Chromatin replication and epigenome maintenance

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Abstract | Stability and function of eukaryotic genomes are closely linked to chromatin structure and organization. During cell division the entire genome must be accurately replicated and the chromatin landscape reproduced on new DNA. Chromatin and nuclear structure influence where and when DNA replication initiates, whereas the replication process itself disrupts chromatin and challenges established patterns of genome regulation. Specialized replication-coupled mechanisms assemble new DNA into chromatin, but epigenome maintenance is a continuous process taking place throughout the cell cycle. If DNA synthesis is perturbed, cells can suffer loss of both genome and epigenome integrity with severe consequences for the organism.

Epigenetics
The studies of heritable changes in genome function that occur without a change in DNA sequence.

Replication stress
General term referring to deregulation of replication. This can include fork problems (change of speed, stalling or collapse) and replication initiation defects.

Epigenome
The epigenome refers to the overall epigenetic state of a cell, including histone and DNA marks, histone variants, nucleosome positioning and higher-order structures.

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Faithful transmission of DNA sequence and maintenance of its organization into chromatin during cell division is fundamental to development and disease avoidance. Chromatin is instrumental for genome function and proper execution of epigenetically defined developmental programmes. Mechanisms that maintain chromatin states during the cell cycle are thus germane to cell fate and identity[1,97]. In S phase of the cell cycle, the chromatin landscape undergoes dramatic alterations as the entire genome is copied[2,74]. Given the many different types of chromatin occupying the nucleus[3], understanding the duplication process and how it is coordinated spatially and temporally with DNA replication is a major challenge. Deregulation of DNA replication, including uncontrolled initiation and fork collapse, can promote DNA damage and genome instability[4,5]. This type of replication stress can result from oncogene activation and probably contributes to carcinogenesis[6]. In addition to genome instability, cancer cells show widespread alterations of DNA and histone modifications, which can jeopardize cellular memory and disable tumour suppressor functions[7]. These epigenome alterations can arise from various cellular defects, and replication stress may contribute, as fork stalling and collapse challenge chromatin replication[8].

In this Review, we discuss how cells replicate DNA and maintain its proper organization into chromatin. We emphasize the role of chromatin and chromosomal architecture in directing the replication programme. During replication, chromatin is disrupted ahead of the replication fork and must be restored behind the fork on the two new daughter strands. Nucleosome assembly is a first step in this process, which also involves nucleosome remodelling, incorporation of histone variants and restoration of marks on DNA and histones[2]. We give special attention to how DNA synthesis is integrated with nucleosome assembly and early steps in chromatin restoration. Chromatin marks can direct gene expression by recruiting effector proteins and modulating genome accessibility[9], with some marks contributing to epigenetic control of genome function[1,97]. We discuss how marks on histones and DNA can be maintained over chromatin domains throughout the cell cycle. Owing to genome-wide replication-coupled chromatin alterations, S phase may provide an opportunity to reset epigenetic controls and at the same time poses a risk of unwarranted chromatin changes. In the final section, we focus on the emerging idea that replication stress may act as a doubled-edged sword that can trigger harmful genome and epigenome alterations with potential consequences for ageing and cancer.

Chromatin and initiation control

Initiation of DNA replication can be divided into three steps. First, as cells exit mitosis, replication origins are recognized by the origin recognition complex (ORC; which consists of six subunits ORC1–6)[10]. Second, in G1 phase, the minichromosome maintenance complex (MCM; which contains the six subunits MCM2–7) is recruited to ORC-binding sites by CDC6 (cell division control protein 6) and CDT1, forming a pre-replication complex (pre-RC)[10] (FIG. 1a). Once the MCM2–7 rings are loaded onto DNA, the origin becomes 'licensed' and is ready to be activated[10]. Third, as cells enter S phase, origins

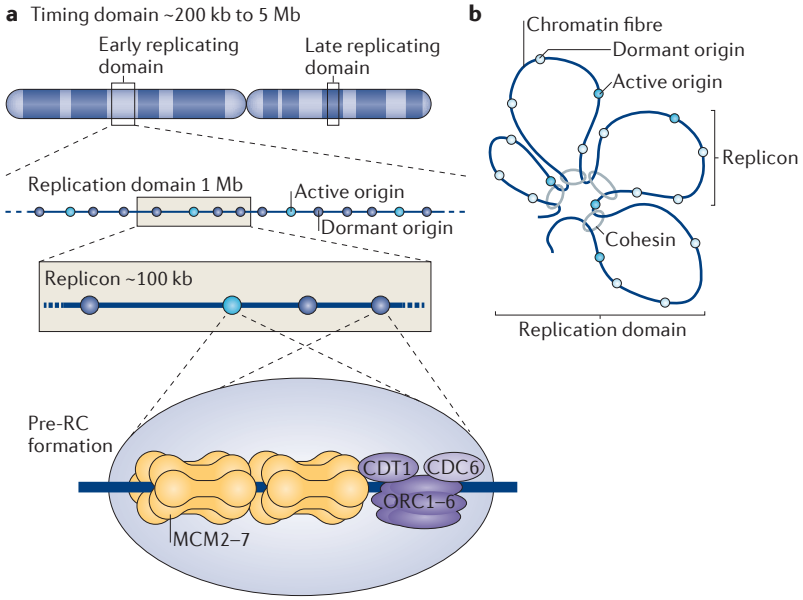


Figure 1 | **Replication initiation and genome organization. a** | Timing domains correspond to large chromosomal regions that replicate at similar times, early or late in S phase. These domains are bordered by so-called transition zones. Each timing domain can include one or several replication domains, which in turn are composed of 5 to 10 adjacent replicons that fire simultaneously. A replicon corresponds to the stretch of DNA that is replicated bi-directionally from a single origin, with nearby dormant origins being replicated passively. Pre-RCs (pre-replication complexes), the ORC (origin recognition complex), CDC6 (cell division control protein 6), CDT1 and MCM2–7 (minichromosome maintenance complex 2–7) double hexamers are assembled on both active and dormant origins, but only selected origins are activated in S phase. **b** | The loop model proposes that replication domains adopt a three-dimensional structure in which replicons are separated into loops by cohesin rings[34].

initiate replication or 'fire' by the sequential action of two S phase kinases, DDK and cyclin-dependent kinases (CDKs), that phosphorylate key fork components and facilitate recruitment of CDC45 and the GINS complex to activate the replicative helicase[11] (BOX 1). Accurate duplication of the mammalian genome relies on sequential activation of 30,000 to 50,000 origins distributed with an average interval of 100 kb. Despite recent advances in origin identification, no consensus sequence with predictive value has emerged in higher eukaryotes. As the features of eukaryotic origins have been thoroughly discussed[10], we focus here specifically on how chromatin and chromosomal architecture control initiation.

Origin selection. Given that origin-poor regions increase the risk of chromosome breakage[12], a crucial question is how ORCs identify binding sites in chromatin. The metazoan ORC does not recognize any specific DNA sequence[10], and its mode of binding seems to depend on the loci. ORC are mainly found in nucleosome-free regions (NFRs)[13–15], but whether NFRs facilitate ORC recruitment or ORC binding brings about nucleosome displacement is still unclear. However, low nucleosome occupancy is clearly not sufficient to recruit ORC[13,14]. Growing evidence indicates that non-histone chromatin factors such as heterochromatin protein 1 (HP1) and high mobility group AT-hook protein 1 (HMGA1) can target ORC to particular regions to specify replication origins[16–18]. Histone post-translational modifications (PTMs), such as histone H4 Lys20 monomethylation (H4K20me1), may also regulate ORC recruitment. Artificial tethering of the H4K20me1 methyltransferase SET domain-containing protein 8 (SET8; also known as PR-SET7 and KMT5A) promotes recruitment of ORC1 and binding of MCM2 and MCM5 to a random locus[19]. After S phase onset, SET8 undergoes PCNA (proliferating cell nuclear antigen)-driven degradation that contributes to both loss of H4K20me1 at origins and inhibition of licensing, unveiling a potential mechanism to prevent re-replication[19–21]. In G2/M phase, this monomethylation mark is re-established by SET8 (REF. 22), perhaps preparing origins that will be used in the next cell cycle for ORC binding.

After ORC binding, CDC6 and CDT1 facilitate the loading of the MCM2–7 helicase[10]. It has been proposed that histone acetylation could stimulate pre-RC assembly and/or origin activity[10], but the exact mechanism remains unclear. An attractive possibility is that histone acetylation facilitates MCM2–7 recruitment[23,24]. CDT1 recruits HBO1 (histone acetyltransferase binding to ORC1; also known as KAT7) to replication origins, and this enhances MCM2–7 loading through a mechanism requiring its acetyltransferase activity[23–25]. As HBO1 preferentially targets the histone H4 residues K5, K8 and K12, it could promote licensing simply by increasing chromatin accessibility. However, pre-RC components including ORC and MCM subunits are also subject to acetylation[25] and could potentially also be HBO1 targets.

