The histone variant composition of centromeres is controlled by the pericentric heterochromatin state during the cell cycle

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ABSTRACT
Correct chromosome segregation requires a unique chromatin environment at centromeres and in their vicinity. Here, we address how the deposition of canonical H2A and H2A.Z histone variants is controlled at pericentric heterochromatin (PHC). Whereas in euchromatin newly synthesized H2A and H2A.Z are deposited throughout the cell cycle, we reveal two discrete waves of deposition at PHC – during mid to late S phase in a replication-dependent manner for H2A and during G1 phase for H2A.Z. This G1 cell cycle restriction is lost when heterochromatin features are altered, leading to the accumulation of H2A.Z at the domain. Interestingly, compromising PHC integrity also impacts upon neighboring centric chromatin, increasing the amount of centromeric CENP-A without changing the timing of its deposition. We conclude that the higher-order chromatin structure at the pericentric domain influences dynamics at the nucleosomal level within centromeric chromatin. The two different modes of rearrangement of the PHC during the cell cycle provide distinct opportunities to replenish one or the other H2A variant, highlighting PHC integrity as a potential signal to regulate the deposition timing and stoichiometry of histone variants at the centromere.

KEY WORDS: Centromere, Heterochromatin, H2A.Z, CENP-A, Cell cycle

INTRODUCTION
The proper genomic distribution of histone variants is important for the specification of chromatin states, which impact on DNA replication, recombination, transcription and repair (Soria et al., 2012; Szenker et al., 2013; Talbert and Henikoff, 2010). The centromere offers an attractive model to address how histone variant composition is transmitted or remodeled, because the identity, propagation and function of this chromosomal landmark depend on its unique histone variant composition (reviewed in Cleveland et al., 2003; Gascoigne and Cheeseman, 2011). Centromeres comprise two adjacent chromatin domains with distinct functional properties and chromatin features. The centric domain serves as the site of kinetochore formation, and the surrounding pericentric heterochromatin (PHC) domains contribute to the cohesion of sister chromatids (Alonso et al., 2010; Bernard et al., 2001; Guenati et al., 2004). Centric chromatin is marked by the histone variant H3 variant CenH3 (CENP-A in vertebrates), which is interspersed with H3 nucleosomes carrying H3K4me2 and H3K36me2/3 marks (Bergmann et al., 2012; Sullivan et al., 2004). The surrounding PHC domains lack CenH3, but are characterized by the presence of canonical H3, H3.3 and several H2A variants, including H2A.Z (reviewed in Boyarchuk et al., 2011). PHC domains also display an enrichment for constitutive heterochromatin marks such as DNA methylation, H3K9me2/3 and H4K20me3, as well as a depletion of acetylated histones and accumulation of heterochromatin protein 1 (HP1) in a non-coding-RNA-dependent manner (reviewed in Boyarchuk et al., 2011; Maisonnais et al., 2010; Probst et al., 2009).

Among histone variants localized at centromeres, CenH3 and H2A.Z have both been reported to be involved in centromere function. CenH3 provides not only the structural and functional foundation for the kinetochore (reviewed in De Rop et al., 2012; Szenker et al., 2013) but also the epigenetic determinant of centromere identity (reviewed in Cleveland et al., 2003). In all species tested, the depletion of CenH3 results in severe chromosome segregation defects, a delay in mitotic progression, an impairment of kinetochore assembly and activation of the spindle checkpoint (Buchwitz et al., 1999; Goshima et al., 2003; Régnier et al., 2005; Stoler et al., 1995). H2A.Z is also necessary for functional centromeres, because its loss leads to chromosome segregation defects, improper heterochromatin formation, loss of centromere cohesion and structural changes in PHC (Carr et al., 1994; Greaves et al., 2007; Hou et al., 2010; Krogan et al., 2004; Rangasamy et al., 2004; Sharma et al., 2013; Swaminathan et al., 2005). Therefore, to fully understand the mechanisms of centromere propagation and kinetochore function, it is important to elucidate the mechanism by which the localization of these variants is regulated.

Previous studies addressing CENP-A propagation at centric regions revealed a controlled multistep process that involves regulation by cell cycle progression, changes in chromatin state and a CENP-A-specific chaperone, HJURP [Holliday junction recognition protein (Dunleavy et al., 2009; Foltz et al., 2009)]. Importantly, CENP-A deposition is uncoupled from centromeric DNA replication in all metazoans studied so far. In human cultured cell lines, it occurs in telophase and early G1 (Hemmerich et al., 2008; Jansen et al., 2007; reviewed in Boyarchuk et al., 2011). In contrast to CENP-A, the deposition dynamics of H2A variants at centromeres remain unknown. Notably, H2A variants constantly exchange at a rapid rate throughout the nucleus (Bönisch et al., 2012; Higashi et al., 2007; Jackson and Chalkley, 1985; Kimura et al., 2006), raising an important question regarding their stable maintenance at a given
locus. Furthermore, given that H2A.Z displays a broader localization across the genome than CENP-A, its dynamics might be distinct in different genomic compartments. Interestingly, in mouse trophoblast cells, the total amount of H2A.Z transiently increases at the centric domain during mitosis, whereas its levels are constant at the pericentric domain (Nekrasov et al., 2012). These recent observations suggest that H2A.Z maintenance at centromeres requires a specific form of regulation during the cell cycle.

Here, by exploiting means to distinguish between parental and newly synthesized histones, we characterize, for the first time, the deposition dynamics of canonical H2A and H2A.Z at PHC. In contrast to a constant and uniform incorporation at euchromatin, we report the deposition of new H2A variants at PHC during two distinct periods of major structural rearrangements in heterochromatin, in S and G1 phases. We further show that induced alterations in heterochromatin state affect the extent and timing of incorporation of H2A.Z, but not of H2A, and lead to the enrichment of endogenous H2A.Z at the PHC at steady state. Finally, we reveal that the PHC state also exerts its influence on the neighboring centric domain, by controlling steady-state centromeric levels of CENP-A. Thus, we propose that dynamic changes in PHC during the cell cycle regulate the histone variant composition at the centromere.

RESULTS

Different dynamics of deposition at PHC

To characterize the dynamics of histone variants, we monitored the deposition of newly synthesized histones in vivo by exploiting the SNAP-tag and CLIP-tag technology that distinguishes parental and new histones and has proven to be effective for analyzing H3 variant deposition (Jansen et al., 2007; Ray-Gallet et al., 2011). These tags can be detected through the use of cell-permeable substrates coupled to the TMR-Star fluorophore, which are covalently bound by SNAP or CLIP tags, correspondingly. We generated two Flp-In TIG-3T3 cell lines stably expressing either H2A or H2A.Z tagged with the SNAP epitope. Consistent with previous observations (Bulykno et al., 2006; Dryhurst et al., 2009; Greaves et al., 2007; Rangasamy et al., 2004), both variants showed a similar nuclear distribution and neither accumulated at the PHC at steady-state levels in our model system (supplementary material Fig. S1A,B). To examine newly synthesized histones globally, we carried out a deposition assay following the 'quench-chase-pulse' approach (Fig. 1A). By analyzing the fluorescently pulse-labeled bands corresponding to H2A.Z and H2A after gel electrophoresis (Ray-Gallet et al., 2011), we could conclude that, on a global level, H2A–SNAP and H2A.Z–SNAP exhibit similar synthesis and deposition dynamics (supplementary material Fig. S1C).

