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Transcription and RNAi in heterochromatic gene silencing

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Recent findings have challenged the longstanding belief that heterochromatin is an inert and transcriptionally inactive structure. Studies in organisms ranging from fission yeast to animals have found that noncoding RNAs transcribed from heterochromatic DNA repeats function in the assembly and function of heterochromatin. In this review, we discuss the roles of RNA and RNA turnover in mechanisms that mediate heterochromatin assembly and keep heterochromatic domains silent.

In the early twentieth century, Emil Heitz recognized cytologically detectable differences in the staining of moss chromosomes during different phases of the cell cycle[1]. In particular, he noted that certain parts of *Pellia epiphylla* chromosomes remain visible throughout interphase, whereas other parts become invisible upon exit from mitosis, at the onset of interphase. To describe these differences in the appearance of parts of chromosomes, he suggested the terms *heterochromatin* and *euchromatin*, respectively. Later studies in plant and animal cells, particularly in *Drosophila melanogaster*, suggested that heterochromatin corresponds to genetically inert chromosome regions. For example, DNA rearrangements that bring euchromatic genes to the vicinity of heterochromatin can sometimes result in silencing of the normally active euchromatic gene[2,3].

Studies over the past few decades have defined the general properties and functions of heterochromatin in gene and genome regulation. Today, we recognize heterochromatin as chromosome domains that have an altered chromatin structure, have a reduced recombination frequency, are localized to specific subdomains near the nuclear periphery, generally replicate late in the cell cycle and are transcriptionally less active[3,4]. Heterochromatic domains are also rich in transposable elements and other repetitive sequences. Although the primary function of heterochromatin seems to involve the regulation of such repetitive sequences, heterochromatin is also associated with landmark chromosome structures such as centromeres and telomeres, tends to associate with specific proteins and distinct histone modifications, and is important in centromere function and the organization of chromosomes in the nucleus (**Fig. 1**). In addition, heterochromatin exerts a striking effect on gene expression, and related chromatin silencing mechanisms are involved in epigenetic regulation of master regulatory genes during development and differentiation[3–7].

Heterochromatin formation involves the assembly of specialized chromatin structures. The fundamental unit of chromatin is the nucleosome, which is composed of an octamer of four core histones (H3, H4, H2A and H2B), around which 147 base pairs of DNA are wrapped[8,9]. The conserved N-terminal tails of histones can be post-translationally modified, and these modifications have profound effects on the interaction of histones with DNA and the association of numerous nonhistone proteins with chromatin[10]. The silent state of heterochromatin has long been thought to result from the tight packaging of nucleosomes into a dense, compact chromatin structure dictated by specific histone modifications and structural proteins, which renders the DNA inaccessible to the transcription machinery[3,4]. More recently, however, this view has been challenged by several studies. Transcription of noncoding RNAs from heterochromatic DNA repeats has been observed in several systems (such as the fission yeast *Schizosaccharomyces pombe*, **Fig. 1**)[11–13]. In fission yeast, these RNAs are substrates for the RNA interference (RNAi) pathway[11], and they seem to participate directly in heterochromatin assembly[14–16], suggesting that active transcription may be a prerequisite for the assembly of heterochromatin. In plants, *Caenorhabditis elegans* and *D. melanogaster*, components of the RNAi pathway also participate in repeat-induced gene silencing and heterochromatin formation[17–22]. Furthermore, active turnover of RNAs that are transcribed within fission yeast heterochromatic regions seems to be an important element of gene silencing within these domains[23]. In this review, we discuss these intriguing and seemingly paradoxical findings and their implications for the mechanism of heterochromatic gene silencing.

Molecular hallmarks of heterochromatin

While initially defined cytologically, heterochromatin is now often defined by the presence and/or absence of specific molecular markers. Chief among these are post-translational histone modifications[3] (**Fig. 1**). At least eight classes of histone modifications have been described, including acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, ADP ribosylation, deimination and proline isomerization[10,24]. The best studied of these modifications are lysine acetylation and methylation. Whereas acetylation of lysines is broadly linked with gene activity[25], lysine methylation of histones can correlate with either transcriptional activation or repression,

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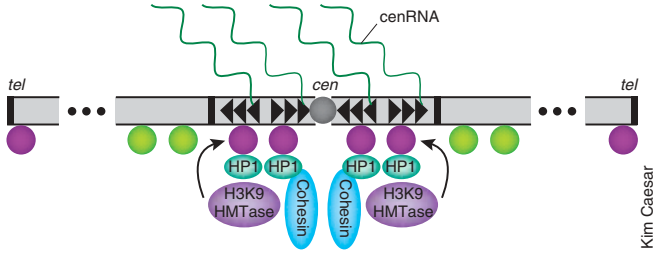

Figure 1 Schematic diagram showing the organization of eukaryotic centromeres. The centromere (*cen*) is surrounded by repetitive DNA elements, which are transcribed in *S. pombe* to produce noncoding cenRNAs. These sequences and the associated cenRNAs attract H3K9 HMTase, HP1 proteins and other complexes that mediate heterochromatin formation. HP1 recruits cohesin to promote sister-chromatid cohesion at centromeres. Nucleosomes associated with heterochromatic regions carry H3K9 methylation and are hypoacetylated (magenta circles), whereas nucleosomes in euchromatic regions carry H3K4 methylation and are hyperacetylated (green circles). Heterochromatic regions at telomeres (*tel*) and other chromosome regions (not shown) share many properties with centric heterochromatin. A role for noncoding cenRNAs in the assembly of centromeric heterochromatin has thus far been established only in *S. pombe*, although components of the RNAi pathway contribute to heterochromatic gene silencing at *D. melanogaster* pericentromeric regions.

depending on the modified residue[26–28]. Methylation of histone H3 on Lys4, Lys36 and Lys79 has been linked with active gene expression, whereas methylation of histone H3 on Lys9 and Lys27 and histone H4 on Lys20 strongly correlates with gene silencing[28,29].

Methylation of histone H3 Lys9 (H3K9) has a well-established role in heterochromatin formation and is therefore often referred to as a hallmark of heterochromatin. Immunolocalization studies in nuclei of metazoans show that methylated H3K9 is highly enriched in condensed heterochromatin[30–32]. Furthermore, the chromodomain protein heterochromatin protein-1 (HP1), which is also enriched in heterochromatic regions of metazoan genomes, specifically recognizes methylated H3K9 and has a distribution overlapping that of the H3K9 methylation marks[27,33–35]. Consistent with a requirement for H3K9 methylation in heterochromatin assembly, H3K9 methyltransferases (Su(var)3 through Su(var)9 in *Drosophila*, Suv39h in human and Clr4 in fission yeast) are required for heterochromatic gene silencing, and substitution of fission yeast H3K9 with arginine, which cannot be methylated, disrupts heterochromatin[35–38].

