Heterochromatin revisited

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Abstract | The formation of heterochromatin, which requires methylation of histone H3 at lysine 9 and the subsequent recruitment of chromodomain proteins such as heterochromatin protein HP1, serves as a model for the role of histone modifications and chromatin assembly in epigenetic control of the genome. Recent studies in *Schizosaccharomyces pombe* indicate that heterochromatin serves as a dynamic platform to recruit and spread a myriad of regulatory proteins across extended domains to control various chromosomal processes, including transcription, chromosome segregation and long-range chromatin interactions.

Knobs
Blocks of heterochromatin other than the centromeres and nucleolar organizer regions.

In eukaryotic cells, genomic DNA is folded with histone and non-histone proteins to form chromatin. Each chromatin unit, or nucleosome, contains 146 bp of DNA, which is wrapped around an octamer of histones[1]. Histone modifying enzymes, chromatin-remodelling complexes and DNA methylation are thought to be components of intricate epigenetic mechanisms that help compact and organize genomes into discrete chromatin domains[2,3]. This organization also underlies many aspects of chromosome behaviour, such as transcription, recombination and DNA repair[4].

In 1928, Heitz first distinguished heterochromatin from euchromatin on the basis of differential compaction at interphase[5]. Euchromatin is less condensed, more accessible and generally more easily transcribed, whereas heterochromatin is typically highly condensed, inaccessible and highly ordered in nucleosomal arrays[6]. Heterochromatin formation and maintenance integrate diverse kinds of information, including chromosomal location, nuclear localization and the presence and density of repetitive DNA elements[7–9]. The chromosomal regions that contain a high density of repetitive DNA elements such as clusters of satellite sequences and transposable elements, which are found at centromeres, telomeres and 'knobs', are the main targets of heterochromatin formation[8–10]. These regions remain condensed throughout the cell cycle, and are referred to as constitutive heterochromatin. However, heterochromatin is also found at developmentally regulated loci, where the chromatin state can change in response to cellular signals and gene activity. These regions are referred to as facultative heterochromatin.

A key feature of heterochromatin is its ability to propagate, and thereby influence gene expression in a region-specific, sequence-independent manner. When heterochromatin spreads across domains, it generally causes epigenetic repression of nearby sequences, in a process that is referred to as silencing. In female mammalian X-chromosome inactivation, heterochromatin spreads from a specific nucleation site, causing silencing of most of the X chromosome, thereby regulating gene dosage[11]. Heterochromatin can also repress recombination, which protects genome integrity by prohibiting illegitimate recombination between dispersed repetitive DNA elements[9]. In fact, control of 'parasitic' transposable elements has been suggested as the original evolutionary benefit of heterochromatic silencing[9,12]. It is now recognized that heterochromatin that is assembled at otherwise parasitic DNA elements can also contribute to several biological processes. For example, centromeres malfunction when heterochromatin formation is disrupted[13,14]. Defects in heterochromatin also affect nuclear organization and developmentally controlled long-range chromatin interactions between *cis*-acting regulatory elements and target loci[15–17].

Although epigenetic gene silencing has become almost synonymous with heterochromatization, there are several reports in the literature in which heterochromatin formation is required for activation of gene expression[7,18,19]. Histone H3 methylated at lysine 9 (H3K9me) and the heterochromatin protein HP1, which are necessary for the formation of heterochromatin, have been found in association with a subset of transcribed genes[20–23]. Furthermore, it has been shown that heterochromatin proteins recruit factors that facilitate the access of RNA polymerase II (Pol II) to heterochromatic loci[24]. In view of the multifaceted role of heterochromatin in diverse cellular functions that in some instances involve opposing cellular activities[24], our accepted concepts about heterochromatin need to be redefined.

An important emerging theme is that heterochromatin provides a mechanism for the recruitment and spreading of regulatory proteins (effectors) that are

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implicated in different aspects of chromosome biology. Although these effectors can be targeted to individual loci in a sequence-specific manner, the ability of heterochromatin to spread provides a sequence-independent platform to allow recruitment of these effectors at the level of chromatin domain. This might facilitate coordinated control of loci that are otherwise incapable of recruiting effectors by themselves.

From an evolutionary point of view, the current multipurpose character of heterochromatin might represent a series of co-optation events. Indeed, although all eukaryotes use epigenetic silencing mechanisms, different lineages have emphasized different aspects of heterochromatin regulation, depending on the chromosomal contexts (BOX 1). It is therefore provocative, but not entirely unexpected, to find that many of the same

Box 1 | **Complexities of heterochromatin in fungi, ciliates, plants and mammals**

Owing to its utility as a model for heterochromatin studies, this review focuses mainly on the fission yeast *Schizosaccharomyces pombe*. We include this brief comparison to highlight differences in other organisms, and to emphasize the evolutionary plasticity of epigenetic silencing effects.

Fungi

Saccharomyces cerevisiae lacks heterochromatin protein HP1, which is essential for silencing in metazoans. Higher-order packaging and silencing at its telomeres and mating-type loci, and recombination suppression in the rDNA repeats are processes that depend on protein complexes that differ from other eukaryotes, with the exception of a highly conserved NAD^+-dependent histone deacetylase, Sir2 (REF. 148). In *S. cerevisiae*, centromeres are small, lack associated repeats and capture only a single spindle fibre each. Traces of homology can be found between proteins of the SKI antiviral system and proteins that are involved in RNAi in other organisms[149].

In *Neurospora crassa*, any repeated sequence is destroyed in a pairwise way by targeted point mutation (RIP)[55]. The RNAi machinery is not required to maintain the silencing of RIPed regions. Whether it is required for the original RIP process is not known. Meiotic silencing of the unpaired DNA in *N. crassa* requires an RNA-dependent RNA polymerase, indicating that dual RNAi-dependent and RNAi-independent mechanisms exist to constrain genome divergence[57]. DNA methylation depends on trimethylation of histone H3 at lysine 9 (H3K9me). HP1 binds H3K9me and serves as an adaptor for the recruitment of DNA-methylation machinery to heterochromatic loci[150].

In *Ascobolus immersus*, silencing of repeats involves H3K9me and targeted CpG methylation, which is induced pre-meiotically (MIP). Although MIP might involve an RNA component to mediate methylation, it has long been considered a model for direct DNA–DNA interaction[57]. It remains formally possible, however, that localized RNA could mediate this pairing, as has been suggested for *S. pombe*[120].

Ciliates

Tetrahymena thermophila breaks, shuffles and selectively eliminates parts of its DNA each time it produces the actively transcribed chromosomal fragments that form the macronucleus. Small RNAs that are produced by the RNAi machinery accumulate during conjugation and are highly enriched in the eliminated sequences[64]. H3K9me and the accumulation of chromodomain proteins, the hallmarks of heterochromatin, occur specifically at sequences that are undergoing elimination, and are dependent on the small RNAs[57,65].

