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Review

New Insights into the Regulation of Heterochromatin

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All living organisms are constantly exposed to stresses from internal biological processes and surrounding environments, which induce many adaptive changes in cellular physiology and gene expression programs. Unexpectedly, constitutive heterochromatin, which is generally associated with the stable maintenance of gene silencing, is also dynamically regulated in response to stimuli. In this review we discuss the mechanism of constitutive heterochromatin assembly, its dynamic nature, and its responses to environmental changes.

Constitutive Heterochromatin Is Traditionally Viewed as a Static Chromatin Structure

In eukaryotes, genomic DNA wraps around histones to form chromatin. According to its compaction levels, chromatin is classified into two categories: gene-rich, less condensed euchromatin and gene-poor, highly condensed heterochromatin. Among heterochromatin regions, facultative heterochromatin often forms at developmentally regulated genes, and its level of compaction changes in response to developmental cues and/or environmental signals [1]. By contrast, constitutive heterochromatin preferentially assembles at repetitive elements such as satellite DNA and transposons, and maintains high compaction levels [2]. The stability of constitutive heterochromatin is exemplified by the classical position-effect variegation (PEV) in *Drosophila*, in which the *white* gene is variably silenced when a chromosome translocation event places it adjacent to pericentric heterochromatin [3]. Remarkably, once a founder cell establishes heterochromatin at the *white* gene during the early stages of development, silencing is maintained in all of the derivative cells through adulthood, resulting in patches of cells without pigmentation in the adult eye [4]. Such stability is essential for repressing recombination between repeat elements and for limiting the transcription of active transposons to maintain genome integrity. However, recent studies show that constitutive heterochromatin is also dynamically regulated and responsive to stimuli. While these changes could potentially help organisms to adapt to new environments, in some cases they also could cause human diseases. In the following sections we highlight a few examples of the dynamic regulation of constitutive heterochromatin domains.

Mechanism of Heterochromatin Assembly

Constitutive heterochromatin harbors distinct chromatin-modification profiles. For example, in constitutive heterochromatic regions histones are generally hypo-acetylated and hyper-methylated at histone H3 lysine 9 (H3K9me) [5–7]. The formation of these heterochromatin domains requires the concerted actions of chromatin-modifying enzymes and comprises three steps: initiation, spreading, and maintenance [8] (Figure 1). Heterochromatin is initiated at nucleation centers by sequence-specific DNA-binding proteins or non-coding RNAs, both of which recruit histone deacetylases (HDACs) and the SUV39 family histone H3K9 methyltransferases (HMTs), resulting in hypo-acetylation of histones and hyper-methylation of H3K9 at the nucleation sites [9–12]. These methyltransferases often contain a chromodomain that binds to existing H3K9me, forming a self-propagating mechanism to methylate adjacent nucleosomes

Trends

Constitutive heterochromatin has long been considered to be a highly stable state. However, recent studies show that it is highly dynamic in nature.

HP1 proteins are in a dynamic equilibrium between chromatin-bound and free forms.

Transcription is essential for RNAi-mediated heterochromatin assembly by providing the source of small RNAs and the scaffold for the recruitment of chromatin-modifying enzymes.

Heterochromatin changes take place during the normal aging process and respond to environmental stresses.

Promiscuous heterochromatin formation is a novel way to adapt to external stresses and permit transgenerational inheritance.

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Establishment

Spreading
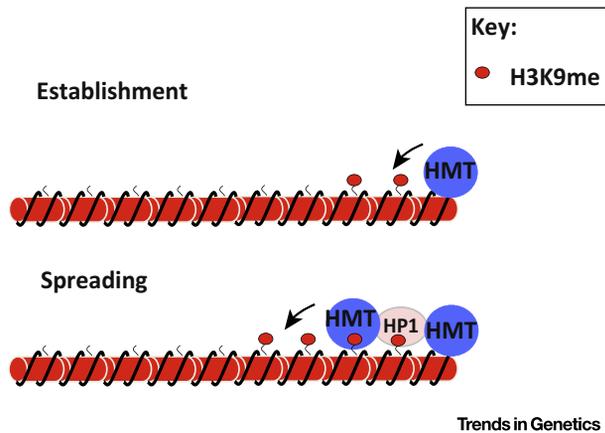
Key:

