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Functional involvement of RNAs and intrinsically disordered proteins in the assembly of heterochromatin

Chikashi Obuse [a,*], Jun-ichi Nakayama [b,c]

[a] Graduate School of Science, Osaka University, Toyonaka 560-0043, Japan
[b] Division of Chromatin Regulation, National Institute for Basic Biology, Okazaki 444-8585, Japan
[c] Basic Biology Program, Graduate Institute for Advanced Studies, SOKENDAI, Okazaki 444-8585, Japan

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ABSTRACT

Heterochromatin is a highly condensed chromatin structure observed in the nuclei of eukaryotic cells. It plays a pivotal role in repressing undesired gene expression and establishing functional chromosomal domains, including centromeres and telomeres. Heterochromatin is characterized by specific histone modifications and the formation of higher-order chromatin structures mediated by proteins, such as HP1 and Polycomb repressive complexes (PRCs), which recognize the specific histone modifications. Recent studies have identified the involvement of non-coding RNAs (ncRNAs) and intrinsically disordered proteins (IDPs) in heterochromatin, leading to the proposal of a new model in which liquid-liquid phase separation (LLPS) contributes to heterochromatin formation and function. This emerging model not only broadens our understanding of heterochromatin's molecular mechanisms but also provides insights into its dynamic regulation depending on cellular context. Such advancements pave the way for exploring heterochromatin's role in genome organization and stability, as well as its implications in development and disease.

1. Introduction

When the nucleus of a mammalian cell is examined under a microscope, densely condensed structures can be easily identified (Fig. 1A). In 1928, Emil Heitz used chromatin-staining techniques to reveal variations in chromosomal condensation and introduced the term 'heterochromatin' to describe these structures, which appeared microscopically distinct during interphase, in contrast to the more decondensed regions referred to as euchromatin [1]. Heitz proposed that heterochromatin represented transcriptionally inactive, gene-poor regions of the genome, while euchromatin was gene-rich and transcriptionally active [1]. This foundational distinction has been validated by subsequent research and remains central to modern chromatin biology. Recent genome-wide analyses confirm that heterochromatic regions are largely gene deserts and closely associated with the nuclear lamina [2]. Furthermore, it has long been recognized that RNA synthesis predominantly occurs outside heterochromatin, with transcription primarily associated with euchromatic domains [3]. These findings support the current understanding that heterochromatin's condensed structure restricts access to transcription factors, reinforcing its repressive nature compared to euchromatin.

The regulatory role of heterochromatin in gene silencing was further elucidated by Hermann Muller, who discovered the phenomenon of position effect variegation (PEV) in the 1930s. PEV demonstrated that genes relocated near heterochromatin could become transcriptionally silenced, highlighting the positional influence of chromatin organization. Subsequent studies confirmed that genes adjacent to heterochromatin are more susceptible to silencing than those located further away

Abbreviations: CBX, chromobox; CD, chromodomain; CSD, chromoshadow domain; ERV, endogenous retrovirus; H2AK119ub1, monoubiquitylation of histone H2A at lysine 119; H3K9me3, trimethylation of histone H3 lysine 9; HBiX1, HP1-binding protein enriched in inactive X chromosome 1; HP1, heterochromatin protein 1; IDR, intrinsically disordered region; IDP, Intrinsically disordered protein; KRAB, Krüppel-associated box; LBR, Lamin B receptor; LLPS, liquid-liquid phase separation; lncRNA, long non-coding RNA; MAC, macronucleus; MIC, micronucleus; miRNA, microRNA; PCGF, polycomb group RING finger protein; PEV, position effect variegation; PHC, pericentromeric heterochromatin; piRNA, Piwi-interacting RNA; PRC1, polycomb repressive complex 1; PRC2, polycomb repressive complex 2; RDRC, RNA-dependent RNA polymerase complex; RISC, RNA-induced silencing complex; RITS, RNA-induced transcriptional silencing; RNAi, RNA interference; SAHF, senescence-associated heterochromatin foci; SIR, Silent Information Regulator; siRNA, Small interfering RNA; Xi, inactive X chromosome.

* Corresponding author.
E-mail address: obuse@bio.sci.osaka-u.ac.jp (C. Obuse).

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[4]. These findings reinforced the concept of heterochromatin-mediated gene silencing and suggested that heterochromatin could "spread" along chromosomal regions, influencing gene expression in a distance-dependent manner.

Recent technological advances, such as Hi-C chromosomal interaction mapping, have provided insights into the higher-order organization of heterochromatin. These studies have shown that heterochromatin not only silences transcription but also organizes the genome into distinct nuclear compartments [5]. For instance, the chromocenter was originally described by Pasquale Baccarini as dark-stained foci in plant nuclei [6]. Later, Heitz proposed that chromocenters primarily consist of heterochromatic chromosome regions [1]. Consistent with Heitz's proposal, recent technological advances, including high-resolution chromatin contact mapping, have revealed that heterochromatin at pericentromeric heterochromatin (PHC) domains derived from multiple chromosomes cluster together to form chromocenters in mouse cells (Fig. 1A), which are enriched in repetitive sequences and contribute to genome stability and compartmentalization [7]. However, the precise mechanisms by which heterochromatin is established and maintained remain elusive. Emerging evidence suggests that non-coding RNAs (ncRNAs) and proteins with intrinsically disordered regions (IDRs) contribute to in heterochromatin assembly and function through diverse mechanisms at multiple stages. One possible mechanism involves liquid-liquid phase separation (LLPS), a ubiquitous phenomenon underlying the formation of membraneless organelles in eukaryotic cells, which is fundamentally driven by the weak intermolecular interactions as these biopolymers and may serve as a core principle for heterochromatin formation.

This article reviews the current understanding of heterochromatin structure and function, with a focus on recent advances related to ncRNAs and IDRs, and provide a comprehensive overview of heterochromatin's roles in gene regulation, genome stability, and cellular differentiation.

2. Molecular signatures of heterochromatin: histone and DNA modifications

Heterochromatin plays a critical role in genome organization and gene regulation, with constitutive and facultative heterochromatin exhibiting distinct structural and functional properties. Constitutive heterochromatin remains consistently condensed across cell types and developmental stages. It is found in centromeric and telomeric regions, where it stabilizes chromosomes by repressing transcription and recombination within repetitive elements and gene-poor regions. This form of heterochromatin is marked by trimethylation of histone H3 at lysine 9 (H3K9me3), which is recognized by Heterochromatin Protein 1 (HP1), a key factor in promoting chromatin compaction and maintaining genome integrity (Fig. 1B) [8–12]. In contrast, facultative heterochromatin represents a dynamic and reversible state, adapting to

