Cell. Mol. Life Sci.
DOI 10.1007/s00018-014-1725-x

Cellular and Molecular Life Sciences

REVIEW

Chromosome boundary elements and regulation of heterochromatin spreading

Jiyong Wang · Stephanie T. Lawry · Allison L. Cohen · Songtao Jia

Received: 30 April 2014 / Revised: 27 August 2014 / Accepted: 29 August 2014

Abstract Chromatin is generally classified as euchromatin or heterochromatin, each with distinct histone modifications, compaction levels, and gene expression patterns. Although the proper formation of heterochromatin is essential for maintaining genome integrity and regulating gene expression, heterochromatin can also spread into neighboring regions in a sequence-independent manner, leading to the inactivation of genes. Because the distance of heterochromatin spreading is stochastic, the formation of boundaries, which block the spreading of heterochromatin, is critical for maintaining stable gene expression patterns. Here we review the current understanding of the mechanisms underlying heterochromatin spreading and boundary formation.

Keywords Boundary element · Heterochromatin · Spreading · Histone modifications · Silencing

Introduction

Eukaryotic genomic DNA is folded with histones and other proteins in the form of chromatin, which regulates every aspect of DNA metabolism, including transcription, replication, and DNA damage repair. Based on the level of compaction, chromatin is divided into euchromatin and heterochromatin. Euchromatin is generally gene rich, less condensed, and associated with active gene transcription, whereas heterochromatin is generally gene poor, highly condensed, and refractory to the transcription machinery. The discovery of position effect variegation (PEV) in the fruit fly *Drosophila melanogaster* in the 1930s paved the way to revealing the importance of chromatin in regulating gene expression [1]. The *white* gene, which is responsible for generating red color pigment in *Drosophila* eyes, normally resides in the euchromatic region. However, when the *white* gene is placed adjacent to pericentric heterochromatin due to chromosomal rearrangement, it is variably silenced and the different expression states are clonally inherited in different cell populations, resulting in mottled eyes [2] (Fig. 1). A similar phenomenon termed telomere position effect (TPE) was later observed in the budding yeast *Saccharomyces cerevisiae*, in which reporter genes placed near telomeres are also variably silenced and clonally inherited, resulting in sectored colonies [3, 4]. A general theme emerging from studies of these phenomena is that heterochromatin can spread variable distances into neighboring regions in a stochastic manner to regulate gene expression and that once established these expression states can be stably maintained through multiple cycles of cell divisions.

The effect of heterochromatin on gene expression is not limited to reporter genes. For example, the similarities between transposon-mediated gene silencing in maize and PEV in *Drosophila* led Barbara McClintock to propose that transposable elements regulate the expression of neighboring genes [5]. Recent studies in *Arabidopsis* show that indeed transposable elements are sites of heterochromatin assembly and influence the expression of nearby genes [6]. In humans, heterochromatin domains expand to cover developmentally regulated genes during the differentiation of stem cells, resulting in large changes in the chromatin landscape [7]. But the variable nature of heterochromatin spreading can potentially lead to inappropriate expression of genes, which has been implicated in a number of serious

J. Wang · S. T. Lawry · A. L. Cohen · S. Jia (✉)
Department of Biological Sciences, Columbia University, New York, NY, USA
e-mail: jia@biology.columbia.edu

Published online: 07 September 2014

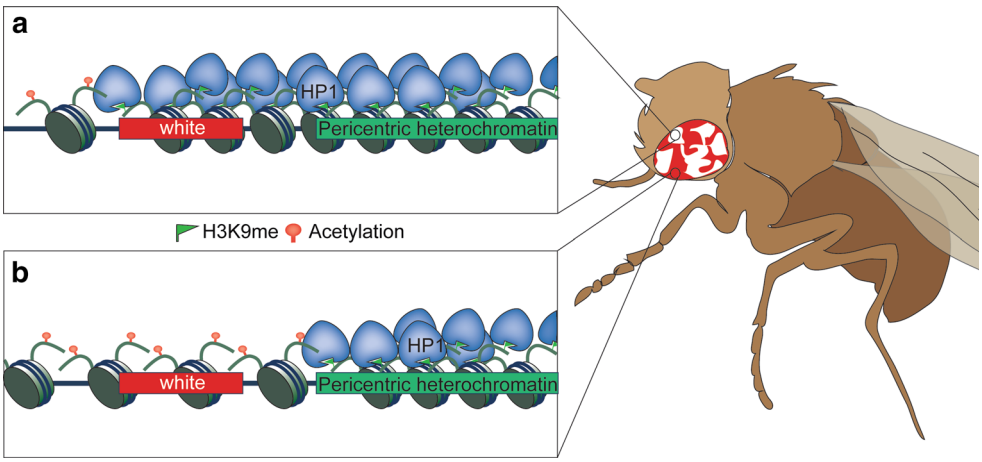

Fig. 1 Position effect variegation in *Drosophila*. The normally euchromatic *white* gene is placed close to the pericentric heterochromatin due to X-ray induced chromosome inversion. During early *Drosophila* development, heterochromatin spreading in some progenitor cells results in the silencing of *white*. Such expression is clonally inherited in all the progenies of the same cell, resulting in white patches of the adult eye

