Basic properties of epigenetic systems: lessons from the centromere
Mariluz Gómez-Rodríguez and Lars ET Jansen

Chromatin-based epigenetic inheritance cooperates with cis-acting DNA sequence information to propagate gene expression states and chromosome architecture across cell division cycles. Histone proteins and their modifications are central components of epigenetic systems but how, and to what extent, they are propagated is a matter of continued debate. Centromeric nucleosomes, marked by the histone H3 variant CENP-A, are stable across mitotic divisions and are assembled in a locus specific and cell cycle controlled manner. The mechanism of inheritance of this unique chromatin domain has important implications for how general nucleosome transmission is controlled in space and time.

Address
Instituto Gulbenkian de Ciência, 2780-156, Oeiras, Portugal

Corresponding author: Jansen, Lars ET (ljansen@igc.gulbenkian.pt)

Introduction
How cellular gene expression profiles and associated phenotypes are inherited is a fundamental question in biology. Inheritance of the cellular state does not solely depend on the DNA sequence, as cells with an identical genetic content, that is, within one organism, often display very different morphologies and functions. Instead, this epigenetic inheritance is driven by non-DNA sequence based factors that have the capacity to carry information across mitotic divisions. Chromatin-based proteins likely include DNA methylation, histone variants and their modifications as well as DNA or histone binding proteins. These candidates are not only proposed to functionally mark chromatin by controlling gene expression in cis, but also to physically maintain a memory of this functional state. We propose that for an epigenetic system to sustain itself, three characteristics will be critical to consider: (1) stability of the mark across cell division, (2) its capacity to template its own duplication and (3) cell cycle control of its duplication. The centromere provides an intriguing yet powerful example of an epigenetic system for which these parameters have been examined in some detail. We will review different chromatin-based epigenetic systems and discuss differences and similarities with centromeric chromatin that may serve as a model for understanding general mechanisms of epigenetic inheritance.

Stable transmission of chromatin across cell division
A simple solution to the problem of stably maintaining cellular information is that the agent carrying this information is itself stably transmitted across the cell cycle. In the case of DNA, the paradigm of molecular inheritance, analysis of cell turnover indicates that individual DNA molecules can be maintained in vivo for decades [1**]. A straightforward hypothesis is that, like DNA, epigenetic marks are stable and by virtue of this can maintain cellular memory. Histones, the DNA-associated proteins that, together with DNA, make up the bulk of chromatin have been shown to be relatively stable in dividing cells. Early work used protein synthesis inhibitors in cells to prevent deposition of new histones, metabolic pulse labeling and in vitro replication assays to demonstrate that parental nucleosomes can be stably maintained or reformed on nascent DNA following DNA replication [2*,3,4] (Figure 1). Recent isotope pulse labeling followed by mass spectrometry analysis of conditionally expressed histone H3 variants in HeLa cells, confirmed the global stability of H3 with significant retention of histones even after one or more cell division cycles [5,6*]. Inclusion of different histone variants or post-translational modifications at specific genomic loci are intimately linked to maintaining differential gene expression patterns [7,8]. Together, these features make histones attractive candidates to carry epigenetic memory. How nucleosomes are maintained on DNA is a matter of intense investigation as propagation of histones is challenged by disruptive processes like transcription and DNA replication. In the latter case, the MCM2-7 helicase disrupts histone–DNA contacts ahead of the replication fork. An attractive model for nucleosome retention involves this same helicase complex that binds parental histones with high affinity and hands them off to the ASF1 chaperone that in turn guides assembly into the nascent strand following replication [9*]. An octamer of histones is composed of one (H3-H4)2 tetramer and two H2A-H2B dimers that together with ~150 base pairs of DNA forms a nucleosome [10]. In vivo FRAP (fluorescence recovery after photobleaching) studies indicate that the inner (H3-H4)2 core of the nucleosome is extremely stable, with
a majority of GFP-tagged H3/H4 showing no turnover within 8 hours following photo bleaching, whereas H2A-H2B dimers are more dynamic [11**]. While nucleosomes are stable in bulk, little is known about the in vivo stability of histones at specific genomic loci. This is of particular interest in the case of the H3 variant H3.3 that is localized to active genes and regulatory regions and implicated in the maintenance of gene activity during development [12–14]. Elegant in vivo pulse-chase experiments in yeast show that parental H3 histones tend to accumulate near the 5′ ends of long genes expressed at a low level [15**]. While the ancestral histone H3 was detected for multiple cell divisions, the absolute rate of histone turnover was not determined in this study or in other model systems.

