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# The Role of Phase Separation in Heterochromatin Formation, Function, and Regulation

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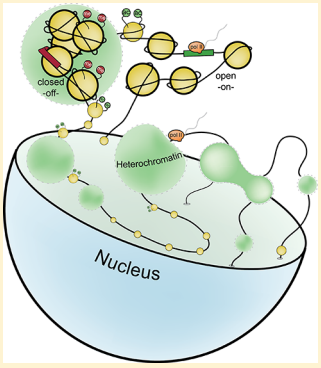
**ABSTRACT:** In eukaryotic cells, structures called heterochromatin play critical roles in nuclear processes ranging from gene repression to chromosome segregation. Biochemical and in vivo studies over the past several decades have implied that the diverse functions of heterochromatin rely on the ability of these structures to spread across large regions of the genome, to compact the underlying DNA, and to recruit different types of activities. Recent observations have suggested that heterochromatin may possess liquid droplet-like properties. Here, we discuss how these observations provide a new perspective on the mechanisms for the assembly, regulation, and functions of heterochromatin.






## ■ "CELLS ARE GELS"

**'Correct for Extract Dilution with Molecular Crowding'.** The seventh of Arthur Kornberg's "10 commandments of enzymology" brings to attention a quality of the cellular environment often neglected in the biochemist's quest to replicate or dissect complicated cellular processes in the microcentrifuge tube.[2] The eukaryotic cell to which Dr. Kornberg was referring to contains complex mixtures of structurally and chemically distinct macromolecules coexisting at high concentrations (100−400 mg/mL).[2] A common property of aqueous solutions of macromolecules at high concentrations is phase separation, where a mixture will demix into phases with different physical and chemical properties. It has been long postulated that the network of charged nucleic acid polymers, myriad proteins, and the mixture of multivalent salts and metals in the cell could not coexist without undergoing such phase separation.[1] Indeed, the observation of cytoplasmic "microcompartmentalization" has been hypothesized to rely on this phenomenon.[9]

The effects of high concentration are expected to be accentuated within the nucleus of eukaryotic cells given the large amounts of chromatin and macromolecular complexes packed within a nuclear membrane. Indeed, simple calculations imply the overall concentration of chromatin alone can range in the order of 10s of $\mu$M, substantially higher than that used in most reconstituted biochemical reactions. Yet within this crowded environment, a multitude of distinct and individually complex processes are carried out with spatiotemporal specificity. Phase-separated compartments provide an attractive model to explain how the reaction conditions for these diverse processes are compartmentalized. The past years have seen an impressive amount of creative work exploring the impact that phase separation may have on organizing macromolecules in and out of the nucleus.[3,4] Here, we highlight advances and yet-unstudied possibilities for the role of phase separation in the context of genome organization and function with an emphasis on repressed regions of the genome called heterochromatin. Below, we first summarize our current understanding of heterochromatin formation, function, and regulation and then discuss how phase separation can add a new perspective to these current models.

## ■ HETEROCHROMATIN: FUNCTIONS AND MECHANISMS

Early visualizations of eukaryotic nuclei carried out several decades ago using DNA intercalating dyes revealed darkly stained regions (termed heterochromatin) and more lightly stained regions (termed euchromatin) that appeared to occupy distinct spatial territories.[11,14] Later studies revealed that the regions termed heterochromatin were often more compact than euchromatin and were integral to gene repression and proper chromosome segregation. In this context, three broad classes of heterochromatic regions can be described. One region is observed at and near centromeres, which typically contain repetitive DNA elements.[17] Heterochromatin in these regions is thought to play a critical role in chromosome segregation and repressing the transcription of foreign repetitive DNA elements. This type of heterochromatin is also the easiest to observe cytologically in certain cell types due to the clustering of centromeres to generate punctate structures called chromocenters.[20] Another region of heterochromatin is found near

