NON-CODING RNA

RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond

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Abstract | A growing number of functions are emerging for RNA interference (RNAi) in the nucleus, in addition to well-characterized roles in post-transcriptional gene silencing in the cytoplasm. Epigenetic modifications directed by small RNAs have been shown to cause transcriptional repression in plants, fungi and animals. Additionally, increasing evidence indicates that RNAi regulates transcription through interaction with transcriptional machinery. Nuclear small RNAs include small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs) and are implicated in nuclear processes such as transposon regulation, heterochromatin formation, developmental gene regulation and genome stability.

RNA interference
(RNAi). Silencing at both the post-transcriptional and transcriptional levels that is directed by small RNA molecules.

Post-transcriptional gene silencing
(PTGS). Silencing achieved by the degradation and/or prevention of translation of a transcript targeted by small RNAs.

Since the discovery that double-stranded RNAs (dsRNAs) can robustly silence genes in *Caenorhabditis elegans* and plants, RNA interference (RNAi) has become a new paradigm for understanding gene regulation. The mechanism is well-conserved across model organisms and uses short antisense RNA to inhibit translation or to degrade cytoplasmic mRNA by post-transcriptional gene silencing (PTGS). PTGS protects against viral infection, prevents transposon mobilization and regulates endogenous genes. Three classes of small RNA can regulate genes by targeting transcripts in the cytoplasm. These are: microRNAs (miRNAs), which are hairpin-derived RNAs with imperfect complementarity to targets and that cause translational repression; small interfering RNAs (siRNAs), which have perfect complementarity to targets and cause transcript degradation; and PIWI-intereacting RNAs (piRNAs), which target transposon transcripts in animal germ lines. Traditionally, the term RNAi has been used to describe siRNA pathways; however, the mechanistic details of diverse small RNA pathways are converging, so in this Review we use RNAi as an umbrella term to describe silencing that is dependent on small RNA.

In plants and fungi, RNAi pathways in the nucleus can repress target genes at the transcriptional level by guiding epigenetic modification of chromatin by, for example, histone and DNA methyltransferases. At first, these pathways were thought to be absent from metazoans, but recently a parallel mechanism has been found in the germ line of several metazoans. These findings have revealed a conserved nuclear role for RNAi in transcriptional gene silencing (TGS). Because it occurs in the germ line, TGS can lead to transgenerational inheritance in the absence of the initiating RNA, but it is dependent on endogenously produced small RNA. Such epigenetic inheritance is familiar in plants but has only recently been described in metazoans.

In this Review, we cover the broad range of nuclear RNAi pathways that have been discovered across organisms so that readers can appreciate the conservation between these pathways while being aware of important differences. As such, we can only scratch the surface of pathways in each organism, so readers are directed to other articles for an in-depth analysis when appropriate. We concentrate on the siRNA and piRNA pathways because their nuclear roles are best understood.

To begin, we outline the biogenesis of small RNAs, focusing on the subcellular localization of these processes. Next, a mechanistic understanding of nuclear RNAi is described in model systems in which it has been elucidated. Nuclear small RNAs function in the germ lines of a broad range of organisms in which mechanistic details are not yet known, so we discuss the biological importance of their roles, including in transposon regulation, epigenetic inheritance and developmental gene regulation, and we suggest parallels that can be drawn to well-understood mechanistic models. Finally, we look forwards by exploring the newly emerging relationship between nuclear RNAi, genome maintenance and DNA repair.

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Table 1 | **Overview of small RNA classes and their functions in the nucleus**

Small RNA class	Size	Nuclear effector*	Biogenesis factors	Genomic origin	Nuclear function
Schizosaccharomyces pombe					
siRNA	21–23 nt	Ago1	Dicer: Dcr1; RdRP: Rdp1	Heterochromatic repeat regions	Centromere function; heterochromatin formation and spreading; RNA Pol II regulation
Arabidopsis thaliana					
siRNA	24 nt	AGO4; AGO6; AGO9	Dicer: DCL3; RdRP: RDR2	Heterochromatic repetitive regions enriched for transposons and retroelements	Systemic RdDM; reproductive strategy (meiosis or apospory)
Caenorhabditis elegans					
22G RNA (siRNA)	22 nt	NRDE-3	Primary siRNA biogenesis: ERGO-1; Dicer: DCR-1; RdRP: RRF-3 (primary), RRF-1 (secondary)	Disperse genomic loci	Systemic heterochromatin formation; RNA Pol II regulation
21U RNA (piRNA)	21 nt	WAGO-9; WAGO-10 with 22G for TGS	PRG-1 loaded with 21U for 22G production	Two clusters on chromosome IV	Heritable heterochromatin formation
Drosophila melanogaster					
siRNA	~ 22 nt	AGO2	Dicer: DCR2	Transposons, repetitive elements, convergent transcription units and structured loci	Heterochromatin formation; chromosome segregation; RNA Pol II regulation
piRNA	23 to 29 nt	PIWI	Ping-pong cycle: PIWI, AUB (primary), AGO3 (secondary), Zucchini	Primary: heterochromatic piRNA clusters antisense to transposons, maternally deposited piRNAs; secondary: sense transcripts of active transposons	Chromosome segregation; heterochromatin formation; telomere homeostasis
Mus musculus					
siRNA	21 to 22 nt	AGO2	Dicer: DCR1	Dispersed naturally occurring dsRNAs, pseudogenes	Speculative
piRNA	24 to 31 nt	MIWI2	Ping-pong cycle: MILI (primary), MIWI2 (secondary), Maelstrom	Primary: sense transcripts of active transposons; secondary: antisense transcripts	*De novo* DNA methylation

*Listed are only the Argonaute proteins that act as the eventual nuclear effectors; other Argonaute proteins may also be involved in these pathways.
AGO, Argonaute; AUB, aubergine; DCR, Dicer; DCRL, Dicer-like; dsRNA, double-stranded RNA; nt, nucleotide; piRNA, PIWI-interacting RNA; Pol, polymerase; PTGS, post-transcriptional gene silencing; RdDM, RNA-directed DNA methylation; RdRP, RNA-directed RNA polymerase; siRNA, small interfering RNA; TGS, transcriptional gene silencing.

Biogenesis of nuclear small RNAs

The siRNA and piRNA pathways differ in the source of the primary RNA that elicits a response and the mechanism by which small RNA is subsequently produced and amplified. The Argonaute family of proteins are the effectors of RNAi, and this family consists of two subclades: AGO proteins, which are ubiquitously expressed and bind miRNAs and siRNAs, and PIWI proteins, which were originally discovered in the germ line and bind piRNAs[1–3]. TABLE 1 summarizes the characteristics of the nuclear siRNA and piRNA pathways for some of the organisms described in this Review, highlighting the nuclear effector, size class and proteins involved in biogenesis.

siRNA biogenesis. dsRNAs are thought to be the trigger for most if not all siRNA biogenesis and can be generated by several means (FIG. 1). When dsRNAs are available, the biogenesis of siRNA requires action of the RNase-III-like Dicer family of enzymes. Dicer cleaves dsRNAs into 20–25-nucleotide (nt) siRNA duplexes with 2 nt 3′ OH overhangs[4] and 5′ monophosphates[4,5]. Dicer-independent mechanisms of siRNA production have also been proposed in *Neurospora crassa*[6], *Schizosaccharomyces pombe*[7] and *C. elegans*[8]. The cellular location in which dsRNA processing occurs has implications for how siRNA biogenesis and nuclear effects are regulated. In *S. pombe*, transcription, processing, amplification by an RNA-dependent RNA polymerase (RdRP) and AGO-mediated target cleavage are all intimately linked in the nucleus[4,9–12] (FIG. 1a). In animals, siRNA processing was originally thought to occur in the cytoplasm[3]; however, recent studies in *D. melanogaster* have shown that Dicer 2 (DCR2) is predominantly found in the nucleus, challenging this view[13]. This is in contrast to *C. elegans*, in which cytoplasmic processing for many siRNA pathways has been confirmed[14] (FIG. 1b).

After they have been generated, the siRNA duplexes are loaded into an appropriate effector Argonaute protein. The subcellular location in which Argonaute loading takes place is not yet fully understood across model organisms. In *Arabidopsis thaliana*, AGO4 loading is

Transcriptional gene silencing
(TGS). Silencing achieved by the formation of a repressive chromatin environment at a locus targeted by small RNA, making it inaccessible to transcriptional machinery.

