Small RNAs in transcriptional gene silencing and genome defence

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Small RNA molecules of about 20–30 nucleotides have emerged as powerful regulators of gene expression and genome stability. Studies in fission yeast and multicellular organisms suggest that effector complexes, directed by small RNAs, target nascent chromatin-bound non-coding RNAs and recruit chromatin-modifying complexes. Interactions between small RNAs and nascent non-coding transcripts thus reveal a new mechanism for targeting chromatin-modifying complexes to specific chromosome regions and suggest possibilities for how the resultant chromatin states may be inherited during the process of chromosome duplication.

RNA interference (RNAi) originally referred to the ability of exogenously introduced double-stranded RNA (dsRNA) molecules to silence the expression of homologous sequences in the nematode *Caenorhabditis elegans*[1]. It has become clear over the past decade that RNAi is mechanistically related to a number of other conserved RNA silencing pathways, which are involved in the cellular control of gene expression and in protection of the genome against mobile repetitive DNA sequences, retroelements and transposons[2–4]. These RNA silencing pathways are all associated with small (~20–30 nucleotide) RNAs that function as specificity factors for inactivating homologous sequences by a variety of mechanisms. At least three classes of small RNA have been identified so far (Table 1). The first two classes, short interfering RNAs (siRNAs) and microRNAs (miRNAs), are ~21–25 nucleotides and are generated from longer dsRNA precursors by Dicer, a ribonuclease III (RNaseIII) enzyme. They are loaded into the RNA-induced silencing complex (RISC) or a nuclear form of RISC, called the RNA-induced transcriptional silencing complex (RITS)[5–10]. RISC and RITS are effector complexes that are targeted to homologous sequences by base-pairing interactions involving the guide strand of the small RNA. The core component of each complex is a highly conserved PAZ- and PIWI-domain-containing protein called Argonaute, which binds to the guide small RNA by means of interactions that involve its PAZ domain, as well as the PIWI and middle (MID) domains, and cleaves the target RNA by means of its RNaseH-like PIWI domain (see page 405 for further information about the structural biology of RNAi proteins).

The Argonaute family of proteins, together with the small RNAs that program them, are the central players in RNA silencing, and seem to participate in all small-RNA silencing pathways thus far described. Phylogenetically, Argonaute-family proteins are divided into the AGO and PIWI clades[11]. The PIWI-clade proteins bind to a third class of small RNAs, called PIWI-interacting RNAs (piRNAs), which have a broader average size (~24–31 nucleotides) than siRNAs and miRNAs and are involved in defence against parasitic DNA elements[12–18]. As discussed later, piRNA-programmed PIWI-clade proteins are also likely to function as RISC- and RITS-like complexes that target the inactivation of homologous sequences (Table 1). With the notable exception of budding yeast, small-RNA-mediated silencing mechanisms and their role in chromatin regulation are conserved throughout eukaryotes, indicating an ancient evolutionary origin.

This Review discusses the roles of diverse small-RNA silencing pathways in the regulation of chromatin structure and transcription in plants, animals and fungi, with particular emphasis on emerging common themes. In addition to their well-known roles in post-transcriptional gene silencing (PTGS), in which silencing is directed at the level of messenger RNA translation or stability, nearly all small-RNA silencing pathways also seem to act at the DNA and chromatin level (Table 1). Studies in *Schizosaccharomyces pombe* (fission yeast) and other organisms suggest that small RNAs access DNA through interactions with nascent RNA transcripts, revealing a close relationship between nuclear and cytoplasmic RNA silencing mechanisms. Moreover, small-RNA silencing pathways seem to be intimately integrated with the RNA surveillance and processing pathways that determine the ultimate fate of RNA transcripts. Together, these studies reveal a broad and previously unsuspected role for RNAi and other RNA-processing mechanisms in the regulation of the structure and expression of eukaryotic genomes. Here, I discuss small-RNA silencing pathways and their role in chromatin regulation, drawing parallels between well-established examples in *S. pombe* and other organisms.

RNA silencing pathways

RNA silencing pathways can be broadly classified into different branches based on their mechanism of action, subcellular location and the origin of the small RNA molecules that they use (Table 1). However, the different branches have common components and intersect in some instances. siRNAs act in both the nucleus and the cytoplasm and are involved in PTGS and chromatin-dependent gene silencing (CDGS). CDGS refers to both transcriptional gene silencing (TGS) and co-transcriptional gene silencing (CTGS)[3]. miRNAs are generated from hairpin precursors by the successive actions of the RNaseIII enzymes Drosha and Dicer, which are located in the nucleus and cytoplasm, respectively (see page 396 for a more detailed discussion of small RNA precursor processing and complex assembly). Although Drosha is absent in plants, the general features of the miRNA pathway are conserved in plants and animals, but not in fungi and other protozoa. Whereas the vast majority of miRNAs seem to act exclusively in the cytoplasm and mediate mRNA degradation or translational arrest[19], some plant miRNAs may act directly in promoting DNA methylation[20]. Furthermore, recent studies describe a role for promoter-directed

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Table 1 | Conservation of small-RNA silencing pathways in eukaryotes

Small RNA	Size (nucleotides)	Mechanism of action	Eukaryotes conserved in
siRNA	~21–25	PTGS (RNA degradation or translational arrest) CDGS	Plants, animals, fungi, ciliates
miRNA	~21–25	PTGS (RNA degradation or translational arrest) CDGS (to a lesser extent)	Plants, animals
piRNA	~24–31*	PTGS (RNA degradation) CDGS (to a lesser extent)	Animals

