Chromatin structure and the inheritance of epigenetic information

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Abstract | Although it is widely accepted that the regulation of the chromatin landscape is pivotal to conveying the epigenetic program, it is still unclear how a defined chromatin domain is reproduced following DNA replication and transmitted from one cell generation to the next. Here, we review the multiple mechanisms that potentially affect the inheritance of epigenetic information in somatic cells. We consider models of how histones might be recycled following replication, and discuss the importance of positive-feedback loops, long-range gene interactions and the complex network of *trans*-acting factors in the transmission of chromatin states.

Histone variants
Structurally distinct, non-typical versions of histone proteins. They are encoded by independent genes and are often subject to regulation that is distinct from that of the canonical histones.

Heterochromatin
The portion of the genome that stays highly condensed throughout the cell cycle. It contains lot of repetitive sequences, is gene-poor overall and is enriched for histone marks, such as histone 3 lysine 9 trimethylation (H3K9me3) and H4K20me3.

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The proper development of multicellular organisms entails the distinct specification of disparate cell types. Despite having identical genomic sequences, different cell types exhibit substantially different profiles of gene expression, and their cellular identity must be conserved during somatic cell divisions. How then are cell-specific gene-expression patterns specified and maintained? It is now recognized that the key is 'epigenetics': the stable and heritable information that is distinct from DNA sequences and fostered by specialized mechanisms. These mechanisms include DNA methylation, small interfering RNAs, histone variants and histone post-translational modifications (PTMs). To date, however, only DNA methylation has been shown to be stably inherited between cell divisions[1]. Although some histone PTMs are expected to contribute to the transmission of epigenetic information, others participate in the process of transcription — the so-called 'active marks' — and others are likely to be restricted to 'structural functions'[2,3]. In this Review, we consider the mechanisms that are involved in the transmission of epigenetic information as cells divide and therefore contribute to the maintenance of cell identity.

To address this phenomenon of epigenetics, we need to consider DNA in the context of chromatin. In eukaryotes, 147 bp of DNA is wrapped around an octamer of histones consisting of two copies of H2A, H2B, H3 and H4. The resulting nucleosomes are further compacted to form higher-order chromatin structures, which remain poorly understood. Chromatin is not simply a packaging tool; it is also a dynamically adjusted entity that reflects the regulatory cues necessary to program appropriate cellular pathways. In particular, each core histone has an amino-terminal tail that protrudes from the nucleosome[4] and can be subject to PTMs, such as acetylation, methylation, phosphorylation and monoubiquitylation, as well as other modifications that are less well studied[5,6]. Chromatin is also characterized by the presence of histone variants, the spacing between nucleosomes (known as nucleosome occupancy) and the position of the chromatin itself in the nucleus.

Genome-wide profiling (using chromatin immunoprecipitation followed by microarray (ChIP–chip) or sequencing (ChIP–seq)) has provided a partial picture of the chromatin landscape, including the localization of histone PTMs and histone variants, DNA methylation patterns and nucleosome occupancy. Moreover, the discovery of protein domains — including chromodomains, bromodomains, plant homeodomains (PHDs), tudor domains and malignant brain tumour (MBT) domains — that specifically recognize a defined histone modification have advanced our understanding of the role of histone PTMs[7,8]. Although specific histone PTMs have been correlated with defined functions, such as gene regulation[8], it is clear that a single type of histone PTM does not dictate a single outcome. For example, histone 3 lysine 9 trimethylation (H3K9me3) is found both in silent heterochromatin and at some active genes[8]. Therefore, it now seems prudent to consider chromatin as a composite of various domains (FIG. 1). These domains are characterized by the local enrichment of a specific combination of histone PTMs, histone variants, nucleosome occupancy, DNA methylation patterns and, possibly, nuclear localization. It is this totality of features that we refer to as chromatin structure or landscape. Although some proteins that regulate
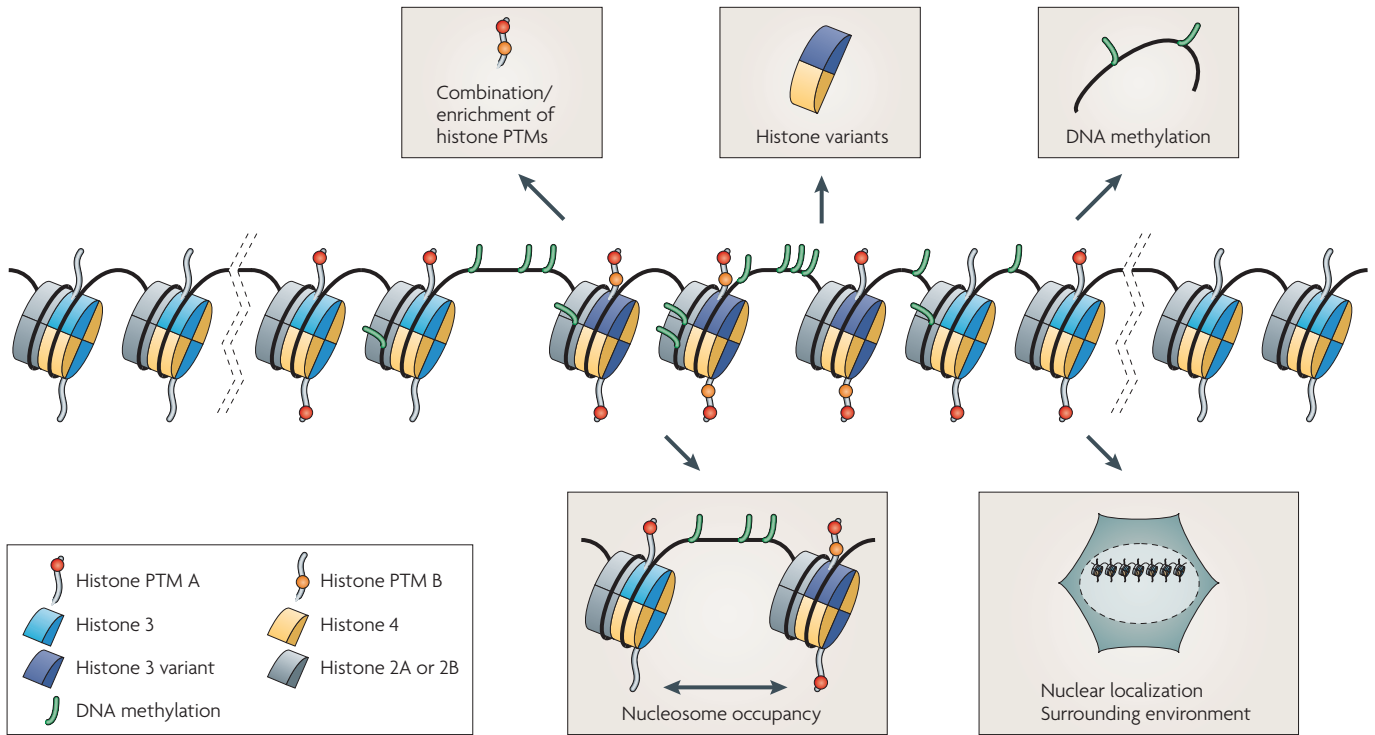
Figure 1 | **Characteristics of a chromatin domain.** Schematic depicting modifications that define different chromatin domains. The range of factors that can contribute to the characteristics of a domain are shown in the shaded boxes. The dashed lines represent the separation between two adjacent domains. PTM, post-translational modification.

chromatin structure are well defined, exactly how the histone-modifying enzymes, histone modifications and modification-recognizing proteins are localized and restricted to specific loci is currently unclear.