However, both H3K9 methylation and HP1 have also been linked to the regulation of some euchromatic genes. One example is the reversible silencing of euchromatic genes, such as retinoblastoma (Rb) protein–mediated repression of the mammalian cell-cycle regulator cyclin E, which involves the recruitment of HP1 and Suv39h[39]. In other cases, H3K9 methylation and HP1 have been found to be associated with transcribed mammalian chromatin[40]. Consistent with these results, high-resolution maps show that, besides forming large domains in pericentric regions, *D. melanogaster* HP1 also has a striking preference for a subset of euchromatic exon-dense genes on chromosome arms and binds along entire transcription units of genes that are actively transcribed[41]. These results suggest that components of heterochromatin may have additional function(s) in the transcription of some active genes and that, at least in mammals, H3K9me and HP1 are not the sole marks that define a chromosomal domain as transcriptionally silent or heterochromatic.

Widespread transcription of eukaryotic genomes

Similar to the unexpected association of H3K9 methylation and HP1 with a subset of euchromatic genes[41], genome-wide gene expression analysis suggests that transcription is widespread throughout the genome. In humans, widespread transcription along chromosomes and across the entire genome has been reported. These studies reveal more polyadenylated transcripts than could be accounted for by the number of predicted protein-coding genes, suggesting that a substantial portion of human poly(A) RNA is noncoding[42]. Tiling array-based whole-genome mapping shows that >50% of observed transcription is intergenic in *Arabidopsis thaliana* and that ~40% of probes in intronic and intergenic areas detect RNA expression in *D. melanogaster*[43]. Furthermore, several studies have revealed the unexpected association of RNA polymerase II (Pol II) with silent regions of the genome[44–48]. In *D. melanogaster*, endogenous Polycomb-repressed genes are associated with general transcription factors[44], and Polycomb-mediated silencing of a heat-shock reporter transgene has been shown to occur after loading of the Pol II pre-initiation complex[45]. Similarly, the results of a recent genome-wide analysis of human cells indicate that most protein-coding genes, including most genes thought to be transcriptionally inactive, experience transcription initiation, but the transcriptionally inactive protein-coding genes show no evidence of elongation, suggesting that they are regulated predominantly at post-initiation steps[46]. Pol II also associates with the vast majority of the budding yeast (*Saccharomyces cerevisiae*) genome, including heterochromatin-like domains and numerous regions conventionally considered to be transcriptional inert[47,48]. Importantly, most of this transcription is not reflected in microarray-based gene expression analysis, as a large portion of these transcripts are unstable and therefore not detectable in wild-type cells[49,50]. Heterochromatic gene silencing in fission yeast (*S. pombe*) is also not always associated with changes in Pol II occupancy or differences in transcription rates as determined by transcriptional run-on experiments[11,51–53]. Fission yeast outer centromeric repeats are composed of two types of repeat sequences, called *dg* and *dh* repeats. Disruption of heterochromatin caused by deletion of RNAi components or of the Clr4 HMTase results in increased Pol II occupancy at the centromeric *dg* repeats but has little or no effect on Pol II occupancy at centromeric *dh* repeats or reporter transgenes[51]. In contrast, deletion of the Clr3 histone deacetylase and its associated factors results in a marked increase in Pol II occupancy of a centromeric reporter transgene[54]. As discussed below, silencing within heterochromatin may involve a combination of mechanisms that interfere with transcriptional initiation or impede later steps such as productive elongation or processing.

Probably among the most remarkable examples of transcription within silent chromatin are those of the *D. melanogaster light* (*lt*) and *rolled* (*rl*) genes. The *light* gene is located in the centromeric heterochromatin of chromosome 2 and is required for viability and normal levels of pigmentation in a number of adult and larval tissues. This gene is unusual in its organization, in that it has a heterogeneous array of repetitive DNA sequences within its intronic and flanking regions[55]. The *rolled* gene is also located deep in heterochromatin, yet its expression is essential for viability[56]. Intriguingly, relocation experiments have demonstrated that *light* and *rolled* do not function well when moved into euchromatin; this contrasts with the findings for euchromatic genes, which are silenced when inserted in or near heterochromatic regions (a phenomenon referred to as position effect variegation, or PEV)[3]. A recent study shows that *D. melanogaster* heterochromatin contains a minimum of 230–254 protein-coding genes[57], the majority of which are highly conserved in insect lineages.

A surprising number of these genes also share high similarity with proteins from vertebrate species. Interestingly, these heterochromatic genes are enriched for domains found in proteins that may regulate chromatin structure or function, including histone variants[57]. This raises the possibility that heterochromatin may contain genes involved in its own establishment or maintenance. The underlying mechanisms that allow essential genes to be expressed and regulated in heterochromatin, however, remain unknown.

These results suggest that the ability of Pol II to associate with silent chromatin is evolutionarily conserved and indicate that this association has a biological function. In the cases of *D. melanogaster light* and *rolled*, genes seem to have evolved within a heterochromatic environment so that their proper transcriptional regulation requires residence in heterochromatin. In other situations, which will be discussed in detail below, transcription of noncoding RNAs from repetitive DNA regions is required for the assembly of repressive chromatin structures.

RNAi-mediated assembly of repressive chromatin structures

RNAi is a highly conserved gene silencing mechanism triggered by double-stranded RNA (dsRNA)[58,59]. The introduction of dsRNA into the nematode *C. elegans* by a variety of methods results in the silencing of cognate messenger RNAs. Silencing is mediated by small interfering RNAs (siRNAs) about 22 nucleotides in size, which are produced from long dsRNA by the Dicer RNase[60–64]. These findings have unified a number of different RNA-based silencing pathways, including RNA-directed DNA methylation[65], cosuppression and paramutation in plants[17], quelling in fungi[66], and microRNA- and siRNA-mediated silencing in plants and animals[67]. In each case, the small RNAs (sRNAs) act as guide molecules that direct Argonaute proteins to target nucleic acids by base-pairing and promote the inactivation of homologous sequences by a variety of mechanisms[59] (**Fig. 2**). In some systems, RNAi is amplified by a positive feedback mechanism that involves the synthesis of dsRNA by an RNA-directed RNA polymerase[17,68] (RdRP; **Fig. 2**). RdRPs seem to be absent in flies and mammals, but other amplification mechanisms may exist in these organisms. In *A. thaliana*, dsRNA promotes the methylation of homologous DNA regions, and DNA methylation as well as histone H3K9 methylation require an Argonaute family member[18,69]. In *D. melanogaster*, multiple copies of alcohol dehydrogenase transgenes induce transcriptional silencing of the transgenes as well as of the endogenous alcohol dehydrogenase gene, a phenomenon that requires Piwi, an Argonaute homolog, and Polycomb, a chromodomain protein involved in the transcriptional inactivation of many developmental regulators[70,71]. In *C. elegans*, silencing of transgene arrays requires components of the RNAi pathway and several proteins involved in transcriptional gene silencing[20,72]. Moreover, Piwi and other RNAi components were recently shown to contribute to the formation of centromeric heterochromatin in *D. melanogaster*[21]. Similarly, in *Tetrahymena thermophila*, programmed DNA elimination that is specified by H3K9 methylation[73] requires Twi1, another member of the Argonaute family[74]; however, until recently it had not been clear whether these effects were direct or indirect.

