# HP1-Driven Micro-Phase Separation of Heterochromatin-Like Domains/Complexes

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Cytologically visible constitutive heterochromatin found at the centromeric and telomeric regions of chromosomes represents the largest differentiated chromatin compartment in eukaryotic nuclei.[1] Outside constitutive heterochromatin, heterochromatin-like domains (HLD, >.1 Mb) and complexes (HLC, <.1 Mb) are found along the chromosome arms.[2] HLD/Cs are present in eukaryotic genomes as divergent as fission yeast and human, with expansions in size and number in mammals. In human, there are ~163 to 859 HLDs and ~18 853 to 32 292 HLCs, depending on cell type.[2] Some of the largest HLDs are the KRAB-ZNF HLDs on chromosome 19. Notably, contacts among these large HLDs emerge as the heterochromatic B4 sub-compartment in Hi-C maps.[3]

Both cytologically visible constitutive heterochromatin and HLD/Cs are associated with the di/tri-methyl modification of lysine 9 on histone H3 (H3K9me2/3) and the HP1-class of chromodomain (CD) proteins.[1,2] The HP1 CD binds to H3K9me2/3 and HP1 proteins dimerise through the chromo shadow domain (CSD), creating 'bridges' between 2 H3K9-methylated molecules in separate nucleosomes.[4] How the HP1 and H3K9me2/3-marked chromatin are involved in partitioning the genome into cytologically visible constitutive heterochromatin and euchromatin as well as into the heterochromatic B-type and euchromatic A-type compartments has been the subject of intense research and led to the notion that the partitioning observed is driven by the same physiochemical process, namely phase separation. However, recent work shows that HP1-driven liquid-liquid phase separation (LLPS) is unlikely to play a major role in the formation and establishment of constitutive heterochromatin. By contrast, HP1 proteins appear to be key players in micro-phase separation and segregation of HLD/Cs that generate heterochromatic B-type compartments.

## Constitutive Heterochromatin: Droplet or Collapsed Globule?

Peri-centric constitutive heterochromatin is necessary for centromere function and proper mitotic chromosome segregation.[1] During interphase, peri-centric heterochromatin from different chromosomes come together to form mesoscopic (0.1-1.0 μm) 'chromocenters'.[1] Based largely on in vitro studies, LLPS of HP1 was posited to form droplets that encapsulate chromocenters and separate them from the surrounding euchromatin, forming a boundary that is selectively permeable to molecules based on their chemical properties[5,6] (Figure 1A). In vivo work, particularly in mammalian cells, has cast doubt on the role of HP1-driven LLPS in establishing constitutive heterochromatin. The mammalian HP1α isoform is thought to be crucial for HP1-driven LLPS of constitutive heterochromatin; under the same conditions as HP1α, HP1β and HP1 γ do not self-phase seprate.[6,7] Yet, HP1α null mutant mice are viable and fertile[8] and loss of HP1α has little effect on chromocenter structure at the mesoscale.[9] Moreover, the defect in HP1α mutants is highly-specific. $T_H1$-specific genes are derepressed in $T_H2$ cells[10] indicating that wild-type HP1α function lies outside constitutive heterochromatin. Further, HP1α opto-droplets do not form in nuclei like other intrinsically-disordered region-containing proteins[11] and, if formed, are unstable.[12] In a study addressing key biophysical properties of chromocenters it was shown that the size, accessibility and compaction of chromocenters were independent of HP1 proteins.[12] This is consistent with work in which all 3 mammalian HP1 isoforms, HP1α/β/γ, were deleted from mouse embryonic liver cells.[13] Histone modifications associated with constitutive heterochromatin were affected and chromocenters moved closer to the nuclear periphery, but the reformation of chromocenters after mitosis, and their global compaction and size at the mesoscale were unaffected by loss of all HP1 isoforms.[13] It seems unlikely that constitutive heterochromatin is established by HP1-driven LLPS. Instead, based on the observation that constitutive heterochromatin 'switches' between 'digital' compacted and de-compacted states without passing through intermediate states, it was suggested that constitutive heterochromatin is a polymer that has collapsed into a globule (Figure 1B).[12] Transition from relaxed to collapsed state is augmented by affinity between homotypic DNA repetitive elements,[14] nucleosome-nucleosome interactions[15] and