Argonaute
The effector proteins of RNA inteference that are composed of three characteristic domains, a PAZ domain and a MID domain, which bind the 3′ and 5′ ends of small interfering RNA respectively, and a PIWI domain, which may possess RNase-H-like slicer activity if the protein is catalytically active.

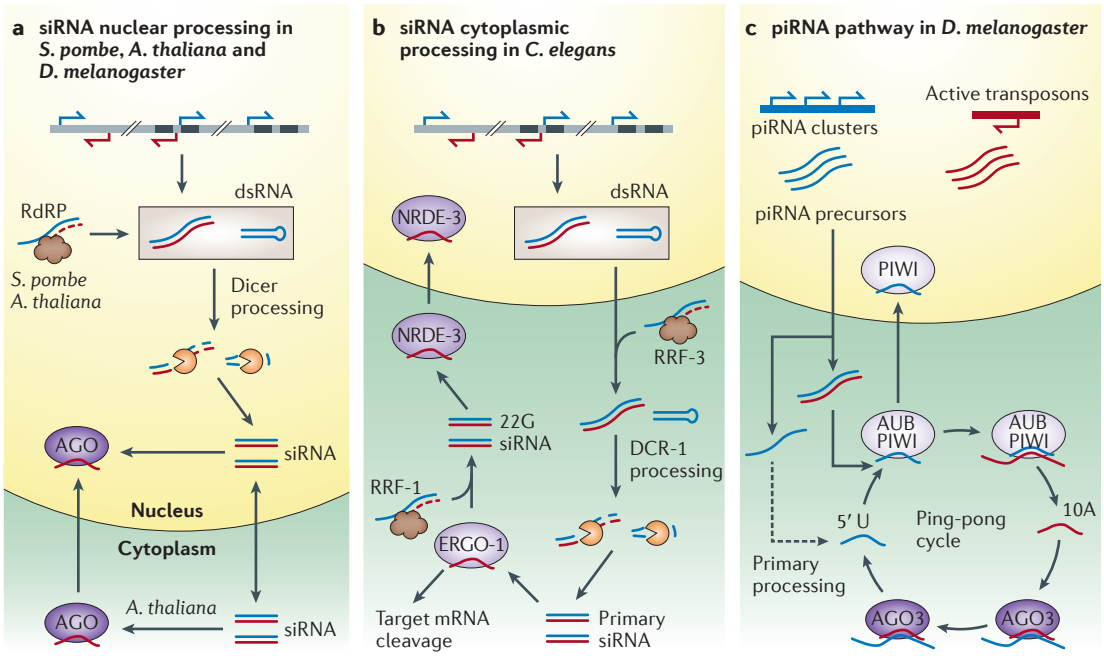

Figure 1 | **Generalized pathways for the biogenesis of nuclear small RNAs.** **a** | Small interfering RNA (siRNA) processing takes place in the nucleus in *Schizosaccharomyces pombe* and *Drosophila melanogaster* and in the nucleolus in *Arabidopsis thaliana*. Double-stranded RNA (dsRNA) can be produced by convergent transcription, complementary transcripts, structured loci or RNA-directed RNA polymerase (RdRP) activity in *A. thaliana* and *S. pombe*. Dicer proteins generate siRNAs that are loaded into an Argonaute protein (AGO). In *A. thaliana*, siRNAs are transported to the cytoplasm, where Argonaute is loaded and then imported into the nucleus. **b** | In *Caenorhabditis elegans*, siRNA processing occurs in the cytoplasm in a two-step fashion. Primary trigger dsRNA arises from nuclear transcription or the RdRP activity of RRF-3, which acts on transcripts in the cytoplasm. Primary processing by DCR-1 produces primary 26-nucleotide siRNAs, which are loaded into the Argonaute ERGO-1. Loaded ERGO-1 can both facilitate post-transcriptional gene silencing (PTGS) in the cytoplasm and with RRF-1 can generate secondary 22G siRNAs. In the cytoplasm, secondary 22G siRNAs are loaded into the nuclear Argonaute NRDE-3, which is then transported into the nucleus. **c** | PIWI-interacting RNA (piRNA) biogenesis via the ping-pong cycle in the *D. melanogaster* female germ line. Primary precursor piRNA antisense to active transposons (blue) is transcribed from heterochromatic piRNA clusters and sense mRNA from active transposons (red). In the cytoplasm, primary processing generates antisense piRNAs (which have a 5′ uridine (5′ U)) from a primary precursor that is then loaded into aubergine (AUB) or PIWI and cleaves sense transposon mRNA to produce sense piRNAs, which have a strong adenine bias at position 10 (10A). Additional antisense piRNA is produced by AGO3-mediated cleavage of antisense primary piRNA transcripts, completing the cycle. Only loaded PIWI is imported into the nucleus.

cytoplasmic and is mediated by heat-shock protein 90 (HSP90), after which it is imported into the nucleus[15]. A requirement for HSP90 in AGO loading has also been observed in *D. melanogaster*, but where this process occurs is not known[16]. Like *A. thaliana*, siRNA processing is nuclear in *S. pombe*; however, it is not known where AGO1 loading occurs. The *C. elegans* nuclear Argonaute NRDE-3 is imported into the nucleus only when it is loaded with secondary siRNAs that are produced in the cytoplasm[17]. If cytoplasmic loading of Argonaute proteins is conserved across species, this would have important implications for the regulation of nuclear RNAi.

piRNA biogenesis. The biogenesis of piRNA primarily occurs by a process known as 'the ping-pong cycle' (FIG. 1c), which was initially described in the *D. melanogaster* germ line[18,19]. First, piRNA genomic clusters are transcribed to produce primary piRNA precursors. In the cytoplasm, an unknown mechanism processes primary piRNA precursors into short 23–29 nt antisense piRNAs that have a strong 5′ uridine bias. These short single-stranded RNAs (ssRNAs) are loaded into the PIWI family Argonaute proteins aubergine (AUB) and PIWI. In the cytoplasm, the loaded AUB or PIWI then targets mRNA of active transposons for cleavage; this produces sense piRNAs, which have a strong adenine bias at position 10. The sense piRNAs are loaded into the PIWI family member AGO3, which then directs cleavage of primary piRNA precursors and the subsequent production of more antisense piRNAs, completing the ping-pong cycle[19]. In the female germ line, AUB is restricted to the cytoplasm, whereas PIWI is predominantly nuclear, indicating that AUB has a larger role in the ping-pong cycle[20]. The nuclear localization of PIWI is lost in AGO3 mutants, suggesting that after it has been loaded with piRNA produced by the ping-pong cycle, PIWI is imported into the nucleus[20]. A lesser-understood, ping-pong-independent piRNA biogenesis pathway operates