The key components of the RNAi pathway, Dicer and Argonaute, as well as RdRP, are conserved in fission yeast (*S. pombe*) but not in budding yeast (*S. cerevisiae*). Deletion of the genes encoding any of these proteins (Dcr1, Ago1 and Rdp1, respectively) results in loss of H3K9 methylation, Swi6 (HP1 homolog) localization, and silencing within the centromeric DNA repeats but not at the silent mating-type loci or telomeres in fission yeast[11]. Moreover, siRNAs corresponding to centromeric repeats have been identified[75]. Considerable progress has been made in elucidating the mechanism of RNAi-mediated

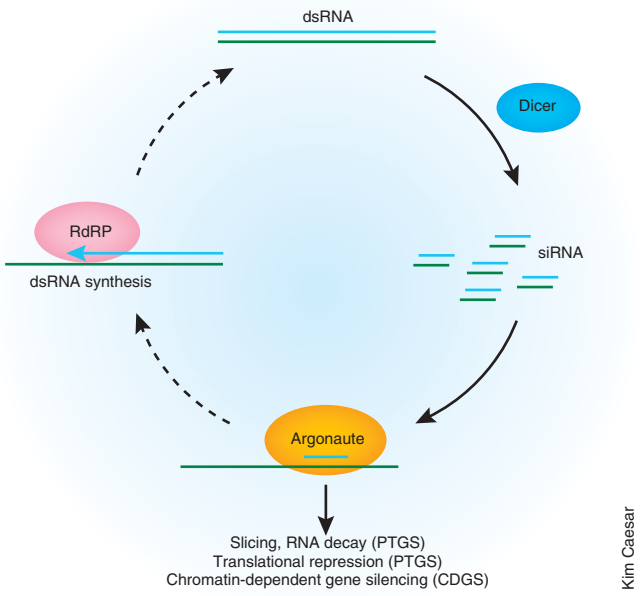

Figure 2 General diagram highlighting the core components of the dsRNA-mediated RNA silencing pathways. RdRPs are conserved in fission yeast, plants and *C. elegans*, but seem to be absent in *D. melanogaster* and mammals.

heterochromatin assembly in fission yeast. In addition to Swi6, heterochromatin formation in fission yeast requires the activity of two other chromodomain proteins, Chp1 and Chp2 (refs. 76,77). All three chromodomain proteins recognize methylated H3K9 and are structural components of centromeric heterochromatin[76]. Biochemical purification of Chp1 has led to the identification of a complex that uses siRNAs to mediate heterochromatin assembly. Chp1 resides in the RNA-induced transcriptional silencing (RITS) complex (**Fig. 3**), which also contains Ago1 and Tas3, a protein of unknown function. In addition, RITS contains siRNAs that originate from centromeric repeat regions and requires siRNAs for efficient binding and spreading throughout centromeric repeats[14]. It has therefore been proposed that RITS uses siRNAs as specificity factors for association with specific chromosome regions. The identification of RITS demonstrates a direct and physical association between RNAi and heterochromatin assembly and suggests possible mechanisms by which sRNAs could initiate and maintain repressive chromatin structures.

The nascent-transcript model for heterochromatin assembly

In principle, siRNAs could target specific chromosome regions by base-pairing with either DNA or nascent RNAs transcribed from the targeted locus[4]. Studies in fission yeast have provided support for the latter mechanism. The fission yeast RdRP, Rdp1, is found in the RDRC complex, which also contains Hrr1, a putative helicase, and Cid12, a member of the poly(A) polymerase family of enzymes[15]. RDRC is an active RdRP and can use a single-stranded RNA template to generate dsRNA *in vitro*. This activity is required for RNAi, as mutations that abolish *in vitro* dsRNA synthesis also eliminate silencing *in vivo*[15,78]. Interestingly, RDRC physically associates with RITS[15]. As RDRC uses centromeric RNA (cenRNA) as a template for dsRNA synthesis, its association with RITS suggests that siRNA-mediated targeting may involve cenRNA (**Fig. 3**). In fact, RITS-RDRC association is Dicer- and

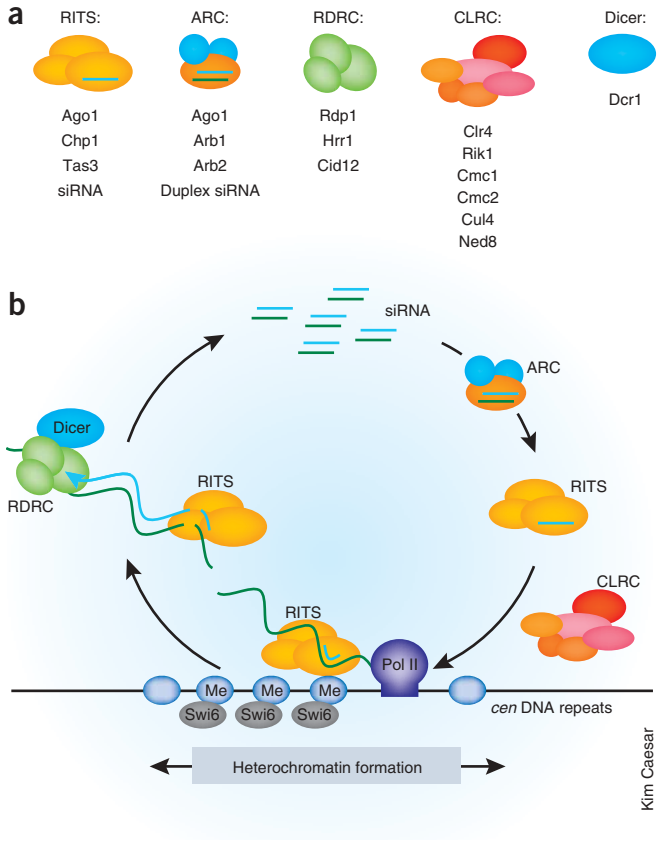

Figure 3 RNAi-mediated heterochromatin assembly in fission yeast. (**a**) Biochemically purified fission yeast complexes required for RNAi-mediated silencing. RITS, RDRC and Dicer are described in the text. ARC is an Ago1 chaperone complex that carries duplex siRNA, and the CLRC complex, which contains the Clr4 H3K9 methyltransferase, is required for H3K9 methylation and siRNA generation (reviewed in ref. 16). (**b**) The nascent-transcript model proposes that the RITS complex mediates heterochromatin formation by associating with nascent transcripts via siRNA base-pairing, and with methylated H3K9 via the chromodomain of its Chp1 subunit. dsRNA synthesis and siRNA generation occur in association with specific chromosome regions and may underlie *cis* restriction of siRNA-mediated silencing. The chromosome-associated siRNA synthesis loop is essential for the spreading of H3K9 methylation and silencing at the centromere. The coupling of the siRNA synthesis loop to H3K9 methylation forms a stable feedback loop that epigenetically maintains heterochromatin.

