RNAi-MEDIATED PATHWAYS IN THE NUCLEUS

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Abstract | RNA interference (RNAi) is an evolutionarily conserved mechanism that uses short antisense RNAs that are generated by 'dicing' dsRNA precursors to target corresponding mRNAs for cleavage. However, recent developments have revealed that there is also extensive involvement of RNAi-related processes in regulation at the genome level. dsRNA and proteins of the RNAi machinery can direct epigenetic alterations to homologous DNA sequences to induce transcriptional gene silencing or, in extreme cases, DNA elimination. Furthermore, in some organisms RNAi silences unpaired DNA regions during meiosis. These mechanisms facilitate the directed silencing of specific genomic regions.

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Double stranded RNA can induce a range of sequence-specific gene silencing phenomena in plants, animals and many fungi. Silencing is initiated when dsRNA is processed into small RNAs, 21–26 nucleotides (nt) in length, by the RNaseIII enzyme Dicer[1]. These small RNAs are then incorporated into silencing-effector complexes, which they guide to complementary nucleic-acid targets. Depending on the nature of the target sequence and the protein composition of the effector complex, different types of silencing can occur[2–4].

RNA interference (RNAi) is initiated by Dicer-generated small interfering RNAs (siRNAs) that target complementary mRNAs for degradation by a ribonuclease-containing RNA-induced silencing complex (RISC). A core component of RISC, and other silencing-effector complexes, is Argonaute, a protein that can bind small RNAs[5] and in some cases execute target-mRNA cleavage[6,7]. Another common, although not universal, protein of the RNAi machinery is RNA-dependent RNA polymerase (RdRP), which synthesizes dsRNA from ssRNA templates to initiate or amplify the RNAi reaction[8,9]. Naturally occurring siRNAs originate from transposons[10–14] and viruses that produce dsRNA during replication[15], as well as other types of bidirectionally transcribed repetitive sequences[16,17] and genes[15,18]. As described in BOX 1, the Dicer, Argonaute and RdRP components of the RNAi machinery — which are also involved in other RNA-mediated silencing mechanisms — are encoded by multigene families in several species.

MicroRNAs (miRNAs) are a second class of small RNA that can induce silencing by targeting mRNA. miRNAs are produced through processing by Dicer of imperfect RNA hairpins, which are typically encoded in non-protein-coding regions of plant and animal genomes. miRNAs are incorporated into a RISC-like complex and, depending on their degree of complementarity to the target mRNA, elicit either translational repression or mRNA cleavage. Gene silencing that is mediated by miRNAs is essential for plant and animal development[19,20], and might also have a role in the pathogenicity of DNA viruses with large genomes that encode miRNAs[21].

Post-transcriptional gene silencing that is induced by siRNAs and miRNAs achieves specificity through RNA–RNA sequence recognition and base pairing. However, RNA can also form base pairs with DNA. Indeed, much of the current excitement in the field of RNA-mediated silencing is being generated by the growing evidence that the RNAi process affects gene function at the level of genomic DNA. In this review, we focus on recent studies that have unveiled an important role for dsRNA and proteins of the RNAi machinery in directing gene silencing in the nucleus. We start by providing a

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Box 1 | **Multigene families that encode components of the RNAi machinery**

Although the fission-yeast genome contains only one copy of each of the genes that encode the core proteins of the RNA interference (RNAi) machinery (Dicer, RNA-dependent RNA polymerase (RdRP) and Argonaute), these proteins are often encoded by multigene families in other organisms.

Dicer

Proteins of the Dicer family are ribonuclease III enzymes that process dsRNA to produce small RNAs. There are four such proteins in *Arabidopsis thaliana*, two in *Drosophila melanogaster*, two in *Neurospora crassa*, one in mammals and one in *Caenorhabditis elegans*.

RNA-dependent RNA polymerases

These synthesize dsRNA from an ssRNA template to initate or amplify the RNAi process. There are four in *C. elegans*, six in *A. thaliana* (at least three of which are expressed) and three in *N. crassa*, but there are none in *D. melanogaster* or mammals.

Argonaute

Members of this family provide the small RNA-binding component of silencing-effector complexes. There are 27 in *C. elegans*, 10 in *A. thaliana*, 8 in humans, 5 in *D. melanogaster* and 2 in *N. crassa*[106].

The proliferation of these gene families in multicellular eukaryotes indicates a subfunctionalization of the encoded proteins into diverse processes, and provides the possibility of separate nuclear or cytoplasmic distributions in the cell. Although much remains to be learned about the extent of functional diversification and/or redundancy of gene-family members, distinct roles in RNA-mediated gene-silencing pathways for some individual representatives of these families have been discovered. Some of these are mentioned in the text and more examples are provided below.

In *A. thaliana*, nuclear DICER-LIKE 1 (DCL1)[107] is required for processing microRNA (miRNA) precursors[19] and DCL2 is required for producing viral small interfering RNAs (siRNAs)[15]. In *D. melanogaster*, Dicer-1 (DCR1) produces miRNAs whereas DCR2 generates siRNAs[108]. The two Dicers in *N. crassa* are functionally redundant in RNAi[109].

In *N. crassa*, meiotic silencing of unpaired DNA (in the nucleus) and RNAi (in the cytoplasm) require distinct RdRPs and Argonaute proteins[93]. In *A. thaliana*, RdRP6 is needed for RNAi that is triggered by sense transgenes[110].

In *D. melanogaster*, Argonaute 1 (AGO1) and AGO2 are implicated in miRNA and siRNA-mediated silencing, respectively[111]. AGO2 in humans contains an RNaseH-like domain that catalyses mRNA cleavage in the RNAi process[6,7].

brief overview of the different RNAi-mediated pathways that operate at the nuclear level. We then describe each mechanism in greater detail.

Overview of nuclear pathways

Four dsRNA-mediated pathways that operate at the level of the nuclear genome have been described so far. Two of these, RNA-directed DNA methylation (RdDM) and RNAi-mediated heterochromatin formation, are EPIGENETIC processes that result in covalent modification of cytosines in DNA or of histones (typically methylation of lysine 9 of histone H3), respectively. RdDM has been described most thoroughly in plants, and RNAi-mediated heterochromatin assembly has been reported in fission yeast (*Schizosaccharomyces pombe*), animals and plants. Although RdDM and RNAi-mediated heterochromatin formation are both initiated by dsRNA, it is not clear whether they represent the products of two separate pathways or a single pathway. In higher organisms, DNA methylation and histone modifications are interconnected in self-reinforcing feedback loops, the details of which are still being unravelled[22]. However, there is no detectable DNA methylation in fission yeast, in which RNAi-based heterochromatin was initially detected[16,23], indicating that histone modifications and DNA methylation are not obligatorially coupled. With this in mind, we will discuss RdDM and RNAi-based heterochromatin separately, and as well as pointing out the overlapping aspects, we will also describe their contrasting features, some of which might reflect mechanistic differences.

In a third mechanism — DNA elimination — ciliated protozoans take the RNAi-mediated heterochromatin pathway a step further: intergenic sequences that have been packaged into silent chromatin by the RNAi machinery are jettisoned during development of the transcriptionally active macronucleus. This initially epigenetic process therefore has a genetic aspect, as the end result is a restructured genome.

Finally, the silencing of unpaired DNA during meiosis that occurs in some organisms relies on proteins of the RNAi machinery. Meiotic silencing, at least in *Neurospora crassa* — where it was first discovered — originates in the nucleus, but unlike the other three mechanisms mentioned above, it is ultimately a post-transcriptional process that does not involve detectable chromatin alterations at the target locus. Indeed, recent evidence indicates that meiotic silencing in *N. crassa* might be an exception to the emerging 'rule' that the RNAi machinery participates in DNA or histone modifications[24,25].

In the following sections, we describe each of these mechanisms in more detail.

RNA-directed DNA methylation

RdDM was the first RNA-guided epigenetic modification of the genome to be discovered. Originally detected in VIROID-infected tobacco plants[26], RdDM was subsequently shown to require a dsRNA that is processed into small RNAs of 21–24 nt, therefore reinforcing a link with RNAi[27]. In plants, dsRNAs which contain sequences that are homologous to promoter regions can trigger promoter methylation and transcriptional gene silencing[27–30].

EPIGENETIC
Refers to mitotically and/or meiotically heritable changes in gene expression that do not involve a change in DNA sequence.

VIROIDS
These are tiny plant pathogens consisting solely of a non-protein-coding, circular rod-shaped RNA that is several hundred base pairs in length.

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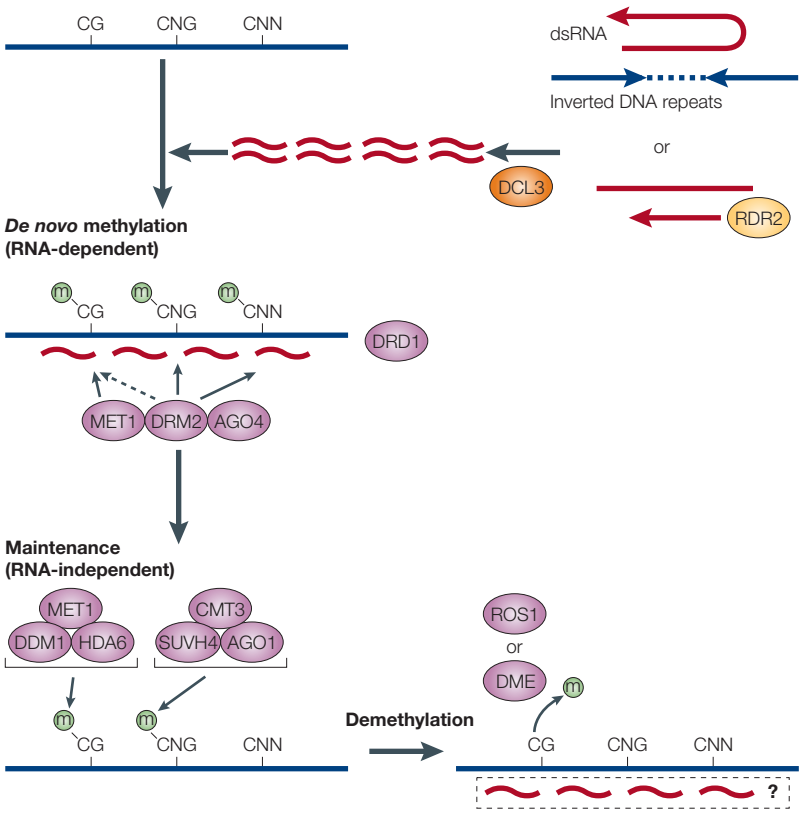