All three of the major RNA silencing pathways identified thus far seem to act in both post-transcriptional gene silencing (PTGS) and chromatin-dependent gene silencing (CDGS) pathways. CDGS refers to chromatin-dependent silencing events that involve the assembly of small RNA complexes on nascent transcripts and includes both transcriptional gene silencing (TGS) and co-transcriptional gene silencing (CTGS) events. The latter involves the chromatin-dependent processing or degradation of the nascent transcript. **Caenorhabditis elegans* piRNAs are 21 nucleotides.

human miRNAs in facilitating repressive chromatin modifications and TGS[21,22]. siRNAs are generated from long dsRNA precursors, which can be produced from a variety of single-stranded RNA (ssRNA) precursors. These precursors include sense and antisense RNAs transcribed from convergent promoters, which can anneal to form dsRNA, and hairpin RNAs that result from transcription through inverted repeat regions[23–25] (Fig. 1a). In some situations the long dsRNA is produced enzymatically from certain aberrant or non-coding RNA precursors. One example of this pathway involves aberrant RNAs that lack processing signals or are produced by Argonaute slicer activity. These RNAs recruit RNA-dependent RNA polymerase (RdRP) enzymes, which recognize free 3′ ends and synthesize dsRNA[2,26,27] (Fig. 1b, c). Here RdRP enzymes are in competition with the TRAMP polyadenylation pathway, which targets aberrant RNAs for degradation by a 3′→5′ exonuclease complex, called the exosome[28–31](Fig. 1b). The siRNA branch of the pathway seems to be conserved from fungi to mammals (Table 1), although *Drosophila melanogaster* (fruitflies) and mammals lack RdRPs and cannot amplify siRNAs.

piRNAs originate from a diversity of sequences, including repetitive DNA and transposons, and like siRNAs they seem to act at both the post-transcriptional and chromatin levels[12–18]. The mechanism(s) that generates and amplifies piRNAs is not yet fully elucidated but involves the slicer activity of the PIWI-clade proteins themselves[4] (Fig. 1d). This class of small RNAs is present in *D. melanogaster*, *C. elegans* and mammals, but seems to be absent in fungi and plants (Table 1).

Small RNAs in DNA and chromatin regulation

An accumulating body of evidence supports an important role for small RNAs in the modulation of chromatin structure and TGS in plants, fungi and animal cells. RNA silencing was first linked to TGS by the discovery that transgene and viral RNAs guide the methylation of homologous DNA sequences in plants[32]. Analysis of the guide RNAs in *Arabidopsis thaliana* revealed that these RNAs were processed into small RNAs of ~25 nucleotides, similar to the size previously described for miRNAs[5,33]. This observation and the realization that exogenously introduced dsRNA in animals is processed into siRNAs[8] established small RNAs as central players in diverse RNA silencing pathways. Later studies in *A. thaliana* indicated that RNA-directed DNA methylation of the *FWA* transgene requires Dicer (DCL3) and Argonaute (AGO4), and is linked to histone H3 lysine 9 (H3K9) methylation, indicating that RNA-directed DNA methylation and RNAi have common molecular mediators[34–36].

Evidence for the role of RNA silencing in mediating changes at the chromatin level also came from studies of silent or heterochromatic DNA domains in unicellular eukaryotes, such as *S. pombe* and the ciliate *Tetrahymena thermophila*. *S. pombe* contains single genes encoding the Argonaute, Dicer and RdRP proteins, called *ago1*, *dcr1* and *rdp1*, respectively. Deletion of any of these genes results in loss of heterochromatic gene silencing, markedly reduced H3K9 methylation at centromeric repeats, and accumulation of non-coding RNAs, which are transcribed from centromeric repeat regions and processed into siRNAs[37,38]. Moreover, RNAi is directly linked to a structural component of heterochromatin through RITS, which in *S. pombe* contains Ago1, the chromodomain protein Chp1, the glycine and tryptophan (GW)-motif-containing protein Tas3 and centromeric siRNAs[10,29,39]. *T. thermophila* cells are binucleate with a germline micronucleus and a somatic macronucleus. Development of a new macronucleus after sexual conjugation and meiosis involves massive DNA elimination of non-genic sequences. This elimination requires TWI1, a *T. thermophila* PIWI-clade protein, and PDD1, a chromodomain protein that binds to both K9- and K27-methylated histone H3 (refs 40–42). In addition, DNA elimination is associated with Dicer-produced small RNAs, called scan RNAs (scnRNAs), giving rise to the idea that a scnRNA RITS-like complex targets sequences destined for elimination into heterochromatin[40]. However, a physical association between chromatin proteins and TWI1 has not yet been reported.

RNAi is also linked to chromatin modifiers in animal cells. In *D. melanogaster*, the introduction of multiple tandem copies of a transgene results in silencing of both the transgene array and the endogenous copies. This repeat-induced gene silencing, which is analogous to RNA-mediated co-suppression in plants[2], requires components of the Polycomb group (PcG) of genes, as well as several RNAi factors, including PIWI and AGO2 (ref. 43). The PcG gene products are chromatin-binding and -modifying repressors that prevent the expression of homeobox (HOX) regulators outside their proper domains of expression[44]. The requirement for both PcG proteins and PIWI in transgene silencing suggested the possibility that in *D. melanogaster*, as in plant cells, RNA silencing could operate at the chromatin level. In fact, later studies showed that RNA silencing factors are also required for the formation of *D. melanogaster* centric heterochromatin, recruitment of heterochromatin protein 1 (HP1) and silencing of transgenes that are inserted in pericentromeric heterochromatin[43,45]. In addition to HP1 and PIWI, efficient silencing requires DCR-1, PIWI, Aubergine (AUB) and the putative helicase HLS (also known as SPN-E)[43]. Moreover, silencing of a mini-*white* gene, which is mediated by a *cis*-acting repeated element from the heterochromatic Y chromosome, requires HP1, SU(VAR)3-9 (the H3K9 methyltransferase), as well as PIWI, AUB, HLS and DCR-1 (ref. 46). Transgene-induced gene silencing in *C. elegans* has also been shown to require RNAi and chromatin modifiers[47,48]. Surprisingly, screens for defects in classical RNAi, mediated by feeding of dsRNA, have also uncovered several chromatin modifiers, suggesting that perhaps the connection between RNAi and chromatin modifiers may not be limited to repeat-induced silencing[49].

