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Review

Phase separation and inheritance of repressive chromatin domains

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Polycomb-associated chromatin and pericentromeric heterochromatin form genomic domains important for the epigenetic regulation of gene expression. Both Polycomb complexes and heterochromatin factors rely on 'read and write' mechanisms, which, on their own, are not sufficient to explain the formation and the maintenance of these epigenetic domains. Microscopy has revealed that they form specific nuclear compartments separated from the rest of the genome. Recently, some subunits of these molecular machineries have been shown to undergo phase separation, both *in vitro* and *in vivo*, suggesting that phase separation might play important roles in the formation and the function of these two kinds of repressive chromatin. In this review, we will present the recent advances in the field of facultative and constitutive heterochromatin formation and maintenance through phase separation.

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Introduction

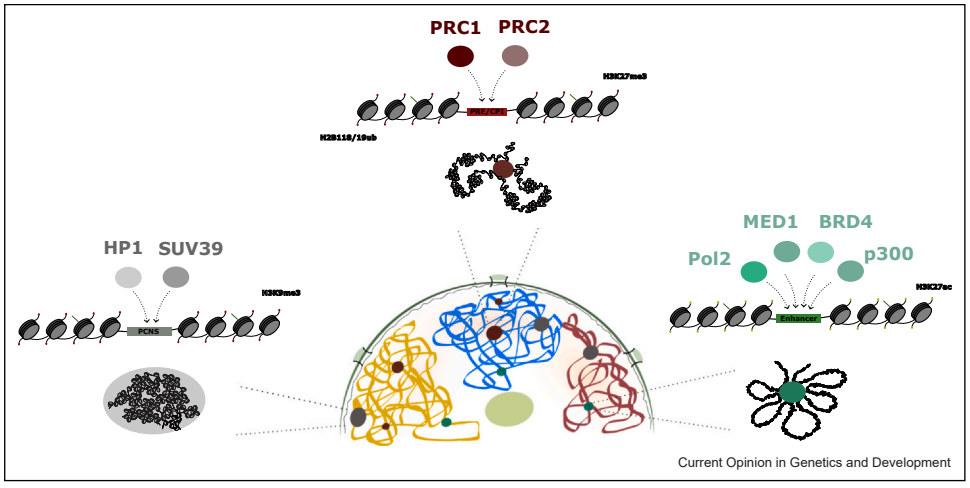
The eukaryotic genome consists of different epigenetic domains separated from each other in 3D in the cell nucleus, and maintenance of the right epigenetic landscape through cell division is critical for homeostasis. Constitutive (HP1-bound H3K9me3 domains) and facultative heterochromatin (Polycomb domains marked by H3K27me3) are two types of epigenetically inherited domains [4]. They repress transcription through higher-order chromatin compaction (Box 1) and are involved in epigenetic processes such as variegated gene expression [19] and in the maintenance of gene silencing throughout development and adult life [9]. Moreover, both Polycomb-associated chromatin and heterochromatin have been shown to be involved in transgenerational (Box 1) inheritance [13,35]. As the enzymes that catalyze the respective histone modifications can read their own reaction product and are allosterically regulated by them, the establishment of these domains has been described as the result of a 'read and write' mechanism [28]. Modeling approaches however argue that the read and write mechanisms alone cannot be enough for the long-term maintenance of heterochromatin domains [1,2,44,50].

Key components of constitutive and facultative heterochromatin have been shown to set up multivalent interactions and form molecular condensates (Box 1) by phase separation, which can affect their function. Phase separation is a general term that indicates the formation of different compartments in which certain types of molecules can segregate. This phenomenon can occur in different physical states, including liquid, solid or gel-like state. Both Polycomb and heterochromatin components were shown to form condensates that can drive a liquid–liquid phase separation (LLPS) process *in vitro*, offering an attractive explanation for the clustering of these and other molecular components into membraneless condensates in the nucleus (Figure 1) [6,42]. Phase separation of biomolecules into condensates has been suggested to provide the cells with several benefits, including sensitivity to reversible inputs (temperature, ion concentrations, concentration of the individual complex components), the generation of an isolated microenvironment, which provides reaction specificity and regulation of reaction kinetics. Intrinsic properties of the components and their interplay with their molecular environment create a robust system where dynamic regulation of biological processes can be achieved without compromising on specificity [21,60,69]. In this review, we will focus on the role of phase separation in chromatin-based epigenetic maintenance and transmission and discuss how the known biophysical properties of these complexes can be relevant for this process.

