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Transcription in heterochromatin seems to be an oxymoron — surely the 'silenced' form of chromatin should not be transcribed. But there have been frequent reports of low-level transcription in heterochromatic regions, and several hundred genes are found in these regions in *Drosophila*. Most strikingly, recent investigations implicate RNA interference mechanisms in targeting and maintaining heterochromatin, and these mechanisms are inherently dependent on transcription. Silencing of chromatin might involve *trans*-acting sources of the crucial small RNAs that carry out RNA interference, but in some cases, transcription of the region to be silenced seems to be required — an apparent contradiction.

Chromatin fibres, which make up chromosomes, are composed of nucleosome arrays, with each nucleosome consisting of an octamer of core histones associated with double-stranded DNA. Great variety in chromatin biochemistry is achieved by a complex system of accessory proteins, which modify, bind and reorganize histone complexes to generate different functional regions in eukaryotic chromosomes. Chromatin can be considered to have two main types of domain: euchromatin, which is gene-rich; and heterochromatin, which is gene-poor. These domains have different patterns of histone modification, are associated with different modes of nucleosome packaging[1] and therefore, presumably, have differences in higher-order packaging[2,3] and nuclear organization (see page 413).

Heterochromatin was initially defined as the portion of the genome that retains deep staining with DNA-specific dyes as the dividing cell returns to interphase from metaphase. Subsequent investigation showed that heterochromatin has a constellation of properties (Box 1). A link between heterochromatin formation and gene silencing has been inferred from the loss of most gene activity on the inactive X chromosome, which is visibly condensed in female mammals, and from the loss of gene expression, correlated with condensed packaging, in position-effect variegation (PEV) in *Drosophila* and other organisms. PEV occurs when a gene that is normally euchromatic is juxtaposed with heterochromatin, through rearrangement or transposition; the resultant variegating phenotype indicates that the gene has been silenced in a proportion of the cells in which it is normally active[1]. Reporter genes that show PEV (packaged in heterochromatin) have a more uniform nucleosome array and, perhaps as a consequence, suffer a loss of 5′ nuclease-hypersensitive sites (that is, regions that are presumed to be nucleosome free and are generally associated with regulatory sequences present in active or readily induced genes)[2,4]. Loss of nuclease-hypersensitive sites depends on Heterochromatin protein 1 (HP1; also known as Suppressor of variegation 205, SU(VAR)205)[5]. Studies in fission yeast, *Schizosaccharomyces pombe*, have shown that HP1-family proteins mediate recruitment and/or spreading of chromatin-modifying factors, such as the multi-enzyme complex SHREC (SNF2- and histone deacetylase (HDAC)-containing repressor complex). Such complexes presumably facilitate the nucleosome modification and positioning needed to organize the higher-order chromatin structures that are essential for diverse heterochromatin functions, including silencing of transcription, suppression of recombination, long-range chromatin interactions and maintenance of genomic integrity[1,3,6].

An important characteristic of heterochromatin is the ability of this form of packaging to spread, as evidenced by the occurrence of PEV in *Drosophila* and as shown in *S. pombe*[7] (discussed later). After heterochromatin has been established, it can be stably maintained through mitosis, as shown by the patchy coat of the tortoiseshell (calico) cat, in which coat colour depends on which X chromosome is inactivated. The general properties of euchromatin are antagonistic to those of heterochromatin (Box 1), although it is anticipated that, in euchromatin, there is much more variation in the modification state of the histones and in the arrangement of the nucleosome array, both of which depend on the transcriptional state of a given gene (see page 407). Indeed, greater expression of a *Drosophila* gene (embedded in heterochromatin) that confers variegation has been reported in response to introducing increased amounts of a transcription factor (Gal4), suggesting that there is constant competition in establishing these alternative states[8]. Furthermore, despite the clear distinctions between heterochromatin and euchromatin, low-level transcription has often been found to occur in heterochromatic regions, and these regions contain several hundred genes in *Drosophila*[9]. Resolving these apparent contradictions will provide new insight into how genomes function. In this review article, we focus on recent findings about how heterochromatin formation is targeted and maintained in specific regions of the genome, examining the potential role of transcription associated with the RNA interference (RNAi) system. We draw mainly on results from studies of fungi and animals; interesting results from plants are reported on page 418.

Heterochromatin assembly in *Drosophila*

A key tool for investigating heterochromatin has been the ability to screen for suppressors of PEV (*Su(var)*): that is, mutations elsewhere in the genome that result in loss of silencing at a variegating locus. About 15 such loci have been characterized in *Drosophila melanogaster*, and many more candidates have been identified[10]. The *Su(var)* genes typically encode either proteins that participate directly in the structure of heterochromatin or enzymes that control changes in the modification of histones. A transition between euchromatin and heterochromatin (as might occur in PEV) can roughly be viewed as a series of reactions in which the histone modifications and the proteins associated with the