In contrast to their apparent requirement for PcG-mediated repeat-induced gene silencing, RNAi components do not seem to be required for PcG-mediated silencing of HOX genes outside their proper domains of expression[50]. Mutations in several RNA silencing factors disrupt the silencing of a tandem mini-*white* gene array and perturb the nuclear clustering of PcG-repressed HOX loci[50]. However, despite their requirement for PcG-mediated repeat-induced silencing, loss of PIWI and RNAi components does not lead to a loss of HOX gene silencing. The simplest explanation for these observations is that RNAi is required for some, but not all, PcG-mediated silencing events.

Linking heterochromatin to RNAi

Heterochromatin is associated with repetitive DNA sequences and transposons, and has important roles in chromosome transmission, maintenance of genomic stability, and regulation of gene expression[51–53]. With the exception of budding yeast, which lacks centromeric DNA repeats, heterochromatin is concentrated at repeats and transposons that surround centromeres, telomeres and other genomic loci (Fig. 2a). Two important defining properties of heterochromatin involve its modes of assembly and inheritance. First, heterochromatin assembly involves nucleation sites, which act as entry points for the recruitment and spreading of repressor proteins. Unlike recruitment, which involves the action of a site-specific DNA-binding protein or RNA molecule,

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spreading occurs in a sequence-independent manner and involves changes in chromatin structure that are mediated by histone-modifying enzymes. The second defining property of heterochromatin is its mode of inheritance. Once assembled, heterochromatin is inherited through many cell divisions, at least partly independently of the underlying DNA sequence. The mechanisms of spreading and epigenetic inheritance of heterochromatin are poorly understood, but, in *S. pombe*, require components of the RNAi pathway[3,53,54].

At the molecular level, heterochromatin is characterized by association with hypoacetylated histones and, in organisms ranging from *S. pombe* to humans, by association with H3K9 dimethylation and trimethylation[3,51]. H3K9 is methylated by SU(VAR)3-9 in *D. melanogaster*, SUV39H in humans and Clr4 in *S. pombe*, and creates a binding site for HP1 (Swi6 and Chp2 in *S. pombe*)[55–58]. HP1 proteins contain a chromodomain that binds to methylated H3K9 and a chromoshadow (CSD) domain, which is involved in other protein–protein interactions[54].

Biochemical isolation of *S. pombe* heterochromatin and RNAi complexes has provided direct physical links between heterochromatin and RNAi proteins, leading to models for how RNAi mediates heterochromatin assembly and participates in gene silencing. In addition to HP1 proteins, heterochromatic gene silencing in *S. pombe* requires the chromodomain protein Chp1 (ref. 59). Chp1 is larger than HP1 and, like the Polycomb (Pc) subfamily of chromodomains, contains only a single chromodomain at its amino terminus. Like Swi6 and Chp2, Chp1 is a structural component of heterochromatin and is required for heterochromatic gene silencing[59]. Unlike Swi6 and Chp2, which are not required for H3K9 methylation within centromeric repeat regions[58,60], a lack of Chp1 in cells leads to a marked loss of H3K9 methylation, indicating that Chp1 has a critical role in heterochromatin formation.

Biochemical purification of Chp1 showed that it is associated with Ago1 in RITS[10]. RITS acts as a specificity determinant for the recruitment of other RNAi complexes and chromatin-modifying enzymes to specific DNA regions. RITS also physically associates with and is required for recruitment of the RNA-directed RNA polymerase complex (RDRC) to non-coding RNAs that are transcribed from centromeric repeats[61,62]. RDRC contains the *S. pombe* RNA-directed RNA polymerase, Rdp1, a putative helicase termed Hrr1, and Cid12, a member of the Trf4 and Trf5 family of polyadenylation polymerases[61], which were first identified in the budding yeast *Saccharomyces cerevisiae* and are involved in the degradation of aberrant transcripts[30,31]. The physical association of RITS and RDRC is siRNA- and Clr4-dependent, suggesting that this association occurs on chromatin and requires histone H3K9 methylation[61]. These observations further suggest that RITS and RDRC localize to chromatin-bound nascent RNA by a mechanism that involves tethering the nascent transcript to chromatin via the bivalent complex RITS (Fig. 2). In addition to RITS, *S. pombe* contains a second Ago1-containing complex, named Argonaute siRNA chaperone (ARC) complex[63]. The Ago1 protein in the ARC complex contains duplex, rather

