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Heterochromatin: new possibilities for the inheritance of structure

Shiv IS Grewal* and Sarah CR Elgin†

Significant portions of the eukaryotic genome are heterochromatic, made up largely of repetitious sequences and possessing a distinctive chromatin structure associated with gene silencing. New insights into the form of packaging, the associated histone modifications, and the associated nonhistone chromosomal proteins of heterochromatin have suggested a mechanism for providing an epigenetic mark that allows this distinctive chromatin structure to be maintained following replication and to spread within a given domain.

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Abbreviations

CAF1	chromatin assembly factor 1
ChIP	chromatin immunoprecipitation
Clr4	<i>S. pombe</i> homologue of SUV39H1
<i>E(var)</i>	<i>Enhancer of variegation</i>
H3-mLys9	Histone H3 modified by methylation on lysine 9
HDAC1	histone deacetylase 1
Mnase	micrococcal nuclease
HMTase	histone methyltransferase
HP1	Heterochromatin Protein 1
HSs	hypersensitive sites
PEV	position effect variegation
<i>Su(var)</i>	<i>Suppressor of variegation</i>
Swi6	<i>S. pombe</i> homologue of HP1

Introduction

Cytologically, the genomic material within the eukaryotic nucleus can be roughly partitioned into euchromatin and heterochromatin. Heterochromatin was originally defined as that portion of the genome which remains condensed and deeply staining as the cell makes the transition from metaphase to interphase; such material is generally associated with the telomeres and pericentric regions of chromosomes [1]. With further characterization, the definition of heterochromatin has been expanded to include a broader set of characteristics [2]. Heterochromatic regions consist predominantly of repetitive DNA, including satellite sequences and middle repetitive sequences related to transposable elements and retroviruses. Although not devoid of genes, these regions are typically gene-poor. Those few genes that are present in heterochromatic regions appear dependent on normal heterochromatic structure for wild-type function [3•]. Characteristically, heterochromatic regions are replicated late in S-phase. Generally these regions show a reduced frequency of meiotic recombination.

Two key observations have linked formation of such a condensed heterochromatic structure with the inactivation of genes normally resident in euchromatic domains. First, X chromosome inactivation in mammals leaves the inactive X as a visibly staining structure, the Barr body. Although the choice of which chromosome to inactivate — either maternal or paternal — appears to be random in most mammalian species, the decision is clonally inherited once made [4]. Second, in *Drosophila*, a similar phenomenon of clonally inherited silencing is observed following chromosome rearrangements with one breakpoint within heterochromatin (position effect variegation [PEV]; see Figure 1). For example, juxtaposition of the *white* gene with such a breakpoint results in silencing of *white* in some of the cells in which the gene is normally active; patches of expressing cells are observed, again suggesting a stochastic ‘decision’ stably inherited through mitosis. Visual inspection of the polytene chromosomes of larvae carrying such a rearrangement shows that the region of the chromosome including the marker gene is indeed packaged as a dense block of heterochromatin, but only in those cells in which the gene is inactive, supporting the correlation between such packaging and gene inactivation [5].

PEV indicates that such rearrangements allow packaging in a heterochromatic configuration to ‘spread’ along the chromosome. Apparently, rearrangement has removed a normal barrier, resulting in silencing of adjacent euchromatic genes. PEV, and/or similar silencing of transgenes inserted into heterochromatin, has been observed in a range of organisms, including yeasts, *Drosophila*, and mammals [6]. Genetic and biochemical studies of chromosomal proteins have recently generated insights that suggest how patterns of heterochromatin formation are inherited, and how heterochromatin formation can spread. Our report here focuses on findings from the fruitfly *Drosophila melanogaster* and the fission yeast *Schizosaccharomyces pombe*; reports in this issue by Dhillon and Kamakaka [pp 188–192] and by Cohen and Lee [pp 219–224] discuss recent findings in *Saccharomyces cerevisiae* and in mammals, respectively.