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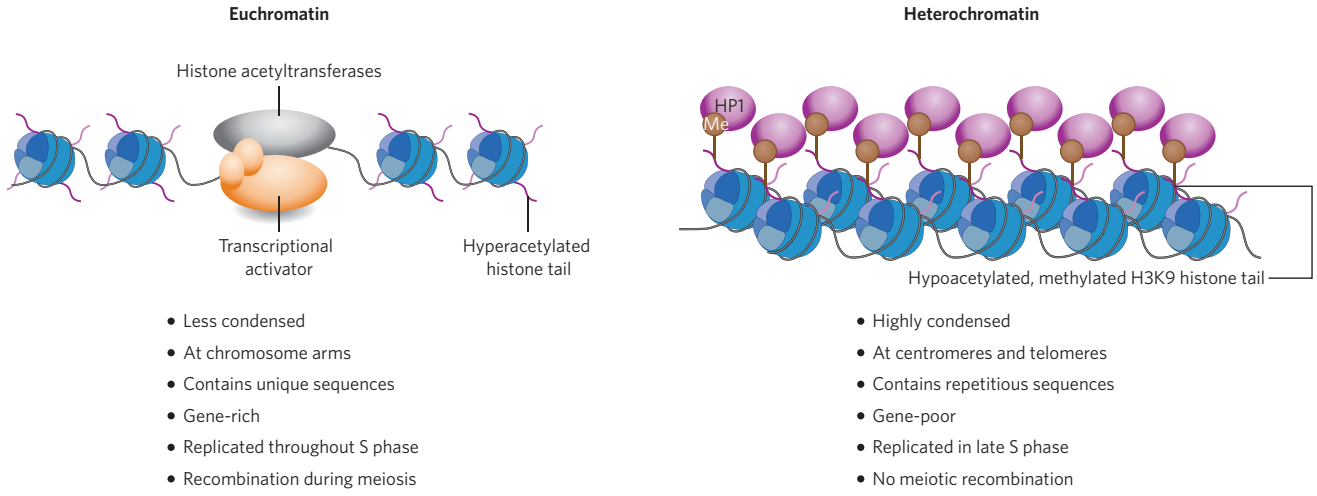
Box 1 | Properties of euchromatic and heterochromatic regions



- Less condensed
- At chromosome arms
- Contains unique sequences
- Gene-rich
- Replicated throughout S phase
- Recombination during meiosis

- Highly condensed
- At centromeres and telomeres
- Contains repetitious sequences
- Gene-poor
- Replicated in late S phase
- No meiotic recombination

Trying to define heterochromatin is like trying to define life itself: a cluster of important properties can be specified, but there are exceptions in every instance. For example, centromeres are usually associated with blocks of flanking heterochromatin. However, the inner centromere of *Drosophila* chromosomes is associated with blocks of nucleosomes that contain CENP-A (also known as CID), a variant of histone H3, interspersed with blocks of nucleosomes that contain H3 with a different modification pattern[78]. The elements at the telomeres of *Drosophila* chromosomes are non-LTR (non-long terminal repeat) retrotransposons, which are transcribed[79]. By contrast, the proximal telomere-associated sequences show more of the properties of heterochromatin. The characteristics listed in the figure are most consistently observed in pericentromeric heterochromatin: that is, in the regions that flank the centromeres of many eukaryotic chromosomes. These regions are rich in remnants of transposable elements. It should be noted that little is known about either the stoichiometry of HP1 or the folding of the chromatin fibre in heterochromatin; the figure is meant to convey only the association of HP1 and the condensation of the chromatin fibre.

active state are removed, and then the histone modifications and proteins associated with the inactive state are added (Fig. 1). A sequential set of reactions is required: for example, the lysine residue at position 9 of histone H3 (H3K9) cannot be methylated until it is deacetylated; the binding of SUV4-20 to heterochromatic loci occurs through interaction with HP1 and requires the activity of SU(VAR)3-9 (ref. 11). This sequential requirement undoubtedly contributes to the relative stability of the alternative packaging states. Although the heterochromatic state can be inherited through mitotic and even meiotic cell divisions, a given site can switch from a repressed to an active chromatin state and vice versa at a low frequency. PEV cannot be scored this way in single-celled organisms such as yeast, but this switching can be observed in the phenotype of sectors of a growing colony (Fig. 2).

In *Drosophila*, a small group of proteins are considered likely structural components of pericentromeric heterochromatin because of the observed dose response: whereas having one copy of the encoding gene results in loss of silencing, having three copies results in increased silencing, presumably due to mass action[12]. This set of proteins includes the following: HP1, the first chromodomain protein to be identified[13]; HP2 (also known as SU(VAR)2-HP2), a large protein with no conserved structural motifs[14]; SU(VAR)3-7, a zinc-finger protein[15]; and SU(VAR)3-9, an H3K9-specific histone methyltransferase[16]. However, a well-defined complex of these heterochromatin-associated proteins has not been isolated (despite HP1 interacting with all of these proteins and other proteins[17]), suggesting that an organized protein assembly is present only on the chromatin fibre.

HP1 is a small protein (206 amino acids in *D. melanogaster*) with two conserved domains, an amino-terminal chromodomain and a carboxy-terminal chromoshadow domain, separated by a hinge region (Fig. 3). The chromodomain, found in many chromosomal proteins, folds to create a binding site for the N-terminal tails of histones. HP1 dimerizes through the chromoshadow domain, forming a peptide-binding surface. HP1 interacts stably with SU(VAR)3-9 through the chromoshadow and hinge domains and with di- or trimethylated H3K9 (H3K9me2 or H3K9me3) through the chromodomain[1]. By interacting with both histone-modifying enzyme and modified histone, HP1 provides a foundation for a self-assembly and spreading mechanism, which has been anticipated from studies of PEV (Fig. 3) (see ref. 18 for a review of possible spreading mechanisms). This core assembly seems to be conserved across animals and fungi[19,20] (Table 1). It should be noted that, in many organisms, there are several homologues of HP1 and multiple H3K9 methyltransferases, suggesting the possibility of alternative protein assemblies[19]. However, in *Drosophila*, only HP1A (referred to as HP1 in this review) seems to be associated with known heterochromatic regions, and the ability of other homologues to mimic HP1 in establishing heterochromatin packaging remains to be determined.