human diseases [8]. For example, facioscapulohumeral dystrophy (FSHD) is a neuromuscular disorder predominantly affecting the skeletal muscles of the face and arms and has been correlated with deletions of D4Z4 repeats in the chromosome 4q35 subtelomeric region. Although the mechanism of this disease is still under debate, one hypothesis is that the loss of these repeats compromises heterochromatin spreading-mediated inactivation of adjacent genes [8, 9]. Therefore, the spreading of heterochromatin needs to be tightly controlled, and the discovery of boundary elements that can shield genes from position effects demonstrates their important roles in regulating gene expression. Given that the mechanisms of heterochromatin spreading and boundary formation are best studied in yeasts, in which precise genetic manipulations can be made, we will focus our review on studies conducted in yeasts and discuss their relevance to mechanisms in higher eukaryotes.

Heterochromatin spreading

It is well established that chromatin structure is regulated by both chromatin remodeling activities and the modification of histones and DNA [10]. Since many factors involved in PEV and TPE have been characterized as enzymes and proteins that regulate chromatin structure, heterochromatin assembly and spreading has been a paradigm for studying the roles of chromatin modifiers in regulating stably maintained chromatin states [2, 4]. The histones within heterochromatin regions are generally devoid of acetylation and are often methylated at H3 lysine 9 (H3K9me) [11–13]. While histone deacetylation can directly affect interactions between nucleosomes to form higher-order chromatin structures [14], histone methylation indirectly affects chromatin structure by either antagonizing acetylation at the same residue [15] or serving as a binding site for the recruitment of chromatin proteins [16]. H3K9me recruits heterochromatin protein 1 (HP1) [12, 17, 18], which acts as both a structural component and an adaptor for the recruitment of chromatin-modifying factors [19]. In addition to histone methylation, the DNA within heterochromatin regions is highly methylated in many higher eukaryotes such as mammals and plants. Although DNA methylation also contributes to heterochromatin functions, the mechanisms by which it enables gene repression are less well-understood [20].

Heterochromatin assembly can be divided into three distinct steps: establishment, spreading, and maintenance [21, 22]. Heterochromatin is established at nucleation centers through the targeting of histone-modifying activities by transcription factors or non-coding RNAs. Subsequently, heterochromatin spreads into neighboring regions, mostly via a network of interactions among chromatin proteins, resulting in the formation of large heterochromatin domains independent of the underlying DNA sequences. While the mechanisms of heterochromatin establishment and maintenance have been extensively studied, those that regulate heterochromatin spreading are less well understood. One of the most attractive models is that heterochromatin spreads by "oozing", in which repeated cycles of histone modifications and the binding of chromatin proteins result in an "inch worm"-like spreading of heterochromatin from the nucleation center until heterochromatin-associated proteins coat the extended domain [23]. Once these domains are formed, they are maintained through interactions among chromatin proteins similar to those involved in heterochromatin spreading [24].

Heterochromatin assembly and spreading in budding yeast

In budding yeast, heterochromatin is formed at telomeres and the silent mating type locus, mediated by the Sir (silent information regulator) protein complex, composed of Sir2,

Sir3, and Sir4 [22, 25]. Sir2 is a histone deacetylase with main activity on H4K16 [26–28], the acetylation of which directly regulates higher-order chromatin folding in vitro [14] and plays a major role in heterochromatin function in vivo [13, 29]. Sir3 and Sir4 preferentially interact with histone tails devoid of H4K16ac [30–32]. At telomere regions, telomere DNA-binding protein Rap1 and the DNA end-binding complex Ku70/Ku80 recruit the Sir complex [33–36]. At the silent mating type locus, Rap1, Abf1, and the origin recognition complex (ORC) recruit the Sir complex to nucleation sites termed silencers [36–41]. In either case, Sir2 subsequently deacetylates histone H4K16, allowing Sir3 and Sir4 to bind. Sir3 oligomerizes and recruits more Sir2 to deacetylate H4K16 in the adjacent nucleosomes and thus facilitates the spread of the entire Sir complex [22, 25]. The main evidence supporting such a model include that Sir proteins cover the entire heterochromatin domain and that silencing spreads continuously through the domain [42–44] (Fig. 2). A distinct silencing mechanism operates at repressive rDNA loci, which is dependent on Sir2, but not Sir3 or Sir4, and spreads in a unidirectional manner controlled by Pol I transcription [45–48].