The MCM2–7 complex is loaded as a double hexamer[26], which upon initiation splits into two single hexamers that progress in opposite directions[27]. However, successful pre-RC assembly does not ensure origin activation. Indeed, only 10% of licensed origins fire, whereas 90% remain 'dormant'[4] (FIG. 1a). This excess of licensed origins may serve as a backup to ensure complete genome duplication under replication stress[28,29]. The origins that will fire are selected in late G1 phase at the origin decision point (ODP). Although the mechanism underlying this choice remains unclear, there is evidence to suggest that the spatial organization of the genome is relevant[30]. Origins are organized into replication domains corresponding to clusters of 5 to 10 adjacent origins that fire almost simultaneously (FIG. 1a). The well-described replication foci[31] observed by immunofluorescence in replicating cells could correspond to one or more replication domains[32]. One hypothesis predicts that there is interdependency between origin usage and the three-dimensional structure of these domains, where replicons may be organized into loops (FIG. 1b)[30,33]. The DNA halo assay has been used to visualize structures that are interpreted as chromatin loops. The ring-shaped cohesin complex is enriched at origins[13] and, given its ability to encircle two chromatin fibres, it could contribute to the spatial organization of replication domains (FIG. 1b). Depletion of the RAD21 cohesin subunit increases the size of chromatin loops and reduces the number of active origins[34]. Faster fork progression that likewise reduces the number

Chromosomal architecture
Three-dimensional organization of chromosomes in the nucleus. For example, each chromosome occupies a territory in the nucleus and will take up a specific higher-order structure of open and compact domains that is partly cell type specific.

Nucleosome assembly
A stepwise process starting with the deposition of two H3–H4 dimers or potentially a $(H3–H4)_2$ tetramer onto DNA to form a tetrasome. This is followed by the incorporation of two H2A–H2B dimers to form a nucleosome core particle.

Histone variants
Replacement histones differ in amino acid sequence from canonical S phase histones to varying extents. They are often incorporated by dedicated pathways to serve specialized functions.

of active origins also increases loop size. Thus, larger loops correlate with longer inter-origin distances, and vice versa[30,34]. Analysis of the well-described oriGNAI3 hamster cell replication origin by the DNA halo assay suggests that active origins may locate close to the base of the chromatin loops[30]. However, our understanding of three-dimensional chromatin architecture and the relationship with origin choice is still rudimentary and awaits new technological developments.

When to fire a domain. If all origins were to fire simultaneously, the entire human genome could be duplicated within 1 hour. Instead, genome replication follows a pre-set timing programme[32,35] with megabases of contiguous DNA, called 'timing domains', replicating at similar times[35,36] (FIG. 1a). Considering their size, each timing domain could comprise more than one replication domain[32]. The replication timing programme is evolutionary conserved and undergoes dramatic changes during development that are related to cell fate decisions (reviewed in REF. 35). Replication timing is in some manner connected to the type of chromatin being replicated[35,37], and replication early in S phase correlates to some extent with DNA accessibility[38,39] and H4K16 acetylation (H4K16ac)[40]. Moreover, replication timing matches well with recent genome-wide maps of long-range genome interactions[35,41,42], suggesting a close link with higher-order chromosomal structure. The well-defined patterns of replication foci in early, mid and late S phase support the idea that chromatin in close spatial proximity replicates synchronously and stays together as a distinct chromosomal unit (FIG. 1b). Moreover, the timing decision point (TDP)[35], the moment in early G1 phase when replication timing is established, coincides with the anchoring of chromosomes as they take up their interphase position and structure. However, it should be underscored that approaches to study chromosomal architecture are limited, and understanding initiation control in three dimensions will be a major challenge. The need to study these mechanisms is further emphasized by the finding that the spatial proximity of regions that replicate simultaneously can help to explain specific patterns of genomic alterations in cancer[43].

Fork progression — making chromatin

The eukaryotic replisome is a multicomponent complex (BOX 1) that drives DNA replication with a speed of approximately 2 to 3 kb min^{-1} (REF. 10). This implies that chromatin is disrupted at a rate of around 10 to 15 nucleosomes every minute ahead of each active replisome. To reproduce a similar chromatin environment on new DNA, histones and perhaps other chromatin-bound factors[44] are transferred from the parental strand to the daughter strands[2]. In addition, new histones are incorporated to maintain nucleosome density, and their PTM signature should be assimilated to nearby old histones in the local chromatin environment. In this section, we focus on how replisome function is integrated with chromatin dynamics to meet this challenge (TABLE 1).

Chromatin disruption. An attractive idea is that large-scale reorganization of chromatin throughout a replication domain paves the way for origin firing and rapid fork progression. Analysis of MCM dynamics in live cells suggests that replicating chromatin is decondensed[45] and, consistently, artificial tethering of CDC45 to a chromosomal site can promote large-scale decondensation independently of DNA synthesis[46]. One mechanism for

Replication origins
Sites in the genome where replication initiates, giving rise to two forks that progress away from the origin in opposite directions.

Nucleosome-free regions
(NFRs). Sites of reduced nucleosome occupancy compared with the immediate surrounding regions. NFRs display sensitivity to DNase I, which is likely to result from high histone exchange or DNA structures that resist nucleosome formation.

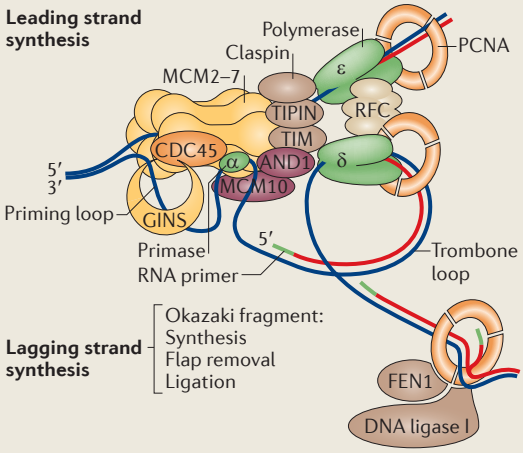
Origin decision point
(ODP). Transition point in late G1 phase that specifies the origins that will fire in the following S phase. It probably represents a change at specific pre-replication complexes (pre-RCs), which potentiates some pre-RCs while preventing others from initiating.

Replicons
Stretches of DNA replicated from a single origin.

DNA halo assay
An approach to visualize DNA loops in interphase nuclei. Nuclei are permeabilized and depleted of histone and soluble proteins on slides, allowing unwinding of supercoiled DNA loops to form a halo around an insoluble scaffold.

Cohesin complex
Ring-shaped multi-protein complex (composed of SMC1, SMC3, RAD21 and sister-chromatid cohesion protein 3 (SCC3)) that by embracing chromatin fibres mediates sister chromatid cohesion and has roles in DNA repair and transcription.

Box 1 | **The eukaryotic replisome**



DNA replication occurs through the coordinated efforts of the replicative helicase that unwinds the double helix and polymerases (Pols) that synthesize DNA in the 5′ to 3′ direction[98]. The CMG complex (CDC45 (cell division control protein 45)–MCM2–7 (minichromosome maintenance complex 2–7)–GINS) is thought to constitute the core replicative helicase in eukaryotes[178]. CDC45 and GINS are likely to associate with the MCM2–7 hexamer in a manner that locks the hexamer onto DNA to allow the helicase to travel along the leading strand[179,180]. The CMG helicase is part of a larger protein complex termed the RPC (replisome progression complex)[27], which in *Saccharomyces cerevisiae* is comprised of the Mrc1–Tof1–Csm3 complex (the claspin–TIM–TIPIN complex in humans), Mcm10 and Ctf4 (known as WDHD1 or AND1 in humans). Pol ε is responsible for continuous leading strand synthesis, whereas the lagging strand is synthesized in a discontinuous manner by ligation of Okazaki fragments[98]. Each fragment is initiated by the primase–Pol α complex by synthesis of a RNA primer with a short DNA extension, which is further extended by Pol δ. The primer and part of the DNA is removed as two Okazaki fragments are ligated together by the action of flap endonuclease 1 (FEN1) and DNA ligase I (REF. 103). The sliding clamp, proliferating cell nuclear antigen (PCNA), tethers the replicative polymerases Pol ε and Pol δ to their template to enhance processivity[98], whereas the replication factor C (RFC) clamp loader orchestrates PCNA loading and probably facilitates coordinated synthesis of leading and lagging strands. Mrc1 may directly tether the leading strand polymerase Pol ε to the CMG complex, whereas Ctf4 together with Mcm10 provides a link to the primase–Pol α complex required for primer synthesis on the lagging strand[181].