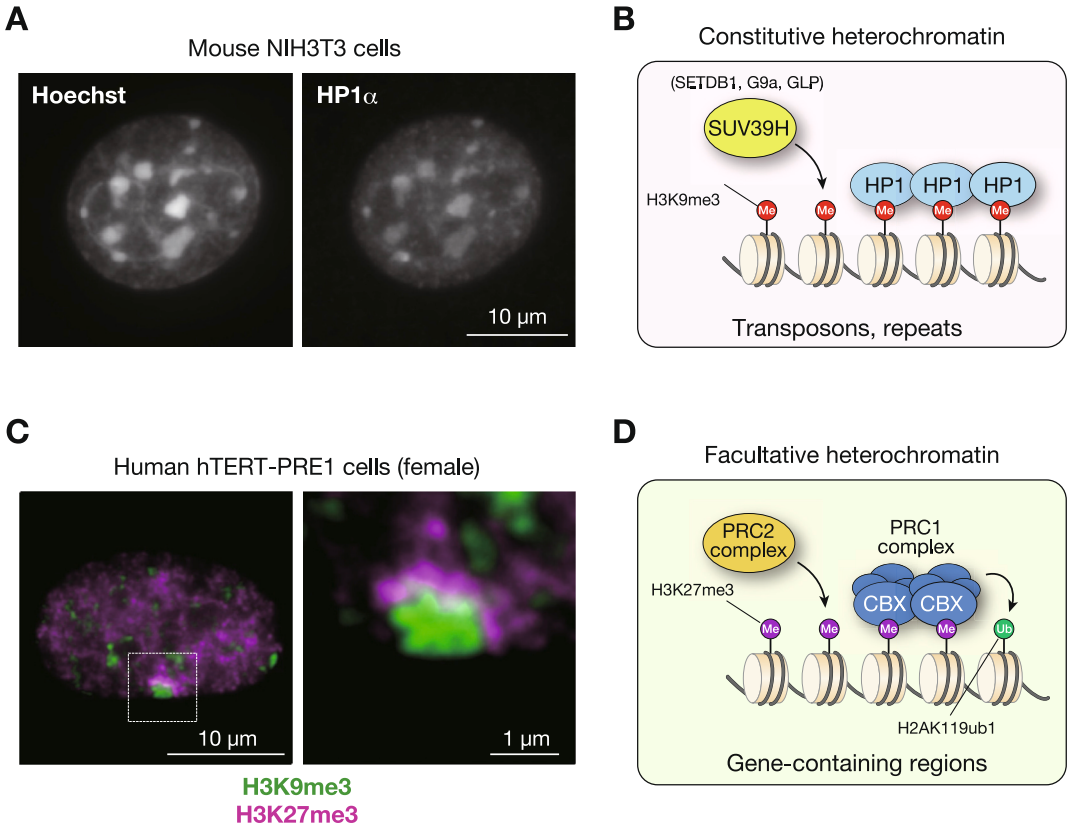

Fig. 1. Constitutive and facultative heterochromatin.
(A) Microscopic images of a mouse NIH3T3 cell. Constitutive heterochromatin corresponding to chromocenters is strongly stained with DAPI (left). Immunofluorescent signals of HP1α (right) colocalize with the DAPI dense regions (left) (scale bar: 10 μm). (B) Schematic diagram of constitutive heterochromatin. Genomic regions containing transposons and repetitive sequences are marked by histone H3 lysine 9 trimethylation (H3K9me3), catalyzed by methyltransferases such as SUV39H. H3K9me3 is recognized and bound by the evolutionarily conserved HP1, forming a higher-order chromatin structure. (C) Microscopic images of a human female hTERT-PRE1 cell. Cells were immunostained for H3K9me3 (green) and H3K27me3 (magenta) (left) (scale bar: 10 μm). An enlarged image of the indicated area is shown on the right (scale bar: 1 μm). Notably, the H3K9me3-dense body largely corresponds to the Barr body, where DNA is highly condensed. (D) Schematic diagram of facultative heterochromatin. Gene-containing regions with potential transcriptional activity become heterochromatinized during development and differentiation. Histone H3 lysine 27 is methylated by the PRC2 complex, and CBX family proteins in this PRC1 complex recognize H3K27me3 and catalyze the mono-ubiquitylation of histone H2A lysine 119 (H2AK119ub1) for transcriptional inhibition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

developmental or environmental cues. Initially, facultative heterochromatin was defined as the selective heterochromatinization of only one of the homologous chromosomes [13], it is now recognized as a mechanism of developmentally regulated gene silencing. A well-known example is the inactivated X chromosome in female mammals, where the long non-coding RNA Xist mediates the heterochromatinization of only one of the homologous chromosomes at an early developmental stage, leading to the formation of a transcriptionally silent heterochromatic structure called the Barr body (Fig. 1C). This process is accompanied by the introduction of epigenetic marks, such as the trimethylation of histone H3 at lysine 27 (H3K27me3) catalyzed by Polycomb Repressive Complex 2 (PRC2), which is a hallmark of