Plants

In *Arabidopsis thaliana*, transposon-rich pericentric regions and knobs are heterochromatic. The RNAi-based antiviral response is strong and systemic. *Arabidopsis thaliana* has at least three distinct small-RNA-based silencing systems. RNAi controls *de novo* DNA methylation, which involves ARGONAUTE 4 (AGO4), DICERLIKE 3 (DCL3), RdRP (RDR2) and plant-specific RNA polymerases (RNA POL IV)[110]. Heterochromatin formation also involves H3K9me, but the role of HP1 remains to be resolved. The maintenance of heterochromatin relies on a complex interdependence between H3K9me and DNA-methylation pathways[57,110].

Maize has repeated sequences throughout the genome, although they are enriched at centromeres, knobs and inactive B chromosomes. It also has high levels of DNA methylation and histones that carry the heterochromatin marks. Neocentromere activity, meiotic drive, changes in developmental timing and recombination frequency are associated with specific knobs. At the B locus, which can be silenced by paramutation, the presence of repetitive elements correlates with the tendency of an allele to silence, and be silenced. Consistent with the involvement of RNAi, RNA-dependent RNA polymerase is required for transposon methylation, transposon silencing and paramutation[151].

Animals

In *Drosophila melanogaster*, simple-sequence repeats and transposable elements make up pericentromeric heterochromatin[8]. Heterochromatin is underrepresented in polytene chromosomes of the larval salivary glands. Some actively transcribed genes lie in heterochromatin. Heterochromatic blocks can have neocentromere activity. Telomeres are maintained by targeted transposition of retrotransposons, not the simple repeats that are generated by telomerase, as is the case in many other organisms. There is no CpG methylation. Nuclear relocation of genes can trigger *trans* silencing. There is RNAi-based silencing of dispersed or chimeric transgenes and related endogenous genes, which, like the formation of pericentric heterochromatin, involves H3K9me and HP1[66]. Heterochromatin components including HP1 are also required for gene activation (see text).

Mammals imprint some genes. Establishment of an imprint can involve temporary demethylation and transcription, as well as remethylation and heterochromatization, of selected regions, indicating that RNAi could be involved. *Xist* RNA is involved in X-chromosome inactivation[11]. The assembly of constitutive heterochromatin involves complex patterns of histone modifications (such as H3K9me, H3K27me and H4K20me), HP1 proteins and an RNA component[2,25]. H3K9me and HP1 bind to actively transcribed genes and could have a role in transcriptional elongation[23].

Co-optation
The adaptation of an existing biological feature for a new purpose.

Spindle
A eukaryotic cytoskeletal structure that is made of bundles of microtubules and functions to segregate chromosomes to the daughter cells during mitosis and meiosis.

Conjugation
A process of sexual reproduction in certain algae and fungi in which temporary or permanent fusion occurs, resulting in the union of the male and female gametes.

Meiotic drive
A process that causes some alleles to be overrepresented in the gametes that are formed during meiosis.

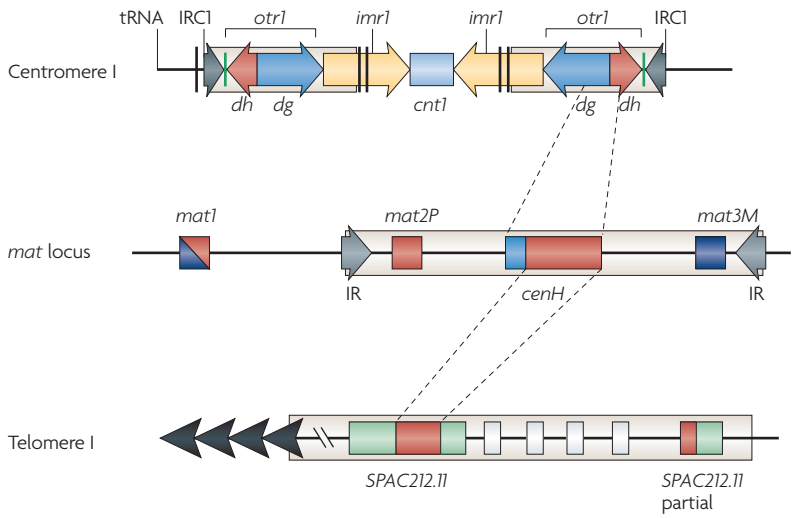
Paramutation
An interaction between two alleles of a single locus, resulting in a heritable change in the expression of one allele, which is induced by the other allele.

Imprinting
The epigenetic marking of a gene on the basis of parental origin, which results in monoallelic expression.

histone modifications and proteins that are required to assemble silent heterochromatin structures are, in other circumstances, instead essential for gene activation.

Studies from diverse systems have contributed to our understanding of heterochromatin and its biological significance (BOX 1). Multiple pathways of histone modifications and DNA methylation in higher eukaryotes contribute to how heterochromatin is assembled[2,3,25] (BOX 1). However, the ever-growing list of factors (which often work redundantly) that are involved in heterochromatin formation[25,26], coupled with the size and complexity of the genomes of higher eukaryotes, complicates further efforts to understand the basic mechanisms that underlie heterochromatin formation. In light of such complications, the fission yeast *Schizosaccharomyces pombe* offers unique advantages for investigating chromatin structure and function. Unlike budding yeast, its genome contains large heterochromatic regions (BOX 2), despite its small genome. Moreover, recent studies have indicated that there are conserved pathways of heterochromatin formation between fission yeast and higher eukaryotes (see below). For this reason, we largely focus on recent advances in understanding the formation of heterochromatin and its functions in *S. pombe*. Brief comparisons are made with other systems in cases in which they are particularly informative (see also BOX 1).

Box 2 | **The main heterochromatic regions in fission yeast**



At centromeres, large inverted repeat structures (*imr* and *otr*) surround the central core (*cnt*) domain, which is the site of kinetochore formation. The *otr* region is composed of *dg* and *dh* repeats, which are targets of RNAi-mediated heterochromatin assembly. Heterochromatin and RNAi machinery coat the entire *otr* region and a small portion of the *imr* repeat structures (shown within a beige box)[34]. Transition between heterochromatin and surrounding chromatin domains is marked by the presence of tRNAs or IRC inverted repeats, which serve as boundary elements. The mating-type region contains three genes; *mat1*, *mat2* and *mat3*. *mat1* is transcriptionally active and determines the mating-type (*P* or *M*) of the cell. However, *mat2P* and *mat3M* are located in a 20-kb heterochromatic domain (shown within a beige box) that is surrounded by IR inverted repeat heterochromatin boundaries[33]. The *cenH* element, which resembles *dg* and *dh* repeats, serves as an RNAi-dependent nucleation centre to assemble heterochromatin across the entire *mat2–3* interval[40,78]. A broad distribution of heterochromatin is also observed at subtelomeric regions[34,82]. A full-length and partial paralogue of *SPAC212.11*, a RecQ helicase gene that has a *cenH*-like element in its coding sequence, is embedded in the heterochromatic domain[152]. All three heterochromatic regions share a common feature — each contains *dg* and *dh* repeats, which are transcribed and are an important source of the siRNAs that are generated by RNAi[34].

