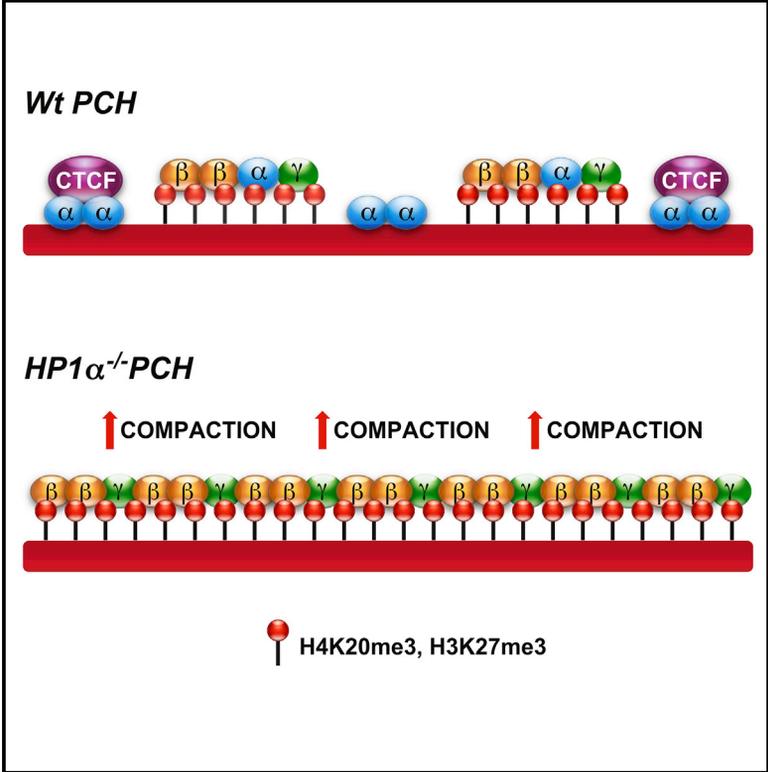


## Mammalian HP1 Isoforms Have Specific Roles in Heterochromatin Structure and Organization

### Graphical Abstract



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### In Brief

Bosch-Presegué et al. find that HP1 $\alpha$  interacts with CTCF in pericentric heterochromatin (PCH) and restricts H4K20me3 and H3K27me3 distribution. Loss of HP1 $\alpha$  results in PCH hypercompaction and a distinctive pattern of mitotic defects. HP1 $\beta$  is functionally related to H4K20me3 deposition and inhibits CTCF distribution, and its deficiency produces decompaction of PCH.

### Highlights

- HP1 $\alpha$  plays a unique role in heterochromatin organization and structure
- HP1 $\alpha$  interacts with CTCF and confines H4K20me3 and H3K27me3 to PCH foci
- Loss of HP1 $\alpha$ , but not HP1 $\beta$  and HP1 $\gamma$ , induces global hypercompaction of chromatin
- HP1 $\beta$  is functionally associated with H4K20me3



# Mammalian HP1 Isoforms Have Specific Roles in Heterochromatin Structure and Organization

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## SUMMARY

HP1 is a structural component of heterochromatin. Mammalian HP1 isoforms HP1 $\alpha$ , HP1 $\beta$ , and HP1 $\gamma$  play different roles in genome stability, but their precise role in heterochromatin structure is unclear. Analysis of *Hp1 $\alpha$* <sup>-/-</sup>, *Hp1 $\beta$* <sup>-/-</sup>, and *Hp1 $\gamma$* <sup>-/-</sup> MEFs show that HP1 proteins have both redundant and unique functions within pericentric heterochromatin (PCH) and also act globally throughout the genome. HP1 $\alpha$  confines H4K20me3 and H3K27me3 to regions within PCH, while its absence results in a global hyper-compaction of chromatin associated with a specific pattern of mitotic defects. In contrast, HP1 $\beta$  is functionally associated with Suv4-20h2 and H4K20me3, and its loss induces global chromatin decompaction and an abnormal enrichment of CTCF in PCH and other genomic regions. Our work provides insight into the roles of HP1 proteins in heterochromatin structure and genome stability.

## INTRODUCTION

The alteration of pericentric heterochromatin (PCH) organization and structure have been linked to cell-cycle-progression defects, DNA damage, chromosomal aberrations, apoptosis, cancer, and aging (Benayoun et al., 2015; Carone and Lawrence, 2013). PCH is defined by several features including specific histone modifications, structural proteins, histone variants, DNA

hypermethylation, and an undefined RNA component (Saksouk et al., 2015). Two histone marks, H3K9me3 and H4K20me3, have been proposed as being hallmarks of PCH structure (Rea et al., 2000; Schotta et al., 2004).

H3K9me3 is mainly catalyzed by the histone methyltransferase Suv39h1 and its close relative Suv39h2 and functions as a docking site for specific factors (Bannister et al., 2001; Lachner et al., 2001), whereas H4K20me3 is catalyzed by Su(var)4-20h2 and is directly involved in chromatin compaction and cohesin recruitment (Hahn et al., 2013). How these marks are distributed throughout heterochromatin and whether they co-localize in the same regions within heterochromatic regions are currently unknown. A key factor in heterochromatin structure is heterochromatin protein 1 (HP1), which was originally described in *Drosophila* as a suppressor of position-effect variegation (Eisenberg et al., 1990). Mammals harbor three HP1 isotypes termed HP1 $\alpha$ , HP1 $\beta$ , and HP1 $\gamma$  (Jones et al., 2000). A growing body of evidence suggests that the role of HP1 proteins in genome stability goes beyond heterochromatin structure as they play a role in gene expression, DNA replication, DNA repair, cell cycle, cell differentiation, and development (Maison and Almouzni, 2004). All three isoforms localize to PCH although HP1 $\beta$  and HP1 $\gamma$  are also found in euchromatic regions (Maison and Almouzni, 2004). HP1 proteins participate in the establishment and propagation of the heterochromatin structure through their specific binding both to H3K9me3 and Suv39h1 (Bannister et al., 2001; Lachner et al., 2001). In this sense, HP1 $\beta$  was suggested to act as a bridge between H3K9me3-enriched chromatin fibers (Hiragami-Hamada et al., 2016). Interestingly, recent studies have suggested that HP1-mediated compaction also involves phase separation from soluble chromatin (Larson

et al., 2017; Strom et al., 2017). HP1 proteins also act as adapter molecules that link other factors to heterochromatin such as Suv4-20h2 or DNA methyltransferases among others (Fuks et al., 2003; Hahn et al., 2013).

Despite these advances, the question remains as to the relative contributions of each of HP1 isotype to heterochromatin organization and structure. This inquiry has been hampered by the strong functional redundancy of HP1 proteins, the abundance of all three isoforms in PCH foci, and their ability to homo- and hetero-dimerize (Canzio et al., 2014). Thus, the role of the three isoforms in heterochromatin has been considered to be more or less equivalent. However, the fact that the three isoforms have a distinct pattern of genomic distribution, specific interaction partners, and post-translational modifications, suggests that they likely perform different functions in cell physiology (Kwon and Workman, 2011; Maison and Almouzni, 2004). This possibility has been supported by recent evidence showing a more direct role of HP1 $\alpha$  and HP1 $\gamma$  in Suv39h1 function in PCH than in HP1 $\beta$  (Raurell-Vila et al., 2017). Furthermore, mutational analyses have shown tissue-specific phenotypes in HP1 $\alpha$ , HP1 $\beta$ , and HP1 $\gamma$  knockout (KO) mice (Aucott et al., 2008; Brown et al., 2010; Maksakova et al., 2011; Singh, 2010).