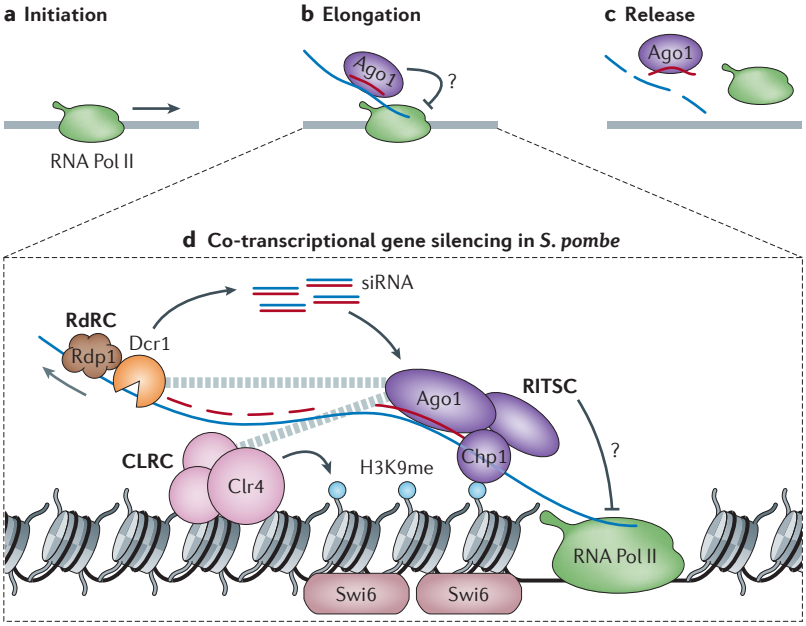

Figure 2 | **Co-transcriptional gene silencing in *Schizosaccharomyces pombe*.** **a** | RNA polymerase II (RNA Pol II) initiates transcription at loci targeted by RNA interference (RNAi). **b** | During the elongation phase of transcription, the Argonaute Ago1 is guided to the nascent transcript and inhibits RNA Pol II transcription by an unknown mechanism (indicated by the question mark). **c** | RNAi can lead to the release of RNA Pol II. **d** | A mechanistic model of RNAi acting during the elongation phase (see panel **b**) in *Schizosaccharmoyces pombe*. The RNA-induced transcriptional silencing complex (RITSC) is localized through siRNA (red) base pairing with the nascent transcript (long blue line) and chromatin interaction mediated by the chromodomain of Chp1. The RNA-dependent RNA polymerase complex (RdRC) couples double-stranded RNA (dsRNA) production by Rdp1 and small interfering RNA (siRNA) cleavage by Dcr1 and is also associated with the nascent RNA Pol II transcript. The RITSC interacts with the cryptic loci regulator complex (CLRC), which catalyses methylation of histone H3 at lysine 9 at target loci. This histone modification serves as a binding site for Swi6, the *S. pombe* orthologue of the highly conserved heterochromatin protein 1 (HP1), which is a defining feature of heterochromatin. The RITSC promotes RNA Pol II release by an unknown mechanism (indicated by the question mark). The dashed grey lines indicate interactions between complexes.

in the somatic follicle cells that surround the female oocytes; this pathway is PIWI-dependent and AUB- and AGO3-independent (discussed in 'Nuclear RNAi in the germ line')[20,21].

Stability and turnover have important roles in the regulation of both the siRNA and piRNA pathways. Both piRNAs and siRNAs are 2′-*O*-methylated by the small RNA 2′-*O*-methyltransferase (known as HEN1) across organisms (for an extensive review, see REF. 22). This methylation protects small RNAs from 3′ uridylation and 3′ truncation, which cause small RNA degradation and turnover. The specificity of HEN1 could therefore contribute to cell-type-specific small RNA profiles and thus could determine targets of RNAi; however, such a mechanism has yet to be uncovered.

Mechanisms of nuclear RNAi

TGS was the first function of nuclear RNAi to be discovered and refers to the process by which RNAi can reduce transcription by guiding localized heterochromatin formation at target genomic loci. A question that arises from this mechanism is 'how is sequence-specific targeting of chromatin modifications achieved?' As in the cytoplasm, the substrate for nuclear RNAi has been shown to be RNA molecules, but these must be in close proximity to the locus from which they arose so that epigenetic modification can be specific. This has led to a model of co-transcriptional gene silencing (CTGS), whereby nuclear small RNAs target nascent RNA molecules from RNA polymerases, and the effector complexes themselves interact with and regulate the transcriptional machinery. In the next section, we discuss the mechanisms of two examples of nuclear RNAi that reveal that positive-feedback loops are involved in chromatin modification. The nuclear RNAi complexes themselves are both attracted to repressive epigenetics marks, and they deposit them, creating robust silencing at target loci.

Nuclear RNAi in* S. pombe*: TGS. A role for RNAi in TGS was identified in *S. pombe*, in which it is required for the formation of constitutive heterochromatin at pericentromeric regions. These regions are highly enriched for histone H3 methylated at lysine 9 (H3K9me) and are composed of varying numbers of repeat units that are bidirectionally transcribed to form dsRNA that is then processed by Dcr1 into siRNAs[23]. The RNA-dependent RNA polymerase complex (RdRC) interacts with both Dcr1 (REF. 4) and Ago1 (REF. 24) to produce dsRNA and siRNA from Ago1-targeted transcripts and to amplify the siRNA response. siRNAs are loaded into Ago1 — the principle member of the RNA-induced transcriptional silencing complex (RITSC) — and guide the RITSC to nascent pericentromeric non-coding RNA (ncRNA) transcripts (FIG. 2). The chromodomain protein Chp1 is also a member of the RITSC and contributes to its localization to heterochromatin by binding H3K9me[25]. After the RITSC has been localized to repeat loci, it facilitates H3K9 methylation by recruiting the cryptic loci regulator complex (CLRC), which contains Clr4, the sole H3K9 methyltransferase in *S. pombe*[26]. Interestingly, the catalytic slicing activity of Ago1 is required for the deposition and spreading of H3K9me, particularly at reporter genes[12]. Catalytic activity is required for passenger-strand release from Ago1-bound dsRNA and thus is required to facilitate base pairing between loaded siRNAs and their targets, explaining this observation[27]. This suggests that nuclear RNAi — specifically, siRNA–target base pairing — is required for the spreading of heterochromatin, a phenomenon originally described as position effect variegation. These interactions place the RITSC in a central role, integrating transcription and chromatin modification. They also create a positive-feedback loop between siRNA generation, RITSC localization and H3K9 methylation. A fascinating consequence of this is that H3K9 methylation itself is required for siRNA generation. The coupling of transcription, siRNA production and silencing in *S. pombe* suggests that TGS occurs in *cis*; however, examples from plants discussed in BOX 1 show that it can also occur in *trans*.

Co-transcriptional gene silencing
(CTGS). The coupling of repressive epigenetic modification with transcription by an RNA polymerase that produces a nascent RNA molecule targeted by small RNAs.

Pericentromeric regions
Sites of constitutive heterochromatin that flank the central kinetochore-binding region of the centromere and are necessary for proper centromere function.

Histone H3 methylated at lysine 9
(H3K9me). H3K9 can be mono-, di- or tri-methylated. Methylation is catalysed by a histone methyltransferase and is highly enriched in repressive heterochromatin. This mark acts as a binding site for heterochromatin protein 1 (HP1; known as Swi6 in *Schizosaccharomyces pombe*), the presence of which is the defining feature of heterochromatic loci.

RNA-induced transcriptional silencing complex
(RITSC). The effector of nuclear RNA interference in *Schizosaccharomyces pombe*. It is composed of an Argonaute protein and other cofactors that may aid in localization to chromatin.

Passenger strand
The antisense small RNA strand in the double-stranded RNA molecule initially loaded by an Argonaute. The passenger strand is released by the catalytic 'slicing' activity of the Argonaute protein (like homologous RNA targets), whereas the guide strand is retained and acts to determine the specificity of the silencing complex.

***Nuclear RNAi in* S. pombe: *CTGS*.** The dependency of RITSC localization on base pairing with ncRNA transcripts presents an interesting paradox in that loci targeted by RNAi for TGS must be transcribed to be silenced. Supporting this idea, genetic screens for loss of silencing in *S. pombe* have identified two point mutations in RNA polymerase II (RNA Pol II) subunits that decouple transcription and the RITSC at the pericentromeres[9,28]. A model linking transcription, RNAi and heterochromatin formation can be formed when these observations are taken in the context of the cell cycle. Studies have shown that transcription of pericentromeric repeats targeted by RNAi occurs during S phase, the same time at which DNA is being replicated and chromatin modifications must be re-established[29,30]. DNA replication and transcription must also be coordinated to prevent collision of the two processes and subsequent replication fork stalling. We found that RNAi is required to facilitate the release of RNA Pol II and to prevent read-through transcription into replicating DNA[31]. This suggests that RNAi, after it has been recruited to an actively transcribing RNA Pol II, may be able to inhibit transcription during the elongation phase, resulting in the release of RNA Pol II (FIG. 2c). These observations support a model of CTGS in *S. pombe* (FIG. 2d), a term first coined by Bühler *et al.*[32].

The CTGS model explains the paradox behind TGS. A nascent RNA transcript is required for the initial targeting of RNAi to a locus; after this has occurred, the nuclear RNAi complex can promote transcriptional silencing at the chromatin level and can release RNA Pol II via an unknown mechanism. It will be interesting to understand how transcription is initiated in what has previously been thought of as a restrictive heterochromatic environment and the mechanism by which the RITSC can promote RNA Pol II release.

There is growing evidence that nuclear RNAi may co-transcriptionally regulate loci outside constitutive heterochromatin in *S. pombe*. It has been shown to have a role in preventing read-through transcription at convergently transcribed genes, presumably though RNA Pol II release[33–35]. Additionally, Dcr1 physically interacts with chromatin at euchromatic genes, suggesting a role in gene regulation without histone modification[36]. Indeed, nuclear Dcr1 has a role in regulating heat-stress-responsive genes through a 'thermoswitch'[37]. In unstressed cells, Dcr1 is localized to the nucleus and negatively regulates stress response genes; however, under heat stress, it is exported out of the nucleus and stress-response genes are activated.

