

Interplay between charge distribution and DNA in shaping HP1 paralog phase separation and localization

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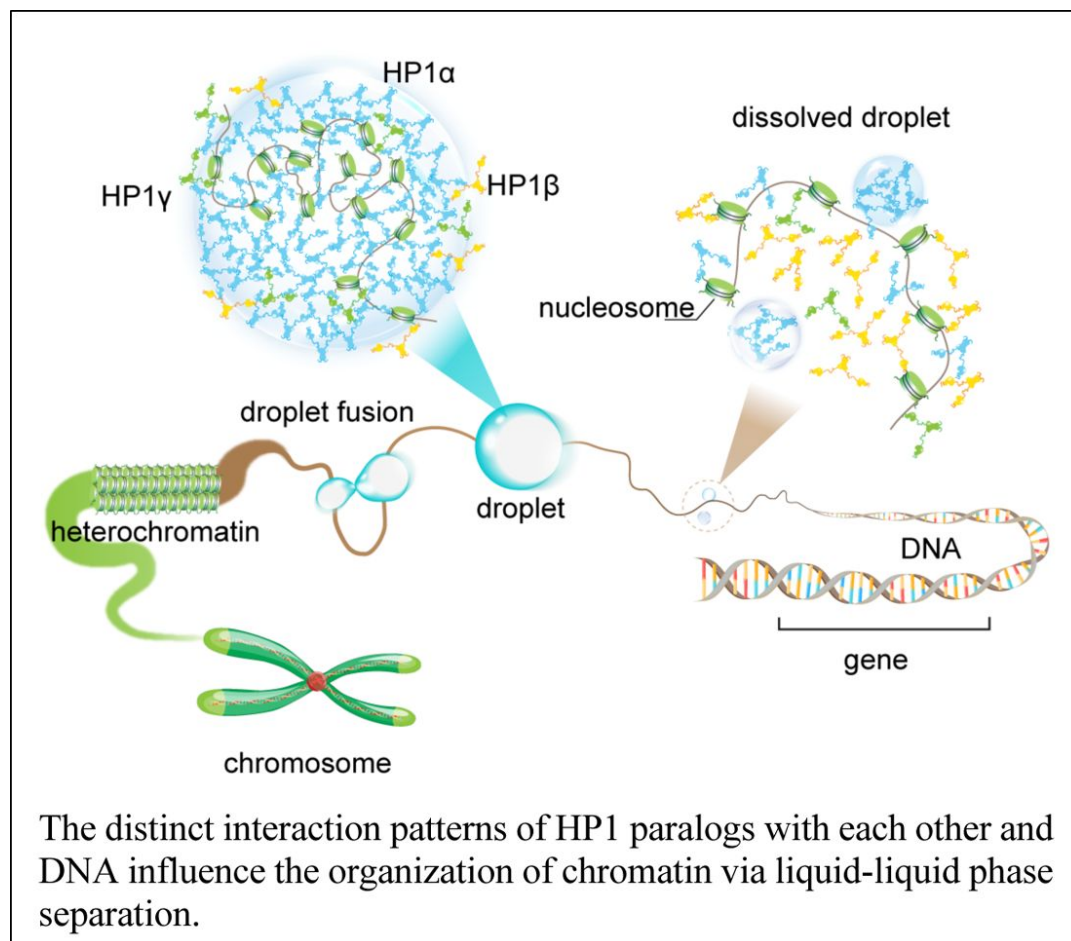
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Abstract

Abstract

The heterochromatin protein 1 (HP1) family is a crucial component of heterochromatin with diverse functions in gene regulation, cell cycle control, and cell differentiation. In humans, there are three paralogs, HP1 α , HP1 β , and HP1 γ , which exhibit remarkable similarities in their domain architecture and sequence properties. Nevertheless, these paralogs display distinct behaviors in liquid-liquid phase separation (LLPS), a process linked to heterochromatin formation. Here, we employ a coarse-grained simulation framework to uncover the sequence features responsible for the observed differences in LLPS. We highlight the significance of the net charge and charge patterning along the sequence in governing paralog LLPS propensities. We also show that both highly conserved folded and less-conserved disordered domains contribute to the observed differences. Furthermore, we explore the potential co-localization of different HP1 paralogs in multicomponent assemblies and the impact of DNA on this process. Importantly, our study reveals that DNA can significantly reshape the stability of a minimal condensate formed by HP1 paralogs due to competitive interactions of HP1 α with HP1 β and HP1 γ versus DNA. In conclusion, our work highlights the physicochemical nature of interactions that govern the distinct phase-separation behaviors of HP1 paralogs and provides a molecular framework for understanding their role in chromatin organization.

Graphical abstract



eLife assessment

This **valuable** study substantially advances our understanding of molecular mechanisms driving the phase separation behavior of HP1 paralogs. The evidence supporting the conclusions is **convincing**, with rigorous and well-designed computational simulations. The work will be of broad interest to biophysicists and biochemists.

Introduction

The heterochromatin protein 1 (HP1) family are evolutionarily conserved nuclear proteins that are essential regulators of chromatin structure and function in eukaryotic cells. In humans, the HP1 family comprises three paralogs, HP1α, HP1β, and HP1γ, encoded by a class of genes known as the chromobox (CBX) genes (CBX5, CBX1, and CBX3, respectively). All three paralogs localize to heterochromatic regions where they mediate chromatin condensation and gene silencing ([1](#), [2](#)). However, HP1β and HP1γ have also been found in euchromatic regions that express active genes ([3](#)–[5](#)). The importance of HP1 proteins in genome regulation and their potential association with cancer development ([6](#)) has prompted increasing endeavors to elucidate the molecular mechanisms underlying their biological activity.

HP1 paralogs are multi-domain proteins, sharing high similarities in amino acid sequence and domain architecture. They possess a basic structure that consists of two highly conserved folded domains, the chromodomain (CD) and the chromoshadow domain (CSD), connected by a disordered hinge of variable lengths and two unstructured N and C terminal extensions (NTE and CTE, respectively). The CD specifically recognizes methylated lysine 9 on the histone H3 tail (H3K9me), an epigenetic mark for gene silencing (7, 8). The CSD mediates the dimerization of HP1 and interacts with other proteins through a PXVXL motif (9–11). Dissociation constant (K_d) values in the nano-molar range have been reported for CSD homodimerization of HP1 α and HP1 β (12, 13). Moreover, *in vitro* and *in vivo* co-immunoprecipitation have shown that mammalian HP1 paralogs can form heterodimers directly with one another (14), which raises the question of the biological functions of these heterodimers. Compared to the CD and CSD, the flexible disordered regions are less conserved, potentially contributing to the distinctive functional properties of different HP1 paralogs. The hinge region has been observed to target HP1 to heterochromatic regions through non-specific binding to DNA and RNA (15, 16). Furthermore, interactions between the hinge region and the CTE are proposed to be responsible for an auto-inhibited dimer conformation (17). By contrast, the NTE has been implicated in mediating the binding affinity of the CD for the methylated histone H3 tail (18). Different HP1 domains contribute to a complex interaction network with specific regions of chromatin, other peptide ligands, and nuclear components. These interactions are attributed to the multi-functionality of HP1 proteins in the context of heterochromatin.

Heterochromatin formation has been suggested to involve the recruitment of HP1 paralogs based on direct binding to H3K9me3, which facilitates the construction of bridges between nucleosomes and potentially induces the formation of compact chromatin states (19–22). However, *in vitro* experiments have shown that HP1 α can undergo liquid-liquid phase separation (LLPS) upon either NTE phosphorylation (pHP1 α) or DNA binding, which provides a new perspective on heterochromatin formation and function through a phase-separation mechanism (15, 17). Despite phylogenetic conservation, HP1 paralogs display different phase separation behaviors. Without phosphorylation or DNA binding, HP1 α can undergo LLPS at high protein concentrations (15, 23) and low salt concentrations (24). While HP1 γ undergoes LLPS at a protein concentration that is multi-fold higher than the threshold concentration for HP1 α (23), HP1 β does not form liquid droplets under any tested conditions (17, 23). However, HP1 β , but not HP1 α or HP1 γ , can form liquid-like droplets in the presence of histone proteins (23). These observations raise fundamental questions regarding the sequence features of HP1 paralogs. For example, what is the physicochemical nature of interactions that dictate the distinct phase-separation behaviors of HP1 paralogs? How does the differential phase separation behavior enable the co-localization of HP1 proteins within heterochromatic regions, and does heterodimerization play a role in regulating this process? Do HP1 paralogs play distinct, cooperative, or largely redundant roles in heterochromatin organization? It is essential to uncover the underlying forces driving HP1 LLPS to understand the mechanisms of heterochromatin formation and regulation.

Addressing the challenges of timescales and length scales inherent in protein LLPS, a recent coarse-grain (CG) framework (25–27) has emerged as a powerful computational tool to provide insights into the molecular interactions behind phase separation of multidomain and intrinsically disordered proteins (IDPs). Here, we use this CG simulation framework to uncover the underlying forces driving phase separation of HP1 paralogs. We examine the intricate interplay between folded and disordered domains in mediating phase separation of HP1 homo- and heterodimers. We further explore the potential co-localization of different HP1 paralogs in multicomponent assemblies and the essential role of DNA in modulating this process. Our findings reveal that strong HP1 α -DNA binding outcompetes other possible heterotypic interactions between paralogs and paralogs and DNA, orchestrating the organization of the multicomponent

condensates. This study presents a comprehensive molecular framework that elucidates the mechanism and regulation of HP1 LLPS, thereby providing invaluable insights into the formation of heterochromatin and its diverse functional implications.

Results

Amino acid composition and distribution of HP1 paralogs

HP1 proteins consist of highly conserved folded domains and less conserved disordered regions (**Fig. 1a**). Compared to the corresponding regions of HP1 α , the NTE, hinge, and CTE of HP1 β share 35%, 33%, and 38% sequence similarity, respectively, while for HP1 γ those numbers are 15%, 36%, and 19%, respectively (**18**). On the other hand, the CD and CSD domains of HP1 α and HP1 β show over 80% sequence similarity, while the similarities between these domains in HP1 α and HP1 γ are 71% and 87%, respectively (**18**). Three-dimensional (3D) structures of the CD and CSD domains have been solved by nuclear magnetic resonance (NMR) spectroscopy (**13**, **28**, **29**) and X-ray crystallography (**30**, **31**). The CD and CSD are both globular domains sharing remarkable similarities. They consist of three anti-parallel β -strands at the N-terminus and an α -helix at the C-terminus. The structural difference between the monomeric units of CD and CSD can be attributed to the presence of an additional α -helix in the CSD (**Fig. 1b**). It should be noted that the CD functions as a monomer while the CSD forms a symmetrical homodimer where the two CSD monomers interact via their α -helices (**Fig. 1c**). Furthermore, in the crystal structure (3i3c) (**32**), an additional CSD-CSD interaction can be observed, which occurs at the hydrophobic β -sheet interface of HP1 α (**Fig. 1c**). This interface has been observed only in this crystal structure so far, and it is not clear if it plays a role in HP1 α oligomerization in solution or under physiological conditions.

HP1 paralogs are enriched in charged residues, with Arg, Lys, Glu, and Asp comprising 39-45% of the total number of amino acids in the protein. Of the three paralogs, HP1 β has the highest percentage of charged residues (45%), with a predominance of negatively charged amino acids (26%). This results in a net charge of -13 , which is more negative than the net charge of HP1 γ (-5) and HP1 α (-3). Ion-exchange chromatography also showed that HP1 β has the lowest isoelectric point (pI 4.85), followed by HP1 γ (pI 5.13) and HP1 α (pI 5.71) (**23**). The three paralogs share comparable composition of other amino acid types such as aromatic residues ($\sim 8\%$), aliphatic residues (21-25%), polar residues ($\sim 15\text{-}18\%$), and other residues (9-11%) (**Fig. 1d**).

As the sequences of HP1 paralogs contain a high fraction of charged residues, we first characterized the charge property differences at the domain level across the three paralogs. Analysis of the charge distribution on folded domains of HP1 paralogs revealed a slight variation in their net charge. Particularly, HP1 α has a smaller net negative charge on its CD and exhibits a CSD with a more negative charge compared to those of HP1 β and HP1 γ (**Figs. 1e,f**). Disordered regions are less conserved and contain the most variable amino-acid sequences. The NTE exhibits variable length and a different fraction of oppositely charged residues organized into a charge-segregated diblock (**Figs. 1g-i**). HP1 β has a longer negative block than HP1 α , resulting in a higher net negative charge. In contrast, HP1 γ has an extended positive block, leading to a net positive charge (**Fig. 1e**). It is important to note that the HP1 α NTE has a unique block of four serine residues (**Fig. 1a**) that are constitutively phosphorylated *in vivo* (**33**). Phosphorylation can dramatically change the charge on the HP1 α NTE and significantly reduce the minimum concentration required for LLPS in the absence of DNA (**12**, **17**, **33**, **34**).

On the other hand, the CTE of all HP1 paralogs is characterized by a net negative charge. The charged residues in the CTE of HP1 β are organized into alternating blocks of opposite charges, whereas they are relatively mixed in the CTE of HP1 α . Notably, HP1 γ has a considerably shorter CTE comprising only negatively charged residues. The hinge region is particularly important for

determining the phase separation properties of HP1 proteins. All three paralogs have conserved basic patches located in the middle and near the end of the hinge, but HP1 α has an additional basic patch at its beginning (**Figs. 1g-i**). Although HP1 β contains a similar number of charged residues within its hinge as HP1 α , it is relatively balanced between oppositely charged residues (13/12). Conversely, HP1 α and HP1 γ contain more positively than negatively charged residues in their hinge regions (17/8 and 13/8, respectively).