Clr4 HMTase–dependent, suggesting that siRNA and H3K9 methylation may be required for localization of RITS to target chromosome regions. Consistent with the idea that both RITS and RDRC associate with cenRNA, subunits of both complexes can be cross-linked to cenRNA as well as to centromeric DNA, providing evidence for recognition of nascent cenRNAs by siRNA-programmed RITS[15].

A central feature of the nascent-transcript model is that it couples the siRNA synthesis loop to H3K9 methylation by tethering the dsRNA synthesis machinery to specific sites on the chromosome. The mutual dependence of siRNA generation and H3K9 methylation, and that of H3K9 methylation and siRNA-mediated targeting, provide a mechanism for the epigenetic inheritance of centromeric heterochromatic domains (**Fig. 3**). In this model, the requirement for H3K9 methylation limits the ability of siRNAs to act promiscuously in silencing homologous sequences throughout the genome[15,51,53,79,80]. In fact, in wild-type fission yeast, siRNAs are *cis* restricted and promote H3K9 methylation only at the locus where they are produced (see below)[51]. Another mechanism that contributes to *cis* restriction involves the physical association of the Dicer RNase with RDRC and RITS complexes[81] (**Fig. 3b**).

A key experiment that supports the nascent-transcript model is based on the artificial tethering of RITS to the transcript of a normally euchromatic gene. Tethering of the RITS complex to *ura4*$^+$ RNA via a site-specific RNA-binding protein (N protein of phage λ) results in heterochromatin assembly and silencing of the cognate *ura4*$^+$ gene[51] (**Fig. 4**). This tethering also results in the generation of *ura4*$^+$-specific siRNAs, and silencing requires proteins associated with both RNAi and heterochromatin. Thus, the initial association of RITS with a nascent transcript can trigger the entire RNAi-mediated silencing pathway.

A prediction of the nascent-transcript model is that transcription of the target locus should be required for silencing. Centromeric repeats are indeed transcribed to produce forward and reverse noncoding cenRNAs[11]. Thus, nascent transcripts are present at centromeric DNA repeats and could, in principle, act as templates for RITS. Furthermore, mutations in two different subunits of Pol II, Rbp2 and Rpb7, result in loss of RNAi-dependent heterochromatin formation at centromeres[52,53]. Neither Pol II mutation seems to affect general transcription, suggesting that the mutations affect structural features of the polymerase that are specifically important for transcription of centromeric repeats and/or the processing of cenRNA into dsRNA and siRNAs. siRNAs are absent in both *rpb2* and *rpb7* mutant cells[52,53], and the temperature-sensitive *rpb7*-G150D mutant also seems to have a specific defect in initiation of cenRNA transcription[52]. Together, these results indicate that Pol II–dependent transcription is important in RNAi-mediated heterochromatin formation.

There are at least four possible explanations for this requirement. First, transcription of centromeric repeats, which produces the cenRNA template for siRNA generation, could simply be more sensitive to certain Pol II mutations. Second, siRNA generation and/or H3K9 methylation could be coupled to transcription of centromeric repeats by Pol II. Third, Pol II could expose an RNA surface necessary for the activity of Rdp1 and/or Dicer. Fourth, transcription-dependent unwinding of the DNA double helix may be required for base-pairing of the siRNA with DNA. Experiments that clearly distinguish between these possibilities are currently lacking, although the absence of siRNAs in *rpb2* mutants[53], even though this mutation has no major effect on cenRNA transcription, suggests a role for Pol II beyond transcription of the repeats *per se*.

siRNA-induced histone modifications *in cis* or *in trans*

In tobacco and *A. thaliana*, hairpin-induced siRNAs act *in trans* to promote the methylation of homologous DNA sequences[69]. In this way, they serve as specificity factors that target methylation to homologous chromosome regions. However, siRNAs cannot always act *in trans* to promote DNA or chromatin modifications. In fission yeast, tethering of the RITS complex to *ura4*$^+$ mRNA using the phage λ N protein results in the *de novo* generation of *ura4*$^+$ siRNAs[51]. These siRNAs load onto the RITS complex and are absolutely required for silencing of the *ura4*$^+$ locus *in cis* (**Fig. 4**). However, in wild-type cells, they are incapable of silencing a second *ura4*$^+$ locus, indicating that their action is *cis* restricted[51]. Silencing of the second *ura4*$^+$ locus is evident when the siRNA RNase gene *eri1* is deleted, indicating that siRNAs can, in fact, act as specificity factors, but their action is under negative control. Importantly, even in *eri1*-knockout cells, silencing of the second *ura4*$^+$ locus is inefficient, suggesting that other factors also contribute to the ability of siRNAs to promote H3K9 methylation *in trans*[51]. A similar situation has also been described in *A. thaliana*.

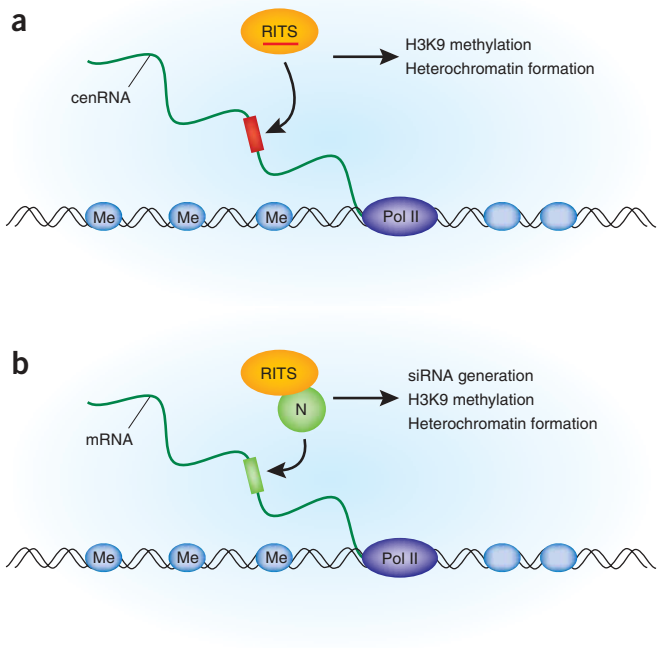

Figure 4 Tethering of the RITS complex to RNA mediates heterochromatin formation. (**a**,**b**) Comparison of the recruitment of RITS by siRNA-cenRNA base-pairing (**a**) with the artificial tethering of RITS by the phage λ site-specific RNA-binding protein (N) to an mRNA containing engineered N-binding sites (green rectangle) (**b**). This figure is based on a drawing by Mark Ptashne.

