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Chapter 14

Chromatin Damage Patterns Shift According to Eu/Heterochromatin Replication

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Additional information is available at the end of the chapter

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1. Introduction

In order to maintain genetic stability, strictly controlled mechanisms are essential to assure the accuracy of genetic functions. Precise genome replication and correct control of gene expression mostly *via* epigenetic mechanisms are critical in maintaining the stability of genomes. Moreover, the characteristic chromatin compartmentalization of mammalian genomes contributes to regulate the housekeeping or tissue-specific genetic activities [1, 2].

Table 1 summarizes the distinct chromatin compartments and their foremost properties. Euchromatin (*eu*: true) and heterochromatin (*hetero*: different) are two major compartments or chromatin states of the DNA originally distinguished by their isopycnotic or heteropycnotic interphase staining properties, respectively [3]. The heterochromatin compartment differentiates in both constitutive (permanent) and facultative (developmentally reorganized) states [4]. Facultative heterochromatin represents chromatin regions being facultatively inactivated (heterochromatinized) because of gene dosage compensation (i.e.: mammalian female inactive X chromosome) randomly silenced at an early stage of embryogenesis or tissue-specific gene expression. Constitutive heterochromatin consists in regions of α- and β-heterochromatin [5, 6].

Distinct features characterize the different chromatin states (Table 1). Interphase open chromatin conformation and transcriptional activity in all cell types distinguish euchromatin. Higher order chromatin compaction characterizes constitutive α- and β-heterochromatic regions while gene silencing differentiates constitutive α-heterochromatin. Tissue-specific transcriptional activity and low or high chromatin condensation, depending on gene expres-

sion, correspond to features of facultative heterochromatin [7, 6]. The mammalian genome compartmentalization can be visualized in both banded metaphase chromosomes and stained interphase nuclei.

Compartments	Euchromatin	Facultative heterochromatin		Constitutive heterochromatin	
Chromatin types	Euchromatin	Tissue-specific	Dosage compensation	α- heterochromatin	β-heterochromatin
Location in metaphase chromosomes	Light G-bands	Dark G-bands	Inactive X chromosome (Xi)	C-bands	C-bands
Location in interphase nuclei	Inner compartment	Peripheral compartment and chromocenters	Peripheral compartment and chromocenters	Peripheral compartment and chromocenters	Peripheral compartment and chromocenters
Interphase chromatin compaction	Open conformation	Low or high order compaction	High order compaction	High order compaction	High order compaction
Presence of genes	Housekeeping genes	Inactivated tissue-specific genes	Dosage inactivated genes	No genes	Transposable elements and heterochromatic genes
Gene expression and relation to chromatin state	Gene activity in euchromatic state in all cells	Tissue-specific gene activity in euchromatic state	Gene activity in euchromatic state until silencing	No gene activity	Low gene activity in heterochromatic state
GC or AT DNA sequences richness	GC-rich	AT-rich	GC- and AT-rich	AT-rich	AT-rich
Repeated DNA sequences	SINEs	LINEs	SINEs and LINEs	Tandem highly repeated DNA sequences	Tandem highly repeated DNA sequences
CpG island methylation	Unmethylated	Unmethylated or methylated	Methylated	Methylated	Methylated
Core histone tail acetylation	Hyperacetylated	Hyperacetilated or hypoacetylated	Hypoacetylated	Hypoacetylated	Hypoacetylated
Replication timing	Early	Early or late	Late	Late	Late or early

Table 1. Distinguishing properties of chromatin compartments.

The C-banding procedure [8] produces a selective staining of specific chromosome regions, mapping at or adjacent to centromeres, telomeres or interstitial arm sites, depending on the species. Occasionally, a chromosome arm is entirely heterochromatic, such as the long arm of the Chinese hamster X chromosome (Figure 1, left). In humans, C-bands are located at centromeres and pericentric regions of all chromosomes, being conspicuous at the pericentric regions of chromosomes 1, 9 and 16 and the distal long arm of the Y chromosome (Yq) (Figure 1, right).

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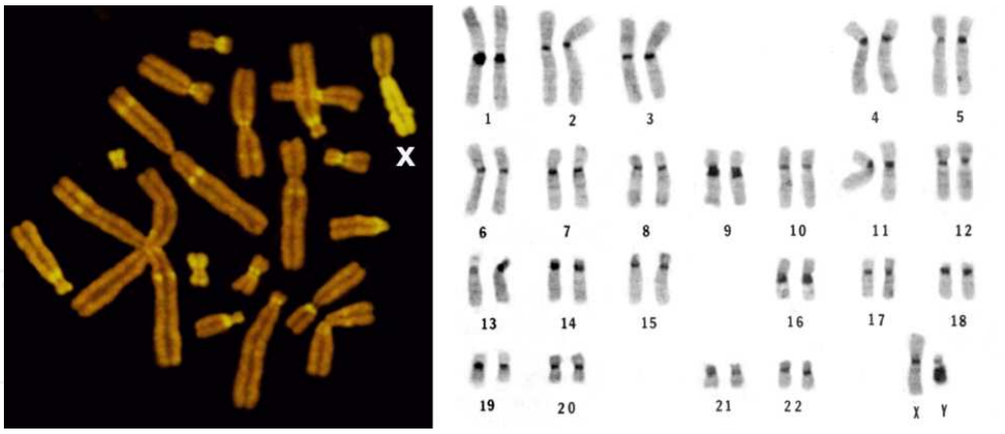

Figure 1. C-banding in CHO and human chromosomes. Left: C-banded metaphase of CHO9 cell line. The CHO cell line was established from a Chinese hamster ovary fibroblast culture [9] and presents a modal number of 21 chromosomes. This cell line contains eight normal and twelve rearranged autosomes with only one X chromosome. Giemsa-stained C-band regions are visualized in yellow (reflected light microscopy). The CHO X chromosome (X) shows an almost entirely heterochromatic long arm. Right: C-banded caryotype of a human peripheral lymphocyte metaphase showing centromeric, pericentric (chromosomes 1, 9 and 16) and distal Yq heterochromatic blocks.