Our sequence comparison shows that HP1 paralogs share a common structure but differ in net charge and charge distribution along the sequence. HP1 β has the most net negative charge with balanced oppositely charged residues in its hinge. HP1 γ has a significantly shorter CTE but possesses an extended charged-segregated diblock in the NTE. HP1 α has the least net negative charge of the three paralogs, and its hinge is rich in clusters of positively charged lysine and arginine residues. The sequence variances may dictate differences in the overall conformation and phase separation behavior of the three paralogs, as discussed below.

Sequence variation of HP1 paralogs leads to differential conformation and phase separation

Sequence variation affects the conformations of HP1 paralogs in the dilute phase. We performed CG simulations of single homodimers using the HPS-Urry model (35), which we recently applied to uncover the molecular interactions driving the phase separation of HP1 α (12). The full-length HP1 α model was constructed as previously described using PDB structural models 3fdt (30) and 3i3c (32) for the CD and CSD domains, respectively. Intrinsically disordered regions (IDRs), NTE, hinge, and CTE, were connected to the folded domains, while the homodimer CSD-CSD α -helix interface was created using the MODELLER software (36). The HP1 α dimer was used as a template to prepare HP1 β and HP1 γ dimers with homology modeling in MODELLER. To mimic the movements of the dimer configuration, we used the simulation protocol described previously (12), in which the folded domains (CD and CSD) were kept rigid to avoid unfolding, the CSD-CSD domains were fixed with respect to each other, and the IDRs remained flexible.

To gain insights into the global conformation of HP1 paralogs (Movie S1), we calculated the radius of gyration (R_g) distributions of the homodimers under dilute conditions (**Fig. 2a**). We found that the HP1 β conformations ($R_g = 3.93 \pm 0.78$) are on average more extended than the HP1 α and HP1 γ structures ($R_g = 3.20 \pm 0.31$ nm and $R_g = 3.48 \pm 0.69$ nm, respectively), which is consistent with observations in previous work (17, 37, 38). Notably, the average dimension of HP1 paralogs expands in order of increasing the net negative charge of the respective homodimer where HP1 α has a negative charge $q = -6$, HP1 γ has $q = -10$, and HP1 β has $q = -26$. To characterize molecular interactions within the homodimer that cause the observed conformational differences, we computed the number of intramolecular (within an HP1 monomer) and intermolecular (between two HP1 monomers) van der Waals (vdW) contacts formed by each residue in the CG simulations. The contacts within the rigid body (folded domain) were excluded from the calculations. As the HP1 paralogs share similar structural topologies, we summed over the time-averaged intra- and intermolecular contact pairs per residue in each domain to create one-dimensional (1D) contact maps (**Fig. 2b**). These 1D contact maps highlight the differences in overall intra- and intermolecular contacts contributed by each domain to the interaction network of HP1 homodimers. Among the three paralogs, HP1 β has the smallest number of contacts at the domain level, presumably due to charge repulsion between extended stretches of acidic residues in its domains. While HP1 α and HP1 γ make similar contacts with their folded domains, the hinge and CTE of HP1 α are much more interaction-prone in both monomer and dimer contexts.

To gain more insights into the interactions between domains, we analyzed two-dimensional (2D) time-averaged contact maps of interactions as a function residue number (**Figs. 2c-e**). These maps display the high and low-contact-prone regions along the homodimer sequence. To highlight

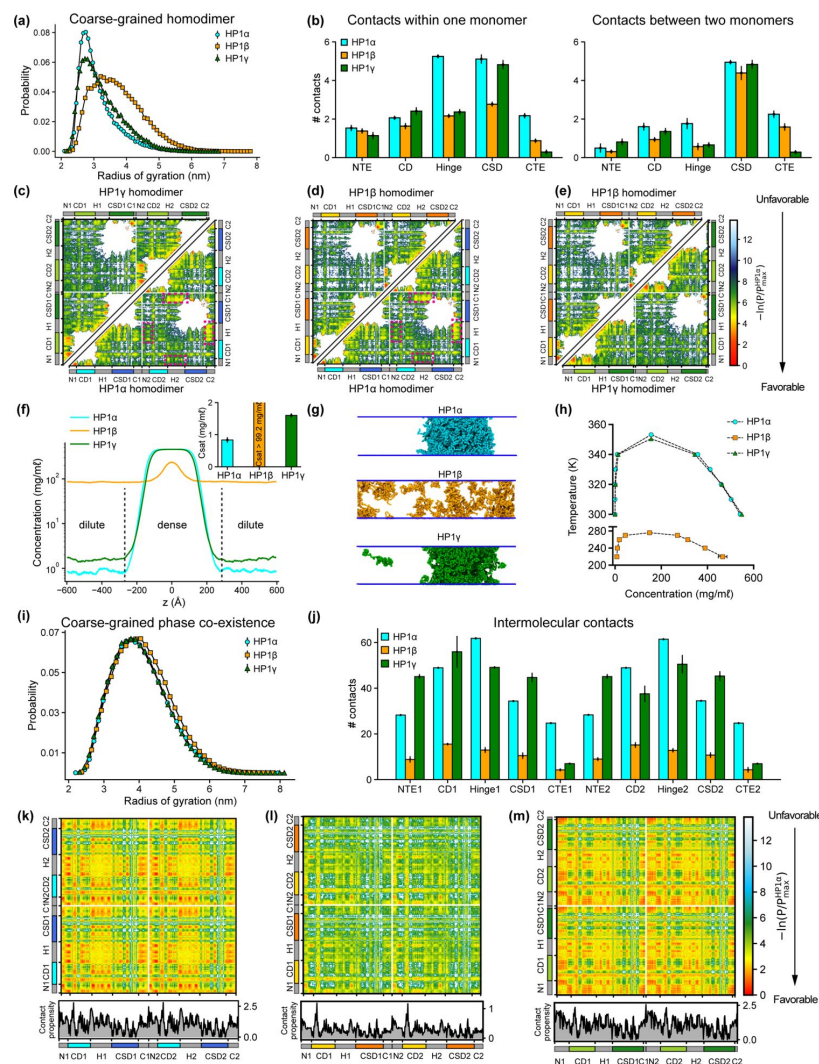


Figure 2

Sequence differences result in differences in conformation and phase separation of HP1 paralogs.

(a) Rg distributions of HP1 paralogs in CG single homodimer simulations using the HPS-Urry model. (b) Average intramolecular contacts within one chain and average intermolecular contacts between two chains on each domain of the three HP1 paralogs. Two residues were considered to be in contact if the distance between them was less than 1.5 of their vdW arithmetic mean. (c, d, e) Pairwise 2D contact maps between two HP1 homodimers. The intramolecular interactions within one monomer and the intermolecular interactions between two monomers are shown in the two small triangles and the off-diagonal quadrant, respectively. The contact propensity of HP1β and HP1γ is normalized to the highest contact propensity of HP1α. The magenta boxes highlight strong electrostatic attractions between the hinge and CTE regions in HP1α. (f) Density profiles of HP1 paralogs in CG coexistence simulations. The inset shows the respective saturation concentrations. (g) Representative snapshots of each system in the CG coexistence simulations. (h) Phase diagrams of HP1 paralogs at different temperatures calculated in CG simulations where a dilute phase of free monomers co-exists with a dense phase. The critical temperatures of HP1α, HP1β, and HP1γ are 353.3 K, 276.1K, and 350.5 K, respectively. (i) Rg distributions of the HP1 paralogs as calculated in the CG phase coexistence simulations. (j) Average intermolecular contacts made by each domain of the HP1 paralogs in CG phase coexistence simulations. The error bars represent the standard deviation from triplicate simulation sets. (k, l, m) Intermolecular contacts within the condensed phase of HP1 paralogs along the sequence of each homodimer. The panels below each map show the average contacts per chain as a function of residue number. The contact propensity of HP1β and HP1γ is normalized to the highest contact propensity of HP1α. **Figs. 2a-e** show the results obtained under dilute conditions, while **Figs. 2f-m** illustrate the conditions of phase coexistence. The CG simulations under dilute and phase coexistence conditions were conducted using the HPS-Urry model at 320K and 100 mM salt concentration.

the distinct molecular interaction differences between the HP1 homodimers, we performed pairwise comparisons of the 2D contact maps. Similar to the 1D interaction map, the interaction network of HP1 α has significant contributions from the hinge and the CTE (**Fig. 2c**). These interactions stem from intra- and intermolecular contacts, primarily between the CTE and the hinge and partially between the NTE and the hinge (magenta boxes). This can be attributed to the charge attraction between oppositely charged residues in the two regions. We also observe intramolecular and intermolecular interactions between the folded CD and CSD domains with disordered segments (NTE and CTE) in HP1 α . HP1 γ shares a similar interaction pattern between folded domains and disordered regions. However, the extended stretch of basic residues in the diblock of the HP1 γ NTE promotes self-interactions within the diblock, as well as intramolecular interactions between the NTE and the CD, and intermolecular interactions between NTEs (NTE1-NTE2). The interactions in the hinge of HP1 γ are less pronounced than those observed in HP1 α , likely due to the absence of an additional attraction hotspot. The most significant difference between the contact maps of the paralogs is the limited and sparse interaction network between HP1 β monomers (**Figs. 2d,e**). The lack of favorable intermolecular interactions between disordered and folded domains causes the extended conformation of HP1 β . The results of our coarse-grained (CG) simulations are in good agreement with those of atomistic simulations performed over a 5 μ s simulation time (**Figs. S1a-h**). Although HP1 paralogs share remarkable similarities in the sequence and structural topology of folded domains, our contact analysis suggests that sequence variations within disordered regions may cause distinct intra- and intermonomer interactions, leading to conformational differences.

Sequence variance in HP1 paralogs leads to their distinct phase separation behaviors. We conducted CG phase coexistence simulations of the HP1 homodimers using slab geometry (39, 40), as done in our previous work (12, 25). We simulated the systems at 320 K and plotted the protein densities as a function of the z-coordinate that separated the dense phase from the dilute phase (**Fig. 2f**). As HP1 can form a homodimer via the α -helix or β -sheet binding interface of the CSD (**Fig. S1j**), we tested the phase separation propensity of these two configurations. In the CG coexistence phase simulations, the CSD-CSD domains were fixed with respect to each other as a single rigid body in their α -helix or β -sheet binding interface. We found that the coexistence densities in the dilute phase (referred to as the saturation concentration, C_{sat}) of the two configurations of HP1 α homodimer were comparable within the error of three independent simulations (**Fig. S1k**). This suggests that the dimerization mode of the CSD domain does not affect the phase separation capability of the HP1 proteins as long as the homodimer can be formed. We note, however, that under physiological conditions, the phase separation propensity of the two configurations may be influenced by the variance in their binding affinities. Therefore, for the rest of the study, we will focus on the α -helix-mediated CSD-CSD conformation as the primary and biologically relevant form of HP1 homodimerization.

Under the simulation conditions, HP1 β did not undergo phase separation, while HP1 α and HP1 γ formed stable condensates (as evidenced in the flat density profiles in **Fig. 2f** and snapshots in **Fig. 2g**, **Movie S2**), but the C_{sat} of HP1 γ was approximately twofold higher than that of HP1 α (inset of **Fig. 2f**). We also constructed the phase diagrams for HP1 paralogs and found that the critical temperature (or the phase separation propensity) decreased in order for HP1 α , HP1 γ , and HP1 β (**Fig. 2h**). This correlates with the conformational expansion trends observed in the CG homodimer simulations under dilute conditions. These observations are consistent with experimental data and the relationship between single-molecule dimensions and phase separation propensity (17, 23, 41). Notably, in the absence of phosphorylation and DNA, HP1 α has been reported to phase-separate at a much lower protein concentration than HP1 γ *in vitro*, while HP1 β did not form phase-separated droplets under any tested experimental conditions. Moreover, SAXS data have shown that HP1 α conformations are more compact than HP1 β structures (17, 37). To date, the SAXS data for HP1 γ has not been reported. Under crowding conditions, the conformational differences among HP1 paralogs are less pronounced (**Fig. 2i**).

To explore the interactions that mediate phase separation of HP1 paralogs, we computed 1D and 2D intermolecular (between homodimers) contact maps within the condensed phase. We found that the number of intermolecular contacts at the domain level of HP1 β homodimers was much lower compared to HP1 α and HP1 γ , as expected (**Fig. 2l**). Moreover, HP1 β does not show noticeable intermolecular interactions between domains that are crucial to establishing multivalent contacts (**Fig. 2l**). On the contrary, NTE-hinge and CTE-hinge interactions are dramatically highlighted in the 2D contact map of HP1 α (**Fig. 2k**). These interaction patterns are also present in HP1 γ , but the overall number of contacts per domain is less than the number for HP1 α (**Figs. 2m,j**). Notably, the inter-dimer NTE1-NTE2 contacts of HP1 γ are substantially enhanced due to the presence of the extended charge-segregated diblock in its NTE (**Figs. 2m**, **1i**).

In summary, NTE-hinge interactions and NTE-NTE (between the charge-segregated diblocks) interactions govern the conformation of HP1 paralogs in the dilute state and drive multivalency under crowding conditions. Additionally, substantial interactions between disordered segments (NTE, hinge, and CTE) and folded domains (CD and CSD) highlight the positive role of folded domains in the condensate formation of HP1 paralogs (**Fig. 3a**).