Table 1 | **The ties that bind: interactions between replisome components and chromatin regulators**

Replisome components*	Chromatin factors*	Proposed function	Interaction details	Organism	Refs‡
ORC	HP1	Replication initiation: ORC recruitment; heterochromatin organization	Direct: ORC1 N-term and ORC3 MIR domain with HP1α chromoshadow domain	Hs, Xl, Dm	182,183
	SIR1	Silencing	Direct: via ORC1 N-term	Sc	184
	HBO1	Replication initiation	Indirect: via ORC1 210–861 aa with HBO1 zinc finger domain	Sc, Hs	185,186
	ORCA (also known as LRWD1)	Replication initiation	Direct: ORC2 with ORCA WD-repeat domain	Hs	187
	EBNA1	Initiation of viral replication	Indirect	EBV-infected human cells	188
	HMGA1a	Replication initiation	Direct: ORC6 with HMGA1 AT-rich domain	Hs	16
ORC1	TRF2	Replication initiation: ORC recruitment at telomeres	Direct: via TRF2 N-term	Hs	17
CDC6	HP1	Replication initiation	Direct: CDC6 N-term with HP1 chromoshadow domain	Sp	189
CDT1	HBO1	Replication initiation	Direct	Hs	24
MCM2–7	H3–H4	Fork progression; histone dynamics	Direct: MCM2 N-term with H3	Hs, Mm	60,62
	ASF1	Fork progression; histone dynamics	Indirect: through H3–H4 dimer	Hs, Mm	60,62
	HBO1	Replication initiation; histone acetylation	Direct: HBO1 zinc finger domain with MCM2 N-term	Hs	186,190
	FACT	Replication initiation and elongation; histone dynamics	Direct: SPT16 and SSRP1 with MCM4	Hs, Sc	27,67
MCM7	SMC1	Sister chromatid cohesion	Direct	Hs	191
RFC1–5	ASF1	Replisome integrity; nucleosome assembly	Direct: possibly via ASF1 N-term	Sc	192
RPA	FACT	Fork progression; histone dynamics	Direct: POB3 M domain with RPA1	Sc	68
Pol α	FACT	Fork progression; histone dynamics	Direct: POB3 with Pol α catalytic subunit	Sc	69
Pol ε	Silencing complex DOS2–RIK1 and MMS19	Heterochromatin maintenance (H3K9me2)	Not known: through catalytic subunit of Pol ε	Sp	136

such priming of replication domains could be the phosphorylation of linker histone H1 by the S phase kinase cyclin A–CDK2 (REF. 47), as this increases H1 mobility and promotes chromatin decompaction[48] (FIG. 2). In the slime mould *Physarum polycephalum*, H1 is transiently lost from replicating chromatin, and H1 depletion accelerates S phase progression and replication timing[49]. In human cells, cyclin A–CDK2 is recruited to sites of replication and interacts directly with both PCNA and MCM7 (REFS 50–52). Moreover, the G2/M phase kinase cyclin A–CDK1, which also phosphorylates H1, can activate late firing origins if overexpressed in early S phase cells[53].

Nucleosome disruption takes place mainly in close vicinity to the replication fork[54,55], perhaps as a result of collision with the replicative helicases[56] (FIG. 2). It is not clear whether additional factors are involved, but positive supercoiling ahead of the fork[5] could potentially aid disruption, as DNA superhelicity is important for nucleosome stability.

Histone recycling. Current evidence supports a model in which parental $(H3–H4)_2$ tetramers segregate randomly to the two daughter strands, forming nucleosomes with either new or old H2A–H2B dimers (FIG. 3a). This model emerged from early studies (reviewed in REFS 56,74) and has been confirmed by sensitive SILAC-based mass spectrometry[57]. Splitting events producing tetramers that contain a mixture of new and old H3–H4 dimers occur only at low frequency. Importantly, most old histone $(H3–H4)_2$ tetramers seem to be maintained in close vicinity to their original locus[58,59]. It is less clear whether histone H2A–H2B dimers are efficiently recycled, in part because of their more dynamic behaviour.

How old histones are transferred to new DNA remains unknown, but the MCM2–7 helicase could be important[56] (FIG. 3a). Histone H3 binds to the amino-terminal domain of MCM2 with high affinity[60] and can likewise interact with the replicative helicase large T antigen encoded by the SV40 DNA tumour virus[61]. In human cells,

DNA superhelicity
Positive or negative supercoiling of DNA molecules.

SILAC
'Stable isotope labelling with amino acids in cell culture' is an approach for *in vivo* metabolic labelling of proteins with amino acids containing light or heavy isotopes and is used for quantitative mass spectrometry.

Table 1 (cont.) | **The ties that bind: interactions between replisome components and chromatin regulators**

Replisome components*	Chromatin factors*	Proposed function	Interaction details	Organism	Refs‡
PCNA	CAF1	Chromatin assembly	Direct: via CAF1 p150 (two PIP boxes)	Hs, Mm, Dm, Sc, Sp	99,100
	DNMT1	DNA methylation maintenance	Direct: via PIP box in DNMT1	Hs, Mm	193
	SMARCAD1	Heterochromatin maintenance	Direct: potentially via two different regions containing PIP boxes in SMARCAD1	Hs	121
	Lamin A, lamin B and lamin C	DNA replication	Direct: via lamin immunoglobulin fold	Hs, Xl	194
	WSTF–SNF2	Preventing unwarranted heterochromatin formation	Direct: via PIP box in WSTF	Hs	128
	HDAC1	Histone deacetylation	Direct: via HDAC1 catalytic domain	Hs	120
	HDAC2	Histone deacetylation	Indirect: part of the SMARCAD1 complex	Hs	121
	ESCO1/2	Sister chromatid cohesion	Direct: via PIP box in yeast Esco1 and PIP box-like motif in human ESCO2	Hs, Sc	195
	SET8 (also known as KMT5A)	Degradation to prevent re-replication	Direct: via two N-term PIP boxes in SET8. One is essential for SET8 degradation	Hs	196,197
	SMCX (also known as KMD5C)	Heterochromatin formation (H3K4 demethylation)	Possibly via SMCX PIP box	Hs	198
	ATRX5 and ATRX6	Prevent re-replication of heterochromatin domains (H3K27me1)	Possibly via ATRX5 PIP box	At	133,134
	p300	Fork progression	Direct: via p300 C-term	Hs	199
	G9A	Silencing (H3K9me1)	Indirect: through SMARCAD1 and DNMT1	Hs	121,131
	SETDB1 (also known as KMT1E)	Heterochromatin maintenance	Indirect: through CAF1	Hs	93,132
CAF1	HP1	Heterochromatin maintenance (H3K9me3) and fork progression	Direct: CAF1 p150 N-term with HP1β	Mm	94,95
FEN1	p300	Okazaki fragment processing	Direct: via P300 catalytic domain	Hs	200
DNA2	p300	Okazaki fragment processing	Direct: via P300 catalytic domain	Hs	201

aa, amino acid; At, *Arabidopsis thaliana*; CAF1, chromatin assembly factor 1; CDC6, cell division control protein 6; C-term, carboxyl terminus; DNA2, DNA2-like helicase; DNMT1, DNA methyltransferase 1; Dm, *Drosophila melanogaster*; EBNA1, Epstein–Barr nuclear antigen 1; EBV, Epstein–Barr virus; ESCO1/2, establishment of cohesion 1/2; FACT, facilitates chromatin transcription; FEN1, flap endonuclease 1; HBO1, histone acetyltransferase binding to ORC1; HDAC, histone deacetylase; HMGA1, high mobility group AT-hook protein 1; HP1, heterochromatin protein 1; Hs, *Homo sapiens*; MCM, minichromosome maintenance complex; Mm, *Mus musculus*; N-term, amino terminus; ORC, origin recognition complex; ORCA, ORC-associated protein; PCNA, proliferating cell nuclear antigen; Pol α, DNA polymerase α; RFC, replication factor C; RPA, replication protein A; Sc, *Saccharomyces cerevisiae*; SET8, SET domain-containing protein 8; SETDB1, SET-domain binding 1; SIR1, silent information regulator 1; SMC1, structural maintenance of chromosomes protein 3; TRF2,telomere repeat factor 2; SMCX, lysine-specific demethylase 5C; Sp, *Schizosaccharomyces pombe*; SPT16, suppressor of Ty 16; SSRP1, structure-specific recognition protein 1; WSTF, Williams syndrome transcription factor; Xl, *Xenopus laevis*. *Human names are used. ‡ For the initial discovery and prime function.

the histone chaperone ASF1 forms a complex with MCM2–7 (REF. 62), and a fraction of ASF1 colocalizes with MCM2 on chromatin[63]. This interaction is bridged by an H3–H4 dimer[62], arguing that MCM2–7 loaded onto chromatin can bind non-nucleosomal histone H3–H4. The key question is whether MCM2–7 binds H3–H4 (dimers or tetramers) released from parental nucleosomes and, if so, what mechanisms ensure transfer of these histones to new DNA. Histones in complex with ASF1 carry modifications that are typical of new histones[63], but chromatin-specific marks that would be present on parental histones can be detected in association with ASF1 when replication is perturbed[62,63]. One possibility is that ASF1 handles parental histones at active forks, in which case H3–H4 would be transferred as dimers (see below)[64,65]. Alternatively, ASF1 mainly takes over when histone dynamics are perturbed at stalled forks[63, 66],while other factors or passive transfer ensure segregation of parental histone $(H3–H4)_2$ tetramers during normal replication. The FACT (facilitates chromatin transcription) histone chaperone binds several replisome components[27,67–69] (TABLE 1) and is probably travelling with the fork. FACT is required for replication in several organisms[70], and fork speed is reduced in chick DT40 cells lacking the small FACT subunit SSRP1 (structure-specific recognition protein 1)[71]. This chaperone interacts

Histone chaperone
Factor that associates with histones and stimulates a reaction involving histone transfer without being part of the final product.