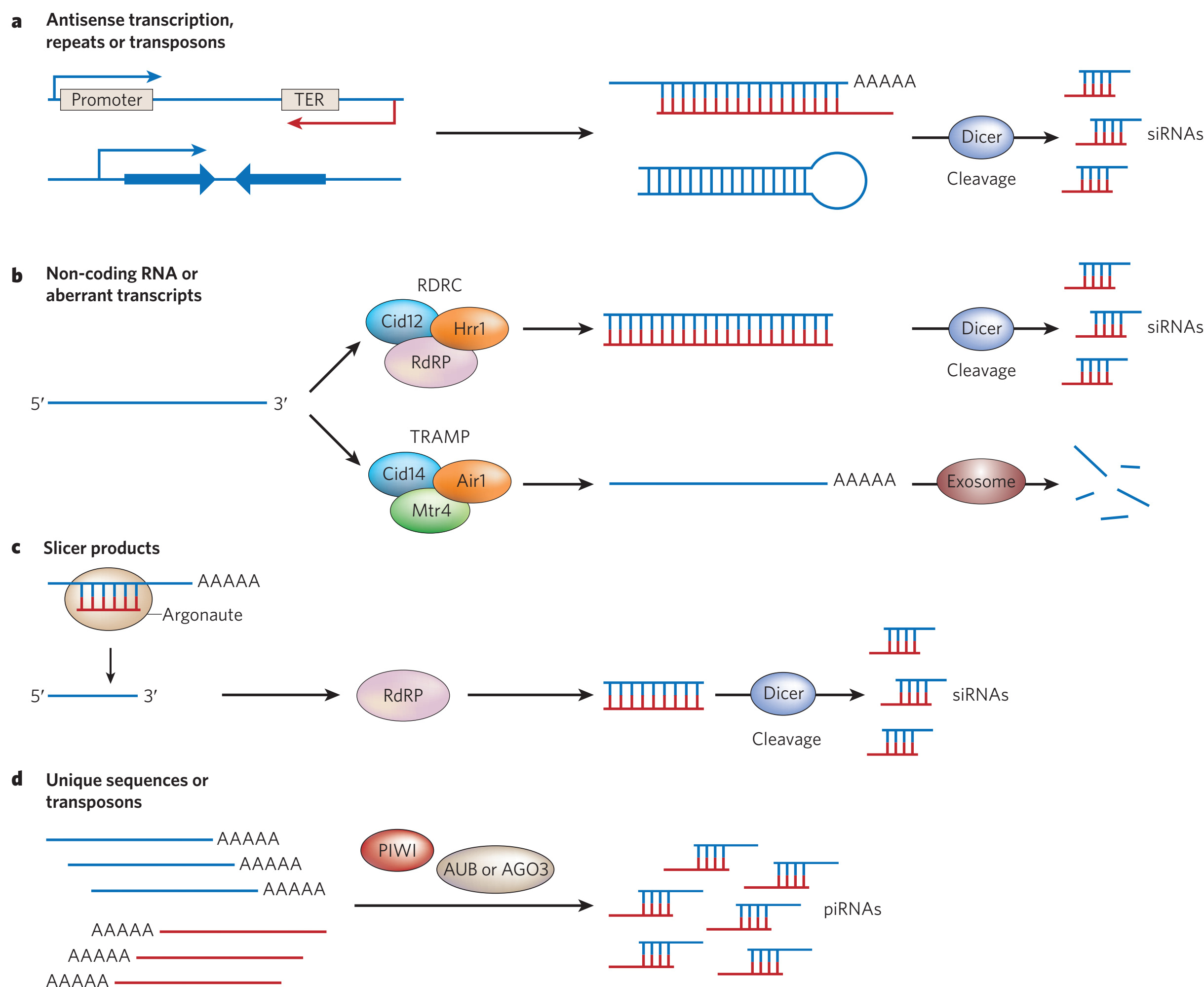

Figure 1 | Pathways of RNA processing and biogenesis of small RNAs. **a**, Generation of endogenous siRNAs from dsRNA resulting from convergent transcription (sense–antisense RNA base-pairing; top) or transcription through inverted repeat sequences (hairpin RNA formation; bottom). TER, transcription termination signal. **b**, Processing of non-coding and aberrant RNAs by the RDRC and TRAMP complexes, containing the Cid12 and Cid14 non-canonical polyadenylation polymerases, respectively; the RDRC/Dicer pathway produces duplex siRNAs, whereas the TRAMP/exosome pathway produces single-stranded degradation products. **c**, Generation of a free 3′ end by the slicer activity of an Argonaute protein, which can be processed into dsRNA by RdRP or targeted for degradation by the exosome (not shown). **d**, Pathway for the generation of piRNAs by the PIWI clade of Argonaute proteins: PIWI, AUB and AGO3.

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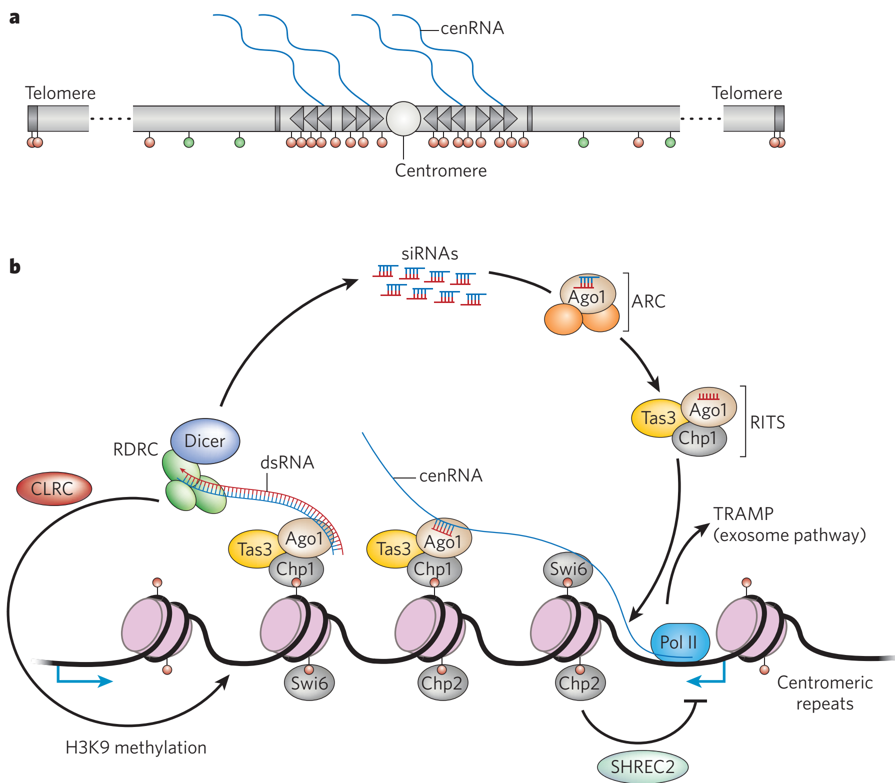