Next, we analyzed the nuclear distribution of newly incorporated histones on a cell-by-cell basis using microscopy (Fig. 1; supplementary material Fig. S1D). In agreement with our global analysis (supplementary material Fig. S1C), and consistent with previous biochemical and FRAP (fluorescence recovery after photobleaching) analyses in human cells (Boëisch et al., 2012; Higashi et al., 2007; Jackson and Chalkley, 1985; Kimura et al., 2006), within the detection range of our method both variants showed a comparable extensive deposition of newly
synthesized histones in euchromatin. However, the situation proved to be different at the PHC domains, where the distribution of newly synthesized histones relative to euchromatin could be categorized into one of three distinct patterns – exclusion, even distribution or enrichment (Fig. 1B). Exclusion of TMR-Star signal from PHC corresponds to the lack of detectable incorporation of newly synthesized histones at this domain, in parallel with clearly distinguishable incorporation of H2A variants at euchromatin. Importantly, this pattern suggests a different mode of regulating the deposition of H2A histone variants at euchromatin and heterochromatin. The other two patterns, enrichment and even distribution of the TMR-Star signal at the PHC with respect to euchromatin, both reflect deposition of the H2A variants at the domain, but likely indicate a difference in the rate of deposition during a given time interval.

We then quantified the fraction of cells that exhibited deposition of the newly synthesized histones at PHC domains with a chase time of 2 h for H2A and H2A.Z. Deposition of newly synthesized H2A at PHC occurred only in ∼30% of cells and, within this population, half (∼15% of total) displayed an enrichment of the newly synthesized H2A at PHC over that observed at euchromatin (Fig. 1C). By contrast, deposition of newly synthesized H2A-Z at PHC observed in ∼50% of the cells showed an even pattern, and we could not detect cells with enrichment of this variant at PHC. Thus, we conclude that, in contrast to their indistinguishable deposition dynamics at euchromatin regions, H2A and H2A.Z show different deposition kinetics at PHC, and only new H2A can become significantly enriched at the domain.

Replication-dependent deposition of newly synthesized H2A at PHC

The non-uniform deposition of the newly synthesized H2A variants at PHC in contrast to constitutive deposition in euchromatin suggests that local properties associated with PHC might influence the dynamics of H2A variant deposition. Indeed, characteristic rearrangements in PHC have been described at two specific time windows during the cell cycle; first, during its replication in mid to late S phase, due to replication fork progression (Corpet and Almouzni, 2009; Maison et al., 2010; Quivy et al., 2004), and second, during mitosis. The latter includes modifications in histone marks and a transient but substantial loss of HP1α, both of which are restored in G1 (Fischle et al., 2005; Hirota et al., 2005; Murzina et al., 1999). These particular time windows were thus crucial to consider in order to investigate whether the deposition of newly synthesized H2A variants at PHC could vary in a cell-cycle-dependent manner. We thus coupled the quench-chase-pulse strategy with the labeling of replication sites using the deoxyribonucleotide analog 5-ethyl-2'-deoxyuridine (EdU) and immunodetection of the cell cycle marker Aurora B (Ray-Gallet et al., 2011; Crosio et al., 2002) (Fig. 2A). Using this strategy, we found that cells exhibiting an enrichment of newly synthesized H2A at PHC were in mid to late S phase, which corresponds to the cell cycle window when this domain is replicated (Quivy et al., 2004). By contrast, newly synthesized H2A.Z was mostly excluded from PHC at this point of the cell cycle (supplementary material Fig. S2A). The enrichment of newly synthesized H2A at PHC in mid to late S phase suggests that its deposition is coupled to DNA synthesis. To test this possibility, we inhibited the latter with aphidicolin and observed a loss of accumulation of newly synthesized H2A at PHC (Fig. 2B), even in the cells arrested in mid to late S phase (Fig. 2C). Notably, this treatment had no significant impact on the deposition of H2A at the surrounding euchromatin, underlining the difference between the regulation of H2A deposition at euchromatin and heterochromatin. Furthermore, inhibiting replication had no effect on H2A.Z incorporation at PHC, suggesting that the loss of H2A deposition does not necessarily cause a compensatory switch towards H2A.Z at PHC. We thus conclude that newly synthesized H2A is deposited at PHC in a replication-coupled manner.

Deposition of newly synthesized H2A.Z at PHC occurs during G1

In contrast to the euchromatic pool, H2A.Z deposition at PHC was not constant and was observed in only ∼50% of cells (Fig. 1C). To narrow down the timing of H2A.Z deposition at PHC, we classified asynchronously proliferating cells according to their cell cycle stage (Fig. 2A). The majority of cells able to deposit H2A.Z at PHC were in G1 (Fig. 3A; supplementary material Fig. S2A). This G1 deposition was further emphasized when we labeled newly synthesized histones 2.5 h after the release from a monastrol-induced mitotic block, a time-point that corresponds to early G1 (Fig. 3B; supplementary material Fig. S2B). In this case, deposition of H2A.Z at PHC was visible in the majority of cells (∼80%, Fig. 3B), whereas H2A deposition was minimal. Importantly, the levels of synthesis and incorporation rate for both variants were similar in gel analyses (supplementary material Fig. S2C), which indicates that the difference in deposition between H2A and H2A.Z cannot result from differential protein synthesis after mitotic exit, rather, it is specific for PHC. Of note, 2 h after release from the monastrol block, about 20% of the cells were still in mitosis (supplementary material Fig. S2B), which could account for the proportion of cells that do not show H2A.Z deposition at PHC in the given time window. Thus, we conclude that, in contrast to H2A, newly synthesized H2A.Z is deposited at PHC in early G1. Taking into account heterochromatin rearrangements during the G2–M–G1 transition, it was tempting to hypothesize that these changes involving disruption and restoration could promote H2A.Z deposition at the domain. According to this hypothesis, once its structure is fully re-established, heterochromatin would work as a barrier to limit further H2A.Z deposition, restricting this deposition to G1.

Alterations in heterochromatin state affect the extent and the timing of H2A.Z deposition at PHC

To test whether changes in heterochromatin state could actually regulate the incorporation of H2A.Z at PHC, we monitored the deposition of newly synthesized H2A.Z and H2A in cells with experimentally altered PHC. We used two different models providing two independent means to disrupt PHC state: (i) Suv39h double-null (Suv39h1−/− and Suv39h2−/−) immuno-detect mouse embryonic fibroblasts (dmMEFs) (Peters et al., 2001), in which H3K9me3 is lost and several other chromatin modifications are altered at PHC and (ii) NIH 3T3 fibroblasts in which DNA methylation was inhibited with 5′-aza 2′-deoxycytidine (5-Aza). We applied 5-Aza, a nucleoside analog that cannot be methylated and that inactivates DNA methylases (Creusot et al., 1982; Jones and Taylor, 1980), for 48 h (over at least two cell cycles) to ensure that the existing 5-methylcytosine was diluted by replication, resulting in a drastic reduction in CpG methylation at pericentric major satellite repeats (supplementary material Fig. S2E). The alterations in PHC in both model systems
are summarized in supplementary material Fig. S2D. Importantly, whereas 5-Aza-treated cells retained typical heterochromatic marks, such as an enrichment of H3K9me3 and HP1a, Suv39h dnMEFs lost these marks (supplementary material Fig. S2D) (Peters et al., 2001; Sugimura et al., 2010; Taddei et al., 2001) but did not display a striking decrease in DNA methylation at PHC (supplementary material Fig. S2E). Furthermore, increased transcription of major satellite repeats occurred in both cases (supplementary material Fig. S2F) (Eymery et al., 2009; Lehnertz et al., 2003; Sugimura et al., 2010), with more pronounced changes in 5-Aza-treated relative to untreated cells than in Suv39h dnMEFs relative to wild-type MEFs. It is also important to note that whereas Suv39h dnMEFs represent a system that has adapted to the heterochromatin perturbations during several cell generations, 5-Aza-treated fibroblasts show an acute response to changes in PHC state.