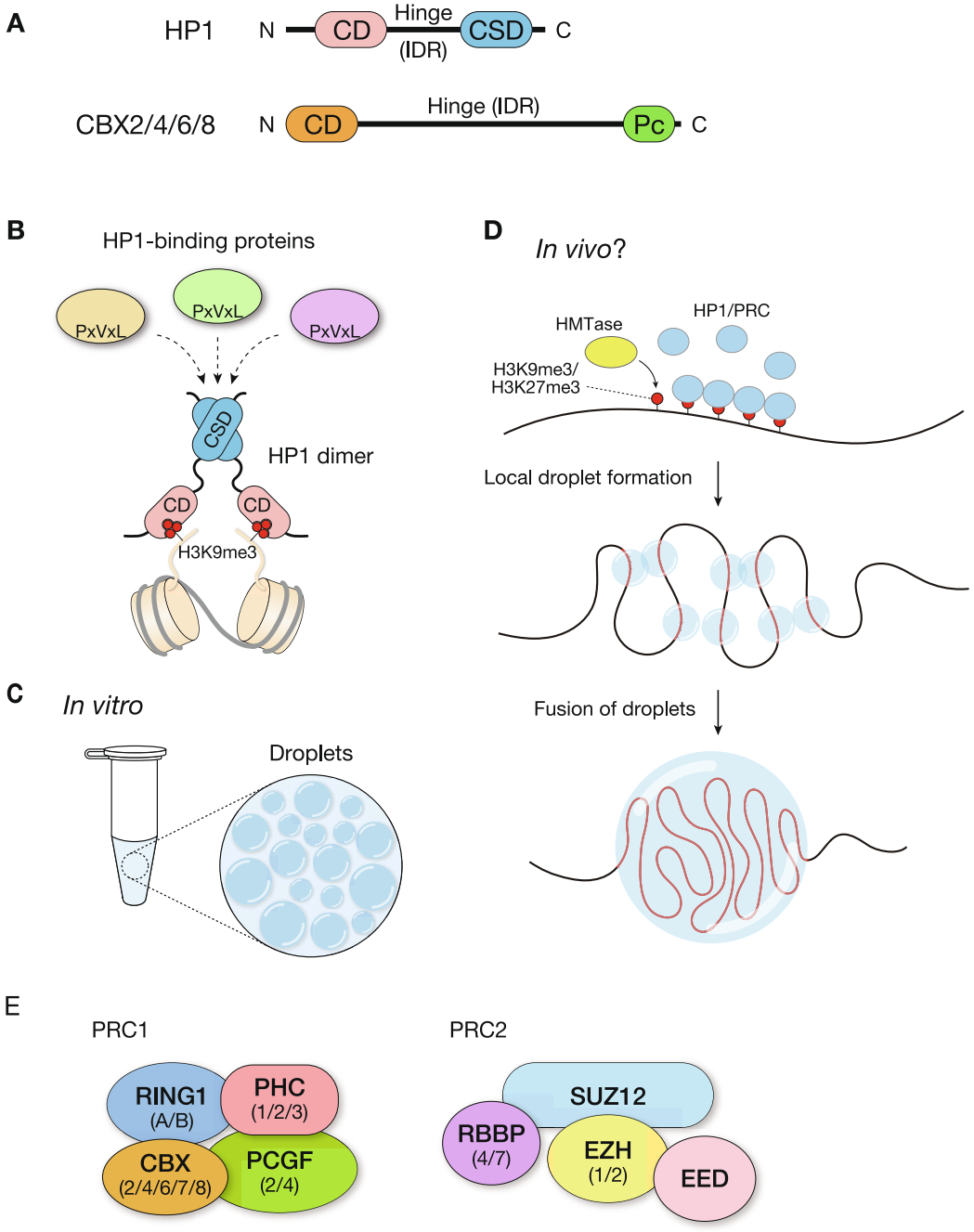

Fig. 2. Methyl-reader proteins and liquid-liquid phase separation.
(A) Schematic diagrams of chromodomain proteins HP1 and CBX2. For HP1, the chromodomain (CD) and chromoshadow domain (CSD) are linked by unstructured hinge region (IDR). For CBX2, the chromodomain (CD) and a carboxyl (C)-terminal polycomb repressor box (Pc) are linked by an unstructured hinge region (IDR). (B) Schematic diagram illustrating HP1 function. HP1 forms dimers, binds to nucleosomes containing H3K9me3, and recruits various chromatin regulators to establish higher-order chromatin structures and functions. (C) Highly concentrated recombinant HP1 proteins form droplets via liquid-liquid phase separation in vitro. (D) (Upper row) Binding of HP1 to H3K9me3 or the CBX subunit of PRC1 to H3K27me3 (introduced by methyltransferases) increases the local concentration of HP1/PRC1. (Middle) Droplet-like structures form via interactions between chromatin-bound HP1/PRC1 or interactions with other factors. (Bottom) Small droplets fuse to form large heterochromatin structures observable under a microscope. (E) Schematic diagram of canonical PRC1 and PRC2. PRC1 (left) is composed of a ubiquitin ligase (either RING1A or RING1B), one of two PCGF family members (PCGF2/4), one of three polyhomeotic family members (PHC1/2/3), and a chromobox (CBX) subunit (CBX2/3/4/7/8). (PRC2 (right) is composed of one of two histone methyltransferases for H3K27me3 (EZH1/2), EED, which recognizes and binds to H3K27me3, SUZ12, which serves as a scaffold for this complex, and one of two histone-binding components (RBBP4 /7).

facultative heterochromatin (Fig. 1D) [14]. In its maintenance mode, PRC1 recognizes this mark and catalyzes mono-ubiquitylation at histone H2A at lysine 119 (H2AK119ub1), further stabilizing the repressive state (Fig. 1D).

The level of histone acetylation is closely linked to transcriptional activity, and heterochromatic regions are generally hypoacetylated, but acetylation states do not exclusively define heterochromatin [15,16]. Another modification that characterizes heterochromatin is histone H3 methylation. As described above, constitutive heterochromatin is enriched with H3K9me3, a modification catalyzed by several methyltransferases, such as SUV39H1, SUV39H2, SETDB1, G9a, and GLP in mammals, and specifically recognized by chromodomain proteins, including those of the HP1 family (Fig. 1B). The core structure characterized by methylated H3K9 and HP1 is highly conserved across species, from fission yeast to humans. In contrast, facultative heterochromatin, primarily observed in higher eukaryotes [17].

In addition to histone modifications, methylation of DNA cytosines is an important mark of heterochromatin in plants and mammalian cells. Studies in model organisms such as fission yeast, ciliates, and plants have also shown that the process of introducing heterochromatin-specific histone methylation involves a mechanism of RNA silencing via small RNA molecules [18–22]. A similar mechanism has been implicated in the repression of transposons in the germline of Drosophila and mammals [23,24]. In budding yeast, which has made important contributions as a model for eukaryotes, heterochromatin formation relies solely on histone deacetylation and the Silent Information Regulator (SIR) proteins, as budding yeast lacks the H3K9 methylation system, HP1, and RNA silencing factors [25].

3. Heterochromatin protein 1 (HP1)

HP1 is an evolutionarily conserved protein that plays a central role in the formation and maintenance of heterochromatin structures. HP1 contains a chromodomain (CD) at its N-terminus and a chromoshadow domain (CSD) at its C-terminus, connected by a hinge region lacking secondary structure (Fig. 2A) [26,27]. The chromodomain specifically binds to H3K9me3, a hallmark modification of constitutive heterochromatin. This interaction is regulated by a cluster of acidic amino acid residues and phosphorylated serine residues immediately upstream of the CD [27–29]. HP1 also forms dimers through the CSD and interacts with various chromatin-associated factors through the hydrophobic surface created by dimerization (Fig. 2B) [30–36].

The structural basis for how HP1 binding to H3K9me3-modified nucleosomes leads to the formation of higher-order chromatin structures remains incompletely understood. Recently, cryo-electron microscopy studies revealed the structure of HP1 bound to two nucleosomes containing H3K9me3, confirming that HP1 dimers can indeed cross-link nucleosomes [37]. Interestingly, these studies demonstrated that HP1 dimers do not directly contact the linker DNA of the nucleosome, suggesting that HP1 binding does not interfere with the binding of linker DNA-associated chromatin regulators (Fig. 2B).

Additionally, studies on Swi6, an HP1 homolog in fission yeast, have shown that Swi6 binding to methylated nucleosomes drives a transition from an auto-inhibited state in which a histone-mimic sequence within the loop structure of the chromodomain of one Swi6 monomer blocks methyl-mark recognition by the chromodomain of another monomer [38]. This transition is regulated by the recognition of the H3K9 methylation and nucleosomal DNA [38]. This mechanism allows Swi6 to form multimeric complexes beyond dimers, enabling the propagation of heterochromatin along chromosomal regions. However, similar chromodomain-mediated interactions have not been observed in human HP1 [39], leaving open the question of whether this represents a conserved property.

