

# Dynamics and Memory of Heterochromatin in Living Cells

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## SUMMARY

Posttranslational histone modifications are important for gene regulation, yet the mode of propagation and the contribution to heritable gene expression states remains controversial. To address these questions, we developed a chromatin in vivo assay (CiA) system employing chemically induced proximity to initiate and terminate chromatin modifications in living cells. We selectively recruited HP1 $\alpha$  to induce H3K9me3-dependent gene silencing and describe the kinetics and extent of chromatin modifications at the *Oct4* locus in fibroblasts and pluripotent cells. H3K9me3 propagated symmetrically and continuously at average rates of  $\sim 0.18$  nucleosomes/hr to produce domains of up to 10 kb. After removal of the HP1 $\alpha$  stimulus, heterochromatic domains were heritably transmitted, undiminished through multiple cell generations. Our data enabled quantitative modeling of reaction kinetics, which revealed that dynamic competition between histone marking and turnover, determines the boundaries and stability of H3K9me3 domains. This framework predicts the steady-state dynamics and spatial features of the majority of euchromatic H3K9me3 domains over the genome.

## INTRODUCTION

In multicellular organisms, cellular identity is defined by distinct profiles of gene expression that are faithfully transmitted through cell division. There are multiple mechanisms that convey heritable transcriptional information independent of DNA sequence. These epigenetic mechanisms are self-sustaining in the absence of the initial stimulus (Bonasio et al., 2010; Ptashne, 2007). Chemical modifications of DNA and nucleosomal histones have been implicated in contributing to epigenetic programs. However, to date, only DNA methylation has been shown to mediate gene repression that is conserved through cell generations (Goll and Bestor, 2005; Wigler et al., 1981). Some post-

translational histone modifications exhibit strong correlations with transcriptional states (Kouzarides, 2007), and mechanisms for their propagation have been proposed (Margueron et al., 2009; Nakayama et al., 2001; Talbert and Henikoff, 2006). However, genetic approaches have not been able to address the cellular dynamics of chromatin regulation and biochemical approaches have been limited by the inability to faithfully reconstitute chromatin in vitro. Hence, new experimental techniques are required to develop a kinetic understanding of chromatin regulation in living cells.

Position effect variegation (PEV) has been a classical paradigm to study the role of histone modifications in inheritance of transcriptional patterns. In flies, PEV describes a mottled eye phenotype caused by random silencing of the *white* gene when translocated into the proximity of a heterochromatic region (Muller, 1930). Patches of red and white cell clones are maintained through cell divisions in the developing fly eye, indicating that silencing of the *white* gene product is clonally inherited. Genetic screens for PEV modifiers in several organisms have identified multiple proteins implicated in methylation of histone H3 at lysine 9 (H3K9me) (Fodor et al., 2010). In mammalian cells, H3K9 trimethylation (H3K9me3) is a hallmark of heterochromatin (Peters et al., 2002) and is also required for transcriptional silencing of genes and retroviral elements (Magklara et al., 2011; Matsui et al., 2010; Nielsen et al., 2001). Transcriptional repression involves heterochromatin protein 1 (HP1), which specifically binds to methylated H3K9 (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001). HP1 can form oligomers, which are thought to bridge neighboring nucleosomes and mediate chromatin condensation (Canzio et al., 2011; Verschure et al., 2005). HP1 also directly interacts with and recruits H3K9-specific histone methyltransferases (HMTs) Suv39h1/2 and SETDB1 (Fritsch et al., 2010; Peters et al., 2003; Rea et al., 2000; Schultz et al., 2002). HMT interactions could facilitate self-propagation and sequential methylation of adjacent nucleosomes consistent with a model of linear spreading (Hall et al., 2002; Schotta et al., 2002). Alternatively, it has been suggested that H3K9 methylation could propagate along the chromosome discontinuously through a mechanism of skipping (Talbert and Henikoff, 2006). In *Drosophila*, spreading of heterochromatin depends on the activity and dosage of heterochromatin components and can be antagonized by euchromatic factors (Ebert et al., 2004; Schotta et al., 2002). Although this

competitive balance appears to determine the boundaries of pericentric heterochromatin, it is unclear whether H3K9me3 repressed target genes are also dosage sensitive and subject to dynamic regulation. Moreover, the persistence of the mark at these genes through cell division may be a result of self-propagation or of renewed targeting of HMTs through recruitment by *cis*-regulatory factors after each replication cycle (Moazed, 2011).

We sought to study the formation of heterochromatin at a well-defined euchromatic target promoter in living cells. In embryonic stem (ES) cells, *Oct4* (*Pou5f1*) is highly expressed, encoding a transcription factor that is critical for pluripotency and self-renewal. Upon cellular differentiation, *Oct4* expression is rapidly and completely silenced through a series of events including histone H3K9 methylation, HP1 binding, and DNA methylation (Feldman et al., 2006). Interestingly, in differentiated tissues *Oct4* repression can be overcome by ectopic expression of pluripotency transcription factors (including *Oct4* itself), which leads to the formation of pluripotent cells (Takahashi and Yamanaka, 2006). However, cellular reprogramming is highly inefficient, possibly due to repressive chromatin structure that presents a barrier to transcription factor binding.

To investigate the kinetics of chromatin modification and the transmission of epigenetic information, we have generated a murine strain that allows rapid addition and removal of chromatin regulatory activities to a genetically modified *Oct4* allele in any cell type by using small molecule-mediated recruitment. Selective recruitment of HP1 $\alpha$  induced H3K9me3 at the *Oct4* reporter locus and subsequent linear spreading in *cis* over a distance of 10 kbp to form a heterochromatic domain with features of PEV. Removal of HP1 $\alpha$  from the locus allowed us to study the epigenetic properties of the histone mark, clearly demonstrating that the H3K9me3 domain was inherited through cell divisions in the absence of the initial stimulus. Transcriptional activators could oppose the maintenance of heterochromatin suggesting that the steady state of H3K9me3 is governed by antagonizing activities of histone marking and turnover. Mathematical modeling based on competitive dynamics enabled us to describe the spatial features of heterochromatic domains and to calculate the rates of histone H3K9 methylation and turnover at the *CiA:Oct4* locus in ES cells and fibroblasts. Finally, when applied to genomic data sets, our quantitative framework predicts the steady-state dynamics of the vast majority of all noncentromeric H3K9me3 domains in the mouse genome.

## RESULTS

### Generation of the Murine Chromatin In Vivo Assay System at *Oct4*

We envisioned an approach where chemically induced proximity (CIP) enables selective addition and removal of different chromatin and transcriptional activities to an endogenous chromosomal locus in vivo (Figure 1). CIP uses bifunctional small molecules that are membrane-permeable and can cause rapid association of two different peptide tags fused to proteins of interest inside cells. Parallel or sequential addition of orthogonal small-molecule ligands has been successfully employed to dissect the mechanism and order of events of various bio-

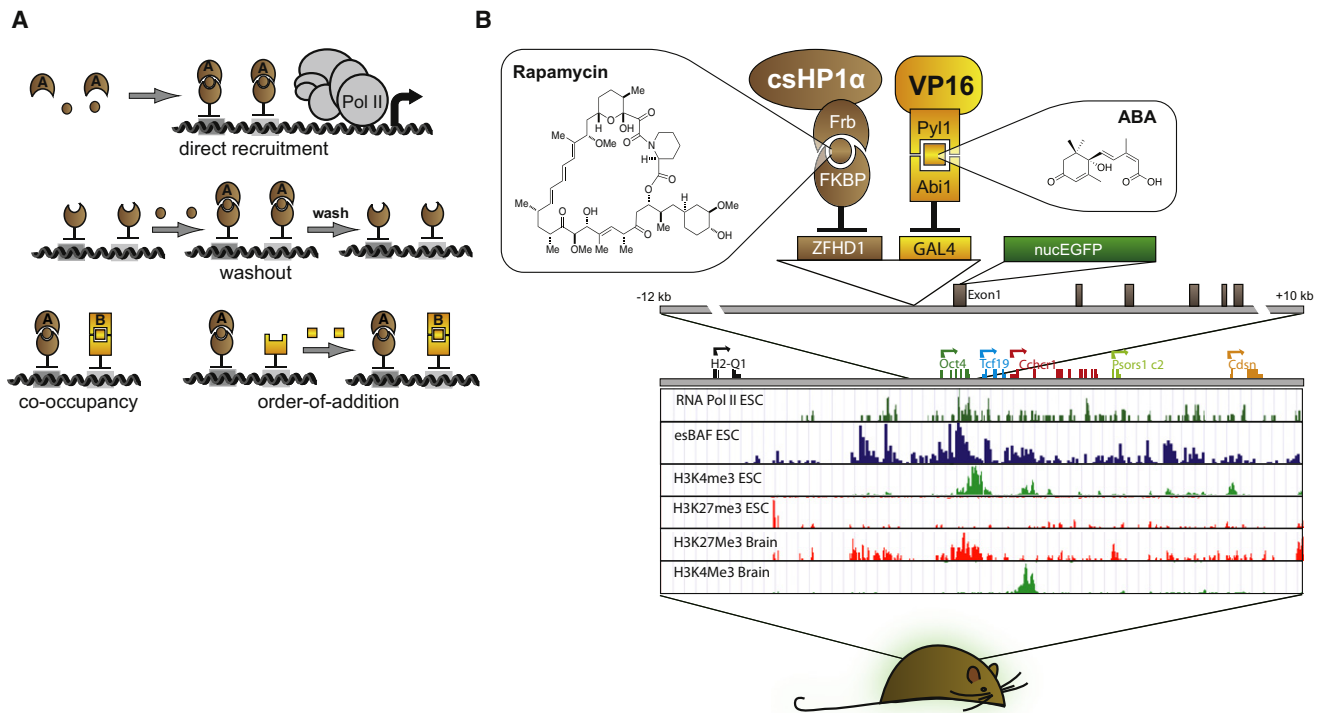
chemical processes (Graef et al., 1997; Gruber et al., 2006; Ho et al., 1996; Spencer et al., 1993). Importantly, induction of protein proximity is readily reversible in cells or animals by washout or specific displacement using one-sided molecules that bind to only one peptide tag.

