Title: Precise re-deposition of nucleosomes on repressive chromatin domains sustain epigenetic inheritance during DNA replication

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Abstract:

Whether chromatin domains display localized strategies to transfer pre-existing nucleosomal $(H3\text{-}H4)_2$ core histones and their post-translational modifications (PTMs) during DNA replication remains unknown, largely due to the limitations of direct and precise methods to follow the fate of parental nucleosomes behind the replication fork. Here, we devised an inducible, proximity-dependent labeling system to irreversibly mark replication-dependent H3.1 and H3.2 histones at desired loci in mouse embryonic stem cells such that their position before and after replication could be determined at high resolution. We found both local and non-local re-deposition of parental histones during DNA replication, with a 'repressed' chromatin state being locally preserved and an 'active' chromatin domain lacking such inheritance.

One Sentence Summary: A method that permanently labels histones at chosen loci revealed that nucleosomes from repressed (but not active) chromatin domains are re-deposited locally after DNA replication.

In order to maintain genome function and cellular identity, the organization of chromatin domains must be conserved during DNA replication and cellular division. Although the semi-conservative model of DNA replication provides resolution for the inheritance of genetic information (*1*), much less is known about mitotic epigenetic inheritance. Epigenetic inheritance encompasses various facets, including the restoration of DNA methylation, small interfering RNAs, segregation of pre-existing (parental) nucleosomes to newly replicated DNA, the incorporation of newly synthesized histones into chromatin and the re-establishment of higher-order chromatin structures (*2, 3*). One of the most fundamental questions in the field of epigenetics is how chromatin domains are inherited upon DNA replication; the bedrock to understanding the propagation of cell identity.

Chromosome duplication requires the replication of DNA and the accurate reassembly of associated histones onto each daughter DNA molecule. This latter process involves a tightly coupled deposition of histones to the replication machinery, as nucleosomes first reappear within 120-300 bp behind the replication fork (*4*, *5*). The founding studies on the structure of replicated chromatin establish that parental histones are segregated onto newly synthesized DNA relatively quickly and that both replicated DNA strands capture equal amounts of parental histones (*2*). It is now accepted that parental histones, starting with the H3-H4 tetrameric core, rapidly re-assemble behind the replication fork, followed by H2A-H2B dimer deposition (*3*, *6-8*). These four histones comprise the nucleosome particle with the $(H3\text{-}H4)_2$ cores being likely candidates to contain most of the epigenetic information as thus far, H3K9me3 and H3K27me3 are the only two modifications with clear evidence for epigenetic transmission (*9*). While only one H4 isoform

has been identified, there are several H3 somatic variants including H3.1 and H3.2 that differ at one amino acid position and are considered the canonical replication-coupled H3 since they are synthesized during S-phase (*10*, *11*). The observation that parental H3.1-containing nucleosomes are re-deposited as intact $(H3.1\text{-}H4)_2$ tetramers upon DNA replication (*7*) supports a model for the local inheritance of histone PTMs. However, direct testing for the local re-deposition of parental $(H3.1\text{-}H4)_2$ tetramers at a particular locus has not been achieved. In particular, the i*n vivo* re-deposition of parental histones within the general vicinity of their original genomic position has not yet been examined through direct methods, but through proteomics and ChIP-sequencing techniques (*12-15*), neither of which can define the precise locale of parental nucleosome segregation as they involve global, genome-wide fluxes, not single loci.

To investigate the segregation of parental core histones, we developed a bio-orthogonal system to irreversibly mark replication-dependent H3.1 and H3.2 *in vivo* at candidate loci and follow their re-deposition at a mononucleosomal level during cellular division in mouse embryonic stem cells (mESCs) (fig. 1A). First, we introduced a Biotin Acceptor Peptide (BAP) motif sequence into endogenous H3.1 and H3.2 loci to biotinylate proximal H3 chromatin using the *Escherichia coli* Biotin Ligase (BirA) (*16-18*). Then, we integrated a transgene encoding the catalytically inactive dCas9 with BirA under an inducible promoter and to maximize the signal-to-noise ratio, we incorporated a FKBP degradation domain (*19*) within BirA. Lastly, expression of chosen gRNAs allowed us to control biotinylation spatially (*20*), thus resulting in the desired, precise localization of the biotin tag, exclusively at the locus of interest and at the desired time. These steps were compounded such that dCas9 was fused at its C-terminus with the FKBP degradation domain (DD) and BirA (dCas9-DD-BirA) and stably integrated into the genome of KH2 mESCs