Figure 1 | **RNA-directed DNA methylation.** This model is based on genetic evidence from *Arabidopsis thaliana*. In the nucleus, dsRNA can be produced by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) activity on ssRNA templates or by transcription of inverted DNA repeats by a DNA-dependent RNA polymerase such as RNA polymerase II (not shown). RNA signals that direct DNA methylation are produced through processing of dsRNA by enzymes of the DICER-LIKE family (in some cases known to be DCL3). These RNA signals (wavy red lines) target site-specific methyltransferases — MET1 for CGs and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) primarily for nonCGs — to catalyse *de novo* methylation of DNA. RNA-directed DNA methylation (RdDM) also requires the activity of the SNF2-like protein DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), perhaps to make DNA accessible to RNA signals. Methylation of CGs and CNGs can be maintained to some extent in the absence of the RNA trigger through the activity of MET1 and CHROMOMETHYLASE 3 (CMT3), respectively. Maintenance of CG methylation requires the activity of the histone deacetylase HDA6 and the SNF2-like protein DECREASE IN DNA METHYLATION 1 (DDM1). CNG methylation is accompanied by H3K9 methylation (not shown), catalysed by SUVH4 (also known as KRYPTONITE). The Argonaute proteins AGO4 and AGO1 are involved in *de novo* methylation or maintenance of methylation of some loci, respectively. The DNA glycosylase domain-containing proteins DEMETER (DME) and REPRESSOR OF SILENCING 1 (ROS1) are needed for loss of methylation — and therefore reactivation — of selected genes, which might also involve small RNAs (see also BOX 2).
m, methyl group

NUCLEOSOME
The basic unit of chromatin. The nucleosome core consists of an octamer made up of two copies of each of the histones H2A, H2B, H3 and H4, around which 147 bp of DNA is wrapped approximately twice. The histone N-terminal tails protrude from the core and are targets of various post-translational covalent modifications, such as methylation and acetylation.

RdDM leads to *de novo* methylation of cytosines that occur in all sequence contexts, not just in symmetrical CG dinucleotides, which are considered the usual targets for methylation. Methylation is largely confined to the region of RNA–DNA sequence homology[31,32]. So, unlike RNAi-based heterochromatin, which can spread over several kilobases from the RNA-targeted nucleation site[23,33], RdDM does not usually infiltrate substantially into adjacent sequences. The minimum DNA-target size for RdDM is ~30 bp[34], allowing a degree of refinement that is not possible with histone modifications, which take place in the context of NUCLEOSOMES, comprising 147 bp of DNA.

RdDM in plants. As mentioned previously, RdDM has been studied most extensively in plants, and both forward and reverse genetic approaches are currently being used to analyse the mechanism of RdDM in *Arabidopsis thaliana*. Here, we summarize findings that have been obtained from *A. thaliana* systems that use transgene-encoded RNA hairpins to trigger methylation of homologous target promoters, as well as studies of several endogenous genes for which methylation is thought to be triggered by small RNAs. These analyses have shown that RdDM requires DNA-cytosine methyltransferases and histone-modifying enzymes, in addition to a novel chromatin-remodelling protein and a growing list of proteins that function in the RNAi pathway.

RdDM is a stepwise process that is initiated by RNA signals and site-specific DNA methyltransferases[35,36] (FIG. 1). After the *de novo* methylation step, histone modifications help to maintain DNA methylation, which can otherwise be lost by passive, and possibly active, processes (BOX 2). In *A. thaliana*, maintenance of CG methylation requires the combined activities of the DNA methyltransferase MET1 (REFS 28,36) and the histone deactetylase HDA6 (REF. 37), whereas maintenance of CNG methylation and histone H3 lysine 9 (H3K9) methylation requires the plant-specific DNA methyltransferase CHROMOMETHYLASE 3 (CMT3) and the H3 lysine 9 methyltransferase SUVH4 (also known as KRYPTONITE), an *A. thaliana* homologue of *Drosophila melanogaster* SU(VAR)3-9 (see later) (REFS 38–41) (FIG. 1). The dispensability of SUVH4 for *de novo* methylation was revealed by experiments in which a target gene in an *suvh4* mutant became methylated and silenced in an RNA-directed process[40].

It should be noted that DNA methylation does not always precede H3K9 methylation: this order of events is reversed in *N. crassa*, in which H3K9 methylation is a prerequisite for all cytosine methylation[42]. The mechanistic relationship between DNA methylation and H3K9 methylation therefore differs in plants and in *N. crassa*[40], perhaps reflecting the fact that DNA and histone modifications are independent of RNAi in *N. crassa*[24,25] or reflecting the different structure of the *N. crassa* DNA methyltransferase compared with plant CMT3 and MET1 (REF. 40).

In *A. thaliana*, the CNG–H3K9 methylation pathway has been further connected to RNAi by the identification of the *ago4* mutant[14]. AGO4 is a nuclear protein[15] that, together with additional components of the RNAi machinery — DICER-LIKE 3 (DCL3), RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), and SILENCING DEFECTIVE 4 (SDE4; a protein of unknown function) (FIG. 1) — establishes the methylation of two tandem repeats in the promoter of a transgene that encodes the seed ENDOSPERM protein FWA, leading to its silencing[43]. DCL3 that is localized in the nucleus, and which is one of four Dicer proteins in *A. thaliana* (BOX 1), produces 24-nt siRNAs that have been implicated in inducing chromatin modifications of transgenes and several endogenous repetitive sequences in plants[10,14,15]. AGO4 also has a role in maintaining DNA methylation and H3K9 methylation at several

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Box 2 | **Cytosine demethylation, imprinting and RNA**

CG methylation is lost passively during successive rounds of DNA replication if it is not maintained by DNA methyltransferases[112]. However, there is growing evidence for active demethylation without DNA replication in animals and plants, possibly through the activity of DNA glycosylases; enzymes that normally function in BASE-EXCISION REPAIR[113]. Several 5-methylcytosine DNA glycosylases in vertebrates are thought to excise methylated cytosines, which are replaced with unmethylated cytosines[114].

In *Arabidopsis thaliana*, DNA glycosylase domains are present in two large proteins — REPRESSOR OF SILENCING 1 (ROS1) and DEMETER (DME) — that have been implicated in the activation of transcriptionally silenced genes, presumably through the removal of methylated cytosines[115–118] (FIG. 1). DME is specifically required for the demethylation and activation of the maternal alleles of the imprinted genes *MEDEA* (which encodes a polycomb-group protein that regulates endosperm development)[117] and *FWA* (which encodes a homeodomain transcription factor that functions in the seed endosperm)[54]. Whether the demethylation of target genes by DME or ROS1 is guided by RNA is unknown, but RNA-directed DNA methylation (RdDM) has been implicated in establishing methylation of *FWA* (REF. 46) and of a transgene promoter that is demethylated by ROS1 (REF. 115). Intriguingly, in vertebrate cells, RNA that is complementary to the methylated DNA strand was reported to be required for active demethylation by DNA glycosylases[119]. In addition, the chicken DNA glycosylase associates tightly with an RNA helicase, p68 (REF. 120). In *Drosophila melanogaster*, the p68 orthologue is required for efficient RNA interference (RNAi)[121]. If the p68 orthologue in chicken is also needed for RNAi, a connection would be established between demethylation by DNA glycosylases and the RNAi machinery in vertebrates.

endogenous and transgene repeats, and in the accumulation of siRNAs that correspond to these sequences[14,15,44]. Whereas AGO4 is proposed to be specialized for chromatin modifications[14], a second *A. thaliana* Argonaute protein, AGO1, is more versatile, acting both in siRNA and miRNA-mediated post-transcriptional gene-silencing pathways[45], and in the maintenance of chromatin modifications at some loci[13,46,47].

A clue as to how the RNAi machinery triggers methylation of DNA in the context of chromatin came from a screen for mutants that are defective in the RdDM of a seed-specific promoter that was expressed transgenically in *A. thaliana*. This screen identified DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), a plant-specific, putative SNF2-LIKE CHROMATIN-REMODELLING PROTEIN[48]. The involvement of DRD1 in RdDM indicates that reconfiguration of chromatin is required to render the target DNA accessible to RNA signals. A second SNF2-like protein, DECREASE IN DNA METHYLATION 1 (DDM1) — which has a mammalian homologue, lymphoid specific helicase (LSH)[49] — contributes to maintenance of RdDM[32,50,51]. DDM1 was originally identified in a screen for mutants that are deficient in DNA methylation of centromeric and rDNA repeats[52] — repetitive regions that provide useful indicators of reduced methylation in populations of mutagenized plants — although it is still not known whether they initially acquire methylation through RdDM. A link between DDM1 activity and RNA-mediated silencing of repetitive elements was revealed by the finding that DDM1 cooperates with MET1 and HDA6 to maintain a large domain of transposon-containing heterochromatin (the chromosome 4 'knob'), a region that is the source of many corresponding transposon siRNAs[46]. Furthermore, DDM1 is needed for MET1-catalysed DNA methylation and silencing of promoter tandem repeats in the endogenous *FWA* gene[53,54]. The trigger for methylation is presumably provided by siRNAs that are homologous to the tandem repeats, which originate from transposons[46]. Subsequent loss of methylation from the maternal allele is important for imprinted expression of *FWA* in endosperm (BOX 2).

FWA provides the first example of an endogenous promoter that is targeted for DNA methylation and transcriptional silencing by transposon-derived siRNAs. Whether other endogenous promoters are similarly regulated is not yet known. Answering this question will require a full survey of small RNA populations and their potential targets. In *A. thaliana*, many endogenous small RNAs are derived from non-protein-coding regions[15] and could potentially target epigenetic modifications to promoter or enhancer elements.