Because *Oct4* gene dosage is haplosufficient (Nichols et al., 1998), we genetically modified one *Oct4* allele to recruit chromatin regulators by CIP and to study the effects on gene expression and chromatin structure. We introduced two arrays of different DNA binding sites (12xZFHD1 and 5xGAL4) upstream of the *Oct4* promoter and an in-frame nuclear enhanced green fluorescent protein (EGFP) reporter replacing the first exon of *Oct4* (Figure 1). Targeted ES cells retained good morphology (Figures S1A and S1B available online) and provided real-time fluorescence-based readout of gene expression at single cell resolution. Protein tethering that uses the CIP system involves expression of two sets of chimeric proteins designed to bind different sides of a CIP molecule. The first set is composed of the respective DNA binding domain (GAL4 or ZFHD1) fused to a CIP anchor partner (e.g., FKBP12). The second set of protein chimeras contains the protein of interest fused to the CIP recruitment partner (e.g., FRB). Addition of bifunctional small molecules (e.g., rapamycin) induces the CIP anchor to reversibly bind the CIP recruitment partner, tethering any given protein to the modified *Oct4* allele. We call this system the chromatin in vivo assay (CiA) system and the allele harboring the recruitment domains and reporter at *Oct4*, *CiA:Oct4*.

Nucleosome modifications at the *Oct4* promoter have been well defined in a variety of cell types including mouse ES cells (Figure 1B), where the active gene is modified with histone H3 acetylation (H3ac) and histone H3K4 trimethylation (H3K4me3) (Mikkelsen et al., 2007). In contrast, *Oct4* is transcriptionally repressed in differentiated tissues and packaged into nucleosomes marked by H3K9me3 and H3K27me3. We induced cellular differentiation of CiA ES cells by removal of LIF and treatment with retinoic acid (RA) (Athanasiadou et al., 2010; Sato et al., 2006). Differentiation of CiA ES cells reduced expression of both the endogenous *Oct4* protein and the green fluorescent protein (GFP) reporter (Figure S1C). Subsequent chromatin-immunoprecipitation (ChIP) analysis showed that GFP repression was paralleled with a loss of H3K4me3 and gain of repressive H3K9me3 and H3K27me3 (Figure S1D). We concluded that the *CiA:Oct4* allele faithfully reflected physiological regulation of wild-type *Oct4*.

### Kinetic Analysis of Heterochromatin Induced by Chemical-Mediated Recruitment

Initiation and maintenance of *Oct4* repression during cellular differentiation involves substantial changes in histone modifications and gain of DNA methylation. To determine the kinetics of H3K9me3-dependent gene repression, we sought to recruit HP1 to the *CiA:Oct4* promoter in ES cells. HP1 $\alpha$  is composed of a chromo-domain (CD), which confers specific binding to methylated H3 lysine 9, and a chromo-shadow domain (CSD), which directly interacts with H3K9-specific histone methylases, including SetDB1 and Suv39h1/2 (Hiragami and Festenstein, 2005; Schultz et al., 2002). We infected CiA ES cells with a lentiviral construct of the DNA binding domain of GAL4 (GAL4) fused



**Figure 1. Design of Chromatin In Vivo Assay at *Oct4* (CiA:Oct4) ES Cell Line and Mouse**

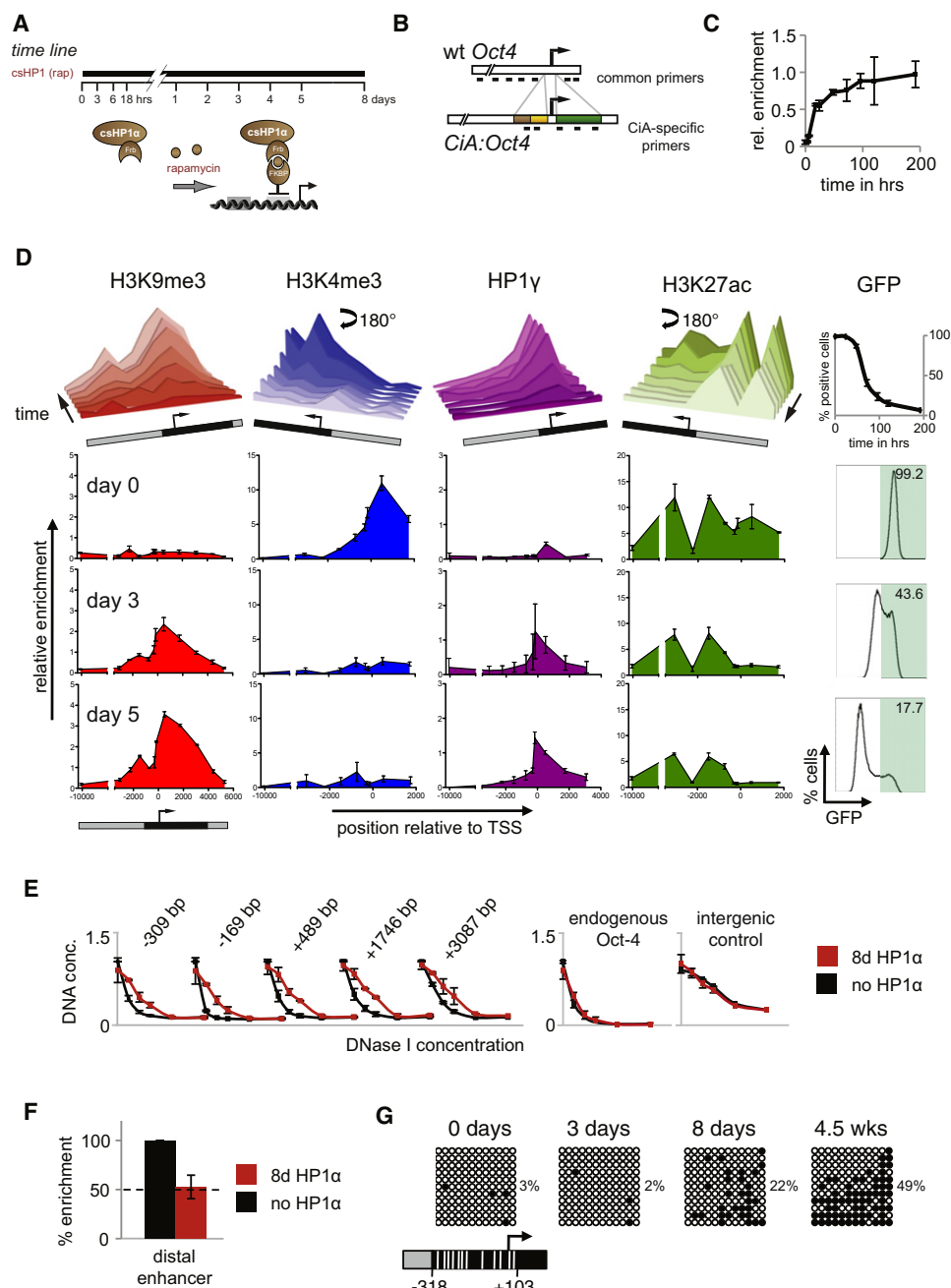
(A) CIP allows direct recruitment, washout, co-occupancy, and order-of-addition experiments.

