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Review

Phase-separated chromatin compartments: Orchestrating gene expression through condensation

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ABSTRACT

Eukaryotic genomes are organized into distinct chromatin compartments, some of which exhibit properties of biomolecular condensates. These condensates primarily form due to chromatin-associated proteins/complexes (CAPs). CAPs play a crucial role in gene expression, functioning as either transcriptional repressors or activators. Phase separation, a well-established biophysical phenomenon, is a key driver of chromatin condensate formation by CAPs. Notably, multivalent CAPs with the ability to engage in diverse interactions promote chromatin compaction, leading to the formation of transcriptionally repressed compartments. Conversely, interactions between intrinsically disordered region (IDR)-containing transcriptional regulators, mediated by their multivalent IDRs, lead to the formation of protein-rich, transcriptionally active droplets on decondensed genomic regions. Interestingly, both repressive heterochromatin and activating euchromatin condensates exhibit spontaneous phase separation and selectively enrich components with concordant transcriptional functions. This review delves into the mechanisms by which transcriptionally repressive CAPs orchestrate the formation of repressed chromatin domains. We further explore how a diverse array of transcription-related CAPs or core histone variants, via phase separation, influence gene expression by inducing erroneous transcription events, regulating expression levels, and facilitating the interconversion of transcriptionally repressed and active regions.

1. Introduction

Eukaryotic cell nuclei are remarkably intricate, densely packed environments characterized by a high degree of compartmentalization (Handwerger et al., 2006). This process spatially segregates the cellular interior, facilitating efficient biochemical reactions and maintaining complex biological processes. Organelles, specialized structures with distinct compartments demarcated by clear boundaries, are the primary drivers of compartmentalization. Traditional membrane-bound organelles, such as the nucleus, endoplasmic reticulum, mitochondria, and lysosomes, utilize phospholipid membranes to ensure functional stability and regulation. However, these membranes can impede processes like organelle assembly/disassembly and macromolecule exchange. Higher eukaryotes also possess membrane-less organelles (MLOs), including nucleoli, centrosomes, Cajal bodies and so on. MLOs maintain distinct boundaries with the surrounding cytoplasm or nucleoplasm, fostering markedly different biochemical environments.

The eukaryotic cell nucleus houses not only a diverse array of nuclear MLOs but also the vast genomic DNA, meticulously organized into chromatin structures. Chromatin exhibits a complex organization that spans multiple levels, ranging from nucleosomes to chromosomes (Quiroga et al., 2022) (Fig. 1). The fundamental unit of chromatin, the nucleosome, comprises 147 base pairs (bp) of DNA wrapped around an octamer of histone proteins (two copies each of H2A, H2B, H3, and H4) (Luger et al., 1997). These nucleosomes are linked by linker DNA, resembling a 10 nm "beads-on-a-string" arrangement. Further compaction occurs through the action of CAPs, ultimately resulting in a gradient condensed chromatin structures (Olins et al., 2003) (Fig. 1A and B). *In*

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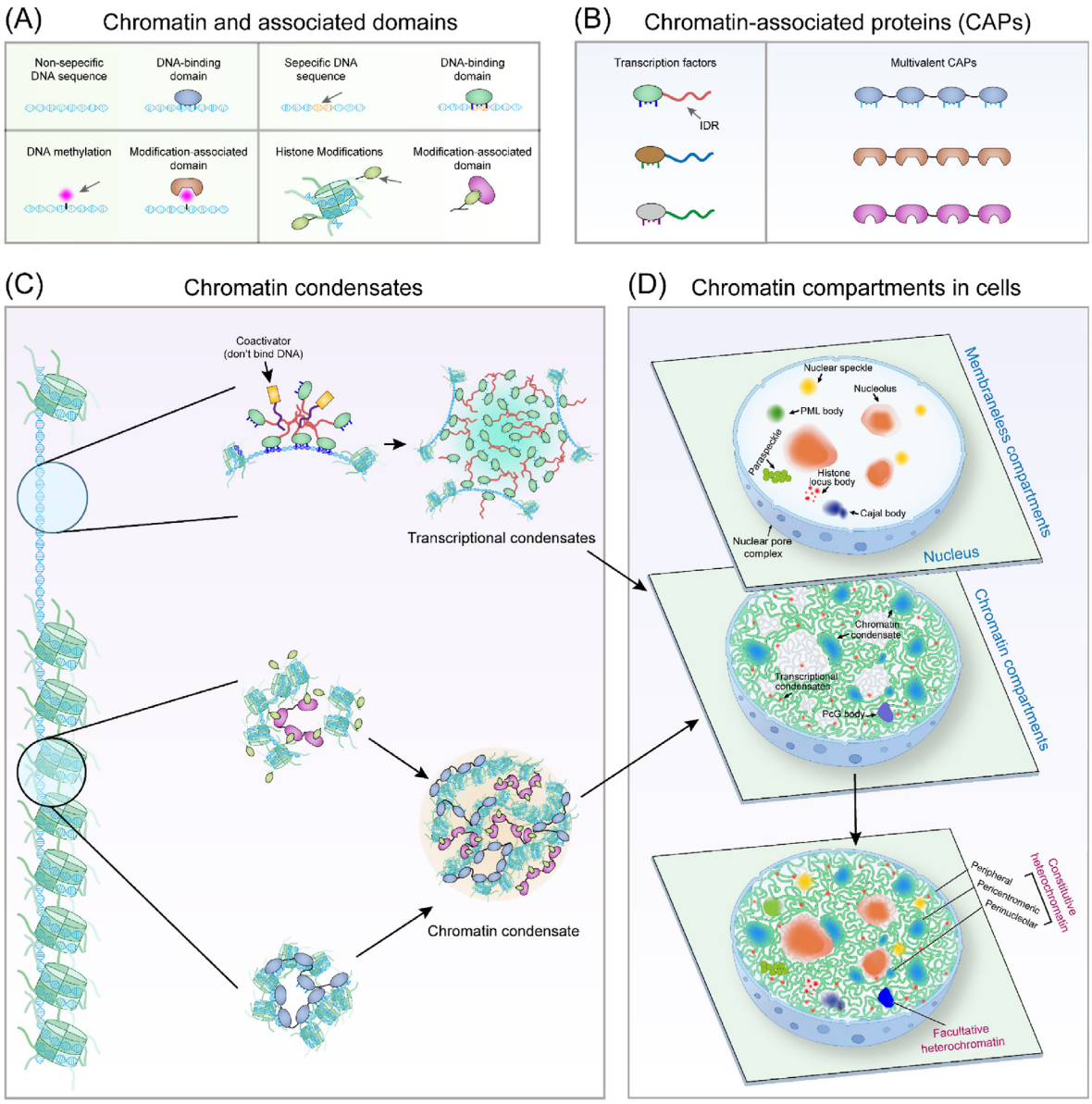