We first describe two examples of heritable transcription regulation from two model systems, yeast and *Drosophila melanogaster*, in which genetic analyses have contributed substantially to our understanding of chromatin regulation. We then analyse how histones are segregated during DNA replication and highlight some of the mechanisms required to maintain defined chromatin domains. Finally, we describe findings that show how chromatin structure acts in concert with *trans*-acting factors to carry the epigenetic information.

Examples of heritable transcriptional regulation
Whether established in response to external stimuli or during development, defined gene-expression patterns should be maintained through cell divisions. In the next sections, we will consider two examples of such maintenance programs and review the current knowledge about the putative underlying mechanisms.

Transcriptional memory. In response to external stimuli, gene-expression patterns can be durably altered. Hence it was reported that artificial gene activation induced by transient exposure to histone deacetylase (HDAC) inhibitors or by modulation of growth media leads to heritable gene regulation.

Histone acetylation is associated with gene activation and histone deacetylation is associated with gene

repression[9]. It has been found that a reporter gene integrated into the centromeric heterochromatin of fission yeast (*Schizosaccharomyces pombe*) cells switches from a repressed to an active state after exposure to the HDAC inhibitor trichostatin A (TSA) for five generations[10]. When the yeast were moved to media depleted of TSA, the reporter stayed active and hyperacetylated through 200 generations, although at each cell division an average of 2% of the cells reverted to the repressed state[10]. Interestingly, this retention of an active state was not observed for genes embedded in euchromatin but was maintained through crosses with yeast that had not been exposed to TSA, suggesting that activation was not solely a consequence of transcription-factor regulation.

A different example of transcriptional memory in yeast has been described for the galactose (*GAL*) regulon. Genes of the yeast *GAL* regulon are repressed in glucose medium, but are strongly induced in the presence of galactose as the only carbon source. The kinetics of *GAL* gene activation are dramatically different depending upon prior exposure of the cells to galactose: whereas galactose induction is slow, requiring up to two hours for full activation, re-induction following a cycle of activation and repression occurs in minutes[11–13]. The Set1 H3K4 methylase is targeted to transcriptionally active genes. H3K4me persists at the *GAL10* gene through the cycle of activation and repression, which has led to the suggestion that H3K4me provides a 'memory' of recent transcriptional activity[14]. However, the enzymes responsible for H3K4me, H3K79me, H2BK123 ubiquitylation

Euchromatin
In contrast to heterochromatin, euchromatin is decondensed and is enriched in active genes and histone marks, such as histone 3 lysine 4 trimethylation or histone 3 acetylation, that are associated with active transcription.

Regulon
A group of transcriptional units or operons that are coordinately controlled by a regulator.

(H2BK123ub) and H3 acetylation (H3ac) were shown to be dispensable for this rapid re-induction[12]. Instead, *GAL* gene memory requires the histone variant H2A.Z and the switch/sucrose non-fermentable (SWI/SNF) chromatin-remodelling complex[11,12].

A possible explanation for the requirement of SWI/SNF came from the observation that the *GAL1* gene is relocated to the nuclear periphery after the first induction; relocation is dependent upon the histone variant H2A.Z[12] and SWI/SNF deletion prevents H2A.Z deposition onto DNA[15]. Interestingly, a transcription-dependent physical interaction between the gene's promoter and terminator (that is, the sequence that regulates polyadenylation) was reported, and this 'gene looping' was suggested to facilitate transcription re-initiation[16]. Hampsey and colleagues reported that gene looping is crucial for the re-induction of *GAL10* (REF. 17), and Proudfoot and colleagues found that it was essential for the re-induction of hexokinase 1 (*HXK1*)[18]. These observations suggest that retention of components of the transcription machinery following active transcription might have an important role in the rapid re-induction of transcription in yeast. By contrast, Tzamarias and colleagues argue that the cytoplasmic level of the Gal1 galactokinase is crucial for transcription memory in yeast. This interpretation is supported by results from heterokaryon experiments, in which naive cells responded quickly to galactose when fused with the cytoplasm of cells induced with galactose before fusion[13].

Whichever mechanism or mechanisms for transcription memory are operational, the above experiments show the existence of this phenomenon and that histone variants (H2A.Z), the chromatin-remodelling complex SWI/SNF and nuclear localization are important players. Moreover, these studies suggest that the actual structure of the transcription machinery and/or a cytoplasmic kinase can affect the process of transcription memory. Most importantly for the topic of this Review, the effects observed were found to be independent of histone modifications, and as the process involved the maintenance of a transcription-permissive environment, we conclude that histone modifications that function in transcription ('active marks', such as H3K4me3, H3K36me3 or H3K79me3) are irrelevant to the process of transcription memory. Further investigations are required to determine whether this observation holds true for other cases of epigenetic regulation and whether other histone modifications, such as those involved in maintaining a repressed state (H3K9me, H3K27me and/or H4K20me, depending on the model), are important players in the establishment of an inherited chromatin domain.

Position-effect variegation. Proper development requires the establishment of defined and heritable patterns of gene expression specific to each cell lineage. Studies of a phenomenon called position-effect variegation (PEV) gave rise to numerous advances in understanding gene silencing during development. PEV was initially described in *D. melanogaster* but has since been observed in other organisms, from yeast to mammals.

PEV reflects either the silencing of a euchromatic gene when artificially moved in proximity to a heterochromatic region or gene activation in the reverse case[19,20]. Using genes with a transcriptional status that can be easily monitored (such as eye colour), this phenomenon was explored by screening for mutations that enhanced or suppressed variegation, and up to 150 loci encoding modifiers of PEV were identified. Importantly, PEV is established early in development (by the end of the first larval instar), but eye cells undergo further divisions, which indicates that this regulation is inherited[20].

Some of the modifiers of variegation — for example, heterochromatin protein 1 (HP1, also known as suppressor of variegation 205 (SU(VAR)205)), SU(VAR)3-7 or SU(VAR)3-9 (REF. 21) — show a dosage-dependent effect, meaning that they have opposite effects on variegation when they are up- or downregulated through genetic manipulation. It is noteworthy that distinct regions of chromatin seem to be regulated differently; some of the genes that modified variegation when a euchromatic gene was positioned at pericentric heterochromatin were ineffectual when this same gene was positioned at subtelomeric heterochromatin[20].