The role of RNAi in *S. pombe*

Genetic and biochemical studies using *S. pombe* as a model system have provided great insight into the mechanisms of heterochromatin assembly. Many of the factors involved in heterochromatin formation in *Drosophila* and mammals are conserved in *S. pombe*[19,20]. In particular, the protein Clr4 (cryptic loci regulator 4) — which is the *S. pombe* homologue of *Drosophila* SU(VAR)3-9 and is present in an E3–ubiquitin ligase complex that contains cullin 4 (also known as Pcu4) — has been shown to methylate H3K9 specifically[21–26]. Methylated H3K9 functions as a binding site for recruitment of chromodomain-containing proteins — including chromodomain protein 1 (Chp1), Chp2 and Swi6 (the last of which is a homologue of *Drosophila* HP1) — to heterochromatic loci[22,27–29]. Heterochromatin-associated factors, including methylated H3K9 and Swi6, were found to map to extended chromosomal regions that are coated with heterochromatin complexes at centromeres, telomeres and the mating-type locus[30]. Interestingly, all three of these heterochromatic regions have a common feature — each contains *dg* and *dh* repeat elements, which are preferential targets of heterochromatin formation[7,31–33]. Recent investigations into mechanisms by which these repeats might trigger heterochromatin formation led to the surprising discovery that the RNAi system is involved in the nucleation and assembly of heterochromatin[7,33].

RNAi was first described as a post-transcriptional silencing mechanism in which double-stranded RNA triggers the destruction of cognate RNAs[34]. Subsequent studies have implicated RNAi-associated

mechanisms in diverse cellular functions. In *S. pombe*, mutations in genes encoding factors that are involved in RNAi — such as dicer (Dcr1; an enzyme that cleaves double-stranded RNA), argonaute (Ago1; a PAZ- and PIWI-domain-containing protein that can bind small RNAs) and RNA-directed RNA polymerase 1 (Rdp1) — result in defects in heterochromatin assembly, as shown by loss of silencing at reporter loci[7,33]. An RNAi-induced transcriptional silencing complex (RITS), which contains both a chromatin-associated protein and an RNAi-associated protein, has been identified[35]. RITS contains Chp1 (a chromodomain protein), Ago1 and a protein of unknown function, Tas3 (RITS subunit 3). In addition, RITS also contains small interfering RNAs (siRNAs) derived from the *dg* and *dh* repeats present at the different heterochromatic loci[30,35]. Genome-mapping analyses have shown that Rdp1 and components of RITS are distributed throughout heterochromatic regions in a pattern that is almost identical to the distribution of Swi6 and of H3K9 methylation[30]. Stable binding of RITS to chromatin depends, at least in part, on the binding of the chromodomain of Chp1 to methylated H3K9 (ref. 36). Deletion of *clr4*, or a mutation in the chromodomain-encoding region of *chp1*, results in delocalization of RITS from heterochromatic loci. Interestingly, there are concurrent defects in the processing of *dg* and *dh* repeat transcripts into siRNAs[30,36], suggesting that siRNAs are produced in a heterochromatic environment.

RITS also recruits an RNA-directed RNA polymerase complex (RDRC) that contains Rdp1; this polymerase activity is essential for siRNA production and heterochromatin assembly[37,38]. The generation of siRNAs also requires an RNaseH-like RNA-cleavage activity (referred to as slicer activity) known to be associated with argonaute-family proteins, such as Ago1, found in RITS. Mutations in conserved Ago1 residues that abolish this activity severely affect the processing of *dg* and *dh* repeat transcripts and result in defects in heterochromatin assembly[39,40]. The slicer function of Ago1 has been suggested to be important for the spreading of heterochromatin[39]. It is also possible that siRNAs generated by Ago1-mediated processing of transcripts have a direct structural role in the assembly of higher-order structures that, in addition to mediating silencing, facilitates the local spreading of heterochromatin. These mechanisms, however, cannot by themselves account for the spreading of heterochromatin across large regions, because this requires the HP1-family protein Swi6, which functions as a platform for recruiting the chromatin-modifying effectors (that is, proteins or complexes) involved in heterochromatin assembly[1,7].

These findings suggest that RNAi-mediated heterochromatin assembly in *S. pombe* might occur through a self-reinforcing loop[36,37]. In this model, siRNAs (possibly generated elsewhere) and/or DNA-binding proteins mediate the initial targeting of heterochromatin-associated factors, resulting in the establishment of H3K9 methylation. The presence of methylated H3K9 and associated silencing factors, in turn, allows stable binding of RITS across heterochromatic regions (Fig. 4). RITS presumably functions as a core for the binding of other RNAi-associated factors, such as RDRC, that are essential for the processing of any *dg* and *dh* repeat transcripts. The siRNA-guided cleavage of nascent repeat transcripts by Ago1 (a component of RITS) is thought to be an important step in producing additional siRNAs. It is possible that cleaved transcripts are preferential targets for Rdp1. Rdp1 generates double-stranded RNAs, which are necessary for the generation of siRNAs by Dcr1. Those siRNAs produced *in cis* can feed back to target more heterochromatin complexes but might also have other functions (discussed in the next section).

