

RNA-mediated epigenetic regulation of gene expression

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Abstract | Diverse classes of RNA, ranging from small to long non-coding RNAs, have emerged as key regulators of gene expression, genome stability and defence against foreign genetic elements. Small RNAs modify chromatin structure and silence transcription by guiding Argonaute-containing complexes to complementary nascent RNA scaffolds and then mediating the recruitment of histone and DNA methyltransferases. In addition, recent advances suggest that chromatin-associated long non-coding RNA scaffolds also recruit chromatin-modifying complexes independently of small RNAs. These co-transcriptional silencing mechanisms form powerful RNA surveillance systems that detect and silence inappropriate transcription events, and provide a memory of these events via self-reinforcing epigenetic loops.

RNA interference

(RNAi). Broadly refers to RNA silencing pathways that use Argonaute and PIWI proteins and small RNAs to silence gene expression.

Small interfering RNAs

(siRNAs). 22–24-nucleotide small RNAs that are generated from longer double-stranded RNA precursors by the ribonuclease Dicer.

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In many organisms, intergenic or antisense transcription gives rise to different classes of small RNAs and long non-coding RNAs (lncRNAs) that have emerged as key regulators of chromatin structure in eukaryotic cells^{1,2}. In addition to their roles in RNA degradation and translational repression, small RNAs modify chromatin and target gene expression via RNA interference (RNAi) pathways^{3–11} (BOX 1). In many instances, nuclear RNAi pathways mediate histone or DNA methylation events that repress transcription. Studies of the mustard plant *Arabidopsis thaliana*^{6–9,12} first demonstrated that post-transcriptional gene silencing, and the accompanying DNA methylation of target loci, correlated with the production of small interfering RNAs (siRNAs) and thus linked RNA-directed DNA methylation to the RNAi pathway, which had previously been described in *Caenorhabditis elegans*¹³. Studies in the fission yeast *Schizosaccharomyces pombe*, the ciliate protozoa *Tetrahymena thermophila*, as well as in animal germline and somatic cells, then revealed a general role for RNAi and related mechanisms in heterochromatin formation or DNA methylation^{10,14–17} (see [Supplementary information S1](#) (figure)).

RNA also regulates chromatin modifications and structure through pathways that do not involve RNAi; some lncRNAs, and even some mRNAs, seem to contain signals that recruit chromatin-modifying complexes independently of small RNAs¹⁸. Early examples include X inactive specific transcript (*XIST*), which coats the entire inactive X chromosome in female mammals, and *RNA on the X 1* (*roX1*) and *roX2*, which coat the

X chromosome in male flies, ultimately leading to increased transcription^{19–21}. More recently, a large number of other lncRNAs have been shown to act at a gene-specific level, rather than at a chromosomal level, to either activate or silence transcription^{18,22} (see [Supplementary information S1](#) (figure)).

A unifying mechanism by which small RNAs and lncRNAs modify chromatin structure and silence transcription is the formation of RNA scaffolds. Although the machineries that use RNA scaffolds have greatly diverged throughout evolution, a number of key similarities enable us to define the common themes and principles that are conserved in eukaryotes from fission yeast to mammals.

In this Review, we discuss recent progress in our understanding of the role of RNA in genome regulation, focusing on the roles of different classes of chromatin-bound RNAs as scaffolds for chromatin-modifying complexes. Moreover, we review recent mechanistic insights into how small-RNA amplification loops are coupled to histone or DNA methylation to form self-reinforcing positive feedback systems that maintain epigenetic states. First, we discuss how small RNAs and Argonaute (AGO) complexes are assembled and how they target specific chromatin regions for silencing, focusing on the better understood *S. pombe* and *A. thaliana* systems where distinct mechanisms have been elucidated by which siRNAs and histone or DNA methylation events form self-reinforcing epigenetic loops. Second, we review nuclear small-RNA silencing pathways in

Heterochromatin

Regions of chromatin that retain the condensed appearance of mitotic chromosomes throughout the cell cycle. Heterochromatic regions are associated with repressive histone modifications and structural proteins, and are transcriptionally silent.

Argonaute

(AGO). A family of proteins that bind to small RNAs and that are conserved in all domains of life. They mediate target recognition via base-pairing interactions between their bound small RNA and complementary coding or non-coding RNAs.

other model systems, including *C. elegans*, *Drosophila melanogaster* and mammals, highlighting conservation and divergence in the roles of these pathways in gene and genome regulation. Finally, we discuss the mechanisms by which lncRNAs and mRNAs interact with RNA processing and chromatin-modifying machineries independently of RNAi pathways.

RNAi-mediated heterochromatin assembly

RNAi-mediated transcriptional gene silencing is best understood in *S. pombe*, in which many basic principles of the pathway were first deciphered. *S. pombe* contains a single gene each for Ago (*ago1⁺*), Dicer (*dcr1⁺*) and RNA-dependent RNA polymerase (RdRP; *rdp1⁺*) (BOX 2). Deletion of any of these genes was shown to result in loss of heterochromatic gene silencing at pericentromeric DNA repeat regions and a reduction in the levels of histone H3 lysine 9 (H3K9) methylation, a conserved marker of heterochromatin in organisms ranging from yeast to plants and mammals¹⁰. Additionally, early sequencing experiments detected small RNAs that mapped to the pericentromeric repeat regions, and Rdp1 was found to associate with centromeric DNA^{10,11}. Based on these

results, it was suggested that RNAi has an important role in the initiation of heterochromatin formation and its subsequent maintenance at pericentromeric DNA repeats via the Rdp1-mediated recruitment of H3K9 methylation. However, later studies indicated that a small-RNA-programmed Ago1 complex mediates the targeting of specific chromosome regions and recruitment of H3K9 methylation²³ (see below).

The RITS complex and the emergence of the ‘nascent transcript’ model. A physical connection between the RNAi pathway and heterochromatin was established by the purification of Chp1, a chromodomain protein that is required for silencing the same heterochromatic regions targeted by RNAi^{23,24}. Chp1 was found to be a component of an RNA-induced transcriptional silencing (RITS) complex that also contains Ago1 and heterochromatic small RNAs (BOX 2). The third RITS complex subunit, Tas3, is also required for silencing and contains a conserved Ago-binding GW domain^{23,25,26}. The RITS complex associates with the RNA-dependent RNA polymerase complex (RDRC), which includes Rdp1, the helicase Hrr1 and the non-canonical poly(A) polymerase Cid12 (REF. 27). Both complexes associate not only with heterochromatin and one another, but also with non-coding pericentromeric RNAs²⁷. The functional importance of this RNA association is made clear by the observation that ectopic tethering of the RITS complex to a euchromatic mRNA triggers H3K9 methylation at its site of transcription²⁸. Together, these results led to the development of the ‘nascent transcript’ model for RNAi-dependent heterochromatin assembly^{27,28} (FIG. 1). Chromatin-associated RNAs are thought to act as scaffolds for the cooperative assembly of complex machineries that couple small-RNA-mediated recognition to chromatin modifications. This co-transcriptional gene silencing mechanism contains two primary components^{28–31}. First, the nascent transcript is degraded by both RNAi-dependent and RNAi-independent mechanisms, with RNAi-independent mechanisms involving TRAMP and exosome complexes²⁹. Second, RNAi-dependent H3K9 methylation leads to heterochromatin formation and transcriptional gene silencing^{30,31}. This small-RNA-based targeting strategy for heterochromatin assembly is in contrast with that used by the distant fungal relative *Saccharomyces cerevisiae*, in which specificity is instead determined by site-specific DNA-binding proteins (reviewed in REF. 32).

In addition to the RDRC and the RITS complex, the nascent transcript heterochromatin assembly platform also includes the Clr4–Rik1–Cul4 (CLRC) complex, the Clr4 subunit of which is the sole H3K9 methyltransferase in *S. pombe*^{33–37}. The CLRC complex subunit Rik1 associates with both the RDRC and the RITS complex, and the peripheral CLRC member Stc1 also provides a link to the RNAi machinery via its interaction with Ago1, although it seems to be dispensable for the interaction of the CLRC complex with the RITS complex subunit Tas3 (REFS 38–40). These physical connections suggest the existence of a feedback loop in which the activities of the RITS complex, the RDRC and the CLRC complex reinforce each other (FIG. 1) (see below).