By digestion with the proteolytic enzyme trypsin followed by Giemsa staining (G-banding procedure) [10], a pattern of alternate light and dark regions along the length of all chromosomes is obtained (light G-bands and dark G-bands, respectively). The G-band pattern is characteristic for each chromosome pair allowing their precise identification and caryotyping. Figure 2 shows the CHO9 and human G-band chromosome patterns.

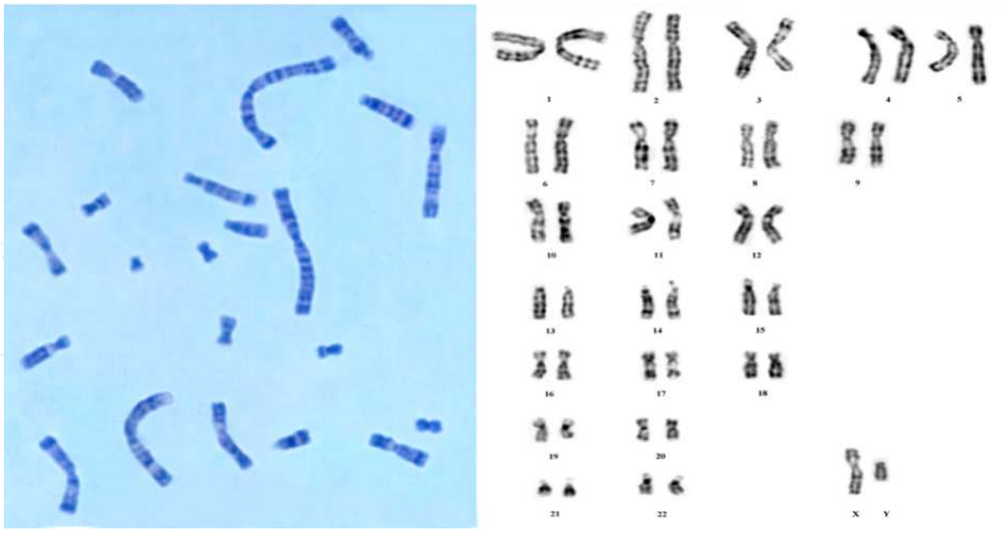

Figure 2. G-banded CHO9 metaphase (left) and a male human peripheral lymphocyte caryotype exhibiting G-bands (right).

C- and G-band patterns reveal the heterogeneous organization of chromatin along condensed chromosomes. C-bands enclose constitutive α- and β-heterochromatin. Regions with

ubiquitously expressed housekeeping genes (euchromatin) reside in light G-bands, while tissue-specific genes (facultative heterochromatin) dwell in dark G-bands [5, 6, 11].

Light and dark G-bands may reflect a differential array of SAR (**S**caffold-**A**ssociated **R**egions), composed by highly AT-rich DNA stretches binding to the chromosome scaffold. Regions of dark G-bands exhibit a tighter chromatin fiber coiling than light G-bands domains [12]. Constitutive heterochromatin has an even more dense conformation.

Moreover, euchromatic light G-bands are GC-rich and gene-dense regions, containing unmethylated CpG islands and moderately repeated **S**hort **In**terspersed **E**lements (SINE), mainly represented by Alu family sequences. Conversely, facultative heterochromatic dark G-bands are AT-rich, gene-poor and harbor hypermethylated CpG and moderately repeated family of Long Interspersed Elements (LINE) sequences. Constitutive α-heterochromatic C-bands are the major locations of tandem non-coding highly repeated satellite DNA sequences, devoid of genes [11, 13]. However, constitutive β-heterochromatin presents inserted middle-repetitive transposable elements between the tandem repeats, some of them transcriptionally active [6]. Moreover, genes residing within regions of pericentric constitutive β-heterochromatin termed "heterochromatic genes" have been reported in *Drosophila,* mammals and plants [14, 15].

In spite of variations according to cell type or function of mammalian interphase nuclei, the corresponding chromatin of light and dark G-bands as well as C-bands is non-randomly distributed in different nuclear compartments, displaying specific chromatin conformation, molecular composition and gene expression patterns.

In most interphase cells, euchromatin (light G-bands) dwells in the inner compartment of nuclei, whereas heterochromatin (dark G-bands and C-bands) resides in the peripheral compartment, chromocenters and around nucleoli [6, 16]. Figure 3 illustrates a HeLa nucleus where the different interphase chromatin compartments can be recognized.

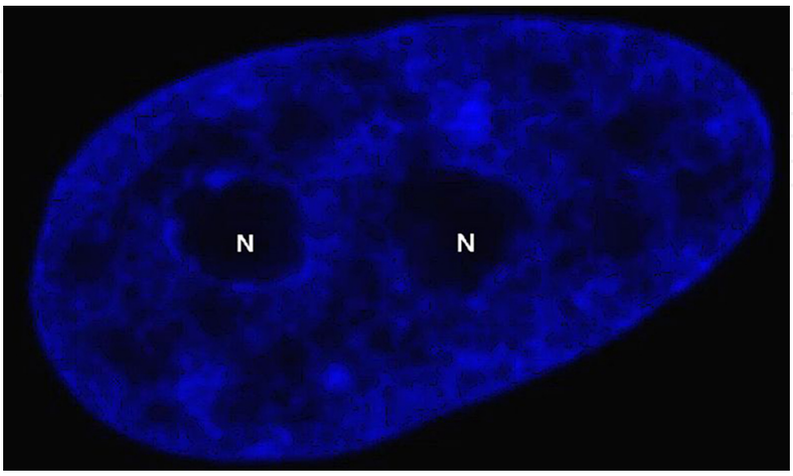

Figure 3. Distinct eu/heterochromatin compartments in DAPI-stained HeLa interphase nucleus. DAPI-bright regions correspond to heterochromatin and dim areas to euchromatin. N: nucleolus.

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Constitutive and tissue-specific genes are only expressed in the euchromatic state. Therefore, facultative heterochromatin behaves as euchromatin in cells where its tissue-specific genes are transcribed, but holds a packed (heterochromatic) conformation when genes remain silent.

However, some transposons and heterochromatic genes of β-heterochromatin are transcriptionally active in heterochromatic state suggesting that distinct epigenetic mechanisms of gene regulation and preservation of eu/heterochromatic states may exist in these regions [6, 14, 15].