The exact mechanism by which siRNAs target histone modifications is unclear. The binding of RITS to heterochromatic regions requires *dg* and *dh* siRNAs to be part of the complex. It has been suggested that RITS, tethered to nascent transcripts by siRNAs, might mediate the recruitment of histone methyltransferases such as Clr4 (ref. 35) or that siRNAs directly facilitate the recruitment of chromatin-modifying effectors, such as the Clr4-containing complex, to heterochromatic repeats[7,23]. It is certainly possible that siRNAs target heterochromatin by base-pairing with nascent transcripts[41]; subunits of RITS and RDRC can be crosslinked to transcripts of non-coding centromeric repeats[38]. However, it is unknown whether this binding simply reflects the roles of these factors in processing repeat transcripts or whether it indicates an additional function in recruiting heterochromatin proteins. Recently, artificial tethering of RITS to nascent transcripts has been shown to induce

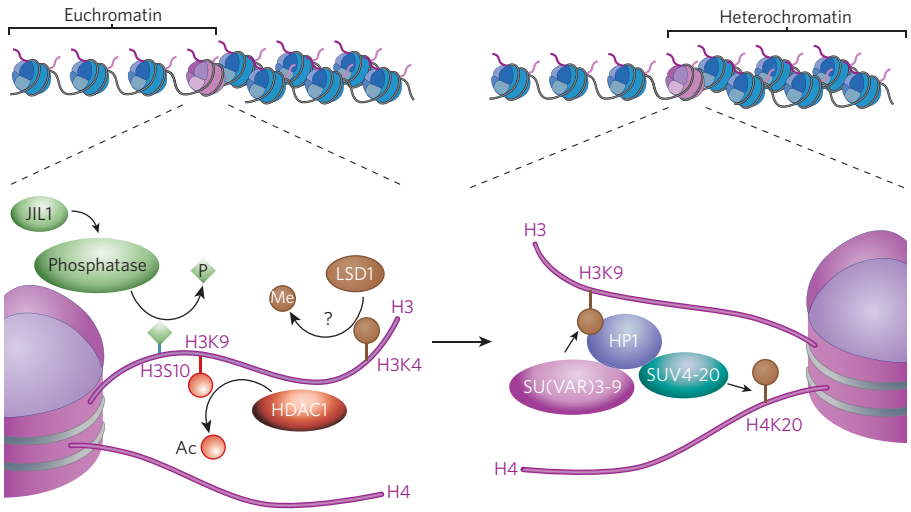


Figure 1 | Changes in histone modification implicated in the switch from a euchromatic to a heterochromatic state in *Drosophila*. Active genes are frequently marked by H3K4me3 (see page 407); this modification is presumably removed by LSD1 (which has not yet been characterized in *Drosophila*). H3K9 is normally acetylated in euchromatin, and this modification must be removed by a histone deacetylase, typically HDAC1. Phosphorylation of H3S10 can interfere with the methylation of H3K9; its dephosphorylation might involve a phosphatase targeted through the carboxy terminus of the protein kinase JIL1 (ref. 10). These transitions set the stage for acquisition of the modifications that are associated with silencing: these include the methylation of H3K9 by SU(VAR)3-9 or another histone methyltransferase, the binding of HP1, and the subsequent methylation of H4K20 by SUV4-20 (an enzyme that is recruited by HP1). Other silencing marks such as methylation of H3K27 by E(Z) (enhancer of zeste; not shown) seem to be relevant in some regions, although this mark is more prominently used by the Polycomb system. Supporting data come from genetic identification of modifiers of PEV, as well as biochemical characterization of the activities of such modifiers and tests of protein–protein interactions[10]. (Figure adapted, with permission, from ref. 10.)

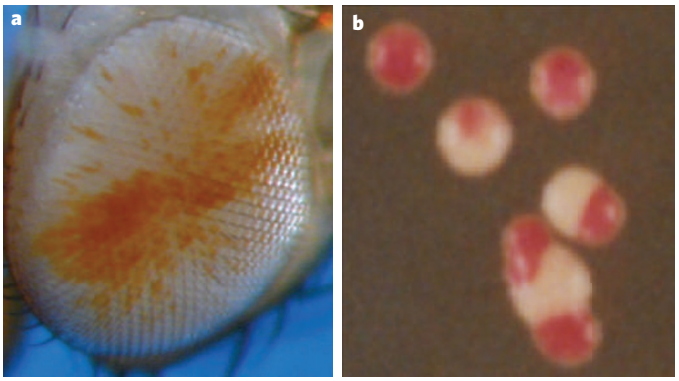


Figure 2 | Variegating phenotypes. Although alternative chromatin packaging states (that is, euchromatin and heterochromatin) can be inherited, they switch at a low frequency. This results in a variegating phenotype in a clonal population of cells. **a**, The image shows a *Drosophila* eye. The *white* gene, expression of which results in a red eye, is active in some eye facets but silenced in others. (Image courtesy of E. Gracheva, Washington University in St. Louis, Missouri.) **b**, The image shows colonies of the fission yeast, *S. pombe*, each of which has differently coloured sectors as a result of variegated expression of the *ade6* gene inserted in a heterochromatic region. (Image courtesy of K. Noma, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.)

local heterochromatin assembly[42]. However, this process requires Dcr1, presumably for the production of siRNAs. Therefore, in addition to the targeting of RITS, other siRNA-dependent steps are required for stable RNAi-mediated heterochromatin nucleation. The emerging view is that, through associations with components of heterochromatin, the RNAi machinery — tethered to specific loci — helps to process transcripts generated from these loci into siRNAs, thereby effectively causing post-transcriptional silencing *in cis*[30,36]. The siRNAs produced in this process also facilitate further targeting of heterochromatin modifications, such as H3K9 methylation. H3K9 methylation enables HP1-family proteins such as Swi6 to localize across heterochromatic regions; these proteins, in turn, facilitate the localization of effectors (such as SHREC) with diverse cellular activities. The HDAC and ATPase (SNF2-like) activities of SHREC are crucial for the proper positioning of nucleosomes to achieve transcriptional silencing[3]. But how can transcription that generates siRNAs occur in a silenced region?