Box 1 | Small-RNA silencing pathways

RNA interference (RNAi) is used broadly to refer to various RNA silencing pathways that use small RNAs, together with a member of the conserved Argonaute (AGO) and PIWI family of proteins, to target genes for inactivation at the post-transcriptional or transcriptional levels^{1,140}. Classical RNAi is triggered by long double-stranded RNA (dsRNA) precursors, which are processed to 22–23-nucleotide duplex small interfering RNAs (siRNAs) with 2-nucleotide 3′ overhangs by the RNase III ribonuclease Dicer^{141–145}. siRNA duplexes contain 5′ monophosphate and 3′ OH groups, and are loaded onto AGO family proteins, which associate with the siRNA 5′ monophosphate-containing nucleotide via their middle (MID) domain, and with the siRNA 3′ nucleotide via their PIWI–AGO–ZWILLE (PAZ) domain^{146–148}. The arrangement and specificity of the MID and PAZ domains in AGO proteins allow them to associate with small RNAs of specific sizes with distinct termini. siRNAs guide AGO proteins and their associated complexes to complementary RNAs, which are then targeted for degradation, translational repression or transcriptional silencing^{3–5}. In addition to the MID and PAZ domains, AGO proteins contain an RNase H-like domain that can cleave or slice target RNAs, promoting their degradation¹⁴⁹. In some organisms, this slicer activity is also critical for the release of one of the two AGO-bound siRNA strands and the conversion of duplex siRNA to mature single-stranded siRNA^{150–153}. In *Schizosaccharomyces pombe*, plants, *Tetrahymena thermophila* and *Caenorhabditis elegans*, siRNA-programmed AGO recruits an RNA-dependent RNA polymerase (RdRP), which uses the targeted RNA as a template to synthesize a dsRNA substrate for Dicer, thereby amplifying siRNAs and the RNAi response^{27,154–156}.

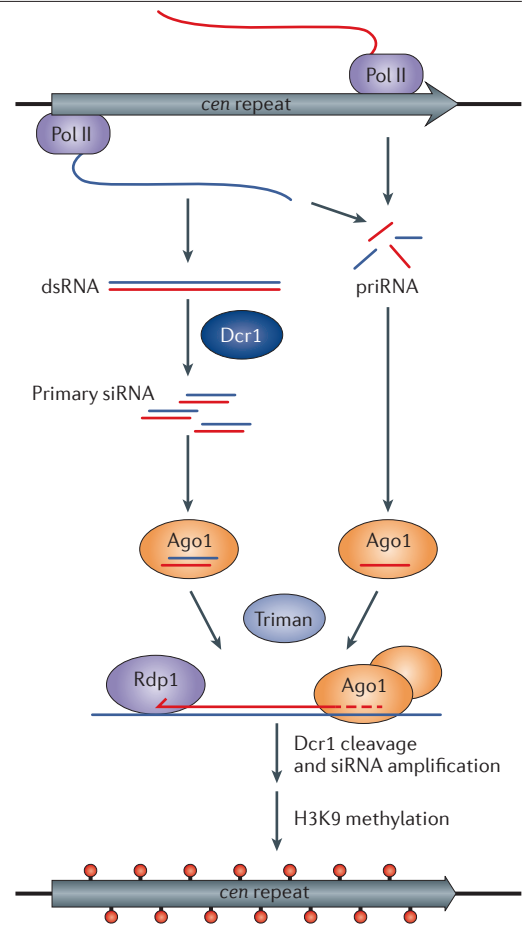
The genomes of *Drosophila melanogaster* and mammals do not seem to encode RdRPs. However, these organisms harbour another class of small RNAs, PIWI-interacting RNAs (piRNAs), which mediate RNA degradation in the cytoplasm and DNA or histone methylation in the nucleus^{157–160}. Metazoan piRNAs originate from single-stranded RNA precursors, and their amplification in the *D. melanogaster* and mammalian germ lines involves the ‘ping-pong’ cycle whereby piRNA-guided cleavage of complementary RNAs by one PIWI paralogue generates the 5′ ends of new piRNAs that are loaded onto another paralogue, and vice versa, in a process that degrades transposon mRNAs^{84,161,162}. It is proposed that piRNA pools are initially derived from random sampling of the transcriptome and then selectively enriched by ping-pong amplification for sequences corresponding to transposons that are actively transcribed and that are able to contribute substrates to the cycle⁸⁵. This idea is reminiscent of primal RNAs (priRNAs) in *S. pombe*. priRNAs also result from cellular RNA sampling by Ago1, but they only direct small-RNA amplification at loci where antisense RNA targets are available¹⁶³. This leads to repeat-specific RNAi-dependent siRNA accumulation¹⁶³.

Box 2 | Heterochromatic small RNAs

The discovery of small RNAs derived from the pericentromeric repeats of the fission yeast *Schizosaccharomyces pombe* marked the first example of heterochromatic small RNAs in any organism¹¹, and studies of *S. pombe* have remained useful for uncovering the modes of biogenesis of this small-RNA class. Non-coding transcripts generated from pericentromeric DNA repeats (*cen*) act as the precursors for heterochromatic small interfering RNAs (siRNAs) in *S. pombe*. Initially, it was hypothesized that heterochromatic small RNAs arise from Dicer 1 (Dcr1) cleavage of double-stranded RNA (dsRNA) formed by the base-pairing of complementary pericentromeric transcripts. According to this hypothesis, the RNA-dependent RNA polymerase Rdp1 amplifies the siRNA pool by generating additional Dcr1 substrates¹⁰. In this scenario, heterochromatic small RNA levels are expected to be higher in cells lacking Rdp1, in which pericentromeric transcripts from opposite strands can still hybridize and undergo Dcr1 processing, than in cells lacking Dcr1. Initially, no differences in small RNA levels in *rdp1*⁻ and *dcr1*⁻ cells were detected on northern blots or in early small-RNA sequencing experiments^{27,163}. However, recent advances in increasing the depth of sequencing have enabled the detection of a small population of Dcr1-dependent, Rdp1-independent heterochromatic small RNAs known as primary siRNAs, which are thought to arise from the base-pairing and Dcr1 processing of bidirectionally synthesized transcripts¹⁶⁴.

Dcr1 can limit the biogenesis of primary siRNAs, as overexpression of Dcr1 not only increases primary siRNA levels but also induces siRNA production from convergently transcribed loci genome-wide¹⁶⁴. Thus, limited Dcr1 availability is likely to represent an adaptation that restricts heterochromatic small-RNA biogenesis to a subset of loci with high levels of bidirectional transcription. The low abundance of Dcr1 and primary siRNAs is compensated for by the Rdp1-mediated generation of secondary heterochromatic siRNAs and also by the cooperation of the two enzymes — Dcr1 associates with Rdp1 and stimulates its dsRNA synthesis activity⁵⁰. Consistent with an adaptive relationship between low Dcr1 abundance and Rdp1 activity, overexpression of Dcr1 suppresses, to a large extent, the requirement for Rdp1 and for Dsh1, a factor required for Dcr1–Rdp1 association, in pericentromeric silencing and histone H3 lysine 9 (H3K9) methylation^{164,165}. These observations also rule out a critical role for Rdp1 in the direct recruitment of H3K9 methylation activity to chromatin as previously proposed¹⁰. Finally, a class of Dcr1-independent heterochromatic small RNAs known as primal RNAs (priRNAs), which are capable of directing H3K9 methylation in an Ago1-dependent manner, has been discovered (see the figure)¹⁶³. This finding, together with the detection of primary siRNAs¹⁶⁴, suggests that self-reinforcing feedback loops involving small RNAs and chromatin modification (BOX 3) may be nucleated by their small RNA components. In support of this idea, priRNAs and siRNAs, which are both trimmed to their mature length by the 3' exonuclease Triman, are required for the maintenance of RNAi-dependent facultative heterochromatin islands and *de novo* establishment of constitutive pericentromeric heterochromatin (see the figure)¹⁶⁶.

Pol II, RNA polymerase II.



Heterochromatic gene silencing

Silencing of gene expression within heterochromatin. It was originally thought to exclusively involve transcriptional gene silencing mechanisms, but recent findings indicate that co-transcriptional degradation of nascent RNA, or co-transcriptional gene silencing, also play important parts in silencing.

Pericentromeric DNA repeat

A repeated DNA sequence that surrounds the centromeres of most eukaryotic chromosomes. These repeats are assembled into heterochromatin, which has been demonstrated to have roles in cohesin recruitment in fission yeast and mammals, and *de novo* centromere assembly in fission yeast.

RNA-induced transcriptional silencing

(RITS). A protein complex first identified in *Schizosaccharomyces pombe*. In addition to Argonaute 1, the RITS complex contains a GW domain protein, Tas3, and a chromodomain protein, Chp1, which tether the complex to the chromosome via interactions with nascent long non-coding RNAs and nucleosomes with methylated histone H3 lysine 9.