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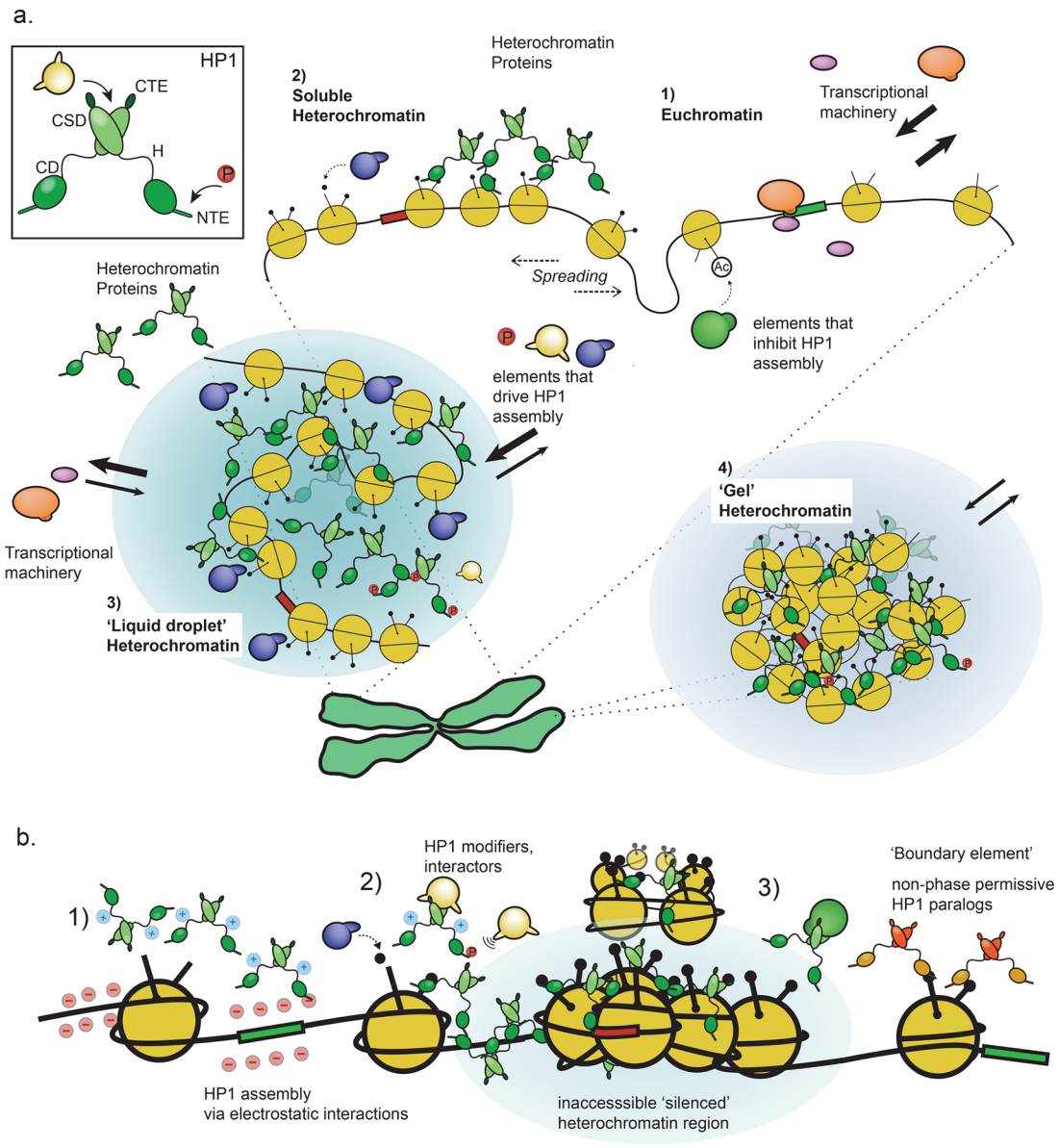


**Figure 1.** Model for the different physical states of chromatin. (a) Physical states accessible to HP1−chromatin complexes in vitro may inform on the organization of functional chromatin states. By altering the physical state of chromatin via tuning local conditions and binding interactions, the chromatin can be made accessible to transcription or made more condensed and refractive to gene activation. (inset) Domain architecture of HP1 proteins (green); the N-terminal extension (NTE), chromodomain (CD), Hinge (H), chromoshadow domain (CSD), and the C-terminal extension (CTE) are shown. Also shown are two pathways of altering HP1$\alpha$ phase separation behavior by a ligand that can bind at the CSD−CSD dimer interface (yellow molecule) and by phosphorylation on the NTE (red P). (1) Active euchromatin is highly acetylated (acetyltransferase in green) and less compact than soluble heterochromatin, resulting in increased accessibility of the underlying chromatin to the transcription machinery (RNA polymerase in orange and transcription factor in pink). (2) Soluble HP1$\alpha$ heterochromatin consists of a complex between HP1$\alpha$ molecules and chromatin with H3K9 methyl marks. The H3K9 methylase is shown in purple. In this state, spreading can occur via oligomerization of HP1$\alpha$ molecules across chromatin coupled to the action of the H3K9 methylase (purple). (3) Increase in HP1$\alpha$ concentration above the critical concentration for phase separation may drive soluble heterochromatin into a phase-separated droplet state. This process can be regulated by ligands that bind the CSD−CSD interface and by changes in phosphorylation of HP1$\alpha$. The droplet state may promote gene silencing by inhibiting accumulation of the transcription machinery inside the droplets, by inhibiting access to the DNA template, or by inhibiting the activity of the transcription machinery. In contrast, accumulation of heterochromatin components such as the H3K9 methylase may be promoted through a combination of specific and nonspecific interactions with other components of the HP1$\alpha$ phase. (4) Certain heterochromatin components, solutes, and counterions may stabilize a gel-like state of HP1$\alpha$ heterochromatin, which may be less permeable than the droplet state and may serve a more structural role. (b) Model for some specific types of interactions affecting HP1$\alpha$ phase separation behavior. (1) The positive residues within the disordered hinge region of HP1 interact with the negatively charged DNA polyanion to form a specific type of coacervate that promotes compaction of the underlying chromatin. (2) Factors such as kinases and methylases that both interact and modify HP1 encourage spreading. In addition to linear spreading, distal chromatin regions with like qualities may partition into the silenced HP1$\alpha$-rich phase while noninteracting components or those displaying qualities not permissive for solvation in the heterochromatin phase may be excluded. (3) Inhibition of HP1$\alpha$ spreading with HP1 paralogs such as HP1$\beta$ (orange) that are not competent to phase separate or with factors that dissolve HP1$\alpha$ assembly (green factor bound to HP1$\alpha$) could serve as boundaries to heterochromatin spread.