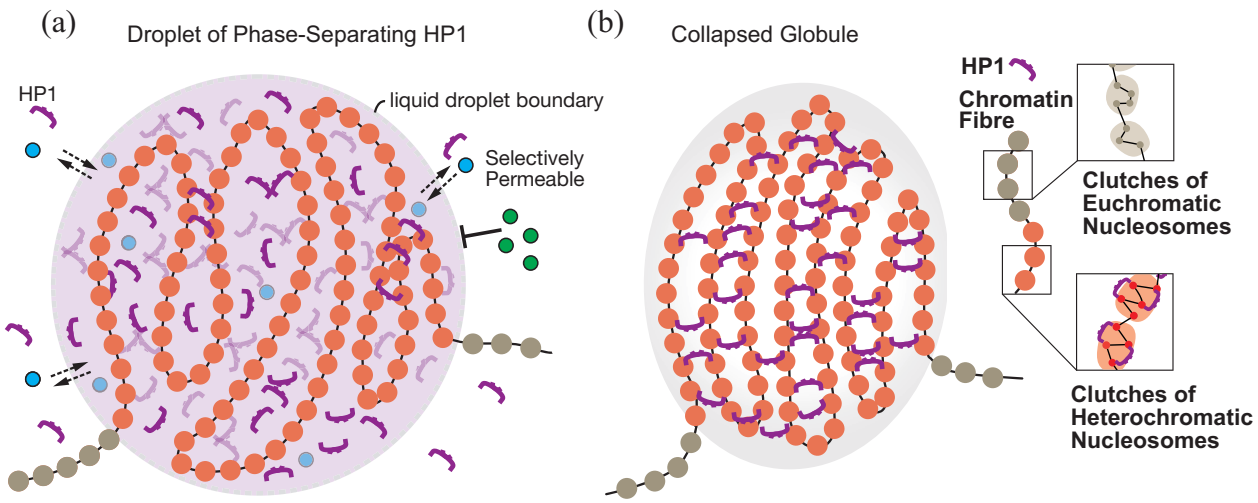


**Figure 1.** Liquid-liquid phase separation (LLPS) vs collapsed globule: (a) A model for HP1-driven LLPS that forms a liquid droplet surrounding constitutive heterochromatin. HP1 dimers 'bridge' within and between 'clutches' of H3K9me3-marked nucleosomes (orange circles). When the concentration of HP1 dimers reaches a critical concentration HP1-driven LLPS (purple sphere with HP1 proteins in the nucleoplasm) drives soluble heterochromatic nucleosomes into droplets that separate constitutive heterochromatin from the surrounding euchromatin (grey circles), forming a boundary that is selectively permeable to molecules based on their chemical properties.[5,6] Depicted are molecules (blue circles) that can cross the droplet boundary and others that cannot (green circles). (b) The collapsed globule model for constitutive heterochromatin envisages the collapsed state as being caused by homotypic DNA repetitive elements,[14] nucleosome-nucleosome interactions[15] and chromatin 'bridging' proteins.[16] HP1 might stabilise the heterochromatic collapsed globule state through bridging between and within between 'clutches' of H3K9me3-marked nucleosomes (orange circles) but is not alone sufficient to establish the collapsed state.[12]

chromatin 'bridging' proteins.[16] HP1 proteins can act as 'bridges' within and between H3K9me3-marked nucleosome fibres[4] and could promote and/or stabilise the collapsed globule state (Figure 1B), albeit this role appears minor given that chromocenters are formed in the absence of HP1.[13] Instead, based on biophysical and mutational analyses in the mouse, the major role of the dynamic, constantly exchanging, pool of HP1 bound to H3K9me2/3 may be as a source of mobilisable HP1 proteins that have functions outside constitutive heterochromatin.[17] It is to the HP1-containing HLD/Cs that lie outside constitutive heterochromatin and their role in the formation of B-type compartments that we now turn.