The central role of nascent RNAs in the recruitment of RNAi and histone-modifying activities has prompted investigations of the interactions between RNA-mediated silencing and other nuclear processes in *S. pombe*. The CLRC complex components Raf2 (also known as Cmc2) and Rik1 were recently found to associate with Cdc20 (the catalytic subunit of the leading-strand DNA polymerase-ε) and Mms19 (a conserved regulator of the general transcription factor TFIIH⁴¹), suggesting the coordination of DNA replication with RNAi-dependent release of RNA polymerase II (Pol II) in the inheritance of heterochromatin⁴². However, this

Raf2–Rik1 complex seems to be distinct from the CLRC complex, and direct evidence of cooperation between the RNAi and DNA replication machineries is lacking. It has also been proposed that the splicing machinery contributes to heterochromatic silencing by directly interacting with the RDRC and the nascent transcript platform to promote siRNA biogenesis^{43,44}. However, earlier biochemical purifications demonstrated that the RDRC subunit Cid12 forms RDRC-independent complexes with splicing factors²⁷. Moreover, the RNAi defect found in several splicing mutants can be partially rescued by the introduction of cDNAs that encode

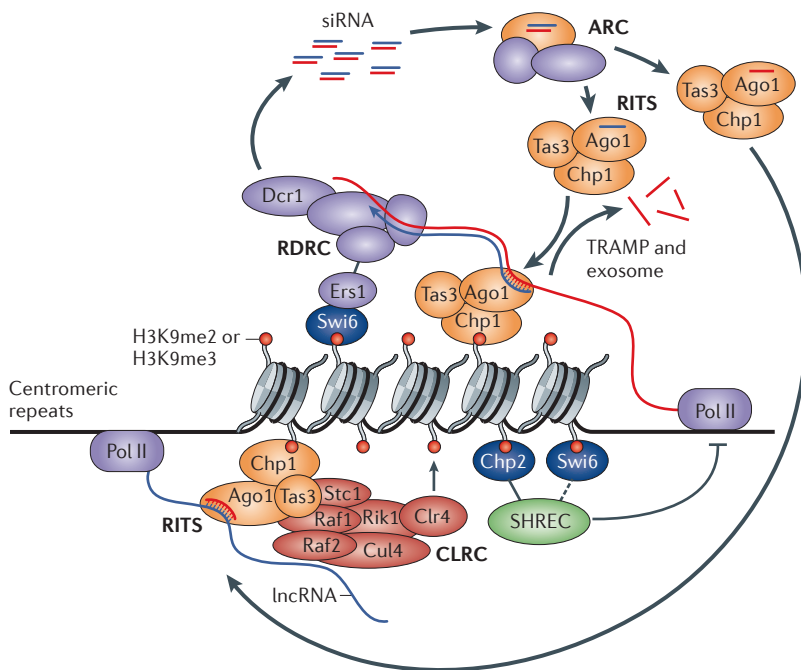


Figure 1 | The ‘nascent transcript’ model and a self-reinforcing epigenetic loop in *S. pombe*. In *Schizosaccharomyces pombe*, the RNA-induced transcriptional silencing (RITS) complex establishes a physical connection between small interfering RNAs (siRNAs) and heterochromatin by targeting a nascent transcript, and forms the basis of a self-sustaining feedback mechanism that couples siRNA production to chromatin modification. A siRNA-targeted centromeric long non-coding (lncRNA) bound to the RITS complex becomes a template for double-stranded RNA (dsRNA) synthesis by the RNA-dependent RNA polymerase complex (RDRC, which is composed of Rdp1, Hrr1 and Cid12) and generation by Dicer 1 (Dcr1) of new siRNAs, leading to further targeting of the RITS complex after passage of Argonaute (Ago) through the ARC (Ago siRNA chaperone) complex. The Chp1 subunit of the RITS complex anchors the complex onto nucleosomes with histone H3 lysine 9 (H3K9) methylation, and the RITS complex recruits the Ctr4–Rik1–Cul4 (CLRC, of which Ctr4 is the methyltransferase) complex via Rik1 and Stc1 to promote the further spread of H3K9 methylation. The heterochromatin protein 1 (HP1) homologue Swi6 binds to methylated H3K9 and promotes RDRC recruitment and siRNA biogenesis via the silencing factor Ers1. Swi6, and particularly the other HP1 protein Chp2, help to restrict RNA polymerase II (Pol II) access by recruiting the Snf2–histone deacetylase repressor complex (SHREC). The TRAMP non-canonical poly(A) polymerase and the exosome also contribute to silencing. Together, the RITS complex and the nascent lncRNA transcript provide a hub for the assembly of machineries that make siRNAs, modify histones and silence gene expression. me2, dimethylation.

RNAi components⁴⁵, suggesting that the effects of splicing mutations on heterochromatic silencing are at least partially due to improper splicing of pre-mRNAs that encode RNAi proteins.

siRNAs form self-reinforcing epigenetic loops with DNA and histone methylation. The observation that silent states of gene expression and their associated chromatin modifications can spread along DNA in *cis* and persist throughout cell division³² led to the idea that positive feedback loops might form the molecular basis for epigenetic memory. Linking the recognition of signals — such as covalent histone modifications, DNA methylation or small RNAs — to the generation of new signals would ensure the stable propagation of silent states^{46–48} (BOX 3).

Indeed, this paradigm of self-reinforcing loops has found consistent support from experimental findings in the small-RNA-dependent epigenetic pathways of both fungi and plants.

In *S. pombe*, the discovery that pericentromeric H3K9 methylation is dependent on the RNAi machinery was quickly followed by the realization that, conversely, pericentromeric siRNA accumulation also requires the H3K9 methyltransferase Ctr4 (REFS 27,49). Several physical interactions are now known to underlie this mutual dependence. First, the bivalent nature of the RITS complex — its ability to interact with nascent RNA via its siRNA-bound Ago1 subunit and with chromatin via its Chp1 subunit — forms the physical basis for a self-reinforcing positive feedback loop (FIG. 1). Methylated H3K9 stabilizes RITS complex binding via Chp1 (REF. 49), promoting siRNA amplification by the RITS complex-interacting factors RDRC and Dcr1 (REFS 27,50). Second, methylated H3K9 enhances siRNA accumulation by helping to recruit the RDRC via the heterochromatin protein 1 (HP1) homologue Swi6 in an interaction that is directly bridged by Ers1 (REFS 30,51,52). Finally, direct associations between the siRNA-programmed RITS complex and the Rik1 and Stc1 subunits of the CLRC^{38–40} ensure that siRNAs in turn feed back on H3K9 methylation states (FIG. 1).

The notion of self-reinforcing epigenetic states that involve multiple types of signals was first proposed by Eric Selker while working with the filamentous fungus *Neurospora crassa*⁴⁶. Initial results revealed that DNA methylation patterns were dependent on histone deacetylation, but recent findings suggest that feedback loops that involve small RNAs also exist in *N. crassa*. The canonical DNA methylation pathway is RNAi-independent⁵³ and acts downstream of, and without feeding back on, H3K9 methylation states⁵⁴. By contrast, a newly uncovered DNA methylation phenomenon exhibits self-reinforcing properties and correlates with the biogenesis of small RNAs. This type of DNA methylation occurs at gene-rich and intergenic loci that are transcribed in a convergent manner and that also give rise to Dicer-independent siRNAs (disiRNAs)^{55,56}. Remarkably, disiRNA loci DNA methylation (DLDM), unlike the well-studied DNA methylation pathway associated with repetitive sequences, is required for H3K9 trimethylation (H3K9me3) and is thus self-reinforcing through the known HP1-dependent mechanism that recruits the DNA methyltransferase DIM-2 to the regions of H3K9 methylation^{54,55}. It was also observed that, for a given DLDM locus, most clones are not methylated; however, when methylation does occur clones are methylated extensively across the disiRNA-producing locus, suggesting that a powerful positive feedback loop leads to the spread of the epigenetic mark⁵⁵. Convergent transcription is critical and, when induced artificially, is sufficient to drive this dynamic DNA methylation pattern⁵⁵. The potential roles of the disiRNAs themselves, and other mechanistic aspects of DLDM, remain to be elucidated, but these results further underscore the prevalence of self-reinforcing epigenetic loops among small-RNA silencing pathways in the nucleus.