Box 1 Some terms in the field, such as droplet or chromatin condensation, are often used with slightly different meaning in the literature. Here, we list some of the potentially confusing terms and define how they are operationally used in this review.

Term	Definition used in this paper
Droplets	Condensates formed via LLPS.
Liquid	A state of matter, characterized by the loose contact between the molecules, enabling them freely moving within the structure.
Condensates	A pool of biomolecules, clustered in a separated volume in the rest of the cyto-/nucleoplasm, which can be detected as a foci by confocal imaging.
Chromatin compaction	A conformation of the chromatin, in which the target loci of the chromatin-modifying enzymes get closer in 3D space, resulting in a smaller volume of the target chromatin, and fostering more confined activity of the enzymes.
Epigenetic inheritance	Transfer of epigenetic information during/after cell division. Long-term epigenetic memory.
Transgenerational epigenetic inheritance	Transfer of epigenetic information to multiple subsequent generations, at least to the F2, when assessing inheritance through males and to the F3, when assessing inheritance through the female germ line.

Figure 1



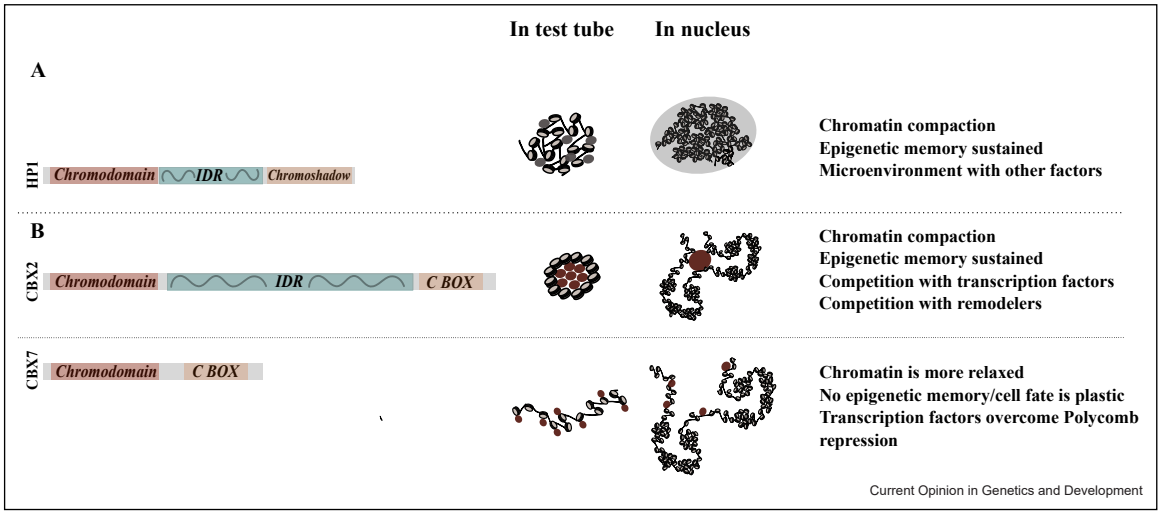
Chromatin compartments in the 3D nucleus and simplified depiction of some of the proteins involved in the formation. Gray: constitutive heterochromatin, which forms large nuclear chromatin domains. Dark red: Polycomb foci. Green: transcription foci. The main proteins or protein complexes and their associated histone marks are depicted.

Constitutive heterochromatin

The main hallmark of constitutive heterochromatin is the formation of densely packed, transcriptionally repressed, H3K9me3-modified chromatin domains, mainly including pericentromeric heterochromatin regions (Figure 1) [19]. Mechanistically, the proteins involved in the formation and the maintenance of heterochromatin rely on a 'writer–reader' system composed of Suvar39, which specifically trimethylates H3K9, and HP1 proteins, which recognize this chromatin mark. The mammalian genome contains three HP1 paralogs: HP1α, HP1β and HP1γ. While the three paralogs show a high degree of homology, HP1α is mostly associated with gene silencing, HP1β plays both gene activating and repressive roles, and HP1γ is more often associated with transcription [28]. Recently, LLPS suggested a possible explanation for how single HP1 molecules, which bind to chromatin with a fast dynamics in the order of seconds [12,23], might promote the formation of repressive chromatin states that are stable for several hours [26,52]. Indeed, the human HP1α protein is able to form phase-separated droplets (Box 1), which is stimulated by either phosphorylation of its N-terminal extension or DNA binding [37]. Similar results have been reported for the *Drosophila* HP1a protein, which forms droplets *in vitro* and nucleates into foci that display liquid properties during heterochromatin domain formation in early *Drosophila* embryogenesis [58]. Moreover, nucleosomal arrays marked by H3K9me2 and H3K9me3 and associated HP1-containing complexes undergo phase separation to form macromolecule-enriched droplets [63]. Altogether, these works suggest that HP1 can be the driver of phase separation of heterochromatin into condensed structures, helping to keep a high local concentration of heterochromatin factors (Figure 2).