Fig. 1. Multivalent interactions of chromatin-associated proteins and phase separation in chromatin organization. (A) Elements providing multivalent binding sites on chromatin. For example, specific DNA sequences can be bound by sequence-specific CAPs. Negatively charged DNA sequences can also be non-specifically bound by positively charged caps. DNA methylation and post-translational modifications (PTMs) on histone tails can be bound by caps specifically recognizing these modifications. (B) Two types of CAPs. Transcription factors (TFs), for instance, may inherently bind to only one site on chromatin but can mediate multivalent interactions through IDRs. Another type of CAPs may interact with multiple sites on chromatin simultaneously through large positively charged regions and multiple chromatin modification recognition domains (schematically represented as tandem repeats of the same recognition domain), facilitating multivalent interactions with chromatin. (C) Phase separation mediated by two types of caps interacting with chromatin. The two types of CAPs described in panel (B) can form different chromatin compartments through phase separation upon interacting with chromatin. (D) The formation of different chromatin compartments as shown in panel (C) may represent the formation of heterochromatin and transcriptionally active condensates on euchromatin in the eukaryotic nucleus.

vitro experiments have demonstrated that 10 nm chromatin fibers can condense into 30 nm fibers (Song et al., 2014). Advances in high-throughput sequencing (such as in situ Hi-C) and microscopy techniques (such as 3D super-resolution and scanning electron microscopy) have revealed a highly structured nuclear architecture characterized by ~200 nm chromatin domains (Chagin et al., 2016; Ferreira et al., 1997; Strickfaden et al., 2020), including topologically associating domains (TADs) (Dekker & Heard, 2015). At larger scales, two major "compartments", A and B, have been identified (Lieberman-Aiden et al., 2009). Functionally, compartment A closely corresponds to euchromatin, while compartment B aligns with heterochromatin, though this is a simplification. Loop extrusion and compartmentalization are the two driving forces behind this 3D chromatin structure (Schwarzer et al., 2017). TADs are delineated by convergent binding of CTCF and cohesin complexes, which facilitate the formation of chromatin loops (Zuin et al., 2014). Cohesin, a ring-shaped protein complex, mediates loop extrusion by extruding DNA until it encounters convergent CTCF proteins, thereby stabilizing the interaction and creating a chromatin loop; however, this model fails to fully explain the selective formation of loops at only a subset of CTCF/cohesin binding sites (Quiroga et al., 2022). Interestingly, despite its role in gene expression, cohesin depletion does not completely disrupt the higher-order structure of chromosomes (Quiroga et al., 2022). Emerging evidence suggests that polymer phase separation, a passive process driven by the intrinsic properties of chromatin components, plays a more pivotal role in shaping the overall 3D chromatin architecture than previously thought. Recent studies have demonstrated

that chromatin self-assembles into distinct compartments through combinatorial interactions of chromatin factors, such as CTCF and cohesion providing a thermodynamically driven explanation for the formation of TADs and other higher-order chromatin structures. Unlike the energy-dependent loop extrusion model, phase separation explains the stochasticity and persistence of TAD structures even without cohesin. While loop extrusion and phase separation may not be mutually exclusive, the growing body of evidence suggests that phase separation provides a more comprehensive framework for understanding the complex and dynamic nature of 3D chromatin organization. Given the computational nature of TADs and A/B compartments, unlike the direct observation of phase separation-mediated membraneless organelles and chromatin compartments via fluorescence microscopy, the chromatin compartments discussed here specifically refer to fluorescently labeled heterochromatin domains (Fig. 1C and D).

Two main mechanisms, polymer-polymer phase separation (PPPS) and liquid-liquid phase separation (LLPS), explain the formation of chromatin subcompartments. In PPPS, specific proteins bridge chromatin segments, creating distinct compartments (Arnould et al., 2023; Conte et al., 2020; Ryu et al., 2021). This forms an ordered, globular chromatin structure. Bridging molecules like histone tails, CTCF, cohesin, condensing, and SMC complexes link different chromatin regions. Experimental studies show these proteins form clusters through bridging, leading to phase separation and chromatin compartments (Arnould et al., 2023; Conte et al., 2020; Ryu et al., 2021; Wei et al., 2022). LLPS is driven primarily by weak, multivalent interactions between soluble factors, resulting in the formation of liquid-like condensates that assemble around chromatin binding sites (Fig. 1). Key differences include the type of interactions and the resulting chromatin structure (Banani et al., 2017; Li et al., 2012). PPPS creates a more ordered, solid-like structure, while LLPS forms more fluid, dynamic condensates. Both mechanisms allow for rapid component exchange, contributing to chromatin's dynamic nature. PPPS doesn't need direct interactions between bridging proteins, unlike LLPS, which is driven by weak multivalent interactions. While many examples involve direct interactions between bridging proteins, they could potentially be categorized under LLPS. Given the complexity of phase separation mechanisms and limited experimental data, it's challenging to definitively differentiate between PPPS and LLPS. Consequently, this review will use "phase separation" to encompass both terms.

Epigenetic post-translational modifications (PTMs) serve as the pivotal in orchestrating the compartmentalization of chromatin within the nucleus (Allis & Jenuwein, 2016). These modifications act as distinctive "markers" that dictate the functional state of different chromatin regions. Repressive modifications attract proteins with "reader" domains (Musselman et al., 2012) (Fig. 1A and B), leading to further chromatin compaction and gene silencing. In contrast, euchromatin, associated with active gene expression, has a more open structure due to activating modifications. Biomolecular condensates, often formed by protein-protein or RNA-RNA interactions, assemble on euchromatin to facilitate active transcription (Shin et al., 2018). Furthermore, this interplay between epigenetic modifications and protein-RNA interactions creates distinct chromatin compartments, ensuring precise gene regulation. Building upon the role of epigenetic modifications in chromatin compartmentalization, recent research suggests that chromatin condensation within the nucleus occurs through phase separation.

A growing body of research on chromatin phase separation compartments has led us to identify two primary mechanisms by which condensates form. These mechanisms differ significantly in the extent to which chromatin is actively involved in the assembly process. The first mode requires CAPs to engage in weak multivalent interactions directly with DNA or nucleosomal arrays (Hansen et al., 2021). In this scenario, chromatin becomes an active participant, being incorporated and compacted alongside CAPs within the condensates. This condensation is further regulated by DNA sequence features and epigenetic modifications like histone or DNA alterations (Fig. 1 C). In contrast, in the second scenario, certain CAPs possess IDRs that inherently promote phase separation through weak, self-associating interactions (Banani et al., 2017). Chromatin serves primarily as a platform, offering binding sites for these CAPs. Consequently, CAP-CAP interactions dominate as the driving force for phase separation. Chromatin involvement is relatively minimal, and its compaction is driven primarily by the self-assembly of CAPs (Fig. 1 C). Building upon the two fundamental chromatin phase separation mechanisms outlined above, this review delves into the intricate interplay between CAPs, phase separation, and their combined impact on chromatin architecture and gene regulation. We initially focus on how CAPs with repressive functions orchestrate the formation of condensed chromatin domains, effectively silencing gene expression. Subsequently, we broaden the scope to explore how a diverse array of CAPs, or even core histones themselves, utilize phase separation beyond repression to influence gene expression in various ways. This includes triggering aberrant transcription events, precisely regulating expression levels, and facilitating the dynamic conversion between transcriptionally repressed and active regions.