Mechanisms of heterochromatin assembly

HP1 and histone modifications in heterochromatin assembly. Originally identified in *Drosophila melanogaster*, HP1 belongs to a highly conserved family of chromatin proteins, with homologues that are found from fission yeast (Swi6, Chp2 and Chp1) to humans (HP1α, HP1β and HP1γ)[6]. These proteins contain an amino-terminal chromodomain, a short variable hinge region and, with the exception of Chp1, a chromoshadow domain. Each HP1 protein interacts with diverse factors that are involved in different aspects of heterochromatin structure and function. The diversification of HP1 isoforms is also indicated by their distinct localization patterns. Whereas HP1α and HP1β are distributed mainly at pericentric chromatin domains, HP1γ is localized to discrete euchromatic sites[6]. The binding of HP1 proteins to chromatin is believed to be highly dynamic[27,28]. In *S. pombe*, two kinetically distinct populations of Swi6 with different residence times at heterochromatin have been described[29], although the significance of this finding remains to be fully explored.

Histones and their modifications have crucial roles in the formation of heterochromatin[2]. Heterochromatin has a characteristic histone-modification profile, which is distinguished by hypoacetylation and H3K9 methylation; euchromatin is characterized by histone H4 acetylation and methylation of histone H3 at lysine 4 (H3K4me)[30–34]. Histone methylation serves as a 'molecular anchor', recruiting proteins that either directly modify chromatin or recruit others that do so[35]. For example, H3K9me provides a binding site for the Swi6/HP1 chromodomain to recruit these factors to heterochromatic loci[31,36,37]. In *D. melanogaster*, chromatin-bound HP1 in turn mediates the recruitment of the histone methyltransferase SU(VAR)3–9, either directly or through the bifunctional binding partner, SU(VAR)3–7 (REF. 38). SU(VAR)3–9 is the main producer of the H3K9me mark[2,39]; as long as it carries out this reaction on an adjacent histone, the heterochromatin assembly process continues. In *S. pombe*, the equivalent process, cryptic loci regulator 4 (Clr4)-dependent methylation of H3K9, is initiated independently of Swi6, but the subsequent spreading of H3K9 methylation across the domain is strictly Swi6-dependent[40]. Given the multimerization of Swi6/HP1 through the chromoshadow domain[41,42], and the ability of Swi6/HP1 to bind to numerous proteins that are implicated in heterochromatin formation, including histone deacetylases (HDACs)[43–46], it has been suggested that Swi6/HP1, when bound to methylated H3K9, serves as an assembly platform for chromatin-modifying factors that are involved in stabilization (maintenance) and spreading of heterochromatin[40,45]. This general strategy of histone methylation functioning as an anchor to recruit effectors is not restricted to heterochromatin. Analogous mechanisms are also used at euchromatic loci, where H3K4me and H3K36me recruit chromatin remodelling and HDAC activities, respectively, to transcribed genes[47–51].

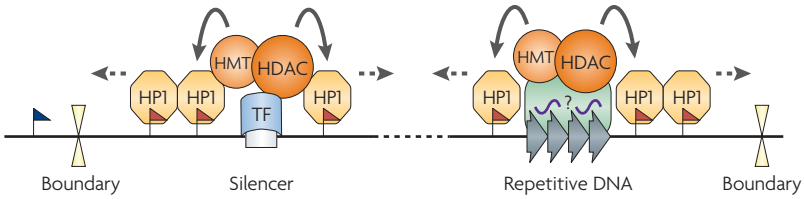


Figure 1 | **Mechanisms for the initiation of heterochromatin assembly.** Heterochromatin structures can be nucleated by factors that recognize specific DNA sequences, such as transcription factors (TF), or by the RNAi machinery that targets repetitive DNA elements. Both mechanisms recruit histone-modifying enzymes such as histone methyltransferases (HMTs) and histone deacetylases (HDACs) to nucleate heterochromatin at a specific site. Heterochromatin proteins such as Swi6/HP1, which bind to modified histone tails, allow heterochromatin to spread to surrounding sequences. Boundary elements prevent heterochromatin from encroaching into neighbouring euchromatic regions, which carry distinct histone modification patterns. The green box indicates an unknown factor(s) involved in siRNA-dependent heterochromatin targeting. Red flags indicate histone H3 methylated at lysine 9, and the blue flag indicates histone H3 methylated at lysine 4.

Heterochromatin nucleation by RNAi and DNA-binding factors. Initial targeting of heterochromatin to nucleation sites is distinct from the subsequent heterochromatic spreading and maintenance steps. The strategies that are used to target heterochromatin differ depending on the chromosomal context (FIG. 1). Local *cis*-acting sequences can promote the establishment of facultative heterochromatin, as exemplified by RB and KRAB–KAP1-mediated recruitment of HP1 and SUV39 proteins[52,53]. However, constitutive heterochromatin formation is associated with the presence of repetitive DNA elements. The repetitive nature of these elements, rather than any specific primary DNA sequence, seems to be the trigger and the target for heterochromatin formation[54–56]. Several lines of evidence indicate that there might be an important and conserved role for non-coding RNAs and RNAi in this context[57].

RNAi was originally defined as a post-transcriptional silencing mechanism[58]. But the core RNAi machinery (which includes Dicer (Dcr), Argonaute (Ago) and RNA-dependent RNA polymerase (RdRP)) also alters chromatin structure and silences genes at the transcriptional level.

The role of RNAi in genome modification is perhaps best studied in *S. pombe*. Before genetic and biochemical studies provided a link between Argonaute proteins and RNAi[59], these proteins were known to be involved in stem-cell maintenance[60]. Because mating-type switching in fission yeast follows a stem-cell lineage-like pattern[61], a single gene encoding Argonaute (*ago1*) was mutated. Loss of *ago1* had no impact on the mating-type switching pattern, but mutant cells showed defects in chromosome segregation — a phenotype that is also observed in cells in which heterochromatin formation is defective[13,62]. Subsequent analyses showed that Ago1, Dcr1 and Rdp1 are all required for heterochromatic silencing, as well as heterochromatin-specific chromatin modifications (such as H3K9me) at centromeres and at the mating-type region[40,63]. Concurrently, the Argonaute family protein Twi1 was found to be required for programmed DNA elimination in *Tetrahymena thermophila*, a process that involves heterochromatin assembly[64,65]. Subsequent studies showed that the RNAi machinery also affects heterochromatin in plants, flies, *Caenorhabditis elegans* and mammals[57,66–70].