● H3K9me

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Figure 1. A Stepwise Model of Heterochromatin Assembly. Histone H3 lysine 9 (H3K9) methyltransferases (HMTs) are first recruited to heterochromatin nucleation centers, leading to H3K9 methylation (red dots associated with the chromosome), which is subsequently recognized and bound by heterochromatin protein 1 (HP1). Additional HMTs are recruited either directly through recognizing H3K9me or via association with HP1, leading to the methylation of adjacent nucleosomes. The repetition of such binding/methylation cycles results in heterochromatin spreading in a sequence-independent manner.

[13,14]. Methylated H3K9 also binds to heterochromatin protein HP1, which in turn serves as a scaffold to further recruit chromatin modifiers, including H3K9 methyltransferases and histone deacetylases [15–19]. These combined actions lead to the spreading of heterochromatin along a large domain of chromatin in a DNA sequence-independent manner [8,14,20–22]. During DNA replication, parental histones, which contain existing modifications, are randomly incorporated onto both daughter strands behind the replication fork [23]. H3K9me-mediated recruitment of H3K9 methyltransferases restores heterochromatin domains on both daughter strands, leading to the stable maintenance of this epigenetic state through generations [20,24–26].

The budding yeast *Saccharomyces cerevisiae* lacks the H3K9 methylation/HP1 system, but assembles functional heterochromatin using the silent information regulator (SIR) complex, composed of Sir2, Sir3, and Sir4 [27,28]. Sir2 is a histone deacetylase with activity mainly towards H4K16 [29–31], the acetylation status of which directly regulates higher-order chromatin folding *in vitro* [32] and plays a major role in heterochromatin function *in vivo* [6,33]. Sir3 and Sir4 preferentially interact with histone tails devoid of histone H4 acetylated on lysine 16 (H4K16ac) [34–36]. Heterochromatin formation starts with the recruitment of Sir2 by factors that recognize specific DNA sequences. Sir2 subsequently deacetylates H4K16, allowing Sir3 and Sir4 to bind. Sir3 oligomerizes and recruits more Sir2 to deacetylate H4K16 of adjacent nucleosomes, and thus facilitates the spreading of the entire Sir complex [27,28].

Even though the protein factors involved in heterochromatin assembly in many organisms are diverse, similar mechanisms of self-propagation underlie heterochromatin spreading. The stepwise spreading model is supported by the fact that heterochromatin assembly factors cover the entire heterochromatin domain, the distance of spreading is sensitive to the dosage of heterochromatin proteins, and silencing spreads continuously [5,20,37–41]. However, heterochromatin sometimes skips particular genomic regions, suggesting that additional mechanisms, such as looping, also aid heterochromatin spreading [21,42–44].

The Dynamics of HP1

Originally identified in *Drosophila*, HP1 belongs to a highly conserved family of chromatin proteins, and homologs are present from fission yeast (Swi6 and Chp2) to humans (HP1α, HP1β, and HP1γ) [45]. While the majority of these HP1 proteins are localized to heterochromatin, some isoforms have diverged from heterochromatin functions. For example, human HP1α and HP1β are distributed mainly at pericentric heterochromatin, whereas HP1γ is localized to discrete euchromatic regions. HP1 proteins are composed of a chromodomain, a chromo shadow domain, and a flexible hinge region [45,46]. The chromodomain binds to

H3K9me [15,16], while the chromo shadow domain mediates dimerization of HP1 [47]. These interactions result in the formation of an HP1 protein network that locks up chromatin in a highly compacted state [48]. Moreover, HP1 proteins recruit a diverse range of factors to further modify heterochromatin [2].