Heterochromatin structure: results from *Drosophila*

A fundamental characteristic of the silencing observed on heterochromatic packaging is that it affects most euchromatic genes tested, being generally insensitive to the properties of individual promoters/enhancers. Heterochromatin is relatively resistant to cleavage by nucleases, whether nonspecific (DNase I) or specific (restriction enzymes), and is less accessible to other exogenous probes, such as *dam* methyltransferase [2,7]. This might reflect a change in the nucleosomal array, or acquisition of some higher-order packaging superimposed on the array found in euchromatic regions. This

Figure 1

A schematic illustration of *white* variegation in the X chromosome inversion $ln(1)w^{m4}$. The *white* locus (w^+), located in the distal euchromatin (dashed line) of the wild-type X chromosome, provides a function essential for normal red pigmentation of the fly's eye. The inversion shown is the result of chromosomal breaks (X-ray induced) that occurred adjacent to the *white* locus and within the pericentric heterochromatin; this inversion places the *white* locus within 25kb of the heterochromatic breakpoint. This abnormal juxtaposition gives rise to flies with mottled (variegated) eyes, composed of both fully pigmented, red facets (*white* active) and less pigmented, white-to-orange facets (*white* completely or partially inactive). Patterns observed as a consequence of this type of rearrangement vary in the number of pigmented cells, the size of the pigmented patches, and the level of pigment in the two different cell types observed. In *Drosophila*, virtually every test locus that has been examined in an appropriate rearrangement has been found to variegate, and rearrangements involving the pericentric heterochromatin of any chromosome can lead to PEV. It has been suggested that in the normal chromosome, the assembly of heterochromatin-specific proteins (represented by the colored geometric symbols) extends

cooperatively from initiation sites 'i' until a barrier 'B' is reached; in the absence of the barrier, the w^+ gene may be so packaged (and silenced), the extent of spreading being the result of competition for the heterochromatic components. The model shown is not intended

to imply a requirement for strict continuous linear spreading, as a gene closer to the breakpoint may escape silencing in a case where a more distal gene is silenced. C, centromere; T, telomere. (Adapted from [17]; figure courtesy of J. Eissenberg.)

issue has been investigated using transgenes inserted into heterochromatic domains. Appropriate lines have been recovered using a *P* element carrying a *white* (or other) reporter gene and a marked copy of a gene for study (e.g. *hsp26* or *hsp70*, heat-shock genes widely used for chromatin analysis). In a line exhibiting a variegating phenotype, *in situ* hybridization shows that, in almost all instances, the *P* element has inserted into pericentric heterochromatin, the telomeres, or the small fourth chromosome — regions shown previously to have heterochromatic characteristics (e.g. [8]).

Such variegating lines show a loss of nuclease hypersensitivity in the 5' regulatory region of the heat-shock gene (loss of hypersensitive sites [HSs]), whether assayed with DNase I or with a restriction enzyme; in the latter, quantitative test, the loss is roughly proportional to the loss in eye pigmentation observed [8]. DNase I footprinting shows a loss of 5' regulatory proteins (GAGA factor and TFIID) from heat-shock promoters of transgenes within telomeric heterochromatin; potassium permanganate cleavage shows a loss of poised polymerase at an *hsp70* promoter in that environment [9]. Analysis of an *hsp26* transgene in pericentric heterochromatin (almost completely silenced) using micrococcal nuclease (MNase) reveals a nucleosome array with extensive long-range order, indicating regular spacing of the nucleosomes (Figure 2). The MNase cleavage fragments are well-defined, suggesting a smaller MNase target than usual in the linker region. The ordered

nucleosome array extends across the 5' regulatory region of the *hsp26* test gene, a shift that could contribute to the observed loss of HSs [10*]. Regular nucleosome spacing is also reported at telomeres [11], and at a variety of endogenous heterochromatic sequences [10*].