The functional differences between HP1 $\alpha$  and HP1 $\beta$  are particularly relevant as both isoforms are enriched within PCH and their combined loss abrogates HP1 $\gamma$  localization in these regions (Dialynas et al., 2007). Aiming to understand the specific role of HP1 $\alpha$  and HP1 $\beta$  isoforms in PCH, we analyzed the impact of each isoform on heterochromatin structure and organization using mouse embryonic fibroblast (MEF) cells derived from KO mice. Our studies suggest that HP1 $\alpha$  plays a key role as an organizer of constitutive heterochromatin regions. Loss of HP1 $\alpha$  results in the enrichment of H4K20me3 and H4K27me3 in PCH foci, whereas HP1 $\beta$  mediates a direct functional link with H4K20me3 and Suv420h2. Consistent with non-overlapping roles in PCH organization and structure, each mutant isoform exhibits a different pattern of H4K20me3 and H3K27me3 distribution in PCH that is associated with different types of mitotic aberrations. Our studies also suggest that HP1 $\alpha$  and HP1 $\beta$  play opposite roles in CTCF distribution in PCH and other genomic regions. These studies provide insight into the specific roles of HP1 isoforms in heterochromatin structure.

## RESULTS

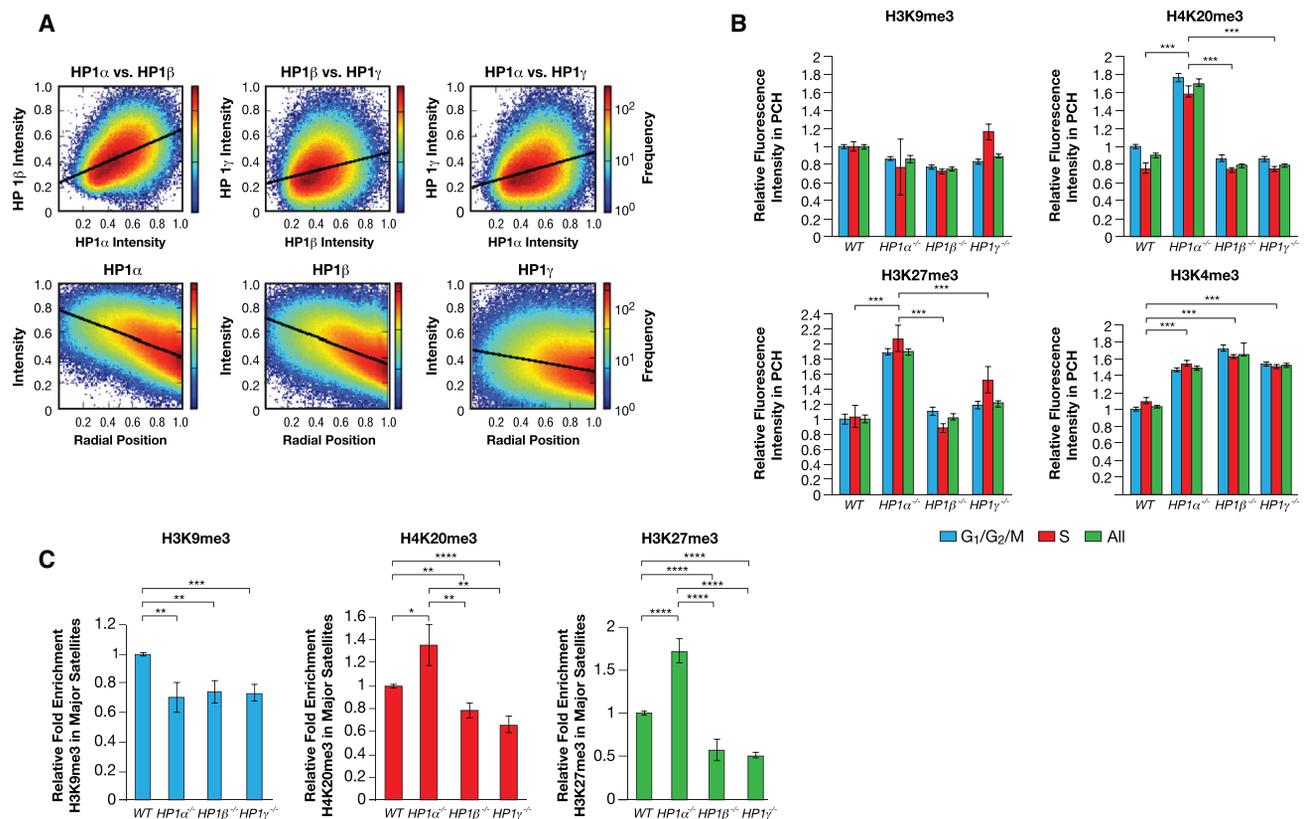
Previous studies have suggested that the localization of endogenous HP1 $\alpha$  and HP1 $\beta$  in PCH was broadly co-incident but not complete (Dialynas et al., 2007). We first aimed to confirm that the pattern of distribution of all three isoforms in PCH foci of NIH 3T3 cells is different. We expressed fluorescence-tagged HP1 isoforms and performed spectral imaging in PCH, which enabled us to correlate the intensity distribution of each isoform (Figure 1A, upper) and their relative localization relative to the foci center or radial position (Figure 1A, lower). As shown in Figures 1A and S1A–S1C, HP1 $\alpha$  and HP1 $\beta$  are enriched in similar regions of PCH, preferentially toward the center of the foci. However, the intensity distribution was not identical, thereby suggesting a distinctive enrichment of both isoforms in PCH structure. HP1 $\gamma$  showed a lower degree of correlation with the other two isoforms

with a rather more dispersed distribution within the foci (Figure 1A). Moreover, the loss of either HP1 $\alpha$  or HP1 $\beta$  did not alter each other's levels in PCH but did induce an enrichment of HP1 $\gamma$ , suggesting that it plays an auxiliary role for both isoforms (Figures S1E and S1F) as has been previously suggested (Raurell-Vila et al., 2017). The interplay between the isotypes is also confirmed by the loss of HP1 $\gamma$  deposition in PCH upon simultaneous loss of HP1 $\alpha$  and HP1 $\beta$  (Figure S1D), which has been seen previously (Dialynas et al., 2007).

### HP1 $\alpha$ Loss Induces H4K20me3 and H3K27me3 Enrichment in PCH Foci

We first analyzed the histone-modification changes in PCH foci that are associated with the specific loss of each HP1 isoform. To consider the possibility of cell-cycle-dependent events, we performed the analysis at different stages of the cell cycle. A loss of each HP1 isoform was correlated with a small decrease in H3K9me3 and a significant increase (1.5- to 1.7-fold) in H3K4me3 levels in PCH foci at all stages of the cell cycle (Figures 1B and S2A), thereby confirming that they are redundant with regard to the deposition of these histone modifications. There was also no significant impact on the DNA methylation levels at major satellite sequences between HP1 $\alpha$  and the other isoforms (Figures S2B and S2C). In stark contrast, a loss of HP1 $\alpha$ , but not of HP1 $\beta$  or HP1 $\gamma$ , resulted in a significant enrichment of H4K20me3 (around 1.8-fold) and H3K27me3 (around 2-fold) in the PCH foci during all cell-cycle stages (Figures 1B and S2A). Notably, increased levels of H4K20me3 were also observed at other genomic regions as well as the PCH (Figures S2A and S2D). Chromatin-immunoprecipitation (ChIP) assays confirmed that both H4K20me3 and H3K27me3 were increased in the major satellites of *Hp1 $\alpha$ <sup>-/-</sup>* MEFs compared to wild-type (WT) cells, while they were decreased in *Hp1 $\beta$ <sup>-/-</sup>* and *Hp1 $\gamma$ <sup>-/-</sup>* cells (Figure 1C).