Disordered and folded domains cooperatively mediate phase separation of HP1 proteins

Multi-domain proteins have been shown to exhibit complex multivalent interaction networks that mediate phase separation ([42](#)). Previous research has demonstrated the influence of electrostatics on the interactions between IDRs and folded domains ([43](#)). For example, it has been observed that folded domains affect the behavior of IDRs in a manner dependent on the accessibility of charged residues on the surface of the folded domain ([44](#)) and the charge properties of IDR sequences ([41](#)).

In studies of HP1 α phase separation, the less conserved disordered regions (hinge, NTE, and CTE) are thought to be sufficient to establish multivalent contacts for higher-order oligomerization, but the role of the highly conserved folded domains (CD and CSD) has not been explored ([45](#)). Given that the CD and CSD contain a significant number of charged residues and possess high net negative charge, we asked whether electrostatic interactions influence the interplay between IDRs and folded domains in the phase separation of HP1 paralogs. To explore this, we performed domain-swapping phase coexistence simulations for the three HP1 paralogs.

Given that HP1 α has the highest phase separation propensity, we first performed swaps of the IDRs (NTE, hinge, and CTE) of HP1 β and HP1 γ into HP1 α , yielding constructs HP1 α - β IDRs and HP1 α - γ IDRs (Table S1), respectively. We found that the HP1 β or HP1 γ IDRs fail to enhance inter-dimer NTE-hinge interactions. These chimeras are less prone to phase separation compared to the wild-type HP1 α (**Figs. 3b,c**). In fact, HP1 α - β IDRs did not undergo phase separation, and the C_{sat} of HP1 α - γ IDRs is twofold greater than the concentration for HP1 α . The results indicate that the IDRs of HP1 α are crucial to establishing multivalent contacts for condensate formation.

We next performed hinge-swapping simulations to explore how individual hinge regions regulate phase separation in the absence of DNA. We proceeded with replacing the hinge of HP1 α with the corresponding hinge segment from either HP1 β or HP1 γ (constructs HP1 α - β Hinge and HP1 α - γ Hinge in Table S1, respectively). We found that neither of the chimeras formed stable condensates (**Figs. 3d,e**). The hinges of HP1 β and HP1 γ failed to provide the necessary multivalent contacts with other HP1 α domains (**Fig. 3f**) due to insufficient segregation of basic blocks compared to the charge pattern of the HP1 α hinge (**Figs. 1g-i**). Indeed, engineered HP1 β , where four acidic amino acids in the hinge region were replaced with basic residues to provide an additional stretch of positive charge, underwent phase separation at protein concentrations

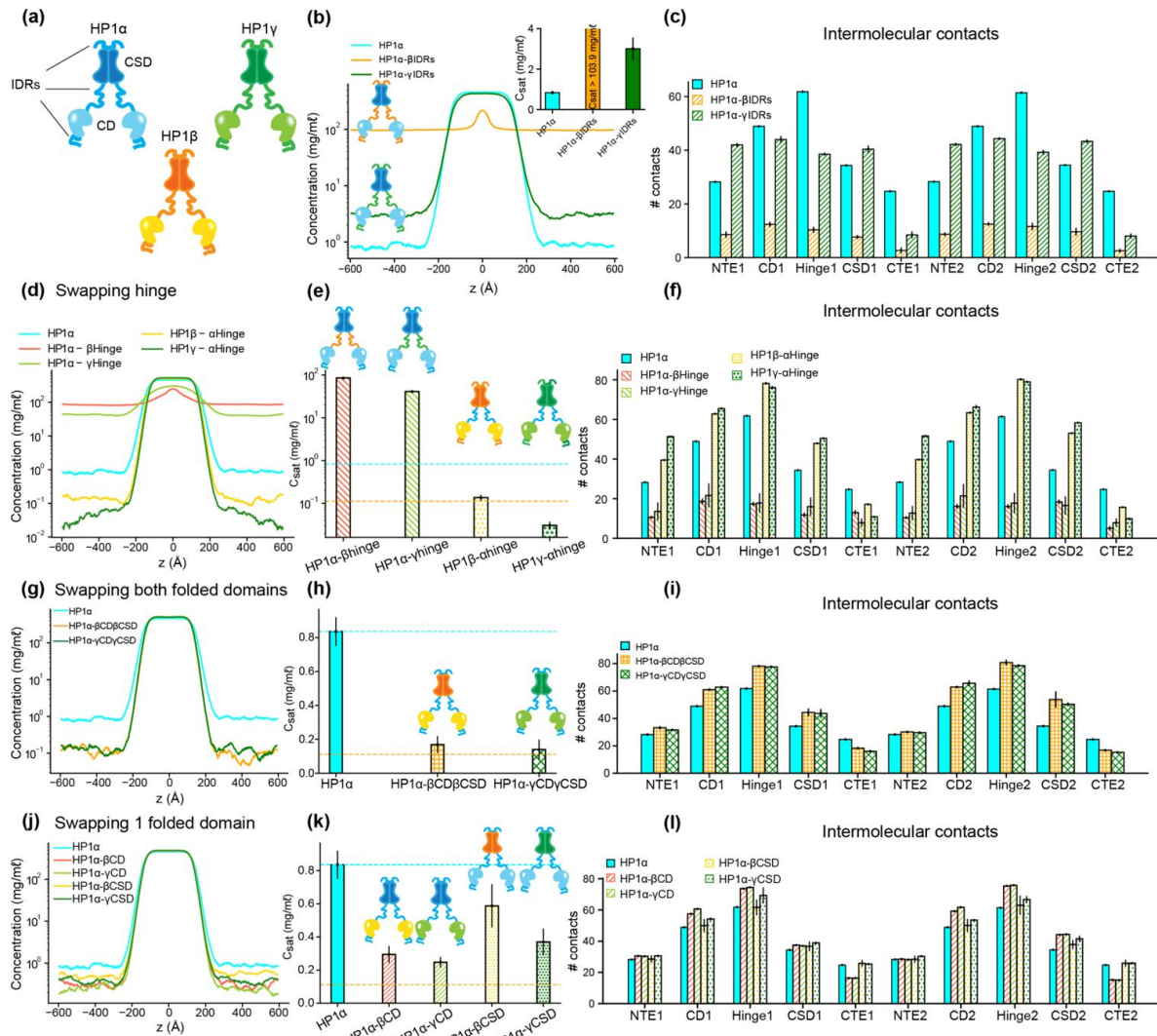


Figure 3

The interplay of IDRs and folded domains in mediating phase separation of HP1 paralogs.

(a) Cartoons representing the three human HP1 paralogs. **(b, c)** Density profiles, saturation concentrations (inset), and average contacts made by each domain of HP1α with IDRs replaced with those from HP1β or HP1γ. **(d, e, f)** Density profiles, saturation concentrations, and average contacts made by each domain of HP1α chimeras whose hinge was swapped with the hinge from either HP1β or HP1γ (HP1α-βHinge and HP1α-γHinge, respectively), and HP1β and HP1γ chimeras whose hinge regions were replaced with the hinge of HP1α (HP1β-αHinge or HP1γ-αHinge, respectively). **(g, h, i)** Density profiles, saturation concentrations, and average contacts made by each domain of HP1α chimeras with folded domains replaced with those from HP1β or HP1γ (HP1α-βCSD and HP1α-γCSD, respectively). **(j, k, l)** Density profiles, saturation concentrations, and average contacts made by each domain of HP1α chimeras with either CD or CSD replaced with the corresponding domain from HP1β (HP1α-βCD and HP1α-βCSD, respectively) or HP1γ (HP1α-γCD and HP1α-γCSD, respectively). The cyan and orange dashed lines show the simulated saturation concentrations of wild-type HP1α and pHP1α, respectively. The error bars represent the standard deviation from triplicate simulation sets. The CG coexistence simulations were conducted using the HPS-Urry model at 320K and 100 mM salt concentration.

comparable to HP1 α (23). Taken together, these results demonstrate that the positively charged lysine/arginine residues clusters in the HP1 α hinge are necessary to induce inter-dimer contacts for condensate formation.

We next performed swaps of the HP1 α hinge into HP1 β (construct HP1 β - α Hinge, Table S1) and HP1 γ (construct HP1 γ - α Hinge, Table S1). We found that both chimeras readily underwent phase separation (Fig. 3d). The HP1 β - α Hinge chimera displayed C_{sat} that was lower than that for wild-type HP1 α , and that was comparable to the C_{sat} of pHP1 α , which is much more prone to phase separation (12). HP1 γ - α Hinge showed an even more remarkable reduction in the C_{sat} , which was nearly threefold lower compared to pHP1 α (Fig. 3e). The presence of the HP1 α hinge led to increased multivalency in almost all domains of the chimeras (Fig. 3f), particularly magnifying NTE-hinge interactions (Figs. S2b,c). The main exception was the CTE region, where contacts were reduced on average. The charge segregation in the HP1 β CTE and the relatively short CTE in HP1 γ may account for the reduced number of contacts in this part of the sequence. These results align well with HP1 α hinge-swapping experiments between different HP1 paralogs in the presence of DNA, which suggest that the HP1 α hinge can make more contacts with the NTE when there is reduced interference from the CTE (15). We also observed a substantial increase in the inter-dimer contacts in the CD and the CSD (Fig. 3f), raising the question of whether the folded domains may also influence the propensity for phase separation outside their wild-type context.

To investigate this question, we proceeded by inserting the folded domains of either HP1 β or HP1 γ into HP1 α (constructs HP1 α - β CD β CSD and HP1 α - γ CD γ CSD in Table S1, respectively). Intriguingly, we again observed that both chimeras readily formed stable condensates, and their C_{sat} values were lower than the concentration for HP1 α and comparable to the concentration for pHP1 α (Figs. 3g,h). Multivalent contacts considerably increased for the folded domains (CD and CSD) and the hinge, but markedly decreased for the CTE region (Fig. 3i), although overall these chimeras share similar interaction patterns as HP1 α (Figs. S2d,e). These results suggest that folded domains from either HP1 β or HP1 γ produce favorable interactions between the HP1 α hinge and the swapped folded domains while disturbing the hinge-CTE interaction.

We, therefore, wondered if these effects stemmed from either the CD or the CSD alone, or collectively from both folded domains. To test these possibilities, we separately replaced the CD and CSD of HP1 α with the corresponding folded domain from either HP1 β (constructs HP1 α - β CD and HP1 α - β CSD, respectively) or HP1 γ (constructs HP1 α - γ CD and HP1 α - γ CSD in Table S1, respectively). We found that swapping the CD domain alone (from either HP1 β or HP1 γ) substantially enhanced interactions between the hinge and the folded domains and reduced competing CTE contacts, hence promoting phase separation more efficiently. Despite sharing high sequence identity and domain architecture similarity, the CD domains of HP1 β and HP1 γ contain more negatively charged residues with a larger solvent-accessible surface area (SASA) than HP1 α (Figs. S2f,g). The CD and the NTE may cooperatively facilitate multivalent interactions with the hinge and substantially contribute to the overall interactions that stabilize the protein condensed phase (Figs. 3l and S2h,i). On the other hand, swapping of the CSD slightly enhanced phase separation compared to wild-type HP1 α . The interactions in the folded domains and the hinge were slightly increased, but the interactions from the CTE remained similar to those of wild-type HP1 α (Figs. 3l and S2j,k). Therefore, our data suggest that the CD domain is more dominant than the CSD in influencing the inter-domain interactions that drive phase separation.

Taken together, our findings reveal the cooperative roles of disordered and folded domains in mediating the condensation behavior of HP1 paralogs. While the condensate formation of HP1 mainly relies on NTE-hinge interactions, the folded domains, particularly the CD domain, also substantially contribute to the multivalent interactions required for the formation of oligomeric networks and phase separation. Moreover, the observations that different domains can influence multivalency across paralog chimeras made us wonder whether we may observe similar effects in HP1 paralog heterodimers.

The HP1 α monomer facilitates multivalent interactions in heterodimer phase separation

Previous literature has shown that the CSD-CSD dimerization of HP1 α occurs with an estimated K_d in the nanomolar range (12, 13). Moreover, the CSDs of HP1 paralogs exhibit a high degree of homology, suggesting that heterodimerization is likely to occur. Indeed, *in vivo* and *in vitro* co-immunoprecipitation studies have indicated that mammalian HP1 paralogs can directly interact with each other to form heterodimers in solution and on chromatin (14).

To explore the phase separation propensity of the heterodimers, we first used MODELLER to perform homology modeling with the HP1 α structure as the template to assemble heterodimers between HP1 α and HP1 β or HP1 γ (HP1 $\alpha\beta$ and HP1 $\alpha\gamma$, respectively), and heterodimers between HP1 β and HP1 γ (HP1 $\beta\gamma$). We next conducted coexistence phase simulations and calculated the coexistence densities and the intermolecular contact maps of the three heterodimers. We found that the three heterodimers formed stable condensates (Figs. 4a,b). The C_{sat} for HP1 $\alpha\beta$ and HP1 $\alpha\gamma$ heterodimers were nearly tenfold higher than the concentration for HP1 α homodimers, while the C_{sat} of HP1 $\alpha\gamma$ heterodimers was comparable to the concentration of HP1 γ homodimers (Figs. 4a-c). We also constructed the phase diagrams for the three heterodimers and plotted them together with the homodimers. The critical temperature for HP1 $\alpha\gamma$ heterodimers (347.0 K) is slightly lower than the temperatures for HP1 γ homodimers (350.5 K) and HP1 α homodimers (353.3 K). The critical temperatures of HP1 $\alpha\beta$ and HP1 $\beta\gamma$ heterodimers (326.1 K and 326.4 K, respectively) are substantially lower but not as low as the temperature for HP1 β homodimers (276.1 K). It appears that the presence of either HP1 α or HP1 γ monomer in heterodimerization with HP1 β can induce the necessary inter-dimer interactions in HP1 β monomers to sufficiently stabilize the condensed phase (Figs. 4e and S3a,b).