HP1 is not a single protein but comprises multiple isoforms in mammals, including HP1α, HP1β, and HP1γ, each of which exhibits distinct nuclear localization and functional specialization. HP1α and HP1β are predominantly associated with constitutive heterochromatin, while HP1γ is more dynamic and interacts with euchromatic regions [40]. This isoform specificity underscores the diverse roles of HP1 in chromatin organization and gene regulation. Moreover, HP1 has been implicated in processes beyond heterochromatin, such as DNA repair, replication, chromosome segregation, and transcriptional regulation, suggesting that its function is context-dependent [30,33,34,41].

Recently, in three patients with developmental delay, hypotonia, and autistic features, de novo variants were identified within the chromodomain of HP1β. Analysis of mice harboring a similar HP1β mutation suggested the possibility of synaptic delay or myelination deficits, and the mutant HP1β exhibited reduced heterochromatin binding in human cells, suggesting that mutant HP1β forms dimers with other HP1 proteins, thereby reducing chromatin binding ability and exerting dominant-negative effects on neurodevelopment [42].

Growing evidence suggests that HP1, in particular HP1α, has the ability to form LLPS droplets in vitro, *which* may explain its role in organizing higher-order chromatin structure and function in cells as discussed in a later section (Fig. 2C and D) [43–47].

4. Polycomb repressive complexes (PRCs)

PRCs are critical regulators of gene silencing and heterochromatin formation, particularly in developmentally regulated regions. Polycomb group (PcG) proteins function through two major complexes, PRC1 and PRC2. PRC2 is primarily responsible for silencing target genes by catalyzing the deposition of H3K27me3, a hallmark repressive histone modification. PRC2 consists of several core components: EZH2 (or EZH1), which serves as the catalytic subunit responsible for H3K27 methylation; EED, which binds to H3K27me3 and enhances PRC2 activity; SUZ12, which stabilizes the complex; and RBBP4/7, which acts as a histone-binding component [48,49] (Fig. 2E). PRC2 is recruited to specific chromatin regions through interactions with DNA-binding proteins, non-coding RNAs, and pre-existing chromatin modifications such as H3K27me3. Once H3K27me3 is established, it serves as a docking site for PRC1 on chromatin. PRC1 reinforces the silenced state by facilitating further chromatin modification and compaction. PRC1 exists in two main forms: canonical PRC1 (cPRC1) and non-canonical PRC1 (ncPRC1), each with distinct subunit compositions and functions. cPRC1 complexes include components such as CBX (Chromobox proteins), which recognize H3K27me3 deposited by PRC2 via their CDs; Polyhomeotic proteins (PHCs), which provide structural support; and PCGF (Polycomb group RING finger proteins), which are essential for the core enzymatic activity of the complex. PCGF also determines how the core complex interacts with auxiliary components, regulating its chromatin targeting and enzymatic activity. Another key component, Ring1A/B, possesses E3 ubiquitin ligase activity and catalyzes the monoubiquitination of histone H2 A at lysine 119 (H2 AK119ub1), a hallmark of Polycomb-mediated repression [48,50] (Fig. 2E). In contrast, ncPRC1 complexes, which lack CBX proteins, are recruited to chromatin through alternative mechanisms independent of H3K27me3 [51].

CBX proteins are characterized by a CD at the N-terminus and an IDR in the middle region, similar to HP1 [52] (Fig. 2A). These structural features enable CBX proteins to bind to H3K27me3 and interact dynamically with chromatin-associated factors. Notably, similar to HP1, CBX2 exhibits LLPS activity in vitro, which is implicated in chromatin organization through the formation of chromatin condensates, chromatin loops, and chromatin compaction, properties previously known as PRC1 characteristics. (Fig. 2C) [53,54]. These properties emphasize the unique role of CBX proteins in mediating higher-order chromatin architecture and maintaining transcriptional repression (Fig. 2D).

Together, PRC1 and PRC2 function in a coordinated manner to maintain long-term gene repression [55]. This pathway underscores the dynamic yet stable repression of genes involved in development, differentiation, and cellular identity.

5. RNAs in heterochromatin

RNA has emerged as a pivotal player in the establishment and maintenance of heterochromatin, influencing chromatin dynamics across diverse organisms. In addition to histone-modifying enzymes and chromatin-associated factors, RNA-mediated mechanisms provide a layer of regulation that ensures the establishment and maintenance of heterochromatin [56].

In fission yeast, heterochromatin structures formed by H3K9me2 and HP1 (Swi6) are maintained by factors central to the RNAi pathway, including Ago1 (Argonaute), Dcr1 (Dicer), and Rdp1 (RNA-dependent RNA polymerase) [18,21,22]. Deletion of any of these genes reduces H3K9me2 levels in heterochromatin, resulting in defects in heterochromatic silencing [57]. Ago1 forms the RNA-induced transcriptional silencing (RITS) complex, analogous to the RNA-induced silencing complex (RISC) in the RNAi pathway, and binds to target heterochromatin through interactions between incorporated small RNAs and nascent RNAs, as well as through recognition of H3K9me2 by the Chp1 protein [58,59]. RITS recruits the RNA-dependent RNA polymerase complex (RDRC), comprising Rdp1, Cid12, and Hrr1, which converts nascent RNAs into double-stranded RNAs. These are then processed into small RNAs by the action of Dcr1 [60]. This process occurs near chromatin, forming a self-enforcing loop, as the loss of these factors reduces H3K9me2 levels, and depletion of Clr4 (a Suv39 family methyltransferase catalyzing the deposition of H3K9me2) disrupts the small RNA-mediated pathway (Fig. 3A) [18,21,22].

A Similar small RNA-mediated transcriptional silencing mechanism has been reported in *Drosophila*. In the germline of multicellular organisms, such as *Drosophila* and mammals, Piwi (an Argonaute family protein) and Piwi-interacting RNAs (piRNAs) repress transcription of repetitive sequences, including transposons. While silencing by piRNAs was initially thought to occur through post-transcriptional RNA degradation, some Piwi proteins localize to the nucleus. Knockdown of Piwi-encoding genes in germline cells abolishes H3K9 methylation at transposon clusters and impairs transposon silencing, suggesting that Piwi and piRNAs contribute to establishing and/or maintaining heterochromatin-specific marks (Fig. 3B) [61]. In mice, Piwi loss prevents de novo DNA methylation on retrotransposons in germ cells, suggesting an RNA-mediated pathway for inducing DNA methylation [62]. Recent studies revealed that Piwi targets chromatin by forming a complex similar to RITS in fission yeast [23,63] (Fig. 3B).

In fission yeast, *Nematoda*, and *Drosophila*, H3K9me2/3 is a major heterochromatin mark, but in other higher eukaryotes, DNA methylation also plays a critical role. For example, in *Arabidopsis thaliana*, small RNAs repress transposons through pathways involving plant-specific RNA polymerases Pol IV and Pol V [20,21,64]. Pol IV transcribes RNA from heterochromatic regions, which RDR2 (an RNA-dependent RNA polymerase) converts into double-stranded RNA. This RNA is processed by DCL3 (a Dicer family protein) into small RNAs that are incorporated into AGO4 (an Argonaute protein). AGO4 targets heterochromatin using Pol V transcripts as a scaffold, subsequently recruiting H3K9 methyltransferase (KYP) and DNA methyltransferase (CMT3) to form repressive chromatin structures. This pathway closely resembles the fission yeast system, where small RNAs and Argonaute proteins introduce repressive chromatin marks.