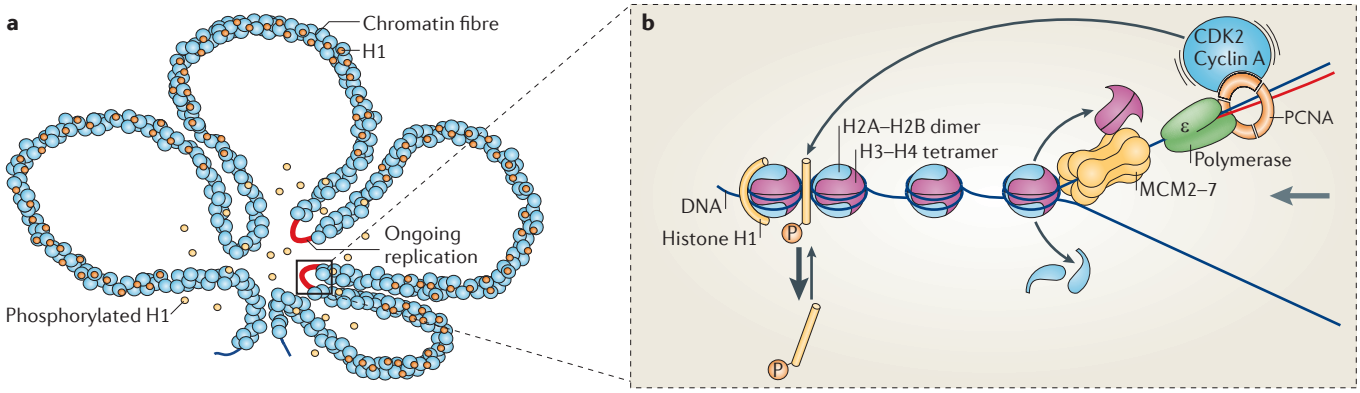


Figure 2 | **Chromatin disassembly during replication.** Model for how large-scale chromatin disruption may prime a replication domain for origin firing and fork progression in chromatin. Release of histone H1 from the chromatin fibre upon its phosphorylation by cyclin A–CDK2 is illustrated at the domain level (**a**) and ahead of an ongoing fork (**b**). Cyclin A– CDK2 is recruited to the fork probably through association with both PCNA (proliferating cell nuclear antigen) and MCM2–7 (minichromosome maintenance complex 2–7), and this may facilitate histone H1 phosphorylation. Nucleosomes are disrupted immediately ahead of the replication fork and histones are kept in close proximity for efficient recycling. Whether histones H3–H4 are released and transferred as tetramers or dimers remains unclear. In either case, the MCM2–7 helicase could act as a transient docking site.

with histone H2A–H2B and H3–H4 in multiple ways to promote both nucleosome disassembly and assembly[70,72], but its exact functions at the fork are not clear.

New histone provision. Duplication of chromatin necessitates efficient supply of new histones to sites of DNA replication. To meet the high demand of histones and at the same time avoid accumulation of potentially toxic free histones, production of canonical histones (H3.1, H3.2, H4, H2A, H2B and H1) in S phase is tightly regulated at the level of transcription, translation and mRNA stability[73]. Shortly after synthesis, histone H3.1 and H4 form dimers that are rapidly shuttled to the nucleus and delivered to chromatin assembly factor 1 (CAF1)[74], a heterotrimeric complex that mediates replication-coupled histone deposition[75] (FIG. 3a). This transport pathway involves a 'line up' of chaperones, including HSC70 (heat shock cognate 70 kDa protein), HSP90 (heat shock protein 90), NASP (nuclear autoantigenic sperm protein), RBAP46 (retinoblastoma-binding protein p46; also known as RBBP7)– HAT1 (histone acetyltransferase 1), importin 4 and ASF1 (REFS 63,76–78). Histone dimers are probably handed sequentially from one chaperone complex to the next and in this process become post-translationally modified. HSC70 and HSP90 may promote folding early in the pathway[76], whereas NASP is required to maintain a pool of soluble H3–H4 available for deposition[78]. Modifications that histones acquire before deposition may facilitate specific assembly steps and/or influence chromatin structure after incorporation. The most prominent and conserved pre-deposition mark is diacetylation of H4 at K5 and K12 (REF. 79), which is present on approximately 70% of soluble histone H3.1–H4 dimers in asynchronous HeLa cells[80]. NASP and the H4K5K12 lysine acetyltransferase RBAP46–HAT1 are proposed to act upstream of ASF1 (REFS 76,77). Consistently, this diacetylation mark is found on more than 95% of histones bound to ASF1 (REF. 63) and may stimulate nuclear import of the H3–H4–ASF1 complex by importin 4 (REFS 63,76,77). Once in the nucleus, ASF1 acts as a histone donor for CAF1 (REF. 81) by binding directly to its p60 subunit[82]. The exact mechanism of histone deposition is not clear, but a transition from H3–H4 dimers to tetramers must be involved, as ASF1 binds histone H3–H4 dimers and prevents tetramer formation[64,65]. The same is true for the CENPA (centromere protein A)–H4 chaperone and assembly factor HJURP (Holliday junction recognition protein)[83], suggesting that such transitions represent a general principle for nucleosome assembly. Whereas upstream chaperones including NASP and ASF1 handle both canonical histone H3.1 as well as the replacement variant H3.3 (REFS 78,84,85), CAF1 is specific for H3.1 (REF. 84). This selectivity explains the exclusive deposition of H3.1 onto newly synthesized DNA[86,87]. A structural explanation of how CAF1 discriminates between the two histone variants, which differ by only five amino acids, is lacking. However, phosphorylation of H3.3–H4 at H4 Ser47 is unfavourable for CAF1 binding and may contribute to H3.3 exclusion[88]. H3.3 is deposited independently of replication through alternative pathways[74], but compensatory incorporation of H3.3 may occur on newly synthesized DNA if CAF1 function is impaired[86].

Histone H3 is also acetylated before deposition onto DNA, but the preferential sites of acetylation differ between species[74]. In budding yeast, H3K56ac is present on most new histones incorporated into chromatin[89], and this mark, as well as K27ac, can promote CAF1-dependent nucleosome assembly[90,91]. In human HeLa cells, H3K14ac and H3K18ac are the major sites[63,80], whereas K56ac is less abundant[63,74,92]. In asynchronous HeLa cells, around 30% of soluble histone H3.1 is also monomethylated on K9 (REF. 80) and, if incorporated, this mark may facilitate the establishment of a repressive chromatin state. Acetylation of K14 and K18 is proposed to prevent K9me1 (REF. 77) and,