***RNA-directed DNA methylation in* A. thaliana.** Transgene DNA methylation directed by viral RNA was discovered in plants long before a role for RNAi was known[38], and later the involvement of small RNAs and RNAi pathways in mediating TGS through cytosine methylation was first proposed in *A. thaliana*[39,40]. There are many parallels between RNA-directed DNA methylation (RdDM) in *A. thaliana* and CTGS in *S. pombe*. For example, the requirement of transcription for silencing is common to both[9,28,41], and both direct silencing at repetitive heterochromatic loci. RdDM differs from CTGS in *S. pombe* in that stepwise transcription by two RNA polymerases (namely, RNA Pol IV and RNA Pol V) is required. Transcripts from RNA Pol IV serve as substrates for siRNA generation, whereas nascent transcripts from RNA Pol V are targeted by RNAi (FIG. 3; reviewed in REF. 42). The initial templates for RNA Pol IV are not known, but they would presumably be the loci that will be subject to RdDM. RNA Pol IV physically interacts with the RNA-dependent polymerase 2 (RDR2), which produces dsRNAs from transcripts[43]; the dsRNAs are subsequently processed into 24 nt siRNAs by DICER-LIKE 3 (DCL3)[44]. These 24 nt siRNAs are exported into the cytoplasm, where they are loaded into an Argonaute complex[15].

At least 3 of the 10 Argonautes found in *A. thaliana* are involved in RdDM, but AGO4 was the first to be identified[45]. After it has been loaded with 24 nt siRNA in the cytoplasm, AGO4 is imported into the nucleus and guided to complementary RNA Pol V intergenic non-coding transcripts through siRNA–target base pairing[46,47]. It is probably aided by direct protein–protein interaction with the RNA Pol V subunit NUCLEAR RNA POLYMERASE E1 (NRPE1)[48] and the RNA Pol V-associated KOW DOMAIN-CONTAINING TRANSCRIPTION FACTOR 1 (KTF1)[49,50].

This co-transcriptional silencing by RNAi ultimately leads to the deposition of repressive cytosine methylation at loci transcribed by RNA Pol V. In *A. thaliana*, *de novo* cytosine methylation is catalysed by the DNA methyltransferase DRM2 at loci targeted by RdDM[51]. It might thus be expected to be a member of the RdDM effector complex alongside an Argonaute protein. Biochemical studies of a new complex member, RDM1, support this notion, as it interacts with both AGO4 and DRM2 and is required for RdDM, thus bridging RNAi and cytosine methylation[52]. The presence of a

Box 1 | *Cis* versus *trans* silencing: paramutation in maize

Although the concept of co-transcriptional gene silencing (CTGS) in *Schizosaccharomyces pombe* implies a role for small RNA silencing in *cis*, there is considerable evidence that RNA-directed DNA methylation (RdDM) in plants can act in *trans*. Perhaps the best known example is the phenomenon of paramutation, which was first discovered in maize (*Zea mays*). Paramutation refers to the silencing of one allele (the paramutated allele) in heterozygous combinations with a silent allele (the paramutagenic allele) in *trans*. The paramutated allele is converted by this process into a paramutagenic allele, and silencing is *trans*-generational, in some cases permanently so. Paramutation is allele-specific and classically occurs at three loci in maize: namely, *Booster* (*B*), *Plant colour* (*Pl*) and *Red* (*R*; for review see REF. 117). Paramutant alleles have rearrangements such as transposon insertions and tandem repeats, which in some cases have been identified as the sequences that are responsible for the effect. Importantly, extensive screens for mutants that are deficient in either the establishment or the maintenance of paramutation at the *B* locus have identified mutants in RdDM, including *mediator of paramutation1* (*mop1*) and *mop2*, which respectively encode orthologues of the RNA-dependent RNA polymerase RDR2 and the RNA polymerase IV (RNA Pol IV) and RNA Pol V subunit NRPD2 (REF. 118). Similar screens for genes required to maintain repression of paramutant alleles of *Pl* have recovered subunits of RNA Pol IV as well as homologues of the chromatin remodellers DRD1 and CLSY1 (REF. 119). DNA methylation changes at paramutant loci have been reported, and some examples at least involve transcriptional silencing[120].

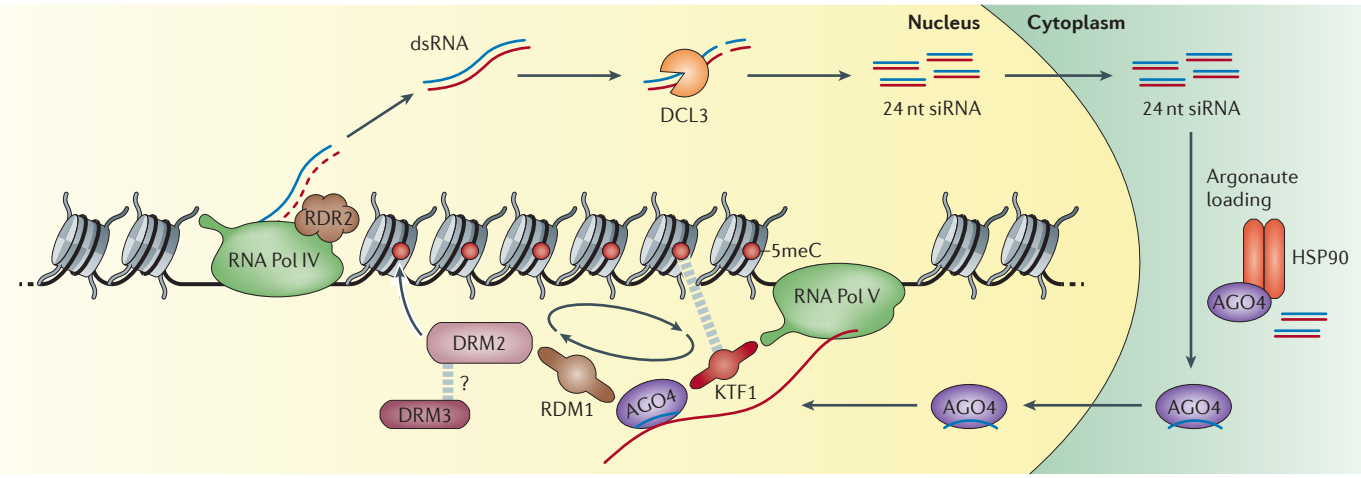

Figure 3 | **The RNA-dependent DNA methylation pathway in *Arabidopsis thaliana*.** RNA polymerase IV (RNA Pol IV) transcribes single-stranded RNA (ssRNA; long blue line) from repetitive heterochromatic loci. RNA-DEPENDENT POLYMERASE 2 (RDR2) physically associates with RNA Pol IV to produce double-stranded RNA (dsRNA). DICER-LIKE 3 (DCL3) cleaves dsRNA to produce small interfering RNAs (siRNAs) that are transported to the cytoplasm for ARGONAUTE 4 (AGO4) loading, which is facilitated by HEAT-SHOCK PROTEIN 90 (HSP90), and the loaded AGO4 is then imported back into the nucleus. In the nucleus, AGO4 targets nascent RNA Pol V transcripts (long red line) through complementarity to the siRNA and forms the RNA-directed DNA methylation (RdDM) complex, which presumably contains the catalytically active *de novo* DNA methyltransferase DRM2. The RNA Pol V-associated GW/WG protein KTF1 may act as an organizer by interacting with AGO4 and 5-methylcytosine (5meC). Similarly, the AGO4-associated protein RDM1 can bind single-stranded methylated DNA and interacts with DRM2. Both proteins could contribute to a positive-feedback loop between AGO4 localization and DNA methylation (circular arrows). DRM3, a catalytically inactive paralogue of DRM2, is required for RdDM; however, its role is unknown (indicated by the question mark). After it has been localized, DRM2 catalyses methylation of cytosine in all sequence contexts. The dashed grey lines indicate interactions.

catalytically inactive DRM2 paralogue DRM3 is also required for RdDM, but its role is not known[53]. After it has been targeted, DRM2 directs cytosine methylation in all cytosine contexts, including at asymmetric CHH sites (where H is any nucleotide), to facilitate heterochromatin formation and TGS[54]. Perhaps analogous to the role of Chp1 in localizing the RITSC to heterochromatin in *S. pombe*, the AGO4-associated protein RDM1 in *A. thaliana* binds single-stranded methylated DNA[52] and thus localizes AGO4 to methylated regions, creating a reinforcing positive-feedback loop.