**CENP-A: a stable component of centromeric chromatin**

The centromere is a chromosomal region that functions as a platform for the assembly of the kinetochore, which drives segregation of chromosomes to daughter cells during cell division [16,17]. Identity and position of the centromere locus in dividing cells is not directly determined by a particular DNA sequence, but largely by its unique chromatin organization involving the histone H3 variant CENP-A [18,19]. Owing to its distinct localization, CENP-A has provided a unique opportunity to assess the in vivo histone turnover rate at a specific locus using imaging-based methods. Turnover has been analyzed by employing SNAP, a self-labeling enzyme that covalently and irreversibly reacts with the small molecule benzylguanine [20,21]. By using fluorescent derivatives of this substrate, specific pools of SNAP-tagged CENP-A can be fluorescently labeled and tracked over time. Such pulse-chase labeling experiments, along with photo bleaching experiments in yeast, human cells, and *Drosophila* have shown that CENP-A remains stably associated with centromeres. Individual molecules are maintained through several cell divisions, apparently only lost by replicative dilution during S-phase [22*,23*,24]. Such high stability in chromatin is consistent with maintaining an epigenetic memory of centromere identity. This is further supported by recent reports showing that artificially generated CENP-A chromatin can nucleate an active kinetochore and a heritable centromere [25*,26,27**]. While centromere and kinetochore function
depend on many components, these experiments show that CENP-A is sufficient to nucleate this structure. Thus, the centromere appears to adhere to a model in which stable transmission of a unique class of nucleosome is central to maintaining epigenetic memory.

**Dynamic nucleosome turnover and epigenetic inheritance**

While stable maintenance of a chromatin mark may be the most parsimonious cellular solution, it is not a necessary property of an epigenetic system. Alternatively, the epigenetic state can be propagated by a cooperative set of components that are individually dynamic but collectively maintain a positive feedback loop (Figure 1). This view is supported by theoretical work showing that, even under rapid histone turnover, cooperative positive feedback allows for stable maintenance of either an active or repressed chromatin state [287]. Indeed, measurement of incorporation rates of de novo synthesized histones by Deal et al. revealed that rapid turnover occurs at specific loci, which include active gene bodies and promoters, regulatory elements and replication origins in *Drosophila melanogaster* reflecting a half-life on the order of 1 hour [29**]. While such fast dynamics was interpreted to argue against a prominent role for histones in epigenetic memory of the transcriptional state, it is possible they form a part of a multi-component feedback loop. Analogous to a relay run, individual histones may carry the burden of epigenetic information part of the time, while passing the baton to other components at other stages (e.g. neighboring histones, histone modifications or non-histone proteins, see below). However, it should be noted that the Deal et al. study [29**] is limited to the detection of the dynamic subset of nucleosomes which may not be representative of all nucleosomes at a locus. It is possible that different subsets of nucleosomes exist with different dynamics. To resolve this it will be important to probe the entire histone population at a locus and have a quantitative measure of its dynamics at multiple time points.

**Transmission of DNA methylation, histone modifications and non-histone proteins**

Modification of DNA and histones (e.g. by methylation) is associated with epigenetic memory of gene expression states [7,8,30]. Cytosine methylation has been tightly linked to long term gene silencing, for example, in X-chromosome inactivation or genomic imprinting [31]. The mechanism of propagation of DNA methylation is well established and is directly coupled to duplication of DNA ([32] and see below). Little is known about the molecular dynamics of the methyl mark itself. While DNA methylation is likely to be a stable modification in general, patterns of CpG methylation can be changed or globally erased during specific developmental stages, for example, during early development. This is driven by several demethylation activities that have only recently been uncovered [30] (Figure 1).