Figure 2 | Chromosome organization and the nascent transcript model for heterochromatic gene-silencing assembly in *Schizosaccharomyces pombe*. a, The structure of *S. pombe* centromeric repeat regions, highlighting the presence of non-coding centromeric transcripts (cenRNA) and association with histone H3 that is dimethylated and trimethylated on lysine 9 (red lollipops) as opposed to histone H3 that is methylated on lysine 4 (green lollipops) in euchromatic regions. **b,** The nascent transcript model for heterochromatin assembly. The RITS is tethered to chromatin through base-paring interactions between siRNAs and nascent non-coding transcripts and interactions with H3K9-methylated nucleosomes, resulting in the recruitment of RDRC–Dicer, dsRNA synthesis and siRNA amplification. This RNAi positive-feedback loop then recruits the CLRC H3K9 methyltransferase. Efficient silencing also requires two HP1 proteins (Swi6 and Chp2), which promote the association of RITS with the non-coding RNA or mediate TGS through recruitment of the SHREC2 deacetylase complex, respectively. Another tier of regulation, involving the degradation of heterochromatic transcripts by the TRAMP/exosome pathway, further ensures full gene silencing. Blue arrows (bottom) highlight convergent transcription resulting in synthesis of sense and antisense RNAs, which may contribute to the production of trigger siRNAs.

than single-stranded, siRNA, indicating that the slicer activity of Ago1 (refs 63, 64), which is required for the release of the siRNA passenger strand, is inhibited in this complex[63].

Nascent transcripts as assembly platforms

In principle, siRNAs in RITS can base-pair with either unwound DNA regions or with nascent non-coding RNAs that are transcribed from their target DNA. The two models are not mutually exclusive and base-pairing with DNA and RNA may contribute to different aspects of the mechanism of siRNA biogenesis and function. However, although a role for siRNA–DNA base-pairing cannot be ruled out at this point, several lines of evidence support siRNA–RNA base-pairing interactions in which the siRNA targets nascent non-coding transcripts (Fig. 2). First, RITS associates with the RDRC, which uses ssRNA as a template to synthesize dsRNA, providing evidence that RITS itself is RNA-associated[61]. Furthermore, the RITS–RDRC interaction requires siRNA and the Clr4 H3K9 methyltransferase, suggesting that it occurs on heterochromatin-bound transcripts[61]. Together with the observation that proteins required for heterochromatin formation — such as Sir2, Swi6, Clr4 and other components of the Clr4 methyltransferase complex (CLRC), as well as RITS and RDRC — are required for siRNA accumulation[10,61,65–67], these studies suggest that siRNA-programmed RITS localizes to nascent chromatin-tethered non-coding transcripts and recruits the RDRC to initiate dsRNA synthesis and siRNA amplification (Fig. 2b). Direct support for this model comes from experiments in which the Tas3 component of RITS was fused to the phage λ N (λN) protein and tethered to the transcript of a euchromatic *ura4*$^+$ gene, which was modified with the addition of five λN-binding sites upstream of its transcription termination sequences (*ura4-5BoxB*)[66]. In cells containing *ura4-5BoxB*, the Tas3-λN protein could efficiently initiate *de novo* siRNA generation and heterochromatin formation[66]. Like the situation at centromeres, siRNA generation in this system is H3K9 methylation-dependent, suggesting that Tas3-λN associates with chromatin-bound nascent transcripts and initiates RNAi-mediated heterochromatin assembly. Finally, several splicing factors associate with RDRC[61,68] and are required for RNAi-mediated centromeric gene silencing[68]. These results provide additional support for co-transcriptional processing of non-coding centromeric RNAs, as spliceosomal components are known to associate with nascent RNA transcripts co-transcriptionally. A role for the nascent transcript in acting as a template for the recruitment of chromatin-modifying activities may be conserved throughout eukaryotes. For example, large non-coding RNAs such as XIST, which is involved in X-chromosome inactivation, are thought to be involved in the recruitment of histone and DNA methyltransferase enzymes[69]. However, the mechanism of recruitment of chromatin-modifying activities to XIST may involve site-specific RNA-binding proteins rather than small RNAs.

Chromatin-dependent processing of siRNAs

A remarkable observation in studies of RNAi in *S. pombe* is that the generation of centromeric siRNAs is a heterochromatin-dependent event[61,65]. In the nascent transcript model (Fig. 2b), RITS associates with methylated H3K9 through the chromodomain of its Chp1 component and captures the nascent non-coding transcript through base-pairing interactions involving siRNAs bound to its Ago1 protein. In cells lacking the H3K9 methyltransferase Clr4 or any component of the CLRC, the levels of centromeric siRNAs are greatly diminished[61,67]. Moreover, one of the two HP1 proteins, Swi6, is required for efficient siRNA generation[61,66,70] and the association of RDRC with centromeric DNA repeats[62] and non-coding centromeric RNAs[71]. Furthermore, the crucial chromatin-dependent step in siRNA generation involves dsRNA synthesis by RDRC, as the introduction of a long dsRNA-containing hairpin into *S. pombe* cells circumvents the requirement for both RDRC and Clr4 in siRNA generation[70]. These results suggest that RDRC is only able to synthesize dsRNA on chromatin-bound templates after it has been recruited by RITS, revealing the existence of a chromatin-dependent step in the activation of the dsRNA biogenesis and siRNA amplification pathway in *S. pombe*. The resultant dsRNA is processed into siRNA by Dcr1, which is also physically tethered to RDRC[72]. Heterochromatin regulation of small RNA production may be conserved in metazoans. X-TAS (transposable *P* elements inserted in telomeric-associated sequences on the X chromosome) and *flamenco*, two major piRNA-producing loci that control the transposition of *P* and *gypsy* elements in *D. melanogaster*, respectively, are embedded