## Micro-Phase Separation of HLD/Cs and the Organisation of the Embryonic Genome

In *Drosophila*, HP1a (most similar to mammalian HP1β), binding within heterochromatic B-type compartments precisely overlaps with H3K9me3.[18] Evidence that HP1 proteins drive formation of B-type compartments comes from knockdown (KD) of maternally provided *Drosophila* HP1a mRNA, which results in a 20% decrease in B-type compartment signal in embryonic nuclei undergoing zygotic genome activation (ZGA), with increased mixing (reduced segregation) of A- and B-type compartments although there was no overt compartment switching.[18] The reduced heterochromatic B-B type contacts in the HP1a KD is due to reduced HP1a levels within the chromosome arms outside constitutive heterochromatin; HP1a localises to 2213 sites in the arms that can be up to several kilobases in size with the median being 730 bp[18]; these are smaller than the HLD/Cs observed in mammals.[2] A generalised block co-polymer model consisting of A- and B-type repeating units that assemble euchromatic A- and heterochromatic B-type chromatin respectively, can be used to explain the effects of the HP1a KD if the HP1a bound to H3K9me3 in the B-type compartments causes preferential interactions between heterochromatic B-type chromatin.[18] Regulation of the 3-D organisation of the *Drosophila* genome during ZGA by HP1a does not extend to differentiated cells where B-type compartmentalisation is unaffected by HP1a KD.[18] However, a similar role for HP1 proteins in regulating 3-D nuclear organisation during mammalian ZGA seems likely. During murine ZGA HP1-marked constitutive heterochromatin undergoes a dramatic re-organisation from a perinucleolar localisation to the typical chromocenter organisation found in somatic nuclei.[19]

Liquid Hi-C experiments have provided evidence that HP1 likely drives B-type compartmentalisation in mammalian cells.[20] Dissociation kinetics of digested chromatin from human K562 nuclei showed that interaction of heterochromatic B-type fragments associated with HP1α and β were more stable than those associated with *Polycomb*-Group (*Pc*-G) complexes (assembled from H3K27me3-marked chromatin).[20] Interactions between the latter complexes were as unstable as that found for loci in A-type compartments.[20] Liquid Hi-C also revealed that chromatin interactions for early replicating domains were unstable compared to late replicating loci that were more stable, as observed for HP1α/β-marked chromatin.[20] Notably, replication timing may regulate the cell-to-cell inheritance of HLD/Cs and heterochromatic B-type compartmentalisation.[21] Disturbing chromatin replication timing

results in loss of smaller H3K9me3-marked regions compared to larger regions that often exhibit increased levels of H3K9me3.[21] Regions that lost and gained H3K9me3 showed corresponding loss and gain of heterochromatic B-type interaction frequencies.[21]

It is not known if HLD/Cs are assembled from nucleosome or chromatosome chromatin fibres, although there is a well-documented interaction of mammalian HP1 with K26H1.4(H1e).[22] Insight into the likely interplay between histone H1 and HP1 in relation to heterochromatic B-type compartments comes from the (inducible) deletion of histone genes in murine cells. Triple *H1c*$^{-/-}$/ *H1d*$^{-/-}$/*H1e*$^{-/-}$ mouse mutants possess 50% of normal H1 levels and do not develop to term, dying at around e7.5.[23] ES cells derived from these mice show negligible changes in HP1 localisation and H3K9me3 and H3K27me3 levels and compartmentalisation is not affected.[24,25] Hi-C maps from triple *H1c*$^{-/-}$/ *H1d*$^{-/-}$/ *H1e*$^{-/-}$ null CD8+ T cells also showed that the majority of A and B compartments are unchanged but affected compartments undergo decompaction and compartment shifting from B to A rather than A to B.[26] Compartments that shift from B to A show greater mixing (increase in local interaction frequency) with other loci. A similar picture emerged using *H1c*$^{-/-}$/*H1e*$^{-/-}$ nuclei from double null germinal centre B cells.[27] In both differentiated cell types H1 deletions resulted in a decrease in H3K27me3[26,27] and the triple null phenocopied loss of EZH2 H3K27 methyltransferase actvity.[27] It would seem that histone H1 regulates compaction of heterochromatic loci (HLD/Cs) in B-type compartments and compartmental segregation, but only in differentiated cells with the majority of H3K9me3-marked compartments little affected compared to those marked by H3K27me3.