(fig. 1A), which constitutively express the Dox-inducible transactivator rtTA (*21*). We then created clonal dCas9-DD-BirA-expressing KH2 mESCs containing Flag-BAP knock-ins to the N-terminus of 13 endogenous copies of replication-dependent H3.1 and H3.2 in the *Hist1h3* cluster (fig. 1A and S1A-B). We found that chromatin immunoprecipitation (ChIP) followed by western blots of Flag-BAP-H3 gave evidence of chromatin enriched in H3K4me3 and H3K27me3 (fig. S1C). Additionally, ChIP-sequencing (seq) of Flag-enriched chromatin demonstrated Flag-BAP-H3 incorporation into the genome (fig. 1B, top panel), suggesting that the endogenous N-terminus Flag-BAP tags did not disrupt H3 metabolism.

To spatially recruit dCas9-DD-BirA and biotinylate local parental H3 incorporated into chromatin, we stably expressed an array of ~29-35 guide RNAs (gRNAs) tiling a 5 kb target for a non-repetitive candidate locus (fig. 1A). We tested the specificity of the system by the introduction of 33 gRNAs in which 5 kb of the *Hoxc6* was targeted. We found that during the last step of a double thymidine G1-block synchronization (fig. S1D), a 6-hr pulse with a minimal amount of doxycycline and exogenous biotin followed by a wash-off step was sufficient to observe specific biotinylation of local *Hoxc6* chromatin (fig. 1B and 1C). Briefly, chromatin from G1-blocked cells with and without a doxycycline pulse was digested with MNase to obtain mononucleosomes and then biotinylated nucleosomes were isolated using biotin antibodies. Subsequently, native biotin ChIP-seq showed a precise labeling at the *Hoxc6* locus as evidenced by a biotin peak upstream from the 5 kb gRNA recruitment site in doxycycline-treated cells (fig. 1B). Validation of the biotin *Hoxc6* peak through native biotin ChIP-quantitative PCR (ChIP-qPCR) also demonstrated accurate biotinylation of the targeted locus in comparison to nonspecific loci and IgG controls (fig. 1C). Furthermore, correct proximity biotin ligation of our

system was confirmed upon recruitment of dCas9-DD-BirA to a second distinct locus, within the *Ebf1* gene. Native biotin ChIP-seq and ChIP-qPCR again showed a biotin peak 5' from the 5 kb gRNA recruitment site and a specific biotin enrichment of *Ebf1* chromatin in contrast to nonspecific loci and IgG controls (fig. S2A and S2B, respectively). Lastly, to verify the extent of temporal control on dCas9-DD-BirA expression, we conducted a time course analysis on G1-blocked and released mESCs (fig. S3A) and observed the targeted recruitment of dCas9 to the *Hoxc6* locus and its consequent loss after the first cell cycle (fig. S3B). Therefore, our system allows for permanent histone labeling *in vivo* with notable spatial (Fig. 1b-c, S2) and transient temporal control of dCas9-DD-BirA expression (Fig. S3B).

To determine the localized strategies for the re-deposition of parental H3 chromatin following DNA replication, we first assayed local biotinylation of Flag-BAP-H3.1 and -H3.2 at repressed chromatin domains. The hallmarks of transcriptionally inactive domains include the *Hox* clusters in ES cells (*22*). Therefore, we tested parental H3 chromatin re-deposition by targeting a 5 kb area upstream of the *Hoxc6* gene in dCas9-DD-BirA expressing cells (fig. 1B). These cells were G1-blocked and given a 6 hr minimal doxycycline and biotin pulse and subsequent wash-off to demarcate parental H3 chromatin. The cells were then released and followed through cell division for 12, 24, and 48 hr. Chromatin from the G1-blocked and released time course was collected, processed through native biotin ChIP, and a 35 kb window spanning 15 kb upstream and downstream from and including the 5 kb recruitment area was assayed at a high resolution of 500 bp through qPCR. To quantitatively analyze the parental biotin ChIP-qPCR through the time points, we used spike-in *Drosophila melanogaster* chromatin and normalized the data to input, spike-in, and minus-doxycycline native chromatin. Similar to the native biotin ChIP-seq (fig.