2. Repressive CAPs: orchestrate heterochromatin silencing

Heterochromatin, a distinct chromatin type characterized by its compact structure and transcriptional repression, plays a crucial role in cellular differentiation and other biological processes (Probst & Almouzni, 2008). The formation of heterochromatin is facilitated by various factors, including phase separation, which contributes to the concentration and compaction of chromatin (Li & Reinberg, 2011). Abnormal heterochromatin formation can lead to developmental delays and diseases, emphasizing the importance of understanding this process.

DNA's negative charge is balanced by positively charged histone tails, which wrap around the DNA (Iwaki et al., 2007). Metal cations in physiological salt solutions further promote chromatin condensation by shielding DNA's negative charge (Davie et al., 1978). The physical properties of these condensates are debated, with some studies suggesting liquid-like behavior and others proposing a more solid-like state. Differentiated cells show regions of both compact heterochromatin and dynamic euchromatin (Eshghi et al., 2021), indicating chromatin condensation may exhibit viscoelastic properties (Muzzopappa et al., 2021). Overall, chromatin condensation is a complex process involving charge interactions, histone modifications, and potentially a combination of liquid and solid-like states within the nucleus.

Hundreds of CAPs exist, containing domains that recognize chromatin/DNA with high affinity (McBryant et al., 2006; Ruthenburg et al., 2007). However, only a limited number have been demonstrably capable of inducing deep compaction of unmodified chromatin into higher-order structures *in vitro* (Li & Reinberg, 2011). These include H1, PRC1, PRC2, L3MBTL1, MENT, MeCP2, and the SIR complex. Notably, most of these proteins are involved in heterochromatin formation and gene silencing within cells. Heterochromatin, a distinct chromatin type, is enriched with repetitive major satellite repeats and marked by high levels of H3K9me3, DNA methylation, and their associated binding proteins (CAPs) (Probst & Almouzni, 2008). Multiple forces contribute to the organization of constitutive heterochromatin, many of which exhibit multivalency and are known to undergo phase separation. These interactions often concentrate and compact chromatin, leading to transcriptional repression (Li & Reinberg, 2011).

2.1. CAPs recognizing multivalent histone PTMs

Heterochromatin Protein 1 (HP1), conserved from yeast to human, are reader proteins for the histone mark H3K9me3 and a central component of constitutive heterochromatin (Wang et al., 2000). In mammals, Suv39h1 is the first identified histone lysine methyltransferase (HMT) that catalyzes H3K9me2/3 formation (Rea et al., 2000). HP1 and Suv39h1 form a tight reader-writer complex essential for establishing and spreading H3K9me3-marked heterochromatin (Bannister et al., 2001).

Mammalian genomes contain three HP1 isoforms (HP1α, HP1β, and HP1γ) with conserved domains: the chromodomain (CD) for H3K9me3 binding, the chromo-shadow domain (CSD) for dimerization and binding partner interaction, and a disordered middle hinge region for nucleic acid binding (Jacobs & Khorasanizadeh, 2002). Notably, HP1 dimerization bridges two H3K9me3 nucleosomes via CD/H3K9me3 interaction without directly contacting linker DNA (Machida et al., 2018), suggesting recognition is dominated by H3K9me3 binding. While the *in vitro* affinity of CD to H3K9me3 is low, HP1 is proposed to form oligomers to increase its CD valence and compact chromatin into heterochromatin (Canzio et al., 2014). Alternatively, HP1 proteins can form multiple CD-containing complexes with binding partners through the CSD-CSD interface. Some partners have multiple CSD binding sites, some self-associate, and others contain additional H3K9me3 reader domains (Iwase et al., 2011; Romeo et al., 2015; Sun et al., 2019). For instance, Suv39h1 with a CD domain recognizes its own product H3K9me3, and TRIM28, an abundant HP1 partner that mediates transposable element repression, forms oligomers via its self-associating RBCC domain (Stoll et al., 2019; Sun et al., 2019). HP1 also interacts with other constitutive heterochromatin proteins like HMT Suv420h2/KMT5C (H4K20me3 modifier), DNA methyltransferase DNMT1, and methyl-DNA binding proteins MBD1 and MeCP2. Additionally, Suv39h1 can interact with DNMT1, MBD1, and MeCP2, forming a heterochromatin-binding network (Muller-et al., 2014).

While human HP1α can self-associate and undergo phase separation at high concentrations *in vitro*, this IDRs-driven behavior is not common to all HP1 isoforms, and its contribution to heterochromatin condensation remains unclear (Larson et al., 2017; Strom et al., 2017). One study suggests that weak multivalent interactions between HP1 complexes and H3K9me3-marked chromatin drive chromatin condensation under physiological conditions (Canzio et al., 2014). The purified human HP1-Suv39h1 or HP1-TRIM28 complex (containing at least 3–4 CDs) can form condensates with H3K9me3-marked nucleosomal arrays in a multivalent CD/H3K9me3 interaction-dependent manner. These findings suggest that phase separation driven by multivalent H3K9me3/CD interactions is a general principle for HP1-mediated constitutive heterochromatin condensation.

Polycomb group (PcG) proteins have an essential role in the epigenetic maintenance of repressive H3K27me3-marked facultative heterochromatin, also called PcG bodies (Reinberg et al., 2018). The PcG bodies exhibit a self-contained write-and-read mechanism, in which PRC2 catalyzes H3K27me2/3, and subsequently enhances the binding of canonical PRC1 to H3K27me3 (Cao et al., 2002), which resemble of Suv39h1 and HP1. Both PRC1 and PRC2 can compact nucleosomal arrays into high-order structures *in vitro*. Canonical PRC1 consists of four proteins: RING, PCGF, CBX, and PHC. Among them, Cbx and PHC are the main subunits that alter chromatin structure (Blackledge & Klose, 2021). Canonical PRC1 complex is also called Cbx-PRC1 (Satijn et al., 1997), and the Cbx subunit contains a CD that recognizes H3K27me3 (Trojer & Reinberg, 2007). Cbx subunit includes Cbx2, Cbx4, Cbx6, Cbx7 or Cbx8. Cbx7 and Cbx8, but not the others such as Cbx2, can be recruited to chromatin by H3K27me3 (Zhen et al., 2016). In addition, Cbx7 contains a positively charged AT-hook-like (ATL) motif that bind DNA and also contribute to its targeting to chromatin (Zhen et al., 2016). Cbx2 contains a positively charged IDR, which inlaid with an AT-hook motif and two ATL motifs, that multivalent binds nucleosomes and compacts unmodified nucleosomal arrays *in vitro* (Lau et al., 2017). Previously reported that Cbx2 forms self-associated phase separation *in vitro*, and is promoted by DNA (Plys, 2019; Tatavosian et al., 2019), while Cbx7 lacks the phase separation activities *in vitro* (Plys, 2019; Tatavosian et al., 2019). The subunit PHC of canonical PRC1 contains an oligomerization domain, SAM, that allows the oligomerization of the entire PRC1 complex (Zhen et al., 2016) and also contribute to the formation of phase-separated droplets with DNA or chromatin (Niekamp et al., 2024; Seif et al., 2020).