On the other hand, the presence of HP1 β monomer globally decreases inter-dimer contacts for the other partner, HP1 α or HP1 γ , compared to their respective homodimer contacts (Figs. S3a,b). We also observed that the presence of the HP1 γ monomer in the HP1 $\alpha\gamma$ heterodimers did not alter the inter-dimer interaction patterns of the HP1 α monomer, while the interactions in the HP1 γ hinge became less favorable than the interactions in its respective homodimer (Figs. 4e and S3c). This could be due to competition with the HP1 α hinge in establishing multivalent contacts. Overall, the results demonstrate the phase separation competence of HP1 heterodimers, raising the possibility that heterodimerization plays an essential role in regulating the co-localization and/or the function of HP1 paralogs. We also note that due to the treatment of folded domains as rigid bodies and the spatial constraints imposed on the CSD dimer, our simulations may not fully capture the biological behavior of HP1 heterodimers.

DNA mediates phase separation of HP1 α homo- and heterodimers

Recent studies have demonstrated that HP1 α can interact with DNA via stretches of basic residues located within the hinge region, mediating the bridging of distinct DNA regions and inducing DNA compaction (15, 17). Furthermore, HP1 α -DNA interactions may induce a local increase in HP1 α concentration, thereby facilitating the formation of higher-order HP1 α oligomers and resulting in condensate formation. HP1 γ has also been shown to form condensates with DNA but requires higher protein concentrations to induce droplet formation than HP1 α (15). Conversely, HP1 β did not undergo condensation with DNA under any tested conditions (15). Inspired by these findings, we further explored the molecular interactions that dictate the phase behavior differences among HP1 homodimers and tested the phase separation competence of HP1 heterodimers with DNA.

To explore HP1-DNA interactions, we initiated condensate formation with the homo- and heterodimer systems described above and added a small mole fraction of dsDNA. Using a recently developed nucleic acid model (46), we first conducted CG coexistence phase simulations of each HP1 homodimer containing 0.01 mole fraction of 147 bp dsDNA. This system is equivalent to an

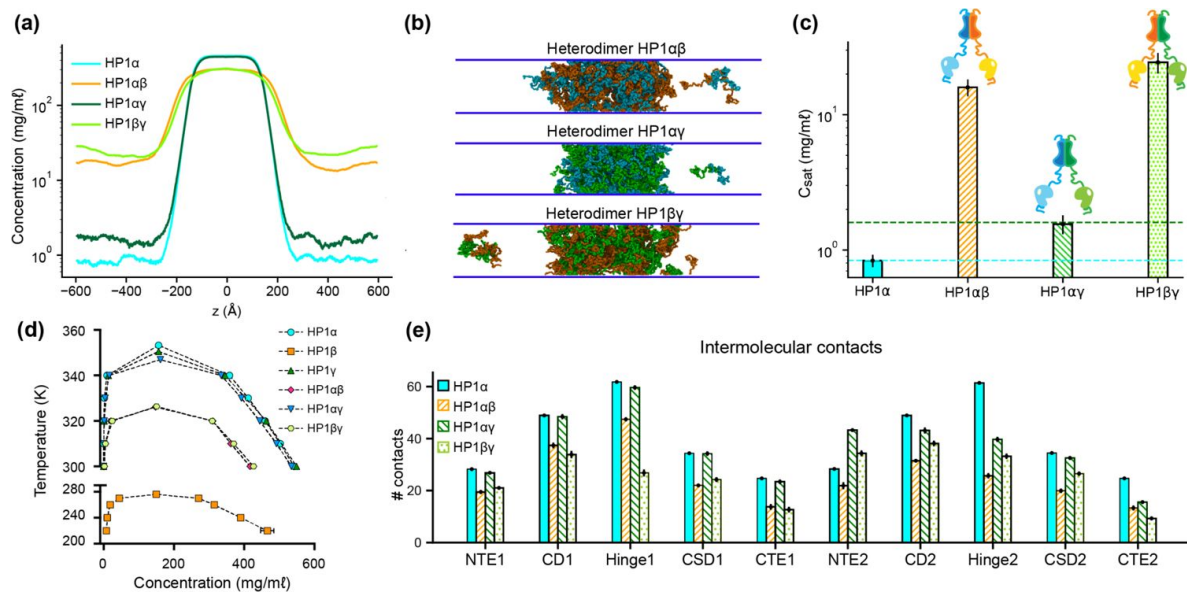


Figure 4

Phase separation of HP1 heterodimers.

(a, b, c) Density profiles, snapshots of the condensates, and saturation concentrations in the CG coexistence simulations performed with heterodimers HP1αβ, HP1αγ, and HP1βγ. (d) Phase diagram of HP1 heterodimer phase separation conducted at different temperatures. (e) Average intermolecular contacts for each domain of the HP1 heterodimers in CG coexistence simulations. The error bars represent the standard deviation from triplicate simulation sets. The CG coexistence simulations were conducted using the HPS-Urry model at 320K and 100 mM salt concentration.

experimental system containing 50 μM HP1 protein with 0.6 μM of 147 bp dsDNA (Table S2). We found that the HP1 β homodimer did not undergo phase separation, while dsDNA partitioned into and stabilized the condensates of HP1 α and HP1 γ (Figs. 5a,b and Movies S3). In the presence of dsDNA, the C_{sat} of HP1 γ is approximately twofold higher than that of HP1 α . It should be noted that increasing the mole fraction of dsDNA in the mixture may significantly enhance the phase separation of HP1 α . Still, excessive addition of dsDNA may lead to unstable condensate due to a large increase of the overall net negative charge as observed in our previous simulations (12). To characterize the protein-DNA molecular interactions, we computed the intermolecular contact maps based on vdW contacts formed between HP1 homodimers and dsDNA as a function of residue number. We found that the electrostatic interactions between dsDNA and the lysine/arginine-rich hinge dominated in the HP1 α homodimer (Fig. 5c). dsDNA also favored the long stretch of lysine-rich NTE of HP1 γ homodimers (Fig. 5e). However, the electrostatic interactions between dsDNA and HP1 β were unfavorable due to extended patches of acidic residues along the HP1 β sequence (Fig. 5d).

We next investigated the phase separation of HP1 heterodimers with dsDNA using the simulation protocol above. We observed that DNA could induce condensate formation of all HP1 heterodimers (Figs. 5f,g). The C_{sat} values for HP1 $\alpha\gamma$, HP1 $\alpha\beta$, and HP1 $\beta\gamma$ heterodimers in the presence of dsDNA increased in progressive order and followed a similar trend as the heterodimers alone (insets of Figs. 5f, a). The presence of HP1 α monomer in complex with either HP1 β or HP1 γ helped facilitate the hinge-DNA interactions necessary for condensate formation. However, the considerable net negative charge of the HP1 β monomer weakened the multivalent interaction network, resulting in expansion and instability within the condensates of HP1 $\alpha\beta$ and HP1 $\beta\gamma$ heterodimers (Figs. 5g,h,j). The NTE of the HP1 γ monomer showed strong binding to DNA and became the main contributor to the condensate formation of HP1 $\beta\gamma$ heterodimers with DNA. However, in HP1 $\alpha\gamma$ heterodimers, this interaction competed with the favorable HP1 α hinge-DNA interactions (Fig. 5i). Overall, the results highlight the emergence of favorable electrostatic attraction between DNA and patches of basic residues in the HP1 α hinge and the HP1 γ NTE and unfavorable electrostatic repulsion between DNA and HP1 β domains within HP1 homo- and heterodimer condensates. These observations have implications for understanding how DNA mediates phase separation of HP1 proteins in the context of heterochromatin organization.

DNA influences the co-localization of HP1 paralogs

Although HP1 paralogs have been found in different regions of the genome (47–50), they localize predominantly to heterochromatic domains and play a vital role in the formation and stabilization of higher-order chromatin structures, which modulate gene expression. Moreover, HP1 α has been shown to form phase-separated droplets and bind most strongly to DNA, followed by HP1 γ and then HP1 β (15, 17). Given that HP1 paralogs show different phase separation behaviors, we wondered how HP1 paralogs behave when they are co-localized in overlapping genomic regions. To explore the co-localization capability of HP1 paralogs, we performed CG coexistence phase simulations of mixtures of HP1 α and HP1 β homodimers and HP1 α and HP1 γ homodimers, respectively. Depending on the cell type, HP1 β is considered to be less abundant than HP1 α , while HP1 γ may be present at comparable levels to HP1 α (51). Therefore, in our simulations, we started with equimolar ratios of the mixed populations (Movie S4) and then decreased the concentration of either HP1 β or HP1 γ while maintaining the same total concentration of proteins (Fig. 6a). We next built the phase diagrams of this two-component system to explore how HP1 β and HP1 γ partition into HP1 α condensates.

We found that the HP1 β /HP1 α homodimer mixtures showed behavior consistent with scaffold-client co-phase separation (Fig. 6b). HP1 α functioned as a scaffold molecule that phase separated on its own, and HP1 β acted as a client that was not able to undergo phase separation on its own. Still, it had favorable heterotypic interactions with HP1 α and was incorporated into HP1 α condensates. Slowly increasing the HP1 β concentration in the system did not significantly affect

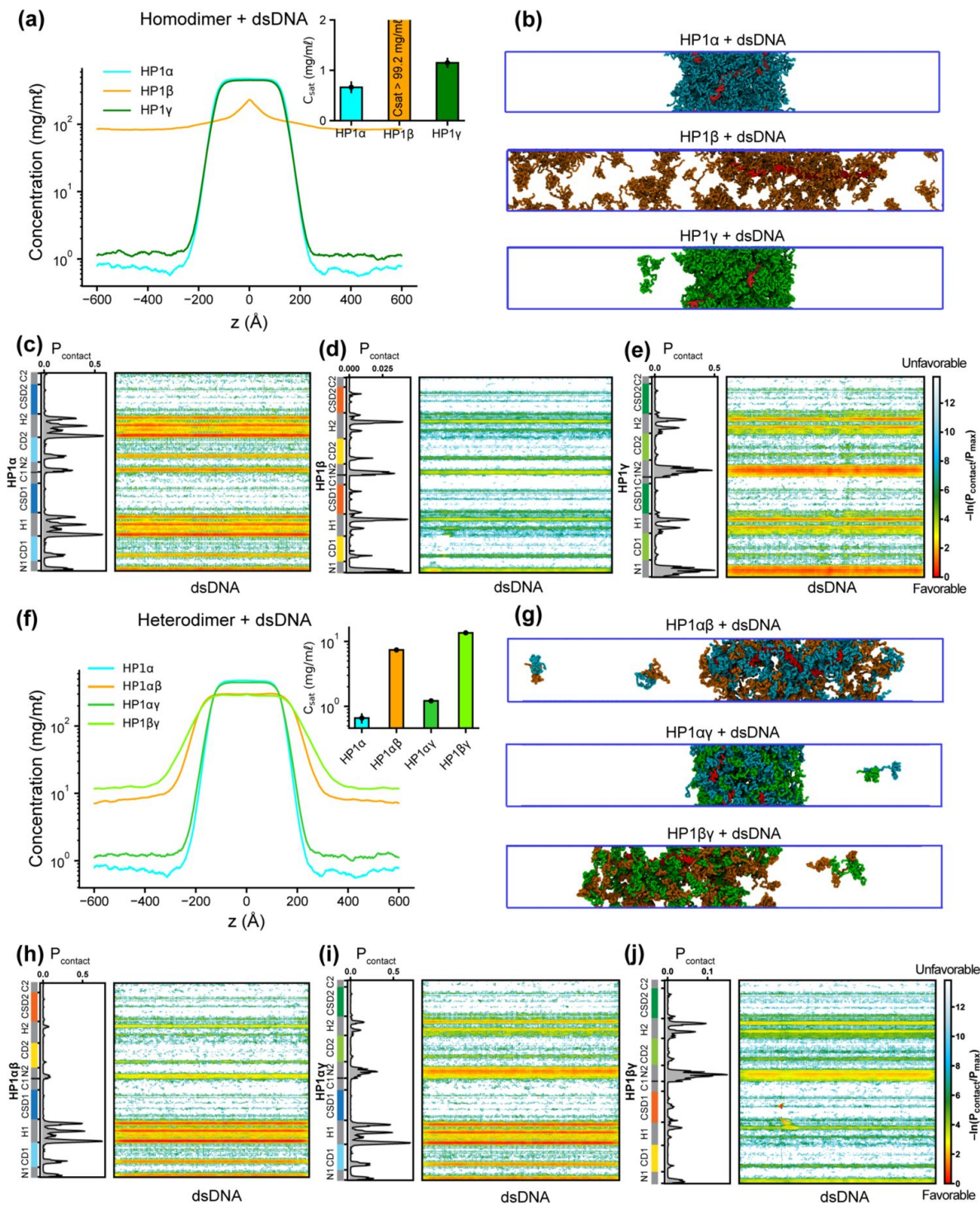


Figure 5

Phase separation of HP1 homo- and heterodimers with DNA.

