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Conserved themes in small-RNA-mediated transposon control

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Abstract

Eukaryotes are engaged in a constant struggle against transposable elements, which have invaded and profoundly shaped their genomes. Over the past decade, a growing body of evidence has pointed to a role for small RNAs in transposon defense. Although the strategies used in different organisms vary in their details, they have strikingly similar general properties. Basically, all mechanisms consist of three components. First, transposon detection prompts the production of small RNAs, which are Piwi-interacting RNAs in some organisms and small interfering RNAs in others. Second, the population of small RNAs targeting active transposons is amplified through an RNA-dependent RNA polymerase-based or Slicer-based mechanism. Third, small RNAs are incorporated into Argonaute- or Piwi-containing effector complexes, which target transposon transcripts for post-transcriptional silencing and/or target transposon DNA for repressive chromatin modification and DNA methylation. These properties produce robust systems that limit the catastrophic consequences of transposon mobilization, which can result in the accumulation of deleterious mutations, changes in gene expression patterns, and conditions such as gonadal hypotrophy and sterility.

Introduction

Transposons are nucleic acid parasites that are capable of both movement and propagation within host genomes [1]. They are found in all phyla but vary, in a species-specific fashion, in character, abundance and activity. In *Saccharomyces cerevisiae*, transposons constitute only 3% of the genome [2], whereas in humans and maize they represent up to 50% and 80%, respectively [3,4].

Eukaryotic transposons can be divided into several classes, according to their strategy for movement. RNA transposons or retrotransposons constitute class 1. These can be further divided into two subtypes: long terminal repeat (LTR) and non-LTR retrotransposons. LTR-retrotransposons are closely related to retroviruses and similarly contain long (several hundred nucleotide) terminal direct repeats (i.e. LTRs) at their 5′ and 3′ ends. The remaining elements lack such terminal repeats and are therefore called non-LTR retro-elements. Both subtypes harbor an internal promoter in their 5′ untranslated region (UTR), and a polyadenylation signal and sometimes a polyA tail in their 3′ UTR. Class 1 elements move via RNA intermediates, which must be converted to DNA copies by reverse transcriptases before integration into the host genome. DNA transposons comprise class 2. Integrated copies of class 2 elements move directly, through the action of transposases. These enzymes recognize the terminal inverted repeat (TIR) and catalyze both the excision of the element from one location in the genome and its insertion elsewhere. Helitrons, comprising a third, less studied class of transposons, replicate through a rolling circle intermediate (see

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Glossary) [5]. These will not be discussed further in this review. Class 1 and class 2 transposons both contain autonomous elements, and their mobility relies on the proteins they encode. However, there are also numerous non-autonomous elements, which can transpose only in the presence of the proteins encoded by autonomous, active elements of the same class.

The complexity of each transposon class varies greatly among organisms. For example, *S. cerevisiae* has only Ty (transposon yeast) LTR retro-elements. In mammals, DNA transposons are mainly vestigial, whereas non-LTR retrotransposons constitute by far the most abundant class. Plants possess a wide variety of DNA and RNA transposons, with LTR retrotransposons being the most common [6].

Owing to their high copy numbers and their ability to move around the genome, active transposons have the potential to be highly disruptive to their host [7,8]. The integration of a transposon in or near a gene can disrupt its coding sequences or perturb its expression pattern [9]. Additionally, the failure to repair DNA breaks associated with transposon excision or integration can lead to chromo-some rearrangements. Lastly, if they are not masked in heterochromatin, repetitive sequences such as transposons can provide sites for non-homologous recombination during meiosis. Despite their disruptive potential, the damage that transposons cause to their host is generally minor. For instance, only 1 in 600 [10] germline mutations in humans can be attributed to transposon insertions. In fact, the damage caused by transposons is largely limited by active repression of these endogenous parasites. Most transposon copies reside in heterochromatin, which by definition contains regions of silent DNA [11,12]. However, the mechanisms by which transposons are selected for packaging into heterochromatin have remained elusive. Transposons are extremely diverse in their sequence character and movement strategies. This raises the fundamental question of how a host cell distinguishes these elements from protein-coding genes and targets them for selective silencing. Recent work points to mechanisms related to RNA interference as key mediators of transposon suppression (see Box 1). The goal of this review is to examine transposon silencing mechanisms that are driven by small RNAs, probing both similarities and differences in silencing strategies among eukaryotes. We divide the challenge of transposon silencing into three parts: detection, amplification and repression; and we discuss the prevailing models for each aspect of the response (Figure 1). We also discuss these models in the broader context of repeat silencing, because other types of repeats present similar challenges to the organism as regards their detection and sequestration into heterochromatin. Data suggest that related mechanisms regulate the silencing of both genomic repeats and transposons.

Box 1. The siRNA pathway

The first RNA silencing process to be biochemically characterized was the cleavage of perfectly complementary targets by siRNAs (Figure I). The process is initiated with the detection of dsRNA by the RNAi machinery. This dsRNA is processed by a Dicer enzyme into 21-nucleotide siRNA duplexes, which are then incorporated into an Argonaute protein. Following incorporation, one strand (referred to as the passenger strand) is cleaved by the catalytic site of the Argonaute protein and degraded. Perfect pairing between the remaining strand (guide strand) and an RNA molecule in turn triggers Slicer cleavage of the targeted RNA.

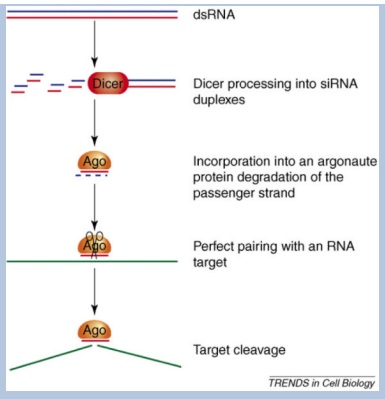

Figure I.
The siRNA pathway.