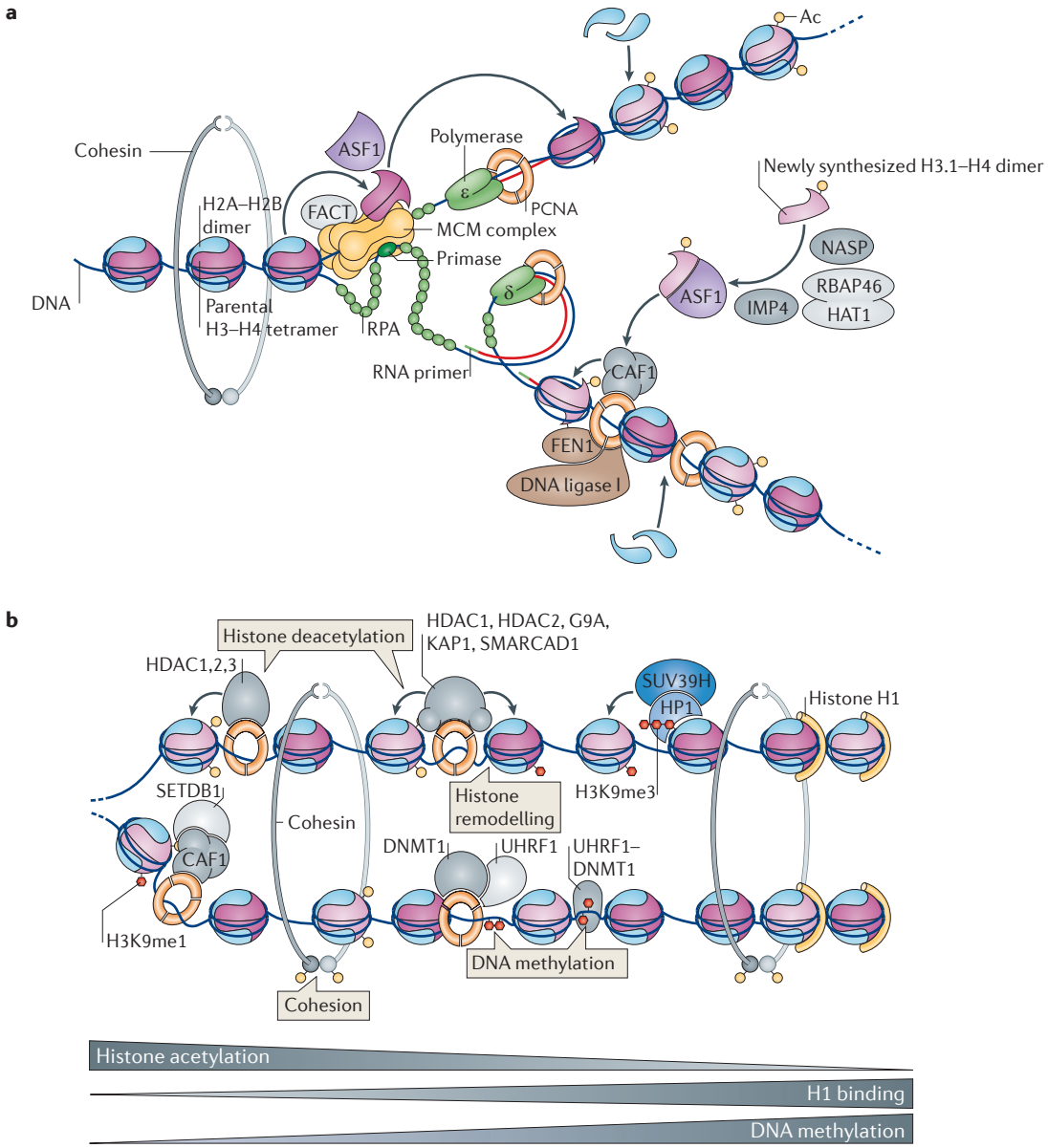


Figure 3 | **Replication-coupled assembly and maturation of chromatin. a** | Nucleosomes are assembled from recycled parental histones and newly synthesized histones. Parental $(H3–H4)_2$ tetramers segregate randomly onto the two daughter strands. How parental H3–H4 dimers or tetramers are transferred remains unclear, but minichromosome maintenance complex 2–7 (MCM2–7) may have a role together with histone chaperones such as ASF1 and FACT. New histones carrying histone H4 Lys5 and Lys12 diacetylation (H4K5K12diAc) are delivered to the trimeric chromatin assembly factor 1 (CAF1) complex by ASF1 through a transport pathway that also involves nuclear autoantigenic sperm protein (NASP), retinoblastoma-binding protein p46 (RBAP46)–histone acetyltransferase 1 (HAT1) and importin 4 (IMP4). CAF1 is recruited to new DNA by proliferating cell nuclear antigen (PCNA) on both leading and lagging strands. On the lagging strand, PCNA must integrate CAF1-dependent histone deposition with Okazaki fragment synthesis and maturation. After assembly of $(H3–H4)_2$ tetramers, two H2A–H2B dimers are added to complete the nucleosome. **b** | Nascent chromatin is highly acetylated and must be rapidly processed by chromatin modifying and remodelling activities to reach a more compact state. Generally this involves deacetylation of H4K5K12 by histone deacetylase 1 (HDAC1), HDAC2 and HDAC3, restoration of DNA methylation by DNA methyltransferase 1 (DNMT1)–UHRF1, nucleosome remodelling and histone H1 binding (illustrated by grey gradients below). In addition, specialized 'domain-specific' enzymes can be recruited in a manner depending on the type of chromatin being replicated. For example, in constitutive heterochromatin the methyltransferase SET-domain binding 1 (SETDB1) monomethylates new H3 at K9 and heterochromatin protein 1 (HP1) is recruited together with the K9me3 methyltransferase SUV39H to parental histones carrying H3K9me3. Moreover, the SMARCAD1 nucleosome remodeller contributes as part of a larger complex with HDAC1, HDAC2, KAP1 and G9A to integrate nucleosome spacing with histone deacetylation and H3K9 methylation. Several of these chromatin maturation factors, including HDAC1, DNMT1 and SMARCAD1, use PCNA as a 'landing pad'. In addition, PCNA guides acetylation of cohesin rings upon fork passage, and this is required for the establishment of sister chromatid cohesion. RPA, replication protein A.

consistently, the K9me1K14ac double mark was not found on ASF1-bound histone H3 (REF. 63). SETDB1 (SET-domain binding 1; also known as KMT1E) can impose H3K9me1 on soluble histones[80], and it may be counteracted by a demethylase, as K9me1 levels fluctuate on cytosolic histone H3.1 (REF. 76). At the replication fork, SETDB1 can interact with CAF1 and directly facilitate H3K9me1 during heterochromatin replication[93] (FIG. 3b). CAF1 also binds and promotes transfer of HP1 proteins[94,95] that bind H3K9me3 and facilitates recruitment of SUV39H1/2 (also known as KMT1A/B) enzymes (reviewed in REFS 96,97). H3K9me1 on new histones primes for K9 di- and trimethylation by SUV39H1/2 and promotes heterochromatin maintenance[93].

At the fork. All DNA polymerases (Pols) synthesize DNA in the 5′ to 3′ direction, and the two antiparallel DNA strands are thus replicated by distinct mechanisms in a coordinated fashion[98] (BOX 1). The heterotrimeric clamp, PCNA, is a central fork component, orchestrating DNA synthesis with nucleosome assembly and establishment of sister chromatid cohesion (FIG. 3a). PCNA recruits CAF1 to promote the first step in nucleosome assembly[99,100], deposition of histone H3.1–H4 dimers onto DNA[84]. Histone H2A–H2B then rapidly associates to complete the nucleosome, probably aided by NAP1 (Nck–associated protein 1)[74] or FACT chaperones. On replicating SV40 minichromosomes, nucleosomes are found on average 225 and 285 nucleotides behind the fork on the leading and lagging strand, respectively[54,55]. On the leading strand, CAF1 may bind PCNA and act simultaneously with Pol ε. However, on the lagging strand, nucleosome formation must be coordinated with Okazaki fragment maturation by flap endonuclease 1 (FEN1) and DNA ligase I (BOX 1). If CAF1 function is similar on the two strands, the prediction is that H3.1–H4 is deposited onto the growing Okazaki fragment or immediately after its completion (FIG. 3a). In this model, Pol δ would run into a nucleosome assembled onto the previous Okazaki fragment, which in turn could trigger termination of DNA synthesis, flap processing by FEN1 and ligation. This model would require that Pol δ is processive and continues strand displacement through 'naked' DNA. In budding yeast, the ligation junctions between Okazaki fragments are found close to the nucleosome dyads rather than in linker regions, and Okazaki fragment length is increased in mutants deficient in nucleosome assembly (D. Smith and I. Whitehouse, personal communication). This supports the idea of a close relationship between Okazaki fragment processing and nucleosome assembly and, indeed, FEN1 and DNA ligase I can operate efficiently on a nucleosomal substrate[101,102]. Interestingly, the lysine acetyltransferase p300 is recruited to PCNA and can acetylate both histones and enzymes that promote Okazaki fragment maturation. Through acetylation of FEN1 and DNA2-like helicase (DNA2), an alternative endonuclease specific for longer flaps[103], p300 may control the length of patch resynthesis — that is, how much of the previous Okazaki fragment is displaced and removed before ligation of the two fragments. p300 can also acetylate H3 at several sites, including K56[92], which could confer plasticity to new nucleosomes[104]. A role for nucleosome assembly and histone acetylation in the regulation of Okazaki fragment processing thus needs further investigation.