(a, b) Density profiles, saturation concentrations (inset), and snapshots of HP1 paralogs with one chain of 147 bp double-stranded DNA in the CG coexistence simulations. **(c, d, e)** Intermolecular contacts between HP1 homodimers and DNA. **(f, g)** Density profiles, saturation concentrations (inset), and snapshots of HP1 heterodimers with one chain of 147 bp double-stranded DNA in the CG coexistence simulations. **(h, i, j)** Intermolecular contacts between HP1 heterodimers and DNA. Preferential interactions between HP1 protein and DNA are shown in red. The error bars represent the standard deviation from triplicate simulation sets. The CG coexistence simulations were conducted using the HPS-Urry model at 320K and 100 mM salt concentration.

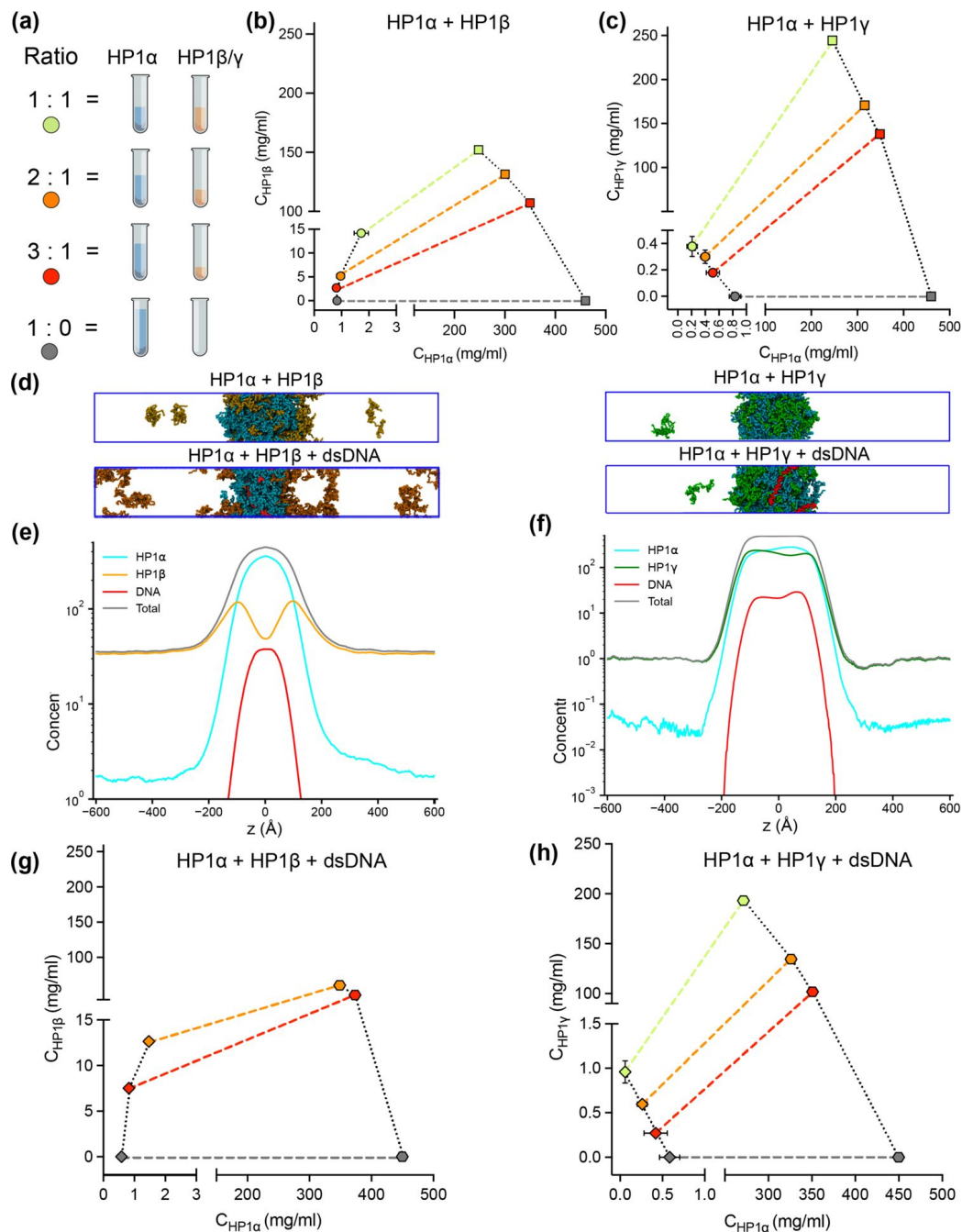


Figure 6

DNA regulates the condensation of HP1 paralogue mixtures.

(a) Schematic of the ratios of HP1 α and HP1 β (or HP1 γ) used to generate multicomponent phase diagrams. The total concentration of the mixture was fixed in all cases. (b, c) Multicomponent phase diagrams of HP1 α mixing with HP1 β (or HP1 γ). The color code corresponds to the ratios shown in the schematic in (a). The circles and the squares show the protein concentrations in the dilute and the dense phases, respectively. The tie line (colored dashed line) connects the concentrations in the dense and dilute phases for each ratio. Phase existence lines (black dotted lines) define the two arms of the phase diagram at low and high concentrations. (d) Snapshots of the condensates in the CG coexistence simulations of HP1 α +HP1 β and HP1 α +HP1 γ mixtures at an equimolar concentration in the absence and presence of dsDNA. (e, f) The density profiles of the mixtures at an equimolar concentration in the presence of dsDNA. (g, h) Multicomponent phase diagrams of HP1 α mixing with HP1 β or HP1 γ in the presence of DNA. The 1:1 ratio data points for HP1 α and HP1 β mixing with DNA were excluded in (g) due to the instability of the condensate. The diamonds and the hexagons show the protein concentrations in the dilute and the dense phases, respectively. The error bars represent the standard deviation from triplicate simulation sets. The CG coexistence simulations were conducted using the HPS-Urry model at 320K and 100 mM salt concentration.

the phase separation propensity of HP1 α (its dilute-phase concentration remained relatively the same ~ 1 mg/ml), and HP1 β was recruited to the HP1 α condensate. However, the excessive presence of HP1 β led to competition between the self-interaction of HP1 α -HP1 α and the cross-interaction of HP1 β -HP1 α , resulting in increased dilute-phase concentration for both HP1 α and HP1 β (**Fig. 6b**, trend in lower left corner of the graph). In contrast, the HP1 γ /HP1 α homodimer assembly showed behavior consistent with cooperative co-phase separation (**Fig. 6c**). Both HP1 α and HP1 γ have a strong homotypic affinity, and they were able to phase separate on their own and cooperatively form heterotypic condensates. This behavior is attributed to the similarity of the two paralogs in sequence properties and phase separation behaviors. However, the condensate was more enriched in the HP1 α component as it displays a stronger self-interaction propensity compared to HP1 γ . In the case of equimolar initial concentration, the dilute-phase concentration of HP1 γ was nearly twofold higher than that of HP1 α (**Fig. 6c**, green circle in lower left corner of the graph).

Given that HP1 paralogs show different preferences in DNA binding, we next asked whether the presence of DNA may regulate the co-localization of HP1 paralogs. We followed the simulation protocol described above and performed simulations in the presence of 0.01 mole fraction of 147 bp dsDNA. These simulations were initiated from a high-density slab, where the two HP1 paralogs and dsDNA were mixed. We first investigated the stability of the condensates of equimolar mixtures in the presence of dsDNA. We observed that HP1 β homodimers were predominantly localized towards the periphery of the condensate while HP1 α and dsDNA occupied the interior (**Figs. 6d,e** and Movie S5). On the other hand, HP1 γ was able to co-localize and form stable condensates with HP1 α and dsDNA (**Figs. 6d,f** and Movie S5). We next constructed the two-component phase diagrams of HP1 α + HP1 β and HP1 α + HP1 γ mixtures in the presence of dsDNA (**Figs. g,h**). We found that HP1 α phase separation was slightly enhanced in the presence of DNA, while HP1 β was more excluded from the HP1 α -dsDNA core due to its electrostatic repulsion with dsDNA. By contrast, dsDNA did not affect the overall cooperative phase separation behavior of HP1 α and HP1 γ . However, compared to the case of mixing HP1 α and HP1 γ alone, the dilute-phase concentration of HP1 γ was found to be twofold higher, whereas the dilute-phase concentration of HP1 α was nearly twofold lower. In other words, the presence of dsDNA substantially enhanced the phase separation of HP1 α but slightly excluded HP1 γ from the HP1 α -DNA complex. Together, these results suggest that HP1 γ may co-partition in HP1 α -DNA condensates while HP1 β mostly remains in the condensate boundary. The observations highlight the role of DNA in modulating the co-localization of HP1 paralogs, which carries significant implications for understanding their distinct biological functions in the context of chromatin.

Discussion

HP1 paralogs are an essential modulator of chromatin architecture, gene regulation, and genomic integrity in mammalian cells (7, 8, 52, 53). Despite their sequence conservation, the three human paralogs, HP1 α , HP1 β , and HP1 γ , exhibit different nuclear localization and interaction patterns that may underlie potentially unique functions in gene silencing and heterochromatin establishment (5, 9, 54). These distinct behaviors may be intimately connected to the proteins' ability to undergo LLPS, a process that can substantially remodel the material properties of chromatin environments (55, 56). Our in-depth *in silico* investigation suggests that the distinct LLPS propensities of the HP1 paralogs result from the precise tuning of the charge distribution along the protein sequence.

In the absence of DNA, the main driver for LLPS is the relative strength of hinge-NTE interactions. HP1 α , the most LLPS-prone paralog, has the most positively charged hinge region, which can interact with negatively charged patches on the NTE and the CTE. While hinge-NTE interactions are dominant, competition from the CTE negatively influences the LLPS propensity of the wild-type protein. This balance can be shifted dramatically by the phosphorylation of serine residues

11-14 on the NTE, which significantly enhances LLPS (12, 17). In HP1 γ , on the other hand, the NTE contains a relatively long stretch of positively charged residues that disfavors hinge-NTE interactions and LLPS. HP1 β has the most negative overall charge of the three paralogs, with a significant number of negative residues in the hinge and a much lower propensity to form hinge-NTE interactions. Importantly, HP1 γ and HP1 β do not have the phosphorylatable serine patch in their NTEs and therefore lack the ability to tune the strength of hinge-NTE interactions. However, when the HP1 α hinge was swapped into HP1 β and HP1 γ , it could enhance global inter-domain interactions (Figs. 3f and S2b,c), especially between the NTE and hinge regions. In this case, the extended stretches of negatively charged residues in the NTE of HP1 β considerably enhanced NTE-hinge interactions and promoted phase separation (Figs. 3e,f and S2b), similar to the effect of NTE phosphorylation in HP1 α (12). In both chimeras, contacts in the CTE regions were significantly reduced compared to the wild-type HP1 α (Fig. 3f), implying that reducing access of the CTE to the hinge dramatically enhances phase separation.

These trends are reflected in the C_{sat} determined in our simulations and concur well with experimental findings. For example, *in vitro* experiments have demonstrated that, in the absence of phosphorylation and DNA, HP1 α undergoes LLPS at elevated protein concentration and reduced temperature (50-400 μM , 4°C) (15, 23) or under low salt concentration (50 mM KCl) (24). Our CG simulations corroborate these experimental observations, indicating that a low salt concentration (50 mM) promotes the LLPS of HP1 α . Raising the salt concentration weakens the electrostatic interactions and increases the C_{sat} , eventually precluding HP1 α 's phase separation at high salt regimes (200-500 mM) (Fig. S5). Phosphorylation allows LLPS at lower protein concentrations, higher salt conditions, and increased temperatures ($\sim 50 \mu\text{M}$, 25°C, and 75 mM KCl) (12, 17). HP1 γ undergoes LLPS at a significantly higher protein concentration than HP1 α (900 μM), while HP1 β fails to form phase-separated droplets under any tested experimental conditions (23). Deletion of the HP1 α CTE in the context of phosphorylation reduced the C_{sat} tenfold, supporting a model where reducing competition from the CTE is key to increasing LLPS propensity. Our previous computational and experimental studies on pHP1 α also agree with these conclusions (12).

While productive and competing interactions of the IDRs of HP1 paralogs are undoubtedly the main determinants of LLPS propensities, our simulations allow us to capture the more subtle contributions of the folded CD domain. When the CD domain of HP1 α was replaced with the corresponding CD domain from either HP1 β or HP1 γ , the phase separation of these chimeras was significantly enhanced (Figs. 3j,k). The average interdimer contacts per region in the CD and hinge substantially increased, while contacts considerably decreased in the CTE (Fig. 3l). Notably, the CD domains of HP1 β or HP1 γ contain more negatively charged residues than the HP1 α CD (Figs. 1e,f), and these residues appear to facilitate multivalent CD-hinge contacts without interfering with NTE-hinge interactions. This also indicates that the NTE and the CD can work in concert to compete with the CTE for access to the positively charged hinge. The CD of HP1 paralogs has a well-established role in recognizing and binding H3K9me2/3, facilitating the targeted recruitment and localization of HP1 to heterochromatic regions (24, 50, 52, 57). Our simulations suggest that the CD also facilitates multivalent electrostatic interactions governing the LLPS of human HP1 paralogs. This mechanism, however, appears to be distinct from the oligomerization capabilities of Swi6 (58), which can promote multivalency through direct CD-CD interactions.