The results indicate that an altered chromatin structure is generated within heterochromatic domains at the nucleosome level; this change may impose (or may reflect) the loss of 5' regulatory proteins, with the concomitant loss of HSs, that is observed for a transgene embedded in heterochromatin. However, genes normally present and active within *Drosophila* heterochromatin (*rolled* and *light*) do not show this pattern, suggesting that the altered chromatin structure is associated with regions that are silent, rather than being a property of the heterochromatic domain as a whole [10*]. The silencing associated with heterochromatin domains can be reversed locally. For example, higher levels of an activator protein will result in greater expression from a GAL4-regulated heterochromatic transgene [12*].

Modifiers of position effect variegation: HP1

Both *Drosophila* and *S. pombe* are particularly well-suited for genetic manipulation, and this attribute has been used to advantage in studies of chromosomal proteins. The variegating line shown in Figure 1 can be used to screen for dominant second site mutations that either suppress (*Suppressor of variegation* [*Su(var)*]) or enhance (*Enhancer of*

Figure 2

MNase digestion reveals long-range nucleosomal ordering at the silenced transgene. **(a)** Nuclei isolated from 6–18hr-old *Drosophila* embryos from a line (39C-X) carrying a marked *hsp26* transgene in a euchromatic domain and a line (HS-2) carrying the same transgene in a heterochromatic domain were treated with increasing amounts of MNase, and the DNA purified and run in an agarose gel. The DNA was transferred to a nylon membrane and hybridized with a probe unique to the transgene. Linker sites cleaved by MNase are indicated by arrows. **(b)** Densitometer scans from the last lane of each sample set are compared (top to bottom of each lane is left to right along the X axis), aligned at the position of the mononucleosome. Although only 5–6 nucleosome arrays can be detected in the euchromatin sample, 9–10 nucleosome arrays can be detected in the heterochromatin sample. **(c)** Although the endogenous *hsp26* gene is known to be packaged in an irregular nucleosome array, with prominent HSs, in euchromatin, the results shown and additional mapping experiments suggest that the transgene in a heterochromatic environment is packaged in a regular nucleosome array, lacking HSs ([10^{*}] and references cited therein). (Adapted from [10^{*}], with permission.)

variegation [*E(var)*]) variegation. Over 50 modifiers of PEV have been characterized, and many more candidates identified using this approach. Where the gene has been cloned and the product characterized, one generally finds a chromosomal protein or a modifier of chromosomal proteins [6]. A subset of these loci (~10%) cause both haplo-abnormal and converse triplo-abnormal phenotypes, implying a dosage-dependent, structural role.

One of the best-studied proteins of this type is Heterochromatin Protein 1 (HP1). HP1 was identified originally in a screen with monoclonal antibodies as a protein primarily concentrated in the pericentric heterochromatin; it is also found in a banded pattern across the small fourth chromosome, at telomeres, and at a small number of sites in the euchromatic chromosome arms [13]. Sequencing of several alleles confirmed that HP1 is encoded by *Su(var)2-5* [14,15], a gene known to cause dosage-dependent shifts in variegation of both euchromatic and heterochromatic genes. Loss of HP1 causes increased expression of a variegating *white* gene, whereas an extra copy of *HP1* causes reduced expression of the same gene. Mutation in *HP1* partially reverses the loss of HSs seen for a euchromatic gene placed in a heterochromatic environment [16]. HP1 is highly conserved: homologues are found in the heterochromatin of organisms from *S. pombe* (Swi6p) to *Homo sapiens* (HP1 α , HP1 β , and HP1 γ) [17]. HP1 is characterized by an amino-terminal chromo domain (homologous between HP1 and Polycomb, a protein required for maintaining the 'off' state of homeotic loci), a short variable hinge region, and a chromo shadow domain (Figure 3). Conservation of HP1 is illustrated by two recent findings: first, that the chromo domain from a mouse HP1 (M31) can functionally replace that of Swi6p in the *S. pombe* gene [18]; and, second, that human HP1s can target heterochromatin and promote silencing in flies [19].