Once acquired, the chromatin states are somatically maintained as stable heritable epigenetic states. Euchromatin remodels during mitosis and restores the original organization in early G_1 phase of each cell cycle. In addition, during DNA synthesis (S-phase) both euchromatin and heterocromatin transiently lose their typical condensation status recovering the previous folding level after replication. Establishment and maintenance of chromatin states involve post-translational modification enzymes that act coordinately to methylate CpG islands and to either acetylate, methylate, phosphorylate, ubiquitinate, poly-ADP ribosylate or SUMOylate the core histone tails of nucleosomes. These epigenetic changes, together with the recruitment of methyl-CpG binding proteins, ATP-dependent chromatin remodeling complexes and the association of specific non-histone proteins, such as HP1 (**H**eterochromatin **P**rotein 1) or RNAi (non-coding interference RNA), also mediate the regulation of DNA replication, transcription and repair [17, 18].

The N- and C-termini of H3 and H4 core histones are particularly involved in epigenetic regulation. Acetyl groups covalently added to lysines, serines or arginines of the N-terminal histone tails reduce the affinity to DNA, promoting the accessibility of chromatin remodeling and activating transcription factors. Therefore, histone hyperacetylation usually characterizes active chromatin regions. Conversely, transcriptionally silenced chromatin regions generally contain hypoacetylated histones (Table 1). For instance, H3 acetylated (ac) in lysine 9 (H3K9ac) is enriched at the promoter region of active genes although, it was reported that the histone H3 acetylated at lysine 4 (H3K4ac) resides in pericentric heterochromatin of *Schizosaccharomyces pombe,* playing a role in the assembly of repressive heterochromatin [19]. On the other hand, histone methylation (me) can be associated with transcriptional activation or repression. For example, methylation of H3 on lysines 4, 36 or 79 (H3K4me, H3K36me, H3K79me) is associated with transcriptional activation whereas methylation of H3 on lysines 9 or 27 (H3K9me, H3K27me) and of H4 on lysine 20 (H4K20me) is involved in transcriptional repression [18]. The concerted action of acetylated and methylated histone core residues is central in creating a "histone code" which delineates distinct genomic loci that recruit factors needed for DNA remodeling, transcription, replication and repair [5, 17].

In general, methylation of CpG islands within 5'regions of genes is associated with hypoacethylated histones, characterizing the heterochromatic state (Table 1). However, DNA methylation is not exclusively related to gene silencing. It was reported that methylation of some imprinting centers can displace trans-acting repressor factors, allowing the expression of the linked imprinted genes [20].

The epigenetic mechanisms involved in the maintenance of eu/heterochromatic compartments and gene expression are connected to DNA replication. There are specific interactions between components of the replication machinery and chromatin related factors, timing the eu- or heterochromatin replication.

2. Replication of eu/heterochromatin compartments

Compartmentalization of vertebrate genomes cooperates in achieving the high fidelity DNA replication necessary for the accurate preservation of the genetic information throughout cell generations. DNA replication is a temporarily and spatially highly ordered and strictly regulated process, occurring during S-phase of the cell cycle, with distinct genome compartments replicating at different times. The replication timing of the genome compartments are highly conserved within consecutive cell cycles and regulated by specific epigenetic chromatin conformation domains, DNA features and transcriptional activity [21, 22, 23].

Mammalian chromosome duplication involves clusters or domains of neighboring replicons named **R**eplication **T**iming **D**omains (RTD) which synchronously start and end replication, according to a deterministic replication timing program [21, 22, 24]. When one domain completes replication, an adjacent domain successively initiates DNA synthesis [25]. Remarkably, mouse and human asynchronous replication timing may function randomly between individual replicons within a RTD and non-randomly between RTD [25]. The random firing of replication origins within a RTD generates a different replication pattern during each S-phase, but it has been reported that some origins fire preferentially and more frequently than others [26]. The RTD are stable structures of mammalian interphase nuclei, replicating and transcribing in temporal and spatial coordination [26].

Pulse labeled interphase nuclei of human, mouse and hamster cells with the base analogues 5-bromo-2'-deoxyuridine (BrdU) or 5-ethynyl-2′-deoxiuridine (EdU) demonstrated the asynchrony and specific spatial distribution of DNA replication. The early replication pattern of S-phase (ES-phase) is characterized by replication foci dispersed throughout the inner environment of the nuclei with scarce or absence of foci at the periphery or adjacent to the nucleoli. The replication pattern changes throughout the progression of S-phase. In mid S-phase (MS-phase) most foci map adjacent to the internal nuclear membrane and around nucleoli, with few foci centrally located. Lastly, late S-phase replication maps next to the nuclear envelope as well as in chromocenters and around nucleoli [16, 27]. Early S-phase and late S-phase replication patterns of CHO9 cells are illustrated in Figure 4.

In general, chromatin with transcriptional activity (euchromatin) replicates early in S-phase whereas constitutive α-heterochromatin duplicates late. Besides, facultative heterochromatin replicates earlier if its tissue-specific genes are being expressed and later if not [6, 28] (Table 1). It has been reported that genes of mouse embryonic stem cells residing within GC-rich and LINE-poor DNA (euchromatin) do not modify their replication timing after differentiation to neural precursors, whereas genes residing in AT-rich and LINE-rich DNA revealed changes in replication timing accompanied by changes in gene expression and

chromatin folding [29]. A change of replication timing from early S to late S-phase is particularly evident in the female mammalian Xi [30]