RNA-directed DNA methylation in *A. thaliana*. In *A. thaliana*, 24-nucleotide siRNAs direct *de novo* DNA methylation and maintenance of DNA methylation at asymmetrical CHH sites (where H represents any base except G) in a manner that depends on two plant-specific polymerase II (Pol II)-related RNA polymerases, Pol IV and Pol V⁵⁷ (reviewed in REF. 58). This phenomenon exhibits many physical connections between pathway components that form the basis of a self-reinforcing epigenetic loop (FIG. 2). First, 24-nucleotide siRNAs are generated by DCL3 processing of double-stranded RNA (dsRNA) synthesized by the RdRP RDR2 using Pol IV transcripts as templates. Recent *in vitro* experiments have shown that RDR2 and Pol IV associate in an RNA-independent manner and that the activity of RDR2, but not of Pol IV, requires this association, which suggests an evolved mechanism for limiting siRNA biogenesis to loci occupied by Pol IV⁵⁹. Loading of siRNAs onto AGO4 in the cytoplasm triggers its import into the nucleus⁶⁰, where it associates directly with siRNA-complementary Pol V transcripts^{61,62}. This sequence-specific recruitment is reinforced by direct interactions between AGO4 and the GW domains of both Pol V and the Pol V transcript-binding protein KTF1 (also known as SPT5L)^{25,63}. While these multiple associations stabilize the localization of AGO4 along a Pol V nascent transcript, the RDM1 protein provides a physical link between AGO4 and the DRM2 DNA methyltransferase, illustrating how the instruction to silence gene expression is conveyed from small RNAs to covalent DNA modifications⁶⁴. RDM1 also has an affinity for methylated DNA, which raises the possibility that it can direct the Pol V–KTF1–DRM2–AGO4 complex to pre-existing sites of DNA methylation. A recent study shows that AGO4 co-purifies with DRM2 *in vivo*, and that DRM2 preferentially methylates the strand of DNA that acts as a template for Pol V transcripts⁶⁵. The authors propose that as AGO4 binds to a siRNA-complementary nascent Pol V transcript, it directs DRM2 specifically to the template DNA strand as it emerges from the Pol V exit channel. This study provides an unprecedented level of mechanistic detail on the feedback between small RNAs and DNA methylation.

Interestingly, at a subset of loci, deletion of DNA methylation by mutations in the histone deacetylase HDA6 or the maintenance cytosine methyltransferase MET1 results in loss of silent locus identity⁶⁶. This observation indicates that, at these loci, silent locus identity is maintained by self-reinforcing interactions involving histone deacetylation and DNA methylation, while siRNA amplification and silencing rely on cooperation between the Pol V–KTF1–DRM2–AGO4 complex and Pol IV machineries (see above) and on pre-existing chromatin modifications⁶⁶.

Embedded in the RNA-dependent DNA methylation pathway are important self-reinforcing feedback mechanisms that have been discovered only lately. For instance, regions of RNA-directed DNA methylation in *A. thaliana* are also enriched in H3K9 methylation⁵⁸, and these two epigenetic signals mutually support one another. Three distinct H3K9 methyltransferases, KYP (also known

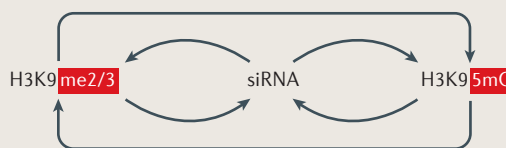
as SUVH4), SUVH5 and SUVH6, contribute to the maintenance of DNA methylation patterns, with KYP having the primary role⁵⁸. KYP is recruited to sites of DNA methylation via its SET and RING finger-associated (SRA) domain⁶⁷ and, conversely, the Pol IV-associated factor SHH1 binds to nucleosomes with methylated H3K9 and unmethylated H3K4 via its SAWADEE domain, thus promoting siRNA-dependent methylation of the underlying DNA^{68,69}. Another example of self-reinforcement involves the SUVH2 and SUVH9 proteins which, like KYP, bind to methylated DNA via their SRA domains but, unlike KYP, lack H3K9 methyltransferase activity⁷⁰. Two recent studies indicate that these SRA domain-containing proteins directly recruit Pol V to sites of pre-existing DNA methylation, thus generating nascent transcript scaffolds for recruitment of AGO4–siRNA complexes and further enhancement of the DNA methylation signal^{71,72}. This dense network of positive feedback in *A. thaliana* suggests that the broad evolutionary relevance of self-reinforcing siRNA-driven epigenetic loops extends beyond the *S. pombe* system in which they were first suggested.

Conservation and divergence of nuclear RNAi

Below, we discuss examples of small-RNA-mediated silencing in the animal germ line, including *C. elegans*, *D. melanogaster* and mammals. Nuclear RNAi in these systems differs from the examples in *S. pombe* or *A. thaliana* in one major aspect. Although animal small RNAs act as signals that trigger the modification of chromatin or DNA, it remains unclear whether small-RNA amplification involves a self-reinforcing

Box 3 | Self-reinforcing positive feedback loops

Self-reinforcing positive feedback loops are formed by the functional coupling of different types of signal generation events. Studies in *Schizosaccharomyces pombe*, *Arabidopsis thaliana* and mammals have uncovered the molecular basis of these coupling events by identifying the proteins that recognize either histone H3 lysine 9 dimethylation (H3K9me2) and H3K9me3, or DNA cytosine methylation (DNA-5mC), and that recruit enzymes which catalyse the other methylation events. In some organisms (for example, *S. pombe* and *A. thaliana*), the methylation events are also physically coupled to proteins that recruit small interfering RNA (siRNA) amplification loops to chromatin, thus forming self-reinforcing loops in which the histone or DNA methylation event promotes siRNA generation and siRNAs in turn promote histone or DNA methylation. Self-reinforcing loops are thought to help to ensure the maintenance of epigenetic information^{47,48}.



relationship with the downstream chromatin or DNA modifications. Recent studies of the *D. melanogaster* Piwi-interacting RNAs (piRNAs), so called because they partner with Ago proteins of the largely germline-specific Piwi subfamily, point in this direction, but mammalian pathways seem to lack this characteristic. Nuclear RNAi-related events associated with the introduction of foreign siRNAs into animal somatic cells are not discussed here.

Small RNAs and chromatin in animal cells: the piRNA system. piRNAs were first discovered in mice and *D. melanogaster*, and their roles in silencing transposons in the germ line of these organisms are well established (reviewed in REF. 73). The *C. elegans* genome also encodes piRNAs, called 21U-RNAs, but they have only been implicated in the repression of one transposable element^{74,75}; we discuss the roles of 21U-RNAs in epigenetic silencing in the next subsection.

In *D. melanogaster*, recent evidence suggests that the nuclear protein Piwi, in addition to mediating

post-transcriptional gene silencing of transposons by contributing to ‘ping-pong’ amplification with its cytoplasmic counterparts Aubergine (Aub) and Ago3, also targets transposons at the transcriptional level^{76–80}. In ovarian somatic cells, which also harbour piRNAs, most of the euchromatic H3K9 methylation islands in the genome correspond to transposon insertion sites and are Piwi-dependent⁷⁹. Furthermore, artificial recruitment of Piwi to a reporter locus induces H3K9 methylation, HP1a (also known as Su(var)205) accumulation and exclusion of RNA Pol II, suggesting that piRNAs may have a direct role in guiding chromatin changes^{76,77}. Piwi and HP1a were previously reported to interact directly *in vivo*⁸¹, but the reproducibility and physiological importance of this result have been debated^{77,80}, raising the possibility that there are unidentified links that transmit instructions from piRNAs to chromatin. Mechanisms involving Piwi engagement with nascent transcripts or even the underlying DNA have been proposed^{76,77}, but understanding the molecular details remains a major challenge for future research.