We first examined the deposition dynamics of H2A variants in cells expressing H2A–SNAP or H2A.Z–SNAP that were treated with 5-Aza for 48 h before performing quench-chase-pulse labeling (Fig. 4A). Although the extent of H2A deposition was not affected, we detected that newly synthesized H2A.Z became enriched at PHC with respect to euchromatin in ~30% of cells, a deposition pattern we very rarely detected in control cells (Fig. 1C). Importantly, treatment with 5-Aza had no significant effect on global H2A.Z–SNAP dynamics, as estimated by gel analysis (supplementary material Fig. S2G). Thus, we hypothesize that perturbations in PHC state specifically affect the dynamics of H2A.Z deposition at PHC and induce the over-incorporation of this histone variant.

To strengthen this hypothesis, we confirmed our findings by transiently expressing FLAG-HA-tagged H2A.Z in wild-type or Suv39h double-null cells (eH2A.Z) (Fig. 4B). Importantly, we observed a higher percentage of cells enriched for newly synthesized eH2A.Z at PHC in Suv39h dnMEFs but not in wild-type MEFs, confirming our hypothesis that the changes in PHC state promote extensive deposition of new H2A.Z at the domain.

To gain insight into the events that could lead to extensive H2A.Z incorporation at perturbed PHC, we further examined whether 5-Aza treatment affected the cell cycle regulation of H2A variant deposition. We found that the enrichment of newly synthesized H2A at PHC was still restricted to the time of its replication in the presence of 5-Aza, arguing that the status of the domain did not affect the timing of H2A deposition (Fig. 4C). Importantly, although deposition of newly synthesized H2A.Z was undetectable under mock treatment conditions during S phase (Fig. 2A; Fig. 4C), 5-Aza treatment led to extensive incorporation of newly synthesized H2A.Z at PHC during this
phase (Fig. 4C). To determine whether this unscheduled mode of H2A.Z deposition was replication coupled, we inhibited DNA synthesis with aphidicolin in 5-Aza-treated cells, but this did not prevent the robust enrichment of new H2A.Z at PHC during S phase (Fig. 4E). Moreover, 5-Aza treatment alone also led to enrichment of new H2A.Z during G1 and in G2 (Fig. 4D). We therefore concluded that alterations of PHC state perturb both the extent and the timing of H2A.Z deposition at the domain.

Heterochromatin state regulates the localization of H2A.Z at PHC

Our data suggest that the heterochromatin state acts as a major regulator of both the timing and extent of H2A.Z deposition and that perturbations in PHC result in unscheduled extensive deposition of this variant at the domain. Therefore, we wanted to determine whether the perturbations in new H2A.Z deposition observed in cells with altered PHC state were replication coupled, as this did not prevent the robust enrichment of new H2A.Z at PHC during S phase (Fig. 4E). Moreover, 5-Aza treatment alone also led to enrichment of new H2A.Z during G1 and in G2 (Fig. 4D). We therefore concluded that alterations of PHC state perturb both the extent and the timing of H2A.Z deposition at the domain.

Restoring heterochromatin structure re-establishes H2A.Z localization

To gain insight into the extent to which changes in heterochromatin state can reversibly affect H2A.Z localization, we focused on the possibility that differences in antigen recognition might have prevented us from assessing the correct distribution of endogenous histone variants. We transiently transfected wild-type MEFs, Suv39h-dnMEFs, and 3T3 fibroblasts with FLAG-HA-tagged H2A (eH2A) and H2A.Z (eH2A.Z) variants. In agreement with data obtained for endogenous histones, staining with anti-HA showed that eH2A.Z, but not eH2A, accumulated at PHC in 5-Aza-treated cells and Suv39h-dnMEFs (supplementary material Fig. S3B,C). Furthermore, total H2A.Z–SNAP, but not H2A–SNAP, also accumulated at PHC at steady state after 5-Aza treatment (supplementary material Fig. S3D). It is important to note that the enrichment of new H2A at PHC during DNA replication does not translate into accumulation of total H2A at PHC. Such enrichment is likely to reflect the extensive H2A deposition to replenish H2A–H2B dimers that are diluted during DNA replication, in order to maintain nucleosomal density. By contrast, extensive and unscheduled deposition of newly synthesized H2A.Z at the domain due to perturbations in heterochromatin state results in accumulation of this variant at PHC at steady state. Therefore, we concluded that the 'heterochromatin state' of PHC contributes to the localization of H2A.Z at the domain.
we tested whether the re-establishment of a normal PHC state would also restore H2A.Z levels at the domain. In agreement with previous studies, 5-Aza treatment affected long-term cell survival (Jüttermann et al., 1994), and therefore we focused on Suv39h dnMEFs. To restore PHC status, we transfected Suv39h dnMEFs with Myc–SUV39H1, whose binding at PHC results in the local reaccumulation of H3K9me3, DNA methylation and HP1α (Lehnertz et al., 2003; Loyola et al., 2009) (Fig. 6A). Following Myc–SUV39H1 expression, loss of H2A.Z accumulation at PHC occurred in the majority of transfected cells by 6 h after transfection, and coincided with the reaccumulation of H3K9me3 (Fig. 6B,C). Importantly, the relatively low level of H2A.Z at PHC was stably maintained after 24 h, that is, after at least one cell cycle (Fig. 6B,C), and there were no changes in the total H2A.Z protein levels (Fig. 6D). Therefore, we conclude that the heterochromatin status of the locus dictates H2A.Z maintenance. Intriguingly, the loss of H2A.Z accumulation in the majority of the cells expressing Myc–SUV39H1 within 6 h also suggests that an active histone

Fig. 4. See next page for legend.
exchange enabled the removal of the accumulated H2A.Z. Taken together, our data suggest that the heterochromatin state might act as a major regulator of both the timing and extent of H2A.Z deposition and, consequently, the maintenance of H2A.Z localization at the domain.

**Heterochromatin state regulates the levels of CENP-A at centromeres**

Given that the deposition of CENP-A at centromeres is similarly restricted to G1 (Jansen et al., 2007), the time at which we observed H2A.Z deposition at PHC, we wanted to determine whether PHC state could also impact on CENP-A deposition. To assay this, we generated a NIH 3T3 cell line stably expressing CENP-A fused to a CLIP tag to be able to monitor newly synthesized histone deposition in vivo (supplementary material Fig. S4A,B). In agreement with previous observations in human cells (Hemmerich et al., 2008; Jansen et al., 2007; Kim et al., 2012), we found that in an asynchronous mouse cell population, newly synthesized CENP-A is deposited at centromeres only in late telophase and/or G1 (Fig. 7A; supplementary material Fig. S4C). Intriguingly, after treatment with 5-Aza, incorporation of newly synthesized CENP-A to centromeres remained restricted to G1 cells (Fig. 7A). Thus, heterochromatin state had no detectable impact on the timing of deposition of CENP-A. However, we noted that the 5-Aza treatment resulted in an increase in the amount of new CENP-A–CLIP at centromeres (Fig. 7A), suggesting that heterochromatin state might regulate the amount of CENP-A deposited in G1.