Transposon detection

There is a vast diversity in the structure of transposons and their movement strategies. As such, detecting these sequences and distinguishing them from its own genes poses significant challenges for the host; indeed, it is a struggle to imagine what set of features might tag transposons as targets for silencing. Insights into small RNA regulatory pathways are beginning to yield at least some answers, revealing a series of elegant, small RNA-based transposon defense systems in plants, fungi and animals.

Double-stranded RNA and aberrant transcripts in plants and yeast

Nucleic acid pathogens such as viruses and viroids produce double-stranded RNA (dsRNA) directly during their replicative cycles. Introduction of plant viroids into hosts carrying homologous sequences within their genomes caused these genomic sequences to be silenced [13]. Such sites became fully methylated in concert with viroid replication [13], and the secondary structure of viroid replication intermediates was proposed as a key methylation trigger. RNA viruses, which also have cytoplasmic dsRNA replication intermediates, elicited the same effect [14–16], as did the introduction of an inverted repeat construct corresponding to the nopaline synthase (NOS) promoter [17,18] in tobacco and *Arabidopsis thaliana*.

These observations sparked the idea that nucleic acid pathogens (viruses, viroids) and parasites (transposons) distinguish themselves by producing dsRNA, which might be detected by a specialized machinery to trigger silencing. Unlike viruses, which produce dsRNA as an obligate replication intermediate, it was not immediately clear how precisely transposon-derived dsRNAs might arise, although several possibilities could be envisioned. First, through integration in cis, transposons tend to form tandem direct and inverted repeats (DRs and IRs, respectively). Read-through transcription (see Glossary) from a single promoter of inverted repeat-containing transposon arrays would produce dsRNA. Second, many transposons have been shown to possess cryptic antisense promoters (see Glossary), enabling dsRNA production through bi-directional transcription [19–21]. DNA sequences neighboring insertion sites could also act as antisense promoters. In *Schizosaccharomyces pombe*, centromeric repeats serve as an example of silencing triggered by dsRNA production through bi-directional transcription [22]. Additionally, even short, structured RNA hairpins such as the class 2 transposon TIRs could be sufficient for recognition by the silencing machinery upon transcription from a single promoter [23]. An example of such TIR-triggered silencing is provided by Tc1 in Caenorhabditis elegans [24].

Box 2. The Argonaute protein family

The Argonaute proteins can be divided into several clades with so-called Piwi and Argonaute family members binding distinct small RNA partners and carrying out distinct functions. For space reasons, only the proteins mentioned in the text, as well as representative Argonautes binding the other classes of small RNAs, are included in Figure I.

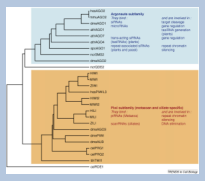

Figure I.
The Argonaute protein family. Abbreviations: ath, Arabidopsis thaliana; cel, Caenorhabditis elegans; dme, Drosophila melanogaster; hsa, Homo sapiens; mmu, Mus musculus; ncr, Neurospora crassa; spo, Schizosaccharomyces pombe; tth, Tetrahymena thermophila.

In accordance with a potential role for dsRNA in transposon detection, Dicer proteins (see Glossary) [25], which are core components of the RNA interference (RNAi) machinery, have proven to be important for transposon detection and silencing in numerous settings (see Box 1, Figure I). In A. *thaliana*, Dicer-like 3 (DCL3) generates 24-nucleotide small RNAs that act in transposon control [26,27], whereas in S. *pombe*, Dicer1 (Dcr1) processes double-stranded centromeric transcripts into 21-nucleotide small interfering RNAs (siRNAs; see Glossary) that direct packaging of these repeats into heterochromatin [22,28,29].

The production of small RNAs from repeat-derived double-stranded RNAs (dsRNAs) is an appealing explanation both for how repetitive sequences are detected and for how silencing mechanisms are activated in plants. However, this model is almost certainly overly simplistic. Transgenes, which have no obvious potential to give rise to dsRNA, can also be silenced in a way that depends upon both their levels of expression [30,31] and their genomic copy number [32,33], although even single-copy insertions are sometimes silenced [34]. Integration next to an antisense promoter could lead to dsRNA production (as described above); however, no clear explanation has yet been given for the silencing of such genes [31–33].

Moreover, the mere production of dsRNA does not seem to be sufficient to trigger transcriptional silencing in plants or fungi. In fission yeast, small RNAs and transcriptional silencing are lost in mutants for the histone methyltransferase, Clr4 [35,36]. Similarly, in Arabidopsis, mutants of either the SWI-SNF homolog Ddm1 or the DNA methyltransferase Met1 are unable to produce detectable amounts of small RNAs against most transposons [37]. Interestingly, even after backcrossing the mutant plants to a wild-type background, these transposons remain active [38,39] despite the presence of functional DDM1 and MET1 proteins. Thus, small RNA-based transposon silencing pathways require the core RNAi machinery but also additional features that distinguish this system from responses mounted against exogenous parasites such as viruses.

Master control loci in animals

In animals, recent studies have increasingly implicated a particular class of small RNAs in transposon detection and control [40–45]. The Piwi-interacting RNAs (piRNAs; see Glossary) were named because of their association with the effectors of metazoan

transposon silencing, the Piwi clade of Argonaute proteins (see Box 2 and Glossary) [46–49]. Unlike the small RNAs that target transposons in plants and repeats in *S. pombe*, piRNAs do not depend upon Dicer [41,44] and do not seem to derive from dsRNA precursors [50] (Figure 1). Instead they seem to arise from single-stranded RNAs that are transcribed from piRNA clusters as a contiguous silencing program that is parsed by processing into individual piRNAs [43,45–47,49,50]. The second but less prominent characteristic is a marked strand asymmetry of the small RNAs mapping to any given cluster, in some cases reflecting the preferential production or stabilization of piRNAs antisense to transposons.