A separate study demonstrated that synthetic PRC1 condensates can accurately mimic the key functions of native PRC1 complexes. These synthetic condensates were shown to directly recognize and establish histone modifications characteristic of facultative heterochromatin. Moreover, they were found to induce chromatin compaction, suggesting a direct link between PRC1 condensation and chromatin structure (Eeftens et al., 2021).

Recent studies have shown that unmodified nucleosomal arrays and Cbx2-PRC1 cooperate to dramatically enhance condensate formation, reducing the critical concentration required by more than 20-fold. In contrast, CBX7 exhibited significantly diminished condensate formation compared to Cbx2-PRC1 under identical conditions, both *in vitro* and *in vivo* (Blackledge & Klose, 2021; Plys, 2019; Tatavosian et al., 2019). These findings suggest that the precise composition of PHC and CBX subunits within PRC1 dictates the initiation, structure, stability, and dynamics of condensates, highlight the crucial role of CBX in condensate nucleation and the importance of PHC for condensate stability. These results collectively suggest that the synergistic interaction between chromatin and PRC1 drives condensate assembly, while variations in PRC1 composition modulates condensate properties. Variations in PRC1 composition can modulate condensate properties, thereby influencing interactions with activating factors and providing crucial regulatory flexibility across developmental stages.

2.2. CAPs binding to DNA directly

Some CAPs such as H1 and MeCP2 can both induce deep chromatin compaction and exhibit multivalent DNA-binding-induced phase separation *in vitro* (Wang, 2020). H1 proteins are the most common CAPs, partially localizes to heterochromatin foci, and promotes chromatin condensation *in vitro* and *in vivo* (Woodcock et al., 2006). H1 proteins contain a globular domain that contacts the nucleosome dyad and both DNA linkers, flanked by a short N-terminal tail and a long positive-charge enriched C-terminal tail (Zhou et al., 2015). The highly positively charged H1 can be simply considered as a multivalent cation that promotes salt-dependent chromatin condensation under physiological conditions (Gibson et al., 2019; Wang, 2020).

MeCP2 is a transcriptional repressor that is highly expressed in adult neurons as an “alternative H1” by binding nucleosomes at the common position (Skene et al., 2010). Numerous mutations in MeCP2 cause a severe postnatal neurodevelopmental disorder, Rett syndrome (RTT) (Amir et al., 1999). MeCP2 recognizes chromatin through multiple DNA-binding domains or motifs (Lewis et al., 1992). MeCP2 contains a methyl-CpG-binding domain (MBD) that selectively binds methylated DNA with higher affinity compared with unmodified DNA (Klose et al., 2005); a transcriptional repression domain (TRD) that represses transcription by mediating self-association and recruiting other gene repressors (Nan et al., 1998); and three AT-hook motifs that distributed downstream of MBD and preferentially bind AT-rich DNA sequences (Baker et al., 2013). We previously reported that both MBD and TRD contribute to the chromatin compaction of MeCP2 *in vitro*, which is positively correlates with its chromatin phase separation capacity (Wang, 2020). The chromatin phase separation also can be enhanced by DNA methylation *in vitro* (Wang, 2020). MBD of MeCP2 is essential for its heterochromatin accumulation, most missense RTT mutations in MBD lost their localize to heterochromatin foci in cells, and compromised their phase separation with chromatin *in vitro* (Wang, 2020). Moreover, the MBD missense RTT mutations dispersed from their targeted heterochromatin in nucleus, while H1 instead accumulated within heterochromatin, which first linked the potential chromatin phase separation-induced functions of CAPs with disease (Wang, 2020).

Many other proteins involving in organizing repressive chromatin (such as Suv420h2 (Muller-et al., 2014), Cbx2 and Cbx7 (Grau et al., 2011; Zhen et al., 2016)) and nucleosome-independent mitochondrial (mitochondrial transcription factor A (TFAM) (Long et al., 2021)) or telomeric genome (TRF1 and TRF2 subunits of shelterin (Jack et al., 2022)), can recognize DNA in multivalent fashions. Most of these proteins have been shown to undergo phase separation with DNA or

chromatin (Keenen et al., 2021; Larson et al., 2017; Plys, 2019; Strickfaden et al., 2019; Tatavosian et al., 2019).

2.3. Multiple repressive CAPs: Reinforcing heterochromatin compartmentalization

Mammalian cells exhibit remarkable resilience in maintaining heterochromatin structure. Loss of HP1 from heterochromatin foci occurs upon Suv39h1/2 double knockout, eliminating H3K9me3, or following rapid depletion of endogenous HP1α (Strom et al., 2021). This seemingly redundant cooperation or competition between chromatin compaction and condensation mechanisms might represent a multifaceted strategy for preserving heterochromatic stability, particularly when key proteins are dysfunctional. Notably, Suv420h2 remains largely localized within heterochromatin foci even in cells lacking HP1α alone (Muller-et al., 2014). However, Suv420h2 disperses in Suv39h1/2 double knockout cells, coinciding with the accumulation of alternative methylations like H3K27me3 and H3K9me1 (Peters et al., 2003). Additionally, other CAPs, such as MeCP2, are highly enriched in heterochromatin foci alongside HP1 (Agarwal et al., 2007). *In vitro* studies demonstrate that MeCP2 droplets preferentially incorporate and concentrate HP1 (Li et al., 2020). Interestingly, a mouse model of Rett syndrome lacking MeCP2 exhibits a more efficient enrichment of H1 and Suv420h2 within heterochromatin foci (Linhoff et al., 2015). Collectively, these known CAPs, along with likely thousands of unidentified CAPs with unknown effects on phase separation, likely cooperate to achieve robust compartmentalization of heterochromatin, enabling the formation of distinct phase-separated assemblies contingent upon the chromatin context (Strickfaden et al., 2019).

Recent studies have revealed that complete removal of H3K9 methylation by mutating all six HMTs can dismantle heterochromatin organization in mammalian cells (Montavon et al., 2021; Padeken et al., 2022). Further comprehensive investigations are necessary to comprehensively evaluate the overall contribution of these factors to heterochromatin organization.

3. Active CAPs: powering gene expression through condensates

In euchromatin, there are regions known to participate in transcriptionally active condensation and likely regions not involving too much chromatin condensation. The processes of gene transcription in eukaryotes require hundreds of regulators, accumulating at the same genomic sites in a coordinated manner. Emerging evidence indicates that RNA and RNA-binding proteins are also playing regulatory roles in gene transcription (Hilbert et al., 2021). Now we appreciate that the transcriptional condensates are actually protein- and RNA-rich droplets on euchromatin. We scrutinize the architectural topology of transcriptional condensates and reveal a clear distinction from that of transcription-repressive condensates.