RdDM in other organisms. Does RdDM occur in organisms other than plants? Some of the known components of the RdDM machinery are present in mammals (BOX 3), indicating that RdDM could potentially occur outside the plant kingdom; however, until now, reports on the occurrence of RdDM in mammals have reached conflicting conclusions. In one of the first reported investigations of RdDM in mammalian cells, long dsRNA that knocked down expression of a target gene by RNAi in mouse oocytes did not induce *de novo* methylation of the corresponding DNA region[55]. Two recent studies[56,57], however, indicate that siRNAs that target the promoters of endogenous genes can induce CG methylation, transcriptional silencing and H3K9 methylation[57] in human cells. Further work is required to determine the generality of these results. Even if dsRNA or synthetic siRNAs do not normally trigger RdDM in mammals, other non-coding and antisense RNAs, the modes of action of which are still not fully understood, have been implicated in chromatin-based regulation in these organisms[58].

RNAi-directed heterochromatin

Heterochromatin has long been recognized as a cytologically visible, genetically inactive component of nuclei. However, the pathway of heterochromatin assembly has come into focus only recently, with the striking discovery of the involvement of RNAi in its formation.

ENDOSPERM
The seeds of flowering plants contain two fertilization products: the diploid embryo and the triploid endosperm, a terminally differentiated tissue that serves as a nutrient source for the developing embryo.

BASE-EXCISION REPAIR
(BER). The replacement of DNA bases that are altered by small chemical modifications through the excision of only the damaged nucleotide (short-patch BER) or through the removal of 2–13 nucleotides containing the damaged nucleotide (long-patch BER).

SNF2-CHROMATIN-LIKE REMODELLING PROTEINS
First identified in budding yeast as sucrose non-fermenter, or mating type switching defective (*Swi*) mutants, these proteins use energy from ATP breakdown to enhance the accessibility of nucleosomal DNA to regulatory factors.

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Box 3 | Does RNA-directed DNA methylation operate in mammals?

Non-CG methylation, a hallmark of RNA-directed DNA methylation (RdDM) in plants, has been detected in DNA of mammalian embryonic stem cells[122] and in human L1 retrotransposons[123], but it is unknown whether this methylation is triggered by RNA.

One issue is whether mammals have the molecular machinery needed for RdDM. Two of the DNA methyltransferases required for RdDM in plants, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) and DNA methyltransferase MET1, have mammalian counterparts (DNMT3A/B and DNMT1, respectively), although the catalytic domain of DRM2 is rearranged relative to DNMT3A/B and there are some differences in their amino termini[104]. The plant-specific DNA methyltransferase CHROMOMETHYLASE 3 (CMT3) is important for CNG methylation[38,39], but whether this enzyme is an absolute requirement for RdDM is not clear. Another plant-specific factor of the RdDM machinery is the SNF2-like protein DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1). The closest non-plant homologues of DRD1 are members of the conserved Rad54/ATRX subfamily of SNF2-like proteins[48]. ATRX (α-thalassaemia, mental retardation, X-linked) is needed for CG methylation of some repetitive sequences in mammals[124]. Whether this methylation is triggered by RNA is unknown, but ATRX could conceivably substitute for DRD1 in the RdDM pathway in mammalian cells.

RdDM clearly takes place in the nucleus, whereas the mRNA degradation step of RNAi occurs typically in the cytoplasm. Are small RNAs that trigger RdDM likely to be present in nuclei of mammalian cells? Plant cells have DICER-LIKE activities that are localized in the nucleus, such as *Arabidopsis thaliana* DCL3 (REF. 15) and DCL1 (REF. 107), which can produce small RNAs in the nucleus. Moreover, in plant cells, small RNAs that initiate RNAi in the cytoplasm can move to the nucleus to induce methylation of homologous DNA[125]. Although the single Dicer of mammals is present in the cytoplasm[126], a mechanism for translocating small RNAs to the nucleus of mammalian cells is indicated by the presence of mature miRNAs in nuclear as well as cytoplasmic cellular fractions[7]. Small RNAs produced in the cytoplasm might also be able to interact with DNA when the nuclear envelope breaks down during cell divisions[57]. So, mammals seem to have much of the machinery for RdDM, but whether this process occurs regularly in mammals remains to be determined.

Properties of heterochromatin. As a general rule, heterochromatin is gene-poor relative to euchromatin and it surrounds the centromeric region in many species. Heterochromatin is usually not conducive to high levels of gene expression; in some organisms, chromosomal rearrangements that juxtapose heterochromatin and euchromatin result in a mosaic pattern of gene silencing called position-effect variegation (PEV), caused by alteration of the chromatin state across the junction between the two[59]. At the DNA level, heterochromatin is composed of degenerate retrotransposon sequences, tandem arrays of a simple repeat unit, or a combination of both[16,60]. Indeed, artificial tandem arrays of transgenes have long been known to mimic PEV-type silencing for the genes that are carried on the repeat[61].

The molecular properties of heterochromatin have been defined most thoroughly in *D. melanogaster*, mammals and fission yeast. Certain conserved proteins are highly concentrated in heterochromatin in many organisms. Heterochromatin protein 1 (HP1) binds at its CHROMODOMAIN to histone H3 that is methylated at Lys9 (REFS 62–64). This methylation of histone H3, which is typical of heterochromatic regions, is mediated primarily by SU(VAR)3-9, the mutant form of which functions as a suppressor of PEV (REF. 65). In fission yeast, the homologues of HP1 and SU(VAR)3-9 are Swi6 and Clr4, respectively[2]. A third protein that has a key function in heterochromatin is cohesin, which is required for the attachment of sister chromatids until they are separated during anaphase[66–68].

RNAi targets centromeric heterochromatin. The DNA component of fission-yeast heterochromatin consists of simple transposon-derived tandem arrays, which surround the central-core centromeric region of each chromosome[16]. This central-core region anchors the KINETOCHORE, whereas the flanking heterochromatin domains promote the cohesion of sister chromatids[69]. The derepression of the centromeric outer-transposon repeats in mutants deficient for components of the RNAi machinery led to the proposal that small RNAs function as guides to target the chromatin modifications that are typical of heterochromatin[16]. Specifically, this evidence came from knockouts for the genes that encode Argonaute, Dicer and RdRP, which are single-copy genes in fission yeast (BOX 1). Usually, transcripts from the forward strand of the repeats are not seen in transcription assays, and a low level is found for the reverse strand. However, in the RNAi mutants, RNA from both strands accumulates, indicating the involvement of the RNAi pathway in transcriptional silencing. The epigenetic basis of this silencing was revealed by reduced amounts of H3K9 methylation and repeat-associated Swi6 in the RNAi mutants.

Further evidence for the involvement of the RNAi pathway in the formation of heterochromatin in fission yeast came from studies in which a transgene that is located in the centromeric repeats, which would usually be silenced, was activated in RNAi mutants. Similar observations were made at the SILENT MATING-TYPE locus, which contains a single copy of the centromeric repeat that is targeted for H3K9 methylation by siRNAs, with subsequent Swi6-dependent spreading of heterochromatin beyond this region[23].

These results led to the proposal that transcription of the two strands of the centromeric repeats generates dsRNA molecules. These molecules might be amplified by the RdRP, which was found to associate closely with the repeats[16]. Processing of the dsRNAs by Dicer is thought to produce small RNAs that guide the histone methyltransferase to the respective sites on the chromosome to catalyse H3K9 methylation. The modified form of H3 is bound by Swi6 (the fission-yeast HP1 homologue), which diminishes transcription capacity. Cohesin subsequently becomes associated with the heterochromatic sites and aids in sister-chromatid association. The sequence of events that is thought to take place in RNA-mediated heterochromatin formation is shown in FIG. 2. In fission yeast, a nuclear-effector complex known as 'RITS' (RNA-induced initiation of transcriptional gene silencing) is involved in targeting chromatin modifications. RITS contains siRNAs that originate from heterochromatic regions, such as centromeres, and three identified proteins — Ago1, Chp1 (a centromere-associated chromodomain protein) and Tas3 (a serine-rich protein that is specific to fission yeast)[70]. RITS is tethered to silenced loci by H3K9 methylation and this tethering is essential for silencing and the continued production of siRNAs[71].

CHROMODOMAIN
(Chromatin organization modifier domain). Initially identified in the *Drosophila melanogaster* HP1 and polycomb proteins, this is an ~50-amino-acid domain that binds to histone tails that are methylated at certain lysine residues.

KINETOCHORE
A large protein complex that mediates the attachment of chromosomes to the meiotic and mitotic spindles.

SILENT MATING TYPE
The unexpressed copy of the mating-type locus.

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The RNAi machinery has also been implicated in heterochromatin formation in *D. melanogaster*. Mutations in two genes that encode members of the Argonaute family (*piwi* and *aubergine*) as well as mutations in the *homeless* (otherwise known as *spindle E*) gene (which encodes an RNA helicase that is important for RNAi[72], meiosis[73] and transposon silencing[74]) suppress PEV. In the studies that revealed this, suppression was manifested by release of the variegated silencing of transgenically inserted tandem arrays of the *white* eye-colour gene or by the reversal of silencing of *white* transgenes that are inserted into the heavily heterochromatic fourth chromosome[75]. The strongest effect was found with *homeless* mutants, which show reduced levels of H3K9 methylation in heterochromatic regions and a reduction in the amounts of HP1 that are associated with these sites.

A recent study showed a similar reliance on components of the RNAi machinery for the formation of centromeric heterochromatin in vertebrate cells. Dicer expression was conditionally inactivated in chicken cells that carry a copy of human chromosome 21 (REF. 76). This system allows a single human chromosome to be analysed without the confounding background of similar centromeric sequences from other human chromosomes. α-Satellite centromere sequences form a repetitive array at all human centromere regions. They surround and include the active kinetochore on human chromosome 21. It has been shown that transcripts from this region are processed by Dicer[76]. Cohesin-GFP reporter proteins — which are usually expected to localize to heterochromatin — were delocalized in the Dicer-deficient cells. The various forms of HP1 that are expressed in chicken cells were also partially delocalized within the nucleus, indicating a disruption of heterochromatin targeting by the RNAi machinery. Abnormal mitoses were also seen in the Dicer mutants, probably as a consequence of the disruption of cohesin localization.