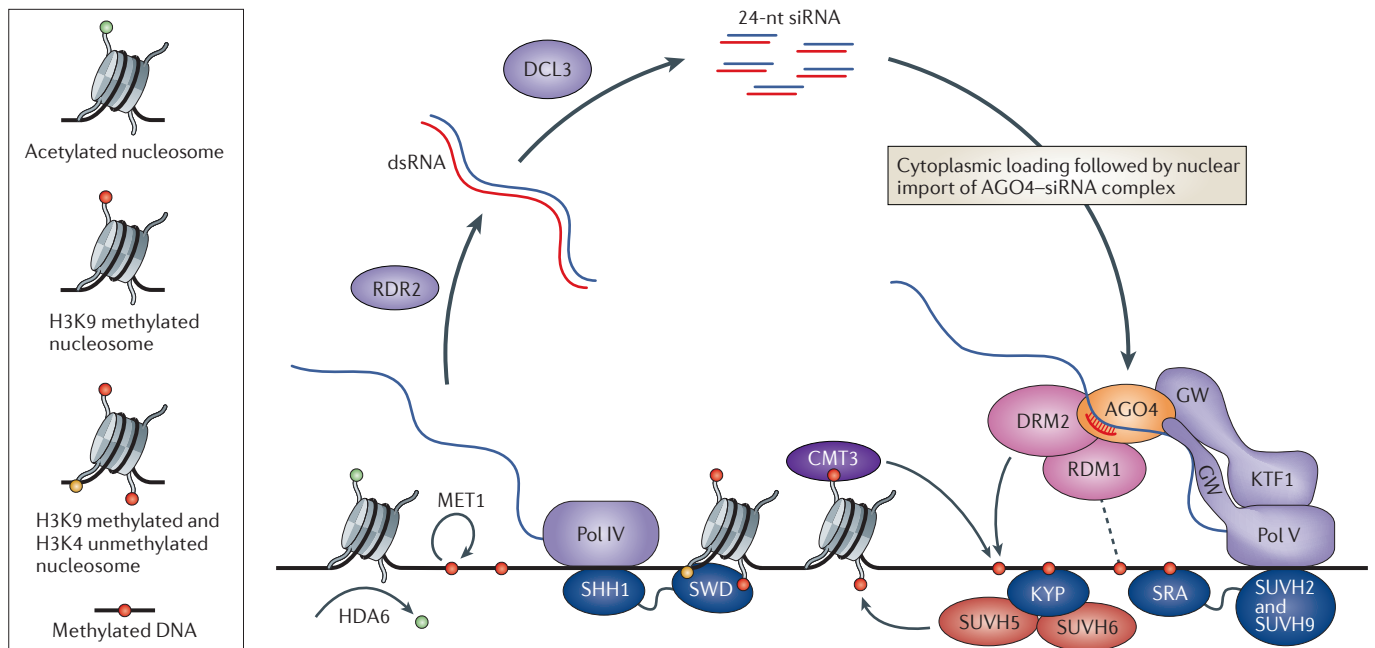


Figure 2 | A self-reinforcing loop linking siRNAs to DNA and histone methylation in *A. thaliana*. Elaborate feedback between small RNAs and DNA and histone methylation underlies a robust silencing pathway at sites of asymmetrical cytosine methylation in the *Arabidopsis thaliana* genome. Two plant-specific polymerases transcribe the critical RNAs. RNA polymerase IV (Pol IV) transcripts are processed by the RNA-dependent RNA polymerase RDR2 and the Dicer protein DCL3 into 24-nucleotide (nt) small interfering (siRNAs), while Pol V transcripts act as their targets. The Argonaute protein AGO4, the siRNA-dependent recruitment of which to Pol V transcripts is reinforced by interactions with the GW domains of Pol V and an associated elongation factor KTF1, in turn recruits the CHH DNA methyltransferase DRM2. RDM1 associates with the Pol V–AGO4–DRM2 complex and may link siRNA amplification to pre-existing DNA methylation. Meanwhile, another DNA methyltransferase that targets CHG sites for maintenance, CMT3, is recruited directly to methylated histone H3 lysine 9 (H3K9). Silencing by DNA methylation is augmented by H3K9

methylation, which is deposited by the enzymes KYP, SUVH5 and SUVH6. These methylation events are coupled to one another and to siRNA activity in several ways. KYP is recruited directly to methylated DNA, where it methylates neighbouring histones, and the H3K9 methylation reader SHH1 recruits Pol IV to promote siRNA generation, while the DNA methylation readers SUVH2 and SUVH9 recruit Pol V to promote AGO4 targeting and further DNA methylation. Thus, the different methylation readers, RNA polymerases and AGO4 act together to create self-reinforcing interactions between pre-existing DNA methylation and siRNA amplification. Erasure of DNA methylation by mutations in either the histone deacetylase HDA6 or the maintenance DNA methyltransferase MET1 results in loss of siRNA biogenesis, emphasizing the importance of these self-enforcing interactions. Altogether, the *A. thaliana* pathway for DNA methylation at asymmetrical sites is one of the most remarkable examples of a recurring theme in epigenetic regulation by small RNAs: self-reinforcing feedback loops. SRA, SET and RING finger-associated; SWD, SAWADEE domain.

Recent findings also show that sites bearing Piwi-dependent H3K9 methylation in turn function as source loci for piRNA production in *D. melanogaster* ovaries. Methylated H3K9 recruits the HP1 family protein Rhino, which, together with the Cutoff protein, promotes non-canonical transcription from these loci to generate precursors to be processed into piRNAs^{82,83}. Therefore, not unlike the plant and fungal systems discussed above, the *D. melanogaster* piRNA pathway seems to exhibit a positive feedback loop in which small RNAs guide histone methylation, which in turn enables biogenesis of more small RNAs.

In mice, piRNAs silence transposons in the male germ line by targeting them for *de novo* DNA methylation during late embryonic and early neonatal development^{84–87}. Consistently, the nuclear PIWI protein, Piwi-like protein 4 (PIWL4; also known as MIWI2), is expressed at this time⁸⁵. Thus, like plants, mammals have an RNA-directed DNA methylation pathway, although few of its mechanistic details are currently understood. Arguing against a self-reinforcing loop is the observation that piRNA levels remain high in DNA (cytosine-5-)-methyltransferase 3-like (*Dnmt3l*) mutants, in which transposon DNA methylation is completely compromised⁸⁵. However, it is unclear to what extent H3K9 methylation at transposons, which could also contribute to piRNA generation, is affected in *Dnmt3l* mutants. One study has suggested that piRNA-directed DNA methylation in mice involves a system that is similar to the nascent transcript model in *S. pombe*⁸⁸. The authors found that piRNAs mapped to a long terminal repeat (LTR) adjacent to a paternally imprinted locus, RAS protein-specific guanine nucleotide-releasing factor 1 (*Rasgrf1*), and that piRNA pathway components are required for methylation of *Rasgrf1*. They also found evidence of sequence-specific piRNA-dependent cleavage of a non-coding RNA overlapping with the LTR, supporting the nascent transcript theory⁸⁸. However, it has also been shown that the endonuclease activity of PIWL4 is completely dispensable for DNA methylation of long interspersed elements (LINE1) and the intracisternal A particle (IAP) family of LTR retrotransposons⁸⁹. This suggests that nascent transcript slicing is not an important event in small-RNA-dependent chromatin silencing in mice, although it has a critical role in piRNA amplification and post-transcriptional gene silencing.

Finally, piRNAs also contribute to epigenetic phenomena in which they are themselves responsible for epigenetic inheritance. This idea first emerged in a landmark paper demonstrating the requirement for maternal deposition of piRNAs, in addition to the presence of genomic piRNA loci, for effective transposon silencing and fertility in the offspring¹⁵. A recent study found that in *D. melanogaster*, strains containing certain *P-lacZ* transgene insertions, but not other strains containing similar insertions at the same site, showed silencing of homologous sequences in *trans*⁹⁰. Remarkably, if maternally inherited, the *trans*-silencing-competent alleles could convert the paternally inherited alleles into strong *trans*-silencers, which was accompanied by *de novo* production of piRNAs corresponding to these

trans-silencing loci. The new allele also acquired the ability to convert other alleles into *trans*-silencers in the same manner; thus, this constitutes a case of paramutation^{90,91}. These results indicate that maternally deposited cytoplasmic piRNAs can direct the conversion of homologous sequences into piRNA-generating loci with *trans*-silencing ability. This work shows how a locus can become incorporated into the piRNA repertoire and how RNA itself can act as a carrier of epigenetic information. Future discoveries regarding the molecular basis of this paramutation process will provide fundamental insights into both piRNA biology and the principles of epigenetic inheritance.