3.1. Transcriptional condensates in gene activation

Gene expression requires various cis and trans elements. Enhancers, a class of cis elements, are sequence-specific DNA regulatory elements that can be simultaneously bound by activator proteins, transcription factors (TFs), in a cooperative manner (Hnisz et al., 2017). Activating TFs typically comprise of a sequence-specific DNA-binding domain (DBD) and an activation domain (AD) that is often an IDR (Boija et al., 2018). Coactivators are a type of transcriptional coregulators that do not bind DNA themselves but contain an IDR and can form heterotypic IDR-IDR interactions with ADs of TFs (Sabari et al., 2018). Multiple enhancers located within a genomic region are called super-enhancers (SEs) that drive strong gene expression (Cho et al., 2018; Sabari et al., 2018). Mediator is a well-known coactivator, and its subunit MED1, together with another well-known coactivator BRD4, marks the presence of SEs (Sabari et al., 2018).

Recent reports have shown that some TFs and coactivators can form IDR-driven condensates *in vitro*, sometimes with crowding agents. While several pairs of TFs and coactivators can form IDR-driven co-separation *in vitro*, without the presence of crowding agents (Boija et al., 2018; Sabari et al., 2018). This common synergy phase-separation property of TFs and coactivators create the initial transcriptional condensation on target genomic regions for further recruitment and condensation of other transcriptional machineries.

RNA polymerase II (Pol II) is a huge transcription machinery that can be recruited to transcriptional condensation, with promoter nearby or together within the condensate, to initiate transcription. The C-terminal domain (CTD) of the largest subunit RPB1 is an IDR, and undergoes phase separation *in vitro* (Boehning et al., 2018; Lu et al., 2018). The Pol II or its CTD can incorporate into many IDRs-driven phase droplets through heterotypic IDR-IDR interactions *in vitro*, such as Mediator-driven condensates, and co-occupied SEs with properties consistent with phase separation in cells (Sabari et al., 2018).

A common property among these positive transcription regulators is that they contain few to none chromatin binding surfaces. Phase separation is largely derived from homotypic or heterotypic dynamic, multivalent, and selective IDR-IDR interactions of TFs, coactivators and Pol II, to establish transcription condensation on chromatin via the low-valent binding surfaces. The fact that components incorporate into transcription condensates through heterotypic IDR-IDR interactions without the necessity of binding chromatin implies that the proportion of chromatin within condensates has no lower limit and can be viewed as solutes within condensates. These properties are in dramatic contrast with repressive transcription regulators, which almost always contain multiple chromatin-binding domains or motifs.

More interesting, the latest research found that condensates formed by MED1-IDR can selectively partition Pol II and other transcriptionally active regulators while excluding transcriptionally silent regulators such as HP1α, MeCP2, CBX2, etc (Lyons, 2023). The authors also demonstrated that selectively partition is driven by the charge patterning in IDRs within these regulators (IDRs with similar patterning have similar partitioning and function, vice versa), and sufficient to activate transcription, and could represent general principles applicable beyond MED1-IDR (Lyons, 2023). Conversely, we also speculate that this selectively partition can partially explain why IDRs-contained transcriptionally active regulators cannot be recruited by HP1α, MeCP2, or CBX2-enriched heterochromatin. Although HP1α, MeCP2, and CBX2 are all used to treated as IDR-containing proteins to study the driving force and function of phase separation (Larson et al., 2017; Li et al., 2020; Plys, 2019; Strom et al., 2017; Tatavosian et al., 2019), their IDRs may be different from most of the IDRs that mediate heterotypic IDR-IDR interactions and drive phase transitions as main driven forces discussed in this section, at least the IDRs of HP1α and CBX2 are to increase their valence to promote weak multivalent interactions between proteins and DNA/chromatin (Larson et al., 2017; Plys, 2019).

We anticipate that further studies with more scrutiny into the properties of transcriptional condensates and those for transcription-repression will enable more comprehensive understanding of the roles of phase separation in regulation chromatin organization and gene expression. It is nevertheless worth pointing out that the clients and scaffolds of transcriptional condensates are challenging to disentangle.

3.2. Fusion TFs and dysregulated condensates

We now appreciate that many transcriptional regulators function at least partly via participating in transcriptional condensates. The relatively promiscuous nature of IDR-IDR of transcription regulators such as Pol II, remodeler, mediator, set the stage for their erroneous recruitment to wrong condensates to cause diseases due to gain of aberrant function or loss of normal functions.

One prominent example of gain of aberrant phase separation is from, often oncogenic, fusion proteins (Pavlaki, 2021; Quiroga et al., 2022; Zuo

et al., 2021). IDRs from members of the FET protein family (FUS, EWS, and TAF15) or nucleoporin NUP98, are reported to undergo strong LLPS (Patel et al., 2015; Schmidt et al., 2015). Pol II CTD and/or other transcriptional regulators can be incorporated into these IDRs-driven condensates *in vitro* (Kwon et al., 2013). These IDRs-contained proteins can generate aberrant chimeras with their IDRs and chromatin/DNA-binding factors, termed oncogenic TFs. Recent reports have shown that these oncogenic TFs formed biomolecular condensates on DBD-binding sites which recruit Pol II to specific loci to promote aberrant gene transcription (Ahn et al., 2021; Boulay et al., 2017; Terlecki-et al., 2021; Zuo et al., 2021), and found in many human cancers as essential oncogenic drivers. Nevertheless, these onco-fusion TFs normally contain a DNA or chromatin binding domain and phase-separated IDRs. Such fusion oncogenic TFs are more prevalent than currently appreciated. More research is underway in this direction.

Meanwhile, this kind of enhanced locally biomolecular interactions have been naturally hypothesized to amplify gene expression. There have been many recent works investigating the effect of manipulating liquid-like TF droplets on transcription of specific endogenous genes, they came to contradictory conclusions. Two works about light-induced phase droplets of TF-IDR can increases global cellular transcription (Schneider et al., 2021; Wei et al., 2020), while another work about light-induced TF droplets didn't enhances transcription activation (Trojanowski et al., 2022) and together with the last work support that IDR-IDR interactions are sufficient to activate transcription independent of phase separation (Chong et al., 2022). The inconsistency of these findings is likely due to differences in the implementation details of non-endogenous operation methods, research targets, goals and judging criteria of each work. For instance, while most studies suggest that phase separation can enhance transcriptional activation, the mechanisms and the extent of this enhancement remain controversial. The specific transcription factors studied in these works exhibit diverse structural features and functional properties, which could lead to varying effects of phase separation on their transcriptional activation. Additionally, the optogenetic approaches commonly used in these studies to control phase separation might introduce artificial interactions that could interfere with the intrinsic transcriptional activation functions of the targeted transcription factors. It is worth mentioning that while overexpression of phase-separated proteins can induce the formation of large, discrete droplets, these droplets may not be fully functional and might even exclude chromatin This suggests that the size and composition of the droplets, as well as the cellular context, could significantly influence the outcome of phase separation on transcription. To address these inconsistencies, future studies should focus on standardizing experimental protocols, utilizing more rigorous controls, and carefully selecting model systems to obtain more reliable and reproducible results.