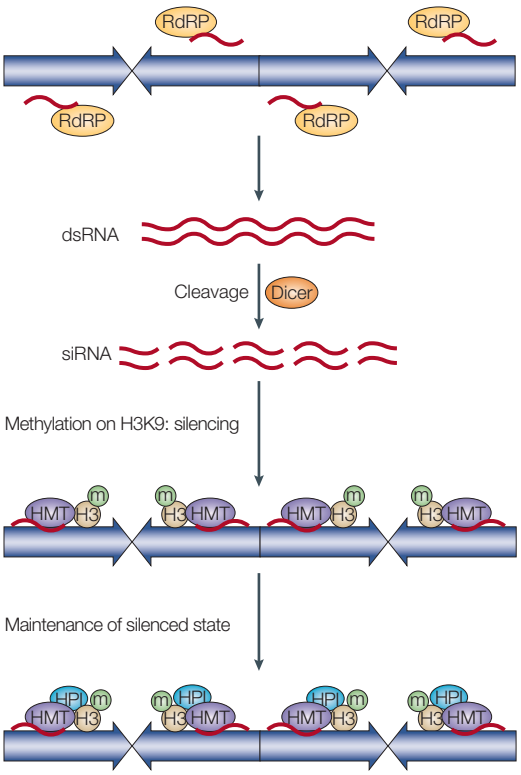

Figure 2 | **RNA interference-mediated heterochromatin assembly.** Tandem repeats or multiple copies of transposable elements in heterochromatin generate dsRNAs, or those that can be converted to a double-stranded form, through the activity of RNA-dependent RNA polymerase (RdRP). The dsRNA is cleaved by Dicer to produce small interfering RNAs (siRNAs). These siRNAs are thought to guide histone methyltransferases (HMTs) to the chromatin to modify histone H3 on lysine 9 (H3K9). The methylated form of H3 is bound by Swi6 or HP1, which also associates with the methyltransferases, to maintain the silenced state. m, methyl group.

Silencing at interstitial sites. In addition to centromeric heterochromatin, the RNAi pathway is also implicated in the targeting of non-centromeric, interstitial sites in euchromatin for silencing. In *D. melanogaster*, dispersed transgenes that are inserted at several sites in euchromatin are silenced at the transcriptional level through association with the POLYCOMB COMPLEX[77,78]. This type of silencing at interstitial sites is also dependent on components of the RNAi pathway[78]. Polycomb-dependent silencing involves histone modifications that are different to those described above for HP1-mediated silencing at centromeric regions (that is, the methylation of lysine 27 rather than lysine 9 of histone H3), but are similar in that they result in a more restrictive chromatin environment that generally prevents high levels of transcription.

A role for RNAi in heterochromatin-like dispersed silencing in otherwise euchromatic regions has also been demonstrated in fission yeast. The transcription of an artificial hairpin RNA was shown to trigger the silencing of homologous sequences throughout the genome[33], and the silenced genes showed increased levels of H3K9 methylation, with attendant Swi6 and cohesin binding. The same study also showed that the long terminal repeats of retrotransposons are targets of this pathway. This type of interstitial RNAi-mediated heterochromatin can spread 7–10 kb from the site that is initially nucleated by siRNAs, and can also spread into endogenous genes and regulate their expression. For example, this was shown to occur for certain genes that are expressed only in cells undergoing meiosis, demonstrating a role for this process in cellular differentiation[33].

Small RNAs are also involved in generating interstitial heterochromatin in *A. thaliana*. A detailed microarray analysis of the transposon-rich heterochromatic knob on chromosome 4 of *A. thaliana* (discussed earlier) revealed high levels H3K9 methylation and DNA methylation that were concentrated in transposon sequences and did not spread to adjacent genes[46]. Small RNAs that are homologous to transposons and repeat sequences in this region presumably direct the target-specificity of these chromatin modifications, although this has still to be shown directly. Methylation of these repetitive elements is dependent on the SNF2-like

POLYCOMB COMPLEX
A group of repressive chromatin proteins that maintain states of gene expression throughout development.

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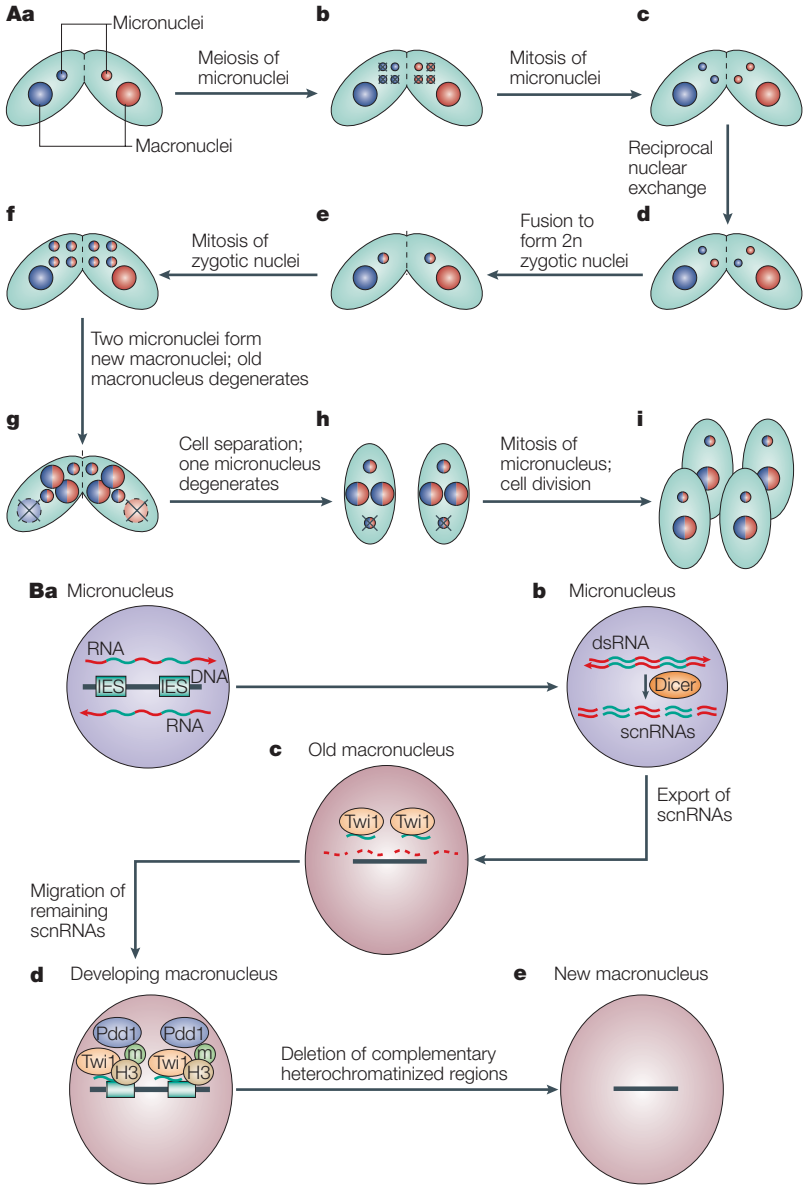

Figure 3 | **RNA interference (RNAi) and DNA elimination in *Tetrahymena thermophila*.** **A** | Conjugation in *T. thermophila*. Vegetative cells of *T. thermophila* contain one micronucleus and one macronucleus. Conjugation begins with pairing of complementary mating types (**a**). The micronucleus undergoes meiosis to produce four haploid nuclei, three of which degenerate (**b**). The remaining haploid micronucleus divides mitotically (**c**). This is followed by reciprocal nuclear exchange (**d**) and fusion to form a new diploid zygote nucleus (**e**). The zygote nucleus undergoes two mitotic divisions (**f**). From the four products of these divisions, two develop into new macronuclei as the old macronucleus degenerates (**g**). It is during this period (**f**, **g**) that internal eliminated segment (IES) elimination and other genome alterations occur. Cells then separate and one micronucleus degenerates (**h**). The remaining micronucleus divides mitotically and subsequent cell divisions produce four daughter vegetative cells (**i**). Modified, with permission, from REF. 130 © (1996) Academic Press. **B** | RNA-scan model for IES elimination. Micronuclear IES sequences destined for elimination during development of the new macronucleus are shown in green. Early in conjugation, the micronuclear genome is bidirectionally transcribed (**a**). The resulting dsRNA is processed by Dicer into small RNAs, termed scan RNAs (scnRNAs) (**b**). The scnRNAs are exported into the old macronucleus, and those that can base pair to genomic DNA (red dotted lines) are degraded. The remaining scnRNAs, which correspond to sequences eliminated in the previous conjugation, migrate to the developing new macronucleus (**c**) and induce H3K9 methylation of the homologous IES sequences (**d**), which are then deleted (**e**). Twi1 is a Piwi-related protein that associates with small RNAs in both the old and new macronuclei[87]. Programmed DNA degradation protein 1 (Pdd1) binds through its chromodomain to H3 methylated on lysine 9 and is essential for DNA elimination. Modified, with permission, from REF. 79 © (2004) Elsevier Science. m, methyl group.

chromatin-remodelling protein DDM1, as the levels of small RNAs that are homologous to a repeated transposon in the knob were reduced in *ddm1* mutants, implicating this protein in the targeting mechanism[46].

Overall, these studies indicate that repeat sequences with the potential to produce dsRNAs are targeted for silencing by an RNAi-mediated pathway. Small RNAs that are generated by Dicer cleavage of dsRNAs function as facilitators in guiding histone methyltransferases to the homologous sites in chromatin, whether they are clustered in heterochromatin or located interstitially in the chromosome. This initial modification provides a platform onto which repressive proteins such as HP1 or the polycomb complex bind, leading to the packaging of the chromatin in such a way that limits the amount of transcription that can occur.

DNA elimination and RNAi

RNAi has recently been implicated in what has been called the 'ultimate form of gene silencing'[79]: the programmed excision of excess DNA. This phenomenon can be observed in ciliated protozoans, which extensively reorganize their genome during the sexual process of conjugation. The single-celled ciliates contain two functionally distinct types of nuclei: a diploid micronucleus provides the germline reserve and is transcriptionally silent during vegetative growth, whereas a polyploid macronucleus serves as the transcribed, somatic nucleus. Both types of nuclei develop from mitotic divisions of a zygotic nucleus, which is formed during conjugation by fusion of two haploid micronuclei (FIG. 3A). Differentiation of the new macronucleus is accompanied by massive elimination of germline DNA sequences — known as internal eliminated segment (IES) sequences. Although no general function has been assigned to this unusual process, it might eliminate 'selfish' DNA elements, such as transposons, that have invaded the micronuclear genome[79]. In *Tetrahymena thermophila*, about 15% of the genome, comprising about 6,000 individual sequences, is deleted in this way[79]. In *Paramecium tetraurelia*, the number of precisely removed IES sequences is around 60,000 (REF. 80). Until recently, the mechanism of coordinated elimination of IES sequences, which do not share common sequences, was unknown. Recent findings implicate the RNAi machinery in targeting modifications that are typical of heterochromatin to IES sequences, therefore marking them for subsequent excision.