Unlike the hairpin siRNAs mentioned above, siRNAs associated with silencing of the *A. thaliana FWA* gene, which controls plant flowering, are restricted in their ability to promote *de novo* DNA methylation[82]. Transformation of plants with an *FWA* transgene results in siRNA generation, but the ability of the siRNAs to direct *de novo* DNA methylation is sensitive to the presence of preexisting methylation at the endogenous *FWA* locus[82]. Thus, in both plants and fission yeast, the local structure of the target locus may enhance the ability of siRNAs to promote further histone or DNA methylation.

Cotranscriptional gene silencing

The above studies demonstrate that what has long been thought to be transcriptionally silent is intrinsically dependent on transcription. At least two distinct steps in RNAi-mediated heterochromatin assembly require transcription (**Fig. 3**). First, transcription is clearly required for synthesis of the precursor single-stranded RNAs that serve as templates for generating dsRNA and siRNA. Second, transcription is required for synthesis of nascent chromatin-bound RNAs that serve as templates for siRNA-dependent recruitment of chromatin-modifying factors. However, it has been unclear how the requirement for transcription in heterochromatin assembly could be reconciled with the silencing of heterochromatic genes.

One possibility is that a trigger round of transcription is required to initiate heterochromatin assembly—but after initiation, transcription is silenced. In fission yeast, transcription run-on experiments have shown that heterochromatin inhibits transcription of the 'forward' but not the 'reverse' transcript of centromeric repeats[11]. This result suggested that transcriptional gene silencing (TGS) mechanisms might cooperate with post-transcriptional gene silencing (PTGS) mechanisms in centromeric (*cen*) repeat regions[11]. However, further analysis indicates that what had been thought to be PTGS of the *cen* reverse transcript actually reflects a more general feature of heterochromatic gene silencing involving the degradation *in cis* of RNAs transcribed from the *cen* regions. One observation supporting this is that, similar to silencing of the *cen* reverse transcript, *ura4*$^+$ H3K9 methylation and silencing induced by tethering of RITS to the *ura4*$^+$ RNA do not affect the rate of transcription[51]. Furthermore, heterochromatin formation results in a marked reduction in Pol II occupancy on the *dg* but not the *dh* centromeric repeats[51–53]. Finally, heterochromatin has little or no effect on Pol II occupancy at *ura4*$^+$ reporter genes inserted into fission yeast heterochromatin, nor on Pol II occupancy at a *ura4*$^+$ locus where H3K9 methylation and gene silencing are initiated by tethering of RITS to the *ura4*$^+$ RNA (although all of these *ura4*$^+$ genes are efficiently silenced)[23,51]. Clearly, some promoters drive transcription within heterochromatic domains, but the resulting RNA is most probably degraded. At heterochromatic loci where RNAi is essential for silencing, RNA degradation could theoretically be executed by the RNAi machinery. Consistent with this idea, recombinant fission yeast Ago1 has been shown to have slicer activity *in vitro*, and siRNAs originating from centromeric *ura4*$^+$ insertions have recently been detected[23,83,84]. Therefore, it is possible that centromeric transcripts, including those originating from centromeric *ura4*$^+$ insertions, are sliced by either RITS or another Ago1-containing complex. Importantly, however, this RNA decay is distinct from RNAi-mediated PTGS. RNAi-mediated heterochromatic gene silencing in wild-type *S. pombe* is *cis* restricted[51]. In contrast, siRNA-loaded RISC acts *in trans* to induce the degradation of cytoplasmic target RNAs during PTGS[59,63]. The mechanism responsible for *cis* restriction of heterochromatic RNA degradation is unknown, but *cis* restriction probably results from the coupling of dsRNA synthesis and siRNA generation to H3K9 methylation and heterochromatin assembly[81]. The simplest explanation for these observations is a cotranscriptional gene silencing (CTGS) model[51], also referred to as *cis*-PTGS[85], in which the targeting of nascent transcripts by the RITS complex mediates their degradation.

Recent results strengthen the experimental evidence for CTGS as a major feature of heterochromatic gene silencing in fission yeast. Silencing of *ura4*$^+$ transgenes inserted at various fission yeast heterochromatic loci is disrupted in mutant cells that encode defective Rrp6 or Dis3 subunits of the exosome[23,86], a multisubunit 3′-to-5′ exoRNase machine[87]. Remarkably, silencing of a *ura4*$^+$ gene inserted at the mating-type locus, which occurs independently of the RNAi pathway, is also dependent on the exosome, suggesting that even at the mating-type locus, heterochromatin formation does not entirely inhibit transcription and an RNAi-independent degradation pathway contributes to full silencing[23,86]. Consistent with this notion, Pol II can be detected readily at a *ura4*$^+$ gene inserted at the silent mating-type locus *mat3M*, although Pol II is normally excluded from native *mat* promoters[23,88] (M.B. and D.M., unpublished data). Furthermore, Pol II occupancy at either *mat::ura4*$^+$ or *cen::ura4*$^+$ does not substantially increase in *clr4Δ* cells, in which heterochromatin is disrupted and silencing of the reporter genes is abolished[23]. In contrast to centromeric heterochromatin, RNAi has a redundant role in heterochromatin formation at the silent mating-type loci of fission yeast[79]. This might explain why exosome mutants have less pronounced silencing defects at centromeric regions than at the mating-type locus[23,86]. In budding yeast, the polyadenylation activity of the Trf4–Air1/Air2–Mtr4 polyadenylation (TRAMP) complex is required for the processing of numerous exosome substrates[49,89,90]. Interestingly, the polyadenylation activity of the fission yeast TRAMP complex (which