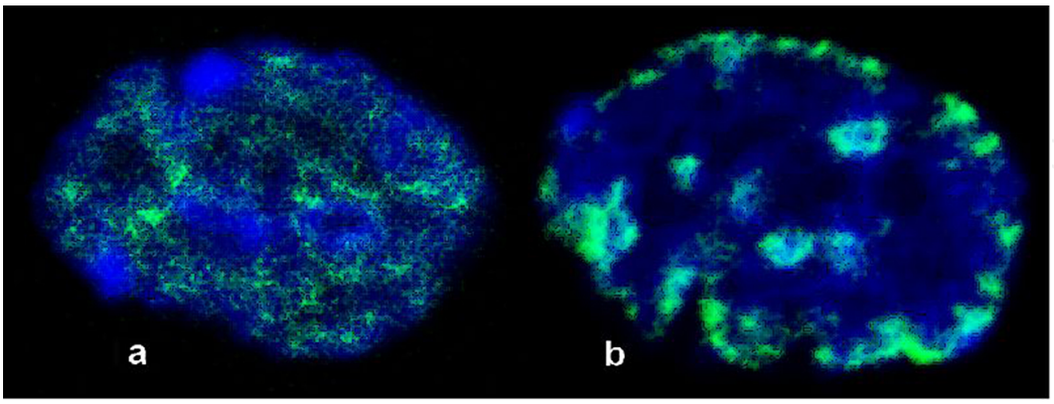

Figure 4. Early (ES-phase) or late (LS-phase) replication patterns of CHO9 nuclei revealed by incorporation of EdU and subsequent detection with an Alexa Fluor 488 (green) conjugated azide (Click-iT EdU imaging kit, Invitrogen). (a) ES-phase nucleus with inner compartment replication. (b) LS-phase nucleus showing replication in the peripheral compartment, chromocenters and around nucleoli.

Early replication seems to be important but not essential for gene transcription. Moreover, late replication is not an obligatory feature of heterochromatin. For example, transcriptionally active transposons of β-heterochromatin replicate late while the heterochromatic centromeres and the silent mating-type cassettes of *Schizosaccharomyces pombe* replicate in early S phase [14, 15, 31]. There are additional cases reported of early heterochromatin replication such as human telomeres [32]. and mouse pericentric heterochromatin and centromeres [33].

The early replicon clusters of higher eukaryotes alternate their replication and transcription activity. However, correlation between replication and transcription does not exist in S*accharomyces cerevisiae* [34]. Employing distinct colored fluorescent labels to recognize early replication foci and transcription foci (factories), it was shown that both labels do not colocalize. In ES-phase, actively replicating foci are transcriptionally inactive and only restart transcription after finishing replication. The replication timing is indirectly related to transcription through the assembly of a higher-order chromatin state [2]. For example, silencing of the mammalian Xi is initially reversible and only stabilizes when an identifiable higher-order chromatin configuration (Barr body) appears and replication is delayed [35].

The chromatin replication timing is reestablished early in G_1 phase of each cell cycle, coincident with the anchorage and positioning of chromosomal segments at specific locations within the nucleus named TDP (**T**iming **D**ecision **P**oint) [36]. Both anchorage and positioning of chromosomes are central in the organization of nuclear eu/heterochromatic compartments and the establishment of replication timing and transcriptional activity [23, 36]. Modifications in subnuclear chromatin organization are associated with changes in replication timing during development [37]. For example, the position of the immunoglobulin heavy chain locus (IgH) in B cells shows that its localization in the interphase nuclei depends on replication timing and gene activity. During early stages of B cell differentiation,

both transcribed alleles of the IgH locus are centrally located in the nucleus and replicate early. Conversely, in advanced differentiation stages the IgH locus is repositioned to the nuclear periphery, repressed and late-replicated [38].

Nonetheless, chromatin positioning at the nuclear periphery is indicative but not mandatory for gene silencing and late replication. In fact, the nuclear periphery is heterogeneous with respect to transcription. For instance, in budding yeast, nuclear pores, which mediate the transport between the nucleus and cytoplasm, enhance the transcriptional activity of genes positioned in their proximity [39]. The dosage compensation complex of the hyperacetylated *Drosophila* male X chromosome interacts with nuclear pore proteins determining its transcription up-regulation and early DNA duplication [40].

Replication clusters correspond to bands of metaphase chromosomes. Tightly coiled C-band (constitutive heterochromatin) replicates in late S-phase. Facultative heterochromatin of the dark G-bands duplicates either early or late depending on its tissue-specific expression. Early replication pattern characterizes the loosely coiled euchromatin of light G-bands. Ubiquitously expressed housekeeping genes (light G-bands) are therefore early replicating [41, 42]. Duplication timing analysis by quantitative PCR of the boundary region between G-light 13q14.3 and G-dark 13q21.1 bands showed that the G-light side of the frontier replicates early whilst the G-dark interface replicates late. However, analysis using PCR primers spaced at approximately 150 Kb intervals showed that the switch in G-light/G-dark band replication timing takes place gradually from early-mid to late S-phase over a 1-2 Mb region [43]. The DNA segments corresponding to large regions between early and late-S phase replication timing domains are termed TTR (Timing Transition Regions) [44].

A correlation between replication timing and epigenetic modification of chromatin has also been shown. Early replication domains are related to specific combination of changes in histone lysine residues (H3K9Ac, H3K27Ac, H3K4me, H3K36me and H3K79me) associated with transcriptional activity. On the other hand, the repressive epigenetic modifications (H3K9me, H3K27me and H4K20me) are linked to late replication [18].

Chromatin epigenetic changes occurring throughout DNA replication may provide a replication timing mechanism (firing early or late replication origins) in the direction of maintaining specific chromatin expression patterns [45]. It was reported that histone hypoacetylation is needed to preserve normal heterochromatin replication dynamics [46] and that histone hyperacetylation may increase the efficiency of replication origins, advancing the replication timing of distinct genomic regions [47]. For instance, removal of acetyl groups by HDAC (**H**istone **D**e**AC**etylase) contributes to mantain late replication at imprinted loci [48] and the generation of neocentromeres [49].

Several proteins, including CpG island-methylating DNMT (**DN**A **M**ethyl **T**ransferase), core histone tail-methylating HMT (**H**istone **M**ethyl **T**ransferase) and HP1 (**H**eterochromatin-associated **P**rotein), colocalize with late replicating DNA regions [45]. HP1 binds to heterochromatin, facilitating the extension of the repressive H3K9me modification [50] and hence delaying replication timing by supporting heterochromatin conformation. HP1 could facilitate the late firing of replication origins within heterochromatin [51]. Furthermore, muta-

tions of DNMT result in earlier replication of normally late replicating DNA. For instance, patients with mutations in the Dnmt3b gene (coding protein DNMT3b) have hypomethylated CpG islands in the Xi chromosome, which replicates at an earlier S-phase stage despite the presence of XIST (**X**-**I**nactive **S**pecific **T**ranscript) RNA [52]. Accordingly, changes in either DNA or histone methylation status in concert with histone acetylation patterns may promote open or tight chromatin conformations and thus modifications in the firing of replication origins and/or replication rates [47].