An initial hint that RNA is involved in programmed DNA elimination came from the observation of bidirectional transcription of the micronuclear genome during conjugation, which produced heterogeneous and potentially double-stranded transcripts that contained IES sequences[81]. Moreover, unusual epigenetic effects, in which an experimentally introduced sequence in the old macronucleus could affect the excision of the homologous sequence in the new macronucleus, indicated the internuclear transfer of a sequence-specific signal, which was postulated to be a diffusible RNA[82]. Indeed, many components of the RNAi machinery are active at the time when DNA elimination occurs. In

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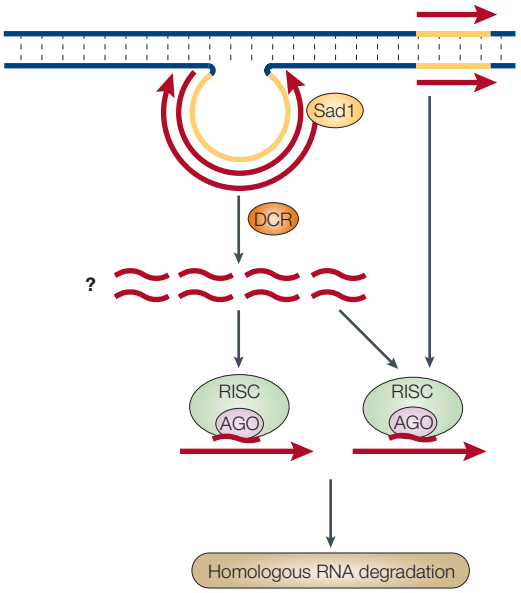

Figure 4 | **Silencing of unpaired DNA during meiosis.** In some species, silencing of unpaired genomic regions takes place during meiosis. Genetic analyses in *Neurospora crassa* have demonstrated that this silencing requires an RNA-dependent RNA polymerase (RdRP) and an Argonaute (AGO)-like protein, which are distinct from the ones required for RNAi in this organism (BOX 1). In *N. crassa*, meiotic silencing involves a *trans*-sensing step at the chromosomal level (dotted lines between the two chromosomes, each depicted as a solid blue line). Any region that is unpaired (yellow loop) is transcribed, presumably by a DNA-dependent RNA polymerase (not shown). The resulting ssRNA (inner red circular arrow) is used as a template by the RdRP Sad1 to synthesize dsRNA (two red circular arrows). This dsRNA is probably processed into small RNAs by Dicer (DCR), however the involvement of small RNAs in this process has not yet been confirmed. Although the precise events that take place in the subsequent steps are not known, the small RNAs are thought to bind to the Argonaute-like protein in a RISC (RNA-induced silencing complex)-like complex, which then targets all transcripts that are homologous to the unpaired region (straight red arrows) — even those originating from homologous paired regions elsewhere in the genome (yellow parallel bars) — for degradation.

T. thermophila, small RNAs that are 28 nt in length appear before elimination[83]. In cells deficient for Twi1 — a Piwi-related protein that is expressed only during conjugation — these small RNAs fail to accumulate and IES excision does not occur[83]. Twi1 is required for H3K9 methylation of IES sequences, which in turn is required for elimination[84]. Two proteins, Pdd1p and Pdd3p, which are also required for deletion of IES sequences, bind through their chromodomains to histone H3 that is methylated on K9 (REF. 85).

A model has been proposed in which small RNAs, generated by Dicer processing of micronuclear dsRNA, scan the genome of the old macronucleus. Any small RNA that lacks a genomic partner for base pairing (that is, those that correspond to regions that were eliminated in the preceding conjugation) is 'subtracted out'[86] and diffuses into the developing macronucleus to guide H3K9 methylation of IES sequences, which are eventually eliminated[79,83,87] (FIG. 3B). Experimental delivery of dsRNA by injection[88] or bacterial feeding[80] triggers the deletion of the homologous genomic DNA, even if it is normally retained, indicating that the mechanism can target any sequence. The role, if any, of DNA methylation in IES elimination is not known; there is no reported evidence that N6-methyladenine — the main modified nucleotide in nuclear DNA of *T. thermophila* and *Paramecium tetraurelia* — is involved in this process (E. Meyer, personal communication).

IES sequences are probably derived from transposons[79,88]. So, the same type of sequence that triggers the nucleation of RNAi-based heterochromatin in other organisms is also targeted by an RNAi-related mechanism in ciliates. However, instead of maintaining silent chromatin, ciliates eject the DNA. A future challenge will be to determine the nature of the DNA-excision machinery and how its activity is induced by heterochromatin-like regions that are established by RNAi-mediated mechanisms.

Meiotic silencing, pairing and RNAi

Throughout the history of gene-silencing research, homologous DNA–DNA pairing has been considered as a possible trigger for silencing. Fueling this idea were studies such as one carried out in the filamentous fungus *Ascobolus immersus*[89]. During meiosis in this organism, DNA methylation can be transferred from one allele to another in a pattern that is characteristic of GENE CONVERSION, indicating that DNA–DNA pairing mediates the process[89]. However, the idea that DNA pairing is involved in other types of homology-dependent gene silencing became less compelling with the discovery of siRNAs, which could function as diffusible *trans*-acting homology signals for silencing. Nevertheless, pairing of homologous genes does seem to be involved in silencing in some cases, although the available evidence still indicates an interrelationship with RNA-mediated silencing mechanisms. A striking example of a relationship between homologous pairing and RNAi is the phenomenon of meiotic silencing of unpaired DNA.

***Meiotic silencing in* Neurospora crassa.** The silencing of unpaired DNA during meiosis was documented through studies of the *Ascospore maturation-1* (*Asm1*) gene in *N. crassa*[90,91]. *N. crassa* is haploid in its vegetative state, but the zygotic form is diploid. This single diploid cell undergoes meiosis, which involves pairing of homologous chromosomes. The endogenous *Asm1* gene, or an *Asm1* transgene placed elsewhere in the genome, is fully active when it has a pairing partner during meiosis. However, a single unpaired copy of *Asm1* is silenced, as is any combination of three copies or even two copies that are located in the genome in such a way that they cannot associate. So, an unpaired copy of a gene in the diploid meiotic cell will trigger silencing of all homologous copies, independent of whether the others are paired (FIG. 4).

A mutation in the *suppressor of ascus dominance-1* (*Sad1*) gene that suppresses this meiotic silencing

GENE CONVERSION
The non-reciprocal transfer of DNA-sequence information during meiotic recombination owing to heteroduplex formation.

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HERMAPHRODITE
Individuals with both male and female sexual organs.

mechanism has been isolated[91]. This mutation reverses the silencing of unpaired DNA and also suppresses the dominant-sterile phenotypes of several other mutations that might cause failure of meiotic pairing, as well as the dominant sterility that is characteristic of chromosomal duplications, which also produce unpaired regions during meiosis. Furthermore, crosses to related species — that might be expected to harbour chromosomal variation and therefore result in unpaired regions in hybrids — are sterile, but this sterility is partially overcome by the *Sad1* mutation. Cloning and sequencing of *Sad1* showed that it encodes an RdRP[91,92], revealing an unexpected connection between DNA pairing and RNAi. An Argonaute-like protein, Sms2 (suppressor of meiotic silencing 2), is also required for meiotic silencing[93], which further implicates the RNAi machinery in this process. Mutations in DNA methyltransferase genes in *N. crassa* do not affect meiotic silencing[92]. However, DNA methylation does — by an unknown mechanism — influence the previous *trans*-sensing step (explained in FIG. 4), which has recently been shown to be genetically separable from silencing[94].

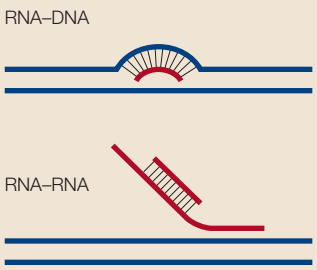
The characteristics of the unpaired regions that are silenced by this mechanism were analysed using mutations of the *Asm1* gene[95]. A range of deletions, which altogether cover the whole gene, were made in a haploid strain, which was crossed to cells of the opposite mating type that carried a normal copy of the gene. This produced diploid zygotic cells, which undergo homologous pairing during meiosis. For a single unpaired copy to silence itself (*cis* silencing), a transcribed region needs to be included in the looped out region that is formed by an unpaired sequence (FIG. 4). Consistent with a need for production of RNA in this mechanism, an unpaired promoter sequence of a gene did not trigger silencing, provided that the structural portion of the gene was paired. Furthermore, silencing of unpaired genes did not spread to paired neighbouring genes[96]. Although silencing could be achieved with as little as 700 base pairs of unpaired DNA, longer unpaired regions induce more efficient silencing. By contrast, when homologous DNA was placed elsewhere in the genome in an unpaired configuration, fragments of more than 2 kb were needed to observe silencing in *trans*[95]. Therefore, *trans* silencing is not as effective as *cis* silencing, although both require unpaired transcription units.

This highly specific type of silencing therefore allows diploid cells to sense and silence newly transposed mobile elements. The mechanism might thereby operate to protect the genome from unchecked transposition and a correspondingly high mutation rate. The process could also function as a powerful speciation mechanism, in that chromosomal changes among separated evolutionary lineages will cause sterility if the lineages are reunited and mate.

Related mechanisms in other organisms. A phenomenon similar to meiotic silencing of unpaired DNA, but which involves chromatin modification, has been documented recently in *Caenorhabditis elegans*. In this species, an unpaired X chromosome is present in males (which have an XO genotype), but not in HERMAPHRODITES (XX genotype). During meiosis, unpaired X chromosomes are targeted for H3K9 methylation regardless of the sexual phenotype of the cell, be it a normal male or an XO animal that has been transformed by sex-determination mutations to phenotypically resemble hermaphrodites[97]. Changes in other histone modifications are not seen. In crosses involving males, the X-chromosome modifications persist for several cell divisions into the new generation, up to the 20-cell stage. It is still not clear why this silencing is needed, but it might reflect a genome-surveillance mechanism that detects new transposable-element insertions. Given that RNA is implicated in meiotic silencing in *N. crassa* and in targeting H3K9 methylation in fission yeast, plants and animals, it is tempting to speculate about its involvement in this epigenetic modification as well. However, there are no data to support this hypothesis so far.