Genetic studies of *Drosophila* have supported a crucial role for at least two piRNA loci as master regulators of transposon silencing. The first corresponds to the *flamenco* locus [43], which was long known to be required for the repression of several transposon families such as Gypsy, Idéfix and ZAM [51–53]. *flamenco* piRNAs associate almost exclusively with one Piwi family member, Piwi itself, and, consistent with their sharing a common function, *flamenco* and *piwi* mutants show overlapping phenotypes, with both showing severe defects in ovary development [54,55]. The other genetically characterized piRNA locus is *X-TAS*, which is located in the pericentromeric region of the X chromosome. X-TAS is involved in the repression of *P* elements, for which it requires another Piwi family member, Aubergine [56–59]. Like *flamenco*, X-TAS controls *P* elements throughout the genome. Evidence for its action in *trans* is best exemplified by an X-TAS variant containing a LacZ-marked *P* element insertion, which can enforce repression of unlinked euchromatic LacZ transgenes [60,61].

Thus, analysis of *Drosophila* piRNAs suggests that metazoan genomes detect transposons at least in part by using piRNA clusters as transposon traps. This model would begin with a non-coding RNA gene that gives rise to piRNAs. The basic properties of transposons — their ability to jump and their presence in high numbers — are such that there is a non-negligible probability that a transposon will jump into such a gene, particularly if, as a whole, piRNA loci occupy substantial genomic space. Instead of transposon insertion into that locus being an inactivating event, as it might for a protein coding gene, insertion would enhance the function of the piRNA locus by conferring the ability to suppress euchromatic, potentially active copies of that same transposon. The benefits of the insertion would promote fixation of the `mutation' within the population.

If piRNA loci represent transposition hotspots, this could enhance the effectiveness of this scheme. Indeed, this is precisely what is observed for X-TAS, which is a preferential target for *P* element insertion [62]. In this way, piRNA loci become an evolutionary record of transposon invasion, which simultaneously provides a genetic reservoir of transposon resistance. The simple model in which insertion into a transposon trap leads to generation of repressive, antisense piRNAs might be true in some cases. However, the system is both more complex and more elegant, as will be discussed below.

Given that the presence of transposon remnants in piRNA loci is likely to be positively selected for, appearance of piRNA clusters as transposon graveyards is a signature of their role in transposon control. However, in mammals, there are distinct populations of piRNAs. piRNAs that initiate expression in the pachytene stage of meiosis are depleted of transposons, and pachytene piRNA clusters are very repeat-poor [46–50]. Thus, they do not seem to act in transposon silencing, leaving their real function obscure for the moment. However, another, less abundant population of mouse piRNAs is expressed in pre-pachytene testes [45]. They have a much higher transposon content and their generative clusters resemble fly piRNA clusters, with many antisense transposon copies. Therefore, in

mammals, the members of only a subset of piRNA clusters seem to act as transposon master control loci.

Other mechanisms of transposon detection

Plants and fission yeast on one hand, and animals on the other, use very different strategies to detect transposons and to generate small RNAs against them. Although plants and fission yeast take advantage of the tendency for transposons to generate dsRNA, animals exploit two different properties of transposons: their high copy number in the genome and their ability to jump. Additional strategies have also evolved to detect and control transposons, including alternative small RNA-based pathways.

Tetrahymena prevents transposon expression by expelling transposon DNA through a process called programmed DNA elimination [63]. *Tetrahymena* possesses two nuclei: a germline, transcriptionally silent micronucleus, and a somatic, transcriptionally active macronucleus. During conjugation, the post-meiotic haploid micronuclei fuse and give rise to a new micronucleus and a new macronucleus. The old macronucleus is then destroyed. Before this happens, however, the patterns of DNA elimination in the old macronucleus are reproduced in the new macronucleus, using a conjugation-specific class of ~28-nucleotide small RNAs called scanRNAs. Although the precise biochemical mechanisms by which this genome comparison occurs are unknown, a simplified model provides a scaffold for future studies. First, the whole genome of the old micronucleus is thought to be transcribed into RNA precursors, which are then processed into scanRNAs by Dicer-like 1 (Dcl1p). The scanRNAs are moved to the old macronucleus, where any small RNA that matches the genome is degraded. The remaining scanRNAs then carry the identity of the sequences to be eliminated to the new macronucleus, where repeat excision is directed. Given that eliminated sequences are strongly enriched for transposons and repeats, this might be considered the ultimate form of small RNA-induced transposon silencing.

Neurospora crassa uses three distinct mechanisms to detect and fight transposons: quelling, repeat-induced point mutation (RIP; see Glossary), and meiotic silencing of unpaired DNA (MSUD; see Glossary) [64]. Although it is not yet clear how repeats are specifically targeted by quelling and RIP, MSUD is known to arise from the detection, during meiotic homologous chromosome pairing, of unpaired DNA sequences. Active transcription of these unpaired sequences during meiosis is required for MSUD to occur. Therefore, transposons that have just integrated into a new region of the genome are very likely to be the primary target of MSUD. Although the small RNAs associated with MSUD have not been characterized, it is likely that an RNAi-related mechanism is responsible for this process, because it requires an Argonaute protein (suppressor of meiotic silencing-2, Sms2), a Dicer enzyme (Sms3) and an RNA-dependent RNA polymerase (suppressor of ascus dominance-1, Sad1). There is also evidence of an RNAi-mediated MSUD mechanism in *C. elegans* and in mammals [65–69].

Mounting a defense

After detecting transposons and generating a primary population of either siRNAs through Dicer processing or piRNAs through the transcription of master control loci, most eukaryotic organisms find a way of amplifying the response and producing abundant secondary small RNAs selectively enriched for species that are antisense to their target elements. These can induce silencing through several mechanisms, including transcriptional (DNA and histone methylation) and post-transcriptional (RNA degradation) modes, the details of which will be discussed in the latter half of this review. To date, two strategies, one involving RNA-dependent RNA polymerases (RDRPs; see Glossary) and one involving

RISC (RNA-induced silencing complex) Slicer activity (see Glossary) have been linked to this amplification process (Figure 1).