In mammals, several distinct discrete or diffuse genomic sequence motifs can potentially act as **O**rigin **R**eplication **I**dentification (ORI), where a large number of proteins bind to load replication complexes. A protein complex, named the pre-**R**eplication **C**omplex (pre-RC) associates with potential replication origins in G_1 phase. This complex includes the **O**rigin **R**ecognition **C**omplex (ORC), which recognizes the replication origins, the helicase MCM2-7 (**M**ini **C**hromosome **M**aintenance 2-7), and other essential factors. Early firing ORI demonstrated to be rich in MCM proteins. Besides, MCM could be more efficient in early firing than in late firing ORI suggesting that heterochromatin could repress MCM activities [53, 54].

Accessibility of replication initiation factors to redundant or discrete replication origins may be regulated by its nuclear localization in relation to chromatin states. For example, the early replicating α-globin locus is located within a light G-band. Deletions that juxtapose the α-globin locus next to a region of late replicating telomeric condensed heterochromatin (repositioning this locus to the nuclear periphery), delay the initiation of α-globin replication by restricting the access of initiation factors to the ORI [55].

There is a complex cell cycle intra-S checkpoint involving the ATR/CHK1-related network in metazoas and ATR/Rad53 in *Saccharomyces cerevisiae* that controls replication asynchrony. The transition from early to late S-phase replication (mid-S replication pause) is coupled with the activation of the intra S-phase checkpoint at mid S-phase which inhibits the initiation of late replicons. It has been reported that inhibition of CHK1 generates earlier firing of a late-firing subset of ORI [56, 57]. Accordingly, the checkpoint function may play a role in regulating replication asynchrony and S-phase progression [25, 58].

Both DNA and histone methylation can affect replication timing *via* the ATR/CHK1 control pathway. There is a complex and so far not completely understood relationship between checkpoint function and epigenetic modifications (DNA methylation, histone methylation and histone acetylation) in the regulation of replication origins firing during S-phase [47, 59].

Following pre-RC loading to ORI, a protein pre-**I**nitiation **C**omplex (pre-IC) assembles upon MCM proteins together with factors required for loading replicative polymerase. The chromatin association of pre-RC and pre-IC is asynchronous, allowing pre-RC inhibition and pre-IC activation (from S-phase initiation toward the end of mitosis) by the cell cycle CDK proteins (**C**yclin-**D**ependent **K**inases). This regulation licenses replication to occur at a specific time, only once per cell cycle, and ensures that cell cycle cannot progress until checkpoints are satisfied. In *Xenopus laevis* and mammalian cells there is an additional system to

control licensing by means of the geminin protein, which also inhibits pre-RC. Degradation of geminin at the end of mitosis is essential for a new license of replication [56, 60].

Completion of replication is necessary for entire chromosome condensation. *Drosophila* ORC mutants unable to complete S-phase have defects not only in DNA replication (with some euchromatic regions replicating even later than heterochromatin) but also in cell cycle progression and chromatin condensation [61]. Although some levels of chromosome condensation occur in the absence of a complete replication cycle, mitotic chromosomes are shorter and thicker than in wild type *Drosophila*. Even though ORC is principally involved in the initiation of DNA replication, additional roles in mitotic chromosome condensation, centromere function as well as the establishment and maintenance of gene silencing and heterochromatin have been suggested [61, 62, 63].

3. Eu/heterochromatin replication and distribution of genetic damage

The S-phase of the cell cycle has proved to be very sensitive to genetic damage. S-phase has been considered as one of the sources of genomic instability. There are several lines of evidence that correlate genomic instability with chromosomal aberrations (CA), birth defects and infertility [64]. Besides, oncogene activation or tumor suppressor gene repression can arise as a consequence of primary DNA damage or CA [65]. Several authors have reported the colocalization of induced CA breakpoints (BP) (sites of chromosomal breaks in a CA) with regions harboring fragile sites, oncogenes or cancer-associated CA [66-72].

The human genome holds long stretches of AT-rich sequences as well as inverted, mirror or direct tandem repeats, prone to be arranged in unusual DNA secondary structures that may inhibit replication. The presence of secondary structures, unstable single-stranded or non-replicated regions could lead to chromosome fragility expressed as gaps or breaks in metaphase chromosomes [73, 74].

DNA replication in mammals slows down significantly when the 1-2 Mb regions of TTR are replicated [57]. It was reported that after replication of euchromatic light G-bands, the replication fork stalls at TTR of the interband regions, restarting DNA synthesis at the adjacent dark G-band after a mid S-phase pause [6]. This interband region devoid of replication origins is often replicated by means of a single replication fork [75]. Such genomic segments could generate damage-prone regions that frequently overlap with DNA fragile sites [43, 76]. For example, the common fragile site FRA3B is devoid of replication origins and thus completes replication very late in S-phase [77]. In addition, it was observed that mutation rates increase with the distance from replication origins [78, 79].

Furthermore, it was reported an increase in mutation rate as S-phase advances. Early replicating housekeeping genes are more conserved than later replicating tissue-specific genes [57, 80]. Genes corresponding to mutational hot spots involved in speciation and adaptive radiation response are late replicating [57]. CpG methylation status of late replicating regions may contribute to the rise in mutation rate mostly due to 5^{me}CpG substitutions [81, 82].

3.1. Eu/heterochromatin replication and induced-damage distribution in a mitotic chromosome model

DNA lesions trigger a **D**NA **D**amage **R**esponse (DDR) characterized by activation of cell cycle checkpoints, damage sensor proteins, DNA repair mechanisms and apoptotic pathways [83, 84]. The DNA **D**ouble-**S**trand **B**reak (DSB) is the critical DNA lesion involved in CA production [85]. DSB can be generated by DNA-damaging agents or spontaneously through the endogenous production of reactive oxygen species (ROS) or cellular processes such as DNA replication, repair, transposition or mitotic recombination. Agents inducing DSB and CA are named clastogens. The S-phase independent clastogens, like ionizing radiation and the radiomimetic agent bleomycin, directly induce DSB. Conversely, S-phase dependent clastogens such as UV-C and alkylating compounds need the intervention of DNA repair and replication in order to generate DSB, which could ultimately lead to CA. Hence, DNA replication constitutes a relevant step in the transformation of DNA lesions into CA. Besides, some clastogenic agents such as the anti-topoisomerase II cleavable complex trappers behave as S-phase independent clastogens. Eukaryotic topoisomerases II alleviate tensional DNA stress by the generation of a DNA topoisomerase II complex (cleavable complex) within which the topoisomerase II component introduces transient breaks in both DNA strands (DSB) allowing the DNA to pass through the breaks [86]. Drugs that act by trapping cleavable complexes hamper the resealing of DSB produced by topoisomerase II and, as a consequence, DNA DSB persist [87, 88].