The role of LLPS in heterochromatin formation appears to be more complex than suggested by initial works.

Figure 2



Putative effects of protein and chromatin compaction on the function and memory of epigenomic domains. **(a)** Left: structural domains of HP1 composed of an N-terminal chromodomain, a middle unstructured hinge region and a C-terminal chromoshadow domain. Middle: Depiction of condensates formed by HP1 and nucleosomal arrays *in vitro*. Right: depiction of the heterochromatin foci observed *in vivo*. Gray shadow around nucleosomes represents HP1 and other heterochromatin factors, such as Su(var)s, MeCP proteins and KAP1, that can vary in different foci. The formation of compact chromatin into a specific compartment can create an isolated reaction environment and enhance the efficiency of heterochromatin inheritance (Grewal et al. [37]). **(b)** Top right: structural domains of CBX2 composed of N-terminal chromodomain, unstructured region in green and a C box. *In vitro*, CBX2 can compact nucleosomal arrays via its intrinsically disordered region (IDR) which is essential for the stability of Polycomb-associated chromatin *in vivo* (**b**, left). CBX7 is a CBX homolog, which does not possess the IDR and is unable to compact nucleosomal arrays *in vitro*. In the absence of IDR, Polycomb-targeted chromatin is less compact [27,34]. Illustration of the organization of the corresponding proteins within chromatin has been inspired by electron microscopy images [55,61], and these representations should be viewed as simplified schemes.

Each HP1 paralog has different ability to form liquid condensates, and it has been shown that HP1α condensates can be readily dissolved by HP1β. The disordered regions of each HP1 paralog are responsible for differences in DNA compaction and phase separation properties [32]. Moreover, different behaviors have been reported for HP1 and for chromatin itself since condensed chromatin exhibits solid-like behavior, whereas heterochromatin proteins display liquid-like behavior and coalesce around the solid chromatin scaffold *in vivo* [57]. The role of LLPS in heterochromatin formation has also been challenged by a work, which reported that HP1 has only a weak capacity to form droplets in living cells. The authors conclude that heterochromatin foci resemble collapsed polymer globules that are percolated with the same nucleoplasmic liquid as the surrounding euchromatin [20]. Finally, by considering the interplay between the *in vitro*–derived LLPS properties of HP1 and those of the viscoelastic chromatin scaffold, a recent work suggests that anomalously slow equilibration kinetics results in the coexistence of multiple long-lived microphase-separated compartments [60]. In sum, although the formation of HP1 droplet *in vitro* is well understood, its role in the context of the cell nucleus is more difficult to address because of the complex nucleoplasmic composition and of its interplay with long chromatin fiber.

Polycomb chromatin

Facultative heterochromatin, or Polycomb chromatin, is characterized by large H3K27me3 domains in which Polycomb group (PcG) proteins bind discrete genomic elements. Two main types of Polycomb complexes are Polycomb Repressive complex 2 (PRC2) that specifically trimethylates H3K27 and Polycomb Repressive complex 1 (PRC1), which deposits the H2AK119ub mark. These two classes of complexes mutually reinforce their function to induce chromatin compaction and robust gene silencing [53]. In the cell nucleus, chromatin associated to H3K27me3 and PcG proteins accumulates in nuclear nanocompartments called Polycomb foci (Figure 1) [11]. Although PRC1 was clearly involved in the formation of these foci, the underlying mechanism remains unclear. Initial works on phase separation and Polycomb biology showed that CBX2 can phase separate *in vitro*. This ability can be abolished by point mutations on the intrinsically disordered region (IDR) of CBX2, which is also required to form Polycomb foci *in vivo* and to compact nucleosomes *in vitro* (Figure 2) [27,47,59]. During spermatogenesis, the CBX2 subunit of PRC1 complex forms condensates via its IDR, and this condensation is essential for cell differentiation, maintenance of Polycomb target gene repression, and chromatin compaction [34]. The SAM domain of Ph, another subunit of PRC1, is required to form Polycomb foci in *Drosophila* [64]. Although SAM domains can