Transcription of heterochromatic repeats

From the results described in the previous section, it can be argued that heterochromatic repeats need to be transcribed to generate the siRNAs that target heterochromatin formation — a circular process. In support of this idea, recent studies have shown that heterochromatic repeats present in the *S. pombe* genome are transcribed by RNA polymerase II (Pol II)[30,43] and that mutations in Pol II impair RNAi-mediated heterochromatin assembly[43,44]. However, an apparent paradox arises in that heterochromatin, in general, is thought to be relatively inaccessible to factors involved in various aspects of DNA metabolism, including the transcriptional machinery[1]. How does Pol II gain access to sequences that are packaged as heterochromatin? Because heterochromatic silencing is thought to be plastic and can be overcome by an increased concentration of transcription factors[8], it can be argued that the promoters driving the transcription of repeats, unlike the promoters of euchromatic genes, have evolved to be somewhat impervious to heterochromatic repression. Indeed, one strand of centromeric repeats in *S. pombe* is always transcribed at a low level[33] but is silenced post-transcriptionally by RNAi-mediated processing of transcripts[33,36].

The transcription of repeats might be facilitated by a specialized mechanism(s) that modulates heterochromatin to provide access for factors involved in different chromosomal processes. In *S. pombe*, Swi6 (*Drosophila* HP1) is thought to function as an 'oscillator' of heterochromatin transcription by directing recruitment of both silencing and antisilencing factors[20]. In addition to factors (such as SHREC) that repress Pol-II-mediated transcription[3], Swi6 also recruits the JmjC-domain-containing protein Epe1 (ref. 40), which was identified in a screen for factors that negatively regulate heterochromatic silencing[45]. Epe1 facilitates Pol-II-mediated transcription of repeats specifically in the context of heterochromatin. It does not seem to have an obligatory role in transcription *per se*, because it is dispensable when heterochromatin is disrupted[40]. The mechanism by which Epe1 counteracts heterochromatic silencing is unknown. Because several JmjC-domain-containing proteins have been shown to catalyse histone demethylation[46], it is possible that Epe1 affects heterochromatin stability through the removal of repressive methylation of lysine residues. However, no such activity has been detected for Epe1 (ref. 47). Epe1 could modulate chromatin by an as yet undefined mechanism. Additional factors targeted to heterochromatic loci by Swi6 or by other mechanisms are also probably important for the transcription of heterochromatin.

In addition to heterochromatin assembly, the transcription of repeats embedded in heterochromatic regions probably has other biological implications. It has been suggested that the transcription of heterochromatic repeats is necessary for continuous production of siRNAs that prime the RNA-induced silencing complex (RISC)-like complexes required to neutralize future invasions by similar sequences[20]. The role of RNAi in destroying viral or transposable element transcripts is conserved in other species, including in *Tetrahymena thermophila* and *Drosophila*[48,49]. Heterochromatin-bound RNAi-associated factors might be components of a memory mechanism that selectively generates a reservoir of siRNAs directed against parasitic DNA elements[20]. It should be noted that, in *S. pombe*, RNAi machinery that is targeted to specific elements can spread to surrounding sequences, including nearby genes, by a process that depends on H3K9 methylation and Swi6 (ref. 36). This might also enable the RNAi machinery to exert heritable control over the expression of sequences located adjacent to repeats.

Silencing of repetitious sequences in metazoans

Although heterochromatin composed of repetitious DNA has become an essential part of the eukaryotic chromosome, maintaining the repetitious sequences in a stable, silent form (repressing both transposition and recombination) is clearly a challenge and a necessity. After it has been initiated, the packaging of heterochromatin occurs in a self-reinforcing manner, through multiple feedback loops[50]. The RNAi machinery seems to be able to detect and respond to repetitious DNA in a variety of ways. But, in metazoans, to what extent might RNAi components be used to target silent regions initially or to maintain these regions? And to what extent might silencing of repetitious DNA depend on transcription *in cis*? The system described in the previous section is unlikely to be universally applicable, because many metazoans, including *Drosophila* and mammals, seem to lack a canonical RNA-dependent RNA polymerase[19].

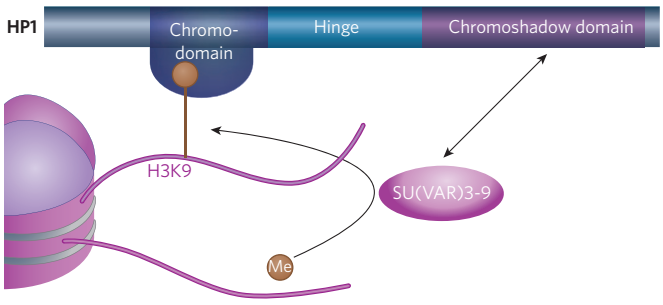


Figure 3 | HP1 and its interactions. HP1 interacts with H3K9me2 and H3K9me3 through its chromodomain, and with SU(VAR)3-9 through its chromoshadow domain. By interacting with both the modified histone and the enzyme responsible for the histone modification, HP1 provides a foundation for heterochromatin spreading and epigenetic inheritance. (Figure adapted, with permission, from ref. 10.)