Heterochromatin suppresses transcription and recombination of repetitive DNA, and a similar molecular mechanism removes repetitive DNA from genomes [19]. In the ciliate *Tetrahymena*, which has two nuclei, the macronucleus (MAC) and micronucleus (MIC). The reproductive nucleus, MIC, retains chromosomes similar to those of other eukaryotes but is transcriptionally inactive, functioning to pass the genome to the next generation during sexual reproduction. In contrast, the somatic nucleus, MAC, is formed during sexual reproduction through large-scale genome reorganization, involving fragmentation of the micronuclear genome and deletion of approximately 12,000 transposon-like sequences. This process relies on small RNAs (scan RNAs) derived from old MIC, and TWI1 (an Argonaute protein) containing scan RNAs targets repetitive DNA regions, leading to the deposition of histone H3 methylation (H3K9me3 and H3K27me3) and their elimination (Fig. 3C) [19,65]. During this process, nuclear structures resembling chromocenters and Barr bodies have been observed, potentially linking LLPS mechanisms to heterochromatin [66].

RNAi and heterochromatin formation are functionally linked across diverse species, suggesting that these connections are evolutionarily ancient, originally serving genome defense by suppressing transposable elements. In higher eukaryotes, including humans, the role of RNAi in heterochromatin formation is less clear. In flies, chicken DT-40 cells, and mouse ES cells, Dicer loss results in abnormal heterochromatin structure, suggesting a functional link to RNAi [67]. In human cultured cells, RNase treatment following weak detergent exposure abolishes HP1 association with heterochromatin and reduces H3K9me3 signals [68]. In mice, PHC, containing transcribed major satellite RNA, relies on this RNA for chromocenter stability, which involves the RNA-binding protein SAF-B [69,70]. Further, the transcribed major satellite RNA forms R-loops and recruits Suv39h1/2 to maintain H3K9me3 (Fig. 3D) [71–73]. These findings suggest that RNA structurally and epigenetically supports heterochromatin in mammals.

Additionally, long noncoding RNAs (lncRNAs; generally defined as those longer than 200 nucleotides) are implicated in heterochromatin. For example, Xist lncRNA mediates X chromosome inactivation by recruiting factors such as SPEN, which removes histone acetylation to silence gene expression. It then recruits ncPRC1 mediated through the hnRNPK-Xist interaction, which subsequently recruits PRC2, and SmcHD1, establishing and maintaining the silenced state and higher-order chromatin structures (Fig. 3E) [14,74–79]. Other lncRNAs, like HOTAIR and ANRIL, recruit PRC2 to repress target loci. Despite these advances, key questions remain about how specific lncRNAs and histone-modifying enzymes target chromatin regions.

Thus, small RNAs, including siRNAs and miRNAs, have been implicated in heterochromatin assembly, highlighting RNA's central role in chromatin regulation. In addition to small RNAs, many lncRNAs serve as scaffolds for repressive chromatin-modifying enzymes, further underscoring the central importance of RNA in chromatin dynamics.

6. Dynamic chromatin organization through heterochromatinization

Genome-wide analyses of histone modifications reveal that heterochromatic regions marked by H3K9me3 are formed during cell differentiation, alongside larger structural changes in the nucleus in certain cell types. In differentiated cells, heterochromatin is typically associated with the nuclear envelope or nucleoli [80]. This localization is thought to result from physical interactions between the nuclear lamina, which forms a network inside the nuclear envelope, and structural heterochromatin factors. Interestingly, in rod cells of nocturnal animals (a type of photoreceptor cell functioning primarily in darkness), heterochromatin accumulates in the nuclear interior, enhancing light penetration throughout the cell [2,81]. Lamin B receptor (LBR) and lamin A/C have been shown to anchor H3K9me3-rich heterochromatin to the nuclear membrane [82]. Additionally, during cellular senescence, lamin B1 contributes to the formation of senescence-associated heterochromatin foci (SAHFs), a heterochromatic structure within the nucleus [83], suggesting that chromatin reorganization through heterochromatinization involves interactions between heterochromatin factors and the nuclear lamina. Such localization of heterochromatin is also evident in the inactive X chromosome (Barr body) in female mammals, which is facultatively established during early embryonic development. The H3K9me3-enriched domain of the Barr body in human cells remains closely associated with the nuclear envelope or nucleolar periphery, maintaining its transcriptionally repressive state [33,84] (Fig. 1C). This example implies that heterochromatin positioning within the nuclear landscape is corelated with transcriptional silencing [5]. Future studies