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telomeres and in these regions heterochromatin is thought to help protect telomeres from the events of DNA repair and recombination.[30] A third type of heterochromatin is found at a subset of specific genes, and in these regions, heterochromatin is thought to enable developmental control of gene expression.[14,20] In many of these cases, it has been observed that the repressive functions associated with heterochromatin can "spread" across large regions of the genome. These functions and behaviors of heterochromatin have raised several fundamental questions, such as: (i) How does heterochromatin contribute to diverse functions ranging from gene silencing to chromosome segregation? (ii) How is compaction achieved? (iii) What is the molecular basis for spreading and for limiting the heterochromatin spread?

A major type of heterochromatin involves two types of modifications to the basic building block of chromatin, a nucleosome, which contains ~150 bp of DNA wrapped around a histone octamer. These two modifications are the methylation of a specific histone residue (histone H3 lysine 9; H3K9me) and the assembly of HP1 proteins, which recognize the H3K9me mark and also bind DNA (Figure 1A, inset).

Biochemical studies of HP1 proteins and the H3K9 methylase enzyme have begun addressing some of the questions raised above. For example, in terms of understanding spreading, it has been shown that HP1 proteins from *S. pombe* can oligomerize and that oligomerization is important for gene silencing in cells[16,22,23] In addition, the *S. pombe* histone methylase can recognize its own product through a noncatalytic domain, providing an opportunity to locally increase histone methylation.[24,25,26] In humans, some HP1 proteins can directly interact with the histone methylase, thus creating the possibility for positive feedback between H3K9 methylation and HP1 binding.[7,27] Together, these studies have begun providing biochemical models for the spreading behavior of heterochromatin in cells. In terms of understanding compaction, the assembly of human HP1 molecules has been suggested to promote condensation of long arrays of nucleosomes as well as promote interstrand interactions.[28]Consistent with the biochemical observations described above, in vivo studies have shown that recruitment of HP1 molecules to an ectopic locus is sufficient to induce large-scale silencing over several kilobases of once active chromatin.[29] Because chromatin compaction in principle can reduce access to the underlying DNA, it has been suggested that compaction is a major contributor to gene repression. However, parallel mechanisms involving RNAi and other small RNA pathways have also been uncovered that contribute to the gene repression seen in heterochromatin.[8] Overall, a model is emerging for a heterochromatin mechanism that relies on two consequences of HP1 assembly: direct effects on chromatin structure and creation of a platform to recruit additional activities.

Two recent studies on HP1 (one from the Karpen group and one from our group) suggest an additional and unexpected capability of HP1 proteins: demixing from solution to form liquid droplets.[12,31] These observations have opened up new ways to conceptualize the assembly, regulation, and mechanisms of heterochromatin, which we discuss below. We emphasize that the previous models summarized above are not mutually exclusive with the use of phase separation-based mechanisms. Rather, these prior models can be revisited from a new perspective. Here, we ask how the fundamental questions raised above can be addressed by including the possibilities presented by phase separation behaviors and where possible we do so by drawing from insights from other better-studied phase separation-based processes in biology. We do not discuss in detail the theories underlying different types of biological phase separation behaviors as these have been thoughtfully addressed in other articles in this issue.