(B) The CiA:Oct4 mouse contains one modified *Oct4* allele harboring two arrays of DNA binding sites (12XZFHD1 and 5XGal4) in the promoter region upstream of an in-frame EGFP reporter. Distribution of histone modifications at the *Oct4* locus in murine ES cells and brain tissue (Mikkelsen et al., 2007) reveals the distinct chromatin substrates for CiA modulation.

to either full-length murine HP1 $\alpha$  or a truncated form of HP1 $\alpha$  containing only the CSD (csHP1 $\alpha$ ). Although infection with GAL4 alone did not alter GFP, both full-length HP1 $\alpha$  and csHP1 $\alpha$  fusion proteins induced complete silencing of gene expression as measured by flow cytometry (Figure S1E). To minimize potential nonspecific effects of ectopic HP1 $\alpha$  expression, we used csHP1 $\alpha$  in subsequent studies.

To gain better temporal resolution of the dynamic processes involved in HP1 $\alpha$ -mediated repression, we used the CIP system to recruit csHP1 $\alpha$ . We infected CiA ES cells with two lentiviral constructs, one containing GAL4 fused to the CIP anchor, FKBP12, and the other encoding the csHP1 $\alpha$  fragment fused to two repeats of the 98 aa FKBP12-rapamycin binding (FRB) domain of mTor with a V5 epitope tag for detection (Figure 2A). After addition of rapamycin, we monitored changes in GFP expression and chromatin structure by flow cytometry and ChIP, respectively. ChIP against the V5 tag revealed significant recruitment of csHP1 $\alpha$  within 6 hr and nearly saturated binding within 24 hr after rapamycin addition (Figure 2C). csHP1 $\alpha$  targeting led to complete repression of the CiA:Oct4 reporter within 5 days (Figure 2D). Interestingly, CiA ES cells did not display a gradual decrease in GFP expression but instead segregated into a bimodal population of GFP-positive and GFP-negative cells (Figure 2D, right panel). The distribution and enrichment of histone modifications was determined by ChIP and real-time

PCR by using sets of common and reporter allele-specific primers, which cover the distal and proximal regulatory regions upstream and the gene body downstream of the CiA:Oct4 promoter (Figure 2B). Prior to csHP1 $\alpha$  targeting (day 0), H3K27ac was broadly enriched at promoter-distal and -proximal sites, whereas H3K4me3 was only enriched downstream of the active transcription start site (TSS). At this time, H3K9me3 was absent and only basal levels of endogenous HP1 $\gamma$  were detectable (Figure 2D). After 18 hr of csHP1 $\alpha$  tethering, we started to observe H3K9me3, which coincided with recruitment of endogenous HP1 $\gamma$  at the CiA:Oct4 promoter. For the next 4 days, H3K9me3 and HP1 $\gamma$  increased and spread upstream and downstream of the GAL4 binding site. After 5 days of csHP1 $\alpha$  recruitment, H3K9me3 had formed a large domain of approximately 10 kbp, which peaked adjacent to the DNA binding site and gradually decreased for 5 kbp to either side (Figure 2D). Interestingly, consistent with the bimodal expression pattern, GFP-negative sorted cells displayed a fully established heterochromatic domain after only 3 days of rapamycin, whereas GFP-positive cells lacked H3K9me3 (Figure S2). This suggests that csHP1 $\alpha$ -dependent repression initiates stochastically in an all-or-nothing fashion in individual cells of the population. Gain of H3K9me3 appeared to involve recruitment of SETDB1 (Figure S3A). Loss of active marks and establishment of the H3K9me3 domain did not significantly affect nucleosome



**Figure 2. Kinetics of Heterochromatin Formation after HP1 $\alpha$  Recruitment in ES Cells**

(A) Experimental design: rapamycin addition recruits HP1 $\alpha$  chromoshadow fragment (csHP1 $\alpha$ ) to the *CiA:Oct4* promoter.

(B) Schematic representation of wild-type and *CiA* alleles depicts location of allele-specific and common real-time PCR primers.

(C) ChIP analysis shows rapamycin-mediated csHP1 $\alpha$  recruitment over time.

(D) ChIP analysis reveals dynamic changes of active (H3K4me3, H3K27ac) and repressive (H3K9me3, HP1  $\gamma$ ) chromatin modifications at the *CiA:Oct4* locus. Upper panel summarizes time course of chromatin remodeling at 0 hr, 24 hr, 48 hr, 72 hr, 96 hr, 120 hr, and 192 hr. Data rotated 180° as indicated to display loss of active marks. Lower panels display ChIP analysis of histone modifications (y axis) across the *CiA:Oct4* locus (x axis) at selected time points. GFP expression was measured by flow cytometry at each time point. Schematic of the reporter allele indicates *CiA:Oct4*-specific primer pairs in black.

(E) DNase I sensitivity across the *CiA:Oct4* locus before and after csHP1 $\alpha$  recruitment.

(F) ChIP analysis of Oct4 transcription factor binding at *Oct4* enhancer before and after csHP1 $\alpha$  recruitment.

(G) Bisulfite sequencing analysis of DNA methylation changes at the *CiA:Oct4* promoter following csHP1 $\alpha$  targeting, with percentage methylated CpGs. White lines in schematic below mark relative positions of CpG dinucleotides.

ChIP and DNase I sensitivity results represent average and SEM of at least two independent experiments. See also Figures S1, S2, and S3.



occupancy at the reporter allele, as measured by total histone H3 ChIP, nor did it alter chromatin structure or expression of the endogenous *Oct4* allele (Figures S3B and S3D).

To test how changes in histone modifications related to chromatin compaction, we analyzed the sensitivity of the *CiA:Oct4* locus to endonuclease digestion. DNase I sensitivity is a hallmark of active promoters, whereas inactive genes and heterochromatic regions are more resistant to digestion (Groudine and Weintraub, 1982; Kerem et al., 1984). We found that the *CiA:Oct4* locus was significantly more resistant to DNase I digestion after 8 days of csHP1 $\alpha$  recruitment, whereas DNase I sensitivity remained unaffected at the endogenous *Oct4* promoter as well as at a control locus (Figure 2E). Reduced accessibility to nuclease digestion was also observed at the *Oct4* transcription factor binding site located at the distal enhancer 2 kbp upstream of the promoter. PCR primers at this region are outside of the knock-in sequence and thus do not allow discrimination between the endogenous and reporter alleles (Figure 2B). We found that *Oct4* binding was reduced by approximately two-fold (Figure 2F). Because *Oct4* expression from the wild-type allele is critical for ES cell maintenance, this decline reflected a near-complete eviction of *Oct4* protein from the distal enhancer of the *CiA:Oct4* allele.

Upon RA treatment and in differentiated tissues, the *Oct4* promoter is subject to DNA methylation, which has been proposed to stabilize maintenance of gene repression through cell division (Athanasiadou et al., 2010; Feldman et al., 2006). We analyzed levels of CpG methylation at the *CiA:Oct4* promoter during the course of gene silencing. Bisulfite-sequencing revealed low levels of DNA methylation in *CiA* ES cells prior to csHP1 $\alpha$  recruitment. Unlike H3K9me3, DNA methylation was only slightly increased after 8 days when transcription at the locus was fully repressed (Figure 2G). However, promoter methylation continued to gradually increase and was significantly higher at 4.5 weeks, similar to levels found in differentiated cells.