1B), *Hoxc6* ChIP-qPCR interrogation of G1-blocked chromatin showed a robust 5' biotin peak from the dCas9-DD-BirA recruitment site with a smaller biotinylated area at the 3' end (fig. 2A, 0 hr). Subsequent release of G1-blocked mESCs through cell division and analysis for biotinylated parental chromatin revealed that the biotin peak remained in the vicinity of the initial *Hoxc6* locus (fig. 2A, time 12-24 hr) until its dilution out at 48 hr (fig. 2A, 48 hr). Furthermore, quantitative analysis of the 5' peak showed a drop in parental biotinylated chromatin enrichment from 1.0 to 0.37 through the first cell division (fig. 2B). To assess whether the dilution in biotin signal at the *Hoxc6* locus was due to the duplication of DNA, G1-blocked biotinylated mESCs were kept in S-phase with 12 hr or 24 hr of Aphidicolin treatment (fig. S4A). The subsequent native biotin ChIP-qPCR of the 5' *Hoxc6* peak exhibited sustained biotin enrichment in the presence of Aphidicolin (fig. S4B). These findings suggest the positional inheritance of parental histones in the *Hoxc* repressed domain.

To further analyze whether the observed positional inheritance of the *Hoxc6* repressed domain was a general phenomenon across repressed chromatin, dCas9-DD-BirA were similarly recruited to two additional repressed chromatin domains on separate chromosomes in ES cells. We selected gRNAs tiling a 5 kb region in the *Ebf1* and *Meis2* genes and observed an analogous biotin enrichment of both loci at the 5' and 3' recruitment areas in G1-blocked mESCs (fig. 2C and 2E, respectively, 0 hr). Similar to the *Hoxc6* locus, subsequent cell division diluted the parental biotin signals within their respective regions (fig. 2C and 2E, 12-48 hr) and quantitative analysis of the 5' peak showed a drop in parental biotinylated chromatin enrichment from 1.0 to either 0.5 or 0.67 through the first cell division (fig. 2D and 2F). These observations from three

independent repressed chromatin domains point to parental histones being re-deposited locally. This local re-deposition of parental histones is attenuated when S-phase progression is blocked.

That parental histones were retained locally during replication of repressed chromatin domains warranted the comparable analysis of active chromatin domains. We employed gRNAs tiling to a 5 kb region upstream of the *Ccna2* gene and during the final step of double thymidine synchronization, induced the transient expression of dCas9-DD-BirA and subsequent biotinylation of Flag-BAP-H3.1 and -H3.2 at *Ccna2* chromatin, as performed above. Native biotin ChIP-qPCR interrogation at a resolution of 500 bp spanning 25 kb of the targeted locus showed a broader and lower extent of chromatin biotinylation of the surrounding *Ccna2* 5 kb recruitment area (fig. 3A, 0 hr), suggesting, not surprisingly, a distinct structure of active versus repressed chromatin. Subsequent release of G1-blocked biotinylated *Ccna2* chromatin upon cell division resulted in the disappearance of biotin enriched parental histones, apparent as early as the first cell division (fig. 3A, 12 hr). This data point to the non-local re-distribution of parental histones in the *Ccna2* active domain. As active domains replicate earlier in S-phase (*23*), we examined whether the loss of *Ccna2* biotinylated chromatin occurs as early as 6 hr, a time at which mESCs are in mid S-phase (fig. S5A) prior to cell division. Indeed, native biotin ChIP-qPCR of *Ccna2* chromatin shows the disappearance of biotin signal at this time point and a more pronounced loss at 12 hr (fig. S5B, 6 hr and 12 hr). Furthermore, transcription inhibition during S-phase progression was ineffectual with respect to the disappearance of the biotin signal (fig. S5D, 6h + Triptolide). Thus, biotinylated parental histones, at least within the *Ccna2* locus, distribute non-locally upon DNA replication.