This observation led us to assay the endogenous CENP-A protein levels and localization at steady state in cells with perturbed PHC. We found that CENP-A levels increased in both 5-Aza-treated fibroblasts and Suv39h dnMEFs with respect to their relative controls (Fig. 7B–D). Importantly, automated quantification of fluorescence intensity of CENP-A staining based on centromere segmentation demonstrated a threefold increase in centromere-associated CENP-A in 5-Aza-treated cells expressing the dominant-negative form of Suv39h. 5-Aza treatment led to a decrease in the levels of CENP-A in both wild-type and Suv39h dnMEFs (Fig. 7B–D).

**Fig. 4. Perturbations in heterochromatin state affect the dynamics of H2A.Z deposition at PHC.** (A,B) Perturbations in heterochromatin state affect the extent of the deposition of newly synthesized H2A.Z at PHC. (A) Upper panel, experimental scheme. Cells expressing H2A–SNAP and H2A.Z–SNAP were treated with 5-Aza for 48 h before and during quench-chase-pulse labeling. Q, quench; P, pulse. Lower panel, quantification of the percentage of cells exhibiting enrichment of newly synthesized H2A variants at PHC (as defined in Fig. 1B). Data show the mean ± s.e.m. (at least three independent experiments; >300 cells per condition). (B) Upper panel, experimental scheme. Wild-type (WT) and Suv39h double-null (dn) MEFs were transfected with H2A.Z–FLAG–HA (eH2A.Z). Cells were treated with Trion X-100 before fixation to visualize only incorporated histones at 6 or 24 h after transfection. Lower panel, quantification of the percentage of cells exhibiting enrichment of eH2A.Z at PHC. Enrichment of tagged eH2A.Z was quantified by counting the transfected (HA-positive) cells and analyzing the colocalization of HA signal with PHC (DAPI-dense regions). Data show the mean ± s.e.m. (three independent experiments). (C–E) Perturbations in heterochromatin state affect the timing of H2A.Z deposition. Cells expressing H2A–SNAP and H2A.Z–SNAP were treated with 0.5 μM 5-Aza for 48 h before and during quench-chase-pulse labeling. Replication sites were labeled with EdU at the time of pulse with SNAP–TMR-Star. Cells were also immunostained for Aurora B to resolve their cell cycle stage. (C) Upper panel, experimental scheme. Lower panel, representative images (single planes) for the H2A–SNAP and H2A.Z–SNAP deposition patterns in mid to late S are shown. (D) Upper panel, experimental scheme. Cells expressing H2A.Z–SNAP were treated with 5-Aza for 48 h (B) were stained with antibodies against H2A or H2A.Z. Representative patterns (single planes) for the H2A.Z–SNAP deposition patterns in mid to late S are shown. Insets represent enlarged images of the selected area. Scale bars: 10 μm.

**Fig. 5. Accumulation of endogenous H2A.Z at pericentric chromatin in cells with perturbed PHC.** Wild-type (WT) versus Suv39h double-null (dn) MEFs (A) and control versus Flp-In™-3T3 cells (3T3) treated with 0.5 μM 5-Aza for 48 h (B) were stained with antibodies against H2A or H2A.Z. Representative patterns (single planes) are shown. Note that, in spite of some decondensation, PHC is still clustered in chromocenters (DAPI-dense domains) after treatment with 5-Aza. Scale bars: 10 μm. (C) Quantification of the percentage of cells with accumulation of H2A variants at PHC. Data show the mean ± s.e.m. (at least three independent experiments; >300 cells in each experiment for each condition). (D) Western blot analysis of total proteins of the cells analyzed in A and B. H2A.Z and H2A were detected with their corresponding antibodies. γ-tubulin and histone H4 were used as loading controls.
fibroblasts relative to untreated cells and a 2.5-fold increase in Suv39h dnMEFs relative to wild-type MEFs (Fig. 7C), arguing that changes in the heterochromatin state of PHC modulate the amount of CENP-A at the locus. We also confirmed the increase in endogenous CENP-A in centric chromatin after 5-Aza treatment by ChIP-qPCR (supplementary material Fig. S3A). Additionally, we detected increased levels of total CENP-A–CLIP at centromeres after 48 h of 5-Aza treatment (supplementary material Fig. S4D). Therefore, we concluded that alterations in PHC state result in an elevation of CENP-A levels at centromeres by deregulating the extent of CENP-A deposition without affecting its timing. These observations highlight the importance of the crosstalk between centric and pericentric domains of the centromere in regulating the propagation of CENP-A.

**DISCUSSION**

**Dynamic changes in PHC state during the cell cycle regulate histone variant composition at centromeres**

We propose that cell cycle changes in PHC are crucial in regulating centromeric chromatin organization. As summarized in Fig. 8, deposition of histone variants at centromeres is restricted to particular time windows during the cell cycle and coincides with transient rearrangements of PHC. H2A.Z and CENP-A are deposited during G1, whereas canonical H2A is incorporated during S phase (Figs 2, 3, 7) and can be compared to the deposition of histones H3.1 and H3.3 (Dunleavy et al., 2011). Notably, whereas at euchromatin both H2A and H2A.Z are deposited continuously, here, we found that a unique cell cycle regulation of the deposition of the H2A.Z variant occurs at PHC loci. This does not exclude possible regulation at subdomains in euchromatin, but this would be beyond our detection sensitivity. Of note, previous studies in trophoblast stem cells did not reveal changes in H2A.Z steady-state levels at PHC during the cell cycle (Nekrasov et al., 2012). Although this could possibly reflect a difference between cell types, we should also stress that steady-state levels do not necessarily reflect deposition rate. Indeed, the measured steady-state levels represent the changes due to both eviction and deposition, whereas our SNAP quench-chase-pulse technique allows us to specifically reveal the deposition aspect. Thus, we suggest that the deposition of new H2A.Z in early G1, if accompanied by the eviction of pre-existing histones, would give rise to a net stable steady state as found previously (Nekrasov et al.; 2012). In support of this hypothesis, a previous study in human cells showed that histone H3.3 is actively evicted from the centromere during early G1 (Dunleavy et al., 2011). We thus propose that it would be interesting to address not only the dynamics of deposition but also of the eviction of H2A.Z during G1.

Remarkably, PHC loci undergo very distinct changes during S phase and the M–G1 transition. During S phase, PHC destabilization is limited to the periphery of the domain where
replication takes place and is fully restored immediately after fork progression (Maison et al., 2010; Quivy et al., 2008). During this transient destabilization of the structure, a DNA-synthesis-dependent deposition of H2A would ensure that the full complement of H2A–H2B histones is present after replication, but H2A.Z would remain excluded from incorporation at the domain. Thus, neither the local destabilization itself, nor the availability of H2A.Z (which is actively deposited at euchromatin throughout the cell cycle) is sufficient to promote H2A.Z deposition at PHC in S phase. By contrast, during mitosis, the reorganization of PHC permits deposition of H2A.Z but not H2A. During this time window, a global rearrangement of the higher-order chromatin structure occurs, as chromocenters dissociate into individual PHC domains corresponding to each chromosome. HP1 proteins are substantially lost and histone marks are highly dynamic, exhibiting transient H3S10 phosphorylation that masks