### Maintenance of Heterochromatin at the *CiA:Oct4* Locus in ES Cells

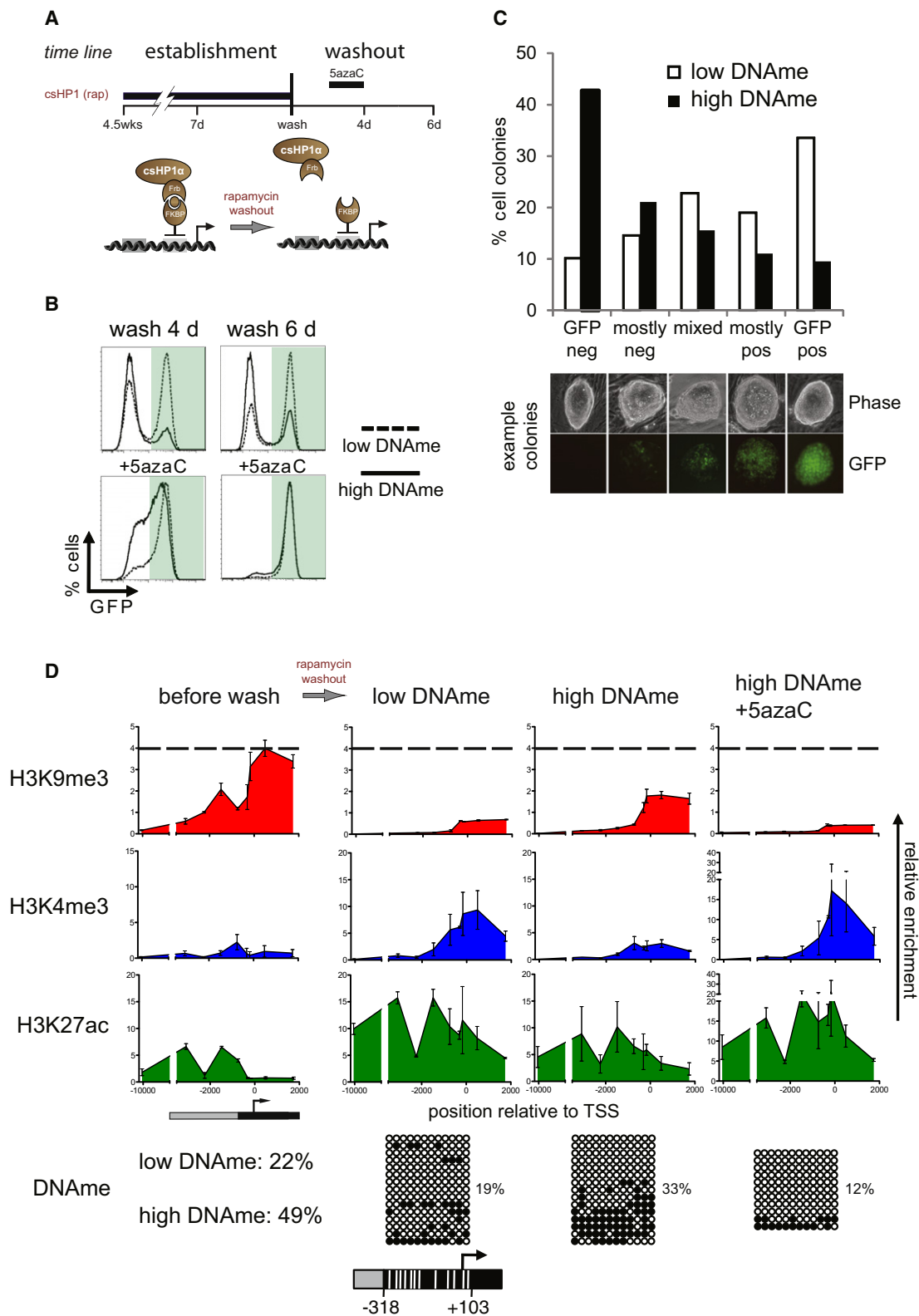
We determined the stability of induced heterochromatin and its transmission through cell generations by the timed removal of rapamycin. The *CiA:Oct4* allele was silenced by rapamycin-mediated recruitment of csHP1 $\alpha$  for either 7 days or 4.5 weeks followed by rapamycin washout (Figure 3A). We observed comparable levels of H3K9me3 after 7 days or 4.5 weeks (Figure S3F), yet the shorter pulse of csHP1 $\alpha$  recruitment induced only low levels of DNA methylation, whereas the extended pulse resulted in high levels of methylation at the *CiA:Oct4* promoter. Although we cannot exclude additional differences, for simplicity, we will refer to the short pulse as “low DNAm” (Figure 2G) and the long pulse as “high DNAm” (Figure 2G). Homogenous populations of GFP-negative cells were obtained by fluorescence-activated cell sorting (FACS) after short (7 days) or long (4.5 weeks) csHP1 $\alpha$  treatment. Cells were then passaged in the absence of rapamycin to release csHP1 $\alpha$  from the *CiA:Oct4* promoter (Figure 3A). To our surprise, a significant fraction of *CiA* ES cells began to re-express GFP. *CiA* ES cells with low DNAm displayed GFP reactivation in 50.7% of the

population 4 days after washout and in 63.2% 6 days after washout (Figure 3B). In contrast, only 18.9% of the *CiA* ES cells with high DNAm displayed GFP reactivation 4 days after washout, and 30.7% after 6 days of washout. To address whether maintenance of GFP repression was controlled by DNA methylation, we treated cells with DNA methyltransferase inhibitor 5-azacytidine (5azaC) (Yoo and Jones, 2006). After a 5azaC pulse for 2 days during washout (days 2–4), GFP repression was rapidly lost regardless of the duration of csHP1 $\alpha$  targeting (Figure 3B, lower panel). To test whether increased DNA methylation enhanced heritable repression from one cell generation to the next or whether reactivation of *CiA:Oct4* was stochastic within the population, we followed GFP expression in individual colonies founded by single parental *CiA* ES cells. GFP-negative cells sorted from low or high DNAm samples were sparsely plated and cultured in the absence of rapamycin for 4 days to form colonies. Colonies were imaged and scored for GFP expression (Figure 3C). Colonies founded by parental cells with low DNAm were 53% GFP-positive or mostly positive, whereas only 10% of colonies were completely GFP-negative. We also observed that many of these colonies contained a mixed population of GFP-positive and -negative cells, indicating that stochastic reactivation occurred in cells with low DNAm. In contrast, colonies formed from high DNAm parental ES cells showed less GFP reactivation. Only 21% of the colonies were GFP-positive, whereas 43% were completely GFP-negative and 21% of the colonies were mostly GFP-negative. These results argue that DNA methylation of the *CiA:Oct4* promoter enhanced maintenance and heritable transmission of gene repression by suppressing spontaneous reactivation in the absence of the HP1 stimulus.

To examine whether the stability of the heterochromatic domain was dependent on DNA methylation, we measured H3K9me3 after 4 days of rapamycin washout and in the presence or absence of 5azaC. Consistent with rapid gain of GFP expression (Figure 3B), cells with low DNAm exhibited a dramatic reduction of H3K9me3 concomitant with an increase in active histone modifications (Figure 3D). *CiA* ES cells with high DNAm also displayed reduced levels of H3K9me3 and increased H3K4me3. However, these changes were less extensive than in cells with low DNAm. H3K9me3 was rapidly and uniformly lost upon addition of DNA methylation inhibitors 5azaC (Figure 3D).

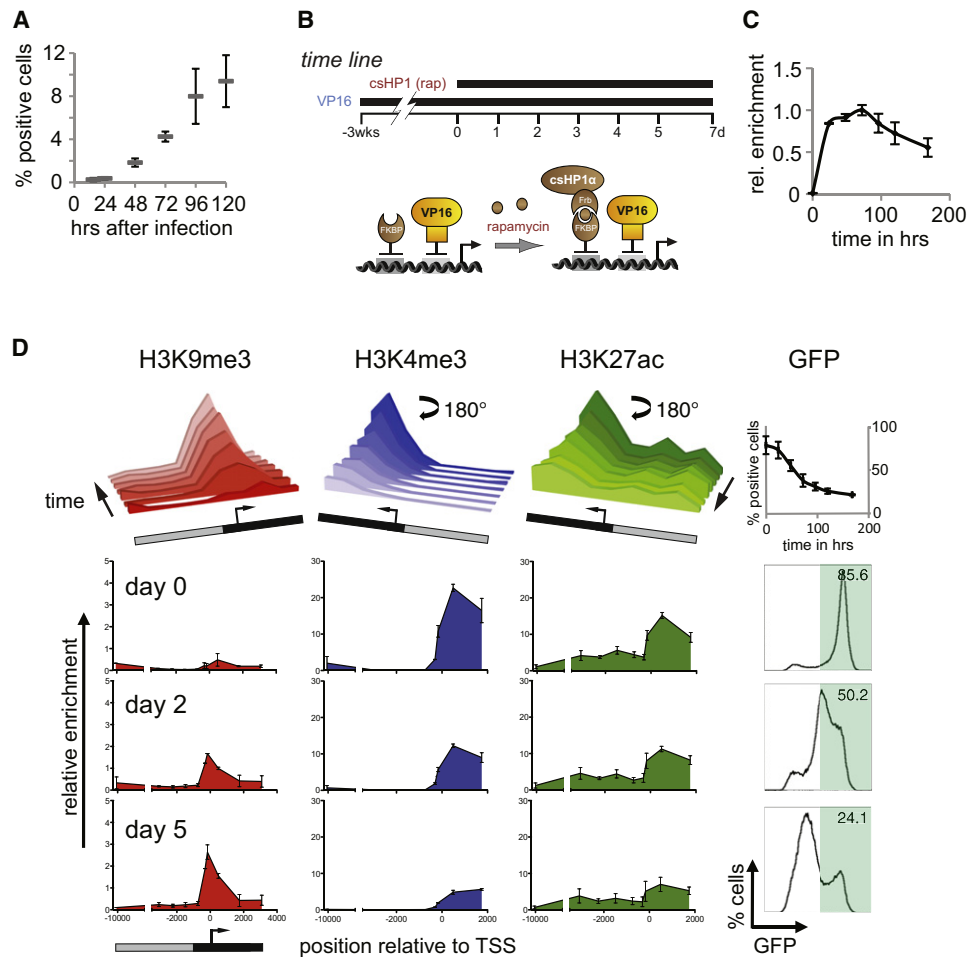
### Activation of the *CiA:Oct4* Locus in Fibroblasts and Dynamics of Re-Establishment of Heterochromatin

To determine whether transcriptional opposition led to H3K9me3 instability, we needed to examine the maintenance of heterochromatin in a tissue lacking ES cell pluripotency factors. We generated a mouse from *CiA* ES cells and prepared mouse embryonic fibroblasts (MEFs). In these cells, the *CiA:Oct4* allele was transcriptionally silent and embedded in repressive chromatin with high levels of H3K9me3 and H3K27me3 (Figure S4). To erase these repressive marks, we recruited transcriptional activator VP16 to the *CiA:Oct4* promoter. Based on studies of reprogramming, we expected *Oct4* reactivation to require 14–16 days or not to be possible at all. Surprisingly, 24 hr after infection we detected a small fraction of reactivated cells by



**Figure 3. Maintenance of Heterochromatin and Heritable *Oct4* Repression in ES Cells**

(A) Experimental design: rapamycin was added for either 7 days or 4.5 weeks and then washed-out with or without Dnmt-inhibitor 5azaC. (B) Flow cytometry analysis after release from csHP1 $\alpha$  after 7 days (low-promoter DNAm) or after 4.5 weeks (high-promoter DNAm).