Given its essential structural role in heterochromatin formation, HP1 is expected to stably associate with heterochromatin domains. However, fluorescence recovery after photobleaching (FRAP) analyses of all three HP1 isoforms in human cells and Swi6 in fission yeast illustrate that binding of HP1 to chromatin is very dynamic, exchanging rapidly between chromatin-bound and nucleoplasm forms, and also among different heterochromatin domains [49–52] (Figure 2). Interestingly, the dynamics of HP1 proteins also change during cell differentiation. For example, human HP1β is more mobile in embryonic stem cells and induced pluripotent stem cells compared to fibroblasts. Similarly, mouse embryonic stem cells are also characterized by higher mobility of HP1 proteins [53,54]. Such differences suggest the existence of regulatory mechanisms for HP1 dynamics, although the molecular basis of such dynamic behavior remains unclear. It is also possible that the increased HP1 mobility in embryonic stem cells is the indirect result of the less compact chromatin structure in these cells.

The dynamic nature of HP1 protein binding provides windows of opportunity for other factors to access the underlying DNA. For example, heterochromatin-mediated gene silencing in yeasts frequently changes between 'on' and 'off' states, and this might be a result of the dynamic binding of heterochromatin proteins [55,56]. HP1 dynamics might also allow cells to maintain the balance between different heterochromatin domains. For example, in fission yeast, disruption of telomeric heterochromatin releases limiting heterochromatin factors, such as Swi6, which can restore defective pericentric heterochromatin due to the loss of RNAi components (see below) [57].

Transcription and Heterochromatin Assembly

Another surprising aspect of the dynamic nature of heterochromatin is the involvement of transcription and non-coding RNAs during heterochromatin assembly [58,59]. Heterochromatin usually forms at repetitive DNA elements and represses the transcription of the underlying repeats. Therefore, it is counterintuitive that heterochromatin assembly actually requires transcription of these repeats. This process was first discovered and is best illustrated in the fission yeast (Figure 3). In this organism, the repetitive sequences are transcribed during the S phase of
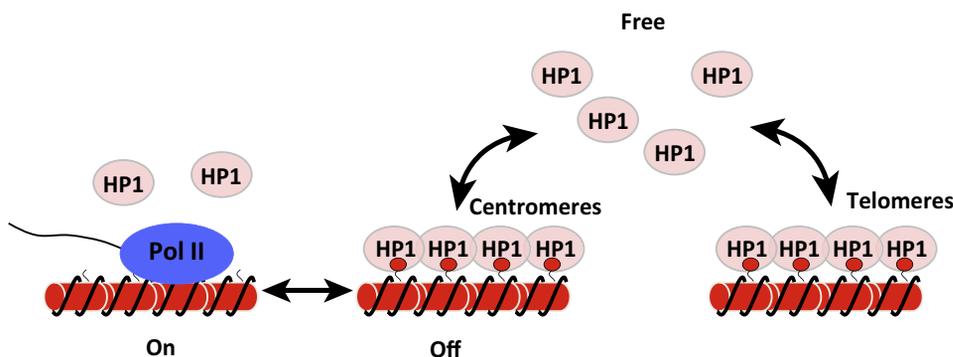
Figure 2. Dynamics of HP1 Proteins. Contrary to the general conception that HP1 is a static component of heterochromatin, it dynamically exchanges between the heterochromatin-bound and free forms, which gives other factors access to the underlying DNA, such as RNA polymerase II (Pol II) shown here. HP1 also exchanges rapidly between heterochromatin domains, such as centromeres and telomeres, to buffer changes in heterochromatin stability.