To interrogate whether this non-local distribution of parental histones is specific to the *Ccna2* locus or represent a wider phenomenon in active domain inheritance, we expanded our system to assay the loci of pluripotent factors Oct4 and Nanog, which are highly expressed in mESCs. Targeting dCas9-DD-BirA to either of these loci in G1-blocked cells again resulted in a broader biotin enrichment surrounding the 5 kb gRNA recruitment area (fig. 3B and 3C, respectively). Furthermore, and similar to the case of chromatin associated with the *Ccna2* locus, the release of G1-blocked biotinylated *Pou5f1* or *Nanog* chromatin led to a loss in biotin signal upon successive cell divisions (fig. 3B and 3C, respectively). Finally, to assess whether the disappearance of biotin signal from active chromatin domains required DNA replication, we treated G1-blocked biotinylated *Pou5f1* mESCs with Aphidicolin for 12 hr or 24 hr to block S-phase progression (fig. S4A). Sustained biotin enrichment was observed in native biotin ChIP-qPCR of *Pou5f1* chromatin when scoring for three distinct areas of the broad biotin signal (fig. S6A). These results underscore the non-local and distinct parental histone distribution of active versus repressed domain inheritance. Such differences might be the result of a dynamic chromatin structure as biotinylation patterns in active domains were lower and broader in comparison to the precise biotin peak evident in repressive chromatin.

Our data demonstrate a degree of spatial conservation in the re-deposition of intact pre-existing H3.1 and H3.2 bearing nucleosomes within repressive chromatin domains that is absent in the case of nucleosomes comprising H3.1 or H3.2 associated with active chromatin domains (fig. 4). In agreement with fundamental studies in *Drosophila (24)*, our data suggest that repressed chromatin is rich in inheritance as parental histones carry a positional memory allowing transmission of their chromatin domain status to daughter cells via a bona fide read-write

mechanism (*9, 25, 26*). Such mechanisms would work along with histone chaperones to sufficiently maintain and propagate repressed chromatin domains. Moreover, these data argue that local re-deposition of parental nucleosomes containing H3.1 and H3.2 are not critical for the inheritance of active chromatin domains. Instead, we speculate that major roles in preserving a transcriptional active domain through cell division entail DNA sequence-specific transcription factors (*27, 28*). In this situation the chromatin is open (euchromatin), as illustrated in our nucleosome labeling experiments wherein a lower and broader area of labeling was observed, compared to the repressed chromatin domains. These differences in biotin labeling might be the result of the three-dimensional structure of chromosomes as detected for distinct epigenetic states (*29*). As replication timing and chromosomal domain structures are intertwined (*23*), it is possible that active and repressed chromatin form self-interacting domains that set thresholds for the accessibility of factors promoting the appropriate chromatin organization during DNA replication.

A feasible explanation for the local conservation of repressed chromatin domains versus active chromatin domains entails the fact that repressed chromatin is replicated late in S phase whereas active chromatin is an early event. This timing difference is known to affect the rate of replication, such that euchromatin is replicated at a faster rate than heterochromatin (*23*). Whether these differences can account for the observed positional inheritance of repressive chromatin domains remains to be elucidated, but we speculate that distinct chaperones might operate during late S-phase, but are absent in early replicating chromatin. These speculations require further investigation, however, our studies clearly demonstrate that nucleosomes

associated with repressed chromatin segregate to the same chromatin domains whereas those associated with active chromatin exhibit a dynamic re-distribution.

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Author contributions: T.M.E., R.B. and D.R. conceptualized the project. T.M.E. performed the experiments and wrote the paper. O.O. helped with formal analysis and development of methodology. N.D. performed bioinformatic analysis. D.R. supervised the relevant study. **Competing interests:** The authors declare no conflict of interest.

Data and materials availability: All sequencing data have been deposited to the Gene Expression Omnibus and will be made available immediately upon publication. All plasmids are available upon request and modified KH2 mES cell lines are available through a materials transfer agreement.

Supplementary Materials:

Materials and Methods

Figures S1-S6

Tables S1-S4

References (*30*-*39*)