The nuclear localization patterns of HP1 paralogs (7, 8, 52) show that there are common overlapping regions in heterochromatin but also distinct territories where only certain paralogs are enriched, e.g., the presence of HP1 γ and HP1 β in euchromatin. These observations suggest the possibility that the LLPS behavior of HP1 proteins may also be influenced by the local distribution and concentration of different HP1 paralogs. The versatility of our CG model allows us to build and analyze multicomponent LLPS environments to capture this complexity and describe the dominant interactions. For example, in mixtures of HP1 paralog homodimers, the HP1 α hinge

region serves as a central hub, connecting adjacent paralogs through electrostatic attraction involving the NTE, CD, or CTE domains. This multivalent network enables the highly negatively charged HP1 β to function as a client, partitioning into the HP1 α scaffold condensate in a concentration-dependent manner. Specifically, at low concentrations, HP1 β can be directly recruited to the phase-separated droplet of HP1 α , consistent with experimental observations (17). However, increasing the presence of HP1 β in the droplets activates repulsive forces between HP1 β molecules, resulting in the overall instability of the condensate. In this scaffold-client phase separation context, HP1 α is central to forming the condensate, with selective recruitment of HP1 β dimers, which can be brought in proximity to specific genomic regions to enhance the efficiency of HP1 β -mediated gene silencing or heterochromatin formation. In contrast, HP1 γ can cooperatively phase-separate with HP1 α due to preferential cross-interactions between its NTE/CD and the HP1 α hinge region. This cooperative phase separation implies that HP1 α and HP1 γ co-condensation may facilitate coordinated gene regulation and chromatin organization activities.

This picture can also be complicated by the formation of heterodimers. HP1 paralogs have been shown to interact directly with each other to form heterodimers in co-immunoprecipitation experiments of mammalian cells (14). Furthermore, the high conservation of the CSD dimerization interface in the three paralogs suggests that heterodimers may form with similar affinities to homodimerization and that such species can be formed under physiological conditions. Our simulations suggest that HP1 α -HP1 β and HP1 α -HP1 γ heterodimers have a lower propensity to undergo LLPS compared to HP1 α alone but display enhanced properties compared to HP1 β and HP1 γ homodimers, respectively. Again, the hinge region of HP1 α is the key to establishing the multivalent networks in such samples. It is important to emphasize that our model is predicated on the assumption that HP1 proteins establish stable chromoshadow domain (CSD-CSD) dimers, a hypothesis supported by their K_d values being in the nanomolar range (13, 53). While this simplification serves as a useful starting point, it may not fully capture the dynamic nature of HP1 dimerization. Further computational and experimental studies are needed to understand better the behavior of the complex mixtures of HP1 paralogs, particularly at phase boundaries.

Our recently developed nucleic acid model (59) also allowed us to evaluate how the interaction patterns and localization of HP1 paralogs change in the presence of DNA. In this context, the distribution of positive charge along the paralog sequence is crucial in defining LLPS. HP1 α displays the most robust phase separation with DNA due to multiple attractive hotspots in the hinge, NTE, and CD (Figs. 5a,c). HP1 γ interacts with DNA primarily through its extended positively charged NTE and through some conserved basic residue stretches in the hinge (Fig. 5e). It undergoes phase separation with DNA but is less competent than HP1 α (Fig. 5a). In HP1 β , contacts with DNA are strongly disfavored due to the overall negative charge of its sequence and LLPS is not observed (Figs. 5a,b,d). These interaction patterns provide a rationale behind the phase separation behavior observed in mixed populations of HP1 and DNA (15, 60). Premixing HP1 β or HP1 γ with HP1 α and DNA inhibits droplet formation in a concentration-dependent manner. When HP1 α -DNA condensates are pre-formed, HP1 β dissolves the condensates, while HP1 γ stabilizes them. Our computational model suggests that when present at low protein concentrations, HP1 β and HP1 γ can co-localize with HP1 α and partition into HP1 α -DNA condensates. However, the increased presence of HP1 β /HP1 γ in the mixture with HP1 α may activate competition for binding sites between paralog-paralog and paralog-DNA, leading to concentration-dependent phase separation inhibition. When HP1 α -DNA condensates are pre-formed, and HP1 β is subsequently added at equimolar concentration with HP1 α , HP1 β molecules mainly remain at the HP1 α -DNA phase boundary. The invasion of HP1 β into the droplet causes binding-site competition between HP1 β -HP1 α and DNA-HP1 α . The repulsive forces between HP1 β and DNA can further destabilize and dissolve the condensate (Fig. 5i, 6d,e,g). In contrast, HP1 γ preferentially interacts with DNA through its NTE and hinge and cooperatively phase separates with HP1 α , thereby stabilizing pre-formed HP1 α -DNA condensates (Figs. 5e, 6c,d,h). It should

also be noted that in HP1 paralog mixtures, heterodimerization likely occurs due to a similarly high CSD-CSD affinity (12, 13), potentially altering DNA-binding propensity and regulating paralog localization with DNA.

Our *in silico* study paints a molecular picture describing how DNA regulates the localization patterns of HP1 proteins, which has general implications for understanding the function of HP1 proteins in heterochromatin organization and spreading. In this picture, HP1 α is central in regulating DNA compaction and initiating protein condensation with DNA or upon phosphorylation. HP1 γ preferentially enters and cooperatively enhances HP1 α condensates, forming heterochromatic domains that silence DNA transactions. In contrast, HP1 β creates a phase boundary (Fig. 7, movie S6) and fine-tunes HP1-DNA phase separation in a concentration-dependent manner, potentially limiting heterochromatin spreading. HP1 β may also be recruited to more open chromatin regions to perform gene-activating associated roles in this context. Thus, our simulations suggest a model of heterochromatin organization whereby HP1 α and DNA initiate condensation. HP1 γ , HP1 β , and heterodimerization can further enhance, dissolve, or fine-tune phase-separated droplets in a concentration-dependent fashion. This mechanism of LLPS fine-tuning is strongly dependent on the charge distribution along the HP1 paralog sequence and likely acts in concert with other components of the HP1 interaction network, including nucleosomes, H3 K9 methylation, RNA, and HP1 partners that specifically bind to the CSD-CSD dimer interface (18, 61). Our *in silico* work sets the stage for dissecting the individual roles and combinatorial effects of these components in a comprehensive effort to understand the molecular forces that determine the fate of genes in the cell.

Materials and Methods

Coarse-grained MD simulation protocol

The CG single homodimer simulations and the CG coexistence phase simulations of HP1 proteins and HP1 proteins with DNA were performed in the HOOMD-Blue 2.9.7 software package (62), using the protocol described in our previous studies (25, 26). To simulate HP1 homodimers and heterodimers, the disordered regions (NTE, hinge, and CTE) remained flexible, whereas the folded domains were constrained using the `hoomd.md.constrain.rigid` function (63, 64). The CD domains were treated as separate rigid bodies, while the CSD-CSD domains were held as a single rigid body, allowing the molecule to mimic the dimer behavior.

The proteins and DNA were modeled using the one-bead-per-residue HPS-Urry model (26) and the recently developed two-bead-per-nucleotide DNA model (65), respectively. The CG coexistence phase simulations were initiated in a slab geometry of 170×170×1190 Å³ with 50 chains of dimers. In all CG simulations, a 5 μ s NVT ensemble was conducted using a Langevin thermostat with a friction factor $\gamma = m_{AA}/\tau$. Here m_{AA} is the mass of each amino acid bead and τ is the damping factor set to 1000 ps. The time step was set to 10 fs. As the coexistence density in the dilute phase was too low at 300 K, we simulated all systems at 320 K to compare the phase separation propensities of homo- and heterodimers and their chimeras. All the CG simulations were conducted at 100 mM salt concentration. When calculating the density profiles and contact maps, the first 1 μ s of the trajectory was skipped and treated as an equilibration period.

Phase diagrams of HP1 homo- and heterodimers were constructed using a method described previously (25, 26). The dilute and dense phase densities (ρ_L and ρ_H , respectively) were extracted from the CG coexistence phase simulations. The critical temperature T_C was estimated by fitting the coexistence densities at different temperatures to the following function:

$$\rho_H - \rho_L = A(T_C - T)^\beta$$

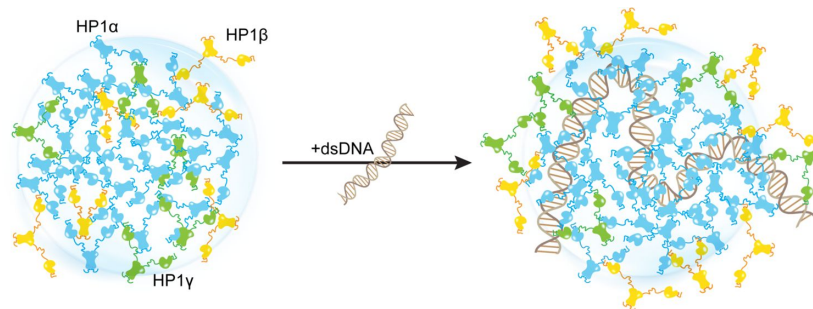


Figure 7

Distinct interaction patterns of HP1 paralogs influence the localization patterns of HP1α, HP1β, and HP1γ in the absence and presence of DNA.

where $\beta = 0.325$ (universality class of 3D Ising model (66)), and A is a protein-specific fitting parameter.

Previous studies have demonstrated that slab geometry can help mitigate finite-size effects and facilitate efficient sampling of the phase diagram (41). To assess the potential impact of finite-size effects with our chosen box dimensions, we conducted a test using the HP1 α homodimer, which serves as a representative system given the comparable sequence lengths of HP1 paralogs and their chimeras. By reducing the system size by 30% and constructing its phase diagram, we observed that both the original system size (50 dimers) and the reduced counterpart (35 dimers) produced similar phase diagrams, with critical temperatures of 353.3 K and 352.1 K, respectively, as shown in Figs. S4a,b.

We further evaluated the influence of the xy cross-sectional area on the measurement of C_{sat} . With the z-direction box length fixed at 1190 Å³, we varied the xy cross-sectional areas (120×120, 150×150, and 200×200 Å²) while maintaining the protein density consistent with the control case (170×170 Å²). Given that HP1 dimers are multidomain proteins, a 120×120 Å² cross-section was the minimum size feasible to prevent particle overlap in HOOMD simulations due to the constraints of the small box size. Our findings indicate that the condensates remained stable across all tested cross-sectional areas and that there were no significant differences in C_{sat} measurements within the margin of error, as depicted in Figs. S4c,d. These results confirm that our chosen box size is sufficiently large to minimize finite-size effects, thus ensuring the robustness of our results.

In the simulations of co-phase separation between HP1 paralogs (HP1 α - HP1 β homodimers and HP1 α - HP1 γ homodimers, respectively), the total number of homodimers in the mixture was kept constant with box dimensions of 170×170×1190 Å³. The number of homodimers for each HP1 component was varied to account for different ratios of HP1 α to HP1 β (or HP1 γ): 1, 2, 3, and HP1 α only. To investigate the effects of DNA on the co-localization of HP1 paralogs, 147 bp of dsDNA was added to each system above. The multi-component phase diagrams were constructed by plotting the extracted coexisting densities of each component in the CG coexistence phase simulation at each ratio of the HP1 paralog mixture. By selecting a box size that minimizes finite-size effects, we can ensure that the spatial segregation observed in our multi-component condensates reflects genuine phase behavior. This finding aligns with Chew et al. (67), who also reported well-separated multilayered condensates and conducted thorough validations to confirm these phases.

All-atom MD simulation protocol

Atomistic simulations of HP1 paralogs were initially prepared using Gromacs 2020 (68). The system topologies were modeled using the force field Amber99SBws-STQ (69). Each HP1 homodimer was placed in an octahedral box of 15 nm in length. The system energy was first relaxed in vacuum and then solvated with TIP4P/2005 water molecules (70) using the steepest descent algorithm. To mimic the physiological salt concentration (100 mM), Na⁺ and Cl⁻ ions were added along with additional Na⁺ counter ions to achieve electrical neutrality. The improved salt parameters from Lou and Roux were used for all simulations (71). The system was first equilibrated in canonical ensemble (NVT) using a Nose-Hoover thermostat (72) with a coupling constant of 1.0 ps at 300 K, then it was further equilibrated in an isothermal-isobaric ensemble (NPT) using the Berendsen barostat (73) with an isotropic coupling of 5.0 ps to achieve pressure of 1 bar. Production simulations were conducted using OpenMM 7.6 (74) in the NVT ensemble at 300K, with Langevin middle integrator (75), friction coefficient of 1 ps⁻¹, hydrogen mass increased to 1.5 amu, and 4 fs timestep. Hydrogen-related bonds were constrained using the SHAKE algorithm (76). Short-range non-bonded interactions were calculated based on a cutoff radius of 0.9 nm, and long-range electrostatic interactions were treated using the Particle Mesh Ewald (PME) method (77). Distance-based contact maps were calculated using a previously published method (78). Two residues were considered to form a van der Waals contact if at least one atom of one residue was within 6 Å of an atom in the other residue.

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Author contributions

TMP, GTD, and JM conceived and designed the research. TMP performed and analyzed computer simulations. YCK provided the computational tools needed to set up the coarse-grained simulations. TMP, YCK, GTD, and JM wrote the manuscript.

Competing Interests

All authors declare they have no competing interests.

Data and materials availability

All data are available in the main text or the supplementary materials.