Variations on the canonical RdDM pathway have been observed. AGO6 plays a partially redundant part with AGO4 (REF. 55), and AGO9 is loaded with 24 nt siRNAs in the female germ line, where its activity is required for transposon silencing, but a direct role in DNA methylation has not yet been established[56]. There is also evidence that transcripts from RNA Pol II (which chiefly transcribes euchromatic genes), as opposed to RNA Pol V, are targeted by RdDM, but the importance of this remains unclear[52,57].

The RdDM pathway may be involved in H3K9 methylation, although it is uncertain whether nuclear RNAi has a direct role, as it does in *S. pombe*. There is substantial crosstalk between the two pathways as DNA methylation is required for the recruitment of the H3K9 methyltransferase SUVH4 (also known as KYP)[58]. At least two SUVH homologues are required for RdDM[59], and small RNAs from inverted repeats have been shown to influence H3K9 methylation to a greater extent than cytosine methylation does, suggesting a direct role[60].

RdDM may not be the only example of nuclear RNAi in *A. thaliana*. There is evidence that another nuclear RNAi pathway involving DCL4 has a co-transcriptional role in transcriptional termination. DCL4 was found to interact directly with chromatin in the 3′ region of an RNA Pol II-transcribed endogenous gene to promote cleavage of the nascent transcript and transcription termination[61]. Further study is needed to identify novel nuclear roles for other RNAi pathways.

A few examples outside *A. thaliana* indicate that miRNAs may influence DNA methylation in plants. In rice, 24 nt small RNAs that arise from miRNA precursors termed long miRNAs (lmiRNAs) are independent of the RNA-directed RNA polymerase RDR2, are processed by DCL3 and are loaded into AGO4, which is normally associated with RdDM in *A. thaliana*[62]. These lmiRNAs are able to direct highly sequence-specific cytosine methylation at their own locus (in *cis*) and at complementary loci (in *trans*). Some lmiRNAs have been identified in *A. thaliana*; however, they have not been shown to influence DNA methylation[63]. Similarly, in the moss *Physcomitrella patens*, several 21 nt miRNAs have been shown to influence cytosine methylation at their targets[64]. Although both examples show that other classes of small RNA can influence DNA methylation, neither uncovers a novel effector pathway outside RdDM.

Position effect variegation
Refers to the variegated expression pattern of a gene that is stochastically inactivated by the spreading of a nearby heterochromatic domain. For example, a pericentromere and an inserted nearby reporter gene.

Cytosine methylation
Covalent modification of a cytosine base catalysed by a DNA methyltransferase that often associates with heterochromatic loci. It can occur in various sequence contexts, including CG, CHG and CHH, which influence establishment and inheritance.

Metazoan somatic nuclear RNAi. Although the germ lines of metazoans have a clear role for nuclear RNAi (see below in 'Nuclear RNAi in the germ line'), some evidence suggests that TGS also occurs in somatic cells; however, the subject is controversial. Feeding *C. elegans* with dsRNA targeting an endogenous gene triggers H3K9 methylation at the target locus in somatic cells in a process that is dependent on the nuclear RNAi pathway (known as the NRDE pathway in *C. elegans*)[17,65–67] and on the RdRP RRF-1 (REF. 68). There are many genes that are targeted by endogenous siRNAs, and some but not all show a reduction of H3K9 methylation in *nrde* mutants[68]. In *D. melanogaster* somatic cells, mutations in siRNA pathway members *Dcr2* or *Ago2* affect the expression of a centromeric reporter and result in a marked reduction of centromeric H3K9 methylation[69–71].

As in the fission yeast, proteins required for nuclear RNAi interact with the transcriptional machinery in metazoan somatic cells, suggesting that CTGS may be conserved. In human and *D. melanogaster* cells, AGO1 directly interacts with RNA Pol II by co-immunoprecipitation[72,73]. In *D. melanogaster* S2 cells, AGO2 and DCR2 directly associate with both chromatin and RNA Pol II and are required to inhibit the expression of heat-shock-response genes under non-stress conditions by maintaining paused RNA Pol II and by preventing elongation[13]. In *C. elegans*, loci targeted by RNAi show a downstream decrease in RNA Pol II occupancy that is dependent on the nuclear RNAi factor NRDE-2 and the Argonaute NRDE-3, suggesting that siRNAs may facilitate transcription termination[65]. Overall, current evidence suggests a conserved interaction of nuclear RNAi and the transcriptional machinery fitting a co-transcriptional model; however, the role of these interactions needs further exploration.

Nuclear RNAi in the germ line

The germ line is the battlefield on which evolutionary wars between selfish DNA elements and their hosts are played out, because transposable element mobilization here would be inherited by future generations. Nuclear RNAi — in the form of the piRNA pathway in animals and various siRNA pathways in plants — is a front-line defence.

***Germline nuclear RNAi in* A. thaliana.** In plants, germline cells arise late in development from somatic stem cells (unlike in animals, in which the germ line is specified early in development), and so transposons must be extensively silenced throughout development. Generally, chromatin marks that are present during somatic development must be reset in the germ line. How this occurs selectively is a question that is actively being pursued. In somatic cells, both the RdDM pathway and maintenance DNA methyltransferases keep transposons silent; however, this changes in the companion cells of the germ line that will not contribute genetically to the next generation. The heterochromatin remodeller DDM1 is a master regulator of transposons[74] and is downregulated in the supportive vegetative nucleus, leading to transposon mobilization and to the production of 21 nt small RNAs antisense to transposons[75] (FIG. 4a). These 21 nt small RNAs can silence reporters expressed in sperm cells so that they appear to act non-cell-autonomously. Regarding DNA methylation, unlike mammals that undergo whole-genome demethylation during spermatogenesis[76], the *A. thaliana* male germ line retains symmetric methylation at levels similar to somatic cells[77,78] but specifically shows a reduction in the levels of asymmetric methylation at a subset of retrotransposons that are later remethylated in the developing embryo[79].

In the female gametophyte, the maintenance DNA methyltransferase MET1 (also known as DMT1) is repressed[80], and the DNA glycosylase DEMETER, which removes cytosine methylation, is expressed[81] in the diploid central cell (which will later become the 'extra-embryonic' endosperm) (FIG. 4b). This leads to global cytosine demethylation in the endosperm accompanied by increased production of 24 nt siRNAs, leading to non-CG hypermethylation at target sites, which are primarily retroelements[82]. These 24 nt siRNAs are bound by AGO9 in the central cell and act non-cell-autonomously to control functional megaspore specification[56]. Currently, there is no direct experimental evidence to show the movement of either 24 nt siRNA or AGO9 from the central cell to the egg cell. However, in *ago9* mutants, transposable elements are activated in the egg cell, where *ago9* is not expressed, and an miRNA expressed in the central cell can silence a reporter gene expressed in the egg cell[78], supporting this hypothesis. These observations suggest a hypothetical model by which transposons are revealed in companion cells and are then used to generate small RNAs that enforce transposon silencing in the germ cells[83]; however, it is not known whether they can also direct TGS through nuclear RNAi. This movement of small RNA between germ cells has implications for epigenetic inheritance that are discussed in BOX 2.

The* D. melanogaster *piRNA pathway. In animals, the role of the piRNA pathway in transposable element silencing has been best described in *D. melanogaster* ovaries. In the ovaries, piRNAs silence transposons in somatic follicle cells that surround the oocyte, the germline nurse cells and the oocyte itself[20,21] (FIG. 4c). The somatic follicle cells produce only antisense piRNAs from the *flamenco* locus; these do not participate in the ping-pong cycle and are instead processed and loaded solely into PIWI. These piRNAs mainly target elements from the *gypsy* family of long terminal repeat (LTR) retroviruses. *gypsy* family elements are able to propagate by producing viral particles in follicle cells that can infect germline cells, thus the *flamenco*-derived piRNA pathway is thought to be an evolutionary counter to this class of transposons[20].

In nurse cells and ovaries, the ping-pong cycle defends against a wide variety of transposable elements using input from all piRNA clusters and mRNA of active transposons[18,21]. Here, the piRNA pathway degrades transposon transcripts and directs H3K9 methylation to silence transposon transcription and to prevent their mobilization[84]. PIWI has been shown specifically to interact with heterochromatin protein 1A (HP1A), a

NRDE pathway
In *Caenorhabditis elegans*, components of the nuclear RNA interference pathway are termed NRDE for 'nuclear RNAi defective' owing to the phenotype of mutants (*nrde*).