oligomerize [31], truncated PH containing its SAM domain phase separates *in vitro* and is able to concentrate DNA as well as other PRC1 subunits from *Drosophila* cell nuclear extracts [54]. Moreover, 1,6-hexanediol, which disrupts liquid condensates, reversibly disrupts PRC1-mediated clustering of Polycomb-associated chromatin in mouse embryonic stem cells [66]. Altogether, these works suggest that Polycomb foci correspond to nuclear condensates formed by LLPS. However, a strict demonstration that PcG proteins phase separate *in vivo* is still lacking because the resolution of optical microscopy prevents characterization of their internal structure, in contrast to bigger assemblies observed *in vitro*. Also, recent work studying compaction of Polycomb chromatin at single cell level concluded that Polycomb domains do not mediate chromatin compaction through LLPS; rather, they possess spatial feedback induced by transient long-range interactions [41].

The composition of the Polycomb machinery is not uniform. Many different Polycomb complexes coexist in the nucleus, with functions that are only partially overlapping [53]. This seems difficult to reconcile with a simple form of LLPS where all the subunits would gather in the same condensates. Recent works focusing on individual CBX subunits shed light on the heterogeneity of Polycomb-associated condensates. Live-cell single-molecule imaging indicates that CBX2 nucleates on chromatin independently of H3K27me3 and other CBX2-PRC1 complex subunits. The formation of condensates on CBX2-PRC1 containing chromatin requires interactions between CBX2 and DNA [59]. A scaffold-client model has been proposed to explain the formation and the regulation of PRC1 condensates, where CBX2 behaves as the scaffold while the other PRC1 subunits are clients [8]. PRC1 subunits are key components driving LLPS associated to the Polycomb machinery. PRC1 was shown to form multicomponent condensates through hetero-oligomerization preferentially seeded at H3K27me3 marks, whereas H2AK119ub marks appear after condensate formation [18]. Since these condensates promote a chromatin compaction, which persists even when the condensates have been removed, the authors concluded that the PRC1 condensates function as 'reaction hubs', where the histone marks, rather than PRC1 condensates, could be the main driver of chromatin compaction. These data were recently extended by a chromatin reconstitution approach and single-molecule imaging, which showed that nucleosomal arrays reduce the PRC1 concentration required to form condensates by 20-folds and that the CBX subunit is required for condensate formation, whereas PHC subunits are required for their stability [43]. Furthermore, individual PHC and CBX subunits modulate condensate initiation, morphology, stability and dynamics [43], suggesting that different PRC1 complex forms might perform specific functions partly thanks to their differential ability to set up higher-order nuclear foci.

Phase separation and inheritance of chromatin states

During replication, histone marks are diluted at each cycle and need to be re-established for proper inheritance. Modeling studies showed that long-term epigenetic memory may require several ingredients. A physical model suggests that sustained epigenetic memory relies on (1) a compartmentalized nucleus, (2) spreading of the epigenetic marks in 3D, (3) limited concentration of read–write enzymes that catalyze epigenetic modification [44]. Other approaches also provided models that supports these three criteria. In addition, they suggest that epigenetic memory requires (4) strong enough positive feedback of reader and writer chromatin modifiers and (5) genomic bookmarking of the chromatin factors for the fidelity of the reestablishment of epigenetic domains [1,50]. As mentioned in the previous sections, several works reported that critical factors involved in the formation of both kind of heterochromatin undergo LLPS, which can serve as a versatile mechanism to provide these criteria. The presence of nucleosome bridges or chromatin domains that can create a compaction allowing communication with distant loci could generate the 3D environment required for spreading of chromatin marks in a particular domain (first and second criteria). The nucleation and condensation of chromatin factors such as HP1, HDAC, and Su(var)s or PRC1 and PRC2 could create an isolated environment enhancing robust feedback mechanisms, where all specifically required elements may cluster in the same condensates (fourth criteria). The clustering of epigenetic factors in a condensate would then prevent the illegitimate spreading of chromatin domains, while allowing the spreading of the marks specifically around the nucleation sites. Preventing factors from freely diffusing out of the condensates would effectively increase their local concentrations and function within their cognate chromatin domains. A recent study provided experimental support for this idea. When the IDR of Ccc1, a member of the PRC2 complex in *Cryptococcus neoformans* , was mutated to prevent phase separation *in vitro*, the ectopic recruitment of PRC2 was shown to induce the deposition of ectopic H3K27me3 at HP1 domains [38]. This suggests that the ability of Ccc1 to recognize H3K9me and the ectopic recruitment of Ccc1 to HP1 domains is suppressed by the phase separation of Ccc1, which therefore only accumulates in Polycomb domains. On the other hand, it has been reported in another study that after excision of PREs in Drosophila, PRC2 can propagate the mark for a few cell divisions, but silencing is gradually attenuated and finally lost. H3K27me3 inheritance alone without local PRC2 recruitment to the PREs is not enough to maintain the off state a target gene, showing that localized activity of the Polycomb machinery is critical [14,36].