RNAi and H3K9 methylation in *C. elegans*. *C. elegans* has an array of 27 AGO homologues that associate with diverse small RNA populations and that act in distinct silencing steps⁹². Classical RNAi, induced by exogenous dsRNA, initially involves the AGO protein RDE-1 and primary siRNAs; however, RdRPs generate secondary siRNAs that are not loaded onto RDE-1 (REF. 92). Among other roles, secondary siRNAs can trigger RNAi in the nuclei of somatic cells by acting with the AGO protein NRDE-3. Upon small-RNA loading, NRDE-3 enters the nucleus, associates with complementary nascent transcripts and recruits the non-AGO silencing factor NRDE-2, which in turn promotes H3K9 methylation and inhibits elongation of RNA polymerases beyond the site targeted by siRNAs^{17,93,94} (FIG. 3). Thus, as in fungi and plants, small RNAs in *C. elegans* can trigger transcriptional gene silencing. However, currently, the biochemical connections remain obscure except for a direct interaction between NRDE-3 and NRDE-2 (REF. 16). More recent work has shown that additional AGO homologues are also required for chromatin targeting¹⁷. Interestingly, RNAi-induced H3K9 methylation is a transgenerational phenomenon, as it is inherited for at least two generations, in the absence of the dsRNA trigger, by a mechanism that involves germline transmission of siRNAs^{17,95}.

In the *C. elegans* germ line, inheritance of H3K9 methylation induced by exogenous dsRNA is mediated by the germline-specific AGO protein HRDE-1 (also known as WAGO-9) in association with RdRP-derived siRNAs^{96,97}. Intriguingly, both HRDE-1 and NRDE-2 are also necessary for germline immortality and fertility⁹⁷. This biological requirement is reminiscent of the piRNA pathway, the widely conserved germline maintenance system discussed above and represented in *C. elegans* by the 21U-RNAs and the PIWI-subfamily AGO protein PRG-1 (REF. 74). Indeed, several studies have concluded concurrently that HRDE-1 is in fact the downstream effector of piRNA-induced silencing in the germ line^{96,98–100}. Another observation was also made — while HRDE-1, NRDE-2 and the HP1 homologue HPL-2 are required to maintain a permanent memory of these silencing events at the chromatin level, PRG-1 and 21U-RNAs are only required for its initiation^{96,99,100} (FIG. 3). Thus, different components are responsible for the establishment and maintenance steps in this example of RNA-mediated epigenetic regulation.

Long terminal repeat

(LTR). A DNA sequence that is repeated at the ends of retrotransposons or pro-viral DNA that is formed from retroviral RNA by reverse transcription. Plant and mammalian genomes contain thousands of LTRs.

Epigenetic phenomena

Phenomena in which changes in gene expression occur without a corresponding change in the DNA sequence; such changes are stable in the absence of initiating signals.

Paramutation

The ability of a silent allele to convert an active allele to the silent (and paramutagenic) form. It was first described in *Zea mays*.

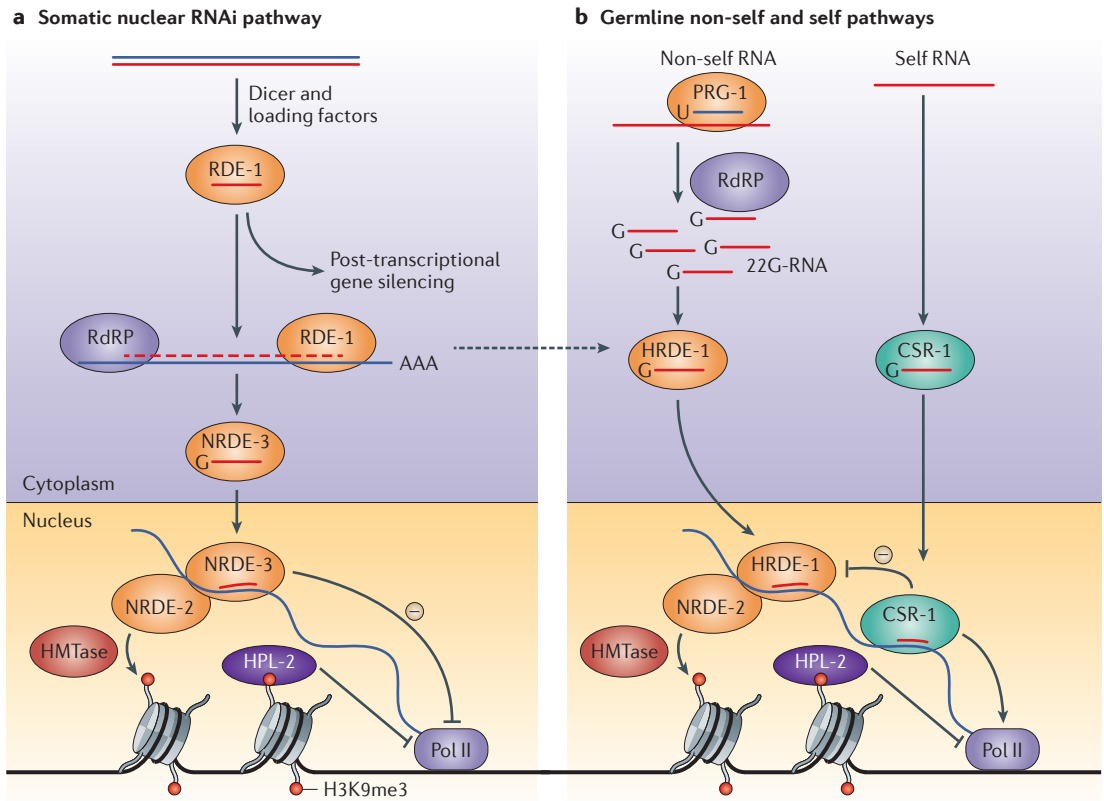


Figure 3 | Small-RNA-driven transcriptional silencing of gene expression in *C. elegans*. **a** | In *Caenorhabditis elegans*, exogenous double-stranded RNA (dsRNA) is processed into primary small interfering RNAs (siRNAs) that are loaded onto the Argonaute (AGO) protein RDE-1 and amplified by RNA-dependent RNA polymerases (RdRPs) to give rise to secondary siRNAs called 22G-RNAs. When loaded with 22G-RNAs, the somatic AGO protein NRDE-3 translocates to the nucleus, where it targets nascent RNA transcripts and silences corresponding genes, acting in concert with the silencing factor NRDE-2. Gene expression is halted by NRDE-2 during the elongation phase of transcription, and silencing involves histone H3 lysine 9 trimethylation (H3K9me3) and recruitment of the heterochromatin protein 1 (HP1)-like protein HPL-2. **b** | In the germ line, small-RNA-directed transcriptional silencing is mediated not by NRDE-3 but by a different AGO protein, HRDE-1, which also acts through NRDE-2, H3K9 methylation and HPL-2. HRDE-1 receives 22G-RNA inputs both from the pathway that responds to exogenous dsRNA and from the PIWI-interacting RNA (piRNA), or 21U-RNA, pathway that scans the transcriptome for foreign RNAs. 21U-RNA-programmed PRG-1 promotes the RdRP-dependent generation of 22G-RNAs, which are loaded onto HRDE-1. In both cases, HRDE-1 maintains a persistent, transgenerational memory of silenced genes in the germ line. Meanwhile, another AGO protein called CSR-1 binds to 22G-RNAs that represent the full complement of endogenously expressed RNAs, and protects the corresponding loci from possible silencing by HRDE-1. Thus, the 22G-RNAs bound by CSR-1 and HRDE-1 transmit a germline memory of ‘self’ and ‘non-self’ RNAs, respectively, to be appropriately licensed for expression or silenced. HMTase, histone methyltransferase; Pol II, RNA polymerase II.

In contrast to piRNAs in *D. melanogaster* and mammals, *C. elegans* piRNAs only silence one transposon family^{74,75}. However, transgene insertions can be silenced in a PRG-1-dependent manner^{96,99,100}, supporting a conserved function for piRNAs in recognizing ‘non-self’ nucleic acids. Interestingly, PRG-1 was found to initiate epigenetic silencing of the same single-copy transgene in some lines but not others¹⁰⁰. Genetic crosses indicate that transgene expression states can be converted in both directions by *trans*-acting silencing and activating factors, leading to the proposal that HRDE-1–siRNA complexes enforce the maintenance of PRG-1-directed silencing, whereas another system prevents or even reverses its establishment¹⁰⁰. The suggested candidate

is the AGO protein CSR-1, the RdRP-dependent small RNA partners of which map not to silenced loci but instead to all mRNAs expressed in the germ line¹⁰¹. This was confirmed by recent experiments in which CSR-1-dependent activity was shown to counteract PRG-1-mediated silencing in a heritable manner^{102,103}. Thus, PRG-1, associated with 21U-RNAs, is proposed to sample cellular transcripts for non-self sequences to be silenced by HRDE-1, while CSR-1 carries a repertoire of self-expressed sequences to be protected from silencing^{96,99,100,102,103} (FIG. 3). CSR-1 seems to have a critical role in sperm, where it promotes spermiogenic gene expression and where its associated small RNAs reflect previous germline expression patterns¹⁰⁴.