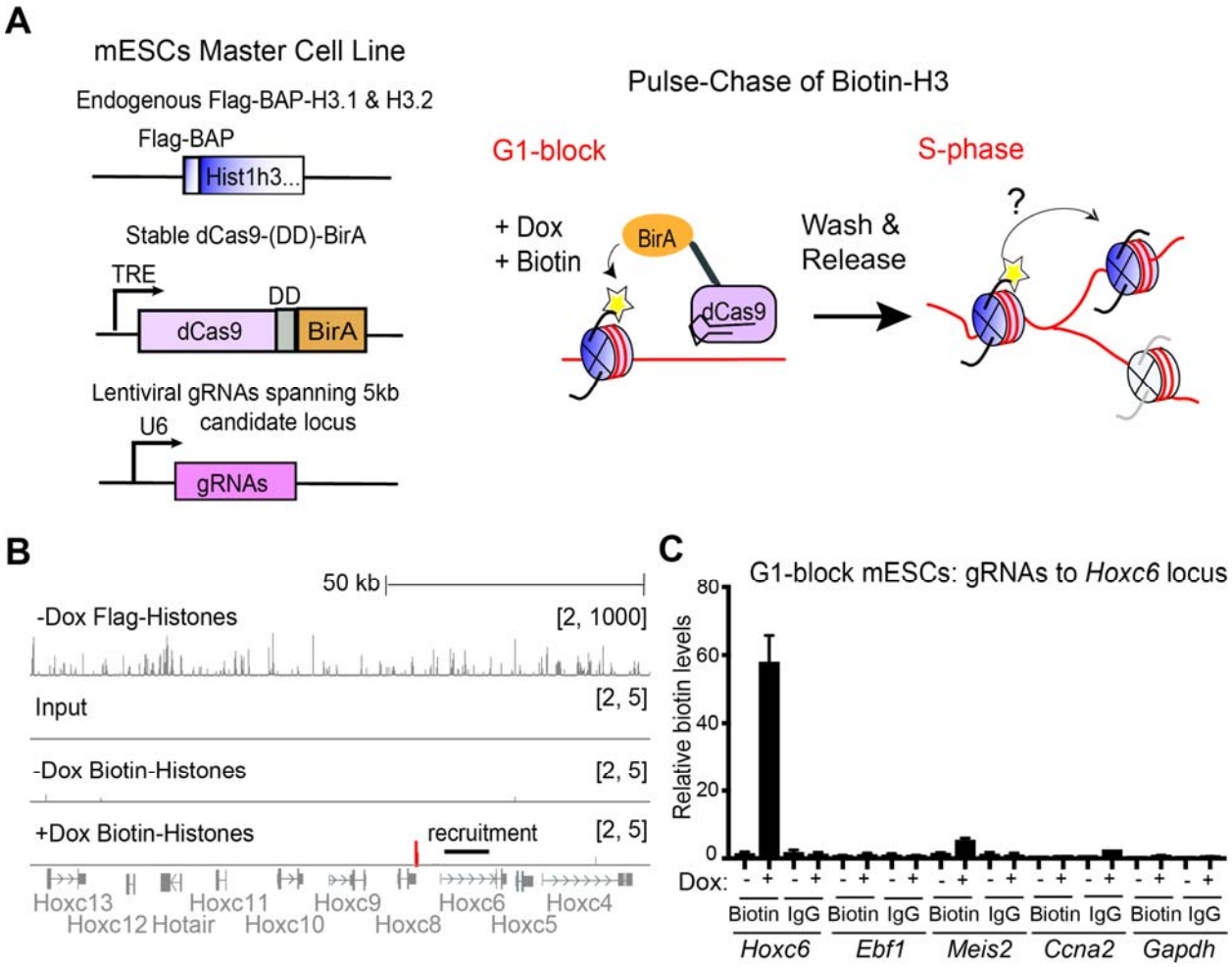

Fig 1. Precise labeling of H3.1 and H3.2 histones in living cells. (A) Overview of the system to assess *in vivo* chromatin domain inheritance in mESCs. A master cell line containing endogenous tags of Flag-BAP H3.1 and -H3.2, stable integration of doxycycline (Dox)-inducible dCas9-DD-BirA, and transducible gRNAs spanning a 5 kb candidate locus are arrested in G1. Following a pulse of Dox and exogenous biotin, nearby tagged parental nucleosomes are biotinylated (blue histones and yellow asterisks). Wash-off of media releases cells into S-phase wherein the re-distribution of biotin-H3 at a mononucleosomal level is assayed in newly synthesized chromatin. (B) Native Flag and biotin ChIP-seq analysis of G1-blocked cells at the *Hoxc* cluster following dCas9-DD-BirA recruitment. (C) Native ChIP-qPCR analysis of biotin-H3 in G1-blocked mESCs showing biotin enrichment at the *Hoxc6* locus compared to *Ebf1*,

Meis2, *Ccna2*, *Gapdh* and IgG controls. Data was normalized to 5% input and error bars correspond to standard deviation of three biological replicates.