Chromatin modification is accompanied by the generation of small interfering RNAs (siRNAs) that match target genomic sequences, directly implicating RNAi in heterochromatin assembly[34,64,71–74]. In *S. pombe*, Pol-II-based transcription of repeat elements is essential to trigger siRNA production and heterochromatin formation[34,75,76]. Notably, RNAi components bind preferentially throughout all the main heterochromatic domains in the *S. pombe* genome[34]. A specific class of repeat elements, referred to as *dg* and *dh* repeats (BOX 2), are an important source of siRNAs from these heterochromatic domains[34]. These repeats are sufficient to nucleate heterochromatin at ectopic sites, in an RNAi-dependent manner[40,77]. Moreover, at the endogenous mating-type (*mat*) locus, a *dg*- and *dh*-like element — *cenH* — serves as an RNAi-dependent heterochromatin nucleation centre[40,78,79].

But DNA-binding proteins can also nucleate constitutive heterochromatin. For example, the sequence-specific DNA-binding transcription factors Atf1 and Pcr1 cooperate with Clr3 (an HDAC) to nucleate heterochromatin at the mating-type region of *S. pombe*[45,80,81]. DNA-based nucleation seems to be a distinct pathway, working in parallel with RNAi-directed nucleation[45]. Redundant DNA- and RNA-based heterochromatin targeting mechanisms also seem to operate at telomeres in *S. pombe*, where telomere-repeat binding protein Taz1 (an orthologue of human TRF1 and TRF2) contributes to heterochromatin nucleation, as does the RNAi machinery through interaction with *dh*-like sequences[34,82,83]. Moreover, factors that bind to repetitive DNA elements, such as CENP-B proteins, contribute to heterochromatin formation at centromeres[84]. DNA-based mechanisms also operate at centromeres and telomeres in mammals[85], and might cooperate with RNA-based mechanisms to efficiently assemble heterochromatin.

Mechanism of RNAi-mediated heterochromatin assembly. A mechanistic insight into RNAi-based heterochromatic silencing was provided by purification of the RITS (RNA-induced transcriptional gene silencing) complex from *S. pombe*[86]. RITS contains Ago1 and siRNAs that correspond primarily to *dg* and *dh* heterochromatic repeats. It also contains the novel protein Tas3 and the chromodomain protein Chp1. When cells lack Dicer, the loss of siRNAs is accompanied by delocalization of RITS from centromeric regions. This observation, and results that show that RITS is required for H3K9 methylation at centromeric repeats, indicate that the presence of siRNAs within RITS provides the specificity for its localization, and for the subsequent recruitment of the Clr4 histone methyltransferase (HMTase). Although it remains to be shown whether RITS directly targets histone-modifying activities, accumulating evidence indicates that RITS is a 'molecular watchdog' that is tethered to heterochromatic loci (in part by the binding of the Chp1 chromodomain to H3K9me[79,87]) to process repeat transcripts into siRNAs[79,88].

RNAi
A mechanism by which dsRNA triggers the destruction of cognate mRNA.

Dicer
An RNase III nuclease that processes dsRNA precursors into small interfering RNAs.

Argonaute proteins
PAZ- and PIWI-domain-containing proteins that are essential components of RNAi effector complexes, which bind small interfering RNAs.

RNA-dependent RNA polymerase
An RNA polymerase that generates dsRNAs from ssRNAs to strengthen the RNAi response.

siRNA
Small interfering RNAs (~22–24 nucleotides), which are derived from the processing of long dsRNA by Dicer.

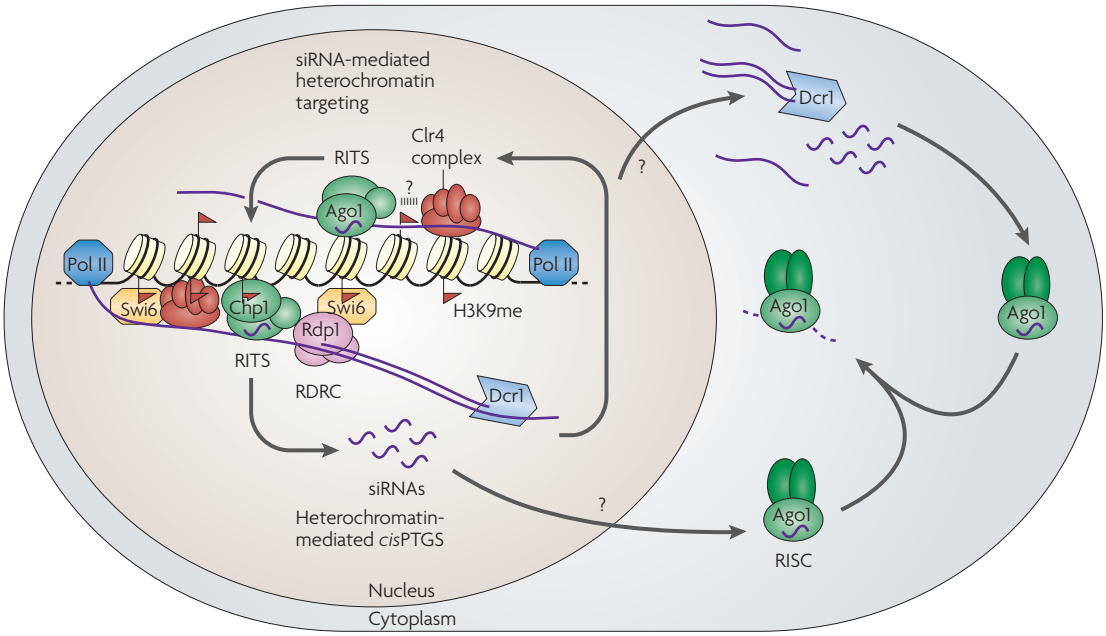


Figure 2 | **RNAi-mediated heterochromatin assembly and silencing in fission yeast.** siRNAs that are generated by RNAi machinery are thought to target histone-modifying activities, such as that of the Clr4 methyltransferase, to repetitive DNA elements to bring about their methylation. Histone H3 lysine 9 methylation (H3K9me) in turn recruits chromodomain proteins such as Chp1, Chp2 and Swi6. Chp1 is part of the RNA-induced transcriptional gene silencing (RITS) complex, which also includes Argonaute (Ago1), and, along with the RDRC complex (which contains RNA-dependent RNA polymerase (Rdp1)) and Dicer (Dcr1), processes nascent transcripts into siRNAs. These siRNAs can further promote Clr4 targeting to repetitive elements to efficiently establish heterochromatin structures. The siRNAs that are produced from heterochromatin might also be distributed to the RISC-like complex to launch RNAi responses in the cytoplasm. *cis* PTGS, post-transcriptional gene silencing in *cis*; Pol II, RNA Polymerase II; siRNA, small interfering RNA.