Characterization of the modifiers of variegation revealed that many are involved directly or indirectly in the regulation of chromatin structure[22]. For example, reduced levels of several histone methyltransferases, such as SU(VAR)3-9, enhancer of zeste (E(Z)), SET domain bifurcated 1 (SETDB1, also known as Eggless), PR/SET domain-containing 7 (PR-SET7) or SUV4-20, or histone demethylases, such as SU(VAR)3-3, were shown to suppress variegation[20,21,23]. The histone modifications that these enzymes catalyse are all associated with repression. Some target distinct regions of heterochromatin, as shown by the selective loss of heterochromatin on chromosome 4 when SETDB1 is deleted[20,24] or loss of pericentric heterochromatin when SU(VAR)3-9 is deleted[21]. Conversely, loss of function of JIL1, the kinase that phosphorylates histone 3 serine 10 (H3S10) — a modification that prevents H3K9me, a mark that is associated with repression — acts as an enhancer of variegation at pericentric heterochromatin[25]. This introduction to PEV shows that chromatin regulation has a crucial role in the heritability of gene expression states, the mechanisms of which are discussed below. Importantly, heterochromatin establishment and maintenance is also regulated by the RNA interference (RNAi) machinery and transcription factors, as discussed later.

Histone deposition following replication

The examples described above show that chromatin has a crucial role in the inheritance of transcriptional regulation. However, for successful cell division, DNA must be replicated. Disruption of chromatin is inherent to replication and replication means that newly synthesized histones will need to be incorporated, as double the amount of DNA needs to be packaged into nucleosomes. Here, we focus on how chromatin modifications and structure are propagated; we first summarize how replication occurs and then consider models of histone segregation.

Chromatin remodelling
An ATP-dependent enzymatic process that alters histone–DNA interactions or regulates the position of nucleosomes. Chromatin remodelling can also be ATP-independent in the case of the facilitates chromatin transcription (FACT) complex.

Nuclear periphery
The area at the edge of the nucleus. It is normally associated with gene silencing.

Heterokaryon
A cell with two nuclei that share the same cytoplasm.

RNA interference
A cellular mechanism involved in gene silencing and 'protection' from retroviral and transposable element invasion. It is regulated by proteins such as Dicer and Argonaute, which are responsible for the production of small interfering RNAs that target mRNAs for cleavage and that localize silencing factors to heterochromatic regions.
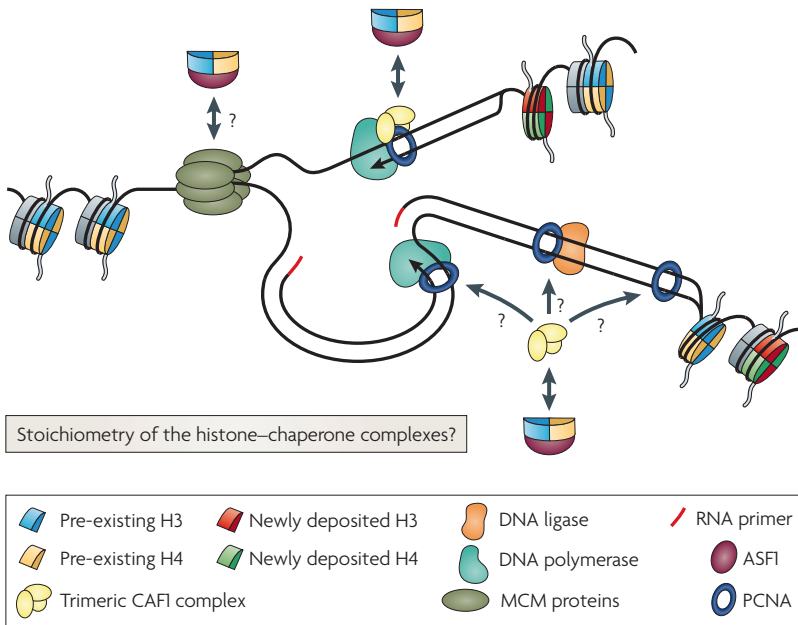
Stoichiometry of the histone–chaperone complexes?

Pre-existing H3	Newly deposited H3	DNA ligase	RNA primer	
Pre-existing H4	Newly deposited H4	DNA polymerase	ASF1	
Trimeric CAF1 complex		MCM proteins	PCNA	

Figure 2 | **The replication fork.** A general and simplified schematic depiction of the replication fork. The mini-chromosome maintenance (MCM) complex is shown at the fork. The possible interaction of MCM proteins with the histone chaperone alternative-splicing factor 1 (ASF1) is shown. The leading strand (upper region) shows a simplified view of the replication fork, depicting DNA polymerase-ε interacting with proliferating cell nuclear antigen (PCNA). The interaction of PCNA with ASF1 is indicated by a double-headed arrow. On the lagging strand (lower region), ASF1 and PCNA could interact at three different steps: first, during Okazaki fragment replication by DNA polymerase-δ; second, during DNA ligation; and third, with PCNA at chromatin following replication. ASF1 interacts with chromatin assembly factor 1 (CAF1), which is composed of three subunits. '?' indicates that we do not know where, when and how the interaction takes place. H, histone.

Mini-chromosome maintenance complex
An oligomeric complex that is suggested to be the helicase involved in replication.

Histone chaperone
A protein that binds and escorts histones. Chaperones contribute to histone deposition into chromatin in an ATP-independent manner.

Replication. The regulation of DNA replication starts in the late M phase of the cell cycle when replication initiation sites are targeted by the origin recognition complex (ORC)[26,27]. This is followed by the loading of the mini-chromosome maintenance complex (MCM complex) and other proteins to form the pre-replication complex (preRC). This complex is activated at the G1/S boundary and the production of new preRCs is prevented, ensuring that replication occurs only once per cell cycle. The activation step leads to the production of a large complex (the replisome protein complex) that contains multiple proteins required for the formation of MCM helicase activity, which catalyses DNA unwinding[28]. Finally, the replicative DNA polymerase and auxiliary proteins — including the clamp loader (replication factor C (RFC)), the clamp (proliferating cell nuclear antigen (PCNA)) and the single-strand DNA-binding protein replication protein A (RPA)[29] — are loaded, together forming the replisome.

Importantly, the initiation of DNA synthesis requires the synthesis of RNA primers by DNA primase; the primers are extended by DNA polymerase-α. Following the loading of PCNA by RFC, DNA polymerase-δ and -ε take over DNA synthesis[29]. PCNA interacts with the DNA polymerases (FIG. 2) and markedly increases their processivity. It should be noted that the leading and lagging strands are replicated differently owing to the 5′ to 3′ directionality of polymerase activity. The leading and lagging strands are synthesized continuously and discontinuously, respectively. Multiple priming and PCNA loading events are required on the lagging strand.

Histones ahead of the replication fork must be removed during the passage of the replication fork, and *in vitro* replication experiments have shown that the DNA is refolded into chromatin close behind the replication fork, with a slightly different timing for the leading and lagging strands[8]. It is now accepted that old histones — first H3–H4 and then H2A–H2B — are deposited on both strands of the newly replicated DNA[8]. Whether in the cytoplasm or during their subsequent import into the nucleus, histones are not free but are bound by histone chaperones (discussed further below), and H3 and H4 are always co-associated. For a long time it was thought that H3–H4 would be deposited as a tetramer, but recent reports have shown that free H3–H4 exists in cells as a dimer[8,30]. This observation was supported by the structure of the histone chaperone alternative-splicing factor 1 (ASF1, also known as SFRS1), which revealed that it interacts with a dimer of H3–H4 (REFS 31,32).