**Figure 4. *CiA:Oct4* Activation and Kinetics of Heterochromatin Formation in MEFs**

(A) *CiA* E14.5 p4 MEFs were infected with lentiviral constructs of either GAL4 alone or a GAL4-VP16 fusion. Puromycin was added at 48 hr and cells analyzed by flow cytometry.

(B) Experimental design: the *CiA:Oct4* allele was reactivated by GAL4-VP16 in transformed *CiA* MEFs. GFP-positive cells were enriched by FACS and treated with rapamycin to induce csHP1 $\alpha$  targeting.

(C) ChIP analysis of rapamycin-mediated csHP1 $\alpha$  recruitment over time.

(D) ChIP analysis reveals dynamic changes of active (H3K4me3, H3K27ac) and repressive (H3K9me3) histone modifications at the *CiA:Oct4* locus. Upper panel summarizes time course of chromatin remodeling at 0 hr, 24 hr, 48 hr, 72 hr, 96 hr, 120 hr, and 192 hr. Data rotated 180° as indicated to display loss of active marks. Lower panels display loss of active and gain of repressive histone modifications (y axis) across the *CiA:Oct4* locus (x axis) at selected time points. GFP expression was measured by flow cytometry analysis at each time point.

ChIP results represent average and SEM of at least two independent experiments. See also Figures S4 and S5.

flow cytometry, and by 48 hr we observed GFP reactivation in approximately 2% of *CiA* MEFs (Figure 4A). Five days following infection with GAL4-VP16, nearly 10% of the cell population stably expressed the *CiA:Oct4* reporter. This is an especially high proportion considering that the artificially reactivated reporter allele conferred no competitive growth advantage. These results demonstrate that directed recruitment of a strong

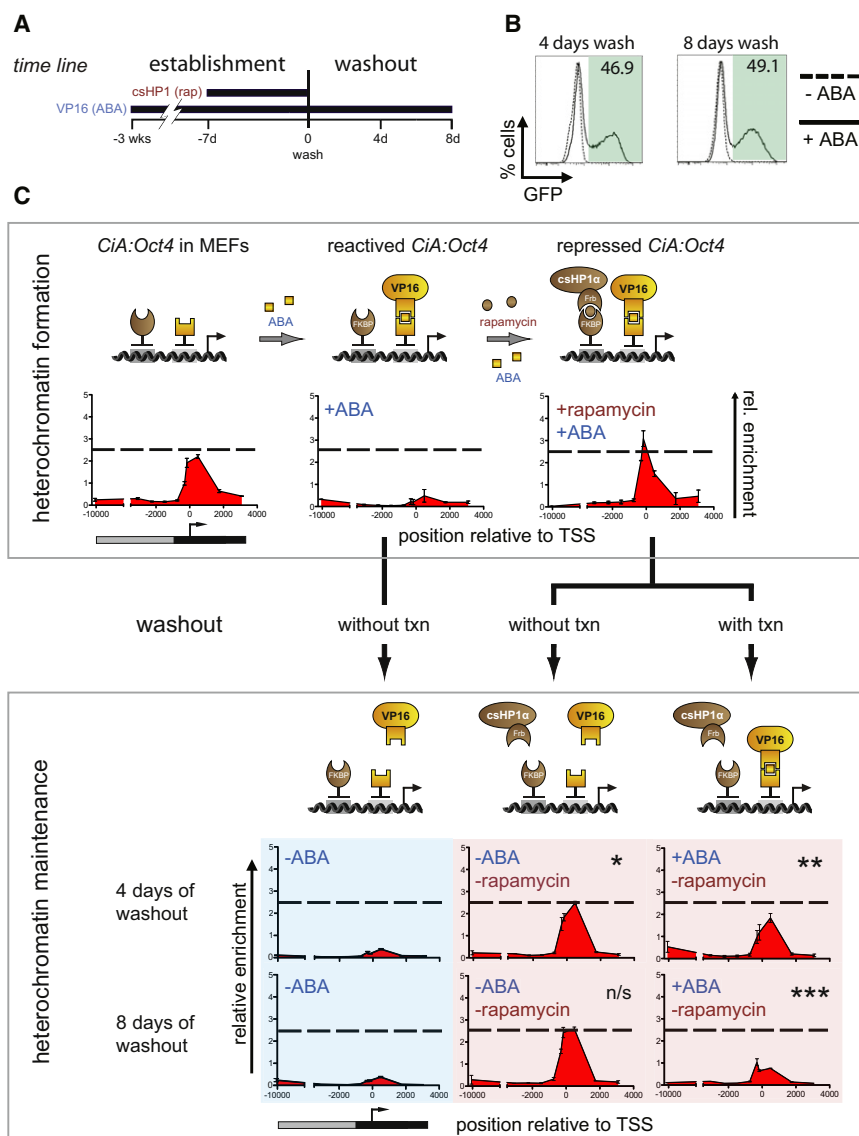
transcriptional activator was capable of rapidly reactivating *Oct4* in differentiated cells.

We generated a uniform cell line by transforming *CiA* MEFs with simian virus 40 large T antigen. We sorted a homogenous population of GFP-positive MEFs and examined the chromatin structure at the *CiA:Oct4* locus. Similar to *CiA* ES cells, sorted VP16-activated MEFs displayed H3K4me3 and H3K27ac and

(C) Colony growth assay tests heritability of *CiA:Oct4* repression. GFP expression of individual colonies was quantified by microscopy (7 days,  $n = 158$ ; 4.5 weeks,  $n = 199$ ).

(D) ChIP analysis of histone modifications after rapamycin washout. The bottom panels depict bisulfite sequencing analysis of DNA methylation at *CiA:Oct4* promoter after csHP1 $\alpha$  washout for 4 days. White lines in schematic below mark relative positions of CpG dinucleotides.

ChIP results represent average and SEM of at least two independent experiments.



**Figure 5. Maintenance of Heterochromatin and Dependence on Transcription**

(A) Experimental design: the *CiA:Oct4* allele was reactivated in transformed CiA MEFs by abscisic acid (ABA)-mediated recruitment of VP16. GFP-positive reactivated cells were enriched by FACS. Rapamycin was added for 7 days to recruit csHP1 $\alpha$ . GFP-negative cells were sorted by FACS. Finally, rapamycin was washed out in the presence or absence of ABA-recruited VP16. Cells were analyzed four and eight days later.

(B) Flow cytometry analysis after removal of csHP1 $\alpha$  in the presence and absence of ABA-recruited VP16.

(C) Cartoon depicts recruitment strategy to form heterochromatin and test its maintenance. ChIP analysis of H3K9me3 along the *CiA:Oct4* allele during heterochromatin formation and after csHP1 $\alpha$  removal with or without ABA-mediated VP16 recruitment for 4 and 8 days. H3K9me3 is maintained after rapamycin washout when not opposed by ABA-mediated transcription (p values: \*p = 0.052, n/s = not significant, \*\*p = 0.007, \*\*\*p = 0.004).