As shown in Figure 1, the CHO9 X-chromosome exhibits an almost entire constitutive heterochromatic long arm (Xq) with the exception of a medial secondary constriction. Besides, Xq replicates in late S-phase whereas the euchromatin of the short arm (Xp) and the Xq secondary constriction duplicates during early S-phase (Figure 5) [89, 90]. Differential replication timing of Xp and Xq of CHO cells provided a valuable experimental model to analyze the relationship between eu/heterochromatin DNA replication and CA induced by different types of clastogens: UV-C light, the methylating agent methylmethane sulphonate (MMS) and the anti-topoisomerase II inhibitor etoposide (a cleavable complex trapper) in BrdU pulse-labeled CHO9 chromosomes [91, 92].

CHO9 cells were treated with MMS (20 mM) or etoposide (20 μM) and simultaneously exposed to 30 mM BrdU (40 min) or otherwise exposed to UV-C (30 J/m2; 0.1 J/m2/s) and immediately labeled with BrdU (40 min). Incorporation of BrdU in Xp or Xq was disclosed by immunolabeling either treated or control CHO9 metaphases with anti-BrdU antibodies coupled to FITC. The relationship between replication timing, chromatin conformation and genetic damage was investigated by mapping induced BP in Xp and Xq in cells treated both in early and late S-phase [91, 92].

Examples of CA induced by MMS, etoposide and UV-C in replicating CHO9 Xp or Xq are shown in Figure 5. Figure 6 illustrates Xp/Xq distribution of etoposide, UV-C and MMS-induced BP in relation to replication.

The application of χ^2 test to analyze the association between Xp/Xq replication pattern and Xp/Xq BP localization showed that when Xp replicates, BP produced by either MMS, UV-C

or etoposide clustered in Xp. On the other hand, during Xq replication, BP induced by the clastogens concentrated in Xq [91, 92] (Figure 6).

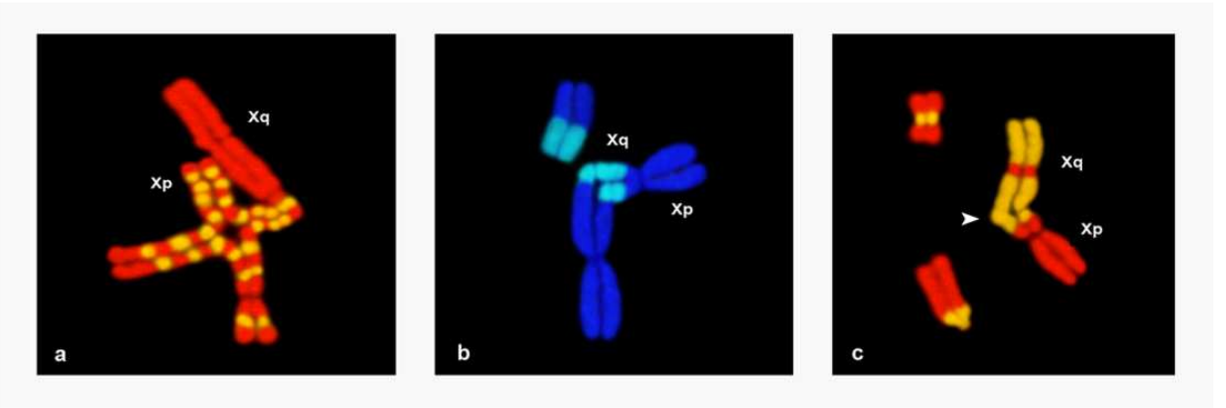

Figure 5. Illustrations of CA involving CHO9 Xp or Xq induced by (a) MMS, (b) etoposide, or (c) UV-C in (a) early (Xp replication) or (b and c) late (Xq replication) S-phase. Different types of CA are shown: (a) symmetric quadrirradial affecting Xp; (b) asymmetric quadrirradial with acentric fragment involving Xq; (c) duplication-deletion in Xq (arrow). Chromosomes exhibit BrdU immunolabeling (yellow) and either PI (red) or DAPI (blue) counterstaining.

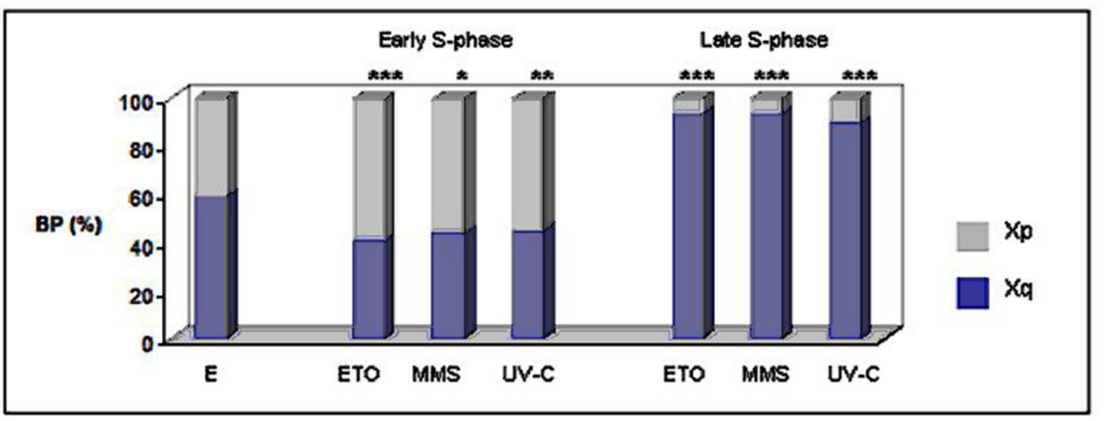

Figure 6. Bar diagram illustrating CHO9 X chromosome BP distribution induced by etoposide (ETO, 20 μM), methylmethane sulphonate (MMS, 20 mM) and UV-C (30 J/m^2; 0.1 J/m^2/s) in Xp (grey) and Xq (blue) during early (ES-phase) and late (LS-phase) cell cycle phases. The bar on the left side (E) indicates the expected frequencies of induced BP according to Xp and Xq relative length.