A process that has the opposite effect occurs in somatic cells of *D. melanogaster*, where the homozygous pairing of certain transgenes results in a reduced level of expression compared with that seen if two copies are present in dispersed, non-allelic positions in the genome. This phenomenon is referred to as pairing-sensitive silencing[98,99]. In *D. melanogaster*, homologous chromosomes are paired throughout much of the life cycle. This allows pairing interactions to occur that might influence gene expression in somatic tissues. Remarkably, pairing-sensitive silencing is affected by

Box 4 | RNA–RNA or RNA–DNA?



Although RNA-dependent RNA polymerase (RdDM) and RNA interference (RNAi)-mediated heterochromatin are initiated by dsRNAs that are substrates for Dicer cleavage, divergent steps in the two pathways might yield distinct primary epigenetic marks — DNA cytosine methylation or histone H3 lysine 9 (H3K9) methylation, respectively. Which of these two processes occurs could depend in part on the way small RNAs interact with homologous target sequences. RdDM is characterized by non-spreading cytosine methylation that can be deposited locally on relatively short, single-copy target regions[34]. By contrast, RNAi-based heterochromatin is often targeted to repetitive sequences[127] and can spread, in a Swi6 (mating type switching defective 6) or HP1 (heterochromatin protein 1)-dependent manner, several kilobases from the RNA-targeted nucleation site. Two models for how small RNAs interact with a homologous target locus have been proposed[128] (see figure): direct RNA–DNA pairing (the DNA-recognition model) or pairing of small RNAs and a nascent RNA that is transcribed from the target locus (the RNA-recognition model). At present, there is no conclusive evidence for either model. To resolve this issue, it will be necessary to determine whether transcription of target DNA sequences is required to elicit silencing and epigenetic modifications. The relatively precise boundaries of many examples of RdDM in plants and of RNAi-mediated DNA elimination in ciliates are most consistent with direct RNA–DNA base pairing, therefore arguing for a DNA-recognition model. However, possibly supporting the RNA recognition model, a recent study in *Arabidopsis thaliana* shows that nascent RNA is needed for miRNA-associated DNA methylation of exons that are downstream of the miRNA complementary region[129].

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mutations in genes that encode components of the RNAi machinery, although not in the way that might be expected from the studies described above. For *engrailed–white* transgenes, which carry an *engrailed* regulatory region adjacent to a copy of the *white* eye-pigmentation gene, homozygous *piwi* and *homeless* mutations increase the level of silencing of paired copies[100]. Unpaired copies of the transgene in the same genomic location are unaffected, indicating that it is specifically pairing-induced silencing that is intensified in RNAi mutants. Curiously, this effect is the opposite to that expected from the requirement for siRNAs as mediators of silencing, because whereas such RNAs are expected to be present at reduced levels or absent in the mutants, the degree of silencing increases. Nevertheless, this example demonstrates a case in which somatic pairing influences gene expression, and the RNAi pathway seems to be involved in this process.

Summary, conclusions and outlook

Findings from diverse organisms have revealed a prominent role for small RNAs and the RNAi machinery in eliciting sequence-specific silencing at the genome level. An impressive variety of effects have been reported: RNA-directed DNA methylation and histone methylation, DNA elimination and pairing-associated silencing. Despite the seeming ubiquity of small RNA-guided mechanisms, however, it is important to keep in mind that there are RNAi-independent pathways for heterochromatin formation[24,25,101] and DNA methylation[24,102–104] in organisms in which RNAi takes place.

Consistent with the role of RNAi in defence against invasive sequences[11,105], transposons and related repeats are preferred natural targets of the RNAi-mediated silencing pathways in the nucleus. Although often considered solely as molecular parasites, transposon sequences that function as foci for RNAi-based chromatin modifications benefit the host through these mechanisms by contributing to gene regulation and to chromosome structure and function.

Although a general picture of RNAi-mediated pathways in the nucleus has emerged, many questions remain. Foremost among these is the manner in which small RNAs interact with a target locus. Do small RNAs base pair with target DNA sequences or with nascent transcripts (BOX 4)? Conceivably, both types of interaction have been exploited in nature, but conclusive experiments are still required. A related issue is the molecular composition of nuclear silencing-effector complexes, which have still not been characterized in most organisms. Defining these will require biochemical approaches similar to those that are used to identify proteins and siRNAs of the aforementioned RITS complex in fission yeast[70,71]. In *A. thaliana*, the reduced accumulation of repeat-associated siRNAs in several chromatin mutants is consistent with recruitment of these factors to the target locus by siRNAs[13,44,46]. However, biochemical analyses that show enrichment of siRNAs in association with chromatin proteins are required to confirm this proposal.

Another question concerns the extent to which gene-family members that encode RNAi proteins are dedicated to nuclear pathways. A subcellular division of activity has been noted for plants and *N. crassa* (BOX 1), but information from other organisms is inadequate. Furthermore, the phylogenetic distribution of the four known RNAi-mediated nuclear pathways is not yet known. Does RdDM occur regularly in mammals? Are there examples of programmed DNA elimination mediated by RNAi in organisms other than ciliates? Meiotic silencing is probably not a universal mechanism, as indicated by the fact that heterozygous deficiencies have no affect on meiosis in plants, *D. melanogaster* or mammals, but does it occur in organisms other than *N. crassa* and *C. elegans*? Are there pairing-sensitive epigenetic phenomena in somatic cells that are dependent on RNAi components, as has been observed in *D. melanogaster*? Are there novel nuclear RNAi pathways that await discovery?

The pervasive involvement of small RNAs in defining chromosome domains and in chromatin-based gene regulation was unknown until only a few years ago. RNAi-mediated chromatin modifications not only determine patterns of gene expression, but also affect chromosome behaviour. Future research developments are likely to reveal further participation of the RNAi pathway in governing chromosome structure and function.

1. Carmell, M. A. & Hannon, G. J. RNase III enzymes and the initiation of gene silencing. *Nature Struc. Mol. Biol.* **11**, 214–218 (2004).
2. Schramke, V. & Allshire, R. Those interfering little RNAs! Silencing and eliminating chromatin. *Curr. Opin. Genet. Devel.* **14**, 174–180 (2004).
3. Matzke, M. & Matzke, A. J. M. Planting the seeds of a new paradigm. *PLoS Biol.* **2**, E133 (2004).
4. Grewal, S. I. S. & Rice, J. C. Regulation of heterochromatin by histone methylation and small RNAs. *Curr. Opin. Cell Biol.* **16**, 230–238 (2004).
5. Yan, K. S. *et al.* Structure and conserved RNA binding of the PAZ domain. *Nature* **426**, 468–474 (2003).
6. Liu, J. *et al.* Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**, 1437–1441 (2004).
7. Meister, G. *et al.* Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol. Cell* **15**, 185–197 (2004).
8. Sijen, T. *et al.* On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**, 465–476 (2001).
9. Martienssen, R. Maintenance of heterochromatin by RNA interference of tandem repeats. *Nature Genet.* **35**, 213–214 (2004).
10. Hamilton, A., Voinnet, O., Chappell, L. & Baulcombe, D. Two classes of short interfering RNA in RNA silencing. *EMBO J.* **21**, 4671–4679 (2002).
11. Vastenhouw, N. L. & Plasterk, R. H. RNAi protects the *Caenorhabditis elegans* germline against transposition. *Trends Genet.* **20**, 314–319 (2004).
12. Aravin, A. A. *et al.* The small RNA profile during *Drosophila melanogaster* development. *Dev. Cell* **5**, 337–350 (2003).
13. Lippman, Z., May, B., Yordan, C., Singer, T. & Martienssen, R. Distinct mechanisms determine transposons inheritance and methylation via small interfering RNA and histone modification. *PLoS Biol.* **1**, E67 (2003).
14. Zilberman, D., Cao, X. & Jacobsen, S. E. ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* **299**, 716–719 (2003).
ARGONAUTE 4 is the first protein of the RNAi machinery that was shown to be required for histone and DNA methylation in a higher organism.
15. Xie, Z. *et al.* Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol.* **2**, E104 (2004).
The authors report on functional diversification and nuclear localization of proteins of the RNAi machinery that produce small RNAs which are involved in DNA and histone methylation.
16. Volpe, T. A. *et al.* Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**, 1833–1837 (2002).
This article describes the discovery of a role for the RNAi machinery in histone methylation and heterochromatin formation at fission-yeast centromeres.
17. Aravin, A. A. *et al.* Dissection of a natural RNA silencing process in the *Drosophila melanogaster* germ line. *Mol. Cell Biol.* **24**, 6742–6750 (2004).
18. Ambros, V., Lee, R. C., Lavanway, A., Williams, P. T. & Jewell, D. MicroRNAs and other tiny endogenous RNAs in *C. elegans*. *Curr. Biol.* **13**, 807–818 (2003).
19. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297 (2004).