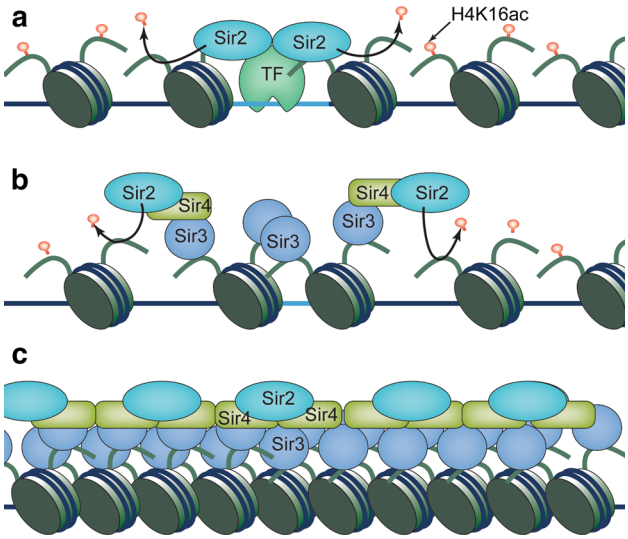

Fig. 2 The stepwise assembly of heterochromatin in budding yeast. **a** Heterochromatin establishment is achieved by targeting of the Sir protein complex to telomeres or silencers at the silent mating type locus through DNA-binding proteins where Sir2 deacetylates H4K16. **b** Deacetylated histones increase the affinity of Sir3 and Sir4 for chromatin and recruit additional Sir complex. Sir2 then deacetylates adjacent nucleosomes to allow heterochromatin spreading. **c** The formation of an extend heterochromatin domain that is covered by Sir complex

Heterochromatin assembly and spreading in fission yeast

In fission yeast, heterochromatin forms at repetitive DNA elements in the centromeres, telomeres, and the silent mating type region [19]. Histones at these regions are not only hypoacetylated by a number of histone deacetylases (HDACs), but are also methylated on H3 Lys 9 (H3K9me) by the histone methyltransferase Clr4 [12, 49]. Similar to budding yeast, these histone-modifying enzymes can be targeted to DNA repeats by sequence-specific DNA-binding proteins to establish heterochromatin [50–53]. Interestingly, the RNA interference (RNAi) pathway is also required for heterochromatin establishment at repeat regions [54]. The DNA repeats are transcribed by RNA polymerase II during the S phase of the cell cycle [55–58]. These transcripts are converted to double-stranded RNAs by the RNA-dependent RNA polymerase complex and then processed by Dicer into small interfering RNAs (siRNAs) [59, 60]. These siRNAs are loaded into the Argonaut protein (Ago1) in the RITS (RNA-induced transcriptional gene silencing) complex, which is targeted to repeat regions through base pairing between siRNAs and nascent transcripts [61–63]. RITS directly associates with the Clr4 complex to initiate H3K9me [64], which further recruits HP1 proteins such as Swi6 and Chp2 [12, 65].

The spreading of heterochromatin from initiation sites requires Swi6, and in its absence H3K9me is restricted to heterochromatin nucleation centers [52, 66]. Because mammalian and fly HP1 interacts with histone H3K9 methyltransferases [67, 68], it was proposed that a similar interaction between Swi6 and Clr4 could result in the recruitment of additional HMTases, which in turn would modify histones of adjacent nucleosomes [66]. In addition, Clr4 contains a chromodomain that recognizes H3K9me, an interaction that could lead to heterochromatin spreading through repeated binding of H3K9me and methylation of the adjacent nucleosomes [69]. Elegant biochemical analyses demonstrate that Clr4 preferentially binds to dimethylated H3K9 while Swi6 prefers trimethylated H3K9, avoiding the potential competition between Clr4 and Swi6 and allowing efficient spreading of heterochromatin [70]. Again, the "inch worm" spreading model is supported by the fact that Swi6 and Clr4 are localized continuously throughout entire heterochromatin domains [69, 71] (Fig. 3).