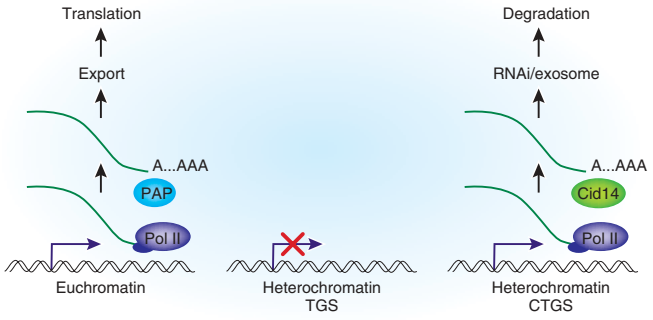

Figure 5 Mechanisms of gene silencing within heterochromatic regions. Assembly of heterochromatin can result in either TGS or the degradation of nascent heterochromatic transcripts (CTGS). Transcript degradation is mediated by RNAi-dependent and RNAi-independent pathways. Unlike transcription in euchromatin (far left), transcription in heterochromatin (far right) results in recruitment of an alternative polyadenylation complex (the TRAMP complex, which contains Cid14 in fission yeast and either Trf4 or its homolog Trf5 in budding yeast) that mediates RNA degradation by the exosome.

contains Cid14) is required for efficient silencing of heterochromatic *ura4*$^+$ genes but does not contribute considerably to the integrity of heterochromatin[23] (**Fig. 5**).

These results suggest that cotranscriptional RNA processing events have a major role in heterochromatic gene silencing in fission yeast (**Fig. 5**). Although it remains to be determined whether CTGS is evolutionarily conserved, it is tempting to speculate that similar mechanisms exist in other eukaryotes, perhaps providing an explanation for the presence of Pol II at other silent chromatin regions (see above; also see, for example, refs. 46–48).

Conservation of RNAi- and chromatin-dependent gene silencing

RNAi- and chromatin-dependent gene silencing (CDGS) are broadly but not universally conserved in eukaryotes. We have discussed examples of RNAi-dependent gene silencing and CDGS in plants, fission yeast, *D. melanogaster* and *C. elegans*. Recent results suggest that analogous pathways may also regulate chromatin structure in mammals.

The introduction of duplex, promoter-directed siRNAs into mammalian cell lines promotes the appearance of H3K9 and H3K27 methylation and transcriptional gene silencing[91–93]. This silencing requires the Ago1 protein, suggesting a function for RNAi components in directing repressive chromatin modifications to homologous DNA regions in mammalian cells[92]. The remaining challenge here is the identification of an endogenous cellular pathway that uses siRNAs and Argonaute to mediate chromatin modifications. In regard to this, a distinct class of sRNAs associated with Piwi members of the Argonaute family, called piRNAs, has been identified[94–96]. piRNAs are generated in a Dicer-independent fashion and map to numerous genomic sites. In *D. melanogaster*, these sites include transposable elements, and the piRNA pathway is required for transposon silencing in the germline[97]. There is some evidence that supports a role for piRNAs in CDGS. *D. melanogaster* Piwi is required for repeat-induced gene silencing, which also requires Polycomb and contributes to heterochromatin formation at centromeres[21,71]. Mutations in *D. melanogaster spindle-E* cause a large reduction in the levels of Piwi-associated sRNAs and a concomitant decrease in H3K9 methylation at retroelements in the germline[98]. Moreover, deletion of the mouse Piwi family member Mili results in loss of CpG methylation at LINE-1 transposable elements[99]. These results suggest that at least two different classes of sRNAs may contribute to CDGS in *D. melanogaster* and mammals.

The role of noncoding RNAs in chromatin regulation extends beyond the RNAi and sRNA pathways. The large noncoding XIST RNA is required for the initiation and maintenance of X-chromosome inactivation in somatic cells of female mammals, and noncoding rox RNAs are required for dosage compensation in flies[100]. In addition, antisense transcription and noncoding RNAs are associated with imprinting of several mammalian genes[100]. Despite the absence of sRNAs, XIST and rox RNAs may have functions analogous to that of the nascent cenRNAs in fission yeast. A common feature of these RNA silencing pathways is that the noncoding RNA is tethered to chromatin and serves as a platform for the recruitment of downstream modifying activities. Whereas siRNAs help in targeting RITS to nascent cenRNAs (**Fig. 3b**), site-specific RNA-binding proteins are likely to serve this function in the case of XIST and other large noncoding RNAs in flies and mammals. The recently identified HOTAIR noncoding RNA, which is transcribed from the human *HOX* cluster and has a role in Polycomb-mediated silencing, may be another example of RNA that mediates recruitment of chromatin-modifying activities[101].

Summary and perspective

Noncoding RNAs are important in the assembly of silent chromatin domains in several eukaryotes. These RNAs not only form positive feedback loops that mediate synthesis of sRNAs, they also act as platforms for sRNA-mediated chromatin modifications. The coupling of sRNA positive feedback loops to histone and chromatin modifications provides an RNA-based mechanism for the epigenetic inheritance of silent chromatin domains. Furthermore, the functions of noncoding RNAs as platforms for the recruitment of modifying enzymes are intimately associated with the transcription of these RNAs from repetitive DNA sequences and with their retention *in cis* at sites of heterochromatin assembly. Transcription in heterochromatin raises questions about how these domains are kept silent. Evidence from studies in fission yeast suggests that heterochromatic gene silencing is achieved, at least in part, through cotranscriptional RNA turnover mechanisms that use components of the RNAi pathway as well as the general nuclear surveillance pathway involving polyadenylation-dependent RNA degradation. The broad conservation of RNAi and RNA processing pathways suggests that these regulatory mechanisms may be widespread.

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1. Heitz, E. Das heterochromatin der moose. *I. Jahrb. Wiss. Botanik* **69**, 762–818 (1928).
2. Muller, H.J. Types of visible variations induced by X-rays in *Drosophila*. *J. Genet.* **22**, 299–334 (1930).
3. Richards, E.J. & Elgin, S.C. Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell* **108**, 489–500 (2002).
4. Grewal, S.I. & Moazed, D. Heterochromatin and epigenetic control of gene expression. *Science* **301**, 798–802 (2003).
5. Moazed, D. Common themes in mechanisms of gene silencing. *Mol. Cell* **8**, 489–498 (2001).