Since UV-C and MMS are S-phase dependent clastogens, the observed predominance of BP produced in Xp or Xq according to replication timing could be explained based on their requirement of DNA synthesis to produce CA. DNA base damage induced by MMS as well as cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4 PP) produced by UV-C are preferentially repaired through **B**ase **E**xcision **R**epair (BER) and **N**ucleotide **E**xcision **R**epair (NER) mechanisms, respectively. Both repair systems create an excision repair **S**ingle-**S**trand **B**reak (SSB) intermediate at the site of DNA lesion which is then filled by DNA

repair synthesis [93]. If DNA replication initiates with an excision repair SSB intermediate, another SSB can be generated in the complementary DNA strand, thus forming a DSB [94, 95]. Additionally, CPD, 6-4 PP or base damage in a single strand (unrepaired before DNA replication) may stall the replication fork and as a result, may produce a SSB in the opposite DNA strand [96, 97]. Furthermore, two nearby SSB in each DNA strand may behave as a DSB [98]. The DSB generated could be ultimately processed and transformed in CA [91, 92].

Nonetheless, the preferential location of CA in replicating Xp or Xq during etoposide treatment (independently of its eu/heterochromatic states) may occur due to the inhibition of topoisomerase II activity during DNA synthesis [87, 88]. The local unraveling and subsequent rewinding of eu or heterochromatin regions undergoing replication require topoisomerase II activities to alleviate DNA torsional stress [86]. Etoposide stabilizes DNA-topoisomerase II cleavable complex and hinders the resealing of DSB introduced by the enzyme generating the accumulation of DSB unable to reach resolution. In addition, chromatin unwinding during replication may turn DNA more accessible to S-independent and S-dependent chemical agents including etoposide and MMS, respectively [91, 92].

3.2. Eu/heterochromatin replication and primary induced-damage distribution in interphase nuclei

Few minutes after exposure of mammalian cells to DSB-inducing agents, the nucleosomal histone variant H2AX is phosphorylated at serine 139 (humans) or 129 (mouse) of C-terminal tails reaching a peak of phosphorylation 30 min later. H2AX phosphorylation (named γH2AX) initiates around the induced DSB and spreads through a large chromatin region (~2000 H2AX molecules) flanking the lesion, which can be visualized as discrete γH2AX foci in interphase nuclei and mitotic chromosomes by means of specific fluorochrome-conjugated antibodies [99].

γH2AX is involved in the DDR by coordination with other damage response proteins to recruit signaling, remodeling, checkpoint and repair proteins. At sites of DSB, the DNA-PK (**DN**A **D**ependent **P**rotein **K**inase) binds to activate the **N**on **H**omologous **E**nd **J**oining (NHEJ) DSB repair pathway. If DSB are produced after replication, RAD51 and BRCA2 are recruited to DSB sites initiating the **H**omologous **R**ecombination repair pathway (HR). Simultaneously, the sensing complex MRN (MRE11, RAD50, NBS1) associates to DSB, facilitating the recruitment and activation (auto-phosphorylation) of ATM (**A**taxia **T**elangiectasia **M**utated), MDC1, BRCA1 and 53BP1 [100].

ATM, ATR (**AT**M- and **R**ad3-related) and DNA-PK are members of the phosphatidylinositol 3-kinase-like family of serine/threonine protein kinases that phosphorylate H2AX. Unlike ATM, which appears to be mainly activated by DSB, ATR seems to be activated by induced SSB and the excision repair SSB intermediates generated during DNA repair. Since ATR activation was observed in replicating cells, it was suggested that the blockage of replication forks by SSB is required to initiate ATR-mediated phosphorylation of H2AX. Besides, it was reported that stalled replication forks may also trigger H2AX phosphorylation when bulky lesions (i.e.: CPD and 6-4 PP) collide with replication forks [101, 102].

NBS1, MDC1, 53BP1, and BRCA1 may all function as mediators and amplifiers of the DDR, recruiting diverse repair and checkpoint proteins (including ATM and ATR) and generating an amplification loop that also extends H2AX phosphorylation [99]. 53BP1 can bind directly to H3K79me and H4K20me accumulated at sites of DSB collaborating with a global chromatin unwinding following the formation of DSB in concert with other proteins like TIP60 (member of an histone acetyltransferases family) and KAP1 [103, 104, 105].

Several immunofluorescence studies have demonstrated that induced-γH2AX foci are located preferentially within euchromatic regions of the genome, suggesting that heterochromatin could be refractory to γH2AX foci formation. Employing immuno-FISH to analyze radiation induced-DSB (γH2AX foci) in chromatin regions with known chromatin compaction (human chromosome 18 versus chromosome 19; RIDGE versus anti-RIDGE region of human chromosome 11), it has been observed that condensed regions of gene-poor chromatin are less susceptible to DSB induction compared with decondensed, gene-rich chromatin [106-109].

Different hypothesis have been raised to explain the non-homogeneous distribution of γH2AX foci in nuclei. The highly condensed state or abundance of binding proteins may reduce the accesibility of chemical DNA damaging agents to heterochromatin. Besides, since condensed chromatin is less hydrated than euchromatin, a lower amount of free radicals could be induced by radiation [110]. Furthermore, compact heterochromatin could contain a lower proportion of H2AX isoform or be less accessible to kinases due to compaction or protein coating [106]. Additionally, a wave of chromatin unwinding starting at DSB sites and spreading throughout the entire chromatin was described (as a result of KAP1 phosphorylation by ATM kinase) implying that the preferential location of γH2AX foci in decondensed chromatin perhaps reflects chromatin reorganization [105, 111-113].