## DIFFERENT PHYSICOCHEMICAL PROPERTIES MAY ENABLE DIFFERENT HETEROCHROMATIN FUNCTIONS

HP1 proteins contain two structured domains, the CD that binds the H3K9me mark and the CSD that contributes a key dimerization interface that acts as a platform to recruit other proteins (Figure 1A, inset). Additionally, there are three regions that do not appear to have a defined structure in the free HP1 protein, an N-terminal extension, a hinge region, which has patches of positively charged residues that bind DNA and RNA, and a C-terminal extension. In humans, there are three major HP1 paralogs: HP1$\alpha$, $\beta$, and $\gamma$. Of these, HP1$\alpha$ is the protein most widely associated with constitutively silenced heterochromatic regions, whereas the other two paralogs also play roles in active gene expression.[16,52] Upon either phosphorylation of its N-terminal extension or addition of saturating DNA, the human HP1$\alpha$ demixes from solution into droplets.[12] The critical (or saturation) concentration for HP1$\alpha$ droplet formation under near physiological ionic strength and temperature is comparable or lower than the local concentration of HP1$\alpha$ in many heterochromatin regions.[12]

Both the phosphorylation- and DNA-dependent demixing of HP1$\alpha$ appears to be electrostatically driven due to the observation that the critical concentration for droplet formation increases with increasing salt concentration.[12] This is consistent with previous findings that HP1$\alpha$ interacts with DNA through conserved positively charged patches of lysines in its hinge region. Indeed, phase separation is greatly reduced when a conserved lysine patch in the hinge of HP1$\alpha$ is mutated.[12] Further, over time the phase-separated HP1 droplets settle into a more gel-like phase.[12] Some of the behaviors of the HP1−DNA complex resemble previous observations made in the context of oppositely charged polymers, which separate out of solution as bodies termed "coacervates".[1,32] Such coacervates have been shown to adopt a range of material properties from liquid-like to more gel-like depending on the components and conditions of the system. Analogously, proteins containing multiple disordered domains and the capability for multivalency through either hydrophobic or electrostatic interactions have been shown to span states from a single homogeneous aqueous phase to a near solid gel phase depending on reaction conditions.[1,33] This large dynamic range of possibilities raises the question of which, if any, phase-separated state is functionally relevant in cells. It is also possible that all states have a function but that there exists tight regulation of transitions between different material states of phase-separated systems.

To date, most of the examples documenting the biological consequences of the equilibria between a soluble protein, a demixed two-phase solution, and a more static gel have suggested that a bias toward gel-like states is associated with pathogenesis. A well- studied example is that of the Crystallin family of proteins.[19,34] These proteins are responsible for numerous functions in the lens of the eye, performing chaperone-like activity thought to cope with age-related stress. They are involved in normal events of phase separation in the mammalian eye, but mutations in $\gamma$-Crystallin that bias its


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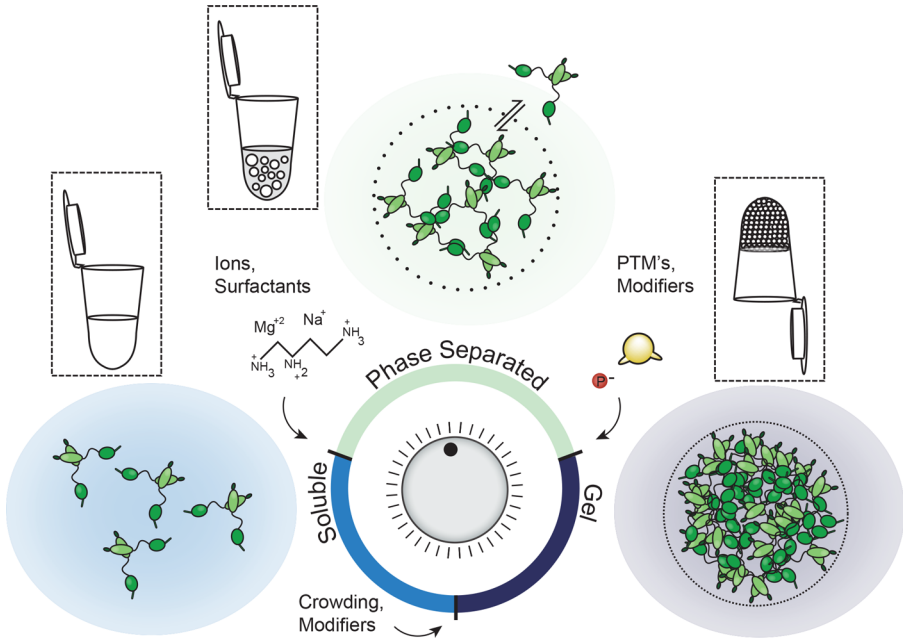


**Figure 2.** Regulated dynamics of phase separation. Multiple physical states of heterochromatin are represented by the dial that can be moved via multiple inputs. The critical concentrations of HP1$\alpha$ required for transitions between soluble to liquid-liquid demixing to gel-like states are proposed to be sensitive to environmental conditions that tune the biophysical characteristics of the protein and binding partners that directly affect protein assembly and solvent conditions.

equilibrium to a more phase-separated composition are linked to the formation of pathogenic cataracts. In vitro, this cataract-forming mutation has no detectable structural rearrangements in the Crystallin protein itself, but the mutant protein phase separates more readily.[34,19] It was further found that the relative amounts of the $\gamma$-Crystallin isoforms can greatly affect the equilibrium and composition of the phases formed in vitro.[35] Such regulation of phase composition through isoform interaction could be an effective general strategy to tune molecular function.