To confirm that the changes in both marks were directly dependent on HP1 $\alpha$ , we overexpressed Cre recombinase (R1) in *Hp1 $\alpha$ <sup>-/-</sup>* MEFs, which excised the promoter-trap Neo cassette that was used to generate the KO and restored HP1 $\alpha$  gene integrity and expression (noKO) (Figure 2A). As expected, the re-expression of endogenous HP1 $\alpha$  by the nuclease-driven removal of the Neo cassette (Figures 2B, S3A, and S3B) restored the levels of both marks in PCH foci. An identical result was obtained upon the overexpression of ectopic HP1 $\alpha$  in *Hp1 $\alpha$ <sup>-/-</sup>* MEFs, demonstrating a direct role of HP1 $\alpha$  in the control of these marks (Figures 2C and S3C). Interestingly, the re-deletion of HP1 $\alpha$  (reKO) in noKO cells by FLP recombinase (R2) restored H3K27me3 levels but did not alter H4K20me3 (Figures 2B and S3B). The reKO generated a short-truncated form of HP1 $\alpha$  (Figure S3A). To rule out any potential effect of the HP1 $\alpha$  short-truncated form on H4K20me3, we knocked down HP1 $\alpha$  by short hairpin RNA (shRNA) in NIH 3T3 cells. Consistently, HP1 $\alpha$  loss resulted in H3K27me3 enrichment in PCH foci (1.6-fold) and no significant increase in H4K20me3 (Figures 2D, S3D, and S3E) (Hahn et al., 2013), suggesting a different deposition mechanism in PCH for both marks. We also knocked down HP1 $\beta$  and observed, in all cell-cycle stages except for G<sub>2</sub>/M, a decrease in both H3K27me3 (25% reduction) and H4K20me3 (20% reduction) in PCH foci, supporting a direct role for HP1 $\beta$  in H4K20me3 deposition (Figures 2D, S3D, and S3E), which prompted us to



**Figure 1. Loss of HP1 $\alpha$  Induces H4K20me3 and H3K27me3 in PCH Foci**

(A) 2D histograms showing HP1-isoform-distribution intensities in cells co-expressing all three HP1 isoforms. Colors represent the observed frequency on a logarithmic scale, and black lines shows the linear regression of the data points. Pairwise-intensity comparisons between indicated isoforms (top) and each HP1-isoform intensity plotted against the radial position are indicated with a value from 0 (chromocenter center) to 1 (periphery). Representative images are shown in Figure S1A. Intensity correlations quantified by Spearman rank-order correlation and linear least-squares regression are included in Figures S1B and S1C.

(B) Mean intensities of histone marks in the PCH foci from WT cells or MEFs *Hp1 $\alpha^{-/-}$* , *Hp1 $\beta^{-/-}$* , and *Hp1 $\gamma^{-/-}$*  through the different stages of the cell cycle. Representative images are shown in Figure S2A. \*\*\**p* < 0.001.

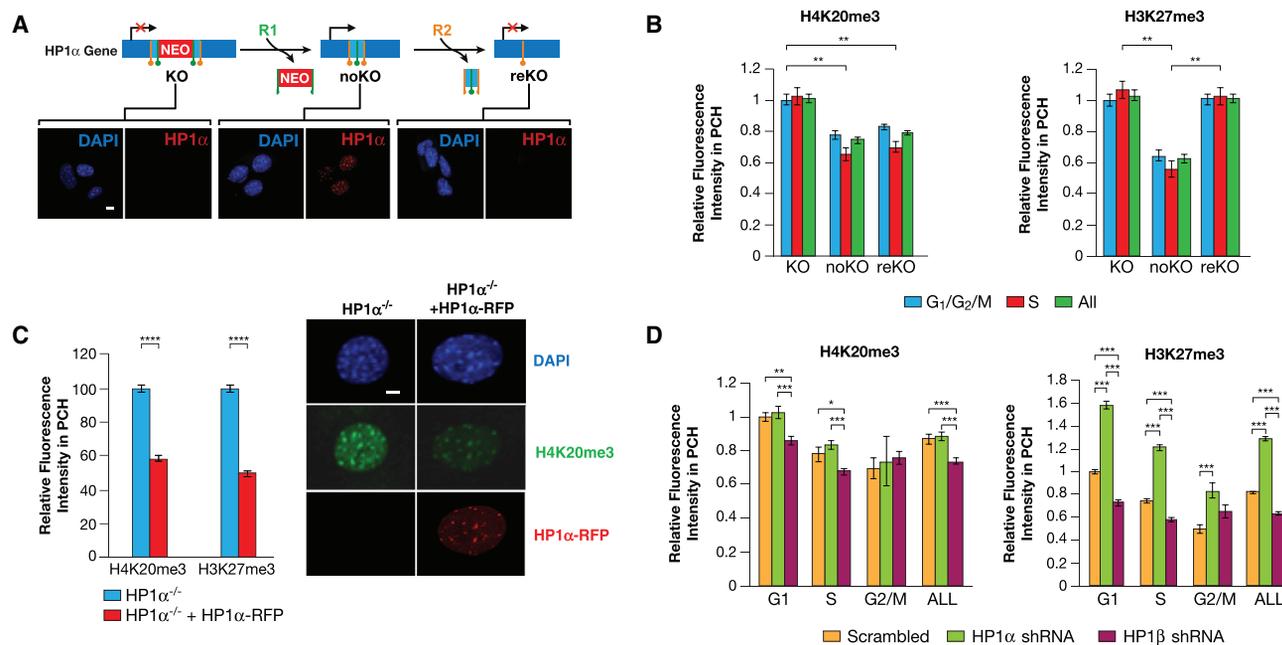
(C) ChIP of H3K9me3, H4K20me3, and H3K27me3 in major satellites of indicated MEFs. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001.

explore the relationship between HP1 $\beta$  and H4K20me3 in more detail.

### HP1 $\beta$ Is Functionally Linked to H4K20me3 and Suv420h2

Previous studies with recombinant HP1 proteins have suggested that all three isoforms may be equivalent in the regulation of H4K20me3 (Hahn et al., 2013). Interestingly, although we confirmed that all three isotypes interacted equally well with the Suv420h2 in nuclear soluble fractions of the transfected cells (data not shown), we observed a specific interaction between Suv420h2 and HP1 $\beta$  compared to the other isoforms in extracts enriched in digested insoluble chromatin upon highly stringent conditions (Figure 3A). Fluorescence resonance energy transfer (FRET) experiments confirmed these observations *in vivo* with a preferential binding of Suv420h2 to HP1 $\beta$  compared to HP1 $\alpha$  and HP1 $\gamma$  (Figures 3B, S4A, and S4C). However, a fluorescence recovery after a photobleaching (FRAP) analysis of the dynamics of Suv420h2 in PCH foci of the WT, *Hp1 $\alpha^{-/-}$* , *Hp1 $\beta^{-/-}$* , or *Hp1 $\gamma^{-/-}$* -deficient MEFs showed a more complex picture. The loss of HP1 $\beta$

resulted in a decreased turnover of Suv420h2 at PCH and did not alter the Suv420h2 mobile fraction, whereas the loss of HP1 $\alpha$  resulted in increased turnover of Suv420h2 in PCH compared to WT (Figures 3C and 3D). The overexpression of HP1 $\alpha$  and HP1 $\beta$  had a small effect on the Suv420h2 residence time (Figures 3D and S4D). The decreased turnover upon HP1 $\beta$  loss contrasted with the increase turnover that was observed in HP1 $\alpha$ -deficient cells, indicating an antagonistic role of HP1 $\alpha$  and HP1 $\beta$  in Suv420h2 dynamics. Notably, HP1 $\gamma$  loss on Suv420h2 dynamics induced an effect between HP1 $\alpha$  and HP1 $\beta$ , but its overexpression decreased its mobile fraction (Figures 3D and S4D). These FRAP analyses suggest that each isoform alters Suv420h2 dynamics in PCH in an isoform-specific manner. Taking our results together, we suggest that the effect of HP1 $\beta$  is likely to have a more direct role in Suv420h2 dynamics. A functional link between HP1 $\beta$ /Suv420h2 might also explain the decreased levels of H4K20me3 that were observed in both *Hp1 $\beta^{-/-}$*  MEFs (Figure 1C) and upon shRNA-driven HP1 $\beta$  knockdown (Figure 2D). To obtain biochemical support for such an interaction, we undertook hemagglutinin (HA)