ChIP results represent average and SEM of at least two independent experiments. See also Figure S6.

replication was required for the transition from the active to repressed chromatin state, we halted cell division in CiA MEFs by serum starvation. Similar to cycling cells; nondividing cells rapidly silenced GFP expression and established a heterochromatic domain within 5 days of csHP1 $\alpha$  tethering (Figure S5). Hence, we conclude that replication-dependent histone exchange was not required for chromatin reprogramming, which may involve replication-independent histone variant incorporation or active demethylation instead. In contrast to CiA ES cells, where the heterochromatic domain

lacked significant levels of H3K9me3 (Figures S4). The ability to reactivate the *CiA:Oct4* locus allowed us to examine initiation and maintenance of csHP1 $\alpha$ -dependent gene repression and compare the dynamics of heterochromatin formation between CiA fibroblasts and ES cells. VP16-activated MEFs were infected with ZFHD1-FKBP12 and csHP1 $\alpha$ -2xFRB fusion constructs (Figure 4B). As in CiA ES cells, addition of rapamycin led to rapid csHP1 $\alpha$  recruitment within 24 hr (Figure 4C). GFP expression was readily repressed in reactivated CiA MEFs, resembling the overall rate of silencing observed in CiA ES cells (Figures 4D and 2D). However, both the mode of GFP reduction and the formation of heterochromatin were different. In CiA ES cells, csHP1 $\alpha$  targeting induced a bimodal transition, whereas a gradual reduction of GFP signal was induced in MEFs (Figures 2D and 4D). During the course of GFP silencing, H3K4me3 and H3K27ac were reduced and H3K9me3 was established (Figure 4D). To examine whether nucleosome displacement during DNA

covered approximately 10 kbp, the extent of H3K9me3 in both cycling and starved MEF cells was much more constricted, expanding only about 2 kbp at the *CiA:Oct4* promoter (Figures 4D and S5). These data demonstrated that csHP1 $\alpha$  targeting could overcome strong transcriptional stimulation by VP16 and induce H3K9me3-dependent repression in MEFs despite forming a smaller heterochromatic domain compared to CiA ES cells.

#### Heterochromatin Is Stable in the Absence of Transcription at *Oct4*

We sought to address the interplay between transcription and H3K9me3 maintenance by sequential recruitment and removal of the transcriptional activator VP16 in CiA MEFs. We made use of an orthogonal pair of CIP partners, PYL1 and ABI1, which dimerize upon binding of the plant hormone abscisic acid (ABA) (Liang et al., 2011). CiA MEFs were infected with lentiviral constructs containing GAL4-ABI1 and PYL1-VP16 (Figure 5A).



Upon addition of ABA, VP16 was recruited to the *CiA* locus, resulting in removal of repressive histone modifications and GFP reactivation to a similar extent as the direct fusion of GAL4-VP16 (Figures 4D and 5C). GFP-positive cells were enriched by FACS and maintained consistent GFP expression in the presence of ABA for weeks without defects in growth or morphology. However, within 4 days after ABA removal, more than 99% lost detectable GFP expression and H3K9me3 (Figure S4). Importantly, ChIP analysis did not reveal reemergence of a H3K9me3 domain, indicating that heterochromatin does not form spontaneously without transcription (Figure 5C, blue panel). In this context, the chromatin state of the *CiA:Oct4* locus could be considered as “neutral” for further experimentation on H3K9me3 stability.

Heterochromatin was induced by rapamycin-mediated recruitment of csHP1 $\alpha$  for 7 days in VP16-activated *CiA* MEFs. We then tested H3K9me3 stability through cell division in the absence of the initiating csHP1 $\alpha$  stimulus. Rapamycin was removed and cells with or without ABA (recruiting VP16) were analyzed after 4 and 8 days of washout (Figure 5). Unlike in *CiA* ES cells, H3K9me3 was stably maintained through cell division for at least 8 days following rapamycin washout in absence of ABA (Figure 5C, red panels). H3K9me3 levels remained unchanged even when maintenance of DNA methylation was inhibited with 5azaC (Figure S6). In contrast, upon VP16 recruitment, GFP expression was significantly increased (Figure 5B) and coincided with a reduction of H3K9me3 close to basal levels after 8 days of rapamycin washout. This result indicated that strong transcriptional activation could overcome repressive chromatin structure in the absence of csHP1 $\alpha$  tethering similar to our results in *CiA* ES cells (Figure 5C, red panel). Most importantly however, these results demonstrated that H3K9me3 could be transmitted undiminished through numerous cell divisions in the absence of the initial stimulus.

### Model of Heterochromatin Dynamics Reveals In Vivo Rates of Histone Modification

We sought a quantitative model for our observations to rationalize the observed kinetics and spatial distribution of these marks and to define the rates of heterochromatin formation in vivo. One model of histone marking has provided insight into metastable H3K9me switching in the yeast *mate*-type locus (Dodd et al., 2007). However, this model does not accurately account for the localized peaks and soft borders of the heterochromatic islands observed in our experiments (Figure S7).

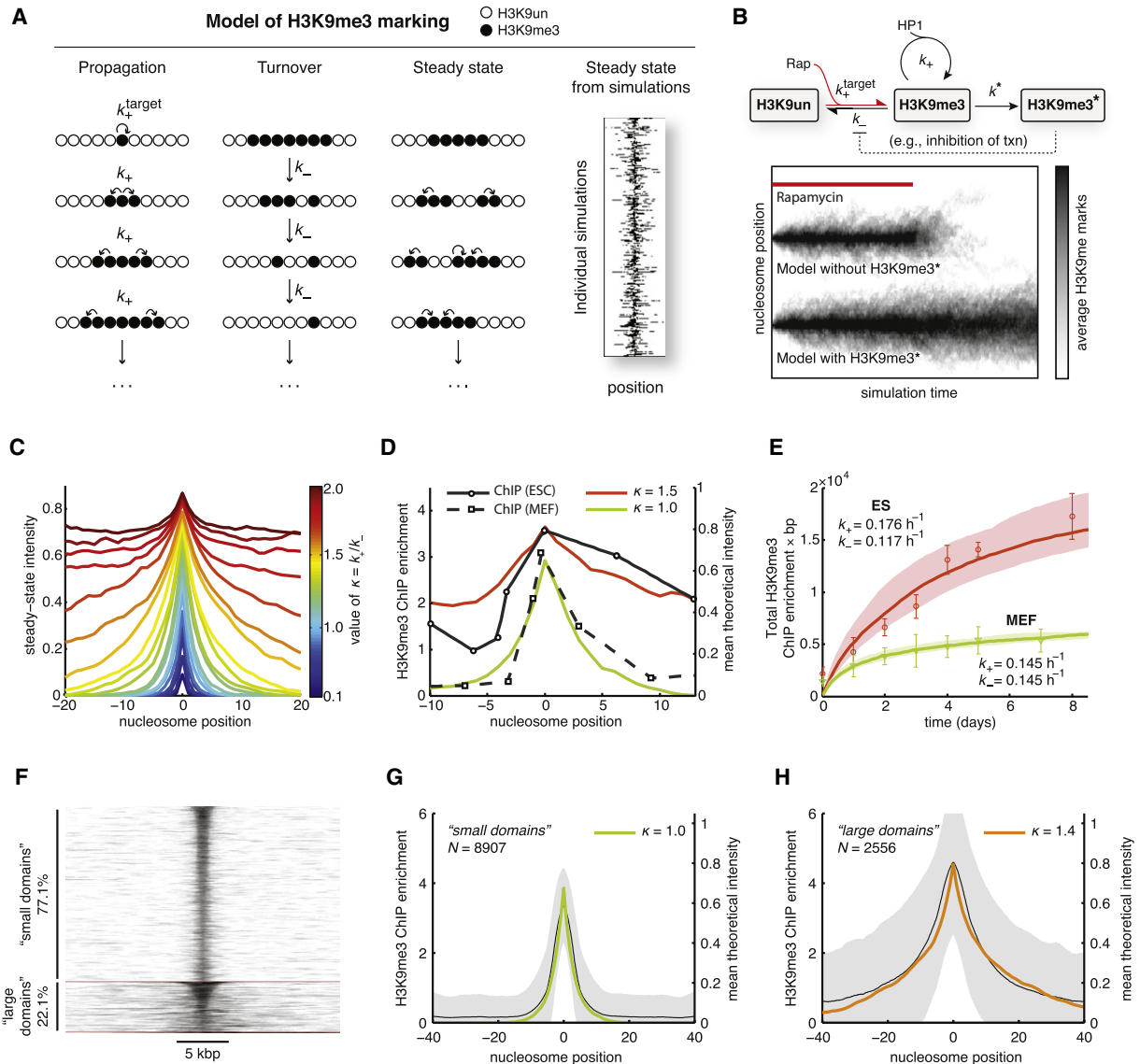
We therefore developed a novel and generalizable steady-state kinetic scheme that can be validated by Monte Carlo simulation. We considered the *CiA:Oct4* locus as a one-dimensional lattice, with each lattice position corresponding to an individual nucleosome (full details in Extended Experimental Procedures). We reasoned that H3K9me3 dynamics at a given locus would be governed by processes involved in either addition or removal of the mark. Our general kinetic scheme integrates all processes that propagate H3K9me3 into a single net propagation rate ( $k_+$ ), and integrates all processes that result in H3K9me3 removal into a separate turnover rate ( $k_-$ , see Figure 6A). In our model,  $k_+$  describes the net rate of H3K9me3 addition at nucleosomes adjacent to H3K9me3-marked sites. In this model, H3K9me3

spreading along the chromosome occurs exclusively through linear propagation to neighboring nucleosomes, consistent with our experimental results and the proposed propagation model via HP1 oligomerization (Bannister et al., 2001; Canzio et al., 2011; Hall et al., 2002; Lachner et al., 2001; Schotta et al., 2002). Unlike propagation, we reasoned that turnover of H3K9me3 is equally likely everywhere, thus  $k_-$  describes the stochastic turnover of H3K9me3 at any marked nucleosome. In our model, the origin corresponds to the site of csHP1 $\alpha$  recruitment; H3K9me3 marks are nucleated at this unique site at rate  $k_+^{\text{target}}$  in the presence of the CIP (i.e., rapamycin).