Methylation of histone H3 (e.g. at lysine 9 or 27) is linked to propagation of silent chromatin. A recent report showed that experimental deposition of H3K9 trimethylation at the Oct4 locus in mouse cells leads to the maintenance of this mark and the silent state for several days in culture in the absence of the initial trigger [33**]. This indicates that histone methylation forms part of the epigenetic memory that heritably maintains gene silencing. The maximum stability of a histone modification will be equal to that of the histone to which it is attached. Classic pulse-chase experiments have suggested that turnover of methyl groups on histones is slow [34]. Recent quantitative mass spectrometry experiments based on isotope pulse labeling, differentiated turnover rates of histones carrying specific marks. Although this study does not assess the dynamics of the modifications themselves, the histone peptides carrying those marks showed different stabilities. These vary from ~2.5-fold turnover per day for active acetyl marks on H3 to a turnover of only 30% of histones H4 trimethylated at lysine 20 that represents repressed chromatin [6]. Analysis of histone modification at active genes during the cell cycle indicates that significant levels of H3 lysine 4 and 79 methylation as well as H3 and H4 acetylation remain present in S-phase or mitotically arrested cells [35**]. However, a recent study in *Drosophila* challenged the view that histone methylation at lysine 4 and 27 are carriers for epigenetic memory, at least during S-phase, as these appear to be depleted from replicating chromatin in embryonic tissues and reappear later in the cell cycle [36**]. While retention of low levels of histone modifications through DNA replication cannot be excluded, this study argues that if histone marks carry any epigenetic memory they may do so only part-time, at least in this system (Figure 1). The molecular dynamics of histone modifications may be highly dependent on the developmental state, as is the case for DNA methylation.

The non-histone Triithorax and Polycomb group proteins that can act as both readers and writers of methylated lysines 4 and 27 on histone H3 are attractive candidates to form part of this relay-run team carrying cellular memory forward. The Polycomb repressive complex 1 (PRC1), implicated in heritable gene silencing, remains bound to DNA through S phase *in vitro* and *in vivo* [36**,37**,38]. Initial reports have indicated that little to no Polycomb is retained on mitotic chromatin [39,40]. However, a recent study has shown that a small but significant fraction of PcG protein complexes remain bound in mitosis with a rate of turnover much slower than that found in interphase [41]. PcG proteins may therefore be maintained around the cell cycle clock at least at some loci. However, it is unlikely that this represents a centromere-like mechanism where individual PcG proteins remain locked in
chromatin at a time scale exceeding that of the cell cycle. Instead, in vivo dynamics of PRC1 subunits on chromatin is very high, with residence times of seconds to minutes [41*,42*].

In summary, histones clearly maintain a capacity to be stably transmitted across the cell cycle which is exemplified by the centromeric histone CENP-A. However, rates of histone retention along with their associated marks may vary across the genome. To what extent histones or histone modifications are stable at loci relevant for gene expression control remains a matter of current debate.

Mechanisms of self-templated duplication

The second characteristic of an epigenetic system that we consider is the ability to copy information into novel structures so as to maintain cellular information through cell division and growth. DNA is a prime example of a self-templating molecule as the bases form a structural template for the opposing strand [43]. Another classic example is structural templating of cortical ciliary units in *Paramecium*, which, once experimentally inverted in polarity, are maintained in this position for more than 700 cell generations [44]. Similarly, self-templating is observed in prion proteins that through a parallel beta sheet structure exhibit the capacity to convert nascent protein into the prion form [45]. These examples of direct templated duplication indicate that inheritance of structural information is a widespread phenomenon. How this works for chromatin based marks, such as methylation of DNA and histone tails is much less obvious. CpG methylation marks do not structurally template the formation of a new methyl cytosine. Instead, the DNA methyltransferase DNMT1 directly binds hemimethylated DNA that is created in the wake of the DNA replication fork and methylates the nascent CpG site on the complementary strand [32]. Reading and writing of the mark is therefore contained within a single enzyme (Figure 2).

The chromatin component HP1, involved in heterochromatic gene silencing, binds to methylated H3K9 as well as the histone methyltransferase SUV39H1 to produce novel methylated H3K9 residues on surrounding nucleosomes [46**,47**]. The Polycomb repressive complex 2 (PRC2) is required for developmentally regulated silencing of gene expression. Analogous to HP1/SUV39H1, PRC2 harbors a component that recognizes methylated H3K27 that leads to the allosteric activation of a methyltransferase activity to methylate novel neighboring H3K27 sites [48**,49]. As a result, models for H3K9me3 and H3K27me3 transmission have been evoked that consist of a closed positive feedback loop involving a limited set of proteins that can, in principle, propagate this histone mark following erosion or DNA replication (Figure 2).