In addition to the self-propagation cycles of H3K9me, heterochromatin spreading in fission yeast also requires complex crosstalk among many chromatin proteins. Like budding yeast, fission yeast also possesses Sir2, which is required for heterochromatin spreading, although functional homologues of Sir3 and Sir4 are absent [72–74]. It is possible that Sir2-mediated deacetylation of H4K16 regulates chromatin compaction, thus bringing Clr4 closer to adjacent nucleosomes [74]. Moreover, Swi6 associates

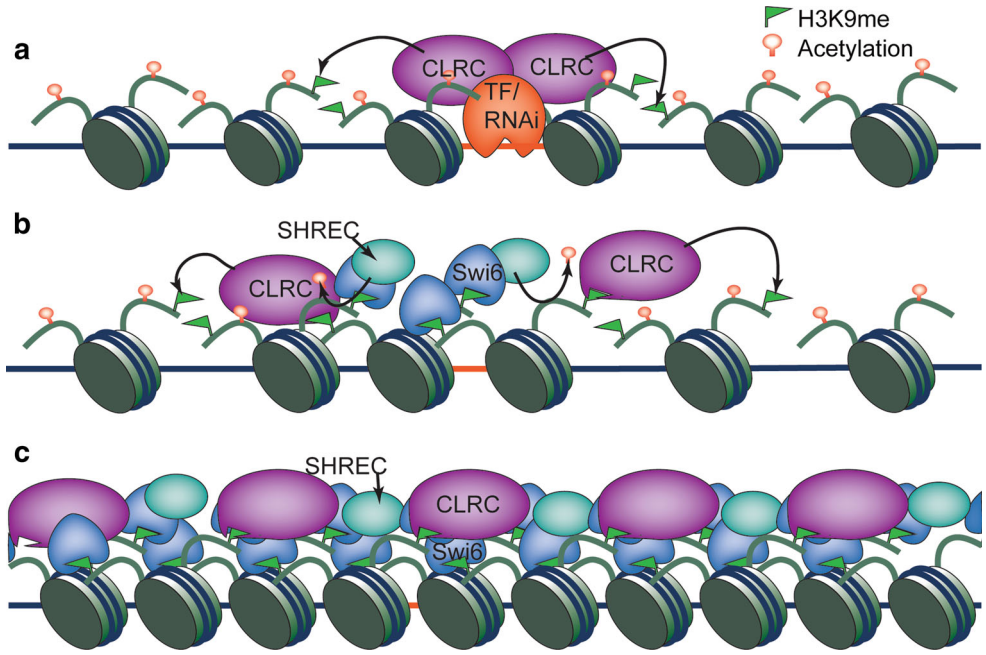

Fig. 3 The establishment and spreading of heterochromatin in fission yeast. **a** Heterochromatin establishment is achieved by sequence-specific DNA-binding proteins or RNAi-mediated targeting of histone methyltransferase CLRC to repetitive DNA elements, leading to local H3K9 methylation. **b** H3K9me recruits Swi6, which might facilitate the recruitment of additional CLRC. The chromodomain of Clr4 also recognizes H3K9me and facilitates CLRC recruitment. CLRC then methylates adjacent nucleosomes, leading to heterochromatin spreading. SHREC associates with Swi6 and deacetylate histones to promote heterochromatin spreading. **c** The formation of an extended heterochromatin domain that is covered by Swi6, CLRC and SHREC

with the histone deacetylase complex SHREC to deacetylate histone H3K14 and remodel chromatin to promote the spreading of H3K9me [75–78]. Meanwhile, structural and kinetic studies reveal that Swi6 undergoes a conformational change to a spreading competent state when it binds to methylated H3K9 [79].

Additional models for heterochromatin spreading have also been proposed based on studies in fission yeast. For example, spreading can be accomplished by direct or indirect coupling of CLRC to RNA polymerase II, allowing H3K9me in the wake of transcription [23]. In addition, the association between the CLRC and DNA polymerase ε suggests that heterochromatin spreads by associating with the leading strand DNA polymerase following RNAi-mediated release of Pol II that restarts stalled replication forks [80, 81].

Heterochromatin assembly and spreading in higher eukaryotes

Heterochromatin spreading in higher eukaryotes is less well defined, mostly due to the highly repetitive nature of the DNA sequences that form heterochromatin prevent precise genetic manipulations. The interactions between HP1 and H3K9 methyltransferase of the SUV39 family and the chromodomain of SUV39 are conserved, so it is possible that the inch worm spreading model functions in other systems as well [11, 68, 82]. In contrast, more is known about Polycomb protein-mediated gene silencing, which shares some similarities with heterochromatin formation and spreading and thus is often termed facultative heterochromatin.

Polycomb-silenced regions are usually characterized by the trimethylation of histone H3 lysine 27 (H3K27me3) [83]. The highly conserved Polycomb Repressive Complex 2 (PRC2) contains the SET domain-containing protein EZH2 (EZ in *Drosophila*) as the catalytic subunit responsible for H3K27me3 [84–87]. PRC2 also contains the histone-binding proteins RbAp46/48, the DNA-binding protein SUZ12, and EED (ESC in *Drosophila*). In *Drosophila*, PRC2 is recruited to Polycomb response elements (PREs) by sequence-specific DNA-binding proteins. In mammals, the binding sequence is less well defined and long non-coding RNAs play important roles in targeting PRC2 to specific sites [83]. The mechanism by which H3K27me3 regulates gene expression is not well understood. H3K27me3 recruits chromodomain protein Polycomb, which is part of the Polycomb Repressive Complex 1 (PRC1) [88, 89]. PRC1 also contains an E3 ubiquitin ligase that ubiquitylates K119 of H2A, which also contributes to gene silencing [90].

