recognizable RdRP homolog, and there is a lack of evidence for transitive RNAi in mammals and flies. These observations suggest that trigger amplification and transitive silencing mechanisms might be restricted to only a subset of organisms that are competent for dsRNA-mediated silencing.

Natural origins of dsRNA

The existence of a potent gene silencing mechanism triggered by dsRNA leads immediately to the question of the biological role for such a response. In many organisms, a major source of dsRNA is RNA viruses. RNA viruses form dsRNA intermediates upon replication that might trigger the RNAi response. Several experiments have confirmed a role for RNAi in antiviral defense (reviewed in [35]). In plants, RNAi targets viral mRNAs for degradation [36]. However, many plant viruses have evolved mechanisms to counteract this host response. Viral suppressors of gene silencing (VSGSs) are essential virulence determinants, which become dispensable when RNAi-related pathways in plants are compromised by mutation (reviewed in [37]). It is important to note that VSGSs are not restricted to plant viruses. One RNA virus that infects *Drosophila* cells – flock house virus (FHV) – also encodes a potential suppressor of silencing that is required for replication. FHV with mutations in b2, a protein that shares genomic organization but not sequence similarity with a plant viral inhibitor of silencing (cucumovirus 2B), can be rescued by suppression of the RNAi pathway in animal cells. Interestingly, FHV b2 also suppresses RNA-induced silencing mechanism in plants, highlighting the conservation of RNAi pathways across kingdoms [38].

What are other targets of RNAi? One emerging theme is that the RNAi machinery provides a conserved mechanism for responding to nucleic acid invaders, in some ways serving as a nucleic acid immune system. Perhaps some of the first indications of this role were experiments from Jorgensen's group, which were designed to overexpress pigmentation genes to enhance flower color in petunia plants. To their surprise, introducing transgenes downstream of a strong promoter caused formation of pigment-free patches in the transformed plants in which not only transgene, but also the endogenous locus was silenced [39,40]. Numerous experiments have suggested that silencing of the pigment gene occurs mainly at the post-transcriptional level. The majority of integrated transgenes form complex arrays. When these transgenic loci are analyzed, a striking percentage contains inverted repeats (IRs) that can form hairpin RNAs upon read-through transcription. Even though IRs appear to be the major inducers of silencing, there are many cases where a single-copy transgene can also induce silencing (reviewed in [41]). These observations led to the hypothesis that so-called aberrant RNAs form especially during highly active transcription, perhaps owing, in part, to premature termination. There might also be specialized proteins that recognize such RNAs and funnel them into the RNAi pathway.

Besides exogenous nucleic acids, there are numerous endogenous parasitic elements that must be kept largely in check to maintain genome stability. In some cases, control of these mobile genetic elements has been linked to the RNAi machinery. Particularly in *C. elegans*, some mutants that affect RNAi affect transposon silencing as well. These mutators have increased endogenous transposon activity [22,42]. However, the precise mechanism by which RNAi controls the activity of transposons is not yet clear.

One emerging theme is that RNAi responds not only to exogenous nucleic acids but also to endogenous DNA