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in heterochromatin, and their genome defence function requires both PIWI and HP1 (refs 73, 74).

siRNA-mediated initiation of chromatin silencing

An important question regarding the role of RNA in gene silencing is whether small RNAs can initiate *de novo* chromatin modifications. Although small RNAs are important components of some CDGS mechanisms, their ability to initiate chromatin modifications seems to be under strict control by other mechanisms. In *S. pombe*, ectopically produced hairpin siRNAs can initiate H3K9 methylation and gene silencing at only a subset of target loci[70]. siRNA-mediated CDGS correlates with chromosomal location and the occurrence of antisense transcription at the targeted locus, and requires overexpression of the Swi6 (HP1) protein[70]. This may be reflecting the importance of cooperativity in the recruitment of RITS and other Argonaute or PIWI effector complexes to chromatin. In addition to siRNAs, stable association of RITS with chromatin requires the binding of the chromodomain in Chp1 to H3K9-methylated nucleosomes[10,65]. In the absence of H3K9 methylation, the initial binding of RITS to chromatin may be inefficient. Swi6 overproduction may help in initial RITS binding by stabilizing low levels of H3K9 methylation that occur throughout the genome, or alternatively by tethering the nascent transcript at the target locus to chromatin[71] (Fig. 2b). Similar limitations may explain the context-dependent ability of siRNAs to promote DNA methylation in plants[75], as well as the observed variability in siRNA-mediated chromatin modifications in animal cells (for example, see refs 76, 77). The ability of siRNAs to act as initiators is reminiscent of the role of DNA-binding transcription factors in the regulation of transcription, which often involves cooperativity between two or more transcription factors and is sensitive to local chromatin structure.

Small RNAs and epigenetic inheritance

Mechanisms that mediate the *cis*-inheritance of chromatin states and their associated gene-expression patterns remain enigmatic. It has long been known that during DNA replication, old parental histones are randomly distributed onto the two newly synthesized daughter DNA strands[78]. This retention of old histones during DNA replication has given rise to the idea that histone modifications mediate the epigenetic inheritance of chromatin states. Histone modifications, such as H3K9 methylation, create binding sites for proteins such as Chp1, Chp2 and Swi6, as well as the methyltransferase Clr4 (ref. 79) (Fig. 2b). Their retention during DNA replication could in principle serve as a mark for the re-recruitment of new chromatin-modifying activities that re-establish old modification patterns. However, the affinity of modified histones for specific binding proteins may be too low to allow the specific re-establishment of chromatin states, and other inputs into the mechanism are required[44]. The nascent transcript model, described above, provides a possible mechanism for epigenetic inheritance of heterochromatin. As in plants and other systems that contain an RdRP-dependent siRNA amplification mechanism[2,80], the siRNA generation mechanism in *S. pombe* is likely to form a positive-feedback loop[61,62]. Two specific features of this loop may underlie the mechanism that ensures the epigenetic inheritance of histone H3K9 methylation and heterochromatin. First, siRNAs can recruit H3K9 methylation to chromatin, possibly through physical interactions with the CLRC or dsRNA[70,81]. Thus, so long as siRNAs corresponding to a specific chromatin domain are present, they can recruit H3K9 methylation to that domain (Fig. 2b). The second feature involves a requirement for H3K9 methylation and chromatin localization in activating the siRNA positive-feedback loop[61,62,65]. This ensures that siRNAs are *cis*-restricted and is central to the role of siRNAs as epigenetic maintenance factors: siRNAs act only on those daughter DNA strands that have inherited old parental histone H3 molecules containing H3K9 methylation. Such cooperativity-based mechanisms involving the dual recognition of histone marks and other specificity factors (siRNAs or DNA-binding proteins) are likely to underlie all epigenetic *cis*-inheritance mechanisms.

RNAi and exosome-mediated RNA degradation

It may seem paradoxical that RNAi, which requires transcription, is required for assembling heterochromatin, a state that is associated with gene inactivation and TGS[3,51]. However, multiple mechanisms seem to ensure that transcription in heterochromatin does not result in the production of mature transcripts, thereby keeping heterochromatic genes off, despite transcription. First, heterochromatic transcripts are degraded or processed into siRNAs by the RNAi machinery itself through a process that has been referred to as CTGS or *cis*-PTGS[65,66] (Fig. 2b). CTGS requires the tethering of the RNAi machinery to heterochromatin by H3K9 methylation. This mechanism makes a major contribution to the silencing of some promoters in centromeric DNA repeats, although TGS is also an important contributing mechanism[66,71,82]. Second, an RNAi-independent RNA surveillance mechanism involving the TRAMP polyadenylation complex, which contains Cid14 (a Trf4/5 homologue), Air1, and Mtr1 in *S. pombe*, also targets heterochromatic transcripts for degradation[28]. In *S. cerevisiae*, TRAMP recognizes aberrant transcripts that lack polyadenylation signals and targets them for degradation by the exosome, a 3′→5′ exonuclease complex[30,31,83]. The presence of another member of the Trf4 polyadenylation polymerase family, Cid12, in the RDRC[61] suggests that RDRC and TRAMP may compete for access to heterochromatic transcripts (Fig. 1b). Furthermore, TRAMP and RDRC may compete more broadly for RNA substrates, because in *cid14* deletion cells new classes of RNAs become RNAi targets and are processed into siRNAs[29]. The involvement of members of the Trf4 family in RNAi processes in *C. elegans* and *T. thermophila* suggests a conserved role for members of this family in the coordination of exosome-mediated RNA surveillance with RNAi[84,85]. Finally, transcription in heterochromatin is cell-cycle regulated and is largely restricted to the S phase of the cell cycle[82,86]. This transcription is associated with high levels of siRNAs during the S phase, which may be important for epigenetic re-establishment of histone H3K9 methylation by the RITS–RDRC–CLRC complexes. However, it remains to be determined whether the increase in centromeric transcription and siRNA levels in S phase is merely a reflection of cell-cycle-associated changes in chromatin structure or has an important role in RNAi-mediated heterochromatin assembly.