Transposable element
Genetic elements that can move their positions within the genome. The mechanism of transposition varies and defines transposon families.

Companion cells
Cells in the germ line of plants that will not contribute genetically to progeny but are produced by meiosis. These are the vegetative nucleus in the male germ line and the central cell in the female germ line. The central cell is fertilized to produce the endosperm that acts as a supportive tissue to the developing embryo.

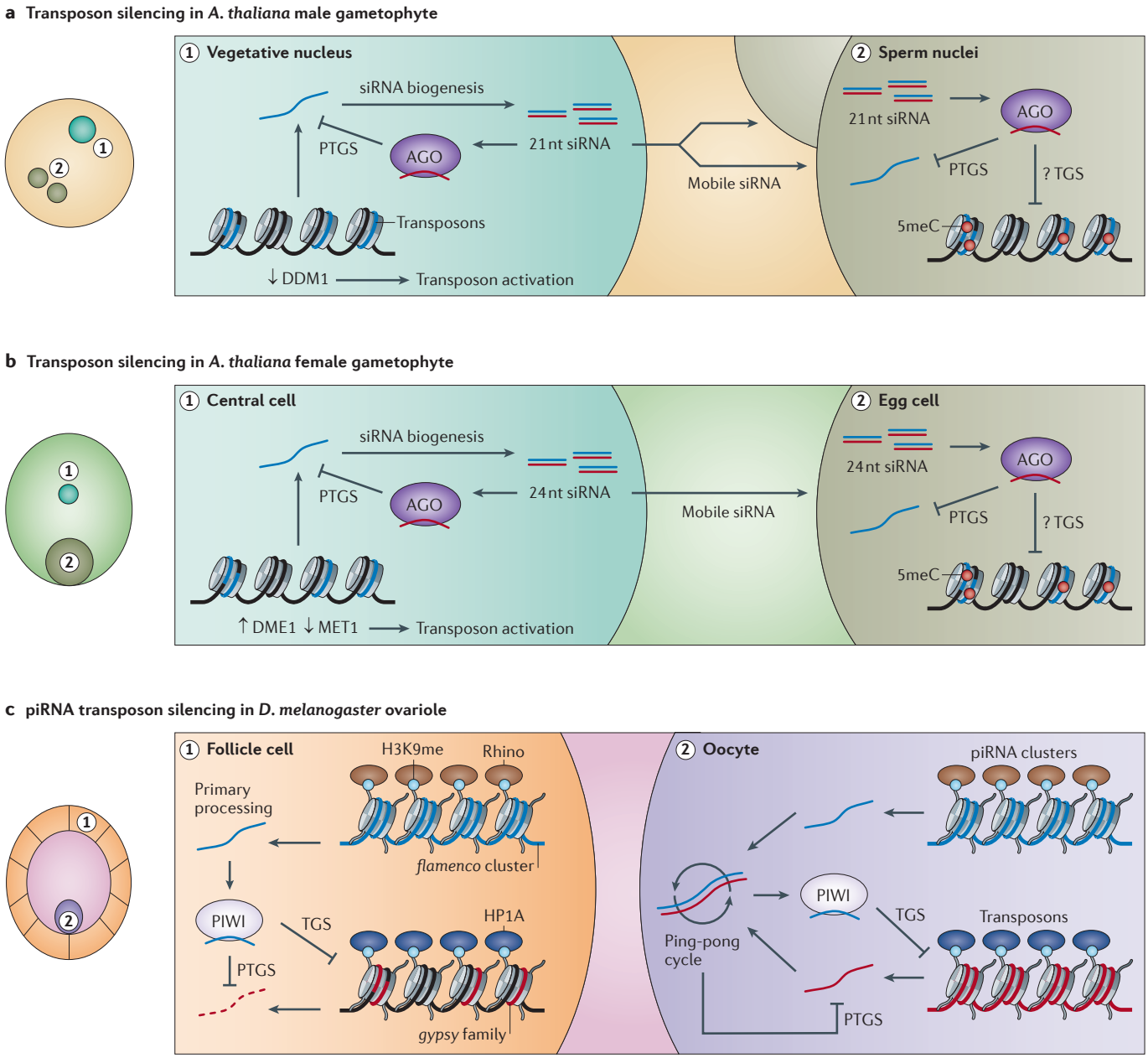

Figure 4 | **RNA-interference-mediated transposon silencing in the germ line.** **a** | In the supportive vegetative nucleus of the *Arabidopsis thaliana* male gametophyte, DDM1 expression is repressed, which allows transposons to be expressed. Transposon transcripts are processed into 21-nucleotide (nt) small interfering RNAs (siRNAs) that are mobile and that can direct post-transcriptional gene silencing (PTGS) in the sperm nuclei. They may also have an impact on transposons transcriptionally by directing or inhibiting epigenetic modification. **b** | In the supportive central cell of the *A. thaliana* female gametophyte, the maintenance DNA methyltransferase MET1 is downregulated, whereas the DNA glycosylase DEMETER is expressed, causing a loss of cytosine methylation and revealing transposons for transcription. This activates the RNA-directed DNA methylation (RdDM) pathway and produces 24 nt siRNAs that can be transported to the egg cell to enforce silencing possibly through AGO9 ('AGO' on the figure). The question mark indicates that more evidence is needed to confirm this. **c** | In the *Drosophila melanogaster* ovariole, the *flamenco* cluster is expressed in somatic follicle cells and generates PIWI-interacting RNAs (piRNAs) independently of the ping-pong cycle. Loaded PIWI silences the *gypsy* family of retrotransposons, which could otherwise form infectious particles. In oocytes and surrounding nurse cells, all piRNA clusters are expressed, and the primary transcripts enter the ping-pong cycle to produce piRNA. Active transposons are post-transcriptionally silenced, and nuclear PIWI promotes transcriptional silencing by methylation of histone H3 at lysine 9 (H3K9me) and heterochromatin protein 1A (HP1A) localization. The HP1A homologue Rhino binds to heterochromatic piRNA clusters in place of HP1A and promotes transcription. TGS, transcriptional gene silencing.

Box 2 | **Systemic transcriptional gene silencing and epigenetic inheritance**

The hypothesis that small interfering RNAs (siRNAs) can move into *Arabidopsis thaliana* germ cells (see 'Germline nuclear RNAi in *A. thaliana*' in the main text) has implications for epigenetic inheritance. Outside the gametophytes, grafting experiments have shown that nuclear silencing signals can be transmitted from the root to shoot[121] and vice versa[122]. Mobile 21 to 24 nt siRNA are the effectors of this systemic silencing and can guide epigenetic modification through RNA-directed DNA methylation (RdDM) in recipient cells[123,124]. These 24 nt siRNAs have been demonstrated to direct DNA methylation in meristematic root stem cells[122], and it is therefore tempting to speculate that they may act similarly in the shoot meristems (where germ cells are produced) to direct heritable epigenetic modification.

Systemic RNA interference (RNAi) is well-known in *Caenorhabditis elegans*, and there is recent evidence for small-RNA-mediated epigenetic inheritance. The progeny of animals exposed to double-stranded RNAs (dsRNAs) show methylation of histone H3 at lysine 9 (H3K9) of target loci and generate complementary small RNAs for multiple generations[66]. The appearance of siRNAs precedes H3K9 methylation in progeny, so it is likely that this inheritance is indirect and is instead re-established by inherited siRNAs in each generation. This process is dependent on the nuclear RNAi pathway, including the Argonaute NRDE-3. Furthermore, small RNAs produced against viral RNAs can be transgenerationally inherited and continue to persist even in the absence of the viral template itself[125]. These studies both point to small RNAs as an epigenetic vector, which can be inherited and direct chromatin modifications through nuclear RNAi in progeny. After they have been established, these chromatin modifications can be maintained and transmitted across generations even in the absence of the original dsRNA trigger[67].