6. Paro, R. & Hogness, D.S. The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of *Drosophila*. *Proc. Natl. Acad. Sci. USA* **88**, 263–267 (1991).
7. Ringrose, L. & Paro, R. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. *Annu. Rev. Genet.* **38**, 413–443 (2004).
8. Kornberg, R.D. & Lorch, Y. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* **98**, 285–294 (1999).
9. Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F. & Richmond, T.J. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389**, 251–260 (1997).
10. Jenuwein, T. & Allis, C.D. Translating the histone code. *Science* **293**, 1074–1080 (2001).
11. Volpe, T.A. *et al.* Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**, 1833–1837 (2002).
12. Fukagawa, T. *et al.* Dicer is essential for formation of the heterochromatin structure in vertebrate cells. *Nat. Cell Biol.* **6**, 784–791 (2004).
13. Kanellopoulou, C. *et al.* Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev.* **19**, 489–501 (2005).
14. Verdel, A. *et al.* RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* **303**, 672–676 (2004).
15. Motamedi, M.R. *et al.* Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. *Cell* **119**, 789–802 (2004).
16. Moazed, D. *et al.* Studies on the mechanism of RNAi-dependent heterochromatin assembly. *Cold Spring Harb. Symp. Quant. Biol.* **71**, 461–471 (2006).
17. Baulcombe, D. RNA silencing in plants. *Nature* **431**, 356–363 (2004).
18. Henderson, I.R. & Jacobsen, S.E. Epigenetic inheritance in plants. *Nature* **447**, 418–424 (2007).
19. Sijen, T. & Plasterk, R.H. Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi. *Nature* **426**, 310–314 (2003).
20. Grishok, A., Sinskey, J.L. & Sharp, P.A. Transcriptional silencing of a transgene by RNAi in the soma of *C. elegans*. *Genes Dev.* **19**, 683–696 (2005).
21. Pal-Bhadra, M. *et al.* Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. *Science* **303**, 669–672 (2004).
22. Bernstein, E. & Allis, C.D. RNA meets chromatin. *Genes Dev.* **19**, 1635–1655 (2005).
23. Buhler, M., Haas, W., Gygi, S.P. & Moazed, D. RNAi-dependent and -independent RNA turnover mechanisms contribute to heterochromatic gene silencing. *Cell* **129**, 707–721 (2007).
24. Kouzarides, T. Chromatin modifications and their function. *Cell* **128**, 693–705 (2007).
25. Kurdistani, S.K. & Grunstein, M. Histone acetylation and deacetylation in yeast. *Nat. Rev. Mol. Cell Biol.* **4**, 276–284 (2003).
26. Bannister, A.J. *et al.* Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410**, 120–124 (2001).
27. 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).
28. Shilatifard, A. Chromatin Modifications by Methylation and Ubiquitination: Implications in the Regulation of Gene Expression. *Annu. Rev. Biochem.* **75**, 243–269 (2006).
29. Lachner, M. & Jenuwein, T. The many faces of histone lysine methylation. *Curr. Opin. Cell Biol.* **14**, 286–298 (2002).
30. Jenuwein, T. Re-SET-ting heterochromatin by histone methyltransferases. *Trends Cell Biol.* **11**, 266–273 (2001).
31. Peters, A.H. *et al.* Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol. Cell* **12**, 1577–1589 (2003).
32. Rice, J.C. *et al.* Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol. Cell* **12**, 1591–1598 (2003).
33. James, T.C. *et al.* Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of *Drosophila*. *Eur. J. Cell Biol.* **50**, 170–180 (1989).
34. 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).
35. Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D. & Grewal, S.I. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* **292**, 110–113 (2001).
36. Rea, S. *et al.* Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**, 593–599 (2000).
37. Melcher, M. *et al.* Structure-function analysis of SUV39H1 reveals a dominant role in heterochromatin organization, chromosome segregation, and mitotic progression. *Mol. Cell. Biol.* **20**, 3728–3741 (2000).
38. Mellone, B.G. *et al.* Centromere silencing and function in fission yeast is governed by the amino terminus of histone H3. *Curr. Biol.* **13**, 1748–1757 (2003).
39. Nielsen, S.J. *et al.* Rb targets histone H3 methylation and HP1 to promoters. *Nature* **412**, 561–565 (2001).
40. Vakoc, C.R., Mandat, S.A., Olenchock, B.A. & Blobel, G.A. Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. *Mol. Cell* **19**, 381–391 (2005).
41. de Wit, E., Greil, F. & van Steensel, B. High-resolution mapping reveals links of HP1 with active and inactive chromatin components. *PLoS Genet.* **3**, e38 (2007).
42. Cheng, J. *et al.* Transcriptional maps of 10 human chromosomes at 5-nucleotide resolution. *Science* **308**, 1149–1154 (2005).
43. Willingham, A.T. *et al.* Transcriptional landscape of the human and fly genomes: nonlinear and multifunctional modular model of transcriptomes. *Cold Spring Harb. Symp. Quant. Biol.* **71**, 101–110 (2006).
44. Breiling, A., Turner, B.M., Bianchi, M.E. & Orlando, V. General transcription factors bind promoters repressed by Polycomb group proteins. *Nature* **412**, 651–655 (2001).
45. Dellino, G.I. *et al.* Polycomb silencing blocks transcription initiation. *Mol. Cell* **13**, 887–893 (2004).
46. Guenther, M.G., Levine, S.S., Boyer, L.A., Jaenisch, R. & Young, R.A. A chromatin landmark and transcription initiation at most promoters in human cells. *Cell* **130**, 77–88 (2007).
47. Pirrotta, V. & Gross, D.S. Epigenetic silencing mechanisms in budding yeast and fruit fly: different paths, same destinations. *Mol. Cell* **18**, 395–398 (2005).
48. Steinmetz, E.J. *et al.* Genome-wide distribution of yeast RNA polymerase II and its control by Sen1 helicase. *Mol. Cell* **24**, 735–746 (2006).
49. Wyers, F. *et al.* Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. *Cell* **121**, 725–737 (2005).
50. Davis, C.A. & Ares, M., Jr. Accumulation of unstable promoter-associated transcripts upon loss of the nuclear exosome subunit Rrp6p in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **103**, 3262–3267 (2006).
51. Buhler, M., Verdel, A. & Moazed, D. Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. *Cell* **125**, 873–886 (2006).
52. Djupedal, I. *et al.* RNA Pol II subunit Rpb7 promotes centromeric transcription and RNAi-directed chromatin silencing. *Genes Dev.* **19**, 2301–2306 (2005).
53. Kato, H. *et al.* RNA polymerase II is required for RNAi-dependent heterochromatin assembly. *Science* **309**, 467–469 (2005).
54. Sugiyama, T. *et al.* SHREC, an effector complex for heterochromatic transcriptional silencing. *Cell* **128**, 491–504 (2007).
55. Devlin, R.H., Bingham, B. & Wakimoto, B.T. The organization and expression of the light gene, a heterochromatic gene of *Drosophila melanogaster*. *Genetics* **125**, 129–140 (1990).
56. Eberl, D.F., Duyf, B.J. & Hilliker, A.J. The role of heterochromatin in the expression of a heterochromatic gene, the rolled locus of *Drosophila melanogaster*. *Genetics* **134**, 277–292 (1993).
57. Smith, C.D., Shu, S., Mungall, C.J. & Karpen, G.H. The Release 5.1 annotation of *Drosophila melanogaster* heterochromatin. *Science* **316**, 1586–1591 (2007).
58. Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811 (1998).
59. Hannon, G.J. RNA interference. *Nature* **418**, 244–251 (2002).
60. Hamilton, A.J. & Baulcombe, D.C. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950–952 (1999).
61. Hammond, S.M., Bernstein, E., Beach, D. & Hannon, G.J. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296 (2000).
62. Zamore, P.D., Tuschl, T., Sharp, P.A. & Bartel, D.P. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33 (2000).
63. Elbashir, S.M., Lendeckel, W. & Tuschl, T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* **15**, 188–200 (2001).
64. Bernstein, E., Caudy, A.A., Hammond, S.M. & Hannon, G.J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366 (2001).
65. Matzke, M.A. & Birchler, J.A. RNAi-mediated pathways in the nucleus. *Nat. Rev. Genet.* **6**, 24–35 (2005).
66. Cogoni, C. Unifying homology effects. *Nat. Genet.* **30**, 245–246 (2002).
67. Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297 (2004).
68. Sijen, T. *et al.* On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**, 465–476 (2001).
69. Mette, M.F., Aufsatz, W., van der Winden, J., Matzke, M.A. & Matzke, A.J. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J.* **19**, 5194–5201 (2000).
70. Pal-Bhadra, M., Bhadra, U. & Birchler, J.A. Cosuppression of nonhomologous transgenes in *Drosophila* involves mutually related endogenous sequences. *Cell* **99**, 35–46 (1999).
71. Pal-Bhadra, M., Bhadra, U. & Birchler, J.A. RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. *Mol. Cell* **9**, 315–327 (2002).
72. Robert, V.J., Sijen, T., van Wolfswinkel, J. & Plasterk, R.H. Chromatin and RNAi factors protect the *C. elegans* germline against repetitive sequences. *Genes Dev.* **19**, 782–787 (2005).
73. 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).
74. Mochizuki, K., Fine, N.A., Fujisawa, T. & Gorovsky, M.A. Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in tetrahymena. *Cell* **110**, 689–699 (2002).
75. Reinhart, B.J. & Bartel, D.P. Small RNAs correspond to centromere heterochromatic repeats. *Science* **297**, 1831 (2002).
76. Sadaie, M., Iida, T., Urano, T. & Nakayama, J. A chromodomain protein, Chp1, is required for the establishment of heterochromatin in fission yeast. *EMBO J.* **23**, 3825–3835 (2004).
77. Partridge, J.F., Scott, K.S., Bannister, A.J., Kouzarides, T. & Allshire, R.C. cis-acting DNA from fission yeast centromeres mediates histone H3 methylation and recruit-