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20. He, L. & Hannon, G. J. MicroRNAs: small RNAs with a big role in gene regulation. *Nature Rev. Genet.* **5**, 522–531 (2004).
21. Pfeffer, S. *et al.* Identification of virus-encoded microRNAs. *Science* **304**, 734–736 (2004).
22. Lund, A. M. & van Lohuizen, M. Epigenetics and cancer. *Genes Dev.* **18**, 2315–2335 (2004).
23. Hall, I. M. *et al.* Establishment and maintenance of a heterochromatin domain. *Science* **297**, 2232–2237 (2002).
24. Freitag, M. *et al.* DNA methylation is independent of RNA interference in *Neurospora*. *Science* **304**, 1939 (2004).
25. Chicas, A., Cogoni, C. & Macino, G. RNAi-dependent and RNAi-independent mechanisms contribute to the silencing of RIPed sequences in *Neurospora crassa*. *Nucl. Acids Res.* **32**, 4237–4243 (2004).
26. Wassenegger, M., Heimes, S., Riedel, L. & Sänger, H. RNA-directed *de novo* methylation of genomic sequences in plants. *Cell* **76**, 567–576 (1994).
This article reports the discovery of RNA-directed DNA methylation.
27. Mette, M. F., Aufsatz, W., van der Winden, J., Matzke, M. A. & Matzke, A. J. M. Transcriptional silencing and promoter methylation triggered by double stranded RNA. *EMBO J.* **19**, 5194–5201 (2000).
This article shows that RNA-directed DNA methylation requires a dsRNA that is processed to small RNAs, reinforcing a mechanistic link with RNAi, and shows that this pathway can target promoter regions.
28. Jones, L., Ratcliff, F. & Baucombe, D. C. RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr. Biol.* **11**, 747–757 (2001).
29. Sijen, T. *et al.* Transcriptional and posttranscriptional gene silencing are mechanistically related. *Curr. Biol.* **11**, 436–440 (2001).
30. Melquist, S. & Bender, J. Transcription from an upstream promoter controls methylation signaling from an inverted repeat of endogenous genes in *Arabidopsis*. *Genes Dev.* **17**, 2036–2047 (2003).
31. Pélissier, T., Thalmeir, S., Kempe, D., Sänger, H.-L. & Wasseneger, M. Heavy *de novo* methylation at symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation. *Nucl. Acids Res.* **27**, 1625–1634 (1999).
32. Aufsatz, W., Mette, M. F., van der Winden, J., Matzke, A. J. M. & Matzke, M. RNA-directed DNA methylation in *Arabidopsis*. *Proc. Natl Acad. Sci. USA* **99**, 16499–16506 (2002).
33. Schramke, V. & Allshire, R. Hairpin RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. *Science* **301**, 1069–1074 (2003).
The authors demonstrate that genes that are normally euchromatic in fission yeast can be targeted for H3K9 methylation and silencing through the RNAi pathway.
34. Pélissier, T. & Wassenegger, M. A DNA target of 30 bp is sufficient for RNA-directed DNA methylation. *RNA* **6**, 55–65 (2000).
35. Cao, X. *et al.* Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. *Curr. Biol.* **13**, 2212–2217 (2003).
36. Aufsatz, W., Mette, M. F., Matzke, A. J. M. & Matzke, M. The role of MET1 in RNA-directed *de novo* and maintenance methylation of CG dinucleotides. *Plant Mol. Biol.* **54**, 793–804 (2004).
37. Aufsatz, W., Mette, M. F., van der Winden, J., Matzke, M. & Matzke, A. J. M. HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double stranded RNA. *EMBO J.* **21**, 6832–6841 (2002).
38. Lindroth, A. M. *et al.* Requirement of *CHROMOMETHYLASE3* for maintenance of CpXpG methylation. *Science* **292**, 2077–2080 (2001).
39. Bartee, L., Malagnac, F. & Bender, J. *Arabidopsis cmt3* chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. *Genes Dev.* **15**, 1753–1758 (2001).
40. Malagnac, F., Bartee, L. & Bender, J. An *Arabidopsis* SET domain protein required for maintenance but not establishment of DNA methylation. *EMBO J.* **21**, 6842–6852 (2002).
41. Jackson, J. P., Lindroth, A. M., Cao, X. & Jacobsen, S. E. Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature* **416**, 556–560 (2002).
References 40 and 41 report a connection between histone methylation and DNA methylation in plants.
42. Tamuru, H. & Selker, E. U. A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature* **414**, 277–283 (2001).
43. Chan, S. W. L. *et al.* RNA silencing genes control *de novo* DNA methylation. *Science* **303**, 1336 (2004).
44. Zilberman, D. *et al.* Role of *Arabidopsis ARGONAUTE4* in RNA-directed DNA methylation triggered by inverted repeats. *Curr. Biol.* **14**, 1214–1220 (2004).
45. Vaucheret, H., Vazquez, F., Crété, P. & Bartel, D. P. The action of *ARGONAUTE1* in the miRNA pathway and its regulation by the miRNA pathway a crucial for plant development. *Genes Dev.* **18**, 1187–1197 (2004).
46. Lippman, Z. *et al.* Role of transposable elements in heterochromatin and epigenetic control. *Nature* **430**, 471–476 (2004).
The authors demonstrate a role for small RNAs that originate from transposable elements in mediating histone and DNA methylation in *A. thaliana* heterochromatin and the transposon-containing promoter of the *FWA* gene.
47. Morel, J. B. *et al.* Fertile hypomorphic *ARGONAUTE* (*ago1*) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* **14**, 629–639 (2002).
48. Kanno, T. *et al.* Involvement of putative SNF2 chromatin remodeling protein DRD1 in RNA-directed DNA methylation. *Curr. Biol.* **14**, 810–805 (2004).
49. Dennis, K., Fan, T., Geiman, T., Yan, Q. & Muegge, K. Lsh, a member of the SNF2 family, is required for genome-wide methylation. *Genes Dev.* **15**, 2940–2944 (2001).
50. Bartee, K. & Bender, J. Two *Arabidopsis* methylation-deficiency mutations confer only partial effects on a methylated endogenous gene family. *Nucl. Acids Res.* **29**, 2127–2134 (2001).
51. Morel, J. B., Mourrain, P., Beclin, C. & Vaucheret, H. DNA methylation and chromatin structure affect transcriptional and post-transcriptional transgene silencing in *Arabidopsis*. *Curr. Biol.* **10**, 1591–1594 (2000).
52. Vongs, A., Kakutani, T., Martienssen, R. A. & Richards, E. J. *Arabidopsis thaliana* DNA methylation mutants. *Science* **260**, 1926–1928 (1993).
53. Soppe, W. J. J. *et al.* The late flowering phenotype of *fwa* mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. *Mol. Cell* **6**, 791–802 (2000).
54. Kinoshita, T. *et al.* One-way control of *FWA* imprinting in *Arabidopsis* endosperm by DNA methylation. *Science* **303**, 521–523 (2004).
55. Svoboda, P., Stein, P., Filipowicz, W. & Schultz, R. M. Lack of homologous sequence-specific DNA methylation in response to stable dsRNA expression in mouse oocytes. *Nucl. Acids Res.* **32**, 3601–3606 (2004).
56. Morris, K. V., Chan, S. W. L., Jacobsen, S. E. & Looney, D. J. Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305**, 1289–1292 (2004).
57. Kawasaki, H. & Taira, K. Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. *Nature* **431**, 211–217 (2004).
Together with reference 56, this paper demonstrates that siRNAs can direct promoter methylation and transcriptional silencing in human cells.
58. Morey, C. & Avner, P. Employment opportunities for non-coding RNAs. *FEBS Lett.* **567**, 27–43 (2004).
59. Wallrath, L. L. & Elgin, S. C. R. Position effect variegation in *Drosophila* is associated with an altered chromatin structure. *Genes. Dev.* **9**, 1263–1277 (1995).
60. Weiler, K. S. & Wakimoto, B. T. Heterochromatin and gene expression in *Drosophila*. *Annu. Rev. Genetics* **29**, 577–605 (1995).
61. Dorer, D. R. & Henikoff, S. Expansions of transgene repeats cause heterochromatin formation and gene silencing in *Drosophila*. *Cell* **77**, 993–1002 (1994).
62. Muchardt, C. *et al.* Coordinated methyl and RNA binding is required for heterochromatin localization of mammalian HP1a. *EMBO Rep.* **3**, 975–981 (2002).
63. Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. & Jenuwein, T. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**, 116–120 (2001).
64. Bannister, A. J. *et al.* Selective recognition of methylated lysine 9 in histone H3 by the HP1 chromo domain. *Nature* **410**, 120–124 (2001).
65. Schotta, G. *et al.* Central role of *Drosophila* Su(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. *EMBO J.* **21**, 1121–1131 (2002).
66. Provost, P. *et al.* Dicer is required for chromosome segregation and gene silencing in fission yeast cells. *Proc. Natl Acad. Sci. USA* **99**, 16648–16653 (2002).
67. Volpe, T. *et al.* RNA interference is required for normal centromere function in fission yeast. *Chromosome Res.* **11**, 137–146 (2003).
68. Hall, I. M., Noma, K.-i. & Grewal, S. I. S. RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. *Proc. Natl Acad. Sci. USA* **100**, 193–198 (2003).
69. Ekwall, K. The roles of histone modifications and small RNA in centromere function. *Chromosome Res.* **12**, 535–542 (2004).
70. Verdel A. *et.al* RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* **303**, 672–676 (2004).
This paper describes the characterization of a nuclear protein complex that is thought to use siRNAs to guide chromatin modifications.
71. Noma, K. *et al.* RITS acts in *cis* to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nature Genet.* **36**, 1174–1180 (2004).
72. Kennerdell, J. R., Yamaguchi, S. & Carthew, R. W. RNAi is activated during *Drosophila* oocyte maturation in a manner dependent on *aubergine* and *spindle-E*. *Genes Dev.* **16**, 1884–1889 (2002).
73. Stapleton, W., Das, S. & McKee, B. D. A role of the *Drosophila homeless* gene in repression of *Stellate* in male meiosis. *Chromosoma* **110**, 228–240 (2001).
74. Aravin, A. A., Naumova, N. M., Tulin, A. A., Rozovsky, Y. M. & Gvozdev, V. A. Double stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in *Drosophila melanogaster* germline. *Curr. Biol.* **11**, 1017–1027 (2001).
75. Pal-Bhadra, M. *et al.* Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. *Science* **303**, 669–672 (2004).
76. Fukagawa, T. *et al.* Dicer is essential for formation of the heterochromatin structure in vertebrate cells. *Nature Cell Biol.* **6**, 784–791 (2004).
Together with reference 75, this paper demonstrates that the RNAi machinery participates in heterochromatin formation in multicellular eukaryotes.
77. Pal-Bhadra, M., Bhadra, U. & Birchler, J. A. Cosuppression in *Drosophila*: gene silencing of *Alcohol dehydrogenase* by *white-Adh* transgenes is Polycomb dependent. *Cell* **90**, 479–490 (1997).
78. Pal-Bhadra, M., Bhadra, U. & Birchler, J. A. RNAi related mechanisms affects both transcriptional and posttranscriptional transgene silencing in *Drosophila*. *Mol. Cell* **9**, 315–327 (2002).
References 77 and 78 show that the RNAi machinery is required for transcriptional silencing that results from chromatin modifications.
79. Mochizuki, K. & Gorovsky, M. A. Small RNAs in genome rearrangement in *Tetrahymena*. *Curr. Opin. Genet. Dev.* **14**, 181–187 (2004).
An up-to-date review covering all key papers about the role of RNAi in DNA elimination in ciliates.
80. Garnier, O., Serrano, V., Duharcourt, S. & Meyer, E. RNA-mediated programming of developmental genome rearrangements in *Paramecium*. *Mol. Cell Biol.* **24**, 7370–7379 (2004).
81. Chalker, D. L. & Yao, M.-C. Nongenic, bidirectional transcription precedes and may promote developmental DNA deletion in *Tetrahymena thermophila*. *Genes Dev.* **15**, 1287–1298 (2001).
82. Meyer, E. & Garnier, O. Non-Mendelian inheritance and homology-dependent effects in ciliates. *Adv. Genet.* **46**, 305–337 (2002).
83. Mochizuki, K., Fine, N. A., Fujisawa, T. & Gorovsky, M. A. Analysis of a *piwi*-related gene implicates small RNAs in genome rearrangements in *Tetrahymena*. *Cell* **110**, 689–699 (2002).
84. Liu, Y., Mochizuki, K. & Gorovsky, M. A. Histone H3 lysine 9 methylation is required for DNA elimination in developing macronuclei in *Tetrahymena*. *Proc. Natl Acad. Sci. USA* **101**, 1679–1684 (2004).
85. Taverna, S. D., Coyne, R. S. & Allis, C. D. Methylation of histone H3 at lysine 9 targets programmed DNA elimination in *Tetrahymena*. *Cell* **110**, 701–711 (2002).
86. Selker, E. U. A self-help guide for a trim genome. *Science* **300**, 1517–1518 (2003).
87. Mochizuki, K. & Gorovsky, M. A. Conjugation-specific small RNAs in *Tetrahymena* have predicted properties of scan (scn) RNAs involved in genome arrangement. *Genes Dev.* **18**, 2068–2073 (2004).
88. Yao, M.-C., Fuller, P. & Xi, X. Programmed DNA deletion as an RNA-guided system of genome defense. *Science* **300**, 1581–1584 (2003).
89. Colot, V., Maloisel, L. & Rossignol, J. L. Interchromosomal transfer of epigenetic states in *Ascobolus*: transfer of DNA methylation is mechanistically related to homologous recombination. *Cell* **86**, 855–864 (1996).
90. Aramayo, R. & Metzenberg, R. L. Meiotic transvection in fungi. *Cell* **86**, 103–113 (1996).
91. Shiu, P. K., Raju, N. B., Zickler, D. & Metzenberg, R. L. Meiotic silencing by unpaired DNA. *Cell* **107**, 905–916 (2001).
This is the first demonstration of an involvement of genes that encode components of the RNAi machinery in meiotic silencing by unpaired DNA.
92. Shiu, P. K. & Metzenberg, R. L. Meiotic silencing by unpaired DNA: properties, regulation and suppression. *Genetics* **161**, 1483–1495 (2002).
93. Lee, D. W., Pratt, R. J., McLaughlin, M. & Aramayo, R. An Argonaute-like protein is required for meiotic silencing. *Genetics* **164**, 821–828 (2003).