It is interesting to note that one of the works considers that such large droplets are formed by phase separation as previous reported, and numerous small puncta do not count (Chong et al., 2022). While another report shown that such submicron-sized puncta are phase condensates, larger condensates appeared with protein level increased in cells (Song et al., 2022). The optogenetic approaches and other manipulation methods provide a framework for the way the transcriptional machinery is organized when studying transcriptional condensates, while when discovery the condensate formation combined with functional significance there strongly suggests a critical need to study in near physiologically relevant contexts (Song et al., 2022).

3.3. Dual-function TFs and gene expression control in condensates

Precise control of diverse eukaryotic gene expression levels is crucial for normal cellular function, achieved by transcription factors with dual activator and repressor roles (Ma, 2005). TFs can exhibit bifunctionality, acting as both activators and repressors in response to cellular context, enabling control of gene expression at intermediate levels, but the underlying mechanisms are still largely unknown.

Recent research employing a novel assay, termed assay for chromatin-bound condensates by exploratory sequencing (ACC-seq) (He et al., 2024), has identified a class of TFs, termed condensate-forming level-regulating dual-action TFs, with dual roles as activators and repressors. These TFs suppress high expression while enhancing low expression, ultimately achieving stable intermediate levels. Notably, these TFs occupy distinct nuclear domains from both the heterochromatin domain and transcriptionally active condensates. Mechanistically, they selectively interact with transcriptional cofactors within condensates, recruiting core transcriptional machinery while simultaneously blocking high-level transcription complexes, such as BRD4 and MED1 (He et al., 2024). This dual functionality is intrinsic to their IDRs with both unique overall charge patterns and the sequence units within them, exhibiting characteristics of both repressors and activators simultaneously.

These findings unveil a previously unrecognized mechanism for precise and robust control of intermediate expression levels, mediated by fine-tuning the phase separation patterns on chromatin. This suggests that the dynamic interplay between these two modes of chromatin organization further influences transcriptional regulation.

4. Distinct active CAPs mediate chromatin phase separation and compartmentalization transitions

Both transcriptionally repressive heterochromatin condensates and permissive euchromatin condensates exhibit spontaneous and selective partitioning, enriching for components with similar transcriptional functions while excluding those with opposing roles (Lyons, 2023). This selective partitioning is further supported by studies demonstrating the targeted recruitment of transcriptionally active, IDR-driven droplets to heterochromatin foci. Notably, optogenetic approaches using CRISPR-dCas9 and lac operator-repressor systems have shown that these droplets nucleate almost exclusively from target regions within heterochromatin, excluding non-specific target chromatin (Shin et al., 2018). Moreover, these droplets enrich markers of active chromatin (H3K27ac) while excluding heterochromatin markers (HP1α and H3K9me3), accompanied by reporter gene activation. This suggests that IDR-driven droplets with specific genomic locus binding can establish an active chromatin environment even within heterochromatin (Lyons, 2023). Importantly, these IDR droplets exhibit local, not global, chromatin binding.

Based on the observation that IDR-driven droplets can convert heterochromatin into euchromatin, we hypothesize that globally chromatin-binding IDR factors could significantly reduce or eliminate heterochromatin domains. Furthermore, the role of natural TFs, particularly "pioneer TFs" or other unique TFs, enabling the conversion of heterochromatin to euchromatin and vice versa.

4.1. Redox-switch multivalency: TMF condensation and gene repression

A single TF either can act as a repressor that insulates a promoter from the influence of all enhancers. How this can be explained by the phase separation patterns we discussed above? Interestingly, a plant transcription factor TERMINATING FLOWER (TMF) is composed of two short IDR fragments and one well-folded DBD (Huang et al., 2021). According to our chromatin-binding module valency hypothesis, TMF should form activity transcriptionally condensates on chromatin. Instead, TMF functions to suppress expression of genes important for flowering. It turns out that TMF responds to metabolically produced oxidative species to form intermolecular disulfide bonds. The concatenation of TMF causes higher valency of the DBD (Huang et al., 2021), which condensates with chromatin and sequesters its target promoter to repress its expression. Therefore, multivalent interactions of oxidized TMF with chromatin, in conjunction with the IDR-IDR interactions, drive TMF phase separation with chromatin, which ultimately represses its target genes (Huang et al., 2021).

4.2. FOXA1: IDRs drive biomolecular condensates and heterochromatin-to-euchromatin conversion

Most TFs require nucleosome-free DNA for target sequence recognition. However, eukaryotic DNA is predominantly packaged into nucleosomes, limiting TF accessibility. This inaccessibility is further compounded by higher-order chromatin structures and phase-separated condensates. Pioneer TFs, a unique class, possess the remarkable ability to access nucleosomal DNA within condensed chromatin, unlike non-pioneer TFs (Iwafuchi-Doi et al., 2016). They bind target sites prior to transcriptional activation, often inducing local chromatin opening to facilitate subsequent binding of cooperating TFs and other regulators. This ultimately establishes competence for gene expression or fate changes during embryonic development.

FOXA1 (formerly HNF-3α) was firstly identified as a pioneer TF critical for liver development from endoderm cells (Cirillo et al., 2002). It reprograms the binding of steroid hormone receptors like estrogen (ER) and androgen receptors (AR), regulating gene transcription (Carroll et al., 2005; Lupien et al., 2008). Dysregulation of FOXA1 expression or function, through amplification, mutation, or upregulation, is linked to prostate and breast cancers (Thorat et al., 2008). FOXA1 has three key regions: a central DBD called the "forkhead" (FH) domain, and flanking N- and C-terminal regions. The crystal structure of the FH domain bound to DNA reveals a helix-turn-helix (HTH) motif for DNA contact and flanking loops that stabilize binding (Ramakrishnan et al., 1993). The "winged helix" structure of FH resembles H1 (Clark et al., 1993). FOXA1 binds tightly as a monomer to DNA or isolated nucleosomes, forming stable complexes (Chaya et al., 2001). FOXA's pioneering activity is evidenced by its ability to bind target sites on nucleosomal arrays *in vitro*, even in the present of linker histone H1, and without ATP-dependent chromatin remodeling complexes. This binding creates locally accessible chromatin regions, allowing other TFs to bind and activate transcription (Crowe et al., 1999). The precise mechanisms by which FOXA1 recruits and decompacts condensed chromatin to execute its pioneer factor function remain elusive. Collectively, these findings support a compelling hypothesis that FOXA1 may act as a natural transcription factor capable of orchestrating the conversion of heterochromatin to euchromatin.

Recently a study unveils a novel mechanism for FOXA1-mediated gene regulation through biomolecular condensate formation and chromatin opening (Ji et al., 2024) (Fig. 2). Live-cell imaging revealed dynamic nuclear puncta, hallmarks of condensates, formed by FOXA1's IDRs. These IDRs, particularly the N-terminal prion-like domain (PrLD), were essential for condensate formation and subsequent chromatin decondensation. Unlike other pioneer transcription factors, FOXA1's DNA-binding domain alone was insufficient for these functions, highlighting the unique role of its IDRs.