ment of silencing factors and cohesin to an ectopic site. *Curr. Biol.* **12**, 1652–1660 (2002).
78. Sugiyama, T., Cam, H., Verdel, A., Moazed, D. & Grewal, S.I. RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. *Proc. Natl. Acad. Sci. USA* **102**, 152–157 (2005).
79. Noma, K. *et al.* RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat. Genet.* **36**, 1174–1180 (2004).
80. Hong, E.J., Villen, J., Gerace, E.L., Gygi, S.P. & Moazed, D.A. Cullin E3 ubiquitin ligase complex associates with Rik1 and the Clr4 histone H3–K9 methyltransferase and is required for RNAi-mediated heterochromatin formation. *RNA Biol.* **2**, 106–111 (2005).
81. Colmenares, S.U., Buker, S.M., Buhler, M., Dlakic, M. & Moazed, D. Coupling of Double-Stranded RNA Synthesis and siRNA Generation in Fission Yeast RNAi. *Mol. Cell* **27**, 449–461 (2007).
82. Chan, S.W., Zhang, X., Bernatavichute, Y.V. & Jacobsen, S.E. Two-step recruitment of RNA-directed DNA methylation to tandem repeats. *PLoS Biol.* **4**, e363 (2006).
83. Irvine, D.V. *et al.* Argonaute slicing is required for heterochromatic silencing and spreading. *Science* **313**, 1134–1137 (2006).
84. Buker, S.M. *et al.* Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. *Nat. Struct. Mol. Biol.* **14**, 200–207 (2007).
85. Grewal, S.I. & Jia, S. Heterochromatin revisited. *Nat. Rev. Genet.* **8**, 35–46 (2007).
86. Murakami, H. *et al.* Ribonuclease activity of Dis3 is required for mitotic progression and provides a possible link between heterochromatin and kinetochore function. *PLoS ONE* **2**, e317 (2007).
87. Houseley, J., LaCava, J. & Tollervey, D. RNA-quality control by the exosome. *Nat. Rev. Mol. Cell Biol.* **7**, 529–539 (2006).
88. Yamada, T., Fischle, W., Sugiyama, T., Allis, C.D. & Grewal, S.I. The nucleation and maintenance of heterochromatin by a histone deacetylase in fission yeast. *Mol. Cell* **20**, 173–185 (2005).
89. LaCava, J. *et al.* RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. *Cell* **121**, 713–724 (2005).
90. Vanacova, S. *et al.* A new yeast poly(A) polymerase complex involved in RNA quality control. *PLoS Biol.* **3**, e189 (2005).
91. Morris, K.V., Chan, S.W., Jacobsen, S.E. & Looney, D.J. Small interfering RNA-induced tanscriptional gene silencing in human cells. *Science* **305**, 1289–1292 (2004).
92. Kim, D.H., Villeneuve, L.M., Morris, K.V. & Rossi, J.J. Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. *Nat. Struct. Mol. Biol.* **13**, 793–797 (2006).
93. Weinberg, M.S. *et al.* The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. *RNA* **12**, 256–262 (2006).
94. Lau, N.C. *et al.* Characterization of the piRNA complex from rat testes. *Science* **313**, 363–367 (2006).
95. Girard, A., Sachidanandam, R., Hannon, G.J. & Carmell, M.A. A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature* **442**, 199–202 (2006).
96. Aravin, A. *et al.* A novel class of small RNAs bind to MILI protein in mouse testes. *Nature* **442**, 203–207 (2006).
97. Brennecke, J. *et al.* Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* **128**, 1089–1103 (2007).
98. Klenov, M.S. *et al.* Repeat-associated siRNAs cause chromatin silencing of retrotransposons in the *Drosophila melanogaster* germline. *Nucleic Acids Res.* **35**, 5430–5438 (2007).
99. Aravin, A.A., Sachidanandam, R., Girard, A., Fejes-Toth, K. & Hannon, G.J. Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science* **316**, 744–747 (2007).
100. Yang, P.K. & Kuroda, M.I. Noncoding RNAs and intranuclear positioning in monoallelic gene expression. *Cell* **128**, 777–786 (2007).
101. Rinn, J.L. *et al.* Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* **129**, 1311–1323 (2007).