While the inheritance of the centromere mark may be limited to the stable retention of a specific histone, the duplication of this structure involves a much more elaborate mechanism. Rather than a post-translational modification, here the mark constitutes an entire variant nucleosome, containing CENP-A. Therefore, the self-templating mechanism involves the recruitment of nascent CENP-A to loci already carrying CENP-A. How does this work? As direct structural templating at the level of CENP-A nucleosomes appears unlikely, one or more adaptors may direct CENP-A deposition (Figure 2). In line with this, CENP-C and CENP-N, two constitutive centromere components, recognize and bind specifically to CENP-A in chromatin and both factors are, partly, required for recruitment of new CENP-A [50,51]. Nascent CENP-A is complexed with the CENP-A specific chaperone, HJURP, that guides CENP-A to the centromere [52,53]. However, how HJURP recognizes the centromere is unclear. HJURP targeting to the centromere depends on a complex of proteins known as the Mis18 complex [25,54,55] which may in turn be recruited through CENP-C [56]. However, with the exception of fission yeast [57] there is no evidence for a direct molecular interaction between the Mis18 complex and HJURP. Instead, Mis18 may change centromeric chromatin structure and this might indirectly result in HJURP recruitment. Consistent with this, the Mis18 complex has been suggested to affect the acetylation state of chromatin [54,58**]. Moreover, HJURP recruitment to engineered centromeres has been shown to depend on transcriptionally active chromatin harboring nucleosomes that are H3K4 dimethylated [59]. The emerging picture is one where multiple factors and chromatin components contribute to a feed forward mechanism in which recruitment of CENP-A to chromatin is promoted at CENP-A containing sites (Figure 2). Importantly, neighboring non-CENP-A containing nucleosome that includes H3.1 and H3.3 nucleosomes may play a role in locally promoting CENP-A assembly [60,61].

Cell cycle control of chromatin inheritance

Finally, we consider the temporal regulation of the propagation of epigenetic systems. The duplication of genetic information contained in genomic DNA is tightly controlled and in synchrony with cell division to ensure precise replication only once per cell cycle. By extension, turnover or dilution of epigenetic marks owing to cell division necessitates control over the extent at which new epigenetic structures are generated in order to prevent overduplication or underduplication (Figure 3). Indeed, both the propagation of CpG methylation by DNMT1 as well as the assembly of canonical H3.1 nucleosomes by the chromatin assembly factor CAF-1 are directly linked to DNA synthesis through an interaction with the replication factor PCNA [62,63], although propagation of CpG methylation by DNMT1 is largely driven through its preference for hemi-methylated DNA [32,64]. However,
cell cycle coupling is not only exerted at the level of availability of new substrate. The chromatin assembly machinery as well as maintenance DNA methyltransferases are both positively regulated by cyclin dependent kinase (Cdk) activity [65,66]. This additional level of cell cycle control may help restrict the propagation of chromatin and DNA methylation thereby maintaining balanced levels throughout the cell cycle.

The stable transmission of CENP-A results in a two-fold reduction of its levels at each cell division [22*,67,68]. Assembly of new CENP-A is also tightly coupled to the cell cycle but in strikingly opposite ways compared to bulk nucleosome assembly. Experiments using SNAP-based fluorescent pulse labeling [22*,69,70*] as well as quantitative fluorescent measurements [24,56,71] in a variety of metazoan systems have shown the same phenomenon. Nascent CENP-A is not deposited into centromeric chromatin during DNA synthesis but is strictly dependent on passage through mitosis. The mechanistic basis for this cell cycle coupling involves inhibition of the CENP-A assembly machinery by cyclin-dependent kinases (Cdk1 and Cdk2) that are active during S, G2, and mitosis, thereby restricting assembly to G1 phase [70*] (Figure 3). This is in sharp contrast to the propagation of CpG methylation and chromatin assembly that are promoted during S phase.

Thus, cell cycle restricted loading of nascent CENP-A provides a direct coupling between the S-phase turnover, cell division and subsequent assembly after mitosis, potentially ensuring a proper homeostasis of centromere size across cell division cycles.

**Cell cycle control of dynamic chromatin components**

Histone modifications, the enzymes that produce them and their associated effectors, may turn over at a timescale much shorter than a cell cycle [41*,42*]. Strict cell cycle control of the formation of modifications or effector binding would therefore not be expected. Nevertheless, there is evidence for the cell cycle machinery controlling aspects of histone modification. For instance, the EZH2 (enhancer of zeste homologue 2) H3K27 methyltransferase subunit of the PRC2 complex is functionally regulated by phosphorylation by Cdk1 and Cdk2 [72] and is recruited to sites of H3K27 methylation during G1 and S phase [48*]. While PRC2 activity may not be restricted to a specific cell cycle phase, its cell cycle regulation may drive waves of propagation of H3K27 methylation, for example, on nascent nucleosomes following DNA replication.