Condensates might be dispersed upon chromosome condensation in mitosis and meiosis, but during the critical phase in which they are dispersed, epigenetic inheritance (Box 1) could be conveyed by the transmission of histone

marks through cell division, as predicted by earlier works [48,68]. In *Drosophila*, maternally inherited H3K27me3 marks a subset of the Polycomb domains in the early embryo and represses the illegitimate activation of enhancers and lineage-specific genes during development [67], highlighting the role of H3K27me3 histone mark in intergenerational inheritance. It has also recently been shown that quick restoration of H2AK119ub is required for accurate and slower restoration of H3K27me3 after replication [25]. These data support the fifth criteria suggested by the modeling studies. It is noteworthy that observation of both spreading, and inheritance of heterochromatin relies on maintenance of a critical density of the domains [15,44], suggesting a mechanistic link between the establishment and the maintenance of chromatin domains, both relying on the phase separation of the factors involved [51].

Enzyme concentration might be regulated by protein homeostasis. However, having several nucleation sites along the genome and condensation of the enzymes around these sites (which might be facilitated by phase separation) can be another way to provide the third criterion [38,50]. Altogether, one might postulate that phase separation is a key process responsible for the compartmentalization of heterochromatin helping the proper and efficient deposition of heterochromatin marks and epigenetic inheritance. We note however, that other models of inheritance which do not imply the essential role of chromatin condensation or 3D compartments [48,68], are also possible, although not the subject of this review.

Regulation of transcription also involves condensation of transcription components via phase separation [30,49,65], yet their inheritance is much less documented. Still, there is evidence that bookmarking of histone marks and transcription factors can lead to their inheritance [5,7,33,45,56]. Sustained promoter–transcription factor interactions can even allow basal level of transcription during mitosis [22]. While the principles discussed above might underlie epigenetic memory of silent chromatin states, an important question is whether similar principles might apply to active chromatin states. Intriguingly, recent research showed that compact chromatin regions marked by H3K9me3 are required for the maintenance of mitotic bookmarking of some transcription factors such as Essrb [16]. This suggests the possibility that LLPS mechanisms might not only regulate heterochromatin and transcription separately, but they might also set up a cross-talk between them.

Conclusion

Although many studies provide insights in the formation of specific nuclear compartment without membranes within the cell nucleus, many questions remain unanswered. A direct experimental data showing the necessity of chromatin and protein condensation for epigenetic inheritance is still missing, mainly due to the technical difficulties and lack of consensus on the biophysical properties of biomolecules. Understanding the biophysical nature of chromatin-associated condensates would provide insights into the regulation and maintenance of the epigenetic domains. For instance, Polycomb-mediated transgenerational epigenetic inheritance is sensitive to the growth temperature in *Drosophila* [3]. Since the properties of Polycomb foci in flies are also temperature dependent, this observation raises the possibility that the biophysical properties of Polycomb foci participate in transgenerational epigenetic inheritance of Polycomb-dependent silencing [10,24].

The role of IDRs of chromatin-binding factors on the sequestration of chromatin domains is difficult to disentangle from other possible roles, such as enabling specific interactions, enzymatic or direct architectural activities (i.e. in driving chromatin compaction). It is thus important to investigate further the sequence grammar of IDRs, to identify which domains are sufficient to drive condensation and to understand whether it is possible to separate this function from other regulatory roles of associated protein domains. Lyons et al. present an example of this kind of study, which shows that a particular IDR charge pattern of MED1 is crucial for selectively partitioning RNA Pol 2 and its positive allosteric regulators while excluding its negative regulators [40]. Another study on cBAF showed that the differences in the sequences of IDRs in different subunits could serve different functions, either in clustering or to drive heterotypic interactions [46].

As a final note, although in this review we focused on LLPS in chromatin-based epigenetic inheritance, other biomolecular condensates such as germ granules, P-bodies or prions, that are not directly associated with chromatin, are also involved in phenomena of cellular memory and transgenerational inheritance [17,29,39,62]. This suggests that the regulation of the physical states of a variety of cellular components might be involved in the inheritance of functional states across cell division and through organismal generations.

Data Availability

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

Declaration of Competing Interest

None.

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