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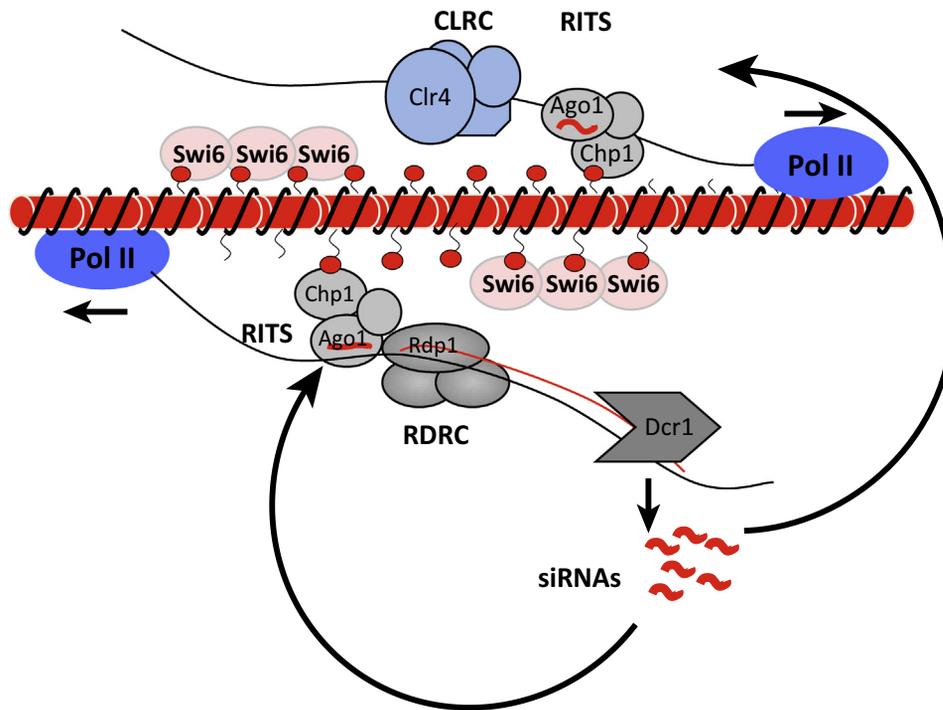
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Figure 3. Transcription-Dependent Assembly at Repetitive DNA Elements in Fission Yeast. During S phase of the cell cycle, DNA repeats are transcribed by RNA polymerase II (Pol II). These transcripts are converted into double-stranded RNAs by the RNA-dependent RNA polymerase complex (RDRC) and then processed into siRNAs by the RNAi machinery. The siRNAs guide the RNA-induced transcriptional silencing (RITS) complex back to nascent transcripts. RITS associates with the Clr4 methyltransferase complex (CLRC), which initiates H3K9 methylation and heterochromatin assembly.

the cell cycle [60,61], possibly as a result of DNA replication, as the passage of DNA polymerases opens up the chromatin structure. These transcripts are converted to double-stranded RNAs with the help of the RNA-dependent RNA polymerase complex (RDRC), and are then processed by the RNase Dicer into siRNAs [62,63]. siRNAs are then loaded into the RITS (RNA-induced transcriptional silencing) complex, which contains the Argonaute protein Ago1 that uses siRNAs as guides to target the RITS complex to nascent transcripts originating from the repeats [64]. RITS then associates with the CLRC complex (containing H3K9 methyltransferase Clr4 and the associated E3 ubiquitin ligase complex Cul4, Rik1, Raf1, and Raf2) to initiate H3K9 methylation [65–68]. The RITS subunit Chp1 contains a chromodomain that recognizes H3K9me, and binding stabilizes its association with heterochromatin, thereby forming a self-reinforcing loop of heterochromatin assembly and RNAi-mediated processing of repeat transcripts [62,69].

Small RNA-mediated heterochromatin assembly has also been discovered in *Caenorhabditis elegans* and *Drosophila*. In addition, small RNAs also mediate DNA methylation in plants, and a class of small RNAs termed piRNAs silence transposons in the germline of animals. Because these topics have been extensively reviewed recently [58,59], we will not discuss them in detail here. Although the chromatin-modifying activities involved differ, a general theme of these different systems is that the nascent transcripts are not only the source of small RNAs but also provide a scaffold for the recruitment of heterochromatin assembly factors, therefore explaining the need for transcription in heterochromatin assembly.