Another well-studied example in the context of liquid-to-gel transitions is that of the FUS protein. The FUS protein is hypothesized to have numerous chaperone-related functions, including the ability to quickly localize to events of DNA damage and form discrete bodies that recruit additional repair factors.[36] This ability to form a reversible microenvironment is assumed to be reliant on the ability of FUS's disordered domain to quickly form multiple weak transient interactions with neighboring FUS molecules. Mutations in this protein are associated with the disease amyotrophic lateral sclerosis (ALS). Interestingly, ALS-related mutations in the disordered domains of FUS accelerate gel formation and eventual pathogenic aggregation.[36] Therefore, it is proposed that, although the "liquid-like" organization of FUS plays a physiological role, the transition to more gel-like fibrous structures contributes to the pathological states associated with ALS.[36]

These examples above raise the question of what might be the consequences of different physical heterochromatin states. By analogy to the examples above, we imagine that HP1-mediated heterochromatin may exist in vivo in at least three types of states: soluble, liquid droplet-like, and gel-like (Figure 1A). As mentioned above, HP1$\alpha$ can adopt all three types of states in vitro.[12] Further, the smallest chromatin-containing droplets observed in vitro are on the submicrometer scale and thus are compatible in principle with nuclear dimensions.[12] Although there is no direct evidence to date for the gel-like state existing in cells, recent in-vivo from the Karpen group suggests that the diffusion of HP1 molecules is slower at the boundary of heterochromatin puncta compared to within and outside the puncta.[31] This result is consistent with the presence of a spatially constrained region such as a phase-separated liquid droplet. Further, recent work from the Zaret group has identified specific regions of heterochromatin that are resistant to sonication-driven dissolution consistent with different material properties between separate regions of chromatin.[37] We propose that the soluble state of heterochromatin may represent the least repressive state in terms of gene expression as it would more readily allow access to the underlying chromatin and DNA. It is also possible that the soluble state is the one in which the parallel mechanisms of RNAi are initiated. Such a state may also be localized to regions of the genome where heterochromatin forms in a developmentally regulated manner. The phase-separated droplet state could then represent a less accessible and more repressed state of developmentally regulated genes as the cells proceed through differentiation. Such a state may also be associated with regions of the genome that have constitutive heterochromatin such as the centromeres. We speculate that a gel-like state of HP1-heterochromatin may play a partial structural role such as providing an anchor for the kinetochore during chromosome segregation or may represent the form taken by hetero-chromatin in terminally differentiated cells. It is also possible that, by analogy to the FUS protein, a gel-like state of HP1 heterochromatin represents a pathological state that locks in repression at locations that need to be developmentally regulated.

For the sake of the discussion above, we described the soluble, liquid droplet and gel-like heterochromatin states as three discrete states. However, in reality there is likely a coexistence of many states ranging from soluble to gel-like with the specific nature of the material state being dependent on the reaction conditions and the component molecules (Figure 2).



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Indeed, if multiple states do occur in cells, there must exist mechanisms to regulate transitions across these states. Some insight into how this might be achieved comes from in vitro observations that phase separation by HP1α is coupled to a large conformational change in the HP1α dimer thought to expose the hinge and N-terminal regions for making multivalent interactions.[12] Interestingly, specific ligands of HP1α can affect this equilibrium and thereby regulate the critical concentration, i.e., the concentration above which the HP1α phase separates. An interacting region of the Shugoshin protein promotes HP1α oligomerization and phase separation, whereas an interacting region of the Lamin B receptor dissolves HP1α phases. Binding of DNA or RNA to HP1α also drives HP1α phase separation, and it will therefore be of much interest to study the interplay between the phase-promoting behaviors of CSD ligands and of DNA. In general, HP1α anchored to the H3K9me[3] mark via its chromdomain presents a molecule with several opportunities for multivalency with numerous epitopes of different nature. These considerations open possibilities for binding events feeding into multiple modes of chromatin interaction. As mentioned above, HP1α also has the capacity to form gel-like states in vitro when the droplets are allowed to settle for longer time-scales or at lower temperatures. This transition is promoted in vitro by deletion of the C-terminal extension of HP1α, raising the possibility that transitions to the gel-like state may also be regulated by CSD ligands. Indeed, in cells, HP1α has been shown to interact with diverse partner proteins through its CSD−CSD interface. It would therefore be interesting to test the effects of these different ligands on the material properties of HP1α phases. Additional components of heterochromatin such as RNA could also affect the material properties by providing scaffolds or templates.