FOXA1 directly binds nucleosomes and unfolds condensed chromatin structures mediated by linker histone H1, independent of its IDRs. However, its IDRs are crucial for its chromatin-unpacking activity *in vitro*. Full-length FOXA1 disrupts condensed chromatin, whereas the DNA-binding domain alone is ineffective. These findings suggest a multifaceted mechanism for condensate disruption by FOXA1's IDRs, potentially involving interactions with H1 or other chromatin components, or modulating internal protein-protein interactions. The reversible nature of this process implies a non-covalent mechanism. This condensate disruption by FOXA1 promotes chromatin accessibility, facilitating transcription factor binding and activation, potentially explaining its diverse roles in development, differentiation, and metabolism. Furthermore, the broad-spectrum condensate disruption suggests a wider role for this process in chromatin regulation. FOXA1's IDRs target specific DNA regions, leading to chromatin opening and impacting genes associated with cell growth. Notably, only full-length FOXA1, retaining its IDRs, suppressed cancer cell proliferation and migration. These findings highlight the critical role of IDRs in FOXA1's anti-cancer properties.

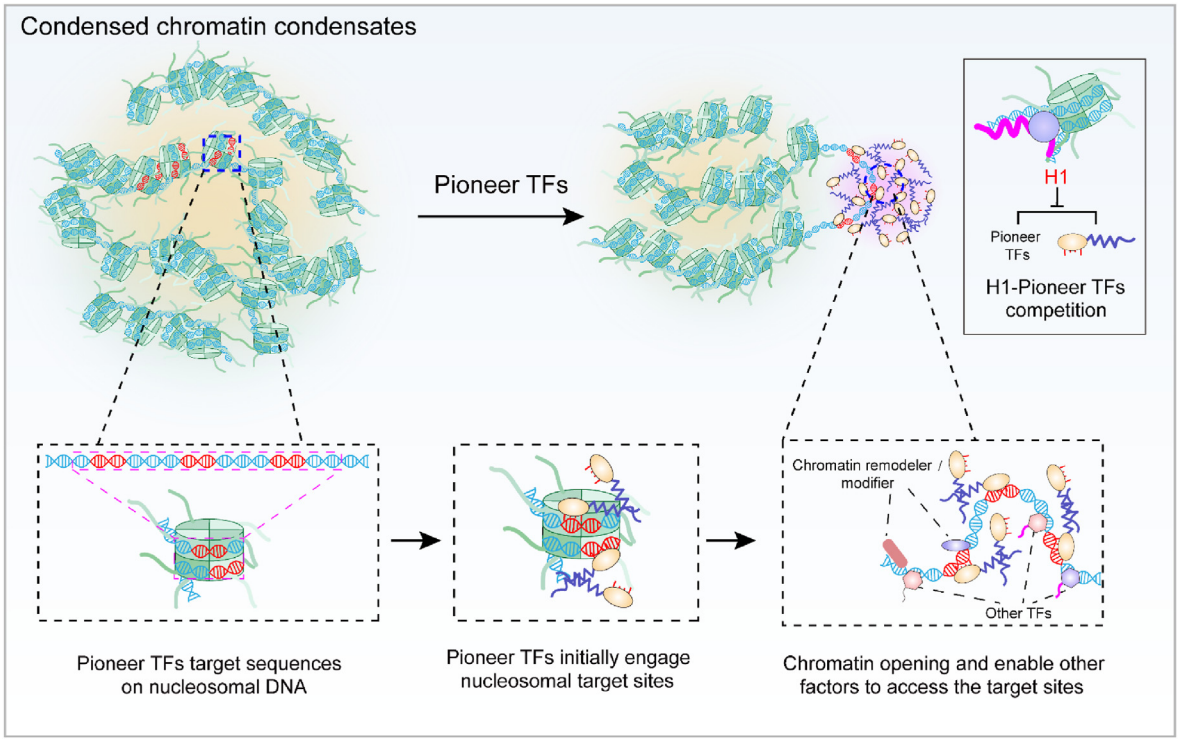

Fig. 2. Pioneer transcription factors: inducing chromatin decondensation and phase separation transitions. Pioneer transcription factors (Pioneer TFs) are a specialized class of eukaryotic transcription factors capable of binding to target sites within compacted nucleosomes, overcoming the repressive effects of linker histone H1. This binding leads to local chromatin decondensation, creating accessible sites for subsequent transcriptional events. Beyond their molecular functions, Pioneer TFs also possess intrinsic phase separation properties that enable them to disrupt heterochromatin condensates formed by linker histone H1 and other CAPs. This disruption involves a transition from "phase separation with chromatin" to "phase separation on chromatin," ultimately facilitating the pioneer function of Pioneer TFs in establishing new gene expression patterns.

In conclusion, this study reveals a novel mechanism by which FOXA1 controls chromatin architecture through IDR-mediated biomolecular condensate formation and subsequent disruption. This disruption increases chromatin accessibility, potentially explaining FOXA1's diverse cellular functions. The study also suggests a broader role for biomolecular condensate disruption in regulating gene expression, offering a new paradigm for understanding FOXA1's function.

5. Core histone variants orchestrate sperm chromatin condensation in flowering plants via phase separation

As previously described, canonical core histones, when assembled into short nucleosome arrays *in vitro*, can exist in a soluble state in low-salt solutions. Nucleosome arrays can also be subjected to weak multivalent interactions with various CAPs (via PTMs, DNA methylation modifications, or DNA/chromatin itself), driving the formation of chromatin phase separation droplets. This process is accompanied by deep compaction of chromatin into droplets, consistent with the mechanism of heterochromatin formation. However, the potential role of core histone variants, as fundamental units of chromatin, in regulating chromatin phase separation has been largely overlooked. These variants not only exhibit alterations in a few key amino acids, potentially leading to changes in PTMs, but may also harbor unique structural domains, such as the C-terminal macro domain of MacroH2A. These unique domains could potentially contribute to phase separation mechanisms, driving or inhibiting the formation of chromatin compartments. As we'll discuss below, H2B.8, a sperm-specific histone variant, exemplifies this alternative strategy.

Sperm chromatin condensation is crucial for male fertility in most organisms. In animals, protamines replace histones during sperm development, compacting DNA into inactive toroidal structures (Steger & Balhorn, 2018). Interestingly, the sperm cells of flowering plants utilize a distinct mechanism for chromatin condensation. Unlike their animal counterparts, flowering plant sperm retain histone-based chromatin, yet they achieve a high degree of compaction through an unknown mechanism that maintains transcriptional activity.

Recently, Buttress et al. identified unique condensed chromatin compartments in flowering plant sperm, distinct from typical heterochromatin (Buttress et al., 2022). A key player in this phenomenon appears to be a sperm-cell-specific histone variant called H2B.8. These variant drives chromatin and nuclear condensation via phase separation of transcriptionally inactive, AT-rich euchromatin into condensates, without affecting transcription (Buttress et al., 2022).