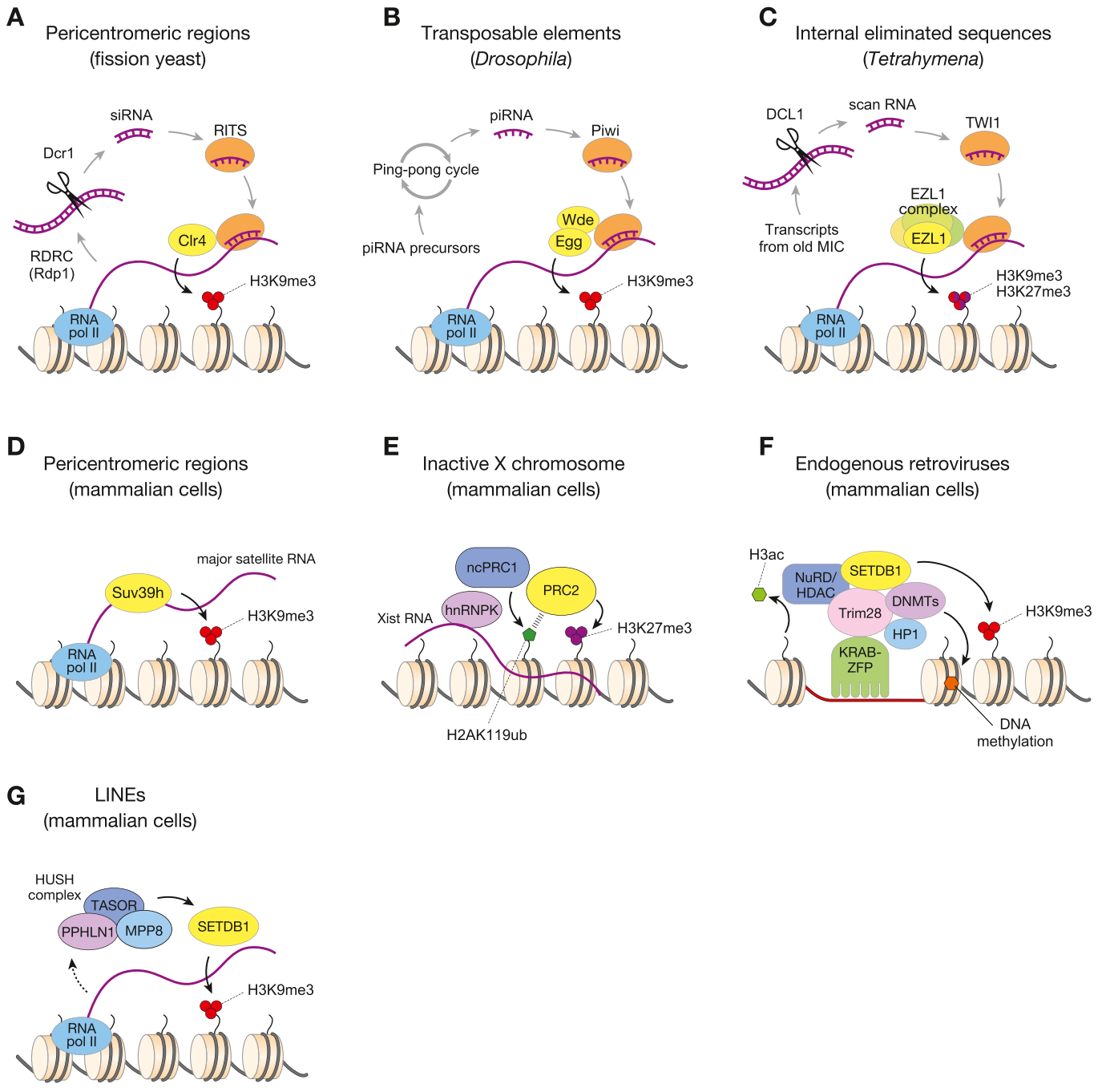

Fig. 3. RNA-mediated heterochromatin assembly.
(A) In fission yeast, pericentromeric repetitive sequences are transcribed by RNA polymerase II. This RNAs are converted into double-stranded RNAs by RNA-dependent RNA polymerase (RdRP) and processed into short double-stranded siRNAs by Dcr1. The d siRNAs are incorporated into the RITS complex, which targets transcribed RNAs via RNA-RNA base pairing. RITS recruitment attracts the methyltransferase Clr4, resulting in the deposition of H3K9me3. (B) In Drosophila germline cells, RNAs transcribed from piRNA clusters are converted into piRNAs via the ping-pong cycle. The piRNAs are incorporated into the Piwi, a member of the Argonaute family, which targets and binds to nascent RNAs transcribed from transposons. Piwi recruitment attracts Wde and Egg, resulting in the deposition of H3K9me3. (C) During sexual reproduction in the ciliate *Tetrahymena*, transcripts produced from the micronucleus genome form double-stranded RNAs, and these double-stranded RNAs are cleaved by DCL1 to produce a small RNAs (scanRNAs). After these scanRNAs are incorporated into TWI1, one of the Aurgonate family proteins, TWI1 scans the old macronuclear genome and then targets the newly developing micronuclear genome. TWI1 further attracts a PRC-like complex containing EZL1, depositing H3K9me3 and/or H3K27me3 at the regions undergoing programmed DNA elimination. (D) In mice, major satellite repeat sequences near the centromere are transcribed into RNA. The methyltransferase Suv39h targets these transcripts, catalyzing H3K9me3 deposition. (E) During X chromosome inactivation in mammalian cells, Xist RNA expressed from the X chromosome inactivation center coats the X chromosome. PRC1, recruited by hnRNPK, provides ubiquitin modifications on H2AK119, further recruiting ncPRC2, catalyzing H3K27me3 modifications, leading to the inactivation of the entire X chromosome. (F) KRAB zinc finger protein (KRAB-ZFP) binds to DNA via their zinc finger domains and recruit Trim28 (KAP1) through the KRAB domain. Trim28 recruits the methyltransferase SETDB1, the NuRD histone deacetylase (HDAC) complex, and DNA methyltransferases (DNMTs), suppressing endogenous retroviral expression by introducing H3K9me3 marks and DNA methylation to nearby chromatin. H3ac: acetylation of H3. (G) In mammalian cells, the human silencing hub (HUSH) complex consisting of MPP8, TASOR, and periphilin (PPHNL1) targets transposable elements such as LINE1 and represses their transcription by recruiting SETDB1, which deposits H3K9me3 at the target regions.

may reveal how interactions between heterochromatin and nuclear lamina or nucleoli components are dynamically regulated during differentiation, senescence, and reprogramming.

The relationship between heterochromatin and cellular stress responses has also been investigated. Using fission yeast as a model system, researchers analyzed how cells acquire caffeine tolerance. Heterochromatin was found to form around gene regions involved in caffeine sensitivity, resembling a phenotype known as epimutation [85]. This suggests that other organisms may also form local heterochromatin in response to environmental stresses. In mammalian epithelial tissues, cells undergo significant morphological changes during development and differentiation, which are often accompanied by mechanical stress. Under such condition, heterochromatin relaxes, with a concurrent decrease in H3K9me2/3 at noncoding genomic regions, increasing nuclear flexibility to cope with external forces [86]. These findings suggest that heterochromatin embodies a dynamic mechanism capable of responding to various environmental stimuli, enabling cells to adapt to changing conditions.

In addition to gene repression, heterochromatin suppresses long terminal repeat (LTR) retroelements, including endogenous retroviruses (ERVs), and non-LTR retroelements, such as LINE1 and Alu elements. ERVs, which account for up to 10 % of the mammalian genome, are repetitive sequences derived from retroviruses. Some ERVs retain the ability to produce infectious viruses, but host cells tightly regulate them to prevent unwanted expression and transposition [87]. While RNA-mediated processes are common in many organisms to repress transposable elements, human KRAB zinc finger proteins (KRAB-ZFPs) appear to recognize ERV sequences to repress their transcription. The human genome contains Approximately 350 KRAB-ZFPs, which originated in early tetrapods [88]. KRAB-ZFPs recognize ERV sequences via their zinc finger domains and recruit corepressors, such as Trim28 (KAP-1) and the histone methyltransferase SETDB1, to deposit H3K9me3 marks (Fig. 3F). For example, ZFP809, one of the KRAB-ZFPs, binds to ERV sequences, and mutations in Trim28 or SETDB1 enable ERV expression [89,90]. While most KRAB-ZFP functions remain unclear, their diversity likely arose from coevolution with various ERVs. KRAB-ZFPs not only suppress ERVs but also contribute to species-specific gene expression networks [88].

A recently identified complex called human silencing hub (HUSH), also acts as an antiviral factor [91,92]. HUSH recruits the chromatin remodeler MORC2 and SETDB1 to deposit H3K9me3 marks, repressing HIV-1, young ERVs, and LINE elements. HUSH specifically targets long, intronless RNA or cDNA, enabling it to distinguish between intron-containing genomic DNA and intronless invasive DNA (Fig. 3G). This mechanism allows HUSH to recognize targets without prior exposure to invasive elements, providing immediate defense against 'nonself' DNA [93]. Notably, HUSH selectively silences evolutionarily young LINE1 elements, while piRNA and KRAB-ZFP pathways confer long-term memory to neutralize similar threats. Collectively, these pathways suppress the expression and insertion of foreign DNA through H3K9me3-mediated heterochromatin formation [94,95].