RITS binding across heterochromatic domains also requires Clr4. Loss of Clr4 or a mutation in the H3K9me-binding chromodomain of Chp1[77] causes defective processing of repeat transcripts, which correlates with loss of the siRNAs that are bound within RITS[79]. Artificial tethering of RITS to a nascent transcript induces heterochromatin assembly, but in a Dicer-dependent way[89]. Therefore, RITS localization is not in itself sufficient to nucleate heterochromatin in the absence of siRNAs.

Genetic evidence indicates that RNAi-mediated processing of repeat transcripts and heterochromatin formation occur through a positive-feedback loop (FIG. 2), in which RITS forms the core of a larger RNAi complex that is assembled on chromatin[79]. RITS recruits the RDRC complex, which contains Cid12, Hrr1 and Rdp1; the RdRP activity of Rdp1 is essential for converting nascent transcripts into siRNAs[88,90].

The processing of repeat transcripts also requires the slicer activity of Ago1; mutations in conserved residues that are essential for Ago1 catalytic function abolish siRNA production, correlating with defects in heterochromatin assembly[91,92].

An emerging view is that the H3K9me mark, which is established by the *trans*-acting RNAi machinery and/or DNA-binding factors, stably tethers RITS to chromatin, which in turn engages RDRC and perhaps Dicer to process nascent transcripts into siRNAs. These siRNAs feed back to trigger further recruitment of heterochromatin machinery. How siRNAs localize histone-modifying activities, in particular the Clr4 HMTase, remains to be determined, although it is known that Clr4 is part of a cullin 4 (Cul4)-based E3 ubiquitin ligase complex[93–96], which could possibly interact with RNAi factors such as RITS. Also, Rik1, another member of this complex, contains WD-β-propeller domains, which are found in some RNA-binding proteins[97]. Rik1 might (directly or indirectly) provide a link between siRNAs and heterochromatin nucleation[40,93]. Once nucleated, however, heterochromatin and RNAi factors spread beyond the initial nucleation site, thereby allowing them to exert control over neighbouring sequences.

Boundaries of heterochromatin domains

The ability of heterochromatin to spread must be limited to prevent inappropriate encroachment into neighbouring euchromatin. Cells have evolved multiple mechanisms to limit the spread of heterochromatin[98]. One such mechanism involves DNA boundary elements, which have been implicated in defining the borders between heterochromatin and adjacent chromatin domains[98,99]. Factors that are recruited to boundary elements might preclude nucleosome assembly, leading to disruption of a contiguous array of nucleosomes, which is believed to be required for heterochromatin spreading[100]. In some cases, boundary elements recruit specialized factors to create an active chromatin environment, which counteracts the propagation of heterochromatin[24,98,101]. This is best shown by studies

of a boundary element at the chicken β-globin locus, in which high levels of H3K4me and histone acetylation, which are targeted by the sequence-specific DNA-binding protein USF, counteract heterochromatin spreading[98]. In *Saccharomyces cerevisiae*, the RNA Polymerase III (Pol III) complex, when bound to a tRNA locus, cooperates with chromatin-modifying factors to create a functional heterochromatin boundary[101]. Recent studies from *S. pombe* indicate that boundary elements confine both heterochromatin and its associated factors within a defined genomic region. For example, inverted repeat (*IR*) boundary elements flank heterochromatin domains at the mating-type region in *S. pombe*, blocking the spread of heterochromatin[33,102] and associated factors including RITS, the Clr3 HDAC and several other proteins[17,24,45,79].

The *IR* boundary elements contain B-boxes: binding sites for the Pol III transcription factor, TFIIIC[103]. Binding of TFIIIC to B-boxes in the absence of Pol III is a feature that is shared by *IR* boundary elements and the so-called 'chromosome organizing clamps', which are thought to tether chromosomes to the nuclear periphery. Tethering of boundary elements to fixed nuclear structures presumably partitions heterochromatin from euchromatin and organizes the genome into specialized higher-order 'loop' domains[99,104,105]. A similar mechanism might operate at *S. pombe* centromeres, where clusters of tRNAs demarcate the borders of heterochromatin domains[34,103,106,107].

The existence of multiple mechanisms to limit the spread of heterochromatin and its associated factors highlights the importance of proper chromatin organization in maintaining genome homeostasis.

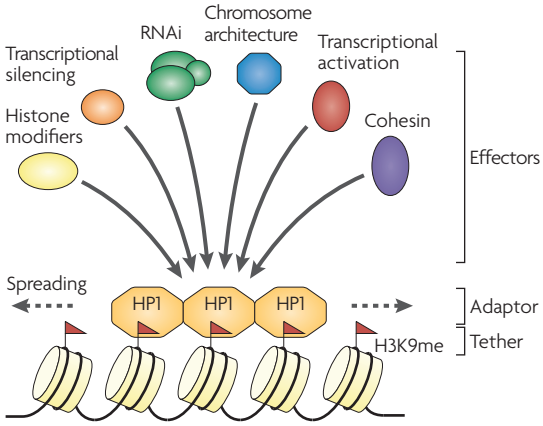


Figure 3 | **Heterochromatin as a platform for the recruitment of effectors across extended domains.** A self-assembling framework of 'tethers' such as histone H3 lysine 9 methylation (H3K9me) or DNA methylation, and 'adaptors' such as Swi6/HP1 (or other chromodomain proteins that are capable of binding to H3K9me), recruit various effectors, such as factors that are involved in chromosome segregation (cohesin), gene silencing (RNAi and histone deacetylases) and transcriptional activation, as well as modifications of histones. The spreading of H3K9me and Swi6/HP1 allows factors that interact with these heterochromatin components to work across large chromosomal domains.

The functions of heterochromatin

Besides repressing transcription and recombination at repetitive DNA elements[9,12], heterochromatin mediates proper segregation of chromosomes and long-range chromatin interactions (see below). But how does heterochromatin perform its diverse functions? Evidence from studies in *S. pombe* and other systems indicates that heterochromatin serves as a self-assembling framework of 'tethers', such as H3K9me, and 'adaptors', such as Swi6/HP1, to recruit effector proteins, which in turn regulate various chromosomal processes (FIG. 3). The ability of heterochromatin to spread in *cis* and to be coordinately regulated in *trans* with other heterochromatic regions[7] offers a powerful tool. It provides a means of targeting factors to sequences that would otherwise lack binding sites for their recruitment and, concurrently, a means of controlling multiple loci in a coordinated manner. Below we discuss some examples of factors that are recruited by heterochromatin proteins to control different chromosomal processes.