Interestingly, newly synthesized histones are modified post-translationally before their deposition: from metazoans to humans, K5 and K12 residues are acetylated on most of the cytoplasmic H4 (REF. 33). These marks are required for histone deposition and are removed during chromatin maturation. A subfraction of human cytoplasmic H3 shows monomethylation of H3K9 and also some acetylation, but the roles of these modifications are unclear[34]. In yeast, a recent study reported that H3K56ac plays an important part in nucleosome assembly by increasing the affinity of the chromatin assembly factor 1 (Caf1) chaperone for H3 (REF. 35). Although this mark was recently detected in mammals[36–38], its function is still not clear.

Models of histone segregation. Two different scenarios can be envisaged by which the 'new' and 'old' histones (the latter of which carry their original PTMs) are distributed after DNA replication. First, chromatin can be formed from a pool of new and old histones that are randomly deposited on the newly replicated DNA; this is the 'random model' (FIG. 3A). This model infringes on the importance of chromatin domain inheritance. Indeed, not only would histone PTMs be diluted by the incorporation of new histones but also their distribution relative to the DNA sequence would probably be modified. This model predicts that a histone PTM (or a combination of PTMs) is only transmitted effectively when it is enriched on several adjacent nucleosomes, and possibly on each copy of the histones (see red circles in FIG. 3A). By contrast, histone PTMs that are present on only a few copies would get diluted and their influence on regulation might be lost (see orange circles in FIG. 3A). In view of this model, whether H3–H4 is segregated as dimers or tetramers is a relatively moot point, as the overall enrichment of the domain for a histone PTM takes precedence.
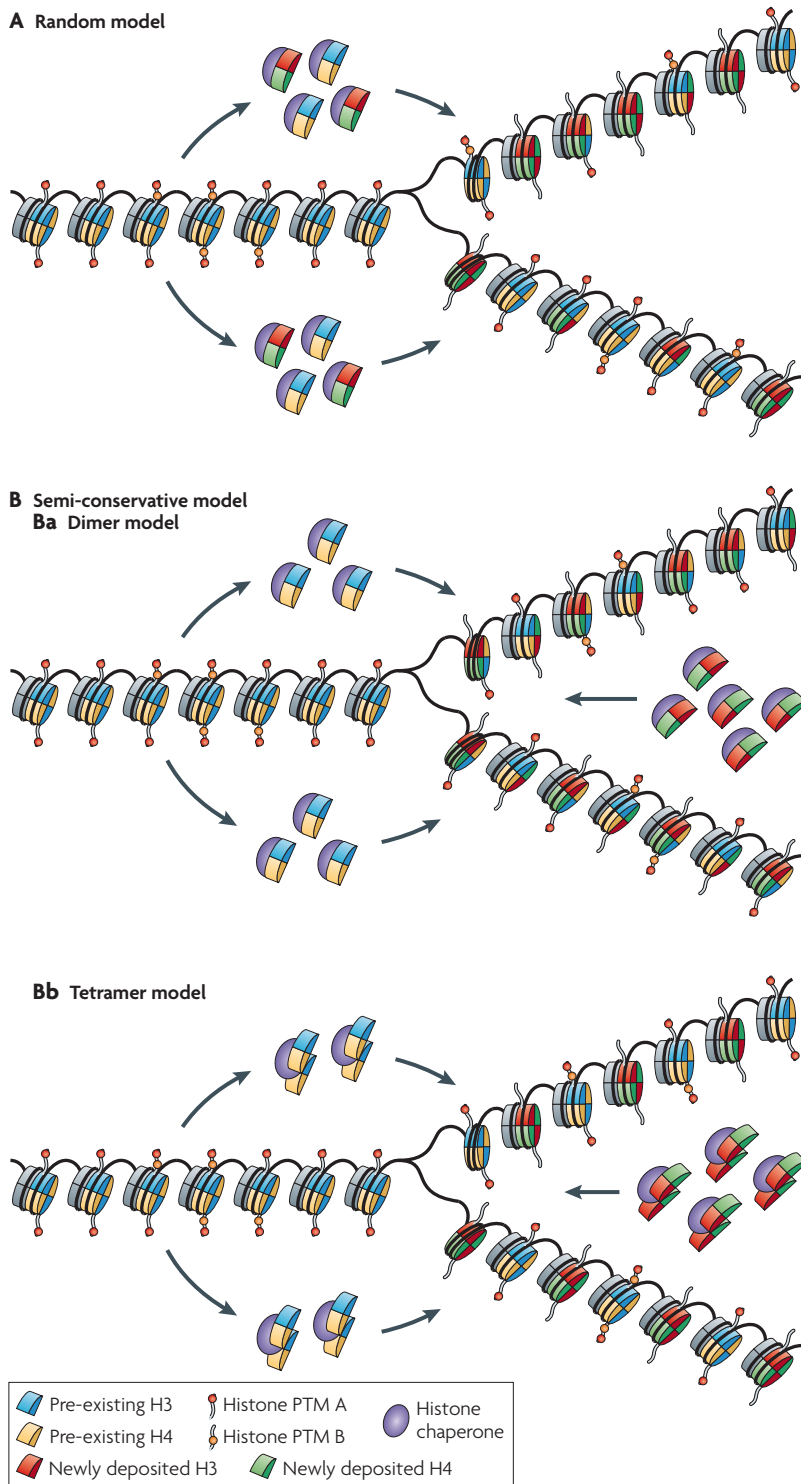
Figure 3 | Models of histone deposition during replication. Here, we illustrate the means by which old and new histones might be deposited following replication. **A** | Random model of histone segregation. In this model, we do not discriminate between dimer or tetramer deposition. The histones segregate randomly between the leading and lagging replicated DNA strands. **B** | Semi-conservative model of histone segregation. **Ba** | the scheme is based on the assumption that the histone 3 (H3)–H4 tetramer is divided during DNA replication and the parental H3–H4 histones segregate as dimers onto the newly replicated DNA strands. The parental histones associate with naive dimers to reconstitute the tetramer. **Bb** | This scheme posits that parental H3–H4 histones segregate as tetramers, resulting in the joint deposition of recycled histones and newly deposited naive histones. PTM, post-translational modification.

The second possibility is the semi-conservative model, which suggests that H3–H4 dimers or tetramers are distributed equally between both strands following DNA replication (FIG. 3B). The propagation of histone modifications (which potentially convey epigenetic information) would then require machinery that duplicates the histone marks between the corresponding tails of a nucleosome if the segregation occurs as dimers, or between adjacent nucleosomes if the segregation occurs as tetramers. Although this hypothesis is attractive in its simplicity with respect to the propagation of histone PTMs, it is difficult to conceive of how each H3–H4 dimer or tetramer would be deposited after replication in an ordered manner on the leading and lagging strands, as both strands are not synthesized simultaneously.

Regarding the question of dimer versus tetramer deposition of H3–H4, although newly synthesized histones are carried by ASF1 as an H3–H4 dimer, several studies have convincingly shown that new and old dimers are not mixed during the replication process. This suggests that H3–H4 is deposited as a tetramer (REF. 39 and B. Zhu, personal communication). The apparent discrepancy might reflect the fact that histone recycling and deposition involves several steps and different histone chaperones. It was reported that ASF1 interacts with CAF1, which is composed of three subunits, p150, p60 and p48, and with PCNA[40], which is a homotrimer. Considering that ASF1 prevents H3–H4 tetramerization, it is proposed that H3–H4 dimers are transferred from ASF1 to CAF1 for deposition[39] (FIG. 2). Although we have information on the steps involved and the participants, the exact mechanisms and, most importantly, the stoichiometry of the protein–protein interactions involved in these transactions require further investigation. Therefore, the exact mechanism of histone segregation during replication remains elusive.