RDRP-mediated amplification

In many organisms, an RDRP is required for repeat silencing. In *Arabidopsis*, *rdr2* mutants lose 24-nucleotide small RNAs and have reduced levels of CNG and asymmetric DNA methylation on some transposons, as well as having elevated transposon expression [26]. In *C. elegans*, the RDRP Ego-1 is required for MSUD, because *ego1* mutants lose histone H3 lysine 9 (H3K9) dimethylation on unpaired DNA [66,70].In *Neurospora crassa*, two RDRP genes, *Qde-1* and *Sad-1*, are required for quelling and MSUD, respectively [64].

The best mechanistic understanding of RDRP action in repeat silencing comes from *S. pombe*. Rdp1p is crucial for the production of siRNAs [36,71,72], for the recruitment of the Ago1-containing effector complex, RITS (RNA-induced transcriptional silencing, see below and Glossary), and the heterochromatic silencing of centromeres [36,73,74]. Rdp1p belongs to a protein complex called RDRC (RdRP complex; see Glossary), which also includes the RNA helicase Hirr1 and the PolyA polymerase Cid12 [36]. RDRC interacts directly with Dicer [75] and RITS [36], with this recruitment depending on Dcr1, Clr4 [36] and the slicing activity of Ago1 [76]. These results indicate that amplification, processing and effector mechanisms are tightly coupled in this context.

A model for RDRP-mediated amplification of small-RNA populations can be inferred from the available data on silencing centromeric repeats in *S. pombe*. In this model, a repeat-derived transcript is first recognized and cleaved by an Argonaute-primary siRNA complex. The resulting cleavage product, perhaps because of its lack of a polyA tail and associated proteins, becomes a substrate for RDRP, resulting in the production of dsRNA. This dsRNA can then be processed by Dcr1 to produce a population of secondary siRNAs. Of course, this model does not address primary siRNA production, with these initiating species perhaps being generated in a Dcr1-dependent fashion from convergent transcription of the centromeric repeats, as described in the section on detection of transposons.

RISC Slicer-mediated amplification of small RNAs

RDRP genes have no clear homologs in *Drosophila* or mammals. However, recent studies suggest an alternative pathway by which expression of transposons can amplify and shape small RNA populations, increasing the effectiveness of small RNA-directed transposon silencing pathways (Figure 1).

This cycle begins with the generation of piRNAs from their source loci (as described above), with cleavage events directed by primary piRNAs to create new, secondary piRNAs [43,77]. Essentially, the first step occurs when antisense primary piRNAs recognize and cleave target transcripts from active transposons. This cleavage event generates the 5′ end of a new piRNA, which is preferentially loaded into Ago3 (with coincident 3′ end maturation through an unknown pathway). Thus, Ago3 becomes abundantly populated with piRNAs that are sense-oriented (as defined by transcripts from transposons themselves) and that correspond to active transposon classes. These sense-oriented piRNAs in Ago3 can then direct cleavage of RNAs that contain antisense transposon sequences, most probably transcripts derived from piRNA clusters. This cleavage event again generates the 5′ end of a new piRNA (loaded into Aubergine or Piwi), which in this case is antisense to the transposon. Successive rounds of this cycle have the effect of increasing the abundance of antisense small RNAs that target active elements through a feed-forward loop.

This cycle, called the ping-pong cycle, creates a set of distinguishing characteristics, including a prevalent 10-nucleotide offset between the 5′ ends of sense and antisense

piRNAs, and a preference for an A at position 10 of sense piRNAs, which mirrors the U bias at antisense piRNA 5′ ends. These same signature features are seen in piRNA populations in zebrafish and mammals, suggesting conservation of this amplification strategy [44,45].

Overall, the Slicer-mediated ping-pong mechanism achieves the same goal of specific repeat-derived small RNA amplification as the RDRP-mediated process. However, unlike RDRP, the ping-pong model does not lead to transitive RNAi (see Glossary), but rather to conservative amplification of functional primary piRNA sequences.

Mechanisms of transposon repression by small RNAs

Throughout eukaryotes, transposon repression by small RNAs relies on Argonaute proteins. Their specificity is conferred by bound small RNAs, which identify silencing targets through conventional Watson–Crick base-pairing. The mechanisms by which Argonaute-small RNA complexes actually repress transposon expression and activity are varied. They include small RNA-directed mRNA cleavage, which will not be discussed in detail here, transcriptional silencing through DNA and histone methylation, and even, as we have mentioned, excision of transposon sequences from the genome. In most cases, such as transposon repression in *Drosophila*, small RNAs can direct silencing of all complementary sequences in the genome (*trans*-silencing), whereas in a few cases (e.g. centromeric silencing in *S. pombe*) repression is restricted to elements neighboring the small RNA-producing locus (*cis*-silencing).

Argonaute proteins mediate transposon silencing

In plants, Argonaute4 (AGO4) binds to 24-nucleotide small RNAs and is required for non-CG methylation of several classes of transposons [27], as well as for *de novo* methylation of the repeat-containing FWA (flowering Wageningen) locus [78]. *S. pombe* has only one Argonaute protein (Ago1) that is required for the formation of centromeric heterochromatin [22] and proper chromosome segregation [79,80]. In *Neurospora crassa*, two Argonaute genes, *Qde-2* (Quelling-deficient-2) and *Sms-2* (suppressor of meiotic silencing 2) are essential for quelling and MSUD, respectively [64].

In *Tetrahymena*, flies and vertebrates, Argonautes are also essential for transposon silencing, although in these organisms, members of a separate clade, the Piwis, are specialized for this task [81]. *Drosophila melanogaster* encodes three Piwi proteins: Piwi, Aub and Ago3; and Piwi and Aub have been genetically linked to transposon silencing [41,54,82–85]. Although no mutant for *Ago3* has been characterized, the Ago3 protein associates with repeat-associated piRNAs [43,77]. Mice also have three Piwis: Miwi, Mili and Miwi2. Although Miwi has not yet been implicated in transposon silencing, Mili and Miwi2 are required for transposon DNA methylation in the male germline [45,86]. Finally, in *Tetrahymena*, Twi1p (*Tetrahymena* Piwi-related protein 1) is essential for elimination of repeat sequences [63].