Heterochromatin and transcriptional gene silencing. Heterochromatin assembly is commonly associated with large-scale chromatin condensation and the reorganization of nuclear domains[7,12]. Both of these factors can reduce the accessibility of transcription machinery to the heterochromatic loci. Adjacent Swi6/HP1 molecules that are bound to different nucleosomes can dimerize through the chromoshadow domain[41,42], mediating condensation. However, according to recent evidence, Swi6/HP1 are not only dynamically bound to heterochromatin[27,28], but also function as a recruiting platform for chromatin-modifying activities that can, in turn, preclude accessibility of the underlying sequences to transcriptional machinery[45]. In *S. pombe*, the chromodomain proteins Chp2 and Swi6 are believed to serve as a platform for the spreading of the HDAC Clr3 from a nucleation site to target distant loci[45]. Deacetylation by Clr3 maintains heterochromatin by stabilizing H3K9 trimethylation, but it also prevents access of Pol II (REF. 45) (FIG. 4). Acetylation of histone tails directly affects the interaction between nucleosomes and reduces chromatin condensation by nucleosome-remodelling factors[108]. Therefore, deacetylation of histones and nucleosome repositioning by remodelling enzymes might be the crucial steps in the assembly of condensed higher-order chromatin structures. Other modifications, such as methylation of histones, or factors that bind to these modifications, facilitate the recruitment and spreading of HDAC and remodelling activities.

Similar mechanisms might operate in higher eukaryotes (for example, plants and mammals) and filamentous fungi, although heterochromatic silencing in these systems relies on a complex cross-talk between DNA methylation and histone methylation (however, see BOX 1)[109,110]. Methylation of DNA and histones are probably complementary marks that recruit gene-silencing activities. In mammals, DNA methylation recruits MBD (methyl-CpG-binding domain) proteins[111], which, analogously to HP1, might serve as loading platforms to recruit chromatin-modifying factors. Indeed, MBD proteins have been shown to form complexes with HDACs[112].

Heterochromatin and post-transcriptional gene silencing in cis. Silencing of heterochromatic sequences in some systems also involves post-transcriptional processing of transcripts by RNAi-related mechanisms. As mentioned above, H3K9me and heterochromatin proteins allow preferential targeting of RNAi effector complexes, such as RITS and RDRC, to repetitive DNA elements, including dispersed repeats[34,79,88,90]. Heterochromatin also allows the RNAi machinery to spread in *cis* and to operate across large chromosomal domains. Once in place, it provides a mechanism to monitor, detect and remove inappropriate, repeat-derived transcripts[34,79,88].

How widespread is the role of post-transcriptional gene silencing in *cis* (*cis*PTGS) in heterochromatic silencing? Loss of Dicer from chicken DT40 cells or mouse embryonic stem cells results in accumulation of transcripts from heterochromatic repeat sequences[68,69,113]. Moreover, a nuclear RNAi-related process degrades intergenic transcripts of the human β-globin gene cluster[114]. Further analysis is required to determine whether RNAi machinery is recruited to genes that are coated with heterochromatin factors, to mediate processing of their transcripts.

What can we make of the close connection between pre- and post-transcriptional silencing? In *S. pombe*, a single Argonaute protein patrols both the nucleus and the cytoplasm, and mediates both *cis*PTGS and classical RNAi responses[79,115]. When heterochromatin mediates the recruitment of RNAi components to repetitive DNA elements, it both silences these loci and creates 'factories' for siRNA production[79,88,90]. These siRNAs have a potential dual purpose. They enforce the silencing of heterochromatin, but they might also be exported to the cytoplasm to prime a RISC-like complex to neutralize future invasion by similar sequences and degrade any transcripts that escape the RITS mechanism (FIG. 2).

Heterochromatic RNAs, higher-order organization and gene silencing. RNA that is derived from repeats might be structurally involved in the assembly of heterochromatin. HP1 requires RNA to assemble condensed chromatin in mammalian cells; its RNA-binding domain lies in the hinge between the chromo- and chromoshadow domains[116,117]. The formation of ectopic chromatin fibres among pericentric heterochromatin and other HP1-binding regions, particularly regions containing repeat elements in *D. melanogaster*[118,119], could in principle also involve RNAs. In *S. pombe*, RNAi machinery is required for the clustering of telomeres into ~2–4 bodies at the nuclear periphery[120]. It has been suggested that siRNAs that are produced in *cis*, along with *trans*-acting factors, might function as a glue to promote folding or clustering of dispersed heterochromatic loci (FIG. 5). Indeed, a recent study revealed siRNA hot spots near the ends of all three *S. pombe* chromosomes, and local enrichment of RNAi components at all of these loci[34]. Due to redundant heterochromatin assembly pathways, the loss of the RNAi machinery has no effect on H3K9me and Swi6 levels at telomeres[82,120]. Nevertheless, when cells with catalytically inactive RdRP lose the ability to produce siRNAs, telomeric clustering is also lost,

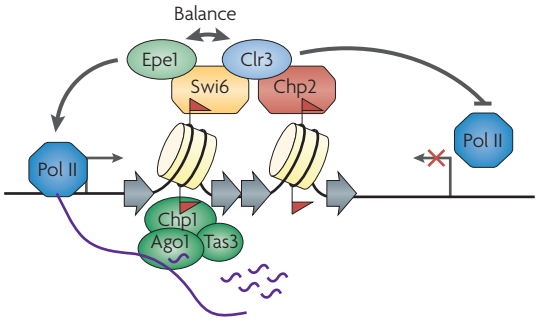


Figure 4 | **Heterochromatin recruits both silencing and anti-silencing factors.** In *Schizosaccharomyces pombe*, methylated histone H3 lysine 9 (H3K9me) recruits chromodomain proteins such as Swi6, Chp1 (part of the RNA-induced transcriptional gene silencing (RITS) complex, with Ago1 and Tas3), and Chp2. Swi6 and Chp2 recruit Clr3 histone deacetylase to limit RNA polymerase II (Pol II) accessibility, thereby causing transcriptional silencing of heterochromatic sequences including repeat elements. Swi6 also recruits Epe1 across heterochromatic domains to facilitate transcription of heterochromatic repeats. These transcripts provide substrate for the RNAi machinery to produce siRNAs, which are essential for the stable maintenance of heterochromatic structures. The balance between the opposing activities of proteins recruited by heterochromatin determines the functional state of a locus.

indicating that clustering might be directly controlled by the RNAi-related process[88]. In *D. melanogaster*, the RNAi components Dicer 2, PIWI and Argonaute co-localize with Polycomb group (PcG) bodies, and are required to potentiate PcG-dependent chromosomal associations between endogenous homeotic genes[121]. Moreover, mutations in RNAi components have been shown to alter pairing-sensitive silencing[121,122], in which two or more PcG response elements (PREs) engage in joint long-range silencing interactions. So, RNAi machinery that is recruited to the target loci seems to contribute to the regulation of the relative location and architecture of chromosomes. It might also help to compartmentalize regions within the nucleus[123]. One or both of these processes might, in turn, promote efficient silencing of target loci[124].