Importantly, PRC2 binds to H3K27me3 via the WD40 repeats of EED and stimulates methylation of nearby histone H3 on Lys 27 [91, 92], indicating that PRC2-mediated H3K27 methylation is propagated in a manner similar to that of HP1-H3K9 methylation. However, high-resolution mapping of H3K27me3 and Polycomb proteins showed that while H3K27me3 marks large chromosome domains, PRC2 is mainly concentrated at the PREs [93]. Thus it is unlikely that an inch worm spreading model applies. The exact mechanism of H3K27me3 spreading is still unknown, but it has been suggested that spreading is achieved by local diffusion of PRC2 or by the formation of chromosome loops [23, 94].

Mechanisms of boundary formation

When heterochromatin spreads into surrounding regions independently of DNA sequences, it can affect the expression of nearby genes to varying degrees depending on the extent of spreading. In certain cases, such variation of gene expression could allow for the development of new traits that help organisms adapt to new environments, facilitating evolution. However, in most cases, the disruption of normal gene expression patterns severely compromises an organism's fitness or health, as seen in a number of human diseases linked to uncontrolled heterochromatin spreading [8]. Additionally, studies in *Neurospora* show that disrupting heterochromatin boundary formation leads to growth defects linked to the unchecked spreading of silenced chromatin and DNA methylation into genes outside of the normal regions, further highlighting the importance of properly restraining heterochromatin spreading for cellular fitness [95]. Thus it is essential for spreading to be tightly regulated in order to maintain stable gene expression profiles. Generally, heterochromatin regions are flanked by DNA sequences termed boundary elements, which form fixed borders accompanied by sharp transitions in histone modification profiles. Such elements result in the precise determination of epigenetic states among closely arranged chromosome loci, even when heterochromatin protein levels change. In other cases, borders are determined by the local balance of heterochromatin and euchromatin proteins, which tends to differ between cells. Such boundaries are termed negotiable borders [96].

Negotiable borders

A distinguishing feature of negotiable borders is that they are not established at a specific DNA sequence, but at a transition region defined by the balance of different proteins and histone modifications associated with heterochromatin and euchromatin [96]. For example, in budding yeast, the balance between histone acetyltransferase Sas2-mediated acetylation of H4K16 and Sir2-mediated deacetylation of the same residue defines the borders of heterochromatin at telomeric regions [29, 97]. Either loss of *sas2*$^{+}$ or overexpression of Sir3 leads to increased heterochromatin spreading [42, 43]. In addition, loss of other histone modifications or proteins usually enriched in euchromatin may also result in increased heterochromatin spreading. For example, loss of the euchromatin-associated bromodomain protein Bdf1 or histone variant H2A.Z results in expanded heterochromatin regions [98, 99]. As a result of such competition, negotiable borders are associated with frequent changes of epigenetic states [100]. The classical example of PEV in *Drosophila*, where *white* is silenced in a portion of progenitor cells during early development, also suggests that heterochromatin spreads over varying distances rather than being constrained to a defined location. In addition, many of the factors identified as regulators of PEV affect heterochromatin spreading in a dosage-dependent manner [101], which also points to the importance of maintaining proper heterochromatin–euchromatin protein balance as a determinant of the distance of heterochromatin spreading.

Thus one important way to regulate heterochromatin spreading is by controlling the availability of heterochromatin proteins. Indeed, heterochromatin protein levels appear limiting in diverse organisms. For example, in fission yeast, ectopic heterochromatin assembly through artificial targeting of Clr4 to DNA or exogenously introduced siRNAs can only succeed when Swi6 is overexpressed or endogenous heterochromatin structures are compromised to release silencing proteins [53, 102, 103]. Moreover, overexpression of Swi6 increases the conversion rate of a less stable heterochromatin domain at the mating type region [104] and allows the cells to bypass the requirement of RNAi for pericentric heterochromatin assembly [53].

On the other hand, endogenous heterochromatin regions with negotiable borders make them ideal as "sinks" to limit the availability of heterochromatin proteins in both budding and fission yeast [53, 105–108]. Increases in heterochromatin proteins predominately localize to telomeres, leading to expansion of telomeric heterochromatin domains. In contrast, compromising heterochromatin assembly at telomeres or rDNA results in the release of heterochromatin proteins and increases the incidence of ectopic heterochromatin assembly.