A prominent marker of pericentric heterochromatin, HP1 has been used in a variety of biochemical screens to identify interacting proteins. Several have been identified, including proteins thought to act in gene regulation, chromatin replication, and nuclear assembly (see [6,17] for tabulation). HP1 also self-associates. Most of these interactions depend on the chromo shadow domain (which forms a homodimer) that is likely to be the interacting species [20^{*}]. The point mutations known to affect HP1 function, however, lie in the chromo domain. This puzzle was recently resolved by the observation that the HP1 chromo domain is a specific interaction motif for the amino-terminal peptide of histone H3 modified by methylation on lysine 9 (H3-mLys9) [21^{**},22^{**}].

Modifiers of position effect variegation: histone modification

During the past several years, there has been an explosion of knowledge concerning histone post-translational modifications, with a developing appreciation of the information

coded, which appears to dictate interactions between the nucleosome array and nonhistone chromosomal proteins (see Berger, this issue, pp 142–148). In general, histones associated with active regions of the genome are hyperacetylated, whereas those associated with the inactive regions are hypoacetylated. As predicted, specific missense mutations in the structural gene of *Drosophila* HDAC1 suppress PEV-dependent silencing [23]. A recent key finding has been the observation that human *SUV39H1* and murine *Suv39h1* — mammalian homologues of *Drosophila* *Su(var)3-9* — encode a methyltransferase that specifically methylates lysine 9 of histone H3 [24•]. This activity, mapped to a conserved SET domain with flanking cysteine-rich regions, is limited to a subset of SET domain proteins, including the products of *Suv39h1* and *Suv39h2* in mouse and *clr4* in *S. pombe*. Suv39h1/SUV39H1 proteins are known to associate with M31, a mouse HP1 homologue [25].

Interaction of the HP1 chromo domain with the H3 amino-terminal peptide is highly specific for the lysine 9 methylated form [21•]. Other chromo domain proteins tested (such as Polycomb), do not exhibit this specific interaction, nor does the HP1 chromo shadow domain [22•]. NMR studies indicate that the Lys9-methylated H3 tail binds in a groove of the HP1 chromo domain formed by conserved residues. A V26M mutation in *Drosophila* HP1 destabilizes the H3-binding interface severely [26•], resulting in a loss of HP1:H3-mLys9 tail interaction. This mutation was isolated originally as a *Su(var)*, demonstrating the link between the ability of HP1 to interact with H3-mLys9 and the ability to achieve silencing. Examination of *Suv39h* double-null primary mouse fibroblasts (using immunofluorescent staining to detect HP1 distribution) indicated that Suv39h-dependent H3 methylation activity is important for HP1 localization [22•]; a similar conclusion was derived by competition experiments with the H3-mLys9 peptide [21•]. Immunofluorescent staining of the *Drosophila* polytene chromosomes indicates that the majority of the H3-mLys9 is localized in pericentric heterochromatin [26•]. However, both the chromo domain and the hinge/chromo shadow domains are reported to localize *Drosophila* HP1 in heterochromatin (see Figure 3 and [27]), suggesting additional routes for assembly.

Heterochromatin and gene silencing in fission yeast

Very similar phenomena to those in *Drosophila* have been observed in the fission yeast *S. pombe*, where the centromeres, telomeres, ribosomal DNA repeats, and the silent mating-type region share many characteristics with heterochromatic regions in higher eukaryotes [28,29]. Marker genes placed either within or adjacent to these heterochromatic locations are subject to transcriptional repression in a metastable epigenetic manner. This results in variegated expression patterns similar to the PEV observed in *Drosophila*, apparently a consequence of heterochromatin protein complexes spreading to the reporter gene [30•,31•].