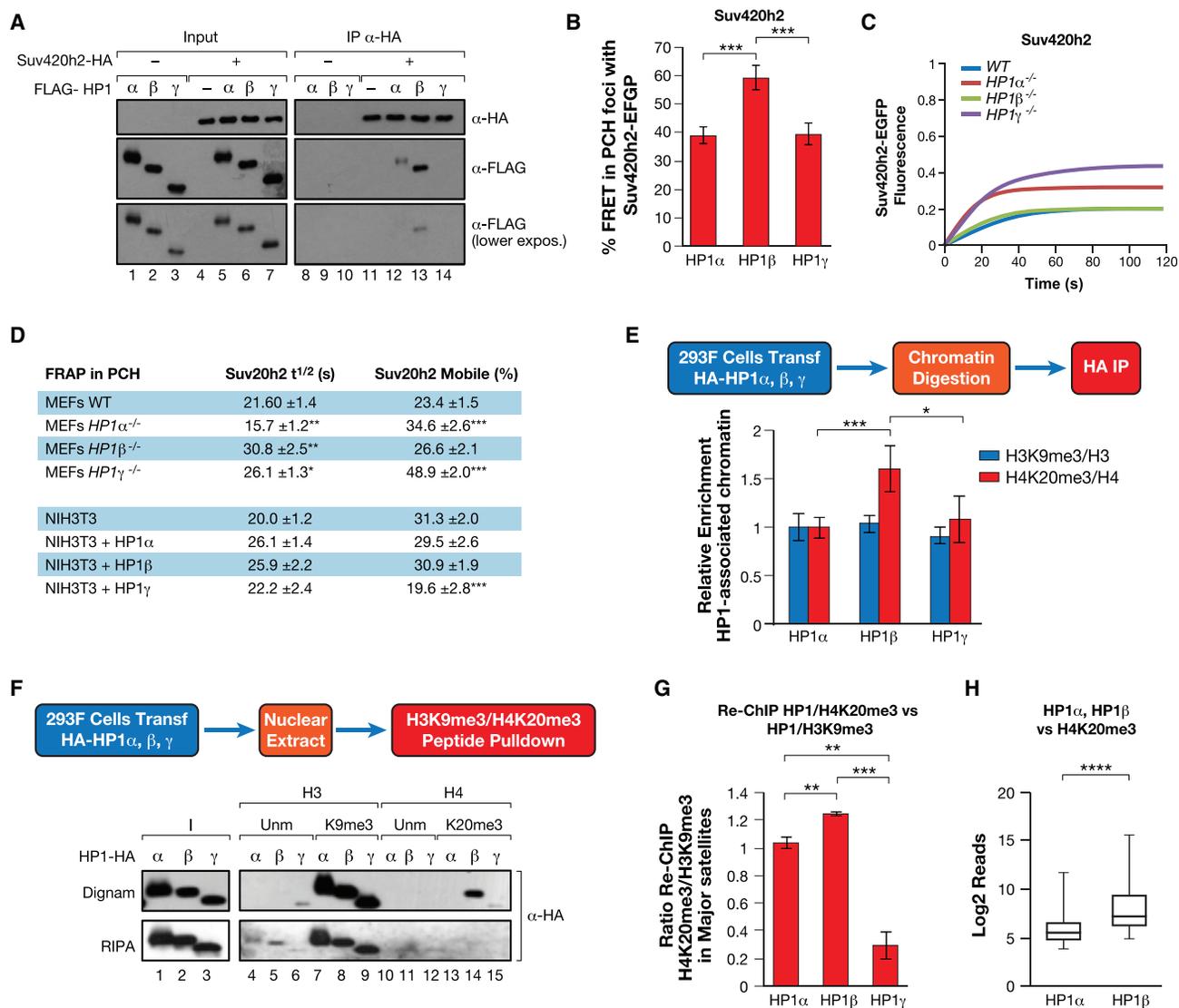
**Figure 2. HP1 $\alpha$  Directly Regulates H4K20me3 and H3K27me3 Enrichment in PCH Structure**

(A) Generation of noKO and reKO cells from *HP1 $\alpha$ <sup>-/-</sup>* MEFs. HP1 $\alpha$  protein levels were determined by IF and western blot (Figure S3A). (B) Quantification of relative fluorescence intensity levels of H4K20me3 and H3K27me3 in HP1 $\alpha$  KO, noKO, and reKO cells through the cell cycle. Representative images are shown in Figure S3B. \*\**p* < 0.01. (C) Relative fluorescence intensity levels of H4K20me3 and H3K27me3 in PCH of KO cells upon ectopic expression of either an empty vector or HP1 $\alpha$ -RFP. Representative images of H4K20me3 (right) and H3K27me3 (Figure S3C) are shown. \*\*\*\**p* < 0.0001. (D) Quantification, as in (B), of H4K20me3 and H3K27me3 levels in PCH of NIH 3T3 cells depleted in HP1 $\alpha$  or HP1 $\beta$  by shRNA throughout the cell cycle (Figures S3D and S3E). \**p* < 0.05, \*\*\**p* < 0.001.

affinity purification of HA-tagged HP1 isoforms, which showed that HP1 $\beta$ -containing chromatin was 1.5-fold enriched in H4K20me3 compared to HP1 $\alpha$  or HP1 $\gamma$ , whereas H3K9me3 was detected at similar levels with all three isoforms (Figures 3E and S4E). We next tested the ability of each isoform to bind to H4K20me3 compared to H3K9me3 in peptide pull-downs using nuclear fractions containing HA-tagged HP1 isoforms (see [Experimental Procedures](#)). We performed these pull-downs under two different buffer conditions, the classical mild Dignam buffer and the highly stringent radioimmunoprecipitation assay (RIPA) buffer. We observed that all three isoforms bound strongly to H3K9me3-methylated peptide, but only HP1 $\beta$  bound to H4K20me3 resin (Figure 3F, Dignam). The binding of HP1 $\beta$  to H4K20me3 was more labile than to H3K9me3 because it was abrogated under very stringent RIPA conditions (Figure 3F, RIPA). These results suggested that, despite a strong redundancy between isoforms, HP1 $\beta$  binds to H4K20me3 with higher affinity than do the other isoforms. Consistently, re-ChIP experiments of endogenous HP1 isoforms (first ChIP) and H3K9me3 or H4K20me3 (second ChIP) of major satellites showed that the ratio H4K20me3/H3K9me3 in HP1 $\beta$  re-ChIP was clearly higher (1.25) than in HP1 $\alpha$  (1) and HP1 $\gamma$  (0.3) (Figure 3G). This increased co-localization between HP1 $\beta$  and H4K20me3 was not an exclusive feature of PCH since a genome-wide analysis of previously reported ChIP sequencing (ChIP-seq) experiments in mouse embryonic stem cells (ESCs) confirmed a stronger overall correlation between HP1 $\beta$  and H4K20me3 compared to HP1 $\alpha$  (Figure 3H).