Though small RNAs appear to form a conserved component of repeat silencing mechanisms, there are also a few cases of Argonaute-independent pathways. For example, *S. pombe* has evolved a partially redundant and RNAi-independent mechanism for heterochromatin formation [87]. The repeat-induced point mutation pathway (RIP; see below) in *N. crassa* might also be RNAi-independent, because Dicer and Argonaute single mutants and Dicer–Argonaute double mutants that are defective in quelling (Neurospora RNAi) retain DNA and histone methylation on sequences that have undergone RIP; however, the meiotic phenotypes of other *Neurospora* RNA-related proteins leave open a possible relationship between RIP and RNAi [88,89].

Transcriptional silencing: DNA methylation and histone modification

The most common effector mode for stable transposon silencing involves heterochromatin formation through DNA methylation and/or histone modification. Abundant evidence suggests a role for small RNAs and Argonaute proteins in establishing the chromatin state of transposons and other repeats in several organisms. Small RNAs, in complex with Argonaute proteins, can identify their targets through sequence-specific recognition, enabling targeting of chromatin-modifying enzymes to the sequences that they modify, in this case for packaging into heterochromatin. Once established, heterochromatic marks need to be faithfully maintained through each replication cycle. The analysis of different eukaryotes also provides examples of small RNA-directed transcriptional gene silencing acting in the maintenance and establishment of silenced chromatin states (Figure 1).

Histone modifications in fission yeast—In *S. pombe*, transposon silencing is accompanied by H3K9 methylation. This depends upon Ago1 forming the RITS multi-protein complex with Chp1, an HP1 homolog, and Tas3, a protein of unknown function [73]. Though the mechanism is yet unclear, RITS is thought to recruit the H3K9 methyltransferase Clr4. Clr4 resides in complex with Rik1, a homolog of the DNA damage-binding protein DDB1, the E3 ligase subunit Cullin-4 (Cul4), the 14–3-3 protein Rad24, and two novel proteins, Cmc1 and Cmc2 [71,90]. Despite the methylated state of histone H3 on centromeric repeats, these repeats are still transcribed by RNA PolII, and this continued transcription is required for small RNA production and heterochromatin formation [91,92].

Mutations in any of the Clr4 complex subunits cause loss of silencing at both centromeres and mating-type loci and coincident loss of corresponding small RNA populations. Additionally, centromeric repeats lose repressive histone methylation in the absence of RNAi components, indicating an ongoing requirement for small RNAs in the maintenance of this state [22] Another region, the mating-type locus, contains a partial centromeric repeat, called cenH, as well as an element, REIII, which is required for mating-type silencing in the absence of cenH [93]. The mating-type locus is not de-silenced in the absence of RNAi components, but *de novo* establishment of silencing at mating-type loci is compromised. This suggests that RNAi is required for heterochromatin maintenance at centromeres, but only for initiation of heterochromatin at mating-type loci [22]. A possible resolution to this discrepancy is that the RNAi machinery acts redundantly with another silencing mechanism at the mating-type locus.

Histone modifications in Drosophila—Although an essential role for chromatin modification in transposon silencing in *Drosophila* has yet to be demonstrated, it is likely that this effector mechanism is based on analogies to other systems. As in *S. pombe*, heterochromatin in *D. melanogaster* contains a high density of methylated H3K9, as well as H3K27 and H4K20. The H3K9 methyltransferase Su(var)3–9 plays a crucial role in heterochromatin silencing and position-effect variegation (PEV) (see Glossary) [94]. Interestingly, Piwi proteins are also involved in PEV and in HP1 localization to heterochromatin on polytene chromosomes [83]. Moreover, HP1 is a direct binding partner of Piwi [95], although recent studies have also implicated Piwi in activation of chromatin [96]. Finally, in the germline, Spindle-E, an RNA helicase required for transposon silencing, is required for H3K9 methylation on several transposon classes [97].

Histone and DNA methylation in plants—*A. thaliana* uses both histone modification and DNA methylation to silence repeats. Cytosine methylation occurs at both symmetric (CG, CNG) and asymmetric (CNN, where N = A, T or C; see Glossary) [98] sites. These marks each depend on different DNA methyltransferases. Met1, a homolog of the mammalian Dnmt1, and the plant-specific Cmt3 are responsible for the maintenance of CG

and CNG methylation, respectively. Additionally, the partially redundant Dnmt3 homologs Drm1 and Drm2 can methylate cytosines *de novo* in all sequence contexts, although they have a preference for non-CG methylation. Met1 mutants gradually lose all CG methylation as well as a significant amount of CNG methylation. As a result, several classes of transposons become more highly expressed [99]. Similarly, transposons are mildly reactivated in *drm1:drm2:cmt3* mutants [99,100]. It is probable that different types of methylation marks cooperate to repress transposons, because expression and mobility of some transposons are synergistically increased in the *met1:cmt3* double mutants [100].

Transposon methylation is also strongly dependent upon chromatin modification and remodeling. The SNF2 protein, Ddm1, is required for transposon repression, and ddm1 mutants show strikingly decreased levels of DNA methylation on transposons [37,100,101]. Mutations in the histone deacetylase gene *Hda6* also affect repeat silencing [102,103]. Recently, two more genes have been implicated in repeat DNA methylation: another SNF2 protein, DRD1 [104]; and a second polymerase IV, PolIVb [105,106]; however, their exact roles are as yet unknown.