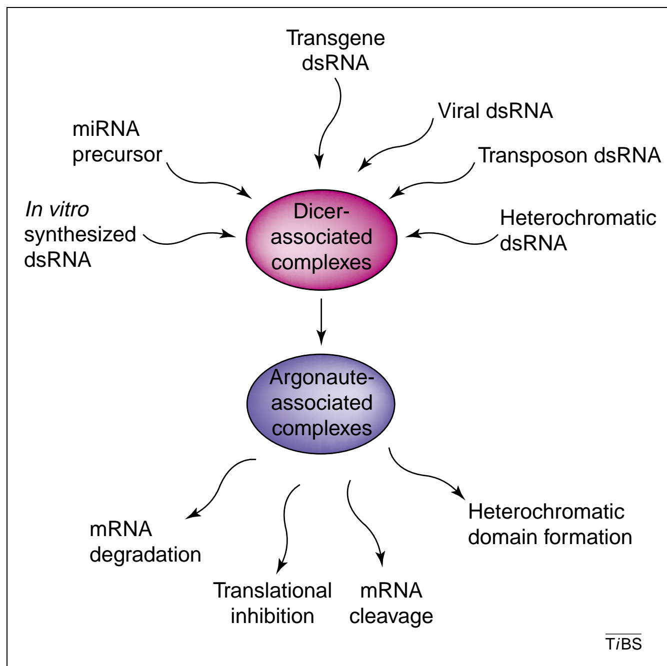

Fig. 2. Multiple inputs to the RNAi machinery can lead to distinct types of silencing events. dsRNA from a variety of sources gets processed by Dicer-associated complexes to guide Argonaute-associated complexes for downstream processes. Dicer and Argonaute family proteins are common denominators in RNA-induced silencing pathways. Genetic studies (reviewed in [57]) suggest that different proteins might be required for silencing by distinct triggers. In addition, biochemical studies suggest correlation of separate Argonaute-associated complexes with different silencing processes (e.g. mRNA degradation versus translational inhibition). One model is that differential interactions of Dicer with trigger-specific proteins trigger the utilization of distinct Argonaute-associated complexes, thus resulting in different outcomes. However, most of the biochemical evidence, to date, suggests that silencing at the level of protein expression and RNA degradation are accomplished by very closely related, if not identical, complexes.

parasites (see Fig. 2). However, recent work has pointed to an even more central role for RNAi in genome maintenance.

dsRNA and heterochromatin

The initial observation of dsRNA-induced chromatin change was methylation of endogenous sequences that shared homology with viroids in infected plants [43]. This observation was later followed by the finding that dsRNA sharing sequence homology with promoter regions was able to induce gene silencing. In this case, silencing occurred at the transcriptional gene silencing (TGS) level and correlated with *de novo* methylation of promoter sequences [44]. Unlike the response of PTGS to dsRNA in plants, TGS is heritable [45], and evidence is only now beginning to accumulate suggesting that TGS and PTGS are biochemically related silencing pathways.

Polycomb proteins are chromodomain proteins that transcriptionally repress genes by favoring the formation of closed chromatin structures. These proteins play a role in RNAi in *C. elegans* and *Drosophila*. (reviewed in [9]). Furthermore, Piwi is required for co-suppression at both the transcriptional and post-transcriptional levels in *Drosophila* [46]. These observations established a link between RNAi and chromatin remodeling, and extended the effect of dsRNA on the genome beyond plant systems.

A more direct link was revealed by a recent study in fission yeast. Volpe and colleagues showed that RNAi proteins, namely Dicer, Argonaute and an RNA-directed RNA polymerase homolog, are required for centromeric silencing [47]. Furthermore, RdRP interacts directly with centromeric repeats [47], and small RNAs complementary to these repeats have been identified [48]. These data strongly implicate the RNAi machinery in the formation of heterochromatin and in the maintenance of genome stability, at least in *S. pombe* [47].

RNAi machinery and repetitive elements in the centromeric region are required for establishment of heterochromatin domains in *S. pombe*, whereas the RNAi machinery is dispensable for its maintenance and inheritance [49]. This is consistent with a model in which heterochromatic domains are formed initially through the RNAi machinery guided by small RNAs. However, once formed, the domains can self-perpetuate through multiple replication cycles.

Tetrahymena is yet another organism in which RNAi-related proteins have been linked both to heterochromatin formation and to the metabolism of repetitive elements in the genome. In *Tetrahymena*, development of the somatic macronucleus from germ micronucleus involves chromosome rearrangements that lead to elimination of certain sequences. Among these sequences are repetitive elements that show characteristics of transposons. This DNA elimination requires an Argonaute homolog, TWI1, and two chromodomain proteins, PDD1 and PDD3. In addition, a hallmark of heterochromatin, H3K9 methylation, is spatially and temporally limited to the eliminated DNA sequences. Remarkably, targeting of regions for elimination correlates with the presence of small ~28-nt RNAs that are homologous to the regions that are lost [50,51].

A central mystery raised by the aforementioned observations is how variants of the RNAi machinery can use small RNA guides to effectively scan either RNA or DNA. Furthermore, how do the effector complexes enforce silencing through changes in RNA metabolism (e.g. mRNA degradation or translational repression) or DNA metabolism (e.g. directing chromatin remodeling)? Answers to these questions might only come as we begin to explore RISC-related complexes from a more diverse group of model systems.

Concluding remarks

Through a combination of genetic and biochemical studies, our rough picture of RNAi and related silencing pathways is coming increasingly into better focus. Studies that began with a response to exogenous dsRNAs have provided insight into a surprising variety of cellular phenomena. Despite an expanding list of players identified by both biochemistry and genetics, we still have very little idea of the precise roles played by any of these components, and only the smallest notion of the true breadth of biological functions for these pathways. Nevertheless, one thing seems certain: we will not have to wait long for the next surprising twist in this increasingly complicated plot.

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