Heterochromatin control of long-range interactions. The ability of heterochromatin to affect nuclear organization[15,16] might be evolutionarily exploited to bring about interactions between regulatory elements that are capable of exerting regional control at a distance and their target loci[19]. Because heterochromatin can be differentially modified in response to cell lineage or developmental signals[32,33,125], differences in heterochromatin structures or their ability to recruit factors could also have wide-ranging implications for developmentally controlled long-range interactions and nuclear organization. *Schizosaccharomyces pombe* has few developmental states. Nevertheless, heterochromatin underlies key developmental changes in this organism[17] (FIG. 6).

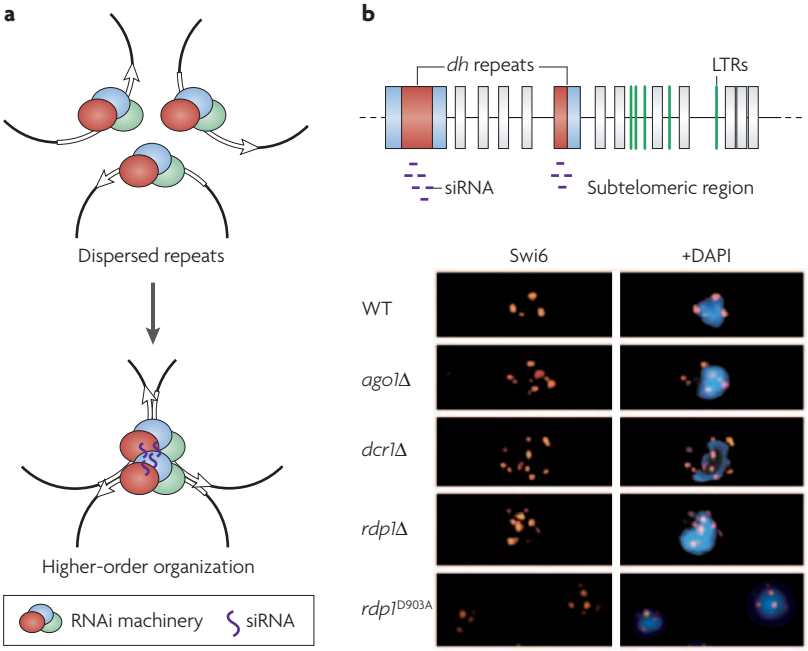


Figure 5 | **A model for a possible role for small RNAs in genome organization.** **a** | Small RNAs produced in *cis* by the RNAi machinery might promote chromosomal association of repeat elements, perhaps by functioning as a glue to hold dispersed repeats in a common structure. **b** | In *Schizosaccharomyces pombe*, loss of small interfering RNAs (siRNAs) in cells that carry either deletions in the genes of the components of the RNAi machinery (*ago1*, *dcr1* and *rdp1*) or a specific mutation in the catalytic site of RNA-dependent RNA polymerase Rdp1 (*rdp1*^{D903A}) correlates with defects in telomere clustering[88,120]. Wild-type (WT) cells contain ~2–3 Swi6 (a chromodomain protein) foci, which represent telomeres and centromeres clustered at nuclear periphery. Defects in telomeric clustering in RNAi mutants result in greater number of Swi6 foci. siRNAs corresponding to centromere repeat (*dh*)-like elements located near telomeric ends have been identified[34]. LTR, long terminal repeats. Images in panel **b** modified with permission from REF. 120 © (2003) National Academy of Sciences, USA.

Schizosaccharomyces pombe switches between the *P* (plus) and *M* (minus) mating-type, by regulated recombination[61]. Information at the expressed *mat1* locus is replaced by sequences that are stored at the transcriptionally silent *mat2P* or *mat3M* donor loci. The choice of donor locus is non-random, and almost always results in a switch to the opposite allele. Consider the challenge: both silent loci lie at a distance from *mat1*, and are embedded within a 20-kb block of heterochromatin. Paradoxically, heterochromatin aids the donor choice by facilitating the spread of a recombination-promoting complex (RPC) in a cell-type specific manner[17]. In *P* cells, RPC binds only to a particular enhancer element that is located near the *mat3M* locus. By contrast, in *M* cells, RPC spreads across a large domain, including the *mat2P* locus. Cell-type dependent protein–protein interactions between RPC and Swi6/HP1, or higher-order folding facilitated by heterochromatin, have been proposed as specific mechanisms to explain differential spreading[17]. Regardless, these findings emphasize that lineage-specific control of heterochromatin-mediated spreading is a potent regulatory mechanism, even in a simple, single-celled organism.

Polytene chromosomes
Chromosomes produced by multiple rounds of replication that remain synapsed together.

HP1-mediated heterochromatic gene expression. Several lines of evidence indicate that Swi6/HP1 carry out a dual function — mediating both transcriptional silencing and activation of target loci[6,18,19,21–23]. For example, two essential genes in *D. melanogaster*, *light* and *rolled*, are embedded in heterochromatin, and their full expression requires both HP1 (REF. 18) and the proximity of large blocks of heterochomatin[7]. More surprisingly, it has been shown that HP1 localizes to actively transcribed heat-shock puffs in polytene chromosomes[21], and, in mammals, both H3K9me and HP1γ associate with coding regions of activated genes[23]. A role for HP1 in the regulation of gene expression is also indicated by mapping of HP1 distribution in *D. melanogaster*[20,22]. The mechanisms by which HP1 proteins mediate gene activation are not fully understood. Heterochromatin might promote the structural organization that is necessary for proper expression of genes, and/or heterochromatin proteins might directly mediate recruitment of gene-activation machinery. In the case of functional *D. melanogaster* genes that are embedded in heterochromatin, heterochromatin formation might simply preserve the relative nuclear location and spacing of a gene and its upstream regulatory elements[19]. Evidence from *S. pombe* indicates that Swi6, in addition to providing a platform or framework for binding of silencing factors, also recruits anti-silencing factors that promote Pol-II accessibility and transcription in a heterochromatin-specific context (FIG. 4). Epe1, a fission yeast JmjC-domain-containing protein that negatively regulates heterochromatic repression[126], is recruited across heterochromatin domains by Swi6 to facilitate transcription of heterochromatic repeats[24]. The balance between the silencing (HDACs) and anti-silencing factors (Epe1) that are recruited by Swi6 is crucial for determining the transcriptional status of a locus. Similar mechanisms could be operating in higher eukaryotes. In particular, HP1 localized to transcribed genes[20,22] might serve as an oscillator of gene transcription by directing the recruitment of both silencing and activator proteins. Heterochromatin proteins that are associated with transcribed genes might also recruit chromatin-modifying activities (such as HDACs and nucleosome-remodelling factors) that are essential for the re-establishment of chromatin structure following transcription, which is essential for the prevention of spurious transcripts initiating from cryptic start sites[49–51], and also to maintain integrity of the genome.