Interestingly, there is interplay between H3K9 and DNA methylation, although the interdependence is not complete [107]. H3K9 methylation on repeats is mainly achieved by the methyltransferase, SUVH4, also known as KRYPTONITE (KYP), and by SUVH5 and SUVH6 to a lesser extent [108]. *Kyp* mutants show reduced levels of non-CG methylation, and *met1* and *ddm1* mutants have abnormally low levels of H3K9 methylation at repeat-rich chromosome regions. Given that AGO4 is required for non-CG methylation, histone methylation and *de novo* CG-methylation of newly introduced transgenes, it is thought to recruit chromatin-modifying enzymes to repetitive DNA.

In *Arabidopsis*, small RNA-mediated transcriptional gene silencing has been implicated in both establishment and maintenance mechanisms. Ago4 is involved in the transformation-induced *de novo* CG and non-CG methylation of the FWA locus [78]. However, mutations in *Ago4* only mildly affect transcriptional silencing of a target transgene when an inverted repeat-containing trigger is introduced. Redundancy could be provided by another of the 10 *A. thaliana* Argonautes or by small RNA-independent processes. Additionally, Ago4 is required for the maintenance of non-CG methylation on several classes of endogenous transposons, but the centromeric repeat CEN, the Ta3 retrotransposons and FWA seem mostly unaffected by *ago4* mutations [27]. In all cases, however, CpG methylation remains largely unaffected in *ago4*, *dcl3* or *rdr2* mutants, which suggests that it is maintained through an RNAi-independent pathway [26,27].

Histone and DNA methylation in mammals—Mammals also use DNA and histone methylation to silence repeats. In contrast to plants, mammalian DNA methylation occurs primarily on CG sequences. In total, around 80% of the CpGs in the mouse genome are methylated, with most modified sites lying in repetitive sequences [109]. Four DNA methyltransferase family members have been implicated in transposon control and methylation [110]. Dnmt1 is the maintenance methyltransferase and modifies hemimethylated DNA. *Dnmt1*-null embryos reduce methylation of LTR-containing IAP (intracisternal-A particle) elements [111]. Similarly, Dnmt3a and Dnmt3b, the *de novo* methylases, are redundantly required for the methylation of IAP elements in embryos [112] and male germ cells [113]. Of all the Dnmts, the role of Dnmt3L in transposon silencing has been described in the most detail. Dnmt3L lacks catalytic activity but is required for transcriptional repression, as well as for DNA methylation of the LTR-class IAP elements and the non-LTR class LINE-1 elements (long interspersed elements-1) in the male germline [114–116]. Again, methylation of H3K9 and DNA seem to be linked in this context. H3K9 tri-methylation and DNA methylation on centromeric repeats and on some elements are

partially dependent on the redundant methyltransferases Suv39h1 and Suv39h2, which are required for transcriptional repression of transposons in embryonic stem cells [117,118]. Additionally, a Ddm1 homolog, Lsh (lymphoid-specific helicase), has been implicated in transposon methylation in embryos and in the female germline [119,120]. Given that both *miwi2* and *mili* knockouts affect transcriptional repression and methylation of L1 and IAP elements [45,86] (A. Girard and G.J. Hannon, unpublished), it is very likely that these proteins target chromatin-modifying enzymes to DNA.

In mammals, the differential role of Piwi proteins in establishment versus maintenance of DNA methylation awaits further analysis. It is noteworthy, however, that Piwi proteins seem to act precisely when global methylation patterns are erased and reestablished in the germline [121]. Dnmt3L is a known partner of the *de novo* methyltransferase Dnmt3a [122–124] and, as such, might be involved in the establishment of DNA methylation patterns in the male germline. The strong resemblance among *dnmt3l*, *miwi2* and *mili* phenotypes [45,86,114–116,125] suggests that these genes all act in *de novo* methylation of transposon sequences.

Chromatin-based repression in other organisms—*N. crassa* and *Tetrahymena thermophila* bring chromatin silencing to another level by mutating transposons or removing them from the genome. *N. crassa* uses RIP to mutate repetitive sequences through a C:G to T:A transition [126]. Given that a putative DNA methyltransferase, RIP-defective, is required for RIP, it has been speculated that RIP is a two-step process: first, RID methylates transposon sequences; second, these sequences are quickly and efficiently deaminated by an unknown activity. Although deamination of methylated cytosines is also thought to occur in mammals, it occurs at a much slower rate than in *Neurospora*.

In *Tetrahymena*, scanRNAs are used to detect DNA insertions (described above). The scanRNAs associate with Twi1 proteins and target complementary sequences for histone H3K27 and H3K9 methylation [63]. These marks are thought to signal for DNA elimination through the chromodomain proteins Pdd1p (programmed DNA-degradation protein 1) and Pdd3p.

Cis versus trans silencing

The high degree of sequence homology within transposon classes makes *trans* action a perfect strategy for transposon silencing, because the detection of one transposon could lead to the silencing of every related sequence in the genome. Indeed *trans*-silencing seems to occur in most organisms. In *A. thaliana*, for example, introduction of an inverted repeat-containing transgene triggers methylation of both the transgene and the homologous sequences elsewhere in the genome [18]. Similarly, the phenomenon of paramutation (see Glossary) in maize suggests the presence of a *trans*-silencing mechanism [127]. In vertebrates and flies, the transposon silencing system relies explicitly on the interaction of two unlinked transposon copies [43,60,61].

Cis silencing does occur, however, in some organisms. In *A. thaliana*, introduction of a methylated FWA epiallele does not result in methylation of the other unmethylated allele [128]. The same restriction *in cis* has been observed with the *SUPERMAN* locus [129]. In *S. pombe*, tethering of RITS to an *ura4* gene leads to the production of *ura4*-specific siRNAs [130]. But these are unable to silence another *ura4* copy *in trans*. One hypothesis is that Argonaute complexes can only repress repeats *in cis*, because of their association with nascent transcripts. However, this does not hold in the case of fission yeast, because a deletion of the small RNA-specific exonuclease Eri1 relieves the cis restriction, perhaps by removing `brakes' on small RNA accumulation. Therefore, it seems that, at least in *S. pombe*, the chromatin-silencing mechanism is actively restricted from exerting its effects

throughout the genome, because the small RNAs are actively degraded. It has been suggested that the purpose of this active restriction is to prevent an uncontrolled amplification of the transposon defense, which might otherwise result in the deleterious silencing of functional genes.