RNA-dependent histone methylation without RNAi

In the RNAi-mediated chromatin silencing pathways discussed above, a recurring feature is the role of RNA as an assembly scaffold. Below, we focus on analogous but RNAi-independent strategies used by both mRNAs and lncRNAs to recruit histone-modifying enzymes. LncRNAs, typically defined as transcripts more than 200 nucleotides in length that do not encode proteins, have been uncovered in large numbers as a result of rapid advances in high-throughput technologies. The function of lncRNAs in genome regulation, although still largely unknown, seems to be diverse and is the subject of a growing field of study (reviewed in REF. 105). A common theme in current models is the ability of many lncRNAs to target chromatin-modifying activities to particular genomic sites. Perhaps the most extensively studied examples are those that involve dosage compensation in metazoans (reviewed in REF. 106), including the mammalian lncRNA *XIST*, which mediates global inactivation of a randomly chosen X chromosome in females in a process known as X chromosome inactivation (XCI). *XIST* has been proposed to function by directing the Polycomb repressive complex 2 (PRC2) H3K27 methyltransferase to chromatin. Below, we examine the evidence and unresolved questions regarding the recruitment of Polycomb to chromatin by *XIST* and other lncRNAs. The basis of specificity for the proposed protein–RNA interactions remains unknown. We also discuss a class of lncRNAs transcribed from enhancers that are thought to activate gene expression *in cis*. Finally, we describe a case in which the mode of specificity is better understood — the targeting of the Clr4 H3K9 methyltransferase by the mRNAs of meiotic genes to their gene loci in *S. pombe*.

lncRNAs and Polycomb recruitment. Polycomb proteins and H3K27me3 were first linked to XCI by genetic and cytological experiments^{107–109}. A more direct biochemical connection was shown by the detection of *XIST* RNA in PRC2 immunoprecipitation experiments and a corresponding *in vitro* test of their interaction¹¹⁰. *REPA* — an independent internal transcript within *XIST*, so called because it spans the conserved A repeat region of *XIST* — was proposed to interact directly with and recruit the PRC2 catalytic subunit EZH2 in the earliest stages of XCI establishment. The same region of full-length *XIST* would then directly recruit PRC2 as silencing spreads *in cis*¹¹⁰. It was also reported that other lncRNAs—such as KCNQ1 opposite strand/antisense transcript 1 (*Kcnq1ot1*) and HOX transcript antisense RNA (*HOTAIR*)—could directly recruit PRC2 to chromatin *in cis* or *in trans*, respectively^{111,112}. However, one study failed to detect an interaction between PRC2 and the *XIST* A repeats using an ultraviolet crosslinking approach¹¹³, and others have found the *XIST* A repeat to be dispensable for PRC2 chromatin association and H3K27 methylation in mice and humans^{107,114}. More generally, the idea that lncRNAs mediate PRC2 targeting through direct interactions has been undermined both by doubts regarding specificity and by the existence of equally compelling alternative

scenarios (reviewed in REF. 115). The number of RNAs found to be associated with PRC2 in cells is enormous^{116–119}, highlighting the risk of interpreting individual interactions as specific. For instance, a recent study revealed that the binding affinity of PRC2 for the bacterial *MBP* mRNA is equal to its binding affinity for *HOTAIR*, and that RNA length is a far better predictor of PRC2 binding affinity than RNA sequence¹¹⁶. Thus, the direct interactions reported between PRC2 and lncRNAs, such as *HOTAIR*, *RepA* and *Kcnq1ot1* (REFS 110–112) among others, offer only minimal insight into the regulation of methyltransferase recruitment to particular genomic loci.

Although the currently prevailing models concerning Polycomb recruitment by lncRNAs remain inconclusive, the reality is likely to involve a much subtler and more complex system of sequentially and/or cooperatively assembled components, in which specific lncRNAs act as scaffolds. For example, the Jumonji family protein JARID2 was recently reported to act as an essential intermediate between *Xist* and PRC2 (REF. 114). While the A repeat of *Xist* is required for gene silencing, it does not seem to be required for the recruitment of PRC2, as previously proposed. Instead, *Xist* recruits JARID2 via its B and F repeats, and JARID2 is required for the localization of PRC2 and methylation of H3K27 (REF. 114). Another study has suggested that the facilitation of JARID2–PRC2 interactions may represent a more general function of lncRNAs¹²⁰, although the amino acid residues that were thought to mediate JARID2 RNA binding differ between the two studies^{114,120}. Thus, the events linking lncRNAs to the recruitment of PRC2, and other methyltransferases to chromatin, are likely to be multiple and intricate, involving the contributions of other protein factors and perhaps other histone modifications¹¹⁵. The increasing scepticism towards widespread assumptions is promising and should ultimately lead to a better understanding of how lncRNAs influence chromatin.

lncRNAs and enhancer function. Transcription at a large number of mammalian enhancers gives rise to lncRNAs, called enhancer RNAs (eRNAs), that seem to have major roles in the regulation of transcription. At least a subset of eRNAs seem to act as nascent transcripts that function *in cis* to promote the transcription of neighbouring genes. Initially, high-throughput RNA sequencing experiments identified thousands of eRNAs that are transcribed from enhancer elements in response to signals that mediate enhancer-dependent transcriptional activation^{121,122}. RNAi-mediated knockdown of several eRNAs was then shown to result in reduced expression of proximally located target mRNAs, suggesting that eRNAs act *in cis* and are required for the activation of target gene transcription^{123,124}.

Insights into the mechanism of action of eRNAs came from the identification of components of the transcription machinery that mediate eRNA function. eRNAs involved in the activation of the developmentally regulated genes T-cell acute lymphocytic leukaemia 1 (*TALI*), snail family zinc-finger 1 (*SNAIL*) and *SNAIL2* require the Mediator transcription co-activator

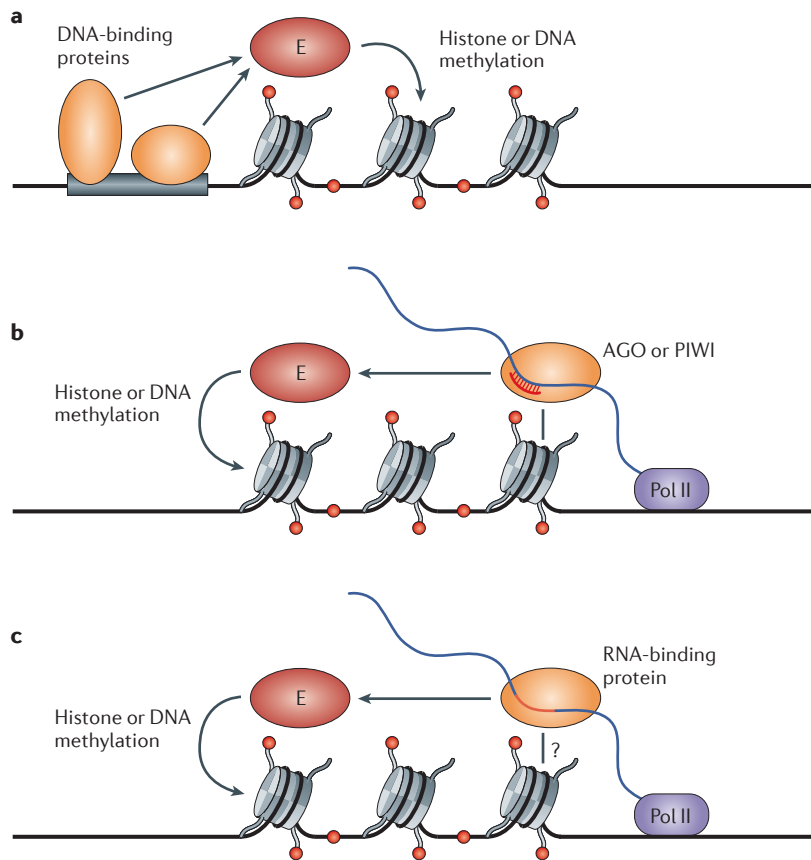


Figure 4 | RNAs, both short and long, represent an alternative to DNA-binding proteins as specificity determinants for epigenetic regulation of gene expression. Enzymes (E) that catalyse methylation of histone tails or cytosine bases in DNA are recruited to chromatin by distinct mechanisms. **a** | Sequence-specific DNA-binding proteins recruit histone- or DNA-modifying enzymes to chromatin. **b** | Small RNAs target an Argonaute (AGO) or PIWI protein to a nascent transcript through base-pairing interactions to recruit modifying enzymes. **c** | Long RNAs act as scaffolds for RNA-binding proteins to recruit chromatin-modifying complexes. In all cases, the binding of the enzyme or the recruiting factors (for example, AGO–PIWI complexes in part **b** and RNA-binding protein complexes in part **c**) to chromatin may be enhanced by interactions with pre-existing modifications, which self-reinforce the epigenetic state. Pol II, RNA polymerase II.