### CTCF Cooperates with HP1 $\alpha$ in PCH Organization

Both Suv420h2 and H4K20me3 have been linked to cohesin enrichment in PCH (Hahn et al., 2013). We next tested whether the changes in H4K20me3 in *HP1 $\alpha$ <sup>-/-</sup>* MEFs also alter cohesin enrichment in PCH. ChIP experiments showed a 2-fold increase in cohesin levels at PCH in *HP1 $\beta$ <sup>-/-</sup>* MEFs in contrast to *HP1 $\alpha$ <sup>-/-</sup>* and *HP1 $\gamma$ <sup>-/-</sup>* (Figure 4A). This result suggested that HP1 $\beta$  may have an inhibitory effect on the accumulation of cohesins in PCH and that this enrichment was not associated to H4K20me3 levels. Cohesin distribution has been directly linked to CTCF, which is a major player in global genomic architecture (Cuddapah et al., 2009; Rubio et al., 2008). This link and the reported link of CTCF to PCH (Mukhopadhyay et al., 2004; Xiao et al., 2015) as well as HP1 $\alpha$  (Agirre et al., 2015), led us to hypothesize that the H4K20me3-independent cohesin enrichment in *HP1 $\beta$ <sup>-/-</sup>* MEFs may be related to abnormal levels of CTCF in PCH. Accordingly, we observed that, although the levels of CTCF in major satellites seem to be under the detection limit of ChIP in WT, *HP1 $\alpha$ <sup>-/-</sup>*, and *HP1 $\gamma$ <sup>-/-</sup>* cells, we did detect a significant enrichment (>7-fold) of CTCF in *HP1 $\beta$ <sup>-/-</sup>* MEFs (Figure 4B). In contrast, no CTCF enrichment was detected in minor satellites (Figure 4B). Although CTCF was not detected in PCH by ChIP analysis, a detailed co-localization analysis of the endogenous CTCF signal within PCH confirmed the presence of CTCF in these regions (Figure S5B). These data are consistent with previous reports (Mukhopadhyay et al., 2004; Xiao et al., 2015) and indicate that CTCF is present in these regions either in limiting



**Figure 3. HP1β Is Functionally Linked to Suv420h2 and H4K20me3**

(A) Interaction between HA-HP1 isoforms and Suv420h2 in HEK293F cells using HA resin. Inputs (I) and elutions (E) are shown.

(B) Relative quantification of FRET analysis analyzing the interaction between HP1 isoforms (RFP) and Suv420h2 (GFP) in PCH foci of NIH 3T3 cells (Figure S4C) \*\*\*p < 0.001. FRET analysis controls are shown in Figures S4A and S4B.

(C) Relative fluorescence intensity of the FRAP assay in PCH foci for Suv420h2-EGFP in WT and HP1 KO cells.

(D) Quantification and statistical analysis of the FRAP experiment in (C) and FRAP in NIH 3T3 cells overexpressing HP1 isoforms (Figure S4D) such as the mobile fraction (Mobile [%]), and half-time of fluorescence recovery (t<sup>1/2</sup>). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

(E) H3K9me3/H3 and H4K20me3/H4 levels in affinity purification of HA-tagged HP1 isoforms. Upper: schematic diagram of the experiment. Chromatin fractions of HEK293F cells expressing HA-tagged HP1 isoforms were digested with Benzoylase and affinity purified with HA resin. Levels of H3K9me3 and H4K20me3 were normalized with histones H3 and H4, respectively, and the ratios in HP1β and γ pull-downs were quantified (n = 3) and represented relative to HP1α. A representative experiment is shown in Figure S4E. \*p < 0.05, \*\*\*p < 0.001.

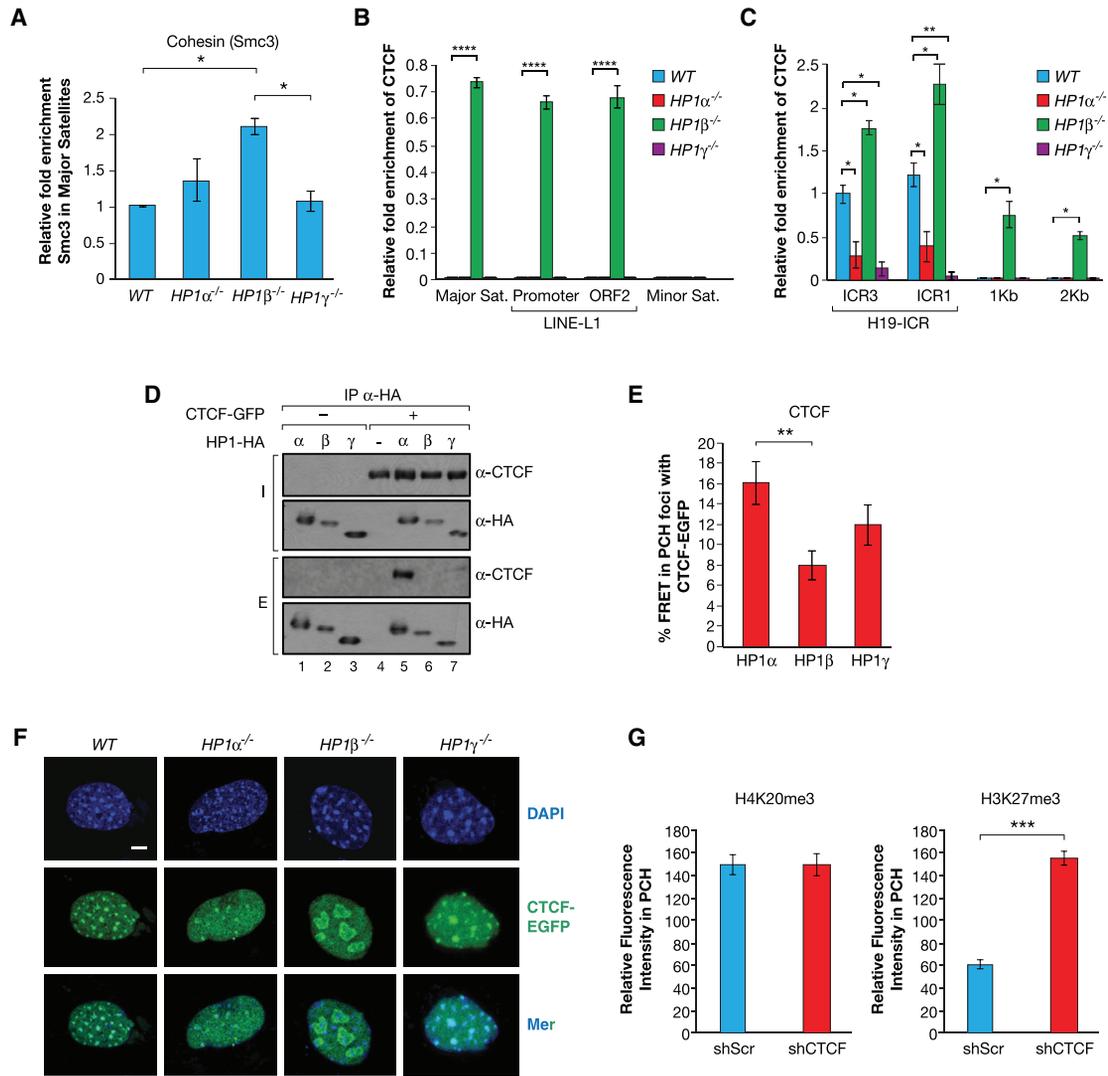
(F) Pull-down of HA-tagged HP1 isoforms with H3 or H4 (unmodified or H4K20me3)-biotinylated-streptavidin-agarose performed with HEK293F cell extracts generated in mild (Dignam) or stringent (RIPA) conditions.