Developmental and inherited aspects of transposon silencing

Germline versus somatic silencing

Different hosts use different global strategies for transposon silencing. Plants express repeat-derived small RNAs in all tissues, and these are, at least partially, required for the ongoing chromatin silencing. In many other species, initiation of transposon silencing takes place in the germ-line or during meiosis. In some cases, the silencing mechanism exploits specific properties of meiosis. In MSUD, meiotic homologous chromosome pairing perhaps enables the detection of unpaired sequences [64]. In DNA elimination in *Tetrahymena*, conjugation leads to the destruction of the old macronucleus and to the formation of a new macronucleus. For the information to be transmitted from the former to the latter, the mechanism has to take place precisely at that developmental time point [63].

In nematodes, flies and vertebrates, transposon silencing is primarily initiated in the germline. Piwi proteins are predominantly expressed in the gonads of these animals [131,132], although somatic expression has also been reported [44,86,125,133–137]. In these organisms, the germline is set aside very early in development. Given that somatic tissues are an evolutionary dead-end for transposons, they might preferentially be active in the germline, where their new insertions can be transmitted to the next generation. This appears to be the case for some mammalian transposons, which seem to be highly active in testes or oocytes [138–140]. In these cases, the most robust defense must coincide with the time of greatest transposon challenge.

Inheritance of transposon silencing

Transposon information and silencing could theoretically be transmitted in at least three ways to the next generation. First, the DNA sequence itself can carry information about transposon sequences. Second, repeat-derived small RNAs can be deposited in the embryo. Last, chromatin modifications can be stably inherited. There are indications that several of these mechanisms are used. In animals, piRNA clusters record, through capture of mobile elements, past transposon challenge and transmit this information to progeny in the form of permissive and restrictive alleles [43]. In flies and fish, Piwi proteins and their associated piRNAs are maternally loaded into embryos [40,44,141,142]. Although the consequences of this can only be hypothesized, the analysis of fly hybrid dysgenic (see Glossary) crosses provides additional clues [143]. When reactive females (i.e. females that have not been exposed to a given active transposon) are crossed with inducer males (i.e. males that have been exposed to and have repressed the same active transposon), this results in sterility. This phenomenon is referred to as hybrid dysgenesis. Surprisingly, the reciprocal cross — inducer females with reactive males — does not lead to the same outcome. This is suggestive of cytoplasmic inheritance and raises the strong possibility of maternally deposited piRNAs being mediators of this phenomenon. Evidence for epigenetic inheritance of chromatin states comes mostly from plants, in which many epialleles are known to be stably transmitted [128,129,144], even in the absence of the RNA trigger. Surprisingly, despite the massive erasure of DNA methylation that takes place in the mammalian germline and in embryos, epigenetic states can also be transmitted in mice. Some epialleles of an agouti locus that contains an IAP insertion can be transmitted at a high frequency through the maternal lineage [145]. Similarly, epigenetic states of the Axin(fu) allele can by inherited paternally and maternally [146]. In both cases, an IAP insertion is responsible for

the differential expression of the loci. LTR-type IAP elements are partially resistant to the loss of DNA methylation [147], which suggests that remaining DNA methylation can influence the expression of the locus in the progeny.

Concluding remarks and future challenges

RNAi-related mechanisms generally act through post-transcriptional regulation or transcriptional regulation of target genes. In the first case, target transcripts are either translationally repressed or cleaved by an Argonaute protein and degraded. In the second case, target transcription is repressed through the formation of heterochromatin as described above. Owing to its durable effects, heterochromatin formation is the most-studied mechanism of regulation of transposon sequences; however, some evidence suggests that several eukaryotes, if not all, also incorporate post-transcriptional regulation in their transposon silencing strategies.

Key challenges for the future include deciphering the relationship between post-transcriptional regulation-based systems and transcriptional regulation-based systems, and the interaction of apparently compartmentalized silencing pathways. Mammals, zebrafish and *Drosophila* all contain both siRNA- and piRNA-based silencing mechanisms, whereas *Tetrahymena* seems to merge siRNA and piRNA pathways into one mechanism that involves both dsRNAs and Dicer and Piwi family proteins. To date, it is not clear whether distinct cell types in each of these systems rely mainly on one class of small RNAs or whether siRNA and piRNAs might act redundantly or even form an interacting network in some cases.

Perhaps the most crucial uncertainty is how different organisms truly distinguish protein-coding genes from mobile elements and repeats. Although studies of small RNAs have revealed much about how such discrimination might occur, we still cannot derive *a priori* rules governing how an organism selectively represses mobile elements.

The growing body of data on transposon silencing in eukaryotes shows that, despite using different proteins and mechanisms, organisms employ strikingly convergent strategies, which can, in many cases, be divided into three intertwined steps (detection, signal amplification and repression; Figure 1). Many eukaryotes also exploit inheritance mechanisms to prime responses in their progeny. These principles are not only common to all transposon repression mechanisms but are also highly reminiscent, at least in concept, to our adaptive immune systems.