for their activity¹²⁵, while HOXA distal transcript anti-sense RNA (*HOTTIP*), an eRNA involved in the activation of HOXA homeobox genes, functions through the WDR5–MLL (also known as KMT2A) H3K4 methyltransferase complex¹²⁴. Chromosome conformation capture experiments show that the eRNA mediates looping interactions between the enhancer and promoter regions of genes. These studies have given rise to an attractive model in which eRNAs recruit co-activator complexes and promote their interaction with gene promoters to activate transcription. Consistent with this model, there is evidence that supports a role for specific enhancer sequences that encode eRNAs, which are different from enhancer sequences that form binding sites for transcription factors, in activation of target genes¹²⁶. These studies provide a new function for nascent RNA transcripts as scaffolds for the recruitment of co-activator complexes that mediate chromosome looping and transcriptional

activation. Large RNAs (>200 nucleotides) may be particularly suited for such architectural tasks that bring enhancer and promoter regions, usually located great distances apart, into proximity.

The identification of both the regions within eRNAs that mediate co-activator complex recruitment and the RNA-binding domains in the subunits of these complexes is required for a better understanding of how different eRNAs activate transcription. In addition to *cis*-acting eRNA, several *trans*-acting lncRNAs that activate transcription have also been identified. They are not discussed here but have recently been extensively reviewed elsewhere^{22,127}.

Silencing of meiotic genes in *S. pombe*. The regulation of certain meiotic genes in *S. pombe* is another well-studied example in which an RNA scaffold promotes the RNAi-independent recruitment of a histone methyltransferase to chromatin. Meiosis-specific genes are silenced in vegetative cells by a post-transcriptional process that targets an RNA *cis*-element called determinant of selective removal (DSR)¹²⁸. Remarkably, DSRs are found within the protein-coding regions of meiotic mRNAs¹²⁸. Therefore, they must exhibit some degree of sequence flexibility to preserve meiotic protein structure but effectively consist of different variants of a hexanucleotide motif¹²⁹. At a subset of DSR-containing genes, notably *mei4*⁺ and *ssm4*⁺, H3K9 methylation is also observed and depends on the DSR and transcription, which suggests that the mRNAs of these genes direct Clr4 to chromatin and may promote transcriptional and post-transcriptional silencing^{130,131}.

How do DSR-containing mRNAs recruit the Clr4 methyltransferase? Much like lncRNAs and Polycomb proteins, the process seems to involve a host of factors rather than direct interactions between the RNA and the histone-modifying enzyme. In the case of meiotic mRNAs, the source of specific recognition is much clearer. DSRs are bound directly by the YTH-domain protein Mmi1, and this binding event is required for meiotic gene silencing and for H3K9 methylation of the *mei4*⁺ and *ssm4*⁺ loci^{128,129,131}. The Mmi1-interacting zinc-finger protein Red1 is also required for silencing and H3K9 methylation at *mei4*⁺ and *ssm4*⁺ (REFS 131, 132). Strikingly, Red1 associates with Clr4, which suggests that it serves as the critical link that targets histone methylation to genes producing DSR-bearing transcripts^{130,131}. Interestingly, this Mmi1–Red1–Clr4 axis of RNA-directed H3K9 methylation also seems to operate outside the meiotic process to target context-specific genes such as *pho1*⁺, which is repressed when phosphate is available. Data from two recent studies show that an upstream lncRNA mediates Red1 chromatin binding and H3K9 methylation at the *pho1*⁺ locus in a phosphate- and Mmi1-dependent manner^{133,134}. Importantly, the lncRNA was found to contain DSR motifs that are critical for this regulation¹³⁴.

While the DSR-bearing mRNAs demonstrate RNA-mediated recruitment of histone modifications without RNAi, silencing itself seems to be independent of H3K9 methylation. This is perhaps made most evident by the observation that some DSR-containing genes are silenced by Mmi1 and Red1 without exhibiting H3K9

methylation¹³¹. DSRs inserted ectopically in other genes produce similar effects¹³⁰. Most conclusively, cells lacking *Clr4* show wild-type silencing of all tested meiotic genes during vegetative growth¹³⁵. Instead, the critical event for silencing of these genes seems to involve degradation by the nuclear exosome through a mechanism that requires polyadenylation of the target RNAs¹³⁶. Consistently, *Red1* interacts with the exosome subunit *Rrp6* (REF. 132), and proteomic analysis recently showed that this association is mediated by the *Mtr4*-like helicase *Mtl1*, another recently identified *Red1* interactor^{133,135}. This poses the question of what fitness advantage is conferred by H3K9 methylation at meiotic genes, if it is not necessary for silencing. The presence of H3K9 methylation might reflect an ancestral pathway that predates the contribution of the exosome and that has not yet been lost¹³⁵. It is also possible that H3K9 methylation could control expression of DSR-containing genes under certain circumstances, for example, during gene induction^{134,135}. Additional work is needed in this area not only to define the biological function of histone methylation but also to further isolate the steps that lead to *Clr4* recruitment, for example, to determine whether *Mmi1* and *Red1* accomplish this task independently.

Studies of the meiotic mRNAs in *S. pombe* have provided a valuable paradigm for investigating the RNA-mediated targeting of histone methyltransferases in all its mechanistic complexity. In this regard, it is worth noting that the *XIST* RNA is thought to have evolved from a protein-coding gene¹³⁷. This *XIST* precursor gene may have shared certain features of the *S. pombe* meiotic RNAs that enabled it to locally recruit repressive chromatin-modifying complexes before it acquired the ability to spread along the entire X chromosome.

Conclusions and perspectives

The mechanisms by which coding RNAs and non-coding RNAs regulate chromatin structure in different organisms share key similarities that allow us to note common principles which unify these ancient pathways (FIG. 4). The first is the principle of recruitment via nascent RNA. In these systems, recruitment of effector complexes that methylate histones or DNA involves the association of small-RNA-guided AGO complexes or site-specific RNA-binding proteins with nascent coding RNA or non-coding RNA scaffolds (FIG. 4b,c), rather than specific sites on DNA and DNA-binding proteins

(FIG. 4a). The second major principle involves the role of RNA as a component of self-reinforcing positive feedback loops. These loops are unique to small-RNA systems that contain an amplification component and have key roles in the epigenetic inheritance of histone and DNA methylation patterns. The key event is the localization of the small-RNA amplification machineries on nascent transcripts, and their activation by the histone or DNA methylation events induced by the small RNAs themselves (BOX 2). Thus, in both *S. pombe* and *A. thaliana*, small-RNA amplification and histone or DNA methylation are co-dependent. Self-reinforcing loops have not yet been described for lncRNAs that act independently of RNAi. However, cooperative recruitment involving the association of chromatin-modifying complexes with both pre-existing chromatin modifications and DNA-binding specificity factors has been suggested to contribute to the maintenance of epigenetic states in budding yeast³². By analogy, it is possible that direct or indirect interactions between RNA-binding proteins that recognize lncRNA and also pre-existing histone modifications help to reinforce lncRNA-mediated changes in chromatin structure. Finally, small RNAs, and their association with positive feedback loops in the germ line, allow them to act as components of transgenerational inheritance mechanisms. The transmission of small RNAs through meiosis seems to act as a signal for the inheritance of internal or environmentally induced changes from parent to offspring^{138,139}.

Despite the considerable progress outlined in this Review, several important questions about the biogenesis and function of non-coding RNAs remain unanswered. The mechanisms that distinguish between different types of transcription and that trigger the generation of different classes of small RNAs remain to be fully understood, although the available evidence indicates a major role for RNA processing events that act co-transcriptionally to determine whether a nascent transcript becomes a functional mRNA or is marked for processing by RNAi and other surveillance mechanisms. Finally, the mechanisms by which lncRNAs participate in the recruitment of Polycomb proteins and other chromatin-modifying activities, particularly the molecular basis of specificity, remain poorly defined. We can look forward to answers to these questions and, if the recent past is a guide, to more exciting and unexpected discoveries about the roles of RNA in gene regulation.

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Competing interests statement

The authors declare no competing interests.

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