Heterochromatin Changes During Aging

The dynamics of constitutive heterochromatin is also reflected in the aging process. It was first found that somatic cells from patients with Hutchinson–Gilford progeria syndrome, a premature aging disease caused by a mutation in the nuclear membrane-associated lamin A protein, show a global loss of heterochromatin, as indicated by a reduction of H3K9me3 and HP1 protein staining [70,71]. Similarly, mutations in the Werner helicase (WRN) cause premature aging, and mesenchymal cells without this helicase also show a reduction in these heterochromatin markers [72]. Most importantly, cells from elderly individuals display a global loss of these heterochromatin marks, suggesting that loss of heterochromatin is part of the normal aging process [72,73]. WRN directly associates with heterochromatin proteins such as the H3K9 methyltransferase SUV39H1 and HP1α as well as with lamina-associated polypeptide LAP2β [72], suggesting a possible direct requirement of the nuclear membrane proteins and WRN in regulating heterochromatin assembly during aging.

These findings were corroborated by genetic studies of aging in *Drosophila* and *C. elegans*, where a global loss of heterochromatin also takes place during normal aging [74,75]. Moreover, in *Drosophila*, overexpression of HP1 extends lifespan, whereas mutation in HP1 reduces it [76]. Similarly, in mammalian mesenchymal stem cells, loss of SUV39H1 activity results in premature aging phenotypes, while overexpression of HP1 proteins alleviates premature aging phenotypes caused by the loss of the WRN protein [72]. Even budding yeast, which assembles heterochromatin by way of Sir2-mediated histone deacetylation, experiences similar effects of modulating heterochromatin on lifespan [77]. Nevertheless, the role of heterochromatin changes during aging is unclear. It is possible that loss of heterochromatin results in the misregulation of gene expression, which contributes to the aging-associated phenotypes [78]. Alternatively, heterochromatin loss might affect other essential functions of heterochromatic domains, such as telomeres [77]. Despite the global loss of heterochromatin during aging, senescent cells often rearrange their chromosomes, leading to the formation of senescence-associated heterochromatin foci (SAHF), which colocalize with heterochromatin hallmarks such as H3K9me and HP1 [79,80]. Therefore, the dynamic changes in heterochromatin might have other unappreciated roles in regulating the aging process.

Heterochromatin Dynamics in Response to Stress

Constitutive heterochromatin domains also change their stability in response to environmental stimuli. For example, gene silencing at constitutive heterochromatin in fission yeast is less stable at elevated temperatures [81]. Interestingly, stress-response transcription factors are directly involved in heterochromatin assembly, providing a possible mechanism for temperature-mediated effects on heterochromatin. The activating transcription factor/cAMP response element binding protein (ATF/CREB) family of transcription factors (Atf1/Pcr1), which regulate the expression of stress-response genes, bind to a specific DNA element within the silent mating-type locus and recruit histone deacetylases and the histone methyltransferase Clr4 to initiate heterochromatin assembly [10,11,82]. Under stress, Atf1 is activated by MAP kinase-mediated phosphorylation to directly activate the transcription of stress-response genes [83]. The fact that the disruption of MAP kinase stabilizes heterochromatin indicates that phosphorylation of Atf1 by MAP kinase may destabilize heterochromatin as part of the stress response [11], although there is no direct evidence to support that phosphorylated Atf1 affects heterochromatin formation in fission yeast. However, a recent study in fruit flies provides a direct link between stress response and heterochromatin assembly. Similar to fission yeast, heterochromatin in flies is also sensitive to temperature fluctuations [84]. dATF-2, the homolog of fission yeast Atf1, colocalizes with HP1 and recruits HP1 to pericentric heterochromatin regions that contain dATF-2 binding sites under normal conditions (Figure 4) [85]. Under stress conditions, dATF-2 is phosphorylated by MAP kinase and released from pericentric heterochromatin, which in turn abolishes HP1 enrichment, resulting in the disruption of heterochromatin [85]. Similar phenomena of stress-mediated
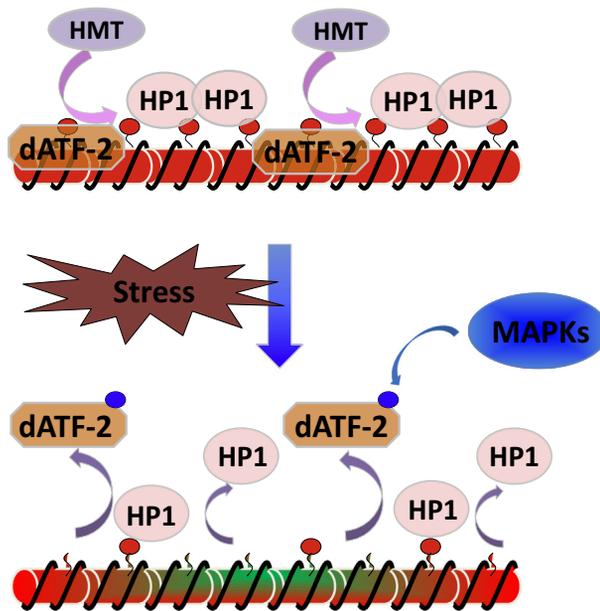
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Figure 4. Pericentric Heterochromatin Disassembly in Response to Stress in *Drosophila*. Under normal conditions, the transcription factor dATF-2 is in a hypophosphorylated form, which recruits HP1 to establish constitutive heterochromatin at pericentric regions. In response to stress, the MAPK pathway phosphorylates dATF-2, thus reducing its binding to pericentric heterochromatin and releasing HP1. Abbreviation: HMT, histone methyltransferase.