Fixed borders

In most cases, specific DNA elements demarcate the borders of heterochromatin regions and function as boundaries

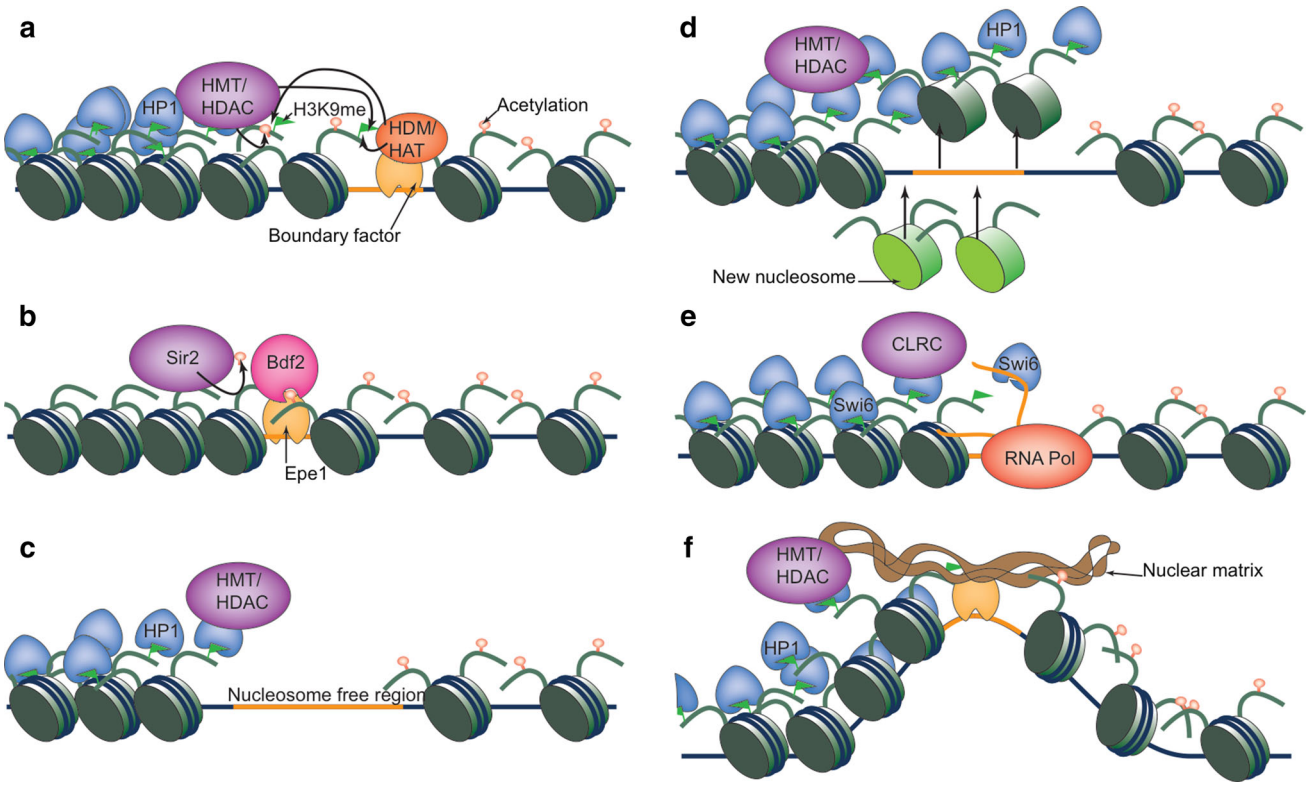

Fig. 4 Mechanism of boundary function. **a** Boundary elements recruit histone-modifying activities. **b** Boundary elements recruit proteins that protect euchromatic modifications. **c** Nucleosome-free regions prevent the spreading of heterochromatin modifications to establish heterochromatin boundaries. **d** High rate of histone turnover prevents the spreading of histone modifications. **e** RNA-mediated eviction of heterochromatin protein Swi6 to prevent heterochromatin spreading. **f** Boundary elements cluster and associate with nuclear structures to form chromatin loops

to prevent spreading of heterochromatin. These boundaries precisely define chromatic regions, resulting in consistent inheritance of epigenetic states, regardless of varying heterochromatin protein levels. A general theme is that these mechanisms all converge on disrupting heterochromatin-associated histone modification cycles (Fig. 4).