(G) Re-ChIP experiments (n = 3) of endogenous HP1 isoforms (ChIP #1) and H3K9me3 or H4K20me3 (ChIP #2) in major satellites of NIH 3T3 cells. The ratio H4K20me3/H3K9me3 is shown for each isoform. \*\*p < 0.01, \*\*\*p < 0.001.

(H) Correlation of the genome-wide co-localization between H4K20me3 and HP1α or HP1β in mouse ESCs. Boxplot of the log reads of H4K20me3 in the regions occupied by HP1α and HP1β based on previously published ChIP-seq experiments. \*\*\*\*p < 0.0001.

levels or under very specific conditions. Prompted by these data, we tested whether CTCF interacts with any of the HP1 isoforms *in vitro* and *in vivo*. We found that CTCF bound specifically to

HP1α in immunoprecipitation experiments (Figure 4D). This result was supported by FRET as the FRET levels between HP1α and CTCF were 2-fold higher than for HP1β (Figures 4E



**Figure 4. CTCF Plays a Role in HP1 $\alpha$ -Dependent Regulation of PCH Organization**

(A) ChIP of cohesin subunit Smc3 in Major satellites of the indicated MEFs. \* $p < 0.05$ .

(B) Representative ChIP ( $n = 3$ ) of CTCF in major satellites, LINE-L1's promoter, and ORF2 and minor satellites in indicated MEFs. CTCF enrichment is represented relative to CTCF levels in H19-ICR of WT cells shown in (C). \*\*\*\* $p < 0.0001$ .

(C) CTCF ChIP, as in (B), of H19-ICR CTCF binding sites ICR3 and ICR1 as well as 1 and 2 kb downstream of ICR1. \* $p < 0.001$ , \*\* $p < 0.005$ .

(D) HA immunoprecipitation of HEK293F extracts expressing HA-tagged HP1 isoforms, CTCF-EGFP, or both. Inputs and elutions are shown.

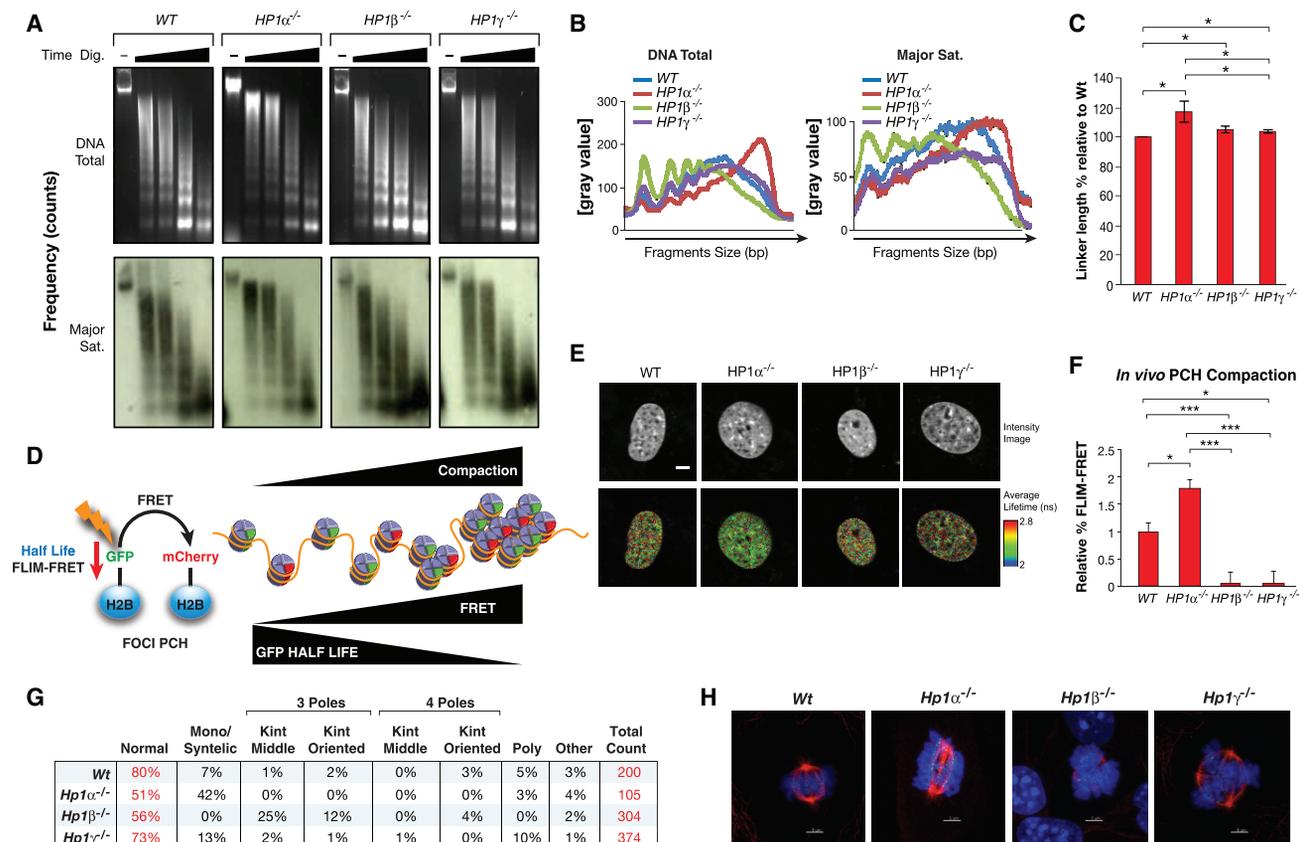
(E) FRET-acceptor photobleaching in PCH foci of NIH 3T3 cells between CTCF-EGFP and HP1-RFP isoforms (Figure S5A). \*\* $p < 0.01$ .

(F) CTCF-EGFP distribution in the nucleus of WT of HP1 KO MEFs.

(G) Quantification of IF mean intensities of H4K20me3 and H3K27me3 for shRNA of CTCF in NIH 3T3 cells. \*\*\* $p < 0.0001$ .

and S5A). Further supporting the antagonism between HP1 $\alpha$  and HP1 $\beta$  in PCH, CTCF EGFP was significantly scarcer in the PCH foci of HP1 $\alpha$ -deficient MEFs, suggesting that HP1 $\alpha$  is directly related to CTCF localization to PCH (Figure 4F). By contrast, a loss of HP1 $\beta$  induced, in around 45% of cells analyzed, a dramatic enrichment of CTCF in PCH regions (Figure 4F), which was correlated with a global increase in CTCF protein levels without altering CTCF gene expression (Figure S5D; data not shown). These data suggest that HP1 $\beta$  loss results in enhanced spreading of CTCF beyond its normal sites of localization. Confirming this hypothesis, CTCF was detected outside the H19

imprinting control region (H19-ICR) binding site in HP1 $\beta^{-/-}$  cells between 1 and 2 kb downstream of H19-ICR binding site 1 (ICR1) (Figure 4C). A similar observation was also observed at LINE-L1 elements (promoter and open reading frame [ORF]2), where a dramatic increase in CTCF levels was observed in HP1 $\beta^{-/-}$  cells. Together, these evidences indicate that HP1 $\alpha$  and HP1 $\beta$  play opposite roles in CTCF distribution. For completeness, we explored the role of CTCF in regulating covalent histone modifications in PCH. As in the case of HP1 $\alpha$ , shRNA-mediated depletion of CTCF induced a significant enrichment of H3K27me3 (2.6-fold) in PCH foci without any change in H4K20me3 levels



**Figure 5. *In Vitro* and *In Vivo* Analysis of Chromatin Compaction in WT and HP1 KO Cells**

(A) Upper panels: representative image of MNase digestion upon time for indicated MEFs. Lower panels: representative experiment of the corresponding southern blot incubated with a [<sup>32</sup>P]-labeled major satellites probe.