modulation of heterochromatin have also been observed in human cells. Upon heat shock, heat shock transcription factor 1 (HSF1) redistributes to a few nuclear foci of pericentric heterochromatin. HSF1 binds directly to satellite repeats and facilitates the transcription of noncoding RNAs (ncRNAs) from these regions [86–88]. In addition, HSF1 regulates global histone acetylation levels by recruiting histone deacetylases HDAC1 and HDAC2 [89]. Therefore, direct involvement of stress-response transcription factors in heterochromatin assembly seems to be a highly conserved process that allows cells to modify constitutive heterochromatin domains in response to environmental stress.

In addition to the involvement of stress-response pathways in heterochromatin assembly, other signal transduction pathways also directly modify heterochromatin proteins to control heterochromatin stability. The diverse histone-modifying activities involved in heterochromatin formation provide ample opportunities to incorporate environmental signals to regulate the stability of heterochromatin domains. One of the key proteins to receive such signals is HP1. For instance, casein kinase 2 (CK2)-mediated phosphorylation of fission yeast Swi6, *Drosophila* HP1a, and mammalian HP1α in all cases promotes binding to H3K9me3 nucleosomes [90], and such phosphorylation is required for heterochromatin function in fission yeast and *Drosophila* [91,92]. Interestingly, a different region of mammalian HP1β is also phosphorylated by CK2 upon DNA damage, resulting in the rapid release of HP1β from the damaged site, facilitating access of DNA repair machinery [93]. These results suggest that the functions of HP1 phosphorylation are diverse in response to distinct stimuli, allowing cells to better adapt to environmental alterations. In addition, the histones themselves are targets of signal transduction pathways that impact heterochromatin stability. For example, in both fission yeast and mammals, the phosphorylation of H3S10 by Aurora kinase releases HP1 from chromatin during the M phase of the cell cycle, allowing proteins involved in chromosome condensation to access the underlying DNA [60,82,94,95].