7. Redundancy of H3K9me3- and H3K27me3-mediated heterochromatin function

Constitutive heterochromatin, defined by H3K9me3, stably occupies PHC that form chromocenters and telomeric regions, playing a key role in chromosome function. Interestingly, it has been reported that knocking out Suv39h1 and Suv39h2 in mouse ES cells leads to the loss of H3K9me3 at chromocenters, with H3K27me3 being introduced in its place [96]. Furthermore, heterochromatin based on H3K9me3 is not necessarily constitutive but can exhibit facultative characteristics. H3K9me3 is crucial for the establishment and maintenance of cell identity, ensuring lineage stability. Genome-wide mapping studies of H3K9me3 have shown that this modification is present in large domains during cellular differentiation, including genes with transcriptional potential [97].

H3K9me3 levels at PHC domains and chromocenter formation undergo dynamic changes during development. In the zygote, both parental PHC domains form rings around the nucleolus-like bodies within their respective pronuclei. However, H3K9me3 distribution differs between the two: while the maternal PHC is enriched in H3K9me3, the paternal PHC lacks this modification. Interestingly, a surge in major satellite transcription from a specific strand at the two-cell stage, concomitant with major zygotic gene activation, is crucial for the incorporation of H3K9me3 into paternal PHC domains and for the reorganization of both parental PHC domains into chromocenters at the late two-cell stage [98,99]. Furthermore, the emergence of early- and late-replicating domains, which are intimately linked to heterochromatin distribution, occurs just after PHC appears in both parental genomes [100]. Even in post-implantation embryos, during the process of differentiation, the establishment of lineage-specific H3K9me3 patterns has been observed, indicating that H3K9me3 is involved in gene regulation, similar to facultative heterochromatin that depends on H3K27me3 [101].

In mouse ES cells, several pluripotency-associated genes are regulated by H3K9me3; KDM3A and KDM4C remove heterochromatin-associated marks (H3K9me2 and H3K9me3), thereby ensuring the maintenance of ES cell self-renewal [102,103]. Interestingly, the status of H3K9me3 has been shown to influence the efficiency of iPS cell production by introducing Yamanaka factors (Oct3/4, Sox2, Klf4, and c-Myc) [104]. In fact, knockdown of the SUV39H1/2 genes, which catalyze the formation of H3K9me3, increases the efficiency of iPS cell production. Therefore, processes that remove established heterochromatin or transiently loosen its structure may be key to future clinical applications of iPS cells. In contrast, while genomic imprinting has traditionally been known to be suppressed via DNA methylation and H3K9me3, recent studies have identified a novel mechanism of non-canonical imprinting. In this mechanism, facultative heterochromatin characterized by H3K27me3 contributes to the metastable repression of imprinting genes in a DNA methylation-independent manner [105]. These findings underscore the distinct yet overlapping roles of H3K9me3 and H3K27me3 in epigenetic regulation, highlighting the complexity in distinguishing their relative stability, dynamics, and reversibility.

8. The ancestral and modern marks for heterochromatin

As discussed so far, and as evidenced by its association with RNA silencing mechanisms, heterochromatin is thought to have evolved to repress foreign genetic elements such as transposons. In fission yeast, transposons accumulated in the PHC are marked by H3K9me2/3 those catalyzed by the evolutionarily conserved Suv39h family methyltransferase Clr4 [106]. In contrast, fission yeast lacks H3K27me3, a mark of facultative heterochromatin in mammalian cells, as well as the E(z)-family enzymes that catalyze this methylation and the related PRC2 complex. These findings suggest that H3K9me3 is an ancestral modification of constitutive heterochromatin. In contrast, facultative heterochromatin regulated by H3K27me3 and PRC2 is an evolutionary modification acquired in multicellular organisms requiring more complex gene expression regulation. Recent findings, however, have blurred the distinction between these two modifications.

In ciliates such as *Tetrahymena and Paramecium*, as described above, both H3K9me3 and H3K27me3 marks are used during development of the somatic nucleus, MAC. Interestingly, both histone H3 methylations are catalyzed by a PRC2-like complex containing E(z)-family enzymes [107]. A similar involvement of PRC2 has recently been reported in a large-scale genome reorganization in the *Paramecium* [108].

Plant heterochromatin studies have focused intensively on angiosperms, particularly *Arabidopsis thaliana*. However, recent comprehensive analyses of the genome and epigenome of the bryophyte *Marchantia polymorpha*, an early diverging land plant, revealed a distinct chromatin landscape. Unlike *Arabidopsis*, *Marchantia polymorpha* lacks

heterochromatin marked H3K9me3, or DNA methylation near centromeres. Instead, transposons widely distributed in its genome are marked by modifications such as H3K9me1, H3K27me1, H3K27me2, and H3K27me3 [109].

Among fungi, basidiomycetes represent a significant group distinct from budding and fission yeasts. Basidiomycete yeasts, such as *Cryptococcus neoformans*, a pathogen causing opportunistic infections, utilize a combination of RNA silencing, H3K9me3, DNA methylation, and H3K27me3. The latter is catalyzed by a PRC2-like complex involved in heterochromatin formation [110]. In species such as *Arabidopsis thaliana*, *Neurospora crassa*, and mammals, which utilize H3K9me2/3 for transposon repression, loss of H3K9me2/3 methyltransferases or DNA methylation-related factors often leads to compensatory use of PRC2-mediated H3K27me3 [111].

These findings challenge the notion that PRC2 and H3K27me3 represent an evolutionarily acquired system for regulating genes involved in development and differentiation. Instead, PRC2 and H3K27me3 appear to represent an ancestral mechanism for transposon repression. Conversely, the Suv39h family of enzymes and the H3K9me3 may be an evolutionarily newer system for constitutive heterochromatin [111]. In fission yeast and *Neurospora crass*, the ancestral PRC2 system may have been lost during an evolutionary arms race with transposons. Understanding the evolutionary roles of H3K9me3 and H3K27me3 will require further chromatin studies in non-model organisms that have yet to be analyzed comprehensively.