Recruitment of histone-modifying activities to directly antagonize heterochromatic histone modifications

Since heterochromatin spreading depends on repeated cycles of histone modifications, installation of incompatible histone modifications at the boundary regions can effectively block heterochromatin spreading (Fig. 4a). Indeed, in budding yeast, the boundary element at the silent mating-type locus recruits histone-modifying activities associated with euchromatic regions, and artificial tethering of Sas2 is sufficient to establish a heterochromatin boundary [109–111]. In fission yeast, the pericentric heterochromatin boundary recruits a histone demethylase complex containing Lsd1 [112]. Lsd1 was originally identified in humans as a demethylase specific for H3K4 [113], but also demethylates H3K9 in specific contexts [114]. The fission yeast Lsd1 complex localizes at the pericentric boundary regions and demethylates H3K9me to prevent heterochromatin spreading [112]. The chicken β-globin gene cluster is adjacent to a ~16 kb condensed heterochromatin region and the 5′ DNase I hypersensitive site HS4 between these two regions also has barrier activity. Transcription factors USF1 and USF2 bind to this element and recruit histone-modifying enzymes such as H3K4-specific histone methyltransferase Set1, and histone H3 acetyltransferase PCAF to block heterochromatin from spreading into the β-globin locus [115].

Protection of preexisting histone modification profiles

In addition to recruiting histone-modifying enzymes to actively counteract heterochromatin-associated histone modifications, protecting existing euchromatic modifications is also critical for establishing a heterochromatin boundary (Fig. 4b). In budding yeast, the bromodomain protein Bdf1, which protects histone H4 tail acetylation, is required for preventing heterochromatin spreading at telomeres to establish negotiable borders [98]. Another budding yeast bromodomain protein Yta7 is involved in

restricting heterochromatin spreading to the silent mating type locus boundary [116, 117]. Importantly, mutations in the bromodomain lead to heterochromatin spreading outside its boundaries [118], although the acetylation events that mediate the binding of Yta7 have not been identified. In fission yeast, a double bromodomain protein Bdf2 is specifically recruited to a repeat sequence termed *IRC* that marks the border of pericentric heterochromatin [74]. Bdf2 is recruited to *IRC* by a JmjC domain-containing protein Epe1, which is highly enriched at the boundary region [74, 119, 120]. Bdf2 protects acetylated H4K16, which is essential for counteracting Sir2-mediated deacetylation to block heterochromatin spreading [74].

Nucleosome-free regions

Since heterochromatin spreading depends on cycles of histone modifications of adjacent nucleosomes, it is reasonable to expect that nucleosome-excluding sequences can function as boundaries due to the separation of substrate from histone-modifying enzymes, thus blocking the spreading of heterochromatin (Fig. 4c). Both the UAS sequence and LexA binding sites, which recruit transcription factors and exclude the formation of nucleosomes, have been shown to block the spreading of heterochromatin in budding yeast [121, 122]. Most importantly, certain DNA sequences that are known to exclude nucleosome assembly can also efficiently establish heterochromatin boundaries [121].

Regulating histone turn over rates

Heterochromatin regions are generally associated with slow turn over of histones [123, 124], which allow stable interaction between H3K9me and HP1/SUV39 to promote heterochromatin spreading. Therefore, increasing the histone turnover rate can effectively form boundary by breaking the histone modifications cycle required for heterochromatin spreading (Fig. 4d). In budding yeast, the boundary regions are indeed associated with high histone turnover rate [125]. In Drosophila, the GAGA factor directs histone H3.3 replacement that prevents heterochromatin spreading [126] and boundaries of cis-regulatory domains and GAGA binding sites are generally associated with high turn over rate of histones [127, 128].

Transcription

Transcription plays two separate roles in regulating nucleosome dynamics [129], which might contribute to boundary function. First, the transcription machinery is associated with diverse histone-modifying activities, some of which can counteract the histone modifications of heterochromatin regions. In addition, transcription increases the rate of histone turnover, which can limit the histone modification cycles required for heterochromatin spreading.

Transcription by RNA Polymerase III, presumably through the tRNA genes it transcribes, is particularly relevant to boundary function. In budding yeast, tRNA genes are required for boundary function at the silent mating type and the rDNA locus [109, 111, 130, 131], and in fission yeast, tRNA genes found at pericentric heterochromatin borders are also critical for limiting heterochromatin spreading [132, 133]. In mammals, tRNA genes also function as boundary elements, indicating an evolutionarily conserved role for tRNA genes in preventing heterochromatin encroachment [134, 135]. Mutation of the RNA pol III machinery in budding yeast, including general transcription factors TFIIIA, TFIIIC, and Pol III itself all resulted in defective boundary function, suggesting that Pol III transcription is essential for proper boundary function [109]. However, tRNA genes may play addition roles in boundary function independent of Pol III transcription. For example, at the silent mating type locus in fission yeast, the boundary region inverted repeat (*IR*) contains B-box sequences that recruit TFIIIC, but no Pol III was detected at this locus. TFIIIC mediates the clustering of chromosome loci at the nuclear periphery, which might contribute to boundary function through the formation of chromosome loops [136]. Similarly, in budding yeast, TFIIIC can also function as a boundary element independent of Pol III transcription [137].