(B) Quantification and intensity versus fragment-size representation of MNase digestion and southern blot line 3 from different experiments (n = 5).

(C) Linker-DNA length calculated from the experiment in (A) and represented in % compared to WT (see [Experimental Procedures](#)). \*p < 0.05.

(D) Schematic representation of FLIM-FRET methodology used for *in vivo* chromatin compaction analysis based on H2B-GFP and H2B-mCherry co-expression ([Lières et al., 2009](#)). Lower GFP half-life means increased FRET levels and increased chromatin compaction.

(E) Representative images of FLIM-FRET experiments in the indicated MEFs. GFP intensity and GFP fluorophore lifetime-average images are shown.

(F) Relative quantification of chromatin compaction (%FRET) in indicated MEFs using FLIM-FRET methodology ([Figures S5E and S5F](#)). Absolute %FRET from [Figure S5E](#) relative to WT values is shown. Controls of these experiments are shown in [Figure S5F](#). \*p < 0.05, \*\*\*p < 0.001.

(G) Analysis of mitotic defects in HP1 KO MEFs.

(H) Representative IF images of the defects included in (H). DAPI (blue), centromere marker, CREST (green), and tubulin (red) are shown.

or HP1 $\alpha$  localization ([Figures 4G and S5C](#)). Altogether, these evidences suggest that CTCF collaborates with HP1 $\alpha$  defining specific chromatin domains within PCH.

### Depletion of HP1 $\alpha$ , but Not of HP1 $\beta$ or HP1 $\gamma$ , Induces Decreased Accessibility and *In Vivo* Hypercompaction of PCH

A key question concerns the role of HP1 proteins in chromatin compaction. Such a role is implied by the observation that H4K20me3 has been directly linked to compaction levels in PCH foci through cohesins and that HP1 proteins recruit Suv420h2, which is the enzyme that is responsible for the trimethylation of H4K20 ([Hahn et al., 2013](#)). Accordingly, we investigated whether the changes that were observed in the HP1 $\alpha$ - and HP1 $\beta$ -deficient cells were associated to changes in the levels of compaction of the PCH foci *in vitro* and *in vivo*.

*In vitro*, we performed a classic micrococcal nuclease (MNase) digestion of the genome followed by a Southern blot with an [<sup>32</sup>P]-labeled probe of the satellite. The loss of HP1 $\alpha$  induced a decrease in accessibility of the PCH foci. By contrast, the loss of HP1 $\beta$  and, to a lesser extent, of HP1 $\gamma$ , induced enhanced digestion of PCH chromatin DNA by MNase ([Figures 5A and 5B](#)). This effect was not restricted to PCH foci but also affected the accessibility of chromatin globally. The loss of HP1 $\alpha$  also resulted in around a 20% increase in linker-DNA length compared to WT cells ([Figure 5C](#)). Longer linker DNA has been associated with higher chromatin compaction ([Szerlong and Hansen, 2011](#)). Next, we investigated the effects of HP1 loss on chromatin compaction within PCH foci *in vivo* using FLIM-FRET assays. This method allowed us to measure the degree of compaction of both PCH foci and the whole genome in live cells by expressing H2B fused to two different fluorophores

(GFP and RFP) (Lières et al., 2009). Higher chromatin compaction was correlated with higher FRET efficiency between H2B-GFP and H2B-RFP, which resulted in a lower half-life of the FRET donor GFP (FLIM) (Figure 5D). A FLIM-FRET analysis of live cells confirmed results that were obtained *in vitro* because HP1 $\alpha$  deficiency produced a decrease in the GFP half-life ( $\pi$ amp) as a consequence of a 1.8-fold increase in FRET efficiency. In contrast, HP1 $\beta$ - and HP1 $\gamma$ -deficient cells showed no significant changes in their GFP half-life (Figures 5E, 5F, S5E, and S5F). These results indicate that HP1 $\alpha$  is a key player in the global organization of PCH by regulating its state of compaction.

Our *in vitro* and *in vivo* studies indicate that, in addition to a common redundant role of all three isoforms, HP1 $\alpha$  and HP1 $\beta$  have unique isoform-specific roles in genome stability. To test this hypothesis, we studied the frequency of mitotic abnormalities in WT, HP1 $\alpha$ <sup>-/-</sup>, HP1 $\beta$ <sup>-/-</sup>, and HP1 $\gamma$ <sup>-/-</sup> MEFs. Our results showed that HP1 $\alpha$ <sup>-/-</sup> and HP1 $\beta$ <sup>-/-</sup> harbored a higher frequency of aberrations compared to HP1 $\gamma$ <sup>-/-</sup> or *Wt* cells. The HP1 $\alpha$ <sup>-/-</sup> aberrations were strikingly different to those found in HP1 $\beta$ <sup>-/-</sup> MEFs. The loss of HP1 $\alpha$  resulted in an increased number of merotelic and syntelic attachment defects (Figure 5G), whereas the loss of HP1 $\beta$  resulted in high frequency of multipolar spindle formation. Interestingly, the loss of HP1 $\gamma$  resulted in less frequent defects that were a mixture of those found in HP1 $\alpha$ <sup>-/-</sup> and HP1 $\beta$ <sup>-/-</sup> MEFs, indicating that HP1 $\gamma$  shares some redundancy with the other isoforms (Figures 5G and 5H).

## DISCUSSION

Our work suggests that each HP1 isoform makes a distinctive contribution to the organization and structure of PCH foci. The individual roles are most clearly manifest in chromosomal abnormalities found in isoform-specific mutant MEFs. The increased frequency of merotelic attachments found in HP1 $\alpha$ <sup>-/-</sup>, which resulted from the simultaneous binding of a single kinetochore to both spindle poles (Figures 5G and 5H), have been previously associated to a deficient Ctr4/Swi6 function in *Schizosaccharomyces pombe* (Gregan et al., 2007). By contrast, HP1 $\beta$ <sup>-/-</sup> MEFs showed defects in mitotic spindle multipolarity (Figures 5G and 5H), which may be related to the de-condensed phenotype observed in these chromosomes. Our work indicates that HP1 $\alpha$  plays a direct role in restraining H4K20me3 and H3K27me3 in PCH. Accordingly, we suggest that HP1 $\alpha$  acts as an organizer of PCH in conjunction with CTCF. This is in agreement with the described localization of CTCF in centromeric/PCH regions (Mukhopadhyay et al., 2004; Rubio et al., 2008). Our data also suggest that HP1 $\alpha$  may recruit CTCF to specific sites within PCH because the loss of HP1 $\alpha$  causes a significant delocalization of CTCF-EGFP. Notably, we did not detect CTCF by ChIP in WT, HP1 $\alpha$ <sup>-/-</sup>, and HP1 $\gamma$ <sup>-/-</sup> cells (Figure 4B) although the co-localization experiments of endogenous CTCF (Figure S5B) and GFP-CTCF distribution and FRET analysis (Figures 4E and 4F) confirmed the presence of CTCF in PCH as shown previously (Mukhopadhyay et al., 2004; Xiao et al., 2015). Our data suggest that HP1 $\beta$  act in opposition to HP1 $\alpha$  with regard to H4K20me3 and H3K27me3 within PCH foci. Moreover, in HP1 $\beta$ <sup>-/-</sup> cells, we observed a global enrichment

of CTCF in large regions of the genome including PCH (Figure 4F), thereby suggesting a role for HP1 $\beta$  in restraining the localization of CTCF to specific loci and, thereby, likely controlling chromatin boundaries. This role is supported by our observation that loss of HP1 $\beta$  results in enrichment of CTCF outside its normal confines. Specifically, we observed that, in HP1 $\beta$ <sup>-/-</sup> cells, CTCF is found in the 6-kb-long LINE-L1 elements at the promoter and also 2 kb away, at the ORF2 (Figure 4C). A similar observation was observed at the H19 ICR in HP1 $\beta$ <sup>-/-</sup> cells (Figure 4C). The latter result is in keeping with the suggestion that HP1 $\beta$  may regulate genomic imprinting (Singh, 2016). Because there does not appear to be any CTCF canonical motifs downstream of the H19 ICR or in LINE-L1s, the observed spreading is unlikely to be related to a direct binding of CTCF to DNA but more likely dependent on other factors.