A parallel study that has provided insight into the mechanism(s) that cause the phylotypic progression,[28] which remains one of the central problems in evolution and developmental biology,[29] could furnish a developmental context for the studies on HP1 and histone H1 in relation to B-type compartmentalisation. Leading up to the phylotypic progression the levels of H3K9me3-marked heterochromatin increase in epiblast cells (at the same developmental stage as ES cells) at around e6.5 of mouse development[30] to reach maximal levels around e8.25[28] when embryos enter the progression. As embryos transit there is a gradual loss of H3K9me3 at genomic sites containing tissue-specific genes that are subsequently expressed thereby directing cellular differentiation. This deployment of HLD/Cs parallels the deployment of *Pc*-G complexes during the phylotypic progression, particularly in the regulation of the *Hox* genes that pattern the anterior-posterior axis.[31] In this scenario in early embryonic stages of the progression HP1 and *Pc*-G complexes contribute to compartmentalisation, but in later differentiated cells histone H1 has a significant role in regulating B-type compartmentalisation, as observed for T- and B-cells.[26,27]

## χ, HP1 Nanodomains and HLD/Cs

Liquid Hi-C experiments revealed that compartmentalisation is stable when chromatin fragments were larger than 10 to 25 kb.[20] Drawing on block co-polymer theory and assuming that nuclear chromatin is a 'polymer melt', the Flory-Huggins parameter χ was estimated to be 0.036 ± 0.013 per nucleosome.[20] The sign and magnitude of χ specifies the degree of incompatibility between the A- and B-type nucleosomes and is what ultimately drives micro-phase separation. The small positive value indicates that B-type heterochromatin tends to micro-phase separate from A-type euchromatin spontaneously. An alternative approach for estimating χ, which is consistent with a concentrated solution of nucleosome fibres dissolved in the nucleoplasm, as opposed to a *solvent-free* polymer melt, departs from the classical approach where the monomer is the polymer repeating unit. Instead, the repeating unit in a HLD/C (B-type heterochromatin) is a 'clutch' consisting of 2 to 10 H3K9me2/3-marked nucleosomes 'bridged' by HP1.[2] Support for this approach comes from a recent study that revealed thousands of H3K9me2/3-marked nucleosome 'clutches' in murine ES cells in the range of 3 to 10 nucleosomes that are bound by HP1 and termed heterochromatin nanodomains (HNDs).[32] The nucleation sites and size of HNDs are determined by separable sequence-specific and thermodynamic parameters. Two classes of HNDs that are strictly sequence-dependent are nucleated by PAX3/9 (85 645 HNDs, 0.9-2.0 kb in size) and ADNP (4673 HNDs, 1.1 kb in size). A third class of ATRX-dependent HNDs (13 113 HNDs, 0.7 kb in size) found at L1 elements are nucleated by ATRX but the size of the HNDs is largely dependent upon the thermodynamics of nucleosome packing.[32] This third class of HNDs is similar to the HP1-containing 'clutches' modelled along the large KRAB-ZNF heterochromatin-like domains that are nucleated by sequence-specific assembly of a small (~1.5 kb) ATRX-containing heterochromatin-like complex[33] (Figure 2A). Spreading from the complex to form the large heterochromatin-like domain involves histone methyl-transferase (HMTase) activity with HP1 binding to the H3K9me3-marked nucleosomes in the wake of the HMTase[33] (Figure 2B). The size of the H3K9me3-marked clutches bound by HP1 is determined thermodynamically by the sum of competing free-energy contributions of interaction energy (CDs of HP1 dimers binding to H3K9me3) and elastic energy (resistance of linker DNA to bending, twisting and stretching deformation and steric exclusion between the nucleosomes and linker DNA).[2] Contacts between KRAB-ZNF HLDs generate the heterochromatic B4 sub-compartment that are observed in Hi-C maps (Figure 2C).[3] There are likely to be many more HNDs in the genome.[32] Much work needs to be done to determine whether HNDs can regulate micro-phase separation of HLD/Cs and whether intra- and inter-chromosomal interactions between micro-phase separated