In mammals, short interspersed nuclear elements (SINEs) also act as boundary elements by regulating transcription [138]. The murine growth hormone (*GH*) gene is regulated by the nearby SINE B2 repeat, which is transcribed by both Pol II and Pol III, though in opposite directions. During early stages of embryonic development, adjacent heterochromatin spreads past the B2 element to silence *GH* expression. In later stages of development, heterochromatin spreading is blocked by B2 element, allowing *GH* expression. Mutations of the promoters compromised boundary function, suggesting that transcription is critical for B2 boundary activity [138]. Similarly, the mouse SINE B1-X35S also has boundary activity, which is dependent on the transcription of this sequence [139].

Although the process of transcription seems to play an important role in boundary function, the transcripts themselves might also directly participate (Fig. 4e). For example, RNA directly competes with H3K9me for binding to the chromodomain of Swi6 [140]. Thus, the RNA transcripts at boundary regions may directly affect heterochromatin-mediated histone modification amplification.

Consistent with such an idea, the pericentric *IRC* boundary of fission yeast is transcribed and mutations of the Swi6 RNA-binding residues result in heterochromatin spreading [141].

Nuclear structures

Another mechanism by which heterochromatin spreading can be blocked is through the spatial organization of chromatin. Physically separated chromatin domains can be achieved by the clustering of boundary elements or by interactions between boundary elements and nuclear structures (Fig. 4f). For example, the gypsy insulator complex in *Drosophila*, which was found to protect transgenes from position effects, localizes to only 20–25 sites in the nucleus despite having over 500 binding sites [142, 143]. Similarly, in fission yeast, the TFIIIC complex binding sites form clusters in the nucleus [136]. Given that the TFIIIC binding sites at the silent mating type region are critical for boundary function without local recruitment of Pol III, TFIIIC-mediated clustering may establish heterochromatin boundaries by separating chromatin domains [136]. In mammals, genome-wide analyses revealed that CTCF (CCCTC binding Factor) binding sites frequently flanked chromosome domains containing the repressive H3K27me3, often in a cell-specific manner, indicating that CTCF may regulate the spreading of facultative heterochromatin domains [144, 145]. Although CTCF is mainly known for its function as an enhancer blocker by regulating the 3D organization of the genome to control interactions between distant loci, it may perform similarly to block heterochromatin spreading [146–153]. CTCF also associates with cohesins, which have been shown to affect chromosomal architecture and organization [154, 155].

Clusters of boundary elements are often found near the nuclear periphery, suggesting that they may be tethered to the nuclear membrane. Nuclear pore proteins have been implicated in tethering DNA and may play a role in boundary activity. In an elegant "boundary trap" genetic screen, Ishii et al. screened a chimeric protein library for proteins that showed boundary activity when fused to a DNA-binding protein. One of the proteins identified, Cse1, was found to localize to the nuclear periphery, but only in the presence of the nuclear pore protein Nup2 [156]. It would be interesting to identify other nuclear membrane or nuclear matrix components that regulate the clustering of other boundary elements.

Conclusions and future directions

Since the major mechanism of heterochromatin spreading is through repeated cycles of histone modifications and binding of chromatin proteins, it is not surprising that most boundary elements function by blocking this cycle by, for example, recruiting antagonizing histone-modifying activities, protecting euchromatic modifications, creating nucleosome-free regions, altering chromatin dynamics through transcription, and tethering DNA to nuclear structures to form chromatin loops. Although each mechanism seems to be sufficient, multiple mechanisms function at each boundary. For example, the well-studied tRNA gene boundary incorporates recruitment of histone-modifying enzymes, generation of nucleosome-free regions, transcription, and TFIIIC-mediated chromatin clustering. Such redundancy might function at other heterochromatin boundary regions to ensure the efficient blocking of heterochromatin spreading.

Although the chromatin modification cycle is an attractive model to explain heterochromatin spreading, there are exceptions that suggest addition mechanisms [23]. For example, in *Drosophila*, H3K27me3 domains are much broader than that of PRC2 and PRC1 [93] and in budding yeast, rDNA silencing requires Sir2, but not Sir3 or Sir4 [45–47]. Moreover, in some cases, heterochromatin spreading is not continuous. For example, at native budding yeast telomeres, the spreading of heterochromatin skips reporter genes flanked by boundary elements [157, 158]. Therefore, a better understanding of the mechanism of heterochromatin spreading will provide further insights into how boundaries are formed.

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