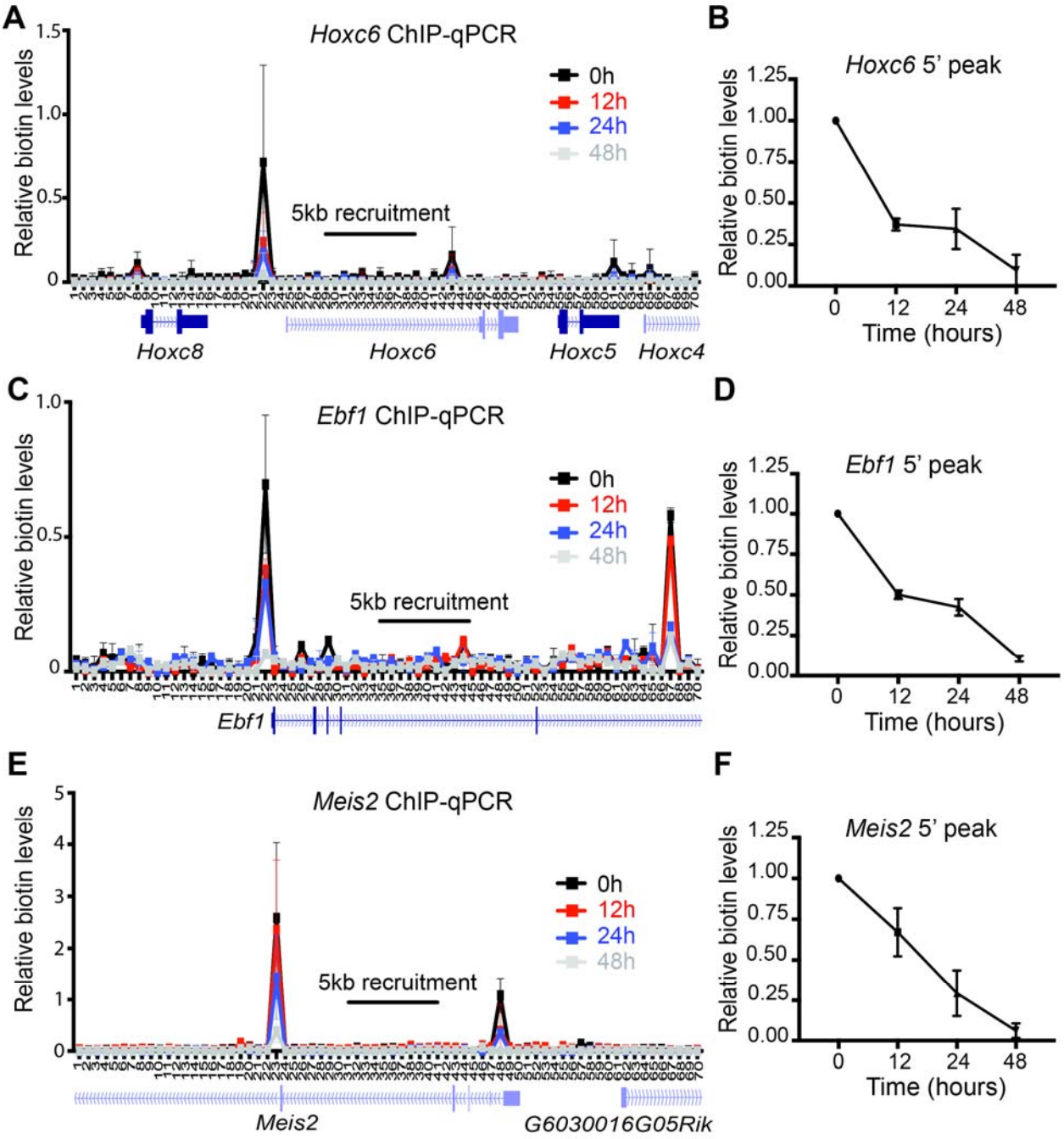

Fig 2. Repressed parental H3 domains are re-distributed locally (A-F) Native mononucleosomal biotin ChIP-qPCR in G1-blocked and released mESCs following a 6 hr pulse of Dox and exogenous biotin in cells targeting BirA to the *Hoxc6* (A-B), *Ebf1* (C-D), and *Meis2* (E-F) loci. The time course represents 0 hr -1 cell, 12 hr - 2 cells, 24 hr - 4 cells, 48 hr - 16 cells. (A, C, and E) Data shows the average of 3 biological replicates spanning a 35 kb area at a