Table 1 | Factors implicated in heterochromatin formation

Component	*S. pombe*	*Neurospora*	*Drosophila*	Mouse	*Arabidopsis*
Repetitious DNA	Yes	Yes	Yes	Yes	Yes
DNA methylation	No	Yes	No*	Yes	Yes
H3K9 methylation	Yes	Yes	Yes	Yes	Yes
HP1	Yes	Yes	Yes	Yes	No*
Small RNAs	Yes	No*	Yes	Yes	Yes
Pol II	Yes	ND	ND	ND	ND
RDR	Yes	No*	No	No	Yes

Yes indicates that the factor has been implicated to have a role in heterochromatin formation in the given organism. No indicates that the factor is not present in the organism. No* indicates that the organism has the factor but that it seems not to have a role in heterochromatin formation. ND means that the organism has the factor but whether it has a role in heterochromatin formation is unknown. *Arabidopsis*, *Arabidopsis thaliana*; *Neurospora*, *Neurospora crassa*; RDR, RNA-dependent RNA polymerase. (Table adapted from ref. 19.)

Post-transcriptional gene silencing mediated by RNAi, either the degradation of mRNA or a block in its translation, is known to occur in all metazoans that have been examined so far. The first suggestion of RNAi-based transcriptional gene silencing in *Drosophila* came from work showing a loss of expression when multiple copies of a transgene are present[51]. Subsequent analysis showed suppression of PEV (that is, a loss of silencing; as monitored through tandem arrays of *mini–white* and through *white* transgenes in heterochromatin) as a result of mutations in factors involved in the RNAi pathway[51–53]. The loss of silencing is associated with decreased levels of H3K9me2 (ref. 52). Similarly to the RNAi system in other organisms (notably plants; see page 418), the system in *D. melanogaster* might have originated as an antiviral defence mechanism[54,55]. About one-third of the genome is considered to be heterochromatic, and much of that DNA consists of remnants of transposable elements, both DNA transposons and retroviruses. The *Drosophila* genome encodes five PAZ- and PIWI-domain-containing proteins — PIWI, aubergine (AUB), AGO1, AGO2 and AGO3 — which are thought to bind small RNAs. PIWI is required for the self-renewal of germline stem cells, apparently having a key role in silencing retrotransposons and blocking their mobilization in the germ line[56]. Both PIWI and AUB are found associated with siRNAs of 24–29 nucleotides that are derived from repetitious sequences in the germ line[49,57]. *In vitro*, PIWI has RNA-cleavage activity[57], and it has been suggested that germline siRNAs might be generated by a unique processing mechanism that depends on cleavage of long single-stranded transcripts rather than double-stranded RNA[49]. How this silencing activity might influence heterochromatin formation in somatic cells (if at all) is unclear at present.

The effects of mutations in *AGO2* are clearly seen in early *Drosophila* embryos as defects in chromosome condensation, nuclear kinesis and spindle assembly, all potentially correlated with defects in the formation of centric heterochromatin[58]. Similar defects are observed when heterochromatin fails to form in *S. pombe* and other species[59–61]. In *Drosophila* with mutations in the genes that encode SU(VAR)3-9, HP1 or DCR-2, cells have disorganized nucleoli, as well as disorganized centric heterochromatin. In these circumstances, there is a substantial increase in extrachromosomal repetitious DNA in mutant tissues[62]. Similarly, mutations in the genes encoding the RNAi machinery in *S. pombe* also result in defects in maintaining chromosome integrity, including high rates of recombination at genes that encode ribosomal RNA[30]. Therefore, although repetitious DNA now contributes to essential chromosome structures, it is crucial to maintain this DNA specifically in a heterochromatic form, and genetic analysis indicates that the RNAi system has a role in this process. In the absence of any recognizable RNA-directed RNA polymerase activity, this might be accomplished by targeting heterochromatin formation to specific sites, either through DNA–protein interactions or through an RNAi-based recognition system, followed by spreading of the heterochromatin modifications and structure. Similarly to *S. pombe*, the spreading of heterochromatin in *D. melanogaster* (as monitored by PEV) depends on HP1 and SU(VAR)3-9.

Targeting heterochromatin formation

Although much of our discussion focuses on RNAi-based mechanisms, it is important to note that heterochromatin proteins can be recruited to specific sites (known as silencers) by DNA-binding factors. For example, in addition to the RNAi-mediated targeting of heterochromatin to a *dg*- and *dh*-like repeat element located in the silent mating-type region of *S. pombe*, the DNA-binding proteins Atf1 (activating transcription factor 1) and Pcr1, which belong to the ATF/CREB (cyclic-AMP-responsive-element-binding protein) family, have been shown to cooperate with components of SHREC to nucleate heterochromatin assembly independently in this region[6,63,64]. Similarly, redundant mechanisms of heterochromatin nucleation also operate at telomeres in *S. pombe*, where the TRF (TTAGGG repeat factor)-family DNA-binding protein Taz1, in conjunction with Ccq1 (coiled-coil quantitatively enriched protein 1), functions in parallel to the RNAi machinery to nucleate heterochromatin[3,32]. Regardless of the nucleation mechanism, heterochromatin targeted to specific sites can spread, and it provides a sequence-independent platform for cellular effectors with appropriate activities (such as SHREC, the RNAi machinery and cohesin) to be recruited across large regions[20].