Fig. 7. The PHC state regulates CENP-A levels at centromeres. (A) Treatment with 5-Aza does not impact on the timing of CENP-A deposition at centromeres. Upper panel, experimental scheme. Cells expressing CENP-A–CLIP were treated with 0.5 μM 5-Aza for 48 h before and during quench-chase-pulse labeling. Replication sites were labeled with EdU prior to pulsing with CLIP–TMR-Star. Cells were also stained against Aurora B to resolve their cell cycle stage. Q, quench; P, pulse; IF, immunofluorescence. Lower panel, Representative images (maximum intensity projections) for the CENP-A–CLIP deposition patterns in G1, late S and G2 phases are shown. See supplementary material Fig. S4A–C for cell-line characterization. (B–D) Accumulation of endogenous CENP-A at centromeres in cells with perturbed PHC. (B) Wild-type (WT) and Suv39h double-null (dn) MEFs, control and 5-Aza-treated Flp-InTM-3T3 cells were stained with antibodies against CENP-A. Centric domains were detected by DNA FISH using locked nucleic acid (LNA) probes for minor satellites. Representative patterns (maximum intensity projections) are shown. Insets represent enlarged images of the selected area. Scale bars: 10 μm. (C) The relative abundance of centromere-associated CENP-A was estimated by quantification of fluorescence intensity of CENP-A antibody signal. DNA FISH signal was used to define centromeres. Fold changes in fluorescence intensity values relative to those of the corresponding control are shown. Data show the mean ± s.e.m. (three different experiments; an average of 55 cells per condition). (D) Western blot analysis of total proteins of the cells analyzed in B. CENP-A was detected with the corresponding antibody. Histone H4 was used as a loading control.
such changes in PHC state do not affect the replication-dependent deposition of canonical H2A in mid to late S phase (Fig. 4). Thus, we propose that the dynamic rearrangements of the organization of PHC that occur naturally during the cell cycle define a window of opportunity for H2A variant deposition at the domain. Therefore, the deposition of a specific H2A variant at this site is dictated by the interplay between two main regulators – cell cycle timing and heterochromatin organization.

**Centromeric chromatin dynamics illustrate the interplay between PHC organization and histone variant composition**

We showed that perturbations in PHC status led to an enrichment of H2A.Z at this domain and to an increase in the amount of CENP-A incorporated next to it at centromeres (Figs 5, 7). Previous studies have proposed that H2A.Z functions as a structural component in the heterochromatin context, where the more-stable hypoacetylated homotypic (H2A.Z–H2A.Z) nucleosomes might influence the folding of the chromatin fiber and facilitate the formation of secondary structures with increased compaction (Fan et al., 2002; Fan et al., 2004; Ishibashi et al., 2009; Suto et al., 2000). This is supported by a series of observations in yeast, where H2A.Z mostly functions as a negative regulator of transcription in the case of heterochromatic regions (Buchanan et al., 2009; Dhillon and Kamakaka, 2000; Hou et al., 2010). We therefore speculate that the enrichment of H2A.Z could function as a compensatory mechanism to maintain heterochromatin structure in the absence of canonical repressive heterochromatin marks.

Intriguingly, we found that the extent of CENP-A deposition is affected by alterations in PHC at established centromeres (thus at its natural endogenous template). Such crosstalk between centric and pericentric domains has previously been demonstrated in *Schizosaccharomyces pombe* on a naïve DNA template (Folco et al., 2008; Kagansky et al., 2009) and in *Drosophila* at artificial heterochromatin boundaries (Olszak et al., 2011). Our data now provide evidence of this crosstalk in mammals and highlight the parallel dynamics of PHC and CENP-A during the cell cycle (Fig. 8). This partial loss of PHC organization during mitosis could potentially provide a window of opportunity to enhance any of the multiple steps of CENP-A deposition, including targeting of the CENP-A chaperone HJURP or stable retention of the deposited CENP-A (Boyarchuk et al., 2011). Alternatively, the increased accessibility of the template, transiently deprived of its repressive heterochromatin marks, could simply facilitate histone turnover, which, in turn, could favor the eviction and replacement with CENP-A of other H3 variants that function as placeholders following the dilution of CENP-A during S phase. Finally, once CENP-A deposition has proceeded in early G1, restoration of PHC organization would act as a barrier to the process and prevent promiscuous spreading and/or overloading of CENP-A.

Importantly, the interplay between PHC state and cell cycle to regulate histone variant composition at the centromere, for which we now provide evidence in mammals, provides means for both maintenance and plasticity of the domain organization. The heterochromatin remodeling that occurs during early development (Probst et al., 2007; White and Dalton, 2005), differentiation (Govin et al., 2007; Solovei et al., 2009; Terranova et al., 2005) or tumorigenesis (reviewed in Carone and Lawrence, 2013) might provide a context for differential incorporation of histone variants. These potential changes in variant distribution could, in turn, further affect higher-order chromatin organization of the centromere, which would then influence mitotic progression and genome stability.

**Fig. 8. Model outlining the timing of the deposition of H2A and CENP-A variants and the cell cycle dynamics of PHC organization.** During mitosis, global histone acetylation, the phosphorylation of H3S10 by Aurora B (which leads to a partial loss of HP1 at PHC) and the formation of the kinetochore result in structural rearrangements at the pericentric domain. These changes provide a window of opportunity for the deposition of H2A.Z and CENP-A, but not H2A, at the domain during G1. The restoration of the PHC structure during G1 fine-tunes the levels of H2A.Z and CENP-A at the domain. Once fully restored, the organization of the domain inhibits further deposition of H2A.Z at the locus, even during transient disruption during replication. By contrast, the temporary disruption of the chromatin structure due to replication fork passage allows deposition of canonical H2A in a DNA-synthesis dependent manner. In turn, at the centric domain, replication results in CENP-A dilution (Hemmerich et al., 2008; Jansen et al., 2007) and incorporation of replicative H3 and H3.3 (Dunleavy et al., 2011).
MATERIALS AND METHODS

Cell lines and plasmids
We generated cell lines stably expressing H2A–SNAP and H2A.Z–SNAP in mouse Flp-In™-3T3 cells, according to the manufacturer’s protocols (Invitrogen). We generated cell lines stably expressing CLIP–CENP-A in mouse NIH 3T3 cells (ATCC) by transfecting cells with the CLIP–CENP-A plasmid, selecting for its stable integration with 500 µg/ml G418 (Gibco) and isolating single-cell clones. We cultured NIH 3T3, wild-type and Suv39h dnMEFs (provided by Thomas Jenuwein) in DMEM (Gibco) containing 10% (v/v) FCS at 37 °C under 5% CO2. We transfected MEFS with Nucleofector Kit MEF-2 (Amaxa), according to the manufacturer’s instructions.

Plasmids encoding H2A–FLAG-HA and H2A.Z–FLAG-HA were obtained from Hideaki Tagami, and Myc–SVU39H1 from Thomas Jenuwein. H2A (accession number NM_003514.2) and H2A.Z (H2A.Z-1, H2AFZ, accession number NM_002106.3) were amplified from cDNA by PCR without their stop codons, and the coding sequences were cloned into pSNAP-tag vector (New England Biolabs) between the ClaI and EcoRI sites. The resulting H2A-SNAP and H2A.Z-SNAP were subcloned into pcDNA5/FRT plasmid (Invitrogen) between the HindII and KpnI sites and used for the generation of cells stably expressing fusion proteins. CENP-A (accession number NM_001809.3) was amplified from a cDNA clone and inserted between the BamHI and XhoI sites of pCLIPm一大批 (New England Biolabs) to generate a CLIP fusion at the N-terminus of CENP-A.