Another way to regulate transitions could be through post-translational modifications as suggested in the context of Cajal bodies. These are nuclear bodies involved in several functions including nuclear ribonucleoprotein biogenesis, and it has been proposed that the formation of Cajal bodies may involve phase separation mechanisms.[15] These bodies are scaffolded by the RNA binding protein Coilin, which contains several putatively unstructured regions, and can form oligomers.[18] These properties of Coilin raise the possibility that Cajal bodies may entail coacervation-like mechanisms between Coilin and RNA, which could aid in maintaining disagregation. Coilin also has numerous phosphorylation sites in its unstructured regions, and these sites appear critical for Cajal body formation. Mutations of these phosphorylation sites results in oligomerization defects in Coilin and its mislocalization to the nucleolus.[18] By comparison, HP1α proteins depend on the electrostatic composition of their unstructured domains to regulate the choice between self-association and DNA association. In vitro, N-terminal phosphorylation inhibits DNA binding but promotes oligomerization as the same region of the hinge appears to make contacts with DNA and with the phosphorylated residues of the neighboring HP1α dimer. N-terminal phosphorylation of HP1α also appears to enable larger droplets compared to those formed with unphosphorylated HP1α and DNA.[12] Thus, in the context of HP1α, phosphorylation may provide a means to control the density of chromatin as well as the size of the heterochromatin bodies. Further, in cells, analogous to Coilin, perturbation of these phospho sites on HP1 results in perturbation of heterochromatin bodies and chromosome defects.[38]

A mechanism for globally controlling heterochromatin states could occur through changes in the overall nuclear volume during the cell cycle, which has the potential to change HP1α concentrations.[39] If HP1 concentrations are maintained at local concentrations hovering around the critical point of separation, then relatively small changes in concentration could lead to large changes in the physical state of heterochromatin. Such changes could explain in part the strong dose-dependent activity of HP1 as a repressor.[40] Analogously, changes in the methylation or phosphorylation state of the nucleosomes throughout the cell cycle could also influence the effective critical concentrations for chromatin-driven phase separation by HP1.

In addition to affecting the overall material properties, phase separation provides an opportunity to regulate the physicochemical environment within heterochromatin. Such regulation could occur through the selective enrichment of specific components and through altering the local chemical environment. How might access be regulated for the partitioned domains? At a gross physical level, it is tempting to imagine that certain phase-separated HP1-chromatin states behave akin to a dynamic matrix with an effective pore size that disproportionately slows the diffusion of larger macromolecules in and out of the heterochromatin region compared to smaller macromolecules.

The polymer-rich environment within the nucleus introduces additional opportunities for segregation of heterochromatin phases based on the different chain lengths and associated chain entropies of coexisting protein and nucleic acid polymers.[41] In several studies of aqueous two-phase systems, partitioning of components into different phases can be greatly varied by altering the chain length or ratio of the components.[5] Within the nucleus, the coexistence of several polymer-rich phases of different composition (pH, ionic strength, molecular weight) could therefore influence segregation of components based on properties such as the effective $pK_a$, hydrophobicity, and size of the solute molecule.[42,5,1]

In another mutually compatible mechanism, macromolecules that make specific interactions with HP1 proteins could be enriched within the phase-separated compartments. In vitro, proteins like Aurora B kinase, which are known to interact directly with HP1 proteins, become concentrated in HP1α droplets.[12] Overall, an increased local concentration of heterochromatin-specific molecules would then play a significant role in affecting binding equilibria and kinetics as well as enzymatic rate constants. Such a possibility could explain why many heterochromatin proteins and enzymes that bind nucleosomes have $K_d$ values in the 10s of micromolar.[21] In terms of enzymatic activity, an enhancement of catalytic activity by concentration within specific droplet phases in the context of an aqueous two-phase system has been observed for the hammerhead ribozyme in vitro.[5] Many enzymes involved in promoting heterochromatin formation such as the ACF chromatin remodeling complex and the Suv39h1 histone H3K9 methylase complex have been shown to directly interact with HP1 proteins.[6] Thus, it is possible that proteins permissive to heterochromatin function are enriched while those permissive to gene activation are excluded. If remodeling complexes and methylases involved in heterochromatin formation could be recruited and retained in a growing heterochromatin phase in preference over the machinery required for gene activation, a specific heterochromatin domain could be assembled and maintained. Such a process would


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provide a qualitatively different mechanism of positive feedback compared to previous models.