Propagation of epigenetic marks

Following DNA replication, the newly formed chromatin carries only part of the epigenetic information. To ensure the stability of chromatin structure, cells need to rapidly duplicate the original information. In the next sections, we discuss some mechanisms that contribute to the proper duplication of histone modifications, which potentially propagate epigenetic information.

Based on the nucleosome modifications that occur at the silent mating-type region in yeast, Thon and colleagues developed a mathematical model of histone PTM regulation in the context of a two-state process[41]. The authors assumed that the model is applicable to bistable conditions — that is, transition from one state to another can occur but does so at a relatively low frequency. Bistability is efficiently maintained through replication if one assumes that histone PTMs are randomly segregated during replication and that newly deposited nucleosomes are naive (that is, they are devoid of the histone modifications that regulate the transition from one state to another and are, therefore, potential targets for these modifications). We mentioned above that newly incorporated histones do not carry the PTMs that are potentially involved in transmitting epigenetic information.

Most importantly, bistability requires cooperativity between the histone modifications and positive feedback for the spreading of a defined mark. Furthermore, this positive feedback should not only propagate the mark linearly — that is, to adjacent nucleosomes — but also reach targets several nucleosomes away, which highlights the importance of higher-order chromatin structure. We consider mechanisms that could be crucial for the propagation of epigenetic information in light of these theoretical predictions, which we believe to be of great importance to understanding the transmission of histone marks and the establishment of inherited chromatin domains. It is important to note that although we have some idea about the propagation of DNA methylation and models are emerging for the duplication of histone PTMs, very little is known about the inheritance of nucleosome occupancy or histone variants.

Duplication of chromatin structure coupled to DNA replication. According to the theoretical model presented above, S phase is an important step for the propagation of epigenetic information, as newly synthesized chromatin is more likely to switch from one state to another. It was therefore suggested that epigenetic information could be rapidly duplicated if the factors that modify chromatin were coupled to the replication machinery. Accordingly, PCNA was suggested to have a pivotal role in orchestrating the various enzymes required to modify the newly replicated chromatin[42,43].

PCNA is a homotrimer that forms a ring-shaped structure that slides along the DNA following the DNA polymerase, and it also remains bound to the lagging-strand chromatin after replication has been terminated[43]. PCNA interacts with many proteins through a hydrophobic pocket situated between its two globular domains[29]. These interacting proteins usually have a conserved motif called the PCNA-interacting protein (PIP) box, and in theory each PCNA ring could contact three different interactors simultaneously.

Propagation of DNA methylation can be used to illustrate this model. Cytosine methylation is required for development in eukaryotes with large genomes[44]. For example, knockout of DNA methyltransferase 1 (*Dnmt1*) in mice results in early embryonic lethality[45] and, at the cellular level, disruption of DNMT1 expression results in cell cycle arrest and cell death[46–48]. Three DNA methyltransferases have been characterized in mammals, DNMT1, DNMT3A and DNMT3B. The classical model is that DNMT3A and DNMT3B are involved in *de novo* methylation and DNMT1 is required for maintenance of DNA methylation and specifically recognizes hemimethylated DNA. However, increasing evidence supports the idea that all three mammalian DNA methyltransferases are involved in DNA methylation maintenance[49]. DNMT1 is localized at replication foci in mid to late S phase[50] and was reported to interact with PCNA[51]. However deletion of this domain does not prevent DNMT1 association with replication foci, and DNMT1 is still able to propagate DNA methylation[47,52]. One explanation for this observation came from recent reports of another protein,

nuclear protein 95 (NP95, also known as UHRF1), that interacts with DNMT1 and PCNA[53,54].

NP95^{−/−} embryonic stem (ES) cells exhibit a global loss of DNA methylation and diffuse staining for DNMT1 instead of localization to the replicative heterochromatin region. Moreover, NP95 binds methylated CpG through its SET- and RING-finger-associated (SRA) domain[55]. Importantly, there is emerging evidence that the distribution of DNA methylation might, in some organisms, be a major determinant of the chromatin landscape through control of histone variant deposition and histone PTMs. In accordance with this notion, it was shown that H3K4me3 and DNA methylation are inversely correlated[56] and, at least in plants, DNA methylation and H2A.Z are mutually exclusive[57] and H3K9me2, H3K9me3 and DNA methylation are highly coincident[58].

The example of H3K9me has been examined in several organisms. Knockdown of DNMT1 in mammals resulted in decreased levels of H3K9me2 and H3K9me3 (REF. 59). Conversely, in *Neurospora crassa*, inactivation of DIM5, which is related to the H3K9 methyltransferase SU(VAR)3-9, or mutation of H3K9 led to decreased DNA methylation[60,61]. In mammals, there are at least five enzymes that catalyse H3K9me, including SUV39H1, SUV39H2, G9A, SETDB1 and SETDB2. Knockdown of SUV39H1, SUV39H2 or G9A has an impact on DNA methylation at defined loci[62,63]. Interestingly, it has been reported that G9A and SETDB1 interact with PCNA[42]. G9A might form a complex with DNMT1 and PCNA, whereas SETDB1 might be associated with methyl-CpG-binding domain protein 1 (MBD1), which interacts with PCNA through CAF1. The exact mechanisms connecting replication to DNA methylation and H3K9me are not completely elucidated and might depend on the genomic locus and time of replication. However they seem to be tightly associated to replication through PCNA.

PRSET7 (also known as SETD8), which is the only histone methyltransferase known to catalyse H4K20me1, has been reported to interact with PCNA in mammals[64–66]. PRSET7 expression and the occurrence of H4K20me1 are cell cycle regulated — both increase during mitosis[67]. Following mitosis, H4K20me1 levels decrease, possibly due to conversion to H4K20me2 or H4K20me3 and/or dilution during S phase when naive histones are incorporated. Interestingly, PRSET7 stability is regulated through ubiquitylation — possibly by the Skp, Cullin, F-box-containing (SCF) ubiquitin ligase complex — and degraded early in the G1 phase[68]. The *D. melanogaster* homologue of PRSET7 was reported to be a suppressor of variegation[23], so therefore it is expected that H4K20me1 conveys some epigenetic information[3]. The idea that PRSET7 would propagate H4K20me1 during S phase through its interaction with PCNA is difficult to reconcile with the low levels of H4K20me1 in S phase and the restriction of PRSET7 expression to the G2/M phase of the cell cycle[69]. However, it should be noted that ongoing studies suggest that the association between PRSET7 and PCNA might be related to the response to DNA damage rather than to replication (H. Oda, M. Hubner, D. Spector and D.R., unpublished data). Furthermore, given that PCNA is present

throughout the genome during DNA replication and DNA repair rather than being recruited to specific loci, it seems appropriate to speculate that PRSET7 has different functions during DNA damage and during mitosis.

Positive-feedback loops. After replication, newly formed chromatin is likely to be acted on by various chromatin modifiers. To ensure the faithful propagation of information by the end of mitosis, a modification should not only serve as a template but also promote further deposition of that modification on newly incorporated nucleosomes. This requires cooperativity between the mark and the enzyme that catalyses it, constituting a positive-feedback loop. A few examples of such loops have been described and are discussed below.