Figure 3

Structure and function of HP1. HP1 is a bifunctional protein with two conserved domains, the amino-terminal 'chromo' domain and the carboxy-terminal 'chromo shadow domain', separated by a 'hinge' of variable length. Whereas nuclear targeting activity has been localized to the carboxy-terminal quarter of the *Drosophila* protein, heterochromatin binding has been associated with both the chromo domain (amino-terminal half) and the chromo shadow domain (carboxy-terminal half). Mutations (from *Drosophila*) that result in suppression of PEV include point mutations in the chromo domain as well as the introduction of stop codons or other defects throughout the transcript. The chromo domain binds specifically to H3-mLys9; the chromo shadow domain interacts with a number of other chromosomal proteins, and might provide a binding site for H3 methyltransferase, either directly or indirectly. See [17] for citations to earlier work and citations given in the text for recent findings.

Centromeres in fission yeast are large complex structures (ranging in size from 38–100kb), bearing a close resemblance to the centromeres of higher eukaryotes. Electron microscopic analysis has revealed that the domain structure, including the organization pattern of centromere-associated proteins, is conserved from fission yeast to human [32]. A central core of unique sequences, surrounded by inner (*imr*) and outer (*otr*) repeats encompassing several kilobases of DNA, are assembled into compact heterochromatic structures. The mating-type region of the fission yeast encompasses a 20kb silent chromosomal domain containing the *mat2* and *mat3* loci, which are used as donors of genetic information to switch the *mat1* locus [28]. In addition to transcriptional silencing, the entire *mat2–mat3* interval exhibits suppression of recombination (see map at top of Figure 4). Interestingly, one third of the 11kb interval between the donor mating-type loci shares strong homology with centromeric repeats [33]. This centromeric homology region, referred to as *cenH*, has been implicated in assembly of the repressive higher-order structure that affects the entire domain [34–36]. In addition, several lines of evidence suggest that silencing of the *mat2* and *mat3* donor loci is also controlled by local DNA elements (REII and *mat3* silencer, respectively) [37–39]. These elements have characteristics of the *S. cerevisiae* silencers, and their effect seems to be localized to sequences around the *mat2* and *mat3* loci.

Figure 4

Distribution of Swi6 and distinct site-specific histone H3 methylation patterns marking the euchromatic and heterochromatic domains in the fission yeast mating-type region. A physical map of the mating-type region with *mat1*, *mat2* and *mat3* loci is shown. The IR-L and IR-R inverted repeats flanking the *mat2*–*mat3* interval are shown as red half arrows. The green box, *cenH*, represents sequences sharing homology to the centromeric repeats. Open boxes indicate the location of open reading frames; arrows indicate the direction of transcription. The graphs below represent results from high-resolution mapping of Swi6, H3-mLys9 or H3-mLys4 levels determined by ChIP experiments [41••]. H3-mLys9 and Swi6 are specifically enriched throughout the 20kb heterochromatic interval that displays transcriptional silencing and suppression of recombination. In contrast, H3-mLys4 is enriched in transcriptionally-poised euchromatic regions containing genes. IR-L and IR-R inverted repeats act as boundaries of the heterochromatin domain and prevent spreading of repressive chromatin complexes into neighboring areas.

Several *trans*-acting factors involved in assembly of heterochromatin in fission yeast have been identified (see Figure 5). Among these, Clr3 and Clr6 belong to a family of histone deacetylases, having strong homology to Rpd3 and Hda1 from *S. cerevisiae*, respectively [40]. Swi6 and Chp2 proteins both contain an amino-terminal chromo domain and a carboxy-terminal chromo shadow domain, and share structural and functional similarities with HP1 from *Drosophila* and mammals [29]. The chromo domain of Swi6 is both necessary and sufficient for its association with heterochromatic loci [18]. Swi6 is present throughout the 20kb silent mating-type interval, but its presence at centromeres is confined to outer centromeric repeats [30••,31••,41••]. The localization of Swi6 and Chp1 (another silencing protein that contains a chromo domain) to heterochromatic loci depends upon the Clr4 and Rik1 proteins [31••,42••]. Clr4, like its mammalian counterpart SUV39H1, contains an amino-terminal chromo domain and a carboxy-terminal SET domain, and possesses intrinsic histone methyltransferase (HMTase) activity [24••,43]. Moreover, it preferentially methylates Lys9 of histone H3, suggesting a possible role for histone methylation in heterochromatin assembly in *S. pombe* [42••]. Although the SET domain and surrounding cysteine rich regions of Clr4 are sufficient for its HMTase activity, both chromo and SET domains are required *in vivo* [42••]. Methylation of H3 Lys9 by the Clr4 enzyme is dependent upon another factor, Rik1, which contains eleven WD40 repeat-like domains [42••,44]. It has been hypothesized that Rik1