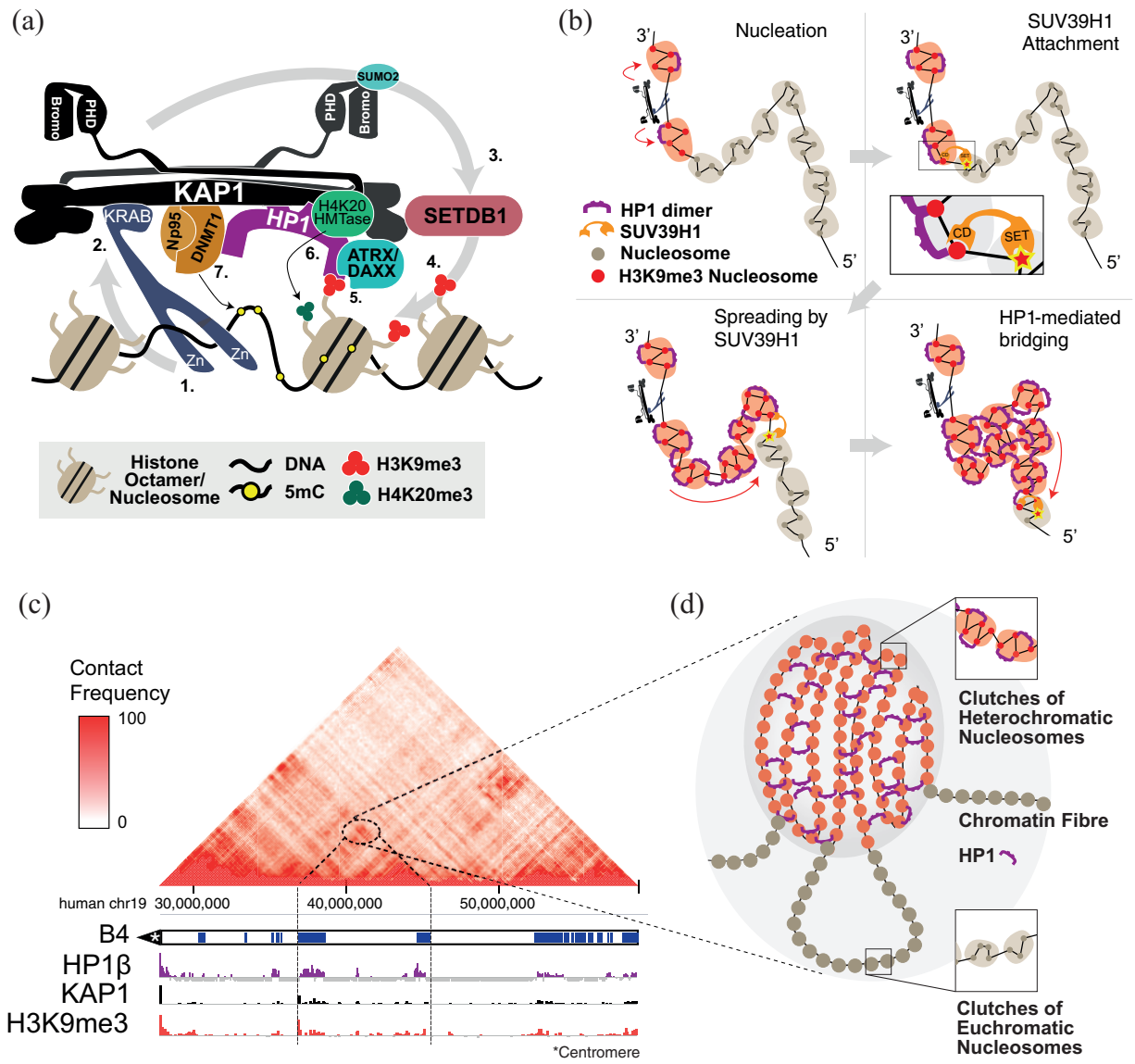


**Figure 2.** Microphase separation of heterochromatin-*like* domains/complexes at the KRAB-ZNF clusters on human chromosome 19 and their relation to the heterochromatic B4 sub-compartment: (a) A small (~1.5 kb) ATRX-containing heterochromatin-*like* complex nucleates the large domains at the KRAB-ZNF clusters on human chromosome 19 (taken and modified from[33]). (1) The KRAB-ZNF binds to its DNA binding site through its zinc-fingers (Zn). (2) The KRAB domain of the KRAB-ZNF interacts with KAP1. An HP1 CSD dimer binds to one molecule of KAP1. The HP1 CD binds to H3K9me3. (3) The SUMOylated bromodomain of KAP1 interacts with SETDB1 H3K9 HMTase. (4) SETDB1 generates H3K9me3 (red circles). (5) The ATRX/DAXX complex is bound to KAP1, HP1 and H3K9me3. ATRX/DAXX incorporates replacement histone H3.3 into chromatin thereby reinforcing nucleation. (6) HP1 recruits a H4K20 HMTase that generates H4K20me3 (green circles). (7) KAP1 binds to the maintenance DNA methylase DNMT1 and its co-factor Np95. DNMT1 maintains cytosine methylation at the site of assembly. (b) Model depicting SUV39H1-mediated spreading of H3K9me3 and HP1 proteins that form the larger KRAB-ZNF domain (taken and modified from[33]). In top left panel, the nucleation complex (shown in (a) generates H3K9me3-marked nucleosomes (red filled circles) in 'clutches' on either side of the complex. In top right panel, the CD of SUV39H1 attaches to H3K9me3-marked nucleosomes within a clutch whereupon the SUV39H1 SET domain catalyses methylation of H3K9, providing a positive feedback loop that enables spreading of the domain in the 5′ direction away from the nucleation site. In bottom left panel, SUV39H1 regulates