**Conclusions and outlook**

A comparison of the different chromatin-based inheritance systems (i.e. histones, their modifications, and
Cell cycle control of replication of epigenetic marks. General overview of different epigenetic marks and the degree of cell cycle control of their assembly. Specific timing of the formation of the mark along the cell cycle is indicated where applicable. Convention of symbols follows those of Figure 1. ‘Cdk’ is indicated when assembly is promoted or inhibited by cyclin dependent kinase activity (Cdk2 for DNA synthesis and associated replication of CpG methyl marks and replication dependent chromatin assembly of H3.1). Conversely Cdk1 and Cdk2 inhibit CENP-A assembly in S, G2, and mitosis. H3.3 assembly is not cell cycle regulated, but can occur throughout the cell cycle, replacing a fraction of the nucleosome pool. Fast turnover of H3.3 is suggested at active genes and regulatory elements [29**] but H3.3 in bulk is stable indicating that not the entire pool is turned over within a cell cycle [5,6*]. There is little evidence for strict cell cycle coupling of H3 methylation but trimethylation of H3K27 has been suggested to occur in G2 phase in the Drosophila embryo [36**]. Possibly, regulation of the H3K27 methyltransferase EZH2 by Cdk1 and Cdk2 plays a role in cell cycle coupled propagation of methylation at this residue [72]. H3K4 methylation at active genes has been shown to increase during mitotic exit [35*]. The non-histone PcG proteins are highly dynamic along the cell cycle but recruitment of PRC2 to H3K27me3 sites may be cell cycle regulated [48**]. Circular arrows indicate dynamic exchange that is not cell cycle regulated.

non-histone effectors) reveals that while centromeres may represent a unique case, specific features are of broad relevance. Firstly, epigenetic memory may be maintained either by a feedback loop involving multiple dynamic components or by stable transmission of a chromatin mark (Figure 1). CENP-A chromatin appears to be maintained by the latter type of mechanism, perhaps owing to its constitutive role in cell division, irrespective of developmental cues. The transfer of parental nucleosomes onto nascent DNA during DNA replication lies at the heart of propagating this chromatin structure. How this works in general, and whether stable retention of CENP-A nucleosomes involves a special mechanism are important future questions. Secondly, another key question involves the dynamic state of chromatin involving broadly distributed H3.1, H3.2 or H3.3 nucleosomes. While there is evidence for rapid histone turnover at specific loci, a challenge for the future will be to determine what proportion of chromatin is stably retained at which loci and in which cell types. This is relevant because whether nucleosomes are dynamic or static has direct consequences for our understanding of the mechanisms of epigenetic inheritance. Third, inheritance of the centromere appears to rely on stable retention of CENP-A nucleosomes. However, in contrast to the simple feedback mechanisms operating to propagate CpG and histone methylation, assembly of CENP-A involves an apparently elaborate series of proteins (Figure 2). Identifying the factors sufficient to self-assemble CENP-A chromatin is a key objective. Finally, different epigenetic mechanisms may be controlled along the cell cycle in different ways. Duplication of DNA methylation, H3.1 and CENP-A nucleosomes is tightly cell cycle restricted (Figure 3). Possibly this reflects the fact
that these components are stable following incorporation, thereby placing constraints on making more of the same. However, while the consequences of overduplication of DNA or misregulation of S-phase histones is well documented it remains to be determined whether tight CENP-A homeostasis along the cell cycle has relevance for proper centromere function.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


Using a mathematical model, these authors show that an active or repressed chromatin state can be stably maintained, even when nucleosomes and histone modifications are dynamic.


The two papers [41,42] show that, while Pcg proteins can remain bound to chromatin during DNA replication, they appear to be highly dynamic in vivo. This indicates that Polycromy based epigenetic memory involves propagation of a dynamic state.


See annotation below Ref. [47].


The two papers [46,47] demonstrated a closed feedback loop between methylated histone H3K9, the methyl transferase that produces the mark and its interacting partner HP1 that binds to the mark, thereby potentially promoting H3K9me spreading.


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Centromeres can form de novo spontaneously on human alpha satellite repeats. This study provides insight into the chromatin requirement for centromere formation. Tethering of histone acetyl transferase activity promotes centromere formation and bypasses the need for the Mis18 complex.


Assembly of CENP-A is directly coupled to the cell cycle. Cdk1 and Cdk2 inhibit CENP-A assembly, thereby restricting formation of centromeric chromatin to G1 phase.