Our kinetic model thus describes a single nucleation site, from which H3K9me3 can be placed at rate  $k_+^{\text{target}}$ , and propagated to neighboring sites at rate  $k_+$ . This propagation is opposed by random turnover at rate  $k_-$ . According to this scheme, a stochastic, bounded steady-state domain of H3K9me3 centered on the csHP1 $\alpha$  nucleation site was established (Figure 6A, right panel). This simple scheme led to the formation of a stable heterochromatic domain, which peaked at the nucleation site and displayed soft continuous borders, consistent with our observations in *CiA* ES cells and MEFs. Moreover, under these conditions the model predicted a rapid collapse of H3K9me3 upon withdrawal of the initiating stimulus (Figure 6B, lower panel). This was similar to the lack of H3K9me3 maintenance observed in *CiA* ES cells. Thus, additional mechanisms might be required to support H3K9me3 stability and epigenetic memory. We extended our dynamic competition model of H3K9me3 marking to include an additional transition that stabilized the H3K9me3 state, which we call H3K9me3\*. We reasoned that this stabilization process occurs at a slow rate,  $k^*$ , and only at sites that are already H3K9me3 modified. In our simulations, this parameter led to sparse transitions to the H3K9me3\* state, which prevented H3K9me3 turnover (Figure 6B, upper panel). Importantly, with this feedback mechanism, the H3K9me3 domain was stably maintained even upon withdrawal of the initial stimulus (Figure 6B). We conclude that besides HP1 $\alpha$ , additional heritable feedback mechanisms (e.g., lack of transcriptional activators, DNA methylation, or chromatin compaction of the locus) are necessary to ensure epigenetic memory of H3K9me3 silencing.

Next, we determined the cellular modification and turnover rates by comparing our empirical measurements to our model of H3K9me3 marking. Our kinetic model indicated that a restricted domain of H3K9me3 naturally arises from the dynamic competition between marking and turnover when the ratio  $\kappa = k_+/k_- \leq 1.5$ . At values of  $\kappa > 1.5$ , the domain of H3K9me3 marks spreads without bounds (Figure 6C). Despite the simplicity of the kinetic scheme, the profiles of the H3K9me3 domains established in simulations under these constraints fit well to our data (Figure 6D). The profile of the H3K9me3 domain in *CiA* ES cells is best described by  $\kappa \sim 1.5$ , and in *CiA* MEFs by  $\kappa \sim 1.0$ . We conclude that slight differences in the relative rates of propagation or turnover can explain the observation that the H3K9me3 domain is characteristically larger in *CiA* ES cells.

We then obtained specific values for nucleosome modification and turnover ( $k_+$  and  $k_-$ ) in each cell type by comparing the empirical rates of H3K9me3 accumulation measured by ChIP. As described above, the ratio  $\kappa$  was obtained by fitting the



**Figure 6. Kinetic Model of H3K9me3 Dynamics**

(A) We consider chromatin as a one-dimensional beads-on-a-string lattice. Three processes: nucleation, propagation and turnover, yield a bounded steady-state island of marks. Nucleation occurs at the target site at rate  $k_+$ . Propagation of the mark continues at sites immediately adjacent to marked sites, at rate  $k_+$ . Turnover of the mark is equally likely everywhere at rate  $k_-$ . When these processes are allowed to occur at the same time, a stochastic, bounded island of H3K9me3 marks is established at steady state. Sample output of the model with H3K9me3 domains at steady state (right panel; each horizontal line represents a single simulation).

(B) Simplified kinetic scheme of H3K9me3 dynamics. Without a feedback mechanism to reinforce placement of H3K9me3 marks, the domain collapses in the absence of continued nucleation (lower panel). In the presence of a feedback mechanism that stabilizes H3K9 methylation (denoted by H3K9me3\*) the domain persists.

(C) The profile of the steady-state island varies with  $\kappa$  (defined in main text). Larger values of  $\kappa$  increase the size of the island until  $\kappa > 1.5$ ; above this value, the island grows without bounds.

(D) Fits of the experimental H3K9me3 ChIP data shown in Figure 2 to the kinetic model. Data from ES cells are best described by  $\kappa = 1.5$ , whereas the data from MEFs are described by  $\kappa = 1.0$ .

(E) Specific values of  $k_+$  and  $k_-$  were obtained by fitting the simulations to a time course of integrated H3K9me3 ChIP enrichment at the locus (see Figure 2). Resulting values of  $k_+$  and  $k_-$  are shown next to the data for each cell type. Our estimated uncertainty in these values is 35% (shaded regions).

(F) Clustering of genomic H3K9me3 domains in ES cells (Bilodeau et al., 2009) by  $k$ -means, with  $k = 3$ . Clustering identified two predominant groups ("small" and "large" H3K9me3 domains), and a very small number of aberrant domains.

(G) Small H3K9me3 domains (mean  $\pm$  SD) are described well by our model with  $\kappa = 1.0$ .

(H) Large H3K9me3 domains (mean  $\pm$  SD) are described well by our model with  $\kappa = 1.4$ .

See also Figure S7.

simulation profile to the in vivo steady-state H3K9me3 domain (Figure 6D). This ratio constrains the relationship between  $k_+$  and  $k_-$ , requiring us only to align the timescale of the simulations to the experimental data to obtain the real rates. To perform this fit, we considered that each simulated nucleosome plus internucleosomal DNA covers a length of 200 bp and integrated our ChIP enrichment values within 3 kbp of the transcription start site. We also used the same relationship between ChIP enrichment and theoretical intensity shown in Figure 6D. We found that the rates  $k_+$  and  $k_-$  in ES cells are respectively  $0.176 \text{ h}^{-1}$  and  $0.117 \text{ h}^{-1}$ , and in MEFs are respectively  $0.145 \text{ h}^{-1}$  and  $0.145 \text{ h}^{-1}$  (Figure 6E). We estimated the uncertainty in these values to be  $\sim 35\%$ . Thus, we conclude that H3K9me3 marks propagate along the chromosome, marking neighboring nucleosomes on average every  $\sim 5.7 \text{ hr}$  in ES cells and every  $\sim 6.9 \text{ hr}$  in MEFs.

### Kinetic Model Predicts Shapes of Genomic H3K9me3 Domains

We next compared the predictions of our H3K9me3 model with previously published H3K9me3 ChIP-seq data from mouse ES cells (Bilodeau et al., 2009). Using  $k$ -means clustering, we clustered 11,556 H3K9me3 domains into three groups (Figure 6F; full details in Extended Experimental Procedures). This clustering separated the H3K9me3 data into similarly sized “small domains” (8,907; 77.1% of the data), “large domains” (2,556; 22.1%), and a small number of “aberrant domains” (93; 0.8%), which did not fit well in either group.

Within the “large” and “small” H3K9me3 domains, we compared the class averages of H3K9me3 ChIP intensity to our model. The average profiles of both large and small domains displayed localized H3K9me3 peaks with shapes that could be readily fitted to our model. Small H3K9me3 domains were described by  $\kappa = 1.0$  (Figure 6G), and large domains fit well with  $\kappa = 1.4$  (Figure 6H). Unlike H3K9me3 peaks, H3K36me3 is broadly distributed over active gene bodies forming plateau-shaped domains (Mikkelsen et al., 2007). Genomic H3K36me3 profiles did not cluster well into groups consistent with our model (not shown). We conclude that the general model described above is consistent with the profiles of 99.2% of noncentromeric H3K9me3 domains.