Antibodies and drugs
We used antibodies against: HP1α (2HP-1H5-AS) from Euromedex; HA (1867423) from Roche; Aurora B (611082) from BD Transduction Laboratories; SNAP (CAB4255) from Open Biosystems; cyclin A (sc-751) from Santa Cruz; Rb (sc-333) from Abcam; pRb (sc-814) from Cell Signaling; H3K4me2 (04-713) and H3K4me4 (04-714) from ActiveMotif; and H3K27me3 (07-449), H4 (05-858) and phospho-H2A.X S139 (05-636) from ActiveMotif; and HA (ab196024), H2A.Z (ab63656), Myc (ab32) and H4 (ab31830) from Abcam; H2A.Z (ab9062, lot NG 1590601 for the blot shown in supplementary material Fig. S1), lamin A/C (2032), Suv39h1 (07-550), H3K27me3 (07-449), H4 (05-858) and phospho-H2A.X S139 (05-636) from Millipore; H3K9me3 (39285) and H2A.Z (39113) from ActiveMotif; and γ-tubulin (T5326) and β-actin (A5451) from Sigma. Antibodies against H3K4me2 and H3K4me4 (Kimura et al., 2008) were a kind gift from Hiroshi Kimura, and anti-p150CAF-1 was created in-house (Quivy et al., 2004). We purchased monastrol (M8515, used at 100 µM), aphidicolin (A0781, used at 4 µg/ml) and 5-aza-2′-deoxycytidine (A3656, used at 0.5 µM) from Sigma.

Analysis of DNA methylation
Genomic DNA was extracted with the QIAGEN DNeasy Blood & Tissue kit and digested with HpaII and HpyCH4IV (MacII) (New England Biolabs), CpG-methylation-sensitive restriction enzymes, as reported previously (Lehnertz et al., 2003). For Southern analysis, we used Hpa II-digestion of genomic DNA to generate a CLIP fusion at the N-terminus of CENP-A.

ChIP-qPCR analysis
We performed ChIP as described previously (Mozzetta et al., 2014), using rabbit polyclonal antibodies against CENP-A (ab33565, Abcam), H2A.Z (ab1174, Abcam), H2A (ab18255, Abcam), H3K9me3 (pAb-056-050, Diagenode), and rabbit IgG (sc-2027, Santa Cruz). DNA was diluted 1/600 and was amplified using PowerSYBR Green PCR Master Mix (Applied Biosystems) and analyzed on the Viia 7 Real Time PCR System (Applied Biosystems). The following PCR primers were purchased from Sigma Genosys: minor satellite forward primer, 5′-GAA-CATATTAGTAGTGAGGTAC-3′; reverse primer, 5′-GGTCTACAATCCCGTTTCAC-3′; major satellite forward primer, 5′-ACGG-GAAATAGGGCGGAA-3′; reverse primer 5′-CAAGTCGTCAAGTGGATGGT-3′.

Quantitative RT-PCR analysis of major satellite mRNA levels
Total RNA was extracted using an RNeasy kit (Qiagen) and treated with TURBO DNA-free kit (am1907, Invitrogen) to remove contaminating DNA. First-strand cDNA synthesis was performed with 4 µg of RNA and random primers using Superscript III (Invitrogen), according to the manufacturer’s recommendations. Following the reverse transcription reactions, 1 µl of cDNA diluted 1:50 was amplified with Power SYBRGreen master mix (Applied Biosystems) and the appropriate primers. Cycling was performed on a 7900HT device (Applied Biosystems) in triplicate for each cDNA sample. Three independent experiments were performed. The following primers for RT-PCR were designed using Primer Express software (Applied Biosystems) and were purchased from Sigma Genosys: GAPDH forward primer, 5′-TGCCCACCACACTGGCTTACG-3′; reverse primer, 5′-GGCATGACTTGGTGCATGAG-3′; major satellite forward primer, 5′-GACGACTTGAAAATGACGAAATC-3′; reverse primer, 5′-CATATTCCAGGTTCTTCAGTGGTCG-3′.

FACS analysis, gel electrophoresis, and western blotting
We performed cell cycle analysis, gel electrophoresis, western blotting and gel-based quantification of TMR signal as described previously (Cook et al., 2011; Ray-Gallet et al., 2011). For the latter, we normalized for protein loading using Imperial Protein Stain (Thermo Scientific).

SNAP labeling in vivo
The SNAP and CLIP labeling protocol is based on that described previously (Ray-Gallet et al., 2011), with modifications in compound concentration and treatment time. For SNAP labeling, we treated cells with 8 µM SNAP-Cell-Block (New England Biolabs) for 30 min at 37 °C to quench pre-existing SNAP-tagged histones. Thereafter, quenched cells were washed twice with PBS for 3 min and once with complete medium for 10 min, re-incubated in complete medium for 30 min to allow excess compound to diffuse from cells and incubated for the indicated chase time in complete medium at 37 °C. We used 3 µM SNAP-Cell TMR-Star for 30 min at 37 °C to pulse label. We carried out CLIP experiments similarly, with the following difference: fluorescent detection of the tag was accomplished after permeabilization with Triton X-100 by incubating in the presence of 3 µM CLIP-Cell TMR-Star, 0.5% bovine serum albumin (BSA) and 1 mM dithiothreitol (DTT) in CSK buffer for 5 min at 37 °C, followed by a rinse and fixation.

Immunofluorescence, DNA FISH, and visualization of replication sites
We performed pre-extraction of cells prior to fixation for 5 min with 0.5% Triton X-100 in CSK buffer as described previously (Martini et al., 1998) and then fixed cells in 2% paraformaldehyde for 20 min. We blocked cells with BSA (3% in PBS plus 0.1% Tween-20) before incubation with primary and secondary antibodies and DAPI staining. We used Alexa-Fluor-conjugated secondary antibodies from Invitrogen. Coverslips were mounted with ProLong Gold anti-fade reagent (Invitrogen).

For fluorescent in situ hybridization (FISH), we post-fixed cells following immunofluorescent staining, equilibrated them in 2× SSC and then fixed cells in 50%-formamide-containing DNA hybridization buffer for 5 min at 75 °C. After incubation for 1 h at 37 °C, cells were washed three times in 0.1× SSC at 60 °C for 5 min each, counterstained with Hoechst 33342 and mounted in ProLong Gold antifade reagent. The Cy3-labeled minor satellite LNA30° probe (5′-GGTCTACAAATGCCTTGTTTC-3′) (Casanova et al., 2013) was obtained from EXIQON. We labeled replication sites in vivo as described previously (Ray-Gallet et al., 2011), by using a 30 min 1 µM EdU pulse concomitantly with the TMR-Star pulse (or prior to, in the case of CLIP). We visualized EdU with the Click-iT_Edu Alexa Fluor 488 imaging kit (Invitrogen).

Microscopy analysis and image processing
We acquired the images shown in Figs 1, 2, 3, supplementary material Figs S1D and S3D using the Deltavision set-up with an inverted Olympus
IX71 microscope equipped with a Photometrics HQ2 camera (Applied Precision; 60× and 100× objectives with a NA of 1.4). The devices were controlled by softWoRx1 5.0 (Applied Precision). The x, y and z motorized stage allowed three-dimensional acquisitions (50 z-slices of 0.2-mm width). We deconvolved z-stack images by the conservative method (15 cycles) using softWoRx1 5.0. The middle plane is shown. The images shown in Figs 5, 6, supplementary material Figs S1A, S3B,C and S4A,C were obtained using an epifluorescence microscope (Axio Imager Z1; Carl Zeiss) piloted with MetaMorph software (Molecular Devices), a 63× PA/1.4 NA objective lens and a chilled charged-coupled device camera (CoolSnap HQ2; Photometrics). The images shown in Figs 4, 7 and supplementary material Fig. S4D were acquired using a Zeiss LSM 780 confocal microscope with a 63× immersion objective (NA 1.4), controlled by ZEN software (Carl Zeiss). We acquired 16 0.39-μm z-slices at optimum voxel size. We used ImageJ software for further image processing. All scale bars correspond to 10 μm. When quantifying the deposition of H2A variants at PHC, we defined cells exhibiting at least three chromatcenters with enrichment of the newly synthesized variant as ‘enriched’. For the cells that did not display an enriched pattern, we defined ‘even deposition’ to be a pattern whereby the majority of the chromatcenters exhibited levels of deposition that were indistinguishable from those of euchromatin. The remainder of cells were defined as ‘excluded’. To quantify the CENP-A fluorescence intensity at chromatcenters labeled by DNA FISH, we used the 3D-FIED macro (Cantaloube et al., 2012).