Nearly all co-transcriptional RNA-processing events studied so far, including pre-mRNA capping, splicing and 3′-end processing, involve association between components of the processing machinery and RNA polymerase II (Pol II). Association with the polymerase is thought to help ensure that processing occurs in an orderly fashion and couples mRNA maturation with mRNA export. In addition, these associations serve to couple RNA quality control with transcription, ensuring that only true mRNAs are exported from the nucleus for translation. There is evidence that RNAi-mediated co-transcriptional heterochromatin assembly also involves interactions with Pol II[87,88]. Point mutations in two different Pol II subunits in *S. pombe*, Rpb2 and Rpb7, have been isolated in screens for defects in centromeric heterochromatin assembly. Neither mutation is associated with a growth defect or general perturbation of transcription[87,88], suggesting that the mutations may affect specific interactions with components of the RNAi machinery or the CLRC. Such interactions may contribute to efficient siRNA generation or H3K9 methylation by stabilizing the association of RITS–RDRC with nascent transcripts. Interestingly, in *A. thaliana*, RNA-dependent DNA methylation involves interactions between an Argonaute protein and RNA Pol IV, a plant-specific DNA-dependent RNA polymerase[89] (discussed below).

Conservation of small-RNA-mediated silencing

As discussed above, RNA silencing mechanisms have critical roles in endogenous chromatin-mediated processes in plants, *C. elegans*, *D. melanogaster*, ciliates and fungi. The role of small RNAs in chromatin silencing can also be extended to mammalian cells, although the mechanisms and physiological pathways are not yet clear. Reports from several laboratories provide evidence for the occurrence of DNA and histone modifications, which are promoted by the introduction

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of siRNAs or hairpin RNAs into mammalian cell lines[76,77,90,91]. In these studies, siRNAs are directed to the promoter regions of target genes and induce the recruitment of repressive histone marks such as H3K9 and H3K27 methylation[91], but silencing is not always associated with CpG methylation[76]. In addition to chromatin-modifying complexes, siRNA-mediated TGS in mammalian cells requires AGO1 and AGO2 when the gene encoding progesterone is targeted[77], and AGO1 when the gene encoding the human immunodeficiency virus 1 co-receptor (CCR5) is targeted[91]. The recent identification of endogenous siRNAs in *D. melanogaster* and mammalian cells, which map to intergenic regions and are produced from dsRNA resulting from antisense transcription or long-hairpin structures, raises the intriguing possibility that some of these siRNAs modulate chromatin structure[23–25].

The PIWI-clade proteins and their associated piRNAs have important roles in the control of transposons in the germline — and possibly somatic cells — of *D. melanogaster* and mammals[4]. The mouse MIWI2 member of this family is required for silencing the long interspersed nuclear element 1 (*LINE-1*) and intracisternal A particle (*IAP*) transposable elements in the testis, and in *Miwi2* mutants both *LINE-1* and *IAP* DNA is demethylated[92], suggesting that piRNAs, directly or indirectly, mediates changes in DNA methylation. It remains unclear how the role of PIWI proteins in transposon silencing in the germline may be related to their function in repeat-induced and heterochromatic gene silencing in somatic cells described in *D. melanogaster*[45,46].

Although the mechanisms that link RNA to chromatin and the biochemical nature of the relevant complexes have not been defined yet, the available evidence allows us to draw some parallels between the nascent transcript model in *S. pombe* and other systems. The common denominator in the RNA silencing pathways operating in genome regulation is the linkage of Argonaute or PIWI proteins to chromatin- or DNA-associated molecules (Fig. 3). Argonaute proteins associate with adaptor proteins containing the conserved GW motif, which binds to their PIWI domain and is required for miRNA-mediated silencing[93] (Fig. 3a). In *S. pombe*, the GW-motif-containing protein Tas3 links Ago1 to Chp1 (refs 10, 94, 95). The binding of Chp1 to a methylated nucleosome then serves to tether nascent non-coding RNA, which is base-paired with siRNA in Ago1, to chromatin (Fig. 3b). This tethering seems to be crucial in that it links RNAi to chromatin and 'activates' the Ago1-bound nascent transcript complex to mediate chromatin modifications[61,65]. A similar Argonaute tethering situation seems to exist in *A. thaliana*, where, in addition to AGO4 and DCL3, RNA-directed DNA methylation requires the plant-specific Pol IV[96–99]. Pol IV exists as Pol IVA and Pol IVB complexes, which differ in their largest component, NRPD1A and NRPD1B, respectively. Pol IVB and AGO4 are thought to act downstream of Pol IVA and DCL3, which are required for siRNA generation, to trigger DNA methylation. NRPD1B contains a GW motif at its carboxyl terminus[89]. This GW-motif-containing domain links Pol IVB to AGO4, providing a parallel with the function of other GW-domain-containing proteins, such as the Tas3 component of RITS in *S. pombe*[89,96] (Fig. 3e). Thus, in plants, the strategy for coupling RNAi to chromatin involves a physical interaction between a repeat- or heterochromatin-specific RNA polymerase and an Argonaute protein. Once an siRNA-programmed AGO4 localizes to a nascent transcript synthesized by Pol IVB, it may trigger histone H3K9 and DNA methylation by recruiting the appropriate methyltransferase enzymes (Fig. 3e).