might form a complex with Clr4 to recruit its HMTase activity to heterochromatic loci.

Establishment and maintenance of heterochromatin domains

The factors that define specific chromosomal domains as preferred sites of heterochromatin assembly are not well understood. Heterochromatin formation might be linked to the presence of repeated DNA sequences rather than any specific DNA sequence identifying these loci. Tandem duplications of *P* elements have been found to induce heterochromatin formation in *Drosophila* [45]. Alternatively, aberrant RNA transcripts produced from repetitive DNA might be recognized by RNA-mediated interference processes and serve as a trigger, targeting chromatin modifiers to the corresponding genomic locations. Interestingly, the components of the RNA interference (RNAi) machinery, such as RNA-dependent RNA polymerase, Dicer and Argonaute, present in higher eukaryotes with complex genomes, are present in *S. pombe* and *Drosophila* ([46,47]; also see review by Hutvagner and Zamore, this issue, pp 225–232). However, these proteins have not been found in *S. cerevisiae*, nor have several other factors involved in the heterochromatin-mediated gene silencing discussed here, including Swi6 and Clr4. Consistent with a role for repeated sequences in heterochromatin formation, a deletion of the *cenH* repeat from the mating-type locus affects recruitment of *trans*-acting factors, such as Swi6, that are essential for heterochromatin

Figure 5

A stepwise model for epigenetic control of heterochromatin assembly in fission yeast. The sequential modifications of the amino-terminal tails of histone H3 by deacetylase and methyltransferase enzymes establish a histone code essential for heterochromatin assembly. Clr6/Clr3 deacetylases remove acetyl groups on lysines 9 and 14 of H3 before methylation of lysine 9 by the Clr4/Rik1 complex. The chromo domain of

Swi6 binds specifically to H3-mLys9 to continue heterochromatin assembly. Both Swi6 and Clr4 are required for spreading of the heterochromatin structure. The dimerization of Swi6 is believed to provide an interface for its interaction with other proteins, including histone-modifying enzymes, and might be a key to its role in cellular memory and inheritance of heterochromatic structures during cell division.

assembly [30^{••}]. Moreover, *cenH* sequences possess the ability to promote silencing at an ectopic site; this depends upon factors involved in heterochromatin formation [39].

The key molecular events leading to the establishment of heterochromatic structures in *S. pombe* have been addressed recently [42^{••}; Figure 5]. The covalent modifications of histone tails by deacetylase and methyltransferase activities are believed to act in concert to establish the 'histone code' essential for assembly of silenced chromatin. Chromatin immunoprecipitation (ChIP) experiments have shown that Clr4 is required for methylation of H3-Lys9 at the heterochromatic loci [42^{••}]. Moreover, Swi6 localization is dependent upon H3-Lys9 methylation. The deacetylation of H3 at Lys9 and Lys14 is apparently required upstream of methylation of H3-Lys9 [24^{••}, 42^{••}]. A mutation in the histone deacetylase Clr3 impaired H3-Lys9 methylation by Clr4 and heterochromatin association of Swi6 at the centromeres and the silent mating-type region. This interaction between deacetylases and methyltransferases is likely to be conserved, as the methyltransferase SU(VAR)3-9 and deacetylase HDAC1 in *Drosophila* associate *in vivo* and cooperate with each other to methylate pre-acetylated histones [48[•]]. The observation that a dominant-negative *HDAC1* mutant effectively represses a triplo-enhancer effect of *Su(var)3-9* on PEV supports the sequence of events suggested above [48[•]].