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Editors

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Reviewer #1 (Public Review):**Summary:**

HP1 plays a pivotal role in orchestrating chromatin packaging through the creation of biomolecular condensates. The existence of distinct homologs offers an intriguing avenue for investigating the interplay between genetic sequence and condensate formation. In this study, the authors conducted extensive coarse-grained simulations to delve into the phase separation behavior of HP1 paralogs. Additionally, the researchers delved into the captivating possibility of various HP1 paralogs co-localizing within assemblies composed of multiple components. Importantly, the study also delved into the critical role of DNA in finely tuning this complex process.

Strengths:

I applaud the authors for their methodical approach in conducting simulations aimed at dissecting the contributions of hinges, CTE, NTE, and folded regions. The comprehensive insights unveiled in Figure 3 compellingly substantiate the significance of these protein components in facilitating the process of phase separation.

This systematic exploration has yielded several innovative revelations. Notably, the authors uncovered a nuanced interplay between the folded and disordered domains. Although disordered regions have traditionally been linked to driving phase separation through their capacity for forming multivalent interactions, the authors have demonstrated that the contribution of the CD cannot be overlooked, as it significantly impacts the saturation concentration.

The outcomes of this study serve to elucidate the intricate mechanisms and regulatory aspects governing HP1 LLPS.

<https://doi.org/10.7554/eLife.90820.2.sa1>

Reviewer #2 (Public Review):

In this paper, Phan et al. investigate the properties of human HP1 paralogs, their interactions and abilities to undergo liquid-liquid phase separation. For this, they use a coarse-grained computational approach (validated with additional all-atom simulations) which allows to explore complex mixtures. Matching (wet-lab) experimental results, HP1 beta (HP1b) exhibits different properties from HP1 alpha and gamma (HP1a,g), in that it does not phase separate. Using domain switch experiments, the authors determine that the more negatively charged hinge in HP1b, compared to HP1a and HP1g, is mainly responsible for this effect. Exploring heterotypic complexes, mixtures between HP1 subtypes and DNA, the authors further show that HP1a can serve as a scaffold for HP1b to enter into condensed phases and that DNA can further stabilize phase separated compartments. Most interestingly, they show that a multicomponent mixture containing DNA, and HP1a and HP1b generates spatial separation between the HP1 paralogs: due to increased negative charge of DNA within the condensates, HP1b is pushed out and accumulates at the phase boundary. This represents an example how complex assemblies could form in the cell.

Overall, this is purely computational work, which however builds on extensive experimental results (including from the authors). The methods showcase how coarse-grained models can be employed to generate and test hypotheses how proteins can condense. Applied to HP1 proteins, the results from this tour-de-force study are consistent and convincing, within the experimental constraints. Moreover, the authors generate further models to test experimentally, in particular in light of multicomponent mixtures. Finally, the authors clearly discuss the computational methods, assumptions and limits of the methodology, which makes

this a strong contribution to our understanding of biophysical basis of condensate formation in gene regulation.

<https://doi.org/10.7554/eLife.90820.2.sa0>

Author Response

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

HP1 plays a pivotal role in orchestrating chromatin packaging through the creation of biomolecular condensates. The existence of distinct homologs offers an intriguing avenue for investigating the interplay between genetic sequence and condensate formation. In this study, the authors conducted extensive coarse-grained simulations to delve into the phase separation behavior of HP1 paralogs. Additionally, the researchers delved into the captivating possibility of various HP1 paralogs co-localizing within assemblies composed of multiple components. Importantly, the study also delved into the critical role of DNA in finely tuning this complex process.

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The outcomes of this study serve to elucidate the intricate mechanisms and regulatory aspects governing HP1 LLPS.

Weaknesses:

The authors do not provide an assessment of the quantitative precision of their model. To illustrate, HP1a is anticipated to undergo phase separation primarily under low salt concentrations. Does the model effectively capture this sensitivity to salt conditions? Regrettably, the specific salt conditions employed in the simulations are not explicitly stated. While I anticipate that numerous findings in the manuscript remain valid, it could be beneficial to acknowledge potential limitations tied to the simulations. For instance, might the absence of quantitative precision impact certain predictions, such as the CD's influence on phase separation?

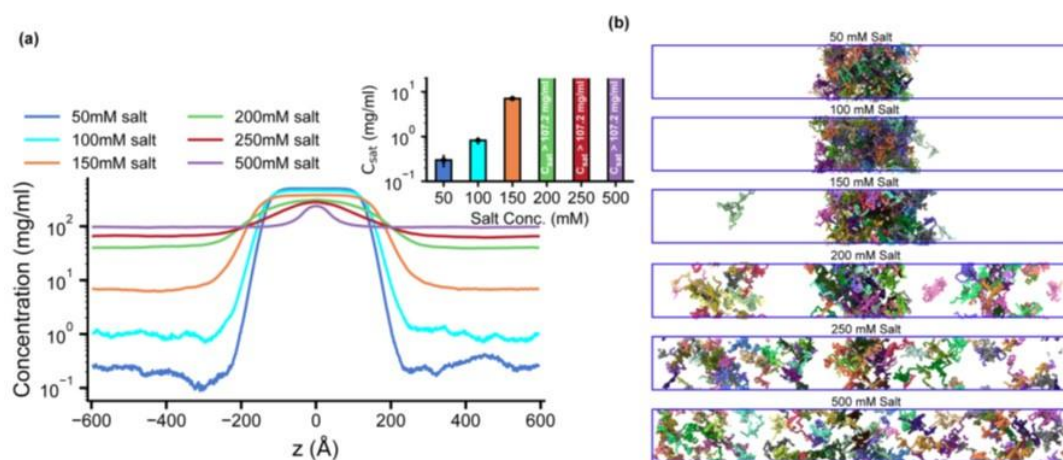
We thank the reviewer for their kind feedback and for highlighting the essential mechanistic insights obtained from our study. We have addressed the concerns raised by the reviewer below, and the specific amendments made in the manuscript are also delineated.

We appreciate the reviewer's comment on our model. Our coarse-grained (CG) physics-based model integrates electrostatic and short-range interactions, parametrized based on the Urry hydrophobicity scale. This approach effectively bridges the timescale gap between simulation and experiment, offering a transferable framework to compute protein phase diagrams in temperature-concentration space that can be compared to experimental phase behavior (1). Additionally, the vdW contact probability per residue correlation between AA and CG simulations (Fig. S1 f-h) underscores our model's capability to uncover the mechanistic insights into the phase separation of HP1 paralogs. Despite its simplicity and widespread adoption for studying sequence-dependent phase separation in biomolecular condensates, we recognize that our CG model does not yet fully replicate experimental observations or the nuanced effects of local secondary structures on phase-separation propensities. We are actively refining our methods and exploring new strategies to enhance the accuracy and efficiency of CG models for the study of biological phase separation.

In assessing the influence of salt on the LLPS of HP1 α , we note that Wang et al. (2) demonstrated that HP1 α can undergo LLPS at a low salt concentration (50 mM KCl). Furthermore, Wohl et al. (3) showed that the CG HPS (Kapcha-Rossky) model can capture the salt-dependent LLPS behavior through the electrostatic screening in HP1a, a *Drosophila* homolog of human HP1 α . In our CG model, the salt concentration is captured by the DebyeHuckle term with tunable screening lengths, which allows for the simulations of salt-dependent effects in the low salt regime. We have added Figure S5 to illustrate the influence of salt on the LLPS propensity of HP1 α . In the low-salt regime (50 mM), the C_{sat} of HP1 α was reduced by twofold compared to that at 100 mM. Increasing the salt concentration to 150 mM raised the C_{sat} and started destabilizing the condensate. In the high salt regime (200–500 mM), HP1 α did not undergo phase separation, consistent with the experimental observations (2, 4–6).

Author response image 1.

Salt-dependent effects on the LLPS of HP1 α homodimer. (a, b) Density profiles and snapshots of HP1 α homodimer simulation with the box dimensions of 170x170x1190 Å³ at differing salt concentrations, 50, 100, 150, 200, 250, and 500 mM, respectively. The simulations were conducted at 320 K using the HPS-Urry model.



However, the primary objectives of our study are to elucidate the molecular interactions and to delineate the domain contributions that dictate the distinct phase-separation behaviors of the HP1 paralogs. To this end, we standardized our simulation conditions to a physiological salt concentration of 100 mM for all paralog constructs, facilitating a direct comparison and enabling physiologically relevant predictions, including those for the CD domain. We have

added the salt concentration used in the CG simulations in the Materials and Methods section, relevant figure captions, and the following sentence in the third paragraph of the Discussions section to improve clarity.

“...Our CG simulations corroborate these experimental observations, indicating that a low salt concentration (50 mM) promotes the LLPS of HP1 α . Raising the salt concentration weakens the electrostatic interactions and increases the Csat, eventually precluding HP1 α 's phase separation at high salt regimes (200-500 mM) (Fig. S5).”

Reviewer #2 (Public Review):

In this paper, Phan et al. investigate the properties of human HP1 paralogs, their interactions and abilities to undergo liquid-liquid phase separation. For this, they use a coarse-grained computational approach (validated with additional all-atom simulations) which allows to explore complex mixtures. Matching (wet-lab) experimental results, HP1 beta (HP1b) exhibits different properties from HP1 alpha and gamma (HP1a,g), in that it does not phase separate. Using domain switch experiments, the authors determine that the more negatively charged hinge in HP1b, compared to HP1a and HP1g, is mainly responsible for this effect. Exploring heterotypic complexes, mixtures between HP1 subtypes and DNA, the authors further show that HP1a can serve as a scaffold for HP1b to enter into condensed phases and that DNA can further stabilize phase separated compartments. Most interestingly, they show that a multicomponent mixture containing DNA, and HP1a and HP1b generates spatial separation between the HP1 paralogs: due to increased negative charge of DNA within the condensates, HP1b is pushed out and accumulates at the phase boundary. This represents an example how complex assemblies could form in the cell.

Overall, this is purely computational work, which however builds on extensive experimental results (including from the authors). The methods showcase how coarse-grained models can be employed to generate and test hypotheses how proteins can condense. Applied to HP1 proteins, the results from this tour-de-force study are consistent and convincing, within the experimental constraints. Moreover, they generate further models to test experimentally, in particular in light of multicomponent mixtures.

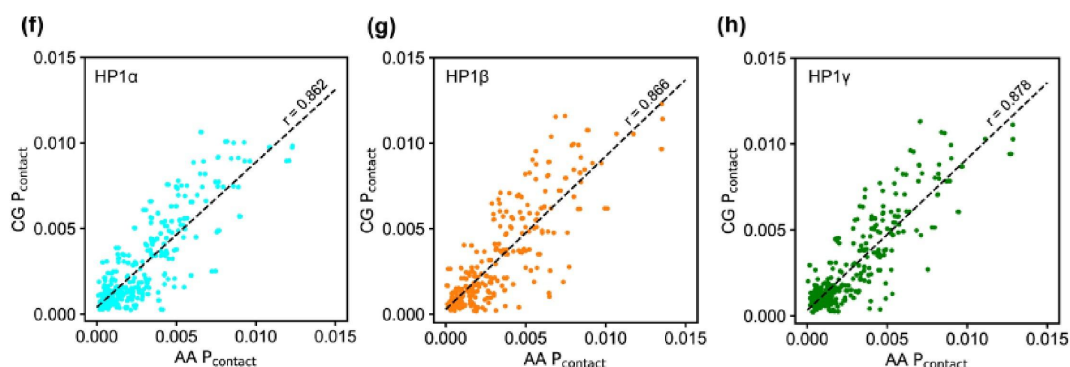
There are, of course, some limitations to these models.

First, the CG models employed probably will not be able to pick up more complex structure-driven interactions (i.e. specific binding of a peptide in a protein cleft, including defined H-bonds, or induced structural elements). Some of those interactions (i.e. beyond charge-charge or hydrophobics) may also play a role in HP1, and might be ignored here. There is also the question of specificity, i.e. how can diverse phases coexist in cells, when the only parameters are charge and hydrophobicity? Does the arrangement of charges in the NTD, hinges and CTDs matter or are only the average properties important?

We thank the reviewer for the thoughtful comments. We also appreciate the opportunity to incorporate the feedback on the reviewer's concerns below.

We agree that the interaction picture becomes more sophisticated, and many interaction modes may be involved in the phase coexistence in the cell environment. However, due to system sizes and required sampling, studying LLPS at an atomistic resolution remains challenging with the current state-of-the-art computer hardware. Our approach employs the CG model to reduce the computational cost but still capture the predominant interactions at the residue level. We have added the plots (Fig. S1 f-h) to show the correlation of the vdW contact probability per residue for each paralog between AA and CG simulation. The Pearson correlation coefficient is approximately 0.86, suggesting a strong positive linear correlation in the contact propensity between AA and CG simulations.

Author response image 2.



Our sequence analysis reveals a high fraction of charged residues in HP1 paralogs, with Arg, Lys, Glu, and Asp constituting 39-45% of the total amino acid count in the sequence. This property may explain why the electrostatic interactions are predominantly involved in the phase-separation behaviors of HP1 paralogs. Our findings on electrostatically driven phase separation and co-localization of HP1 paralogs are consistent with experimental observations by Larson et al. and Keenen et al. (5, 6). Significantly, we observe that the charge patterning in the disordered regions (NTE, hinge, and CTE) plays a critical role in the LLPS of HP1 paralogs, as articulated in the second paragraph of the Discussions section. Modifying this charge patterning, such as by phosphorylating serine residues in HP1α, excising the HP1α CTE, or substituting four acidic residues with basic ones in the HP1β hinge, can profoundly augment the LLPS of these proteins (4, 5, 7). Our in silico molecular details, complemented by in vitro observations, lay a solid foundation for future experiments. These future investigations may delve deeper into the specificity of interactions and the role of structural elements in modulating HP1 phase separation.