In addition to concentration effects, the multitude of binding and chemical processes occurring inside a phase-separated region are also expected to be sensitive to the local electrostatic and redox environment, pH, and the extent and type of water solvation. The core components of a nucleosome, histones, and DNA are held together largely through electrostatic interactions. Higher-order chromatin folding, which entails internucleosomal interactions, also relies on electrostatic histone−histone and histone−DNA interactions.[43] Correspondingly, in vitro, all the steps associated with chromatin assembly and folding are particularly sensitive to the nature of the ionic strength of the medium.[44] Thus, it is possible that the specific local electrostatic environment within a heterochromatin phase directly affects chromatin stability and compaction. In addition, a substantially different chemical environment inside the heterochromatin phase compared to outside provides an opportunity to control enzyme activity. For example, the H3K9 methyl transferase from Neurospora, Dim-5, has an unusual pH dependence in vitro with maximal activity around pH 9.8.[10] It is thus tempting to speculate that some of these pH dependencies may reflect the natural environment in which these enzymes function. Further, reactants that are concentrated within a phase could themselves affect reaction kinetics by altering the local electrostatic potential and thereby feed back on activity. Such local control could be used both to create reaction centers by providing optimum physiochemical conditions and to poise inactive molecules for contributions at a later time or condition. Overall, understanding how different heterochromatin states regulate the local chemical environment could help inform how they accomplish their function. If these heterochromatin compartments do regulate their internal components by creating unique environments, the ability to specifically mark this composition would be very useful. There are an array of dyes known to stain the cross $\beta$-sheet structure of amyloid fibrils used for years as diagnostics for pathological progression of amyloid formation.[13] Of great use would be analogous environmentally sensitive dyes that could specifically mark nuclear structures and environments based on their composition without direct perturbation of the system with genetically encoded tags.

## COUPLING PHASE TRANSITIONS TO CHROMATIN COMPACTION

Several previous studies have shown that assembly of HP1 proteins in vivo is correlated with chromatin compaction. Previous models have proposed that oligomerization of HP1 on chromatin may be coupled to compaction.[16] Recent work has uncovered an intrinsic DNA compaction property of HP1$\alpha$. HP1$\alpha$ is able to rapidly compact, at a speed of 2 $\mu$m/s, several kilobases of DNA into dense puncta.[12] Mutations in the positively charged regions of the hinge of HP1 that are important for DNA binding inhibit droplet formation and the associated DNA compaction. N-terminal phosphorylation of HP1$\alpha$ also inhibits DNA binding and slows the rate of compaction. How such a rapid and large degree of compaction is achieved is not fully understood, but substantial bending of the DNA would be required to enable its compaction to such dimensions.

We can imagine three mutually compatible mechanisms to explain such DNA bending. First, specific interactions between HP1$\alpha$ and the DNA could promote bending analogous to the bending achieved by the architectural eukaryotic HMG proteins.[45] Second, the physical properties of a phase-separated HP1$\alpha$ coacervate might also influence the process of chromatin compaction. In many systems of DNA or RNA packing, such as some instances of viral packaging, a motor must provide the actuation energy to drive bending of the fiber. However, in systems where there exists a viscosity difference between two solutions, such that a high-energy interface introduces surface tension, a filament with more affinity for one solution will use the discrepancy in surface energies as the actuator to buckle and pack the fiber.[46] As the viscosity of nucleoli have recently been measured to be on the order of honey ($10^6$ cP), it is possible that heterochromatin domains also use viscosity differences to their advantage.[47] Here, it is important to also note that while complex coacervates are generally associated with low surface energy, strong electrostatic interactions between components can increase the surface energy.[48] A third mechanism can arise from coupling between incorporation of the DNA in an HP1$\alpha$ with an increase in the local ionic strength. Increasing ionic strength increases the elasticity and decreases the persistence length of DNA due to screening of the Coulombic self-repulsion.[49] In the context of chromatin, increasing the elasticity of the linker DNA between nucleosomes could influence compaction by changing the forces required to bend the chromatin fiber.

The DNA compaction ability of HP1$\alpha$ is most reminiscent of the DNA compaction achieved during the packaging of human sperm DNA, where phase separation driven processes have also been invoked.[50] During the final stages of spermatogenesis, histone proteins are replaced with small arginine-rich proteins called protamines, which are thought to directly enable DNA condensation. Interestingly, protamines display N-terminal phosphorylation analogous to HP1$\alpha$, and this phosphorylation results in a reduction of overall DNA binding affinity with a concomitant enhancement of interstrand cross-linking during genome packaging.[51] We propose that the DNA compaction activity of HP1$\alpha$ is major contributor to the chromatin compaction observed in heterochromatin. In the cell, however, this process would need to be tightly regulated. Consequently, we imagine HP1$\alpha$ proteins as modular protamines where the additional CD and CSD domains help regulate the potent DNA compaction activity through interactions with histones and other proteins. In addition, the observation that N-terminal phosphorylation of HP1$\alpha$ slows DNA compaction raises the possibility that phosphorylation could serve to control the extent of DNA compaction.