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94. Pratt, R. J., Lee, D. W. & Aramayo, R. DNA methylation affects meiotic *trans*-sensing, not meiotic silencing. *Genetics* (in the press).
95. Lee, D. W., Seong, K.-Y., Pratt, R. J., Baker, K. & Aramayo, R. Properties of unpaired DNA required for efficient silencing in *Neurospora crassa*. *Genetics* **167**, 131–150 (2004). **A thorough characterization of the requirements for meiotic silencing.**
96. Kutil, B. L., Seong, K.-Y. & Aramayo, R. Unpaired genes do not silence their paired neighbors. *Curr. Genet*. **43**, 425–432 (2003).
97. Bean, C. J., Schaner, C. E. & Kelly, W. G. Meiotic pairing and imprinted X chromatin assembly in *Caenorhabditis elegans*. *Nature Genet*. **36**, 100–105 (2004).
98. Kassis, J. A. Unusual properties of regulatory DNA from the *Drosophila engrailed* gene: three 'pairing sensitive' sites within a 1.6 kb region. *Genetics* **136**, 1025–1038 (1994).
99. Kassis, J. A., VanSickle, E. P. & Sensabaugh, S. M. A fragment of *engrailed* regulatory DNA can mediate transvection of the *white* gene in *Drosophila*. *Genetics* **128**, 751–761 (1991).
100. Pal-Bhadra, M., Bhadra, U. & Birchler, J. A. Interrelationship of RNAi and transcriptional gene silencing in *Drosophila*. *Cold Spring Harb. Symp. Quant. Biol.* (in the press).
101. Jia, S., Noma, K. & Grewal, S. I. S. RNAi-independent heterochromatin nucleation by the stress-activated ATF/CREB family proteins. *Science* **304**, 1971–1975 (2004).
102. Laayoun, A. & Smith, S. S. Methylation of slipped duplexes, snapbacks and cruciforms by human DNA (cytosine-5) methyltransferase. *Nucl. Acids Res*. **23**, 1584–1589 (1995).
103. Tamaru, H. & Selker, E. Synthesis of signals for *de novo* DNA methylation in *Neurospora crassa*. *Mol. Cell Biol*. **23**, 2379–2394 (2003).
104. Groll, M. G. & Bestor, T. H. Eukaryotic cytosine methyltransferases. *Ann. Rev. Biochem*. (in the press).
105. Matzke, M. A., Mette, M. F. & Matzke, A. J. M. Transgene silencing by the host genome defense: implications for the evolution of epigenetic control mechanisms in plants and vertebrates. *Plant Mol. Biol*. **43**, 401–415 (2000).
106. Carmell, M. A., Xuan, Z., Zhang, M. Q. & Hannon, G. J. The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes Dev*. **16**, 2733–2742 (2002).
107. Papp, I. *et al.* Evidence for nuclear processing of plant microRNA and short interfering RNA precursors. *Plant Physiol*. **132**, 1382–1390 (2003).
108. Lee, Y. S. *et al.* Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* **117**, 69–81 (2004).
109. Catalanotto, C. *et al.* Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in *Neurospora crassa*. *Mol. Cell Biol*. **24**, 2536–2545 (2004).
110. Baulcombe, D. RNA silencing in plants. *Nature* **431**, 356–363 (2004).
111. Okamura, K., Ishizuka, A., Siomi, H. & Siomi, M. C. Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev*. **18**, 1655–1666 (2004).
112. Kress, C., Thomassin, H. & Grange, T. Local DNA demethylation in vertebrates: how could it be performed and targeted? *FEBS Lett*. **494**, 135–140 (2001).
113. Jost, J. P. & Jost, Y. C. Transient DNA demethylation in differentiating mouse myoblasts correlates with higher activity of 5-methyldeoxycytidine excision repair. *J. Biol. Chem*. **269**, 10040–10043 (1994).
114. Zhu, B. *et al.* Overexpression of 5-methylcytosine DNA glycosylase in human embryonic kidney cells EcR293 demethylates the promoter of a hormone-regulated reporter gene. *Proc. Natl Acad. Sci. USA* **98**, 5031–5036 (2001).
115. Gong, Z. *et al. ROS1*, a repressor of transcriptional gene silencing in *Arabidopsis*, encodes a DNA glycosylase/lyase. *Cell* **111**, 803–814 (2002).
116. Choi, Y. *et al.* DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in *Arabidopsis*. *Cell* **110**, 33–42 (2002).
117. Xiao, W. *et al.* Imprinting of the *MEA* polycomb gene is controlled by antagonism between MET1 methyltransferase and DME glycosylase. *Dev. Cell* **5**, 891–901 (2003).
118. Choi, Y., Harada, J. J., Goldberg, R. L. & Fischer, R. L. An invariant aspartic acid in the DNA glycosylase domain of *DEMETER* is necessary for transcriptional activation of the imprinted *MEDEA* gene. *Proc. Natl Acad. Sci. USA* **101**, 7481–7486 (2004).
119. Jost, J.-P., Frémont, M., Siegmann, M. & Hofsteenge, J. The RNA moiety of chick embryo 5-methylcytosine-DNA glycosylase targets DNA demethylation. *Nucl. Acids Res*. **25**, 4545–4550 (1997).
120. Jost, J. P. *et al.* A chicken embryo protein related to the mammalian DEAD box protein p68 is tightly associated with the highly purified protein-RNA complex of 5-MeC-DNA glycosylase. *Nucl. Acids Res*. **27**, 3245–3252 (1999).
121. Ishizuka, A., Siomi, M. C. & Siomi, H. A *Drosophila* fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev*. **16**, 2497–2508 (2002).
122. Ramsahoye, B. H. *et al.* Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc. Natl Acad. Sci. USA* **97**, 5237–5242 (2000).
123. Woodcock, D. M., Lawler, C. B., Linsensmeyer, M. E., Doherty, J. P. & Warren, W. D. Asymmetric methylation in the hypermethylated CpG promoter region of the human L1 retrotransposon. *J. Biol. Chem*. **272**, 7810–7816 (1997).
124. Gibbons, R. J. *et al.* Mutations in *ATRX*, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. *Nature Genet*. **24**, 368–371 (2000).
125. Jones, L. *et al.* RNA–DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* **11**, 2291–2301 (1999).
126. Billy, E., Brondani, V., Zhang, H., Muller, U. & Filipowicz, W. Specific interference with gene expression induced by long, double stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc. Natl Acad. Sci. USA* **98**, 14428–14433 (2001).
127. Lippman, Z. & Martienssen, R. The role of RNA interference in heterochromatic silencing. *Nature* **431**, 364–370 (2004).
128. Grewal, S. I. S. & Moazed, D. Heterochromatin and epigenetic control of gene expression. *Science* **301**, 798–802 (2003).
129. Bao, N., Lye, K. W. & Barton, M. K. MicroRNA binding sites in Arabidopsis class III AD-ZIP mRNAs are required for methylation of the template chromosome. *Dev. Cell* **7**, 653–662 (2004).
130. Mpoke, S. & Wolfe, J. DNA digestion and chromatin condensation during nuclear death in *Tetrahymena*. *Exp. Cell Res*. **225**, 357–365 (1996).

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The authors declare no competing financial interests.

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