H2B.8 has a long, negatively charged IDR in its N-terminal tail (Fig. 3 A). Unlike standard histones, H2B.8 appears to drive chromatin condensation via a novel form of phase separation involving interactions with other histones, potentially through its unique IDR. Histone tails, rich in positively charged amino acids, are essential for forming higher-order chromatin structures and chromatin phase droplets (Fig. 3 B). This mechanism differs from CAP-mediated heterochromatin formation and warrants further investigation.

6. Conclusion

This review highlights phase separation as a key driver for chromatin compartmentalization. Chromatin structure itself plays a crucial role,

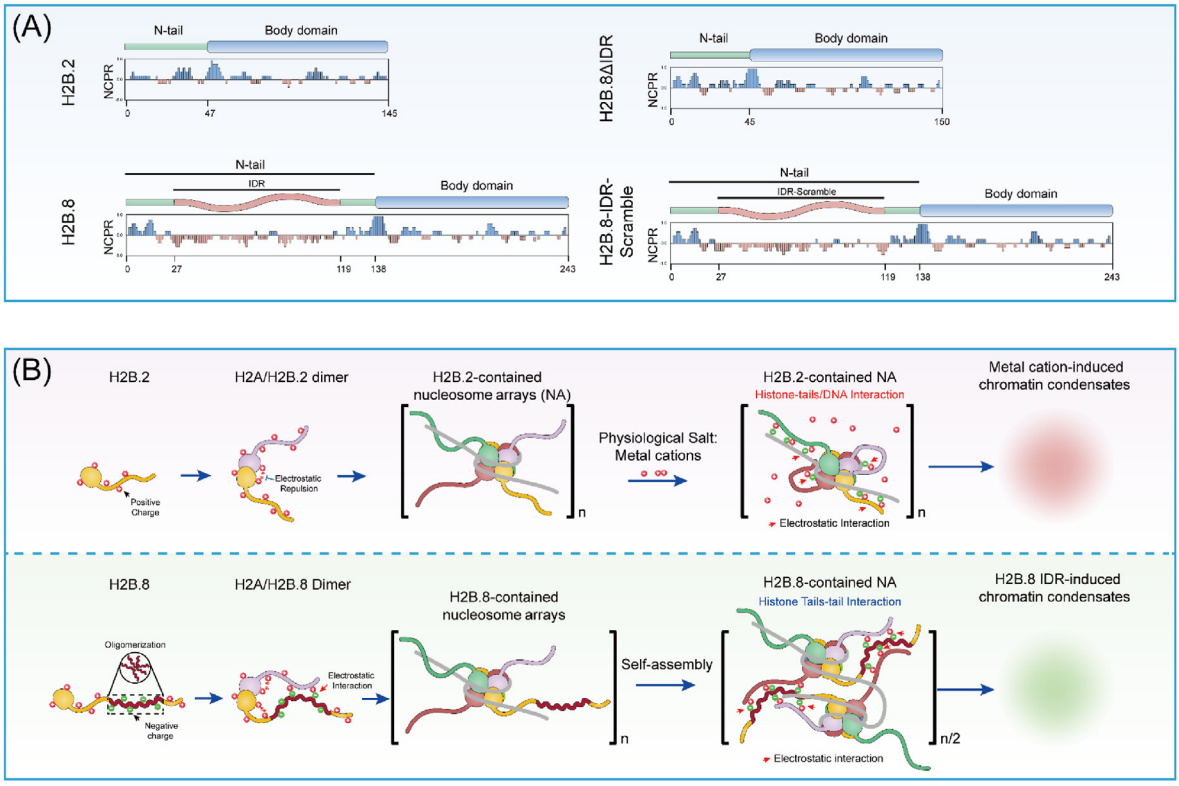

Fig. 3. The sperm-specific histone variant H2B.8 and its IDR-mediated chromatin phase separation. (A) Unique IDR in the sperm-specific histone variant H2B.8. The sperm-specific histone variant H2B.8 possesses a distinct 93-amino-acid intrinsically disordered region (IDR) at its amino-terminal tail. Net charge per residue (NCPR) analysis reveals that this additional IDR is predominantly negatively charged, contrasting with the positively charged nature of other histone tails. Removal of this IDR from H2B.8 results in an amino acid sequence and NCPR profile nearly identical to that of H2B.2. Additionally, NCPR analysis of an IDR-scrambled H2B.8 variant is also shown for comparison. (B) IDR-mediated chromatin phase separation by H2B.8. Classical nucleosome arrays require metal ions at near-physiological salt concentrations to drive chromatin phase separation. In contrast, H2B.8 can induce chromatin phase separation in low or even no salt conditions, likely mediated by its IDR. The mechanism may involve interactions between the negatively charged IDR and the positively charged regions of neighboring histone tails, promoting intra- or inter-chromatin interactions and driving phase separation.

with its biophysical properties influencing the formation of both repressive and activating condensates. Multivalent CAPs promote chromatin compaction and formation of transcriptionally repressed compartments, while weak multivalent interactions mediated by IDRs lead to protein-rich, transcriptionally active droplets. Notably, these distinct types of condensates selectively enrich components matching their transcriptional function.

However, it's important to acknowledge that not all genomic regions are regulated by phase separation. Additionally, the multifaceted functions of CAPs extend beyond their role in condensate formation. Future research should focus on precisely quantifying the contribution of phase transitions to local and global chromatin organization within the nucleus.

As we delve deeper into the realm of chromatin phase separation, several intriguing questions and avenues for future research emerge. These inquiries aim to unravel the intricate mechanisms underlying this fundamental biological process and its implications for gene regulation and human health.

To what extent do intrinsically disordered regions (IDRs) contribute to gene regulation beyond their role in phase separation? Are there additional mechanisms, such as direct DNA binding, through which IDRs influence gene expression?

How does phase separation influence chromatin architecture, and what are the underlying molecular mechanisms driving this interaction?

What are the physical properties of chromatin *in vivo*, and how can we develop more accurate methods to measure these properties to better understand chromatin dynamics and gene regulation?

How do different types of phase-separated condensates interact with each other within the nucleus, and what are the functional consequences of these interactions?

What molecular mechanisms govern the formation, maturation, and dissolution of phase-separated condensates?

How do phase-separated condensates contribute to the spatial organization of chromosomes within the nucleus, and what are the molecular mechanisms underlying this process?

How does dysregulation of phase separation contribute to the pathogenesis of human diseases?

By addressing these questions, we can gain a more comprehensive understanding of the role of phase separation in chromatin organization and gene expression, and develop strategies to target phase separation-related processes for therapeutic purposes.

CRediT authorship contribution statement

Xin Li: Writing – original draft. **Chengzhi Liu:** Writing – original draft. **Zhichao Lei:** Writing – review & editing. **Huan Chen:** Writing – review & editing, Writing – original draft. **Liang Wang:** Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors 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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