Acknowledgements
SUPPLEMENTAL INFORMATION FOR

The distinct cell cycle control of centromere organization entails crosstalk between pericentric heterochromatin state and histone variant composition

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Characterization of H2A- and H2A.Z-SNAP Flp-In™-3T3 cell lines.
(A) Visualization by fluorescence microscopy of steady-state levels of H2A- and H2A.Z-SNAP with TMR-Star, and total H2A and H2A.Z detected with corresponding antibodies in cell lines stably expressing H2A- or H2A.Z-SNAP. Single planes are shown. Note the similar homogenous distribution (except for the low staining at the nucleoli) for TMR signal or antibody staining in all cases. (B) Western blot analysis of total extracts (Triton-) and the chromatin-bound (or detergent-resistant, Triton+) fraction from H2A-SNAP, H2A.Z-SNAP, and a parental control cell line (P). Endogenous H2A variants were detected with H2A and H2A.Z antibodies, epitope-tagged histones were detected with an anti-SNAP antibody. Note that the H2A.Z antibody recognizes both endogenous and the SNAP-tagged version of the protein, indicated by an asterisk. The levels of lamins A/C are used as a loading control. Levels of Cyclin A are used as a control for the efficiency of Triton X-100 extraction. The estimated total level of H2A.Z (endogenous and H2A.Z-SNAP) is about 1.5 times higher than that of H2A.Z in the parental cell line. We were unable to detect the tagged version of canonical H2A using antibodies against H2A and therefore to estimate the levels of the canonical H2A overexpression. However, taking into account that H2A is expressed in much higher levels than H2A.Z (Wu and Bonner, 1981), and that the levels of H2A-SNAP and H2A.Z-SNAP are equal, we suggest that the total H2A level is even less affected by the presence of the tagged version of this histone variant. (C) Gel analysis of total H2A-SNAP and H2A.Z-SNAP after pulse and quench-chase-pulse experiments (left), and total or incorporated (detergent-resistant) H2A-SNAP and H2A.Z-SNAP after quench-chase-pulse experiments as in Fig. 1 A-C (right). Extracts from equal numbers of labeled cells were used for NuPAGE gel electrophoresis. The fluorescent bands corresponding to the labeled H2A- and H2A.Z-SNAP were visualized, quantified, and the values were normalized to Coomassie staining. To access the rate of synthesis of SNAP-tagged histone variants in our experimental setup, we quantified the ratio between newly synthesized and the entire pool of SNAP-tagged histones (18% and 23% for H2A.Z- and H2A-SNAP correspondingly). The ratio of newly synthesized tagged histone variants (New
H2A.Z / New H2A) expressed within 2.5 h is close to 1 (0.81), reflecting comparable amounts of the two variants. The ratio of chromatin-incorporated (detergent-resistant) and total populations of newly synthesized histones evaluates their efficiency of incorporation. This value is similar for both variants (71% for H2A.Z- and 75% for H2A-SNAP). The ratio of newly incorporated histones H2A.Z/H2A is close to 1 (0.76) reflecting a similar incorporation level. Of note, the almost equal incorporation of new H2A.Z and H2A is in contrast to the case of newly incorporated histones H3.1 and H3.3, whose ratio of 0.53 is reflecting the more limited incorporation of H3.1, restricted to replicating cells, whereas deposition of H3.3 occurs throughout the cell cycle (Ray-Gallet et al., 2011). The summarizing table contains ratios that correspond to the average value derived from three independent experiments ± s.d. (D)

Visualization by fluorescence microscopy of incorporated H2A- and H2A.Z-SNAP with TMR-Star in “pulse,” “quench-pulse,” and “quench-chase-pulse” experiments. The “pulse” labels pre-existing H2A- and H2A.Z-SNAP, “quench-pulse” quenches pre-existing H2A-SNAP variants with non-fluorescent SNAP-Cell-Block and prevents their subsequent labeling with SNAP-Cell-TMR-Star. “Quench-chase-pulse” labels newly synthesized and incorporated H2A- and H2A.Z-SNAP during the 2-hour chase. In all cases cells were pre-extracted with Triton X-100 before fixation to remove soluble histones. Scale bar, 10 µm.

Figure S2.

(A) Quantification of the deposition patterns of H2A variants at PHC during cell cycle. Cell cycle phases were identified as described in Fig. 2A. More than 200 cells of each cell line were quantified. Note that S-phase includes all EdU-positive cells without partition into early, mid-late and late S.

(B-C) Characterization of global deposition dynamics of newly synthesized H2A- and H2A.Z-SNAP in G1 cells. (B) Cell-cycle profiles and their quantification in asynchronous H2A.Z-SNAP cells, those arrested by monastrol treatment, or released from the mitotic block for 2, 6, and 24 hours. (C) Top left: Experimental scheme (as in Fig. 3B). Bottom: Gel analysis of total or incorporated (detergent-resistant) H2A-SNAP and H2A.Z-SNAP 2.5 hours after release from mitotic arrest, labeled by quench-chase-pulse method. Extracts from equal numbers of labeled cells were used for NuPAGE gel electrophoresis. The fluorescent bands corresponding to the labeled H2A- and H2A.Z-SNAP were visualized, quantified, and the values were normalized.
to Coomassie staining. The summarizing table contains ratios that correspond to the average value derived from three independent experiments ± s.d.

(D-F) Characterization of the model systems used in the study. (D) Changes in chromatin marks in cells with perturbed pericentric chromatin. We consolidated and complemented characterizations of Suv39h dn MEFs (Lehnertz et al., 2003; Maison et al., 2002; Peters et al., 2001) and 5-Aza-treated mouse embryonic fibroblasts (Sugimura et al., 2010; Taddei et al., 2001; Takebayashi et al., 2001) published previously. A + signifies enrichment at PHC, while a — indicates lack thereof. (E) Analysis of DNA methylation at centromeres by digestion with methylation-sensitive restriction enzymes with one recognition site per repeat, followed by detection by Southern blot. At minor satellites, consisting of 120 bp repeats, the high degree of DNA methylation is only slightly affected by either absence of Suv39h or 5-Aza treatment. Major satellites, consisting of 234 bp repeats, are hypomethylated in MEFs compared to 3T3 control cells, consistent with alterations in DNA methylation upon long-term culture (Razin and Cedar, 1991). In 3T3, 5-Aza treatment induces massive loss of DNA methylation at the domain. (F) RT-PCR quantification of major satellite transcripts. Mean fold changes ± s.d. of transcript levels relative to GAPDH are shown. (G) Characterization of global deposition dynamics of newly synthesized H2A- and H2A.Z-SNAP in 5-Aza treated cells. Top: Experimental scheme. Cells expressing H2A- or H2A.Z-SNAP were treated with 0.5 µM 5-Aza for 48 hours before and during quench-chase-pulse labeling. Bottom: Gel analysis of total or incorporated (detergent-resistant) H2A-SNAP and H2A.Z-SNAP in control and 5-Aza treated cells after quench-chase-pulse experiments. The summarizing table contains ratios that correspond to the average value derived from three independent experiments ± s.d.