Although methylation of H3-Lys9 is required for heterochromatin association of Swi6, mutations in Swi6 have no effect on H3-Lys9 methylation [42^{••}], suggesting that Swi6 is dispensable for H3-Lys9 methylation and most likely acts downstream of Clr4 in *S. pombe*. Collectively, the observations described above define a temporal sequence of events leading to heterochromatin assembly that may be conserved from fission yeast to humans (see Figure 5). The deacetylation of the histone H3 tail precedes the methylation of H3-Lys9, which creates a binding site for recruitment of Swi6. As discussed above, HP1 specifically recognizes the H3-mLys9 'mark' through the conserved chromo domain [21^{••}, 22^{••}]. The dimerization of HP1 through its chromo shadow domain may contribute to formation of heterochromatic structures [18, 20[•], 49]. Interestingly, methylation of H3-Lys9 is not only important for initial recruitment of Swi6 to nucleation sites, but also seems to be required for its spreading into neighboring chromatin [41^{••}].

Genetic and biochemical experiments suggest that chromatin-based epigenetic imprints marking the mating-type region and centromeres contribute to stable inheritance of heterochromatic structures at these loci [28]. During replication, the histones originally present are distributed at random to the daughter chromatids, potentially maintaining the histone modification pattern in a 'diluted' state as new histones are incorporated. Swi6

remains stably associated with the silent mating-type region throughout the cell cycle [30^{••}]; moreover, Swi6 is found physically associated with the DNA replication protein Pol α in fission yeast [50,51], and HP1 is associated with the replication-coupled chromatin assembly factor 1 (CAF1) in mammals [52]. The bifunctional nature of Swi6/HP1 proteins, described above (Figure 3), might be the key to a role in recapitulating the specific chromatin configuration for both sister chromatids following DNA replication, thus clonally propagating the silent state. A self-perpetuation mechanism can be suggested, in which recruitment/maintenance of Swi6/HP1 at heterochromatin occurs through its interaction with H3–mLys9, while the recruited Swi6/HP1 stabilizes the localization of other factors, including histone deacetylases and histone methyltransferases, promoting maintenance of the heterochromatic state [21^{••},30^{••}]. A similar mechanism could be invoked to explain the spreading of heterochromatin complexes into neighboring domains (see Figure 1).

Boundaries of heterochromatin domains

Given a mechanism to perpetuate heterochromatin assembly, how is spreading limited? The existence of barriers has long been inferred from observation of the consequences of their removal, as seen in PEV in *Drosophila* (see Figure 1); the presence of interspersed euchromatic and heterochromatic domains observed along the fourth chromosome of *Drosophila* demands the presence of such barriers [53^{••}]. In their normal chromosomal contexts in *S. pombe*, a heterochromatic domain can be easily distinguished from a neighboring euchromatic domain on the basis of their distinct histone-modification patterns [41^{••}].

High-resolution mapping across 47kb including the silent mating-type region of fission yeast has revealed that H3–mLys9 and Swi6 are localized strictly to the 20kb heterochromatic interval ([41^{••}]; Figure 4). In contrast, H3 methylated at lysine 4, only a few amino acids away, is specific to the surrounding euchromatic regions. Importantly, two inverted repeat (IR) elements that flank the silenced domain define the borders between heterochromatin and euchromatin, as shown by a marked transition in histone methylation. Deletions of the inverted repeats lead to spreading of H3–mLys9 and Swi6 into adjacent euchromatic regions, concomitant with a decrease in H3–mLys4. Moreover, the complex of H3–mLys9 and Swi6 apparently prevents H3–Lys4 methylation in the silenced domain [41^{••}]. Therefore, differential methylation of histone H3 might serve as a marker for specific euchromatic and heterochromatic domains, separated by the IR barriers.