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Glossary

Argonaute Family of proteins with a crucial role in silencing mechanisms. Argonaute proteins possess two recognizable domains: the PAZ domain, which binds the 3′ end of the small RNA, and the Piwi domain, the RNAseH-like fold of which confers Slicer catalytic activity to the protein. Argonaute proteins bind to a small RNAs and can often cleave complementary RNA targets. They can also

	serve as a sequence-specific recruitment platform for other silencing activities such as histone and DNA methyltransferase. The Argonautes can be divided into the Argonaute and Piwi clades. The Piwi clade is found exclusively in animals, where its members bind piRNAs and are involved in silencing of transposons. The Argonaute clade binds a wider variety of small RNA classes and is required, in particular, for repeat silencing in plants and fungi and for siRNA and microRNA function.
CG/CNG/CNN methylation	DNA methylation occurring in defined sequence contexts. CG, CNG and CNN (or asymmetric) methylation refers to the modification of the cytosine in CG dinucleotide, CNG trinucleotide and CNN trinucleotide contexts, respectively (where N=A, T or C)
Cryptic promoter	promoter diverged from the consensus core promoter sequence and generally overlooked by prediction programs. Microarray analysis has revealed that a substantial fraction of our genome is transcribed from such promoters. In particular, transposons often contain cryptic antisense promoters in their 3′ sequence.
Dicer	Endonuclease of the RNaseIII family, specific for dsRNA substrates. Dicer proteins generate fixed-size small RNA duplexes from 21 to 24 nucleotides in length, with 2-nucleotide 3′ overhangs and 5′ monophosphates. Small interfering RNAs, as well as many other small RNA classes, are produced by Dicer proteins.
Hybrid dysgenesis	the sterility observed when females flies that have not been exposed to a particular active transposon (reactive females) are mated with males that have been exposed to the same transposons and have successfully repressed it (inducer males). Surprisingly, an inducer female mated to a reactive male is fertile, which suggests that inducer females transmit a protective agent to their progeny.
Meiotic silencing of unpaired DNA (MSUD)	meiosis-specific mechanism found in *Neurospora*, *C. elegans* and mammals, by which unpaired DNA sequences are detected and silenced. Any paired DNA with an identical sequence is also repressed in the process.
Paramutation	stable mutation triggered by an allele on the other allele of a heterozygous pair.
Position-effect variegation (PEV)	mosaic pattern of expression of a gene, often because of its placement near a heterochromatin domain
Piwi-interacting RNAs (piRNAs)	small RNAs associated with Piwi proteins and responsible for transposon silencing in animals. piRNAs are 24–30-nucleotides in length and originate from discrete loci in metazoan genomes.
Piwi	refers to a clade of the Argonaute family more closely related to one Argonaute founding family member, *Drosophila* Piwi. These are often involved in transposon control in animals. Piwi proteins associate with piRNAs and are specifically expressed in the germline (and, in some cases, in gonadal somatic cells). Piwi also designates the RNAseH-related catalytic domain of Argonaute proteins (see Slicer)

RdRP complex (RDRC)	refers to the complex containing the RdRP enzyme Rdp1p in *S. pombe*.
RNA-dependent RNA polymerase (RdRP)	Polymerase enzyme capable of primed or unprimed synthesis of a complementary RNA strand from an RNA template. In many cases, RdRPs are required for the amplification of a small-RNA population and for efficient silencing.
Read-through transcription	transcription through an inverted repeat. The two halves of the resulting transcript are complementary and can fold back into an RNA hairpin.
Repeat-induced point mutation (RIP)	high frequency accumulation of C:G to T:A mutations in repetitive DNA in *N. crassa*.
RNA-induced silencing complex (RISC)	refers to small RNA-containing silencing complexes. In addition to a small RNA, RISC complexes always contain an Argonaute protein. In particular, RISC often designates the small-interfering RNA-containing complex responsible for the cleavage of complementary RNA targets.
RNA-induced transcriptional silencing (RITS) complex	the RISC complex involved in transcriptional gene silencing in *S. pombe*.
Rolling circle replication	DNA polymerase-mediated amplification of a circular genome, which leads to the production of linear concatamerized versions of this genome.
Small interfering RNAs (siRNAs)	siRNAs are distinguished from other types of small RNAs through their particular biogenesis mechanisms. Most siRNAs are produced from dsRNAs by Dicer cleavage.
Slicer	endonucleolytic activity of some Argonaute proteins. When a complementary RNA target is recognized by the Argonaute-bound small RNA, the Piwi domain cleaves the target 10 nucleotides downstream of the 5′ end of the small RNA. The 5′ end of the downstream cleavage product carries a monophosphate. Not all Argonaute proteins are capable of Slicer activity, because some of them carry mutations in crucial catalytic residues.
Transitive RNA interference (RNAi)	extension of silencing to the sequences 5′ of the siRNA-trigger pairing site.

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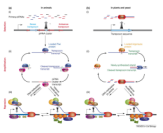

Figure 1.

Eukaryotic small RNA-based transposon silencing relies on three linked steps: detection, amplification and repression. (**a**) (i) In animals, 24–30-nucleotide primary piRNAs are processed from long RNA precursors transcribed from defined loci called piRNA clusters. Any transposon inserted in the reverse orientation in the piRNA cluster can give rise to antisense piRNAs (in red). (ii) Antisense piRNAs are incorporated into a Piwi protein (in flies, this is mostly Aubergine or Piwi) and direct its Slicer activity on sense transposon transcripts. The 3′ cleavage product is bound by another Piwi protein (Ago3 in flies) and trimmed to piRNA size. This sense piRNA is in turn used to cleave piRNA cluster transcripts and to generate more antisense piRNAs. (iii) Eventually, antisense piRNAs can target the Piwi complexes to cDNA for DNA methylation and/or histone modification. (**b**) (i) In plants and *S. pombe*, transposon expression leads to dsRNA formation, through a process that is still largely unexplained. One possible source of dsRNA is the read-through transcription of inverted repeats. Another is the synthesis of the reverse strand from transposon RNA templates by an RDRP. This dsRNA is then processed into 21–24-nucleotide small RNAs by a Dicer protein. (ii) Transposon-derived siRNAs can then bind an Argonaute protein and direct cleavage of transposon transcripts. These cleaved RNAs are potential templates for RDRP-mediated reverse strand synthesis and processing of more siRNAs by Dicer. (iii) As in animals, siRNA-Argonaute complexes can target DNA and histone-modifying complexes to cDNA sequences.