Similarly to nucleosome assembly, establishment of sister chromatid cohesion takes place at the fork and involves PCNA and acetylation[105] (FIG. 3b). The ring-shaped cohesin complex is loaded onto DNA in G1 phase, and cohesion is thought to be established as replication forks slide through these rings[105], allowing them to embrace the two daughter strands. The acetylation of SMC3 (structural maintenance of chromosomes protein 3), a component of the cohesin ring, by ESCO1/2 (establishment of cohesion 1/2) acetyltransferases during replication stabilizes the ring on DNA and facilitates cohesion (reviewed in REF. 105). ESCO1/2 can be recruited to the fork by interaction with PCNA and the alternative clamp loader complex, CFT18–RFC–CTF8–DCC1 (REFS 106,107). Acetylation of SMC3 is required for replication fork progression in human cells[108], which suggests that the cohesin complex may present a barrier to the replisome. Relaxation of the trombone loop on the lagging strand during Okazaki fragment maturation might be important for fork passage, and here the CTF18 complex could play a part. How nucleosome assembly and chromatin maturation (see below) is coordinated with cohesion establishment remains largely unexplored.

Maturation of nascent chromatin. Maturation of chromatin from a nuclease-sensitive nascent (newly synthesized) state into a structure that shows a similar resistance to nucleases as bulk interphase chromatin takes around 10 to 20 minutes[74]. Given the speed of replication, maturation is complete about 40 kb or 200 nucleosomes behind the fork. Maturation does not imply that epigenetic states are fully restored in this short time window. However, removal and acquisition of certain histone PTMs, DNA methylation and nucleosome remodelling take place in nascent chromatin, and these processes are often guided through interactions with the replication machinery (FIG. 3b). The PCNA clamp recruits several chromatin-modulating activities and is in an ideal position to integrate chromatin assembly and maturation with replication and fork repair. Live cell analysis of PCNA dynamics has suggested that clamps, once loaded, are surprisingly stable and stay on replicated DNA for up to 20 minutes[109]. Consistent with this, PCNA is left on new DNA after replication of SV40 DNA is completed in cell-free systems[99]. It is thus plausible that nascent chromatin contains PCNA rings that are not actively engaged in replication but rather orchestrate chromatin maturation (FIG. 3b). Whether old clamps remain mainly as a result of discontinuous DNA synthesis on the lagging strand and hence create an asymmetry[99] between the two sister chromatids remains to be directly addressed. Given that PCNA and CAF1 are required to generate neuronal bilateral asymmetry in *Caenorhabditis elegans*[110], and thus to specify cell fate, it is attractive to speculate that in certain cases replication-coupled chromatin assembly may allow distinct epigenetic states to be established on sister chromatids.

Sister chromatid cohesion
The joining of two sister chromatids upon chromosome replication that enables proper chromosome segregation.

Okazaki fragment maturation
Okazaki fragments are short DNA molecules of approximately 100 to 200 nucleotides in eukaryotes. They are initiated by primase–Pol α (DNA polymerase α) on lagging strands by the synthesis of an RNA primer with a short DNA extension, which is then further extended by Pol δ. The primer and part of the DNA is removed as two Okazaki fragments are ligated together.

Nucleosome dyads
Axes of symmetry in the nucleosome.

Nascent chromatin is highly acetylated owing to the incorporation of new histones (FIG. 3). This highly acetylated state probably creates a 'window of opportunity' for DNA repair, transcription factor binding and transcriptional activation, as the DNA is more easily accessible[111–114]. In human cells, acetylation of nascent chromatin is proposed to attenuate H1 deposition and thus counteract higher order compaction[74,115]. In line with this, failure to remove these acetylation marks jeopardizes silencing and pericentric heterochromatin organization, leading to severe chromosome segregation defects[116]. Deacetylation and proper chromatin maturation may also be required for fork progression and stability[117,118]. In human cells, short-term treatment with a histone deacetylase (HDAC) inhibitor can slow fork speed, and specific knockdown of HDAC3 partly recapitulates this phenotype[117]. In mouse embryonic fibroblasts, conditional knockout of HDAC3 leads to accumulation of marks that are typical of new histones, and this correlates with S phase DNA damage, chromosome fragility and, when deleted specifically in the liver, development of hepatocellular carcinoma[118]. These dramatic effects may at least in part be due to chromatin maturation defects, as HDAC1, HDAC2 and HDAC3 are found on nascent chromatin by iPOND technology[119]. HDACs can be recruited directly through PCNA or as part of large repressive complexes[120,121], providing the possibility to integrate deacetylation with other maturation steps. Deacetylation kinetics differ in eu- and heterochromatin[122], and this might relate to the preferential assembly of repressive chromatin on DNA microinjected in late S phase[123] when heterochromatin is replicated.

Replication-coupled restoration mechanisms specific to distinct types[37] of chromatin require additional layers of regulation beyond PCNA binding. For the maintenance DNA methyltransferase DNMT1, this involves UHRF1 (also known as NP95), a multi-domain protein that binds hemimethylated CpGs and directs DNMT1 to these sites (reviewed in REF. 124). An attractive model is that PCNA binding enhances the local concentration of DNMT1 and facilitates rapid recognition of hemimethylated sites in the open structure of nascent chromatin (FIG. 3b). Consistent with this view, lack of PCNA-dependent DNMT1 recruitment does not reduce DNA methylation dramatically but slows methylation kinetics on newly replicated DNA[125,126]. An open question is to what extent methylation patterns are copied immediately after replication and whether this is subject to cell-type- and loci-specific differences. The recently described TET1, TET2 and TET3-dependent oxidation of 5-methylcytosine (5mC) to 5-hydroxy-methylcytosine (5hmC), which may influence maintenance of DNA methylation[127], adds to the complexity of this question.

Chromatin maturation also involves nucleosome remodelling (FIG. 3b). The human SMARCAD1 and Williams syndrome transcription factor (WSTF; also known as BA21B)–SNF2 remodelling complexes can both be recruited to newly replicated chromatin by PCNA, but they seem to have almost opposite roles. WSTF-SNF2, which belongs to the ISWI family of remodelling enzymes, localizes to replication sites throughout S phase and seems to counteract unwarranted heterochromatinization[128]. SMARCAD1, a SWI/SNF-like remodelling factor, is required to restore heterochromatin silencing probably by facilitating histone deacetylation and H3K9me3 (REF. 121). How remodelling may prime chromatin for deacetylation remains unknown. However, SMARCAD1 is part of a large repressor complex together with HDAC1, HDAC2, the H3K9 methyltransferase G9A (also known as KMT1C) and heterochromatin factor KAP1, and thus is well suited to coordinate nucleosome spacing with deacetylation and H3K9 monomethylation. Mass spectrometry analysis of PTMs on new and old histones at different cell cycle phases indicates that H3K9me1 and H3K27me1 are established on a fraction of new histones in S phase[129,130]. Although the exact kinetics and loci-specific differences need to be worked out, it supports a stepwise mechanism for establishment of H3K9me3 and H3K27me3, which are key repressive marks with central functions in constitutive heterochromatin and developmental gene regulation, respectively. G9A and SETDB1 are probably responsible for H3K9me1 (FIG. 3b). G9A interacts with both DNMT1 (REF. 131) and SMARCAD1 (REF. 121), whereas SETDB1 is recruited in complex with CAF1 to promote H3K9me1 primarily in heterochromatin domains[80,93,132]. The human enzyme responsible for H3K27me1 remains to be defined. However, in *Arabidopsis thaliana*, the H3K27 monomethyltransferases ATXR5 and ATXR6 bind PCNA[133], and their histone methyltransferase activity is required on replicating DNA to prevent re-replication of heterochromatin[134].

In *Schizosaccharomyces pombe,* restoration of pericentric heterochromatin requires RNA interference (RNAi) to facilitate loading of heterochromatin factors, and recruitment of chromatin modifiers by the replication machinery. A burst in transcription of pericentric repeats in early S phase triggers RNAi-dependent H3K9me2 (REFS 113,114). One function of RNAi is to release RNA polymerase II at sites of inefficient termination and prevent interference with DNA replication[135]. During replication, the Cdc20 subunit of Pol ε binds and recruits the Dos2–Rik1 silencing complex and Mms19, a factor required for heterochromatin transcription[136]. Heterochromatin silencing and recruitment of Dos2–Rik1 and Mms19 is compromised in Cdc20 mutants, supporting a function of the replication machinery in establishment of marks on newly incorporated histones and restoration, or spreading, of silencing. In mammals, transcription of pericentric repeats seems to mainly be important for the initial establishment of heterochromatin domains during development (reviewed in REF. 96). For maintenance of heterochromatin during somatic cell division, spreading of H3K9me3 from parental to new histones and crosstalk between DNA methylation and H3K9me3 have central roles[96].

Maintenance of epigenetic states

In a simplified view, restoration of epigenetic states after replication involves three processes. As we have discussed above, the first two involve: transmission of chromatin marks to the two new DNA strands, relying on the symmetrical nature of mCpGs and random segregation of parental $(H3–H4)_2$ tetramers; and maturation processes linked to replication, such as histone deacetylation, DNA

iPOND
'Isolation of proteins on nascent DNA' is a technology to isolate proteins on newly synthesized DNA by combining EdU labelling with Click-iT chemistry.