In *Drosophila melanogaster*, reciprocal crosses have shown that progenies inherit the maternal PIWI-interacting RNA (piRNA) composition, and this composition persists into adulthood[126]. The maternally deposited piRNA may prime the ping-pong cycle and determine its specificity, or it could potentially direct epigenetic modification to enforce a specific piRNA transcription programme. A similar situation is seen in the *A. thaliana* endosperm, where maternally deposited 24 nt siRNAs silence transposable elements and transposable-element-associated genes during its development[127,128].

defining component of heterochromatin that is chromatin-associated itself[85]. Furthermore, the nuclear localization of PIWI is required for chromatin-mediated repression of a subset of transposons, suggesting a direct role[86]. Silencing of the piRNA clusters themselves would be detrimental, as this would prevent primary piRNAs from entering the cycle. This is solved by the HP1 variant Rhino, which is restricted to germline nuclei and specifically localizes to piRNA clusters and promotes transcription of the heterochromatic clusters[87]. How Rhino is localized to piRNA clusters and not to active transposons remains unexplained.

The mouse piRNA pathway. The PIWI pathway is highly conserved in animals and has a similar role in the mouse germ line. In mice, two PIWI homologues, MILI (also known as PIWIL2) and MIWI2 (also known as PIWIL4), are required for transposon silencing in the male germ line. Loss of either protein causes transposon mobilization and sterility[88,89]. The piRNA pathway, however, operates differently from *D. melanogaster*. In the mouse male germ line, transposons are globally derepressed by cytosine demethylation during early development. The piRNA pathway is then primed with individual transposons and re-establishes methylation patterns during development[90,91]. As MIWI2 is found in the nucleus, it is likely to be the effector Argonaute of RNA-directed DNA methylation in mice[92]. The role of MIWI2 in establishing DNA methylation in the germ line may not be direct (unlike the role of AGO4 in *A. thaliana* or Ago1 for H3K9me in *S. pombe*) as co-immunoprecipitation experiments have failed to show interaction between MIWI2 and the *de novo* methyltransferases DNMT3A and DNMT3B.

The role of nuclear RNAi in directing DNA methylation in mammals is nicely demonstrated at the imprinted Ras protein-specific guanine nucleotide-releasing factor 1 (*Rasgfr1*) locus, where the piRNA pathway is required for *de novo* methylation in the male germ line[91]. Upstream of the differentially methylated region is an LTR that matches piRNAs with a typical ping-pong signature; these piRNAs can probably be generated owing to the presence of another copy of the LTR in a piRNA cluster. The LTR is contained within an ncRNA that is specifically transcribed during spermatogenesis when *de novo* methylation occurs. This nascent ncRNA is targeted by piRNAs and is co-transcriptionally silenced by the deposition of DNA methylation. This may facilitate the spread of targeted silencing into the nearby *Rasgfr1* locus, leading to imprinting, in a similar manner to CTGS in *S. pombe*. The authors do not rule out the possibility that silencing by piRNAs may be indirect, and this is a single-locus example. The *Rasgfr1* locus is, however, unlikely to be the only example of RNAi directing imprinting or silencing of an endogenous gene, and this example hints that nuclear RNAi and transposon acquisition have roles in imprinting across organisms. Further genetic and biochemical dissection is needed to discern whether the piRNA pathway has a direct role in DNA methylation and, if so, what the mechanistic details are. Specifically, interactions between piRNA effectors and cytosine methyltransferases and the use of exogenous reporters containing sequences complementary to known piRNAs would provide convincing evidence.

***Germline nuclear RNAi in* C. elegans.** A class of small RNAs termed 21U has been proposed to be the piRNA of *C. elegans*[93–95]. They associate with the PIWI family protein PRG-1, which is required to silence Tc3 mariner transposons in the germ line and is required for fertility[93,96]. *C. elegans* 21U RNAs originate from more than 5,700 loci that are dispersed over two broad clusters on chromosome IV[94]; however, no evidence of a ping-pong cycle has been observed. The 21U pathway has been suggested to function by determining the specificity of the 22G siRNAs and NRDE pathways (see 'Metazoan somatic nuclear RNAi' and FIG. 1b) that direct TGS in the form of H3K9 methylation at piRNA targets (FIG. 5). Two avenues of study have validated this model. In *C. elegans*, single-copy transgenes with long exogenous DNA sequences, such as GFP, are stably silenced at a high frequency. This silencing correlates with H3K9me3 accumulation and is dependent on PRG-1 and 21U RNA accumulation for its establishment, and on the germline-specific nuclear Argonautes WAGO-9 and WAGO-10 that bind 22G RNA for its maintenance[97]. Studies with reporter transgenes that contain sequences

Cohesin
Large protein rings that predominantly localize to heterochromatic regions of the genome. They function to keep sister chromatids connected during mitosis, facilitate spindle attachment to chromosomes and are involved in DNA repair through recombination.

complementary to known 21U small RNAs (known as piRNA sensors) have revealed identical requirements for silencing and additionally implicated the heterochromatin protein 1 (HP1) orthologue HPL-2 and putative methyltransferases SET-25 and SET-32 in establishing H3K9me3 at loci targeted by piRNA[98]. Silencing at endogenous loci mediated by piRNA probably functions by the same mechanism. Indeed, many endogenous loci that are targeted by 21U small RNAs and that are silenced exhibit increased mRNA expression and a loss of corresponding 22G RNA in a *prg-1* mutant background[99]. RNAi also acts to establish repressive heterochromatin during meiosis at unpaired chromosomal regions in *C. elegans*. Specifically, the RdRP EGO-1 and the PIWI family Argonaute protein CSR-1 are required for this process[100,101].

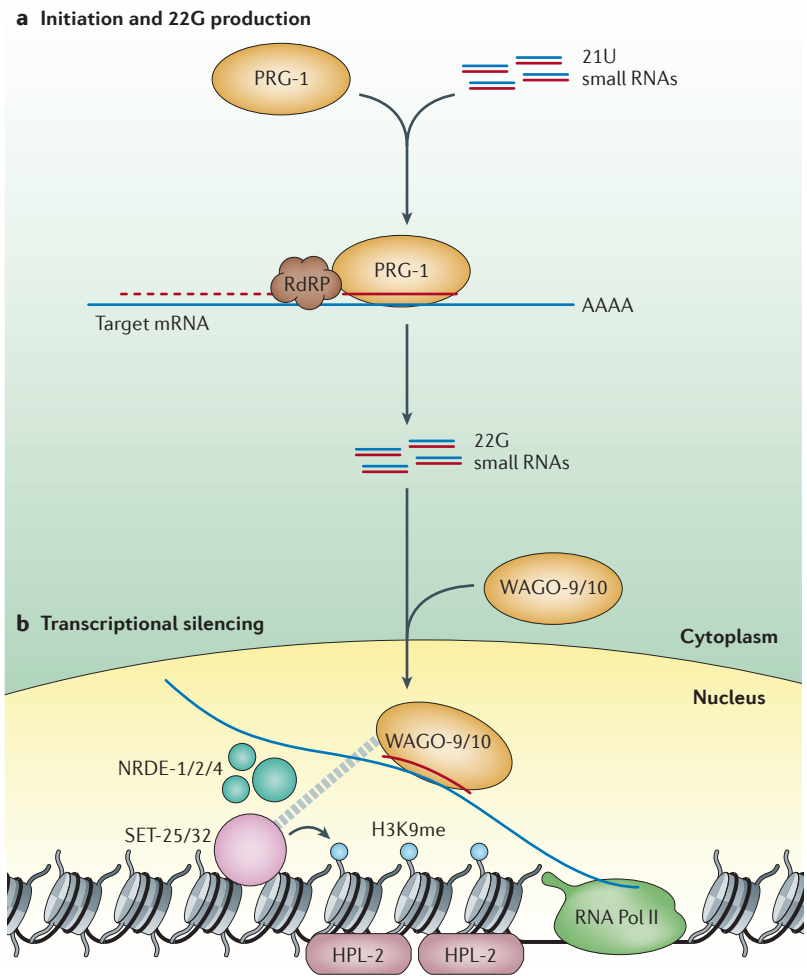

Figure 5 | **The 21U small RNA pathway in the *Caenorhabditis elegans* germ line.** **a** | The 21U small RNAs of *Caenorhabditis elegans* originate from two broad clusters on chromosome IV; however, little is known about their biogenesis. They act with the PIWI family Argonaute PRG-1 to target mRNA in the cytoplasm. Targeting of PRG-1 to mRNA recruits an RNA-directed RNA polymerase (RdRP) to produce abundant 22G small RNAs. **b** | 22G small RNAs are loaded into the germline-specific nuclear Argonautes WAGO-9 or WAGO-10, which are closely related to NRDE-3, the nuclear Argonaute that is involved in somatic transcriptional gene silencing (TGS). Loaded WAGO-9 or WAGO-10 (shown as WAGO-9/10) is transported into the nucleus, where it targets nascent transcripts of RNA polymerase II (RNA Pol II) and directs methylation of histone H3 at lysine 9 (H3K9) in a manner that is dependent on the nuclear RNA interference (RNAi) components NRDE-1, NRDE-2 and NRDE-4 (NRDE-1/2/4). H3K9 methylation is catalysed by two putative histone methyltransferases, SET (Trithorax/Polycomb) domain containing 25 (SET-25) and SET-32 (SER-25/32). The heterochromatin protein 1 (HP1) orthologue HPL-2 binds the H3K9me mark and is required for multi-generational silencing.