9. LLPS and intrinsically disordered proteins (IDPs) in heterochromatin

Significant progress has been made in understanding the molecular structure and function of heterochromatin proteins, such as HP1 and PRCs. However, a substantial gap remains in our knowledge of how large nuclear structures, like those observed under a microscope, are formed from these components. As mentioned above, LLPS may contribute to heterochromatin formation [43–47,79]. LLPS is a principle gaining attention as a mechanism underlying the formation of membrane-less intracellular structures, such as nucleoli and RNA granules. Proteins with intrinsically disordered regions (IDRs), which lack characteristic secondary structures, play a central role in this aggregation process. HP1 contains naturally disordered N-terminal and hinge regions, and both *Drosophila* HP1α and human HP1α have been shown to form droplets in vitro (Fig. 2C). Cellular analyses have confirmed the dynamic movement of HP1 within heterochromatin regions, aligning well with the LLPS model. The fusion of small heterochromatin foci during *Drosophila* development and their sensitivity to aliphatic alcohols suggest that heterochromatin observed under the microscope shares features with other intracellular structures formed through LLPS [43] (Fig. 2D). This mechanism for compartmentalization may also explain how heterochromatin attracts repressors while excluding transcription-related factors such as RNA polymerase. Furthermore, as mentioned above, among the three human HP1 isoforms, only HP1α efficiently forms droplets in vitro, while HP1γ does not [44,46]. The different LLPS capacities of the three HP1 isoforms in vitro suggest that isoform-specific properties in LLPS are critical for their distinct roles in chromatin dynamics and function in cells, with HP1α and HP1β being associated with heterochromatic regions, while HP1γ is associated with euchromatic regions [40,44,46]. The biological implications of these isoform-specific properties remain unclear, underscoring the need for further research into the differences between in vitro behavior and intracellular functionality.

RNA is also implicated in heterochromatic LLPS, akin to its role in other non-membranous nuclear organelles. As earlier described, major satellite RNA and the RNA binding protein, SAF-B contribute to PHC and chromocenter formation. Major satellite RNA enhances the LLPS activity of SAF-B, facilitating chromocenter formation [70].

CBX2, a key component of cPRC1, also exhibits LLPS properties in vitro (Fig. 2C). Even before the concept of biological LLPS gained prominence, PRC1 was recognized for facilitating chromatin looping, enabling interactions between distant genomic regions and contributing to chromatin organization [112,113]. Recent studies have shown that CBX2's LLPS properties are driven by multivalent electrostatic interactions involving its charged regions. This allows facultative heterochromatin to form distinct nuclear compartments enriched in PRC1 components [53,54] (Fig. 2D). Through LLPS, PRC1 is thought to establish repressive domains that exclude transcriptional activators while shielding chromatin from transcriptional machinery. This LLPS state is suggested to complement PRC1's chromatin looping ability by creating physical barriers that isolate silenced regions from transcriptionally active zones [52]. By integrating LLPS and LLPS-associated chromatin looping, PRC1 exhibits adaptive responses to nuclear regulatory demands and environmental cues. Furthermore, CBX2's LLPS properties highlight a potential parallel mechanism between constitutive and facultative heterochromatin in organizing higher-order chromatin structures [52].

The establishment and maintenance of Xi, a transcriptionally silent heterochromatic structure Barr body, require Xist RNA, which coats the entire inactive X chromosome. Xist interacts with various IDR-containing proteins, forming condensates essential for its function. During early X-chromosome inactivation, SPEN recruits HDAC3 to Xi through Xist, removing histone acetylation. SPEN also interacts with chromatin remodelers, transcriptional machinery, and RNA modification factors, forming condensates through its IDR [114]. Additionally, Xist-binding proteins such as MATR3, PTBP1, CELF1, and TDP-43 are critical for gene silencing and Xist stabilization on Xi. These proteins form liquid droplets in vitro in an Xist-dependent manner and assemble condensates with XIST in cells [115]. In human somatic cells, Xi chromatin alternates between H3K9me3-rich and H3K27me3-rich domains [33,84]. HBiX1/LRIF1, an HP1-binding protein associated with SMCHD1, links H3K9me3 domains via HP1 and H3K27me3 domains through SMCHD1, which is enriched on Xi by the Xist-hnRNPK-PRC1 axis, facilitates spatial proximity of these domains across Xi [33], aligning with the chromatin cross-link model [116]. Indeed, transient depletion of HBiX1/LRIF1 or SMCHD1 impairs Barr body formation in human cells. In Xi, in interphase nuclei, the distribution of H3K27me3 wraps around the nucleoplasmic side of H3K9me3 body which is attached to the nuclear envelope [33,78,84] (Fig. 1C), suggesting that HBiX1-SMCHD1-mediated chromatin cross-linking enhances local concentrations of H3K9me3 and H3K27me3 chromatin, promoting the LLPS activities of HP1 and PRCs. This likely contributes to the distinct nuclear architecture of the Barr body.

MeCP2, known for binding methylated DNA and localizing to heterochromatin, exhibits LLPS activity dependent on methylated DNA in vitro [117]. MeCP2 droplets incorporate HP1 while excluding transcription factors like MED1 and BRD4, which also exhibit LLPS properties. This suggests that self-assembling proteins like MeCP2 create chromatin compartments that exclude transcription factors, repressing gene expression. MeCP2 dysfunction causes Rett syndrome, with disease-associated mutations impairing its self-assembly activity. In cells, these mutations reduce MeCP2 and HP1 localization to heterochromatin, weakening heterochromatin integrity and causing widespread gene expression abnormalities.

These findings indicate that chromatin compartmentalization, driven by LLPS mediated by the IDRs of factors such as HP1, CBX2, MeCP2, and XIST-binding proteins, function as an autonomous epigenetic memory. This compartmentalization not only recruits and concentrates heterochromatin-associated factors but also excludes transcription factors, representing a hierarchical layer for regulating gene expression.

10. Conclusions and perspectives

After outlining the basic features of heterochromatin, several recent

advancements were introduced. Although it has been around 100 years since heterochromatin was first named and around 30 years since its major factor, HP1, was identified, the molecular mechanisms underlying the formation and maintenance of this structure remain enigmatic. Recent discoveries have highlighted the pivotal roles of LLPS and the involvement of IDRs in heterochromatin organization. These findings provide a framework for understanding how LLPS create functional nuclear compartments that selectively recruit repressors while excluding transcriptional machinery. Additionally, non-coding RNAs have emerged as critical regulators in heterochromatin formation, acting as scaffolds for chromatin-modifying enzymes and influencing chromatin architecture through RNA-protein and RNA-RNA interactions. These insights underscore the importance of RNA-mediated pathways in the dynamic regulation of heterochromatin, offering new avenues for exploring how gene expression and chromosomal stability are modulated across diverse cellular contexts. Heterochromatin serves a dual role as both a genome guardian and a regulator of gene expression. On one hand, it silences transposons and other repetitive elements, maintaining genome stability. On the other hand, it contributes to the spatial and functional organization of chromosomes, regulating gene expression during development and differentiation. Disruptions in heterochromatin structure have been implicated in various diseases, including cancer and neurodegenerative disorders. Further studies integrating detailed molecular analyses with cell biological approaches are expected to deepen our understanding of heterochromatin. Such research will not only elucidate its fundamental roles in genome organization and stability but also provide critical insights into its involvement in health and disease.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 4o in order to English editing. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

CRediT authorship contribution statement

Chikashi Obuse: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Jun-ichi Nakayama:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Chikashi Obuse reports financial support was provided by MEXT & JSPS in Japan. Jun-ichi Nakayama reports financial support was provided by MEXT & JSPS in Japan. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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