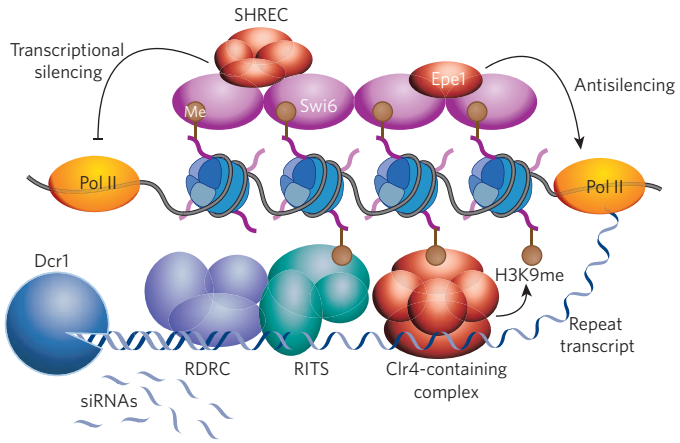


Figure 4 | Model showing RNAi-mediated heterochromatin assembly and silencing in *S. pombe*. Centromeric repeat (*dg* and *dh*) transcripts produced by Pol II are processed by the RNAi machinery, including the complexes RITS and RDRC (which interact with each other and localize across heterochromatic regions). The slicer activity of Ago1 (a component of RITS) and the RNA-directed RNA polymerase activity of Rdp1 (a component of RDRC) are required for processing the repeat transcripts into siRNAs. The siRNA-guided cleavage of nascent transcripts by Ago1 might make these transcripts preferential substrates for Rdp1 to generate double-stranded RNA, which in turn is processed into siRNAs by Dcr1. The targeting of histone-modifying effectors, including the Clr4-containing complex, is thought to be mediated by siRNAs. This process most probably involves the base-pairing of siRNAs with nascent transcripts, but the precise mechanism remains undefined. siRNAs produced by heterochromatin-bound RNAi 'factories' might also prime the assembly of RISC-like complexes capable of mounting a classic RNAi response. Methylation of H3K9 by Clr4 is necessary for the stable association of RITS with heterochromatic loci, apparently through binding to the chromodomain of Chp1. This methylation event also recruits Swi6, which, together with other factors, mediates the spreading of various effectors, such as SHREC. SHREC might facilitate the proper positioning of nucleosomes to organize the higher-order chromatin structure that is essential for the diverse functions of heterochromatin, including transcriptional gene silencing. Swi6 also recruits an antisilencing protein, Epe1, that modulates heterochromatin to facilitate the transcription of repeat elements, in addition to other functions. A dynamic balance between silencing and antisilencing activities determines the expression state of a locus within a heterochromatic domain.

In several cases documented in mammalian cells, HP1 can be targeted to specific promoters by interaction with DNA-binding complexes and seems to contribute to silencing at these loci (see ref. 65 for an example). However, in these cases, a different histone methyltransferase seems to be responsible for the accompanying H3K9 methylation, and spreading is generally not observed. These findings suggest that the interactions of HP1 with both the modified histone (that is, H3K9me3) and the modifying enzyme (usually SU(VAR)3-9) are crucial for heterochromatin spreading (Fig. 3).

Repetitious DNA is a hallmark of heterochromatin. In the case of satellite DNA (simple sequence tandem repeats), it can be suggested that a specific DNA-binding protein recognizes the satellite DNA sequence, thereby triggering heterochromatin assembly. In *Drosophila*, the protein D1, which when mutated results in a loss of silencing, preferentially binds satellite III DNA, which is (A+T)-rich[66,67]. Similarly, the heterochromatin-associated protein DDP1 (dodeca-satellite-binding protein 1; also known as DP1) binds to a conserved dodeca-satellite DNA sequence; however, this protein, which has 15 tandemly organized KH domains, also binds strongly to single-stranded nucleic acids with this sequence, including RNA[68]. Recent work has shown that DDP1, which also causes a loss of silencing when mutated, has a crucial role in the deposition of HP1 and methylated H3K9 at centromeric heterochromatin[68]. Given the ability of DDP1 (and its mammalian homologues, the vigilins) to bind RNA, it is possible that RNA mediates this interaction. Except for the blocks of satellite DNA, the repetitious sequences present in the *Drosophila* genome (which are mainly remnants of transposable elements and DNA transposons) are diverse. Consequently, a recognition process based on RNA (rather than specific protein binding) seems to be most parsimonious, and this suggestion has been supported by studies on the fourth chromosome of *D. melanogaster*.