spreading of H3K9me3 along the chromatin fibre (red arrow). The size of the 'clutches' formed in the wake of the newly-deposited H3K9me3 is determined by the sum of competing free-energy contributions of interaction energy and elastic energy that result from binding of HP1 dimers to H3K9me3-marked nucleosomes. In bottom right panel, H3K9me3 continues to spread by SUV39H1 activity (red arrow) and HP1-mediated bridging of H3K9me3-marked nucleosomes within and between 'clutches'. (c) The heterochromatic B4 sub-compartment overlaps exactly with chromosomal regions enriched for HP1β, KAP1 and H3K9me3 (only the q arm is shown; taken and modified from Ref.[33]). The interactions of 2 KRAB-ZNF domains that contribute contact enrichments to the B4 sub-compartment are depicted as an oval with dotted lines. The inset in (d) depicts segregation of micro-phase-separated HP1-containing 'blocks' that results in the contact enrichment shown by the oval in (c). Micro-phase separation and segregation of the blocks is driven by HP1-mediated 'bridging' of H3K9me3-marked nucleosomes within and between clutches.

domains/complexes could result in collapsed globules as envisaged for constitutive heterochromatin (*c.f.* Figure 1B with Figure 2D).

Work in this area is moving at a rapid pace and we suspect that understanding the physiochemical mechanisms that drive micro-phase separation and segregation of HLD/Cs will

provide considerable insight into the biophysics of the 4D genome during embryonic development.

## Contributions

PBS wrote the paper. AGN drew the figures. Both authors made comments and approved the final version. PBS thanks Stephanie C Weber for discussions.

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