The role of the *D. melanogaster* PIWI protein in repeat-induced gene silencing and heterochromatin assembly seems to involve a direct association between PIWI and HP1 (ref. 100) (Fig. 3c). PIWI–HP1 may function as a RITS that targets nascent transcripts in repeat DNA elements and tethers these transcripts to chromatin by means of base-pairing interaction between piRNAs in PIWI and the association of PIWI with HP1 (Fig. 3c). Unlike the case with RITS and AGO4, this tethering does not seem to involve a GW-domain-containing protein and is mediated by the HP1 CSD and a conserved CSD-binding PXVXL motif (where X

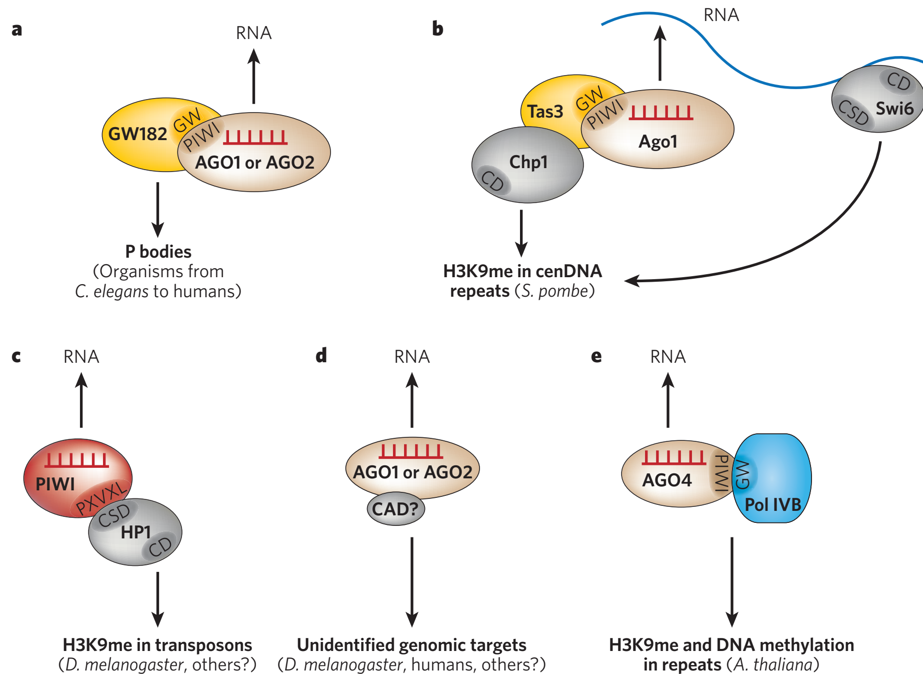

Figure 3 | Argonaute complexes that link RNA silencing to chromatin modifications. Argonaute proteins in different silencing pathways, including miRNA- and siRNA-mediated PTGS, are associated with conserved GW-motif-containing adaptor proteins, which help direct them to different targets. **a,** In many organisms, GW182 (a GW-motif-containing protein) or one of its homologues associates with the AGO1 and AGO2 proteins and directs them to P bodies. **b,** In *S. pombe*, Ago1 in the RITS is linked to heterochromatin through its association with the GW protein Tas3, which also binds to Chp1. Chp1 in turn associates with H3K9 methylated nucleosomes (H3K9me) through its chromodomain (CD). Swi6 (a homologue of HP1) acts as an accessory factor that helps tether the non-coding RNA to heterochromatin. The chromoshadow domain (CSD) is involved in protein–protein interactions. cenDNA, centromeric repeat DNA. **c,** In *D. melanogaster*, PIWI is targeted to heterochromatin through direct interactions with HP1; the association of PIWI with HP1 is mediated through the PXVXL motif, present in many HP1-binding proteins, rather than through a GW motif. **d,** In *D. melanogaster* and possibly other organisms, AGO1 and AGO2 have been implicated in mediating chromatin modifications, but the putative chromatin adaptor (CAD) protein has not been identified. **e,** In *A. thaliana*, AGO4 is linked to Pol IVB, which contains a GW motif at its carboxyl terminus and is specifically required for DNA methylation and silencing of heterochromatic repeats.

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is any amino acid) present in *D. melanogaster* PIWI[100]. The PIWI–HP1 complex may be required for the recruitment and spreading of H3K9 methylation or possibly for the co-transcriptional degradation of RNAs that may escape heterochromatic TGS. It remains to be determined whether this PIWI–HP1 complex acts more broadly in piRNA-mediated silencing of transposons in the germline. Similarly, the possible role of AGO1 and AGO2 in siRNA-dependent gene silencing may be mediated by interactions with unidentified chromatin adaptors (Fig. 3d).

Future prospects

RNAi and related RNA silencing pathways have emerged as new mechanisms for the regulation of the structure and activity of genes and genomes. Our understanding of the mechanisms that allow some small RNAs to act at the DNA and chromatin level, and restrict other small RNAs to mRNA regulation in the cytoplasm, is still at an early stage. Although accumulating evidence suggests that nuclear small-RNA pathways are conserved, the endogenous pathways that may use small RNAs for genome regulation in animal cells remain for the most part unknown. Another gap in our knowledge of nuclear small-RNA pathways in animal cells involves the biochemical identification of the molecular networks that link different types of small RNA to chromatin proteins. Whereas Argonaute and PIWI proteins, as well as small RNAs, have been implicated in mediating chromatin or DNA modifications, it remains unclear how specific chromosome regions are targeted and how modifying enzymes are recruited. Future studies are likely to provide new and surprising insights about the way in which small and large non-coding RNAs regulate chromatin structure and how this ability is, in turn, regulated. ■

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