The small fourth chromosome of *D. melanogaster* is considered to be entirely heterochromatic by the criteria described in Box 1, but it has 88 genes in the distal 1.2 megabases. Mapping with a *white* reporter transgene showed the presence of interspersed heterochromatic regions (inducing a variegating phenotype) (Fig. 2a) and euchromatic regions (allowing expression that results in a full red eye). Detailed examination of the region around the *Host cell factor* (*Hcf*) gene resulted in the *1360* element, which consists of remnants of a DNA transposon, being identified as a potential site for heterochromatin initiation: *D. melanogaster* with reporters lying within 10 kilobases of a *1360* element showed a variegating phenotype, indicating heterochromatin packaging and silencing, whereas *D. melanogaster* with reporters farther away from a *1360* element showed a red eye, indicating euchromatin packaging and full expression[69]. A direct test — using a *P* transposon carrying one copy of *1360*, upstream of a *white* reporter — demonstrated that *1360* contributes to silencing, because silencing of the reporter is largely lost when the adjacent *1360* is deleted. However, stable heterochromatin (resulting in a variegating phenotype) is only observed when that *P* element is located in a region close to the centromere, indicating a requirement for a high density of repeats locally and/or proximity to the pericentromeric heterochromatin, where HP1 is most abundant. Genetic analysis indicates that this silencing depends not only on HP1 and SU(VAR)3-9 but also on RNAi-pathway components, notably AUB[53]. Whether transcription occurs at the target element *1360* is unknown. Small RNA products have been recovered from *1360* and from ~40 other transposable elements in *Drosophila*[70]. It is probable that other transposable elements, in addition to *1360*, are targets for heterochromatin formation. However, it seems unlikely that all transposable-element remnants are targets, given the mapping results obtained on chromosome *4* with the *white* reporter[69]. The crucial characteristics of targets are unknown but could include the presence of start sites for transcription[53]. Many *1360* remnants contain a sequence known to function as a promoter at the multi-copy *Su(Ste)* locus, resulting in the generation of inverse transcripts that are used in the suppression of the multi-copy *Stellate* gene[71]. These results suggest that remnants of transposable elements could be targeted for silencing by a mechanism using a small RNA and that transcription of some of these elements might be involved.

Concluding remarks

Eukaryotes that tolerate large amounts of repetitious sequences in their genomes generally have both the RNAi machinery and the enzymes and structural proteins required to generate a heterochromatin structure based on H3K9 methylation. Whereas some features of the RNAi system (such as RNA-dependent RNA polymerase) and some features of the heterochromatin structure (such as DNA methylation) are used in only a subset of metazoans, this key shift in histone modification from euchromatin to heterochromatin seems to be universal (Table 1; Fig. 1). The RNAi system *per se* can limit gene expression through post-transcriptional gene silencing and can therefore eliminate some sources of damage from invading repetitious elements. However, by itself, it cannot generate the compact chromatin structures that are required to maintain chromosome integrity and chromosome function in mitosis. Hence, the suggestion that post-transcriptional gene silencing is sufficient to explain the silencing of repetitious elements seems unlikely. So, taking into account our new knowledge (described here) of the delicate balance between the need for expression and the need for silencing, an attractive model remains one in which the RNAi machinery has a key role by generating small RNAs involved in specifically targeting chromatin components (including HP1 and H3K9 methyltransferase) to silence repetitious DNA.

Although an assembly of heterochromatin structure based on binding of HP1 proteins to methylated H3K9 provides a foundation for spreading, the molecular mechanisms by which heterochromatin exerts long-range repressive effects are not fully understood. The oligomerization of chromatin-bound HP1 through the chromoshadow domains might mediate condensation. However, recent evidence suggests that HP1 binding is dynamic[72,73]. An alternative emerging view is that HP1-family proteins facilitate recruitment of regulatory proteins (effectors) that are involved in silencing and other chromosomal processes[20]. Indeed, as described earlier, HP1-family proteins mediate preferential binding of SHREC, which has HDAC activity[3]. The deacetylation of histones, which is a universal property of heterochromatic regions, might result in a lower affinity of transcription factors for target loci or could be crucial for higher-order packaging of nucleosomes, both of which would contribute to silencing. The HP1 and H3K9-methylation system might use several routes to minimize H3K9 acetylation, a key characteristic of the active state.

Evidence from different systems suggests that once triggered, a repressive chromatin structure can be sustained for many cell generations. In *S. pombe*, heterochromatin structures established by RNAi and/or DNA-binding factors are inherited *in cis* for many generations in a manner dependent on Swi6 and histone-modifying activities[7,74]. Moreover, a recent study in *Caenorhabditis elegans*, an organism known to silence repetitious DNA by using RNAi and chromatin-associated factors[75,76], showed that a single exposure to RNAi resulted in dominant silencing of a reporter gene in ~30% of the progeny for many generations[77]. A screen for mutations that affected the maintenance of silencing identified four essential genes: *hda-4* (which encodes a histone deacetylase), *K03D10.3* (which encodes a histone acetyltransferase), *isw-1* (which encodes a homologue of the chromatin-remodelling protein ISW1) and *mrg-1* (which encodes a chromodomain protein). Coupled with the observation that trichostatin A (a histone deacetylase inhibitor) relieves silencing, the results imply that maintenance of silencing is a consequence of heterochromatin formation, heritable even in the absence of the initial RNAi stimulus[77]. Although much remains to be learned about the mechanisms involved, it is clear that proper interplay of the RNAi and heterochromatin systems is crucial for the maintenance and function of our genomes. ■

Note added in proof: Two recent publications have shed light on the production of small repeat-associated RNAs in the germ line of *Drosophila*. PIWI and AUB are found associated with RNAs that are mainly antisense to transposons, whereas AGO3 is found associated with RNAs arising mainly from the sense strand. Complementary relationships between these sense and antisense RNA populations suggest that the

slicer activities of the three proteins work together to produce significant amounts of small RNA from endogenous transcripts[80,81]. Such small RNAs, which are maternally inherited, might promote both transcriptional and post-transcriptional silencing of repetitious DNA.

The demethylation of H3K4 has been suggested to be crucial for heterochromatin formation (Fig. 1), and this has now been shown in *Drosophila*[82].

In addition, a histone H2B ubiquitylation ligase complex (HULC) that facilitates Pol-II-mediated transcription of repeat elements in *S. pombe* has been identified[83]. HULC ubiquitylates H2BK119, and this, in addition to promoting euchromatic gene expression, contributes to the transcription of heterochromatic repeats.

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