RNA interference
(RNAi). Processing of transcripts into small double-stranded RNAs that can silence gene expression. Small RNAs can work by interfering with translation to induce post-transcriptional gene silencing or induce chromatin-dependent gene silencing by interacting with nascent transcripts and targeting chromatin-modifying complexes.

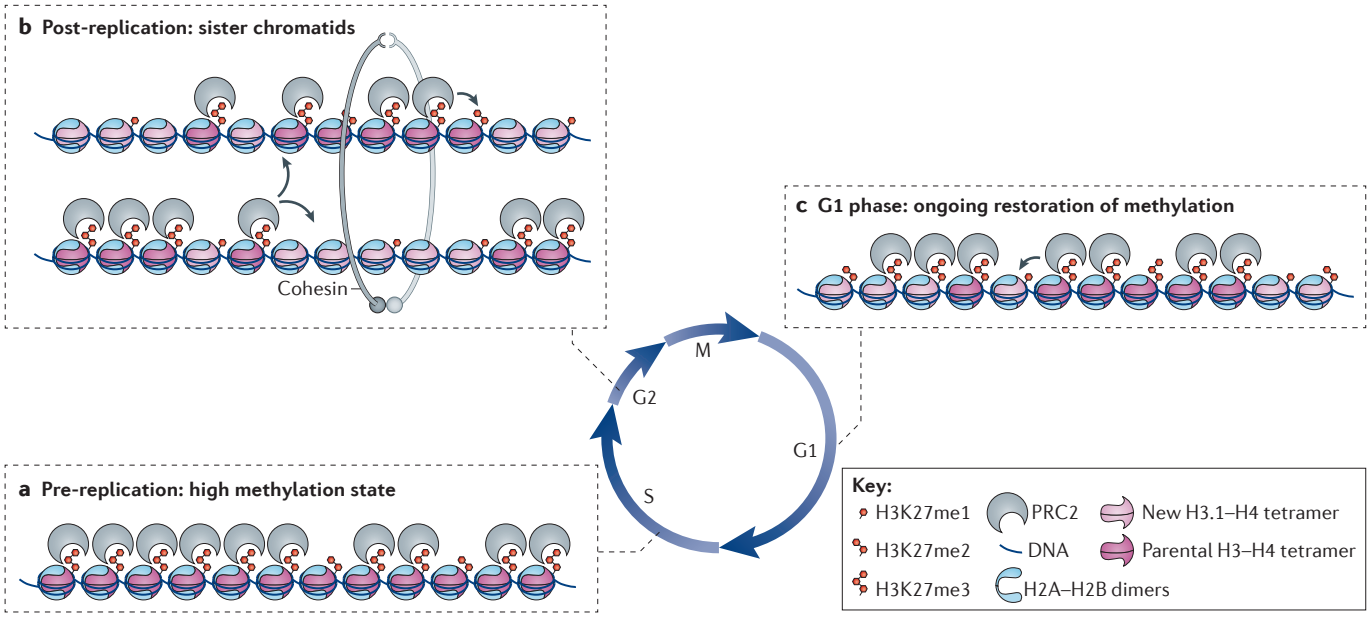


Figure 4 | **Oscillation of histone H3K27 methylation during the cell cycle**. **a** | A chromatin domain containing high levels of histone H3 Lys27 trimethylation (H3K27me3) is shown before replication. PRC2 (polycomb repressive complex 2) that mediates H3K27me3 is recruited to its own mark and perpetuates the epigenetic states. **b** | During replication, H3K27me3 is transmitted with parental histones randomly to the two daughter strands and new naive histones are incorporated and partly monomethylated at H3K27. **c** | During the next G1 phase, H3K27me3 levels are gradually restored and this process is potentially reinforced by massive recruitment of PRC2 before the next round of replication (**a**).

methylation and H3K9me1. In addition, chromatin states are shaped by a broad range of general chromatin maintenance mechanisms, including transcription-based processes, non-coding RNAs (ncRNAs), spreading of histone PTMs and crosstalk between marks. The prevailing view has been that rapid restoration of chromatin states after replication would be necessary for transmission of epigenetic information to daughter cells. However, emerging evidence shows that establishment of some methylation marks on new histones is a slow process that continues in daughter cells[129,130,137,138]. Here, we highlight the cell cycle dynamics of chromatin restoration, and readers are referred to reviews on epigenetic memory for in-depth discussion of general maintenance mechanisms[1,97,124,127].

Random distribution of parental $(H3–H4)_2$ tetramers on the two daughter strands is a cornerstone in current models for transmission of histone PTMs (FIGS 3a,4). Therefore, it is key to uncover the molecular mechanisms responsible for parental histone transfer and address whether all histone marks are maintained in the process. Modelling based on genome-wide analysis of histone protein inheritance using a tag-swapping approach in yeast has predicted that old $(H3–H4)_2$ tetramers reincorporate within 400 bp of their pre-replication loci[59]. This argues that inheritance of chromatin states must involve blocks of nucleosomes carrying similar types of modification. A similar conclusion has been drawn from a theoretical analysis of nucleosome modification and epigenetic memory using the *S. pombe* silent mating-type locus as a model[139]. This study also predicts that marks must be able to spread beyond their neighbouring nucleosomes, perhaps through higher-order structures.

The H3K9me3 and H3K27me3 marks have the potential to contribute to epigenetic cell memory because the enzymes in charge, SUV39H1/2 and enhancer of zeste homologue 2 (EZH2), respectively, can be recruited to their own mark (FIG. 4). SUV39H1/2 interacts with H3K9me3 via HP1α[140] and EZH2 binds H3K27me3 as part of polycomb repressive complex 2 (PRC2)[141,142], thereby forming self-reinforcing loops that can propagate the repressive state (FIG. 4). Detailed dissection of the liaison between PRC2 and H3K27me3 argues that marks on parental histones may suffice to recruit and activate PRC2 (REF. 142). However, higher order structures, ncRNAs and crosstalk with other marks may also contribute to maintaining these domains (reviewed in REFS 96,97)

In general, restoration of histone trimethylation after replication is not achieved before mitosis, but continues in daughter cells[129,130,137,138]. The methylation state of a domain can thus be envisioned to oscillate with the cell cycle; it would be reduced in S phase owing to new histone incorporation and then gradually increase until the next round of replication (FIG. 4). Mass spectrometry-based profiling of marks on new histones in HeLa cells shows slow restoration of H3K9me3 and H3K27me3 that continues into the next G1 phase[129,130]. This is in contrast to monomethylation of K9 and K27, which increases more rapidly after replication[129,130]. Chromatin immunoprecipitation (ChIP)-based analysis of H3K27me3 at silenced homeotic genes in *Drosophila melanogaster* embryonic cells corroborates this view and shows that PRC2-dependent H3K27me3 is reinforced immediately before replication when PRC2 levels peak[138]. Notably, variations in H3K27me3 during the cell cycle were not

accompanied by loss of transcriptional repression[138], arguing that the methylation state does not fall below the threshold required for silencing. Given the high complexity of histone marks, further studies of global and local restoration kinetics will be required to judge whether such continuous fluctuation throughout the cell cycle is a general principle. The emerging model predicts that epigenetic states are not fully restored when cells divide (FIG. 4) and suggests that passage through S phase could prime for changes in gene expression, and hence differentiation, in the following G1 phase. Moreover, with regard to restoration of chromatin states, the notion that new histones must assimilate the modification of old ones probably represents too simplified a view. PTMs on old histones are not static and, once marks are diluted during replication, modification of both old and new histones contributes to restoration of the epigenetic state[130]. Active demethylation will also contribute to the final equilibrium, although in general old histones tend to have a higher methylation state than new ones[130,143,144].

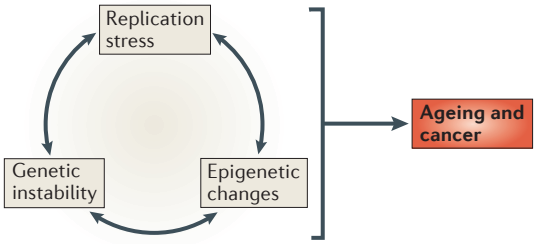


Figure 5 | **A vicious circle of (epi)genome instability may add to tumour heterogeneity.** In recent years, replication stress has been proposed as a source of genetic instability in carcinogenesis[6,43]. New evidence highlights that replication stress may also challenge the epigenome, giving rise to chromatin aberrations that can be transmitted to daughter cells and affect gene expression. It is also possible that genetic and epigenetic alterations in turn will fuel each other such that the mutational landscape of cancer cells may be mirrored by alterations in chromatin environment.