The hallmark of heterochromatin from fission yeast to mammals is H3K9me. Although several histone methyltransferases target this residue in mammals, it was shown that SUV39H1 and SUV39H2 are responsible for H3K9me3 at constitutive heterochromatin[70]. HP1 — a highly conserved protein[71] that is associated with heterochromatin[72] and modulates PEV in *D. melanogaster*[73] — has been shown to be important for linking H3K9me3 and heterochromatin formation. In yeast, it was found that H3K9me is crucial for the recruitment of the orthologue of HP1, Swi6 (REF. 74), as the chromodomain of Swi6 interacts with H3K9me2 and H3K9me3 (REFS 75,76). HP1 also directly interacts with SUV39H1 and SUV39H2 through its chromoshadow domain[21,77]. Therefore, H3K9me3 and heterochromatin could spread through the positive loop constituted by HP1 recognition of H3K9me3 and its subsequent recruitment of SUV39H1 and SUV39H2. The role of non-coding RNA in this positive loop will be discussed below and, as mentioned above, in mammals DNA methylation contributes to this loop.

Another example of positive feedback involves polycomb repressive complex 2 (PRC2), the enzymatic component of which, enhancer of zeste homologue 2 (EZH2), catalyses H3K27me. Although some discrepancies exist in the literature as to whether the H3K27me mark can be self propagated (REFS 78,79 and R.M. and D.R., unpublished data), a recent study using a multidisciplinary approach — including biochemistry, structural biology and genetics — showed a mechanism for the propagation of H3K27me3. This study showed that the PRC2 component embryonic ectoderm development (EED) specifically binds to the repressive histone marks H1K26me3, H3K9me3, H3K27me3 and H4K20me3 through an aromatic cage. EED is responsible for the specific recognition of trimethylated repressive lysines by PRC2, although other components of this complex contribute to the overall affinity for nucleosomes[80]. This specific binding by EED leads to an allosteric stimulation of PRC2 activity. Stimulation is optimal when EED interacts with amino acids preceding H3K27, such that H3K27me3 enhances PRC2 activity more than H3K9me3 (even though EED has a slightly higher binding affinity for H3K9me3 and other repressive marks) (FIG. 4). In *D. melanogaster*, mutation of residues in EED that are important for binding repressive histone PTMs

caused polycomb phenotypes and reduced levels of H3K27me[80]. Together, these results show that the ability of a histone PTM to promote its own duplication is required for its maintenance[80].

Cooperativity between chromatin modifiers. Cooperativity between chromatin modifiers is likely to be a consequence of the mutual exclusivity of some PTMs at a defined histone residue. For example, acetylation and methylation cannot occur on the same residue. Therefore, HDACs have been found to be associated with histone methyltransferases: for example, SUV39H1 associates with either HDAC1 (REF. 81) or sirtuin 1 (SIRT1)[82]. However, cooperativity might also occur for the same type of modification at a particular residue. For example, SUV39H1 and SUV39H2 dimethylate H3K9 and H4K20, and SUV4-20H1 and SUV4-20H2 trimethylate H3K9 and H4K20, but each of these histone methyltransferases requires a monomethylated substrate[70,83]. Consistent with this, deletion of PRSET7 (which catalyses H4K20me1) is associated with a loss of H4K20me2 and H4K20me3 (REF. 69). More work is required to understand how monomethylation is targeted and whether this substrate contributes to the regulation of the propagation of the di- and trimethylated versions. Histone PTMs on different residues have also been reported to be exclusive in some cases, such as H3S10 phosphorylation and H3K9me3 (REF. 84) — one modification must be erased to establish the other. Therefore, it is likely that cooperativity between chromatin modifiers is a general mechanism.

Nuclear architecture and long-range interactions. Our current knowledge about the relationship between nuclear architecture and chromatin regulation is mostly correlative and, as yet, little is known about the involvement of nuclear architecture in the propagation of epigenetic information. However, the development of technologies such as chromosome conformation capture (3C) has led to a better characterization of long-range chromatin interactions, and several studies support the involvement of such interactions in maintaining epigenetic silencing.

In *D. melanogaster*, insertion of the *Fab7* transgene leads to a variegated repression of neighbouring genes, and this provides an example of long-range chromatin interactions that occur in *trans*[85]. *Fab7* transgene silencing is enhanced in homozygous female flies and requires the endogenous *Fab7* locus. *Fab7* contains a polycomb response element that is recognized by polycomb group (PcG) proteins, and it was shown that the transgene and endogenous locus were physically associated in a manner dependent on the PcG proteins. Also, the RNAi machinery was required for the long-range interaction between transgenic *Fab7* copies, although no clear evidence supports the involvement of small non-coding RNAs in PcG regulation[86,87]. Physical interaction between chromatin regions for which silencing is maintained by PcG proteins has been reported to be a general mechanism[88,89]. Given that PcG proteins form subnuclear structures called polycomb bodies[90], it is tempting to speculate

Chromosome conformation capture
A technique used to study the long-distance interactions between genomic regions. These interactions can be used to study the three-dimensional architecture of chromosomes in a cell nucleus.

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that higher-order chromatin structure could promote the spread of chromatin-mediated gene silencing.

The role of *trans*-acting factors

A crucial issue for determining the mechanism of epigenetic inheritance is understanding how chromatin modifiers are targeted to specific loci. We have already seen that pre-existing marks are required, and in this next section we discuss how they work hand-in-hand with *trans*-acting factors.

Non-coding RNA. Genome sequencing and advances in genome-wide analysis of RNA have recently revealed that only a small proportion (1.2%)[91] of the human genome encodes proteins, although a large part of the genome is transcribed[92]. An explanation for these results is the existence of a large repertoire of short and long non-coding RNAs (ncRNAs) that includes many new RNAs in addition to previously characterized ncRNAs (for example, tRNA, ribosomal RNA (rRNA) and

small nuclear RNA (snRNA)). Increasing evidence suggests that the newly discovered ncRNAs are functional and important for gene regulation[93].