Second, the authors fix CSD-CSD dimers, whereas these interactions are expected to be quite dynamic. In the particular example of HP1 proteins, having dimerization equilibria may change the behavior of complex mixtures significantly, e.g. in view of the proposed accumulation of HP1b at a phase boundary. This point would warrant more discussion in the paper. Moreover, the biological plausibility of such a behavior would be interesting. Is there any experimental data supporting such assemblies?

We appreciate the reviewer's insightful comment regarding the dynamic nature of CSD-CSD interactions in HP1 proteins. Our assumption of fixing CSD-CSD dimers is grounded on reported dissociation constant (K_d) values for HP1α and HP1β, which are within the nanomolar range, indicative of strong dimerization affinity (4, 8). While the precise K_d values for HP1γ are not available, a study has demonstrated that HP1γ dimerization is crucial for its interaction with chromatin, suggesting a similar strong dimerization tendency as its paralogs (9, 10). Furthermore, evidence from the literature underscores the dimeric functionality of HP1 paralogs facilitated by their ChromoShadow Domains (CSD), which are instrumental in forming stable genomic domains and engaging in crucial interactions within chromatin architecture (5, 6, 11).

However, we acknowledge that despite the strong dimerization affinity, the CSD-CSD interactions exhibit dynamics, which may influence the behavior of complex mixtures, particularly at phase boundaries. A study by Nielsen et al. (12) shows that mammalian HP1 paralogs can interact directly with one another to form heterodimers. Moreover, the CSD-CSD interface has been shown to act as a hub for transient interactions with diverse binding partner proteins (5, 13). These experimental observations reflect the dynamic nature of CSD-

CSD interactions. However, due to the computational constraints and the focus of our study, a simplified static model was employed to gain initial insights into the phase separation behaviors of HP1 paralogs. We believe that the dynamic nature of CSD-CSD interactions and its implications for phase behavior in complex mixtures form an exciting avenue for future computational and experimental studies.

In light of the reviewer's comment, we have expanded our discussion in the 6th paragraph of the Discussions Section:

"... It is important to emphasize that our model is predicated on the assumption that HP1 proteins establish stable chromoshadow domain (CSD-CSD) dimers, a hypothesis supported by their Kd values being in the nanomolar range (13, 53). While this simplification serves as a useful starting point, it may not fully capture the dynamic nature of HP1 dimerization. Further computational and experimental studies are needed to understand better the behavior of the complex mixtures of HP1 paralogs, particularly at phase boundaries."

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Recommendations for the authors:

In this important work, the authors apply a residue-resolution protein coarse-grained model to investigate the differences in molecule dimensions and phase behaviour of three HP1 paralogs, HP1 paralog mixtures, and HP1/DNA mixtures. The simulations are well designed to investigate the impact of HP1 sequence on its phase behaviour. The work reveals that electrostatic interactions are a key determinant of HP1 paralog phase behaviour; hence advancing our understanding of the molecular mechanisms driving the phase separation behaviour of HP1 paralogs. Notably, the authors uncovered a nuanced interplay between the folded and disordered domains of HP1. Although disordered regions have traditionally been linked to driving phase separation through their capacity for forming multivalent interactions, the authors demonstrate that the contribution of the CD cannot be overlooked, as it significantly impacts the saturation concentration.

Essential revisions (based on reviewers assessment below):

1. *The manuscript describes the results of both single-molecule simulations and direct coexistence simulations. However, it is not very easy for the reader to determine which types simulations were performed in each section. The details on the simulations input parameters are also missing. Such details are needed throughout, i.e. to allow readers to follow the work and its implications. For instance, the specific salt conditions employed in the simulations are not explicitly stated. Since HP1 charge is presented as a key regulator for the modulation of HP1 paralogs radii of gyration and their phase behaviour, it is crucial for the authors to explicitly describe the salt concentration used for the different simulations and highlight how the relative differences observed are expected to change as the salt concentration decreases/increases.*

We have turned the first sentences in the paragraphs into subtitles to describe the results of single homodimers in dilute phase and multi-dimers in phase coexistence simulations.

“Sequence variation affects the conformations of HP1 paralogs in the dilute phase.”

“Sequence variation in HP1 paralogs leads to their distinct phase separation behaviors.”

To improve the clarity, we have also added the following sentence to Fig. 2 caption.

“... Figs. 2a-e show the results obtained under dilute conditions, while Figs. 2f-m illustrate the conditions of phase coexistence.”

We have specified the salt concentration used in the CG simulations in the Materials and Methods section and the relevant figure captions to improve clarity. We also addressed the reviewer's comment on salt concentration in the public review above.

1. Since direct coexistence simulations suffer from important finite-size effects, especially for multi-component mixtures as those investigated here, describing how many proteins/DNA copies were used per system, the size of the simulation, and which checks were done to check for finite-size effects is important. Regarding this point, estimating C_{sat} from Direct Coexistence simulations is extremely challenging, given the sensitivity of the dilute phase concentration to the box dimensions. Hence, it would be valuable if the authors clarify that the differences on C_{sat} provided represent a qualitative comparison and are sensitive to the simulation conditions. Importantly, the observation of spatial segregation of components in multi-component condensates could be an artefact of the box dimensions, relative copies of the various components, and overall system density.

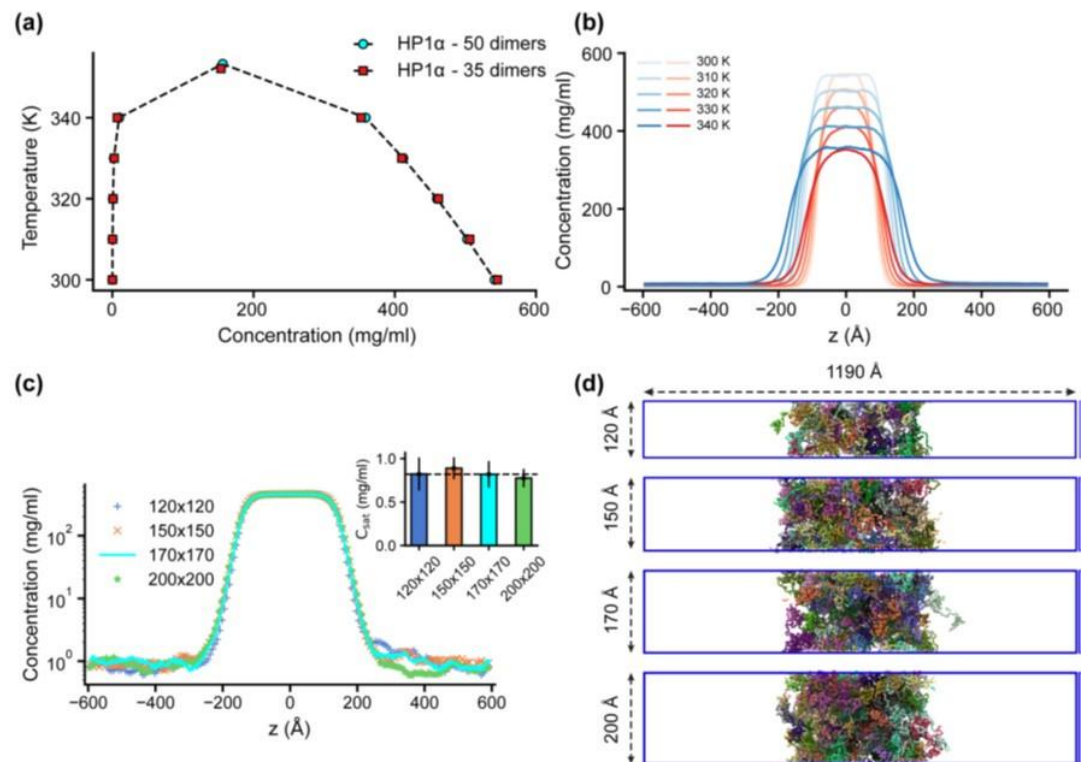
We appreciate the reviewer's concern regarding the finite-size effects in phase coexistence simulations and potential artifacts arising from box dimensions and system composition. In response to this, we have expanded the Materials and Methods section to elaborate on the specific checks to examine the finite-size effects. The new texts and additional SI figures are shown below.

"Previous studies have demonstrated that slab geometry can help mitigate finite-size effects and facilitate efficient sampling of the phase diagram (41). To assess the potential impact of finite-size effects with our chosen box dimensions, we conducted a test using the HP1 α homodimer, which serves as a representative system given the comparable sequence lengths of HP1 paralogs and their chimeras. By reducing the system size by 30% and constructing its phase diagram, we observed that both the original system size (50 dimers) and the reduced counterpart (35 dimers) produced similar phase diagrams, with critical temperatures of 353.3 K and 352.1 K, respectively, as shown in Figs. S4a,b.

We further evaluated the influence of the xy cross-sectional area on the measurement of C_{sat} . With the z-direction box length fixed at 1190 Å³, we varied the xy cross-sectional areas (120x120, 150x150, and 200x200 Å²) while maintaining the protein density consistent with the control case (170x170 Å²). Given that HP1 dimers are multidomain proteins, a 120x120 Å² cross-section was the minimum size feasible to prevent particle overlap in HOOMD simulations due to the constraints of the small box size. Our findings indicate that the condensates remained stable across all tested cross-sectional areas and that there were no significant differences in C_{sat} measurements within the margin of error, as depicted in Figs. S4c,d. These results confirm that our chosen box size is sufficiently large to minimize finite-size effects, thus ensuring the robustness of our results."

Author response image 3.

Finite-size analysis. (a) Phase diagrams for the HP1 α homodimer (50 dimers) and for a system reduced in size by 30% (35 dimers), with critical temperatures of 353.3 K and 352.1 K, respectively. (b) Density profiles of HP1 α and its reduced size counterpart at various temperatures. (c, d) Density profiles and snapshots of HP1 α homodimer simulation with box dimensions of 170x170x1190 Å³ and for systems with z-direction length fixed at 1190 Å and varying cross-sectional areas: 120x120, 150x150, and 200x200 Å². The black dashed line shows the simulated saturation concentration of wildtype HP1 α homodimer in the box dimensions of 170x170x1190 Å³. The simulations were conducted at 320 K and 100 mM salt concentrations. The error bars represent the standard deviation from triplicate simulation sets.



In response to the observed spatial segregation in our multi-component condensates, we have carefully considered finite-size effects and are confident that the segregation reflects genuine phase behavior rather than an artifact of simulation parameters. This interpretation is supported by findings from Chew et al. (14), who observed similar multilayered condensates and conducted thorough validations to verify these phases. To clarify our approach, we have included additional details in the Materials and Methods section of our manuscript.

“... By selecting a box size that minimizes finite-size effects, we can ensure that the spatial segregation observed in our multi-component condensates reflects genuine phase behavior. This finding aligns with Chew et al. (66), who also reported well-separated multilayered condensates and conducted thorough validations to confirm these phases.”

1. The authors should provide a clearer assessment of the quantitative precision of their model. For instance, the authors use all-atom simulations to compare with CG interaction maps. The all-atom maps are sparser due to less sampling, but the authors state that the maps are 'in good agreement'. How do the authors judge this? The issue of model validation is very important: to illustrate, HP1α is anticipated to undergo phase separation primarily under low salt concentrations. Does the model effectively capture this sensitivity to salt conditions? While numerous findings in the manuscript likely remain valid, it could be beneficial to acknowledge potential limitations tied to the simulations. For instance, might the absence of quantitative precision impact certain predictions, such as the CD's influence on phase separation? The CG models employed do not consider the specific binding of a peptide in a protein cleft, including defined H-bonds, or induced structural elements. Thus, the authors should discuss whether specific interactions (i.e. beyond charge-charge or hydrophobics) may also play a role in the phase behaviour of HP1, and why it makes sense to ignore them in this study. If the only important parameters are charge and hydrophobicity, how can diverse phases coexist in cells? Does the arrangement of charges in the NTD, hinges and CTDs matter or are only the average properties important?

This is similar to the point made by Reviewer 2 in the Public Review. We have addressed these questions in the public review and incorporated new plots (Fig. S1 f-h) in the SI.

1. *The authors fix CSD-CSD dimers, whereas these interactions are expected to be quite dynamic. In the particular example of HP1 proteins, having dimerization equilibria may change the behaviour of complex mixtures significantly, e.g. in view of the proposed accumulation of HP1b at a phase boundary. This point warrants more discussion in the paper.*

We have addressed the comment in the public review and extended the discussion in the Discussion section.

Reviewer #2 (Recommendations For The Authors):

The authors use all-atom simulations to validate their CG model. In Figure S1, they compare interaction maps. Of course, the AA maps are sparser due to less sampling, but the authors state that the maps are 'in good agreement'. How do the authors judge this (they do not look very similar to me, e.g. the NTD-hinge interactions are mostly lacking)?

This is similar to Reviewer 1's concern. We agree that the AA simulations are moderately limited over 5 μ s due to the large size of the HP1 proteins (~400 residues in a dimer). However, the expansion trends of the average dimensions of the HP1 paralogs agree with the CG simulations (Fig. S1 a,b). Regarding the AA contact maps, we agree that they are relatively sparse, which makes it difficult to compare them to the CG maps. We have added new plots (Fig. S1 f-h) to show the correlation of the vdW contact probability per residue for each paralog in the AA and CG simulations. The Pearson correlation coefficients are approximately 0.86, suggesting a strong positive linear correlation in the contact propensity between AA and CG simulations.