Heterochromatin-Mediated Epigenetic Adaptation

Although heterochromatin formation is highly organized and heterochromatin domains are well defined, recent findings suggest that heterochromatin formation is much more promiscuous than expected. In fission yeast, two factors, Epe1 and Mst2, negatively regulate heterochromatin assembly. Epe1 contains a JmjC domain, which is typically associated with histone demethylase

Key Figure

Heterochromatin-Mediated Epigenetic Adaptation in Fission Yeast
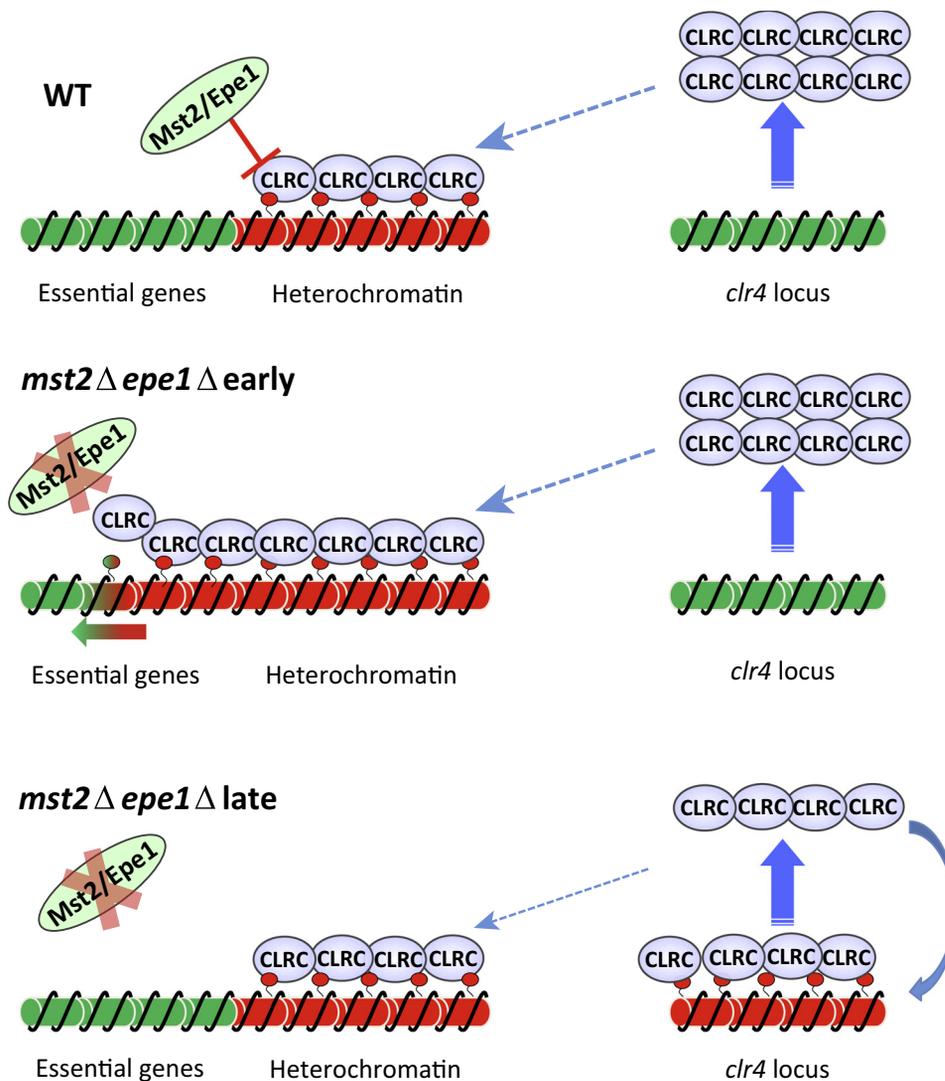
Figure 5. The negative regulators of heterochromatin, Mst2 and Epe1, prevent promiscuous heterochromatin spreading in wild-type (WT) cells. However, in *mst2Δ epe1Δ* cells, uncontrolled heterochromatin spreading inactivates essential genes, resulting in severe growth defects during early stages of development. Gradually, cells form heterochromatin at the *clr4*+ locus to reduce Clr4 expression levels, resulting in a new equilibrium that maintains heterochromatin at key locations, but which minimizes heterochromatin spreading, leading to normal growth during late stages of development.