resolution of 500 bp. (B, D, and F) Graph highlighting the 5' peak of corresponding assays: *Hoxc6* primer set 22 (B), *Ebf1* primer set 22 (D), and *Meis2* primer set 23 (F). All biotin enrichment levels are relative to input, normalized to *Drosophila* chromatin spike-in followed by subtraction of the minus-Dox (-Dox) control, and error bars represent standard deviation. For figures B, D, and F, the dataset for 0 hr was set to 1.

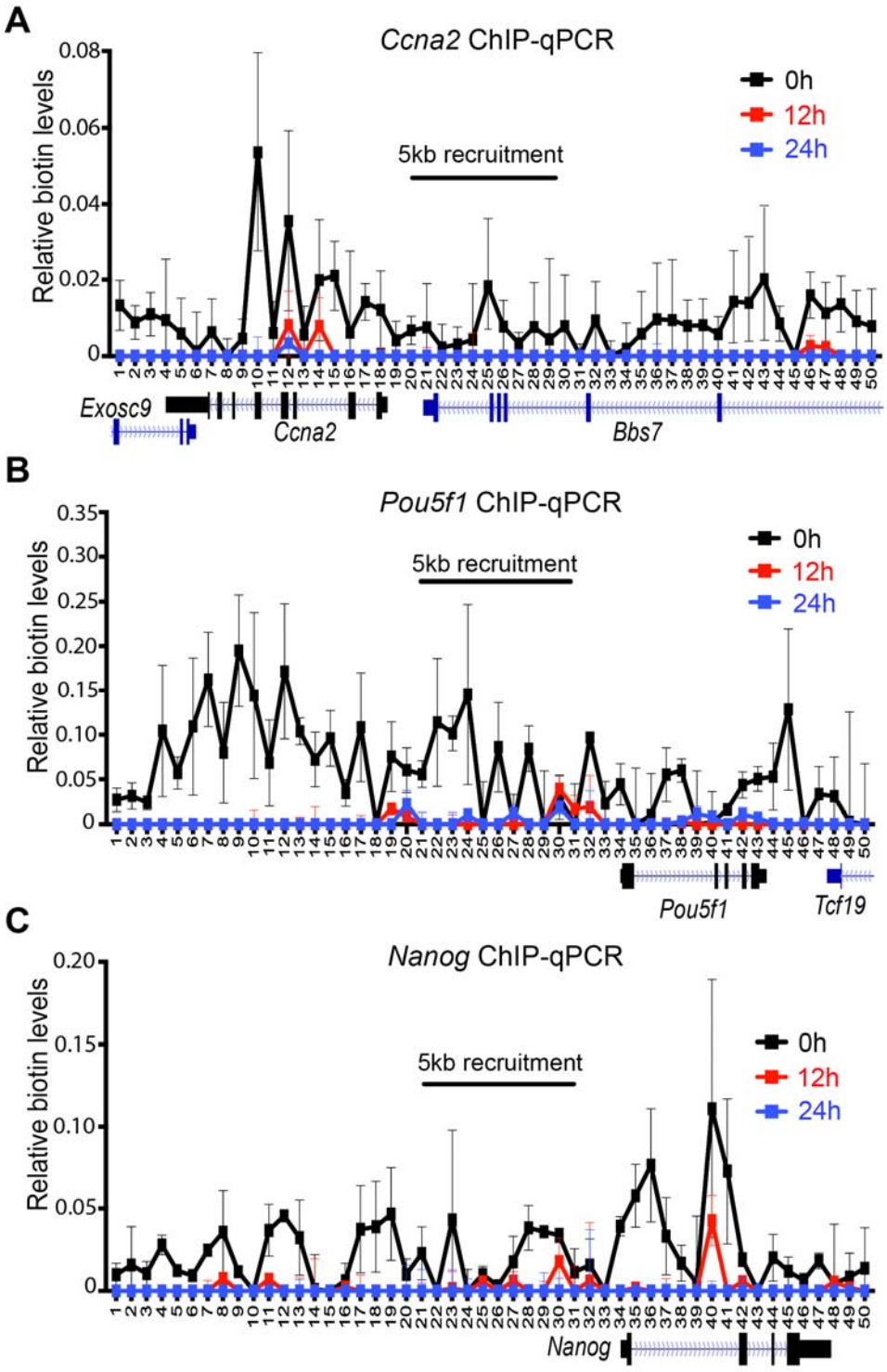

Fig 3. Dynamic distribution of active parental H3 domains. (A-C) Native mononucleosomal biotin ChIP-qPCR from G1-blocked and released mESCs following a 6 hr Dox and exogenous biotin pulse in cells targeting BirA to the *Ccna2* (A), *Pou5f1* (B), and *Nanog* (C) loci. The time

course represents 0 hr -1 cell, 12 hr - 2 cells, 24 hr - 4 cells. Data shown is the average of 3 biological replicates spanning a 25 kb area