How such barriers protect against the encroachment of repressive chromatin complexes is currently under investigation (see review by Dhillon and Kamakaka, this issue, pp 188–192). A recent study of the chicken β -globin locus has suggested that the presence of sharp peaks of H3 Lys4 methylation and acetylation at barriers might prohibit the spread of silenced chromatin [54]. This mechanism is

unlikely to operate at the *S. pombe* mating type locus, however, as no such preferential enrichment of H3–mLys4 is observed at the IR elements (Figure 4). Instead, H3 Lys4 methylation is correlated tightly with transcriptionally-poised regions containing genes, outside the heterochromatin boundaries [41^{••}]. Insulators operating within euchromatic domains and heterochromatin barriers may well use distinct mechanisms to mark the borders between adjacent chromatin domains.

Heterochromatin and maintenance of genomic integrity

Heterochromatin formation may have originated simply as one of several modes of defense against parasitic DNA elements that can invade the genome (reviewed in [55]). However, both the pericentric heterochromatin, and the mechanism of silencing that evolved, appear to have seen specific utilization. The same mechanism of histone modification and HP1 association that appears critical for gene silencing in heterochromatin is also used to regulate a subset of euchromatic genes ([56]; see review by Kouzarides, this issue, pp 198–209). Formation of pericentric heterochromatin, or at least the proper function of many of its components, appears to be required to generate fully functional centromeres in higher eukaryotes. Higher rates of chromosome loss and aberrant mitotic figures are observed in the presence of mutations in heterochromatin components such as HP1 in *Drosophila* and Swi6 in *S. pombe* [57,58]; Suv39h-deficient mice display chromosomal instabilities and perturbed chromosome interactions during male meiosis [59[•]]. Recent studies [60^{••},61^{••}] suggest that the role of heterochromatin in chromosome segregation in *S. pombe* might be coupled to its involvement in preferential recruitment of cohesin, which is required for proper kinetochore assembly and to preserve the genomic integrity of the *mat* locus.

Conclusions and speculations

Certainly, the work of the past year has provided major insights into the structure and biochemistry of heterochromatin, at the same time generating the outlines for a mechanism of epigenetic inheritance and spreading. The latter depends on two basic premises, first the use of the histone modification code to dictate the pattern of associated non-histone chromosomal proteins, and second, the use of Swi6/HP1 as a bifunctional reagent, able to bind both the modified histone H3–mLys9, and to interact (either directly or indirectly) with the enzyme that produces that modification. Creating such a linkage between the modified histone and the capacity to modify the histone may be the basis of epigenetic inheritance (see also [62]). Whether the specific pattern of histone modification and/or the presence of the HP1 complex dictates the uniform nucleosome array observed in *Drosophila* heterochromatin is unknown. The ability of HP1 to form homodimers may play an important role in compacting the nucleosome array. In addition to the specific binding to the Lys9 methylated H3 tail, it has been reported that mammalian HP1 can

interact with the H3 histone fold, again through the chromo domain, whose integrity is required [63]; such an interaction might also contribute to condensation.

The genetic analysis in *S. pombe* of the chromosomal proteins required to establish silencing and maintain genomic integrity has provided numerous insights into the relationships between the structural proteins required for heterochromatin formation and the key modifying enzymes; however, it has also identified other components whose role is as yet unknown, as has the identification of *Su(var)* and *E(var)* mutations in *Drosophila*. Thus, although the outlines of the model, based on interactions of Swi6/HP1, H3-mLys9, and Ctr4/Su(var)3-9 appear clear, there is no doubt that much remains to be discovered. Application of ChIP to map additional heterochromatin domains, both in *S. pombe* and in *Drosophila*, should identify additional barriers at the heterochromatin/euchromatin junctures, further elucidating the properties of these elements. Most important, one can anticipate growing understanding of the RNAi system, which may provide critical insights into how heterochromatin packaging is targeted, and into the evolutionary processes that led to the accumulation of heterochromatin in our genomes.

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