Figure S3. Localization of H2A variants in cells with perturbed PHC.

(A) ChIP-qPCR analysis of endogenous H2A.Z, H2A, CENP-A, and H3K9me3 occupancy at major and minor satellites in control and 5-Aza treated NIH 3T3 cells. The immunoprecipitated material was quantified by qPCR, and results are expressed as a percentage of input DNA immunoprecipitated (% input). IgG was used as negative control. Data are represented as mean ± SEM, n≥3.
(B) Top: Experimental scheme. WT and Suv39h double-null MEFs were transfected with plasmids encoding either H2A-Flag-HA or H2A.Z-Flag-HA (eH2A and eH2A.Z correspondingly). Cells were treated with Triton X-100 before fixation to visualize only incorporated histones 48 hours after transfection. Bottom: Representative images (single planes) for both cell lines. eH2A and eH2A.Z were visualized with anti-HA antibodies. (C) Top: Experimental scheme. Flp-In™-3T3 cells were transfected with plasmids encoding either eH2A or eH2A.Z. 5-Aza (or PBS) was added 4 h after transfection. Cells were treated with Triton X-100 before fixation to visualize only incorporated histones after 48 hours of 5-Aza treatment. Bottom: Representative images (single planes) for control and 5-Aza treated cells. eH2A and eH2A.Z were visualized with anti-HA antibodies. Scale bar, 10 µm. (D) Left: Experimental scheme. Flp-In™-3T3 cells, stably expressing H2A- or H2A.Z-SNAP, were treated with 0.5 µM 5-Aza for 48 hours. SNAP-tagged histones were labeled with TMR-Star at steady state, pre-extracted with Triton X-100, fixed, and co-stained with DAPI. Right: The representative images (single planes) for both cell lines with or without treatment with 5-Aza are shown. Scale bar, 10 µm. Note accumulation of exogenous H2A.Z but not H2A in all cases.

Figure S4. Characterization of the CENP-A-CLIP NIH3T3 cell line.

(A) Visualization by fluorescence microscopy of incorporated CLIP-CENP-A with TMR-Star in “pulse” and “quench-pulse,” experiments. Co-staining with anti-CENP-B antibodies was used to identify centromeres. Maximum intensity projections are shown. Scale bar, 10 µm.

(B) Western blot analysis of total cell extracts from CLIP-CENP-A and the parental cell line. Endogenous and CLIP-tagged CENP-A were detected with anti-CENP-A antibodies. The levels of β-actin are used as a loading control. The estimated total level of CENP-A (endogenous and CLIP-CENP-A) is about 4 times higher than that of CENP-A in the parental cell line.

(C) Analysis of the deposition of newly synthesized CLIP-CENP-A during the cell cycle. Top: Experimental scheme. Bottom: Patterns of incorporation of CLIP-CENP-A after in vivo labeling by the quench-chase-pulse assay combined with co-staining with Aurora B and CAF p150 to resolve the cell cycle stage of individual cells. Cells are scored as telophase and early G1 (mid-body staining for Aurora B), G1 (negative for CAF p150 and Aurora B), S-phase (CAF p150-positive) or G2 (CAF p150-
negative, Aurora B-positive), mitosis stages were discriminated based on DAPI staining. S-phase designations are based on the CAF p150 distribution (Quivy et al., 2004). Maximum intensity projections are shown.

(D) Top: Experimental scheme. Cells expressing CLIP-CENP-A were treated with 0.5 µM 5-Aza for 48 hours, then labeled with TMR-Star at steady state, and co-stained with antibodies against CENP-A. Bottom: Representative images (maximum intensity projections) are shown.
SUPPLEMENTAL REFERENCES


**Steady-state**

A

H2A-SNAP (TMR)

H2A.Z Ab

H2A Ab

H2A.Z-SNAP (TMR)

H2A.Z Ab

H2A Ab

**Newly synthesized**

C

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<th>Pulse</th>
<th>QCP</th>
<th>Total</th>
<th>Incorporated</th>
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<td>4x 2x 1x</td>
<td>4x 2x 1x</td>
<td>4x 2x 1x</td>
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**Quench-Chase-Pulse**

H2A (TMR)

Coomassie

H2A.Z (TMR)

Coomassie

**Incorporated (+Triton)**

D

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<th>H2A.Z-SNAP</th>
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<tr>
<td>DAPI</td>
<td>TMR (H2A.Z)</td>
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</table>

**Synthesis rate (QCP/P), %**

| H2A  | 23 ± 5 |
| H2A.Z | 18 ± 2 |

**Incorporation efficiency (Incorporated/Total), %**

| H2A  | 75 ± 6 |
| H2A.Z | 71 ± 04 |

| Ratio of total new histones (H2A.Z/H2A) |
| Ratio of new incorporated histones (H2A.Z/H2A) |
| 0.81 ± 0.08 |
| 0.76 ± 0.14 |

**Boyarchuk et al, Figure S1**

- Triton

+ Triton

- Triton

+ Triton

SNAP

H2A.Z

H2A

Lamins A/C

Cyclin A
Boyarchuk et al, Figure S2

A

Deposition at PHC

G1  S  G2  G1  S  G2

new H2A  new H2A.Z

% of cells

Exclusion
Even deposition
Enrichment

B

H2A.Z-SNAP

DNA content (PI)

24h release

Asynstr.

H2A-SNAP

G1%  S%  G2%  

Asynchr.

41.3  44.8  12

Monastrol

0.2  6.7  88.3

Release

2h  42.8  12.5  38.4

6h  59.7  12.5  38.4

24h  18  52.5  24.3

H2A.Z-SNAP

Asynchr.

44.5  38.6  12.5

Monastrol

0.2  6.5  87.8

Release

2h  46.1  10.7  40.3

6h  59.8  16.9  20

24h  22.4  50.9  22.4

C

Monastrol

Shake-off  wash-out  TMR

(NEW H2As)

Monastrol

Monastrol

Q  Chase  P

M  G1

-15 -0.5 0 2 2.5 (h)

D

Enrichment of marks at PHC

<table>
<thead>
<tr>
<th>Control</th>
<th>Adapted</th>
<th>Acute</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA meth.</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>H3K9me3</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>H3K4me2</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>H4K12ac</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>HP1a</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>H3K9ac</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ring1B</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

E

MEF

Suv39h

3T3

Suv39h

3T3

wt  dn  mock5-aza

wt  dn  mock5-aza

HpaII

minor satellites

HpyCH4IV

major satellites

F

Relative expression level

(major satellite/GAPDH)

G

+/− 5-Aza

TMR = New H2As

Q  Chase  P

-48 -0.5 0 2 2.5 (h)

<table>
<thead>
<tr>
<th>Ratio of new total histones (Mock/5-Aza)</th>
<th>Ratio of new incorporated histones (Mock/5-Aza)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2A  0.71 ± 0.07  0.71 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>H2A.Z  0.87 ± 0.22  0.89 ± 0.16</td>
<td></td>
</tr>
</tbody>
</table>
Boyarchuk et al Figure S3

A

H2A.Z-SNAP
H2A-SNAP
5-Aza
Mock

H2A.Z-SNAP
H2A-SNAP
Mock

DAPI
merge
TMR = Total H2As

B

WT or Suv39h dn

48h

Localization assay

MEFs

3T3

+C

WT
Suv39h dn

3T3

+/- 5-Aza

48h

Localization assay

C

D

H2A-SNAP

H2A.Z-SNAP

Mock
5-Aza
Mock
5-Aza