activity [96]. Although the enzymatic activity of Epe1 has not been demonstrated *in vitro*, genetic evidence is consistent with the idea that Epe1 acts as a functional H3K9 demethylase, and loss of Epe1 results in the expansion and stabilization of heterochromatin domains [25,26,97–99]. Mst2 is a component of an acetyltransferase complex that is highly specific to H3K14 [100]. Loss of Mst2 results in a reduction of H3K14 acetylation, which in turn slows histone turnover to preserve parental histone modifications required for heterochromatin maintenance [24,101]. In *mst2Δ epe1Δ* cells there is massive upregulation of heterochromatin, resulting in severe growth defects at an early stage of development as a result of inactivation of essential genes (Figure 5, Key Figure) [101]. Surprisingly, these cells quickly adapt to such a heterochromatic stress by accumulating heterochromatin at the *clr4+* locus, which in turn downregulates the levels of the Clr4 histone H3K9 methyltransferase to constrain heterochromatin domains in the genome. Such epigenetic changes can be inherited through mitosis and meiosis, conferring upon future generations greater resistance to heterochromatic stress. Interestingly, when heterochromatin is unable to form at the *clr4+* locus via genetic manipulations, cells accumulate heterochromatin at the *rik1+* locus, which encodes another subunit of CLRC complex required for its activity [101]. These observations indicate that epigenetic alteration is a rapid and efficient way to adapt to a new environment in response to stress.

In plants, a process termed vernalization silences the flowering repressor gene FLOWERING LOCUS C (*FLC*) in response to prolonged low temperature in winter, allowing flowering to occur in the following spring. Such silencing requires polycomb repressive complex 2 (PRC2)-mediated H3K27 methylation, which forms facultative heterochromatin [102], demonstrating an important role for heterochromatin in integrating environmental signals. Similar epigenetic changes in heterochromatin might also enable tumor cells to survive certain therapies. For example, populations of tumor cells show both genetic and epigenetic heterogeneity, and these contribute to the variations of almost every phenotype in these tumors [103]. A recent study demonstrates that, in a non-small cell lung cancer cell line model, there are consistently subpopulations of drug-resistant cells which contribute to the development of drug resistance [104]. The establishment of drug resistance requires histone H3K4 demethylase KDM5A and histone deacetylase activities, indicating that a more repressive heterochromatic environment favors the adaptation of drug-resistant tumor cells. More importantly, these cells restore drug sensitivity after 20–30 passages, suggesting that this epigenetic adaptation is reversible [104]. Therefore, heterochromatin-mediated epigenetic adaptation offers greater flexibility for cells to tolerate environmental insults and seems to be an evolutionarily conserved phenomenon.

Concluding Remarks

Constitutive heterochromatin has traditionally been viewed as a highly-stable structure that represses the transcription and recombination of repetitive DNA elements. However, recent studies have demonstrated that constitutive heterochromatin domains are also highly dynamic. The function of such dynamics is only beginning to be appreciated (see Outstanding Questions), and it might be part of the cellular response to outside stimuli by modifying chromatin structure to cushion against adverse effects [105]. The silencing of gene expression by heterochromatin in a sequence-independent manner makes heterochromatin formation one of the most versatile forms of epigenetic changes. Adaptive changes of heterochromatin in response to numerous stresses take place in diverse species from yeasts to humans. Because a crucial step in tumor development is the inactivation of tumor-suppressor genes, the discoveries of epigenetic inactivation phenomena in different systems provide invaluable clues for studying the adaptation of tumor cells and designing new strategies to counteract such effects.

Outstanding Questions

What is the molecular mechanism that regulates HP1 dynamics?

What is the mechanism of small RNA-mediated chromatin modifications in higher eukaryotes?

What are the signals that trigger heterochromatin changes during normal aging? What are the roles of these changes during aging?

What are the roles of heterochromatin changes in cellular adaptation to stress?

What is the molecular mechanism of ectopic heterochromatin formation in response to uncontrollable heterochromatin spreading? Is it programmed or does it reflect the selection of low-frequency events?

Are there chromatin-based mechanisms for transgenerational inheritance in higher eukaryotes?

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