Regulation of chromosome segregation. Defects in heterochromatin formation, especially those affecting the pericentromeric regions, result in mis-segregation of chromosomes[13,14,62,127]. In *D. melanogaster*, centromeric heterochromatin can facilitate the achiasmate (non-exchange) segregation of chromosomes during meiosis[128]. Through Swi6, heterochromatin can directly recruit factors such as cohesin, which is essential for sister-chromatid cohesion[129,130]. Cohesin precisely co-localizes with the heterochromatin components in the 20-kb silent mating-type interval (K. Noma and S.I.S.G., unpublished observations). Mutants that are defective

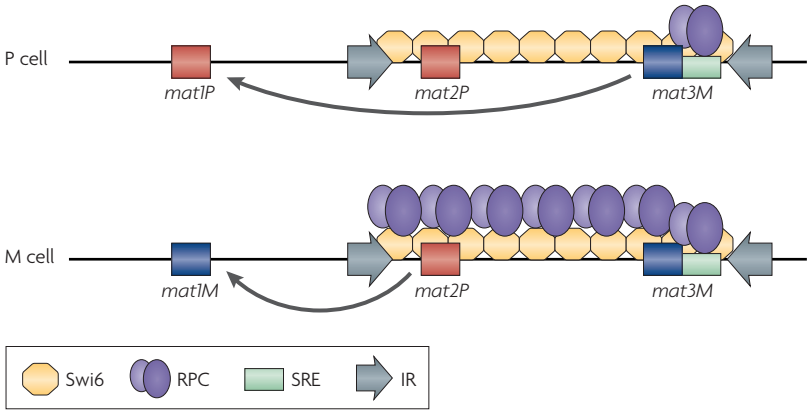


Figure 6 | **Heterochromatin regulates cell-type specific spreading of a protein complex.** A recombination-promoting complex (RPC) that is involved in mating-type switching binds specifically to an enhancer element, SRE, which is located near *mat3M* locus in the *P* mating-type cells. However, in the *M* mating-type cells, the RPC spreads across a 20-kb region, including the *mat2P* locus. This cell-type-specific differential spreading of the RPC is regulated by heterochromatin, in particular Swi6 protein (see text for details). IR, inverted repeat boundary elements.

in Swi6 localization lose cohesin from their centromeres and show chromosome-segregation defects[120,131]. Although a high density of cohesin at centromeres might properly orient kinetochores[132], recruitment of cohesin by heterochromatin also protects genomic integrity by preventing chromosome rearrangements[130]. HP1 also facilitates recruitment of kinetochore proteins such as Mis12 and Mtw1[133], and interacts with the inner centromere protein (INCENP)[134], which regulates chromosome segregation[135].

Other functions that build on the heterochromatin platform. Heterochromatin interacts with many other effector proteins and complexes[25]. These include the origin recognition complex (ORC), which marks and potentiates replication origins, and chromatin assembly factor (CAF1), which couples chromatin assembly to replication[136,137]. These interactions indicate that ORC and CAF1 complexes might recruit HP1 and encourage the formation of heterochromatin, or that heterochromatin facilitates recruitment of these proteins to regulate replication and promote inheritance of epigenetic states through cell division. A conserved protein kinase, Hsk1–Dfp1, which is implicated in DNA replication, interacts with Swi6 in *S. pombe*[138]. It is conceivable that Swi6 recruits this kinase complex to activate replication origins within heterochromatic regions.

Epigenetic reprogramming

Among the increasing number of factors that are known to interact with heterochromatin are proteins for which chromatin association is cell-cycle dependent, or regulated in response to developmental signals[26]. The dynamic nature of Swi6/HP1[27–29] might facilitate rapid transition in chromatin states in response to changing environment conditions and/or developmental signals, by providing an opportunity for rapid exchange of associated regulatory proteins. In other words, occupancy levels of Swi6/HP1-associated factors at target loci probably reflect a dynamic equilibrium that has important regulatory consequences, and might explain concurrent recruitment of various factors by Swi6/HP1.

Perhaps the most extreme case of the reprogramming of heterochromatin-nucleated events is provided by the demethylation of H3K9 by the JmjC family of histone demethylases[139–141], which are expected to completely erase heterochromatin structures. Another attractive model for dynamic heterochromatin effects is provided by the rapidly reversible phosphorylation of histone 3 at serine 10 (H3S10). Modification of H3S10 by Aurora B and other kinases interferes with binding of Swi6/HP1 chromodomains to H3K9me[45,142,143], and offers a potential model for the regulation of heterochromatin stability.

Modifications of heterochromatin proteins represent another pathway for regulating the targeting of effectors to heterochromatic loci. In this mechanism, modifications are thought to change the relative affinities of heterochromatin proteins for alternative interaction partners. In particular, HP1 proteins are known to be modified by various post-translational modifications, including phosphorylation, acetylation, ubiquitylation, sumoylation and methylation[138,144–146]. In mammals, three distinct HP1 isoforms undergo specific modifications that profoundly alter their behaviour[145,147]. It will be interesting to test whether enzymatic activities that modify histones also target heterochromatin proteins to regulate their binding to effector proteins.

Conclusion

Genomes of higher eukaryotes contain large amounts of repetitive DNA sequences, such as transposons and retrotransposons. Any mechanism that evolved to protect the genome from invasion by such elements had to recognize the common features that these parasitic elements use to integrate and spread through the host genome. It is therefore not surprising that an RNAi pathway that uses small RNAs as specificity determinants to degrade transcripts and target heterochromatin to repetitive elements has evolved into a powerful genome-defense mechanism in diverse organisms.

Heterochromatin formation protects genomic integrity by rendering repetitive structures recombinationally inert and prohibiting potentially mutagenic transposition events. But the role of heterochromatin in genome maintenance is not limited to overcoming the harmful effects of repetitive elements. The ability of repeat-rich, gene-poor sequences to be a preferential target of heterochromatin assembly has been exploited during evolution to carry out various cellular functions. The formation of heterochromatin in association with repetitive sequences is crucial for functional organization of vital chromosomal structures such as centromeres and telomeres. The ability of heterochromatin to promote long-range interactions in a cell-type specific manner, and to clonally propagate gene expression patterns, is likely to contribute to the development of diverse cell lineages.

Kinetochore
A multiprotein structure that is assembled on centromeric DNA to mediate the attachment and movement of chromosomes along the mitotic spindle.

The realization that heterochromatin assembled at repetitive sequences provides a platform for the recruitment and spreading of various cellular activities, further to facilitating nuclear organization, underscores the regulatory potential of this specialized form of chromatin. This platform is probably a highly dynamic one that can change in response to cellular and environmental signals. Heterochromatin no longer represents the ultimate form of gene repression. Recruitment of regulatory proteins by heterochromatin offers a potent mechanism for coordinated regulation — involving both activation and repression of genes — at the domain level.

If evolution makes sense only in the context of the regulatory control of genes, we propose that heterochromatin, which is the main form of chromatin in higher eukaryotes, is positioned to be a deeply effective target for evolutionary change. Future investigations into assembly, maintenance and the many other functions of heterochromatin will shed light on the processes of gene and chromosome regulation.

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