at a resolution of 500 bp. All biotin enrichment levels are relative to input, normalized to *Drosophila* chromatin spike-in followed by subtraction of the -Dox control, and error bars represent standard deviation.

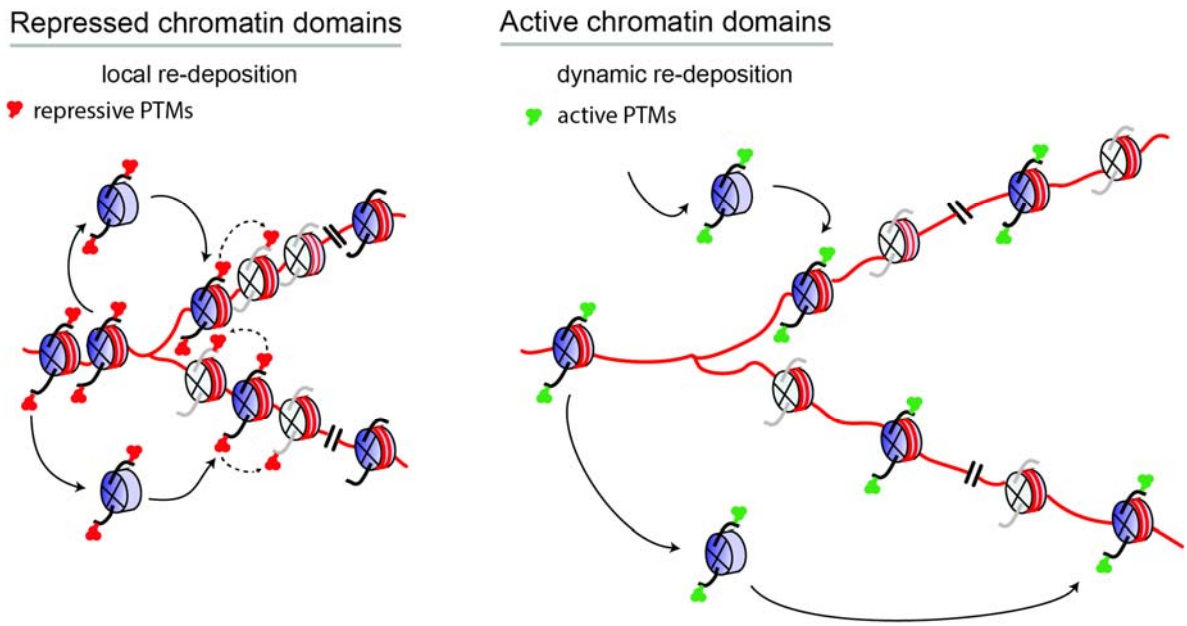

Fig 4. Parental H3 nucleosomes segregate locally in repressed chromatin domains. In repressed chromatin domains, a degree of spatial conservation in the re-deposition of intact pre-existing $(H3.1\text{-}H4)_2$ or $(H3.2\text{-}H4)_2$ tetrameric core-bearing histone PTMs (blue histones and solid arrows) are sufficient to transmit chromatin states to daughter cells if maintenance enzymes are available and can restore the transmitted modification(s) to neighboring newly synthesized histones (gray histones and dashed arrows) (*9*). This local re-deposition of parental H3.1 or H3.2 nucleosomes is dynamic in active chromatin domains.