## DISCUSSION

### Kinetics and Bounding of Heterochromatic Domains

The temporal control achieved with the CiA system provided the motivation to develop a mathematical model of the kinetics and spreading of H3K9me3. We reasoned that at steady-state H3K9me3 is governed by dynamic competition of opposing activities with a rate of addition ( $k_+$ ) and a rate of H3K9me3 turnover ( $k_-$ ). Thus, the ratio of these two rates weighs the relative contributions of various cellular processes, including csHP1 $\alpha$  recruitment, H3K9 methylation, demethylation, and nucleosomal turnover. Remarkably, by modeling different ratios of these two rates ( $\kappa$ ), we identified good fits to the spatial distributions of H3K9me3 in both ES cells and fibroblasts. Based on these results, we were able to define the net rates of H3K9 methylation as well as the turnover rates of modified histones at the CiA:Oct4 locus in ES cells and fibroblasts. The estimated net turnover rate

$k_-$  approximates previously measured rates of global histone turnover in HeLa S3 cells (Zee et al., 2010) and rates of histone H3 displacement from chromatin in *Drosophila* S2 cultures (Deal et al., 2010), supporting the validity of our model (Figure S7). Furthermore, our model suggests that heterochromatic boundaries need not necessarily be limited by local insulator elements. Instead, transitions between heterochromatin and euchromatin might reflect a gradual shift in the balance of activities that add and remove histone modifications. In CiA cells, H3K9me3 marking gradually decreases to either side of the csHP1 $\alpha$  recruitment site. According to the model, this is consistent with a reduction in the maintenance of heterochromatin with increasing distance from the initiation site.

In addition to heterochromatin formation at the CiA:Oct4 locus, our model accounts for endogenous steady-state distribution profiles of H3K9me3 domains throughout the mouse genome. Indeed, an overwhelming majority (99.2%) of noncentromeric H3K9me3 domains can be described well by our model with a single variable parameter. This general parameter,  $\kappa$ , describes the rate of H3K9me3 propagation relative to mark turnover at a given nucleosomal position. Although these individual rates may vary considerably throughout the genome, we find that the value of  $\kappa$  falls within a narrow range for nearly all H3K9me3 domains. This may reflect that H3K9me3 domains are compacted and less accessible, and therefore the turnover rates within the domains are expected to be quite slow. In this case, even modest variation in the propagation rate will result in larger or smaller H3K9me3 domains.

### Memory and Propagation of H3K9me3 through Cell Generations

The ability to initiate heterochromatin formation and then to terminate csHP1 $\alpha$  recruitment allowed us to examine the epigenetic properties of H3K9me3 through cell division. We found that the stability of H3K9 methylation domains differed between cell types and varied in the context of transcription and DNA methylation. Chemically induced H3K9me3 was stably maintained through cell divisions upon removal of the csHP1 $\alpha$  stimulus in CiA MEFs. In the absence of induced transcription, this maintenance did not rely on DNA methylation, as enrichment and extent of H3K9me3 was retained at low levels of promoter methylation and also after 5azaC treatment. However, upon washout of the csHP1 $\alpha$  stimulus and recruitment of a potent transcriptional activator, H3K9me3 retention was compromised. The collapse of heterochromatin upon transcriptional activator recruitment resembled the rapid loss of H3K9me3 after washout in CiA ES cells with low DNAm or after treatment with 5azaC. Although GFP reactivation was also detected in ES cells with high DNAm, this population was significantly smaller, suggesting that high levels of DNA methylation could enhance heterochromatin stability in these cells. We conclude that H3K9me3 is an epigenetic mark in the strict sense that it persists after removal of the initial stimulus. However, recruitment of transcriptional activators could disrupt heritable maintenance indicating the reversible nature of this modification. This scenario might occur naturally in the context of signaling-dependent gene activation, in which a transcription factor enters the nucleus to induce transcription and erases a repressive histone mark.

In our model of dynamic competition, maintenance of the heterochromatic domain after rapamycin withdrawal required the establishment of a stabilized state, which we refer to as H3K9me3\*. One could imagine that loss of transcriptional activators, gain of DNA methylation, changes in nuclear localization, and/or higher-order chromatin structure may contribute to the increased stability of the H3K9me3\* state. Our experiments in MEFs indicated that the absence of transcriptional opposition is an important component of the H3K9me3\* state. Along these lines, enhanced H3K9me3 stability in ES cells with high DNAm likely reflects the ability of CpG methylation to interfere with transcriptional activation at the *Oct4* promoter.

### Implications for Artificial Modulation of Mammalian Gene Expression Programs

Our model faithfully captures the steady-state dynamics of heterochromatin with the ratio  $\kappa = k_+/k_-$ , where the opposing effects of histone marking and mark turnover are sufficient to establish an inherently bounded H3K9me3 domain. Synthetic recruitment of the transcriptional activator VP16 to the *CiA:Oct4* locus in MEFs illustrates how disruption of steady-state conditions can compromise the maintenance of chromatin and expression state. In keeping with our model, we speculate that this rapid reactivation reflects a sudden shift in the dynamic balance in favor of  $k_-$  due to recruitment of transcriptional machinery. Even in terminally differentiated primary mouse fibroblasts, we observed GFP reactivation within 24 to 48 hr of direct recruitment of VP16 to the *CiA:Oct4* locus. This demonstrates that strong transcription alone can rapidly override the multiple epigenetic mechanisms involved in silencing a single allele.

We demonstrate that the tight temporal control made possible by chemically induced proximity enables quantitative studies in different cell types, which in the future could be extended to different chromatin regulatory mechanisms. Furthermore, the *CiA* system is adaptable for high-throughput screening for small molecules and natural modifiers of chromatin regulation. Such studies could lead to a better understanding of the mechanisms involved in establishment and maintenance of stable gene expression. We anticipate that integrative models of chromatin dynamics in living cells will be required to understand how gene regulation is achieved through modulation of chromatin structure.

## EXPERIMENTAL PROCEDURES

### Construction and Culture of Chromatin In Vivo Assay at *Oct4*

#### (*CiA:Oct4*) ES Cells

A BAC containing the mouse *Pou5f1* locus was manipulated through recombining (see details in [Extended Experimental Procedures](#)). Cells were cultured by using standard conditions (see [Extended Experimental Procedures](#)).

### Generation and Culture of Chromatin In Vivo Assay MEFs

*CiA* ES cells were injected into BI/6 derived blastocysts and implanted into surrogate mothers. *CiA:Oct4* allele presence was confirmed by agouti coat color and Southern blot. MEFs were produced and cultured by using standard conditions. See [Extended Experimental Procedures](#) for full details.

### Construct Design and Chemical Induction of Proximity

All constructs were created in a modified lentivirus backbone with EF1- $\alpha$  promoter driving the gene of interest and a second PGK promoter driving

production of a gene resistant to selection. All essential plasmids can be obtained from Addgene. Details on lentiviral production and chemical induction technique in [Extended Experimental Procedures](#).

### RA Assay and Western Blots

*CiA* ES cells were treated with Retinoic Acid at 5  $\mu$ M for indicated time. Lysates were collected in RIPA buffer (150 mM NaCl, 1% triton, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0). 30  $\mu$ g/lane total protein was run on 4%–12% Bis-tris gel, transferred to PVDF membranes and imaged by Infrared fluorescence (Li-Cor Biosciences) with the following antibodies: Oct-3/4 (Santa Cruz Biotech, SCBT-9081), GFP (Clontech, 632375), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotech, SCBT-32233 or SCBT-25778).

### Flow Cytometry Analysis and Sorting

All Flow cytometry analysis was performed on an LSR II (BD Biosciences) and analyzed with FlowJo software, individual cells were gated based on forward and side scatter, autofluorescent cells were omitted, and remaining cells were then analyzed for GFP levels. Cells were sorted by using an Aria or Aria II (BD Biosciences).

### ChIP Analysis

ChIP was essentially performed as described previously ([Mohn et al., 2008](#)); complete details are in [Extended Experimental Procedures](#). Antibodies used for ChIP are as follows: H3K4me3 (Millipore, 05-745R), H3K27me3 (Millipore, 07-449), H3K9me3 (Abcam, ab8898), H3K27ac (Abcam, ab4729), HP1 gamma (Millipore, 05-690), V5 (Invitrogen, 46-0705), GAL4 (Santa Cruz Biotechnology, sc510), and Oct-3/4 (Santa Cruz Biotech, SCBT-9081). Primers used for real-time PCR listed in [Table S1](#).

### Bisulfite Sequencing Analysis of DNA Methylation

One microgram genomic DNA was bisulfite converted with the EpiTect Bisulfite Kit (QIAGEN). Endogenous and knock-in-specific *Oct4* promoter sequences were amplified by PCR and PCR products were cloned by using TOPOTA cloning kit (Invitrogen) followed by sequencing. Methylation profiles were analyzed by using BiQ Analyzer software ([Bock et al., 2005](#)). Primers for PCR amplification are listed in [Table S1](#).

### DNase I Sensitivity Assay

DNase I sensitivity assay was carried out as previously described ([Lu and Richardson, 2004](#)); see [Extended Experimental Procedures](#).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at [doi:10.1016/j.cell.2012.03.052](https://doi.org/10.1016/j.cell.2012.03.052).

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