Epigenome integrity and disease

Cancer development is characterized by global genetic and epigenetic alterations[5–8]. Here we discuss the interplay between genetic and epigenetic instability, highlighting that chromatin abnormalities may be the consequence of replication defects.

When chromatin maintenance fails. Defects in heterochromatin can promote genome instability and carcinogenesis. This is seen in patients with ICF (immunodeficiency, centromeric instability, and facial anomalies) syndrome, which is caused by mutation in DNMT3B[145] and in mice lacking SUV39H[146] or DNMT1 (REF. 147). Hypomethylation of DNA is frequent in cancer[7], and genome-wide analysis has identified large blocks of hypomethylation affecting up to half of the genome in colon cancer[148]. The chromatin changes that are observed in cancer include loss of H4K16ac and H4K20me3 over repetitive regions[149] and a reduction of H3K9me2 domains[150]. How these aberrations arise and whether they fuel genetic instability is not clear, but their impact on replication control needs to be considered given emerging links between chromatin structure, initiation and chromosomal instability[12,134].

Defects in chromatin assembly can also endanger genome integrity. In yeast, impaired nucleosome assembly can lead to replication fork collapse, DNA damage, hyper-recombination and large chromosomal rearrangements[151–153]. The H3K56 acetyltransferase regulator of Ty1 transposition 109 (RTT109) and the Asf1 histone chaperone are also important for DNA repeat stability[154]. In humans, mutations in a gene that can regulate ASF1 function is associated with a rare type of anaemia involving severe chromatin abnormalities and replication defects (K. Ask, Z. Jasencakova, P. Menard, Y Feng, G. Almouzni and A.G., unpublished observations). Furthermore, chromatin assembly defects have been linked to senescence in human cells and replicative ageing in yeast[155,156] (FIG. 5). This work suggests that chromatin 'lesions' can accumulate during cellular life span, potentially leading to DNA damage. Heterochromatin domains pose a particular challenge to genome stability. Failure to restore these domains after replication owing to lack of histone deacetylation or chromatin remodelling can lead to chromosome breakages and jeopardize segregation in mitosis[116,121,157].

Replication stress fuels epigenetic instability. Deregulation of replication presents a dual threat to the organism by challenging the integrity of both DNA and chromatin[8]. Given that oncogenic activity can trigger replication stress[6,158], including unscheduled initiation, fork stalling and collapse, this could be relevant to epigenetic aberrations in cancer[8] (FIG. 5). In light of the tight coupling of histone dynamics to fork progression, several types of 'chromatin injuries' can be envisaged in response to replication stress: first, recycling of parental histones may be impaired upon fork stalling[63], potentially leading to unwarranted loss or gain of epigenetic information[159]; second, replication stress may alter the modifications on histones and DNA[63,160]; and last, fork collapse could lead to more dramatic chromatin reorganization[135], also leaving behind a potential epigenetic imprint.

Replication defects can lead to loss of gene silencing if DNA synthesis becomes uncoupled from parental histone recycling. This was shown in chick DT40 cells deficient for REV1, a Y family translesion DNA polymerase that can facilitate replication of G-quadruplex structures (G4 structures)[159]. On REV1 deletion, cells gradually lost repression of the β-globin locus that harbours a G4-forming structure, correlating with loss of H3K9me2 and gain of H4 acetylation over the locus. Once lost, silencing could not be restored by re-expression of REV1, consistent with an epigenetic change. Damage bypass most likely occurs in REV1-deficient cells, leading to unreplicated gaps of 400 to 3,000 kb[161]. This suggests that DNA synthesis may be uncoupled from parental histone recycling, resulting in loss of repressive histone marks[159]. In *Saccharomyces cerevisiae*, filling of unreplicated gaps can be delayed until G2 phase[162],

Senescence
A state of irreversible cell cycle arrest that occurs as a consequence of continued cell division in primary mammalian cells in part owing to erosion of telomeres. Senescence contributes to organismal ageing and at the same time provides a barrier to carcinogenesis.

Replicative ageing
Accumulation of genetic and epigenetic defects at each round of replication during life span in yeast.

G-quadruplex structures
(G4 structures). Guanine-rich DNA sequences capable of forming four-stranded secondary structures by square arrangement of guanines.

Chromatin remodeller
A large multi-protein machine that, through ATP hydrolysis, enables access to nucleosomal DNA by altering the structure, composition and/or position of nucleosomes.

when new histones would probably be incorporated[163]. Whether this is the case in mammals and how it would affect epigenetic gene regulation remains unknown.

Impediments to replication fork progression may also promote gene silencing owing to unscheduled histone modification and recruitment of silencing factors. In *S. pombe*, replication stress can promote spreading of heterochromatin beyond the silent mating type locus[164] and CENPB heterochromatin proteins that are known to recruit HDACs stabilize replication forks paused at long terminal repeat retrotransposons[165]. In *S. cerevisiae*, the recruitment of SIR proteins to forks that are 'paused' at artificial or natural pause sites can contribute to silencing[166]. Removal of the rRNA recombination mutation 3 (Rrm3) helicase, which normally alleviates protein–DNA barriers, enhances SIR recruitment, suggesting that longer pausing increases the probability of silencing. Whether this response is somehow beneficial remains unclear, but there is evidence of a similar phenomenon in mammals in which triplet repeat expansions can confer variegated silencing to a reporter transgene independently of chromosomal location[167]. Because trinucleotide repeats are prone to form hairpin secondary structures and interfere with replication[168], this mouse study supports a link between fork stalling and unscheduled gene silencing. When fork progression is blocked, parental and new histones cannot be loaded onto newly replicated DNA normally. They accumulate temporarily in complex with ASF1 (REFS 62,63,85), and quantitative mass spectrometry has shown that the proportion of H3K9me1 marks increases[63]. Incorporation of these histones upon fork restart[63] can in turn lead to an elevated level of H3K9me1 on nascent chromatin (C.A. and A.G., unpublished observations). This may contribute to unscheduled gene silencing, as H3K9me1 counteracts H3K9ac and may prime for H3K9me3 (REF. 80). Increased levels of DNA methylation have also been reported in cells that are exposed to severe replication damage[160]. In primary cells, premature senescence in response to replication stress and oncogenic stimuli correlates with a global increase in H3K9me3 (REF. 169). As H3K9me3 does not increase if senescence is induced by replication-independent damage[169], it is tempting to speculate that unscheduled silencing is initiated at sites of fork stalling.

These lines of evidence underscore that chromatin integrity and epigenetic gene regulation is susceptible to replication stress. Severe replication damage leading to fork collapse and DNA repair by homologous recombination has probably even more dramatic effects on the chromatin landscape. But little is known about the molecular mechanisms that orchestrate chromatin dynamics during repair of damaged forks. In yeast, the Ino80 chromatin remodeller is recruited to arrested forks, where it may reorganize nucleosomes during replication restart and DNA repair[170–172]. In human cells, the TONSL–MMS22L complex can be recruited to damaged forks[66,173]. MMS22L is thought to mediate homologous recombination repair by displacing replication protein A (RPA) from single-stranded DNA and promoting RAD51 loading[66,173,174]. Interestingly, TONSL interacts with ASF1, FACT and histones[66,173–175], suggesting that MMS22L function is coupled to nucleosome disruption and/or assembly. Mutation of the *A. thaliana* TONSL homologue highlights its dual function in epigenome and genome maintenance, as BRU1 (also known as TONSOKU) mutants are highly sensitive to DNA alkylating agents that stall replication forks and in addition show stochastic gene silencing and developmental defects[176].

Outlook

The interplay among chromosomal architecture, chromatin structure and DNA replication is elaborate and has a bearing on epigenome maintenance during development as well as in the adult organism. Major questions of a basic nature and of disease relevance remain unresolved. What rules govern replication timing and the link to three-dimensional organization of chromosomes? When will we move beyond speculation with respect to the organization of replication domains and the mechanisms that orchestrate the intrinsic order of origin firing throughout development? The answers clearly require bridging between the fields of nuclear structure and replication. With respect to the underlying mechanisms whereby replication forks move through chromatin and leave properly packaged daughter strands behind, the intense interest in epigenetic inheritance and new techniques to probe chromatin replication in cells[119,177] holds promise of rapid progress. It is plausible that chromatin replication and restoration processes are in part loci specific. Quantitative means to follow replication at a given locus and the distinct kinetics of chromatin restoration throughout the cell cycle are thus desired. Understanding the mechanisms that underlie epigenome maintenance in dividing cells should also give insights into how perturbations may challenge cellular memory. Indeed, chromatin seems vulnerable to replication stress. Future research should unveil whether epigenetic variation driven by replication stress can contribute to cellular ageing and cancer.

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Competing interests statement

The authors declare no competing financial interests.

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