The manner in which DNA compaction appears to occur, i.e., via partitioning of DNA into the compacting complex, also suggests a new way to conceptualize heterochromatin spread. It is possible that some of the heterochromatin spreading behavior observed in cells arises from partitioning of non-HP1$\alpha$-bound chromatin into a nearby HP1$\alpha$ phase (Figure 1B). Overall, these new findings suggest that, in addition to the spreading and compaction mediated by oligomerization of HP1 proteins across chromatin as previously proposed, compaction and spreading could also be enabled through the intrinsic DNA-coupled phase separation behavior of HP1$\alpha$.

## A MECHANISTIC WINDOW INTO UNDERSTANDING HP1 PARALOGS

An interesting characteristic of the HP1 family of proteins is the presence of paralogs that have different biological functions. In humans, the other two HP1 proteins, HP1$\beta$ and HP1$\gamma$, show



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>90% conservation of sequence in their CD and CSD domains yet perform functions quite different than HP1α. Although both HP1β and HP1γ have been implicated in gene repression, they have also been shown to play direct roles in increasing transcription at either the initiation or elongation steps.[52] Further, the three HP1 paralogs also localize to different regions of the nucleus in a variety of mammalian cell types.[53,54] Why the three human HP1 paralogs have such different biological functions is not clear. At a biochemical level, HP1α has been shown to bind most strongly to DNA followed by HP1γ and then HP1β.[21] Consistent with these findings, HP1β does not appear to form phase-separated droplets in the presence of DNA or phosphorylation and also does not compact DNA.[12] These results have suggested that the different functions of the HP1 paralogs may arise in part from differences in their intrinsic biophysical capabilities. The different biophysical properties in turn appear to be conferred by sequence differences in the unstructured N-terminal, hinge, and C-terminal regions. Consistently, swapping the N-terminal and hinge regions of HP1α into HP1β causes the chimeric protein to form droplets in a phosphorylation-dependent manner.[12] The more potent DNA compaction and phase separation activities of HP1α compared to HP1β can help explain the dominant role of HP1α in heterochromatin as opposed to gene activation. Correspondingly, the absence of such activities in HP1β may allow this protein to recruit gene activators in the context of more open chromatin states.

A consequence of the high conservation in HP1's CSD dimerization domain is the potential for heterotypic dimers that are readily formed in vitro.[16] Such observations raise the question of whether heterotypic dimers function to inhibit oligomerization and thereby act as boundary elements that limit the spread of heterochromatin. Thus, for example, a compacting HP1α DNA droplet surrounded by a pool of HP1β at a suitable concentration may act as a barrier to spreading, insulating nearby domains from silencing in three dimensions by diluting out the HP1α assembly through heterotypic dimerization.

## HP1α PHASE SEPARATION UNDER PHYSIOLOGICAL CONDITIONS AND MOLECULAR STIR BARS

Currently, in the field of genome organization, there is a vigorous debate about the phase separation phenomenon. The questions being asked range from if phase separation of chromatin actually occurs in the nucleus to what might be the functional significance of different manifestations of phase separation. In terms of the second question, i.e., functional significance, we have shared our speculations in the previous sections. In terms of the first question, several others have pointed out that the evidence already exists in the form of phase-separated chromatin within the nucleolus, the most cytologically obvious nuclear membrane-less organelle.[47,55] Studies with the nucleolus have strongly suggested liquid-like behavior of this organelle. A question to ask then is whether the phenomenon observed for the nucleolus extends to other forms of chromatin organization. In this context, it would be further useful to ask what the smallest size of a phase-separated chromatin domain would be for demixing to still be an accurate description as opposed to the formation of a large macromolecular assembly.

Existing biochemical data strongly suggests that phase separation of HP1α mediated heterochromatin is likely to be an energetically downhill process under physiological conditions. This is because HP1α droplet formation occurs in a test tube under physiological temperatures and ionic strength without any molecular crowding agents and at concentrations that are well within the physiological concentration range of HP1 proteins.[12] In the nucleus, the environment will be substantially more crowded due to the presence of additional macromolecules. Thus, mechanisms such as depletion and volume exclusion forces will likely make the phase separation process even more favorable.[1] We believe the question therefore is not whether phase separation occurs within cells but rather how the cell works to prevent such processes from occurring all the time. We propose that the many different types of ATPases working on specific substrates within the nucleus, including chromatin-remodeling motors, act as molecular stir bars to increase the local kinetic energy of chromatin and the molecules bound to it in a manner that prevents irreversible phase separation.

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### Notes

The authors declare no competing financial interest.

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