Small ncRNAs are known to be involved in heterochromatin assembly. Heterochromatin formation has been extensively studied in fission yeast, in which it shows low histone acetylation, high methylation of H3K9 and disruption upon interference with the RNAi machinery[94]. Similar properties were described for heterochromatin in *D. melanogaster*[95], in plants[96] and, to some extent, in mammals, although in plants and mammals DNA methylation provides another layer of regulation[97]. The RNAi machinery has been shown to be important for H3K9me at the centromeres in *S. pombe* and *D. melanogaster*. In plants, mutations in the RNAi machinery affect H3K9me and DNA methylation; however, studies reporting the knockout of Dicer in mammalian ES cells come to different conclusions[98,99]. In *S. pombe*, it was shown that H3K9me is required for stabilizing the RNAi machinery at chromatin[100].
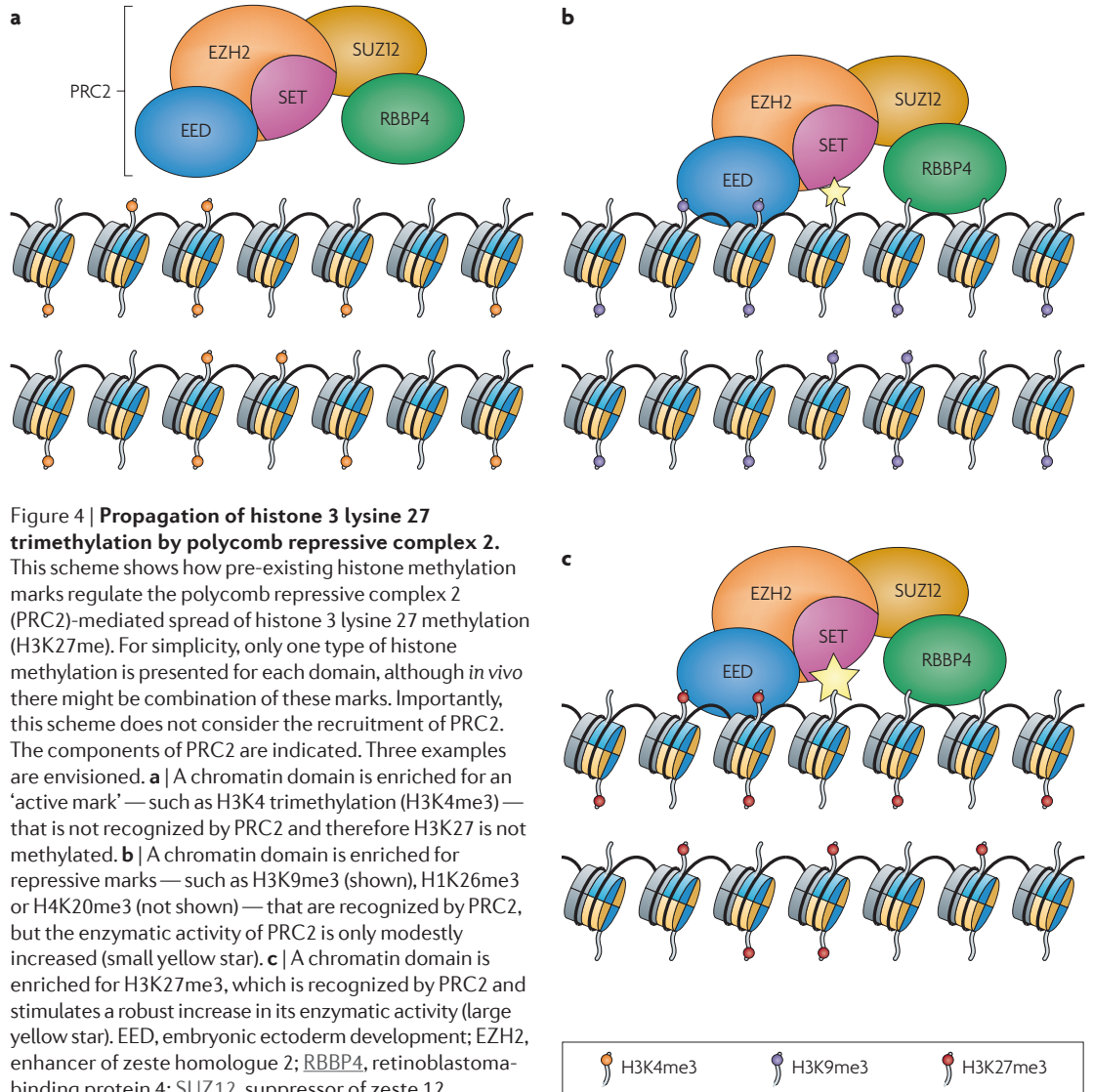

Figure 4 | **Propagation of histone 3 lysine 27 trimethylation by polycomb repressive complex 2.** This scheme shows how pre-existing histone methylation marks regulate the polycomb repressive complex 2 (PRC2)-mediated spread of histone 3 lysine 27 methylation (H3K27me). For simplicity, only one type of histone methylation is presented for each domain, although *in vivo* there might be combination of these marks. Importantly, this scheme does not consider the recruitment of PRC2. The components of PRC2 are indicated. Three examples are envisioned. **a** | A chromatin domain is enriched for an 'active mark' — such as H3K4 trimethylation (H3K4me3) — that is not recognized by PRC2 and therefore H3K27 is not methylated. **b** | A chromatin domain is enriched for repressive marks — such as H3K9me3 (shown), H1K26me3 or H4K20me3 (not shown) — that are recognized by PRC2, but the enzymatic activity of PRC2 is only modestly increased (small yellow star). **c** | A chromatin domain is enriched for H3K27me3, which is recognized by PRC2 and stimulates a robust increase in its enzymatic activity (large yellow star). EED, embryonic ectoderm development; EZH2, enhancer of zeste homologue 2; RBBP4, retinoblastoma-binding protein 4; SUZ12, suppressor of zeste 12.
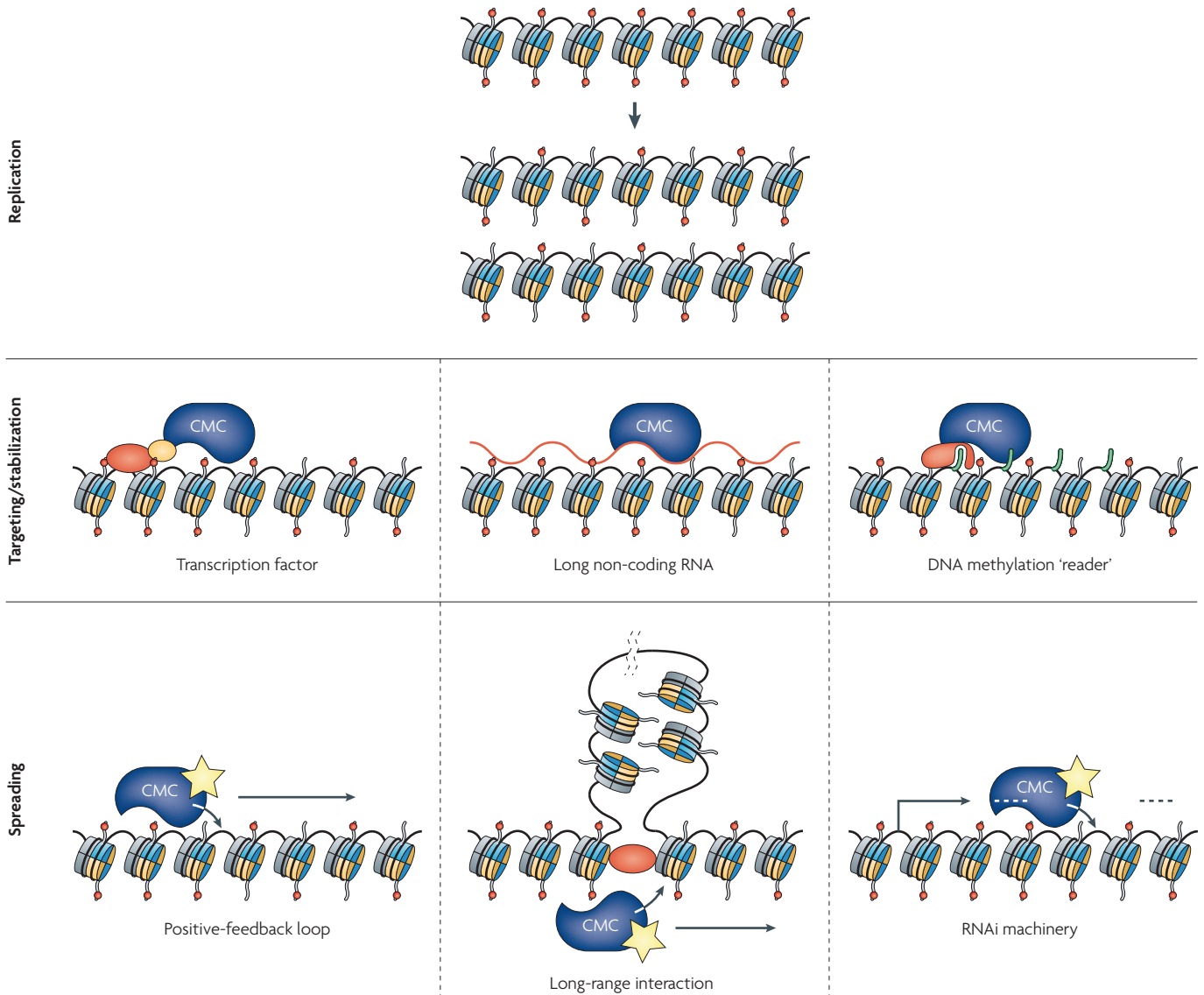
Figure 5 | **Different phenomena that contribute to propagation of regulatory information.** This figure illustrates the different factors that contribute to the propagation of epigenetic information through the regulation of chromatin structure. Regulatory information is represented as a histone post-translational modification (red circle) that is diluted by the incorporation of newly synthesized histones during replication (top panel). We distinguish two steps in the propagation process: the first step is the targeting of a chromatin-modifying complex (CMC) at a defined locus, and the second is the spreading of a putative modification throughout a defined domain. Based on known examples, we have attributed defined mechanisms to one step or the other; however, it is clear that this distinction is not a strict one and that each of these mechanisms probably contributes to some extent to both steps. The yellow star represents enzymatic activity. RNAi, RNA interference.