Nuclear RNAi in genome maintenance and repair

Nuclear RNAi has a crucial role in maintaining genome integrity by preventing transposon mobilization. However, more direct roles in genome maintenance and DNA repair are emerging.

Chromosome structure and function. Proper chromosome condensation is required for segregation during mitosis. In *S. pombe*, the loss of RNAi causes a high incidence of lagging chromosomes and sensitivity to a microtubule-inhibiting drug[102]. Also, in the *D. melanogaster* germ line, the DEAD box RNA helicase VASA facilitates condensin I localization; this promotes chromosome condensation and is dependent on the piRNA pathway components AUB and spindle E (SPNE)[103]. A VASA paralogue, BELLE, acts analogously in somatic cells and requires the siRNA pathway components AGO2 and DCR2 (REF. 104). Interestingly, a role for RNAi in cohesin localization has also been proposed in *S. pombe*[35], suggesting a conserved role for RNAi in facilitating cohesion and/or condensin localization to ensure proper chromosome condensation.

The telomeres of *D. melanogaster* are unique in that they rely on a transposon-based elongation mechanism[105]. The piRNA pathway has been found to regulate these telomeric transposons in the germ line, and thus it can regulate telomere length[106]. Specifically, *Ago3*-mutant embryos show an increase in telomeric transposition and a subsequent increase in telomere length[107]. Additionally, *aub* and the RNA helicase *armitage* are involved in the production of telomere-specific piRNAs, and their loss results in increased telomere fusion, suggesting another role for the piRNA pathway in telomere cap assembly[107]. Nuclear RNAi is also required for proper telomere function in *S. pombe*. Subtelomeric regions contain a region that is homologous to the pericentromeric repeats, and this region facilitates RNAi-dependent heterochromatin formation[108]. It is possible that nuclear RNAi may have a conserved role in telomere maintenance across organisms.

Eukaryotic genomes contain extensive regions of repetitive DNA that, if engaged in recombination, can cause detrimental changes to chromosome structure. There is evidence that RNAi pathways may act to repress recombination in repetitive regions. The loss of RNAi in *S. pombe* cells leads to both an increase in meiotic recombination[109] and a dependence on mitotic recombination in repetitive pericentromeric regions, as double mutants between RNAi components and the master regulator of homologous recombination *rhp51* are synthetically lethal[31]. This observation has also been made in *D. melanogaster*, in which RNAi-mediated suppression of recombination is required to maintain stability of repetitive DNA[71].

Recombination
The joining of similar or identical DNA sequences to produce a novel molecule. Homologous recombination is used as a mechanism to repair damaged DNA in cells; however, at repetitive regions, it can be detrimental by leading to copy number changes of repetitive elements.

Double-strand breaks
(DSBs). A deleterious form of DNA damage that occurs when the covalent bonds of both strands of a double helix are broken at a locus. It can be repaired by homologous recombination or by error-prone non-homologous end joining.

DNA damage response. A role for small RNAs in DNA damage response (DDR) was first observed in *N. crassa*, where small RNAs are generated from ribosomal DNA (rDNA) repeats when cells are treated with DNA-damaging agents[110]. More recently, RNAi has been shown directly to mediate DNA repair in *A. thaliana*. Double-strand breaks (DSBs) were found to induce a population of 21 nt small RNAs[111]. These small RNAs originate from the vicinity of the DSBs, and their biogenesis requires the siRNA biogenesis factors RNA Pol IV and DCRL proteins. They are recruited to DSBs by AGO2 and mediate repair, as mutations in *Ago2* or biogenesis factors cause a reduction in DSB repair efficiency. The authors suggest[111] that AGO2 recruits the DSB repair complex to damaged loci analogously to the localization of DNA methylation complexes in RdDM. Importantly, the results were validated in human cell lines, pointing to a conserved role for RNAi in DSB repair. A similar finding has been reported in *D. melanogaster* cells, in which DSBs induce a localized production of siRNA that is dependent on AGO2 and DCR2, which are members of the endogenous siRNA (endo-siRNA) pathway[112]. After DSB formation, the DDR pathway is activated and can arrest cell proliferation. Focus on this pathway has revealed that Dicer- and Drosha-dependent small RNAs are required for DDR activation in humans, mice and zebrafish[113]. It is therefore likely that the DDR pathway may link RNAi and DNA repair, although the specific function of the small RNAs themselves remains a mystery.

Targeted genome elimination. Perhaps the most extreme role for nuclear RNAi in genome stability is in targeted genome elimination in *Tetrahymena* spp. and *Paramecium* spp. *Tetrahymena* spp. retain two nuclei, a germline micronucleus and a somatic macronucleus. After zygote formation, a new macronucleus develops by the deletion of ~6,000 internal eliminated sequences (IESs). These IESs are enriched for H3K9 methylation before deletion[114] and produce a population of 28 nt scan RNAs (scnRNAs) that associate with the Argonaute Twi1 (REF. 115). A RNA helicase called Ema1 facilitates the interaction between loaded Twi1 and chromatin by promoting base pairing with nascent transcripts, fitting a co-transcriptional model[116]. It is hypothesized that this leads to the deposition of H3K9 methylation — by a mechanism similar to that occurring in *S. pombe* — which then serves as a mark for DNA elimination in the macronucleus.

These examples show that nuclear RNAi has a conserved role in maintaining genome stability by participating in various pathways across different organisms. In particular, the link to DSB repair shows that Argonaute effector complexes can be directly involved in DNA repair. In other examples, it is not clear whether RNAi has a direct role or whether it simply maintains genome integrity through H3K9 methylation. Higher eukaryotes have numerous Argonaute proteins, many of which are uncharacterized. Further investigation of these Argonautes may reveal novel roles in genome maintenance outside classical RNAi.

Conclusions

Although a role for RNAi in the nucleus was first described in *A. thaliana* and *S. pombe*, observations in key model organisms suggest that it is evolutionarily conserved. RNAi-mediated transcriptional gene silencing has now been observed in plants, fungi and metazoans, and evidence is mounting that it operates co-transcriptionally, as in *S. pombe*. Across organisms, nuclear RNAi predominantly operates at heterochromatic loci, where it facilitates sequence-specific silencing through the direction of histone H3K9 methylation and/or cytosine methylation. Differences are seen, however, in small RNA biogenesis, particularly in the subcellular localization of small RNA processing and loading of Argonaute proteins and could represent alternative approaches to regulating nuclear RNAi. Mechanistically, it is still unclear in the context of the co-transcriptional model how nuclear RNAi complexes regulate transcriptional machinery. Outside constitutive heterochromatin, RNAi co-transcriptionally regulates some genes, and experiments are underway to determine whether this is a widespread phenomenon across organisms.

The part played by nuclear RNAi in the germ line to prevent the propagation of selfish DNA elements in future generations is substantial and highly conserved. In mammals as well as in plants, there is often a link between imprinted genes and nearby transposons, and this may be important in the evolution of some aspects of imprinting from germline transposon control. This field of study will be particularly fruitful in parallel with work on co-transcriptional models that could explain the spreading of silencing at transposon targets into nearby genes associated with ncRNAs and RNAi. Beyond imprinting, it is likely that small RNAs themselves have a conserved role in epigenetic inheritance. As the ability to profile germline cells improves, these questions will be addressed.

Finally, the participation of nuclear RNAi in genome maintenance and DNA repair shows that there are other roles that nuclear small RNAs and their effectors have outside those involved in classical transcriptional silencing. Biochemical purification of novel Argonaute effectors in the context of DNA repair will help to identify the players. The more we learn about nuclear RNAi, the more apparent it becomes that RNAi has a fundamental role in gene regulation and genome maintenance from one generation to the next.

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