Our work suggests that the relationship between H3K27me3 and H4K20me3 with HP1 $\alpha$  is different. The increased levels of H3K27me3, but not of H4K20me3, that were observed upon downregulation of HP1 $\alpha$  suggest that the specific regulation of H4K20me3 may be restricted to an earlier developmental stage prior to the specification of the fibroblast lineage. In support of this idea, the establishment of both HP1 $\alpha$  and H4K20me3 seems to take place at the same time during late development (Wong-tawan et al., 2011). Recent work has suggested that HP1 $\alpha$  has a more significant role in the establishment of H3K9me3 mark than in its maintenance (Hathaway et al., 2012). This function may also be true for H4K20me3 since the re-expression of HP1 $\alpha$  in HP1 $\alpha$ <sup>-/-</sup> could re-establish the H4K20me3 levels in PCH, but its re-deletion did not have any clear effect (Figures 2A–2C).

We have also revealed an unexpected link between HP1 $\beta$  and H4K20me3. *In vivo* HP1 $\beta$  preferentially interacts with Suv420h2 within the PCH foci and regulates its dynamics. Consistently, HP1 $\beta$  recognizes H4K20me3-methylated peptides and HP1 $\beta$ -containing chromatin is enriched in H4K20me3 and tends to localize with H4K20me3 in major satellites compared to other isoforms. Moreover, a ChIP-seq analysis shows a higher genome-wide correlation in ESCs between HP1 $\beta$  and H4K20me3 compared to HP1 $\alpha$ .

One of the most striking observations that we found was the hypercompaction of the PCH structure in HP1 $\alpha$ -deficient cells. Hypercompaction in HP1 $\alpha$ -deficient cells was associated with an increased enrichment in H4K20me3 and H3K27me3 and a longer linker DNA (Figure 5C). This result was surprising because a previous study reported that artificial binding of LacR-tagged HP1 $\alpha$  or HP1 $\beta$  to Lac operon-regulated transgenes resulted in chromatin compaction (Verschure et al., 2005). These apparently conflicting observations may be reconciled if one population of HP1 $\alpha$  molecules is involved in PCH compartmentalization while another plays, along with the other isoforms, a more redundant role.

Based on our data, we propose that, despite a well-established functional redundancy between isoforms in PCH, HP1 $\alpha$  and HP1 $\beta$  play different roles in the organization and structure of PCH. Our studies also suggest a model of heterochromatin organization whereby HP1 $\alpha$  maintains, together with CTCF, the internal structure and compaction of PCH foci by restricting the distribution of H4K20me3 and H3K27me3. These findings offer

insight into the structural organization of the genome and provide a perspective on the role of HP1 isoforms and their functional link with heterochromatin structure, genome organization, and stability.

## EXPERIMENTAL PROCEDURES

### FRET, FLIM-FRET, and FRAP Assays

Leica SP5 confocal and Acceptor photo-bleaching methods were used to measure the FRET in PCH foci. %FRET was calculated taking 100% as the FRET value that was obtained for GFP-RPF (positive control) and 0% as the value obtained for the FRET value that was obtained for the donor construct alone. RFP protein from PCH foci was bleached by using a maximum laser 561 power obtaining ~80% of acceptor-intensity bleaching. The FLIM-FRET experiments were performed as indicated (see [Supplemental Experimental Procedures](#)). All experiments were performed at least in 10 independent assays and on 50 different cells. FRAP experiments were carried out as previously described (see [Supplemental Experimental Procedures](#)).

### Generation of noKO and reKO Cells

The generation of *Hp1 $\alpha$ <sup>-/-</sup>*, *Hp1 $\beta$ <sup>-/-</sup>*, and *Hp1 $\gamma$ <sup>-/-</sup>* mouse and associated MEFs was previously described (Aucott et al., 2008; Brown et al., 2010; Mak-sakova et al., 2011). The process of conversion from KO to reKO was similar as it was shown for *Hp1 $\gamma$ <sup>-/-</sup>* (Brown et al., 2010). *Hp1 $\alpha$ <sup>-/-</sup>* (*Cbx5<sup>-/-</sup>*) (KO) MEFs were converted into noKO (WT) by the overexpression of Cre recombinase (R1) in the *Hp1 $\alpha$ <sup>-/-</sup>* MEF cells resulting in the release of the Neo cassette and the restoration of HP1 $\alpha$  gene integrity and expression (noKO). Subsequently, the generation of reKO cells was performed by overexpression of FLP recombinase (R2) in noKO cells, which resulted in a partial deletion of the *Hp1 $\alpha$ <sup>-/-</sup>* gene and complete abrogation of HP1 expression.

### ChIPs and re-ChIPs

ChIPs were performed with 3–5 × 10<sup>6</sup> cells as previously described (Rodríguez-Ubrea and Ballestar, 2014). In re-ChIP experiments, the first ChIP (HP1) was eluted with 10 mM Tris-EDTA (TE) and 20 mM DTT and diluted 20 times in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl 8.1, 167 mM NaCl, and protease inhibitors) and proceeded to the second ChIP (H3K20me3 or H3K9me3).

### Peptide Pull-Down of HP1 Isoforms

Biotinylated peptides spanning histone H4 residues 1–23 or 1–21 of H3 (unmodified or H3K9me3) were obtained from Anaspec (Fremont, CA). 100  $\mu$ g of peptides were pre-bound to streptavidin agarose (Millipore) and then incubated at 4°C overnight (O/N) with nuclear extracts from 293F cells expressing the HA-tagged HP1 isoforms either prepared according to the Dignam or RIPA method (see [Supplemental Experimental Procedures](#)).

### Statistical Analysis

The statistical analysis was performed using a multivariate ANOVA (immunofluorescence [IF] analysis, ChIP-seq, FLIM-FRET) or Student's t test (rest of analysis). Graph values represent mean values of n  $\geq$  3 experiments and include SEs except in the case of ChIP-seq (SDs). The specific n of each quantification and p values are indicated in the corresponding figure legends.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2017.10.092>.

## AUTHOR CONTRIBUTIONS

A.V. and L.B.-P. conceived the study and designed the experiments. A.V. supervised the work. A.V. and P.B.S. wrote the manuscript. L.B.-P. performed the experiments. H.R.-V. and J.G. supported the performance of the experiments; L.S., P.B.S., G.S., and J.A. collaborated in the discussion. L.S.,

J.K.T., and N.K.-G. performed FRAP and IF quantifications. P.B.S. and J.P.B. generated the MEF KO cells. G.S. generated the Suv420h2-EGFP vector. C.C., L.B.-P., and T.Z. carried out the FLIM-FRET experiment. M.V. and M.E. carried out methylation assays. A.G. performed the ChIP-seq bioinformatic analysis.

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