Chromatin readers
Protein domains that show high binding affinity for histone post-translational modifications and function in downstream effects.

Considering the interdependence between chromatin modifiers/chromatin readers and the RNAi machinery, both of which are required for the maintenance of heterochromatin, it is important to understand how these two distinct systems are targeted to chromatin and how they act jointly. The current model in fission yeast suggests that the RNA-induced transcriptional silencing (RITS) complex is recruited to nascent RNA in an RNAi- and H3K9me-dependent manner[100]. Artificial recruitment of RITS to nascent transcripts resulted in subsequent methylation of H3K9, and

recruitment of the RNA-directed RNA polymerase complex (RDRC) and Swi6 resulted in heterochromatin silencing[101,102]. Increased H3K9me and RNAi production would be expected to generate a positive-feedback loop that enables heterochromatin spreading in *cis*. Therefore, heterochromatin formation could be initiated during late S phase of the cell cycle by an RNA polymerase II-dependent increase in transcription of the non-coding repeats that flank the central centromeric chromatin[103,104]. However, how this transcription is regulated remains unclear. It should be noted that

in *S. pombe*, sequence-specific transcription factors, such as Atf1, Pcr1 and Taz1, are required for heterochromatin formation at the mating-type loci (Atf1 and Pcr1) and telomeres (Taz1)[100]. In *D. melanogaster*, the zinc-finger protein SU(VAR)3-7 was shown to be required for heterochromatin formation[105], and binding of transcription factors to DNA could also be crucial for this process in mammals (T. Jenuwein, unpublished data).

The function of long ncRNA in chromatin silencing is only just being realized, and there is much to learn from future experiments. Preliminary results suggest that long ncRNAs might be involved in the recruitment of chromatin modifiers, among other functions. This phenomenon may be important in gene imprinting[106–108], although the mechanisms by which RNA and chromatin modifier(s) interact are unclear, as their association seems to be cell-specific[106]. Many questions remain, such as: how is the expression of long ncRNAs regulated? Do they correlate with active transcription? Is their function to remove DNA-associated factors? And do they provide a novel mechanism for the recruitment of factors that silence active genes? Importantly, specific patterns of histone methylation are associated with their promoters as a function of their expression[109], and common transcription factors (such as MYC and SP1) are involved in their regulation[110]. What the importance of this correlation with histone modifications is remains elusive, but the study of these long ncRNAs promises to be fruitful. Although interdependence among chromatin modifications, long ncRNAs and transcription factors has been observed, the mechanism that ensures locus-specific targeting is unclear. However, this issue might not be relevant if it is the simultaneous presence of chromatin modifications, transcription factors and ncRNAs that is required to maintain the epigenetic information of defined chromatin domains.

Transcription factors. Nucleosome occupancy, histone PTMs and DNA methylation are pivotal to determining which response elements will be bound by a particular transcription factor either directly, by regulating the affinity of a transcription factor for its binding site, or indirectly, through factors that recognize a defined chromatin environment. However, the relationship between transcription factors and chromatin can also work another way: transcription factors can form regulatory loops (including positive-feedback loops) that impose epigenetic regulation[111]. Also, as noted above, transcription factors can have an indirect effect through the control of ncRNA expression. Furthermore, the importance of transcription factors in the chromatin landscape has been shown by reprogramming experiments. Transforming cells from their fully differentiated state into pluripotent ES cells requires the transient expression of only a few transcription factors, which initiate a dramatic restructuring of the chromatin landscape[112].

Conclusion

In this Review we have emphasized that the transmission of epigenetic information results from the contributions of several different mechanisms. These mechanisms include: recycling of old histones during replication, cooperativity in histone mark duplication, long-range interactions, defined chromatin architecture and the complex network of *trans*-acting factors, whether they be proteins or RNAs (FIG. 5).

Probably the most striking examples of the complexity inherent to epigenetic regulation are X-chromosome inactivation in mammals or dosage compensation in *D. melanogaster* and worms, which have recently been reviewed[113,114]. Indeed, in female mammals, one of the two X chromosomes is silenced early in embryogenesis, and this silencing is inherited through cell divisions. The establishment and maintenance of the X-chromosome silencing program requires the spectrum of mechanisms that we have discussed in this Review: DNA methylation, histone modifications, histone variants, nuclear localization, ncRNA and transcription factors.

Importantly, these regulatory mechanisms do not work in isolation but instead function through positive and negative cooperativity, the mechanisms of which remain poorly understood. Understanding how a defined chromatin landscape promotes the recruitment of *trans*-acting factors or modulates the enzymatic activity of chromatin-modifying complexes will be important for refining the models of epigenetic inheritance.

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DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/gene
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FlyBase: http://flybase.org
Fab7
UniProtKB: http://www.uniprot.org
DNMT3A | DNMT3B | EED | Eggless | E(Z) | EZH2 | JIL1 | PCNA | PR-SET7 | RBBP4 | Set1 | SFRS1 | SUV4-20 | SU(VAR)205 | SU(VAR)3-3 | SU(VAR)3-7 | SU(VAR)3-9 | SUZ12 | UHRF1

FURTHER INFORMATION
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