Finally, a short-range migration of DSB from packed chromatin toward specific decondensed DSB repair domains could also take place [106, 110]. Using carbon ion microirradiation to induce DSB combined to a modified TUNEL assay to directly visualize these lesions and γH2AX immunodetection, a bending of the linear ion-induced γH2AX track around heterochromatic regions was observed [114]. The γH2AX foci migration from the interior to the periphery of heterochromatin appears to initiate within 20 min post-irradiation and be almost complete 1 h after damage induction. The decondensation of heterochromatin at sites of ion hits possibly promotes the movement of DSB to peripheral regions of lower chromatin density where repair may potentially proceed [114].

To assess the influence of replication in the distribution of chromatin damage, we analyzed the localization of bleomycin-induced γH2AX foci in relation to replication of eu- or heterochromatin interphase compartments in 5-ethynyl-2'deoxiuridine (EdU) pulsed-labeled CHO9 nuclei. Bleomycin (BLM) is a radiomimetic S-independent clastogen that induces oxidative damage, SSB and mainly DSB as well as a rapid phosphorylation of H2AX [115].

Asynchronously growing CHO9 cultures were pulse-exposed (30 min) to EdU (controls) or simultaneously (30 min) treated with BLM (40 μg/ml). Early and late replication regions and γH2AX foci were detected with an azide conjugated to Alexa Fluor 488 (Click-iT EdU, Invi-

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trogen) and mouse anti-γH2AX (Abcam) followed by Cy3-conjugated antimouse antibodies, respectively. Single-cell z-stacks from control (n=25) and treated (n=63) nuclei were obtained by confocal microscopy and processed with Image J software. Using binary masks for each channel, the relation (ratio) between the percentage of damaged (γH2AX) area in replicating chromatin (EdU) area and the percentage of damaged area in the whole nuclear area (DAPI) was calculated for each nucleus. Finally, the arithmetic mean of the ratios corresponding to early S (n=30) and late S (n=33) nuclei was calculated.

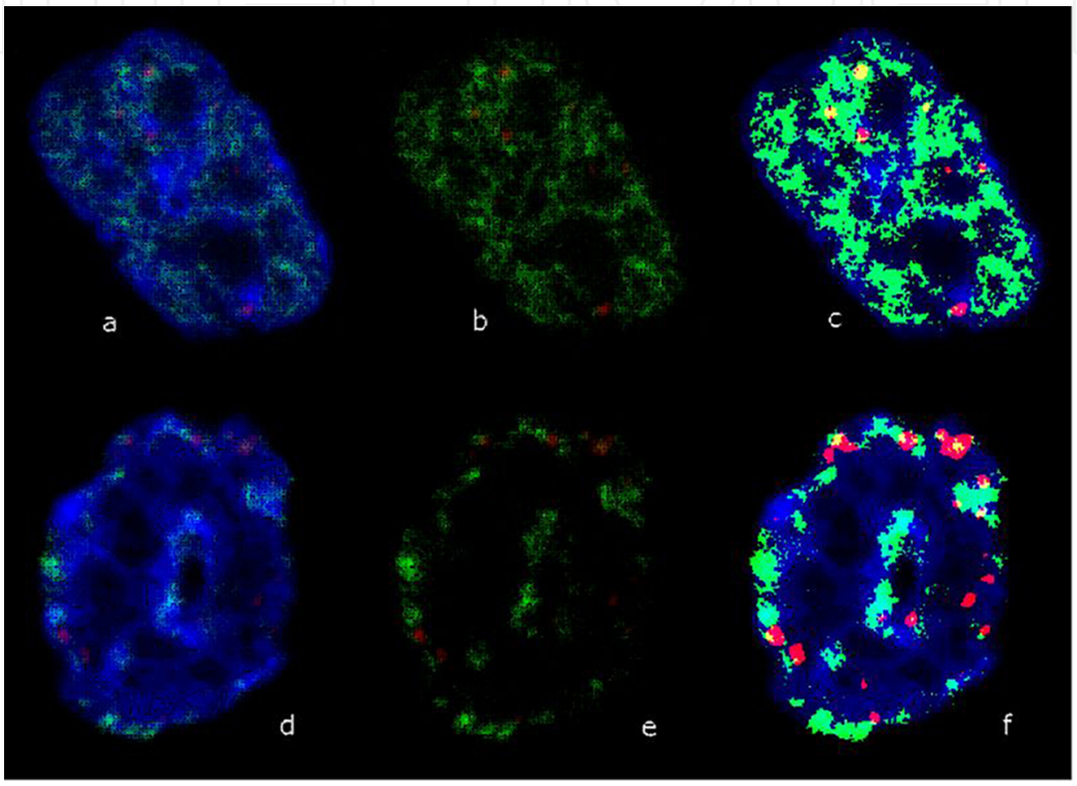

Figure 7. Distribution of BLM induced-γH2AX foci revealed by immunolabelling (Cy3; red) in early (top) or late (bottom) S-phase CHO9 nuclei. Replicating patterns were obtained by EdU incorporation and chemical detection (azide-Alexa Fluor 488; green). Nuclei were counterstained with DAPI (blue). Early S (a-c) and late S replicating nuclei are shown. Panels (a, d) and (d, e) contain DAPI/γH2AX/EdU and γH2AX/EdU merged images, respectively. Panels (c) and (f) illustrate binary masks of red (γH2AX) and green (EdU) channels overlaying the respective DAPI images.

Preliminary results (arithmetic mean of the ratios: 1.57 in early S- and 1.45 in late S-nuclei) suggest a bias in damage distribution towards replicating areas (~50 % higher than expected) probably due to local unwinding of chromatin down to naked DNA in both eu- and heterochromatin during DNA synthesis. Chromatin decondensation may increase the susceptibility to DNA damage as well as the accessibility of kinases that phosphorylate H2AX. Noteworthy, detailed visual analysis of fluorescent images or the corresponding binary masks in both early and late S-phase revealed that these results were not due to a large amount of γH2AX foci dwelling within replicating area and few of them outside. Instead, γH2AX foci recurrently mapped to the interfaces between replicating and non-replicating regions (Figure 7; Liddle P, unpublished observations).

The fact that in late-replicating cells γH2AX foci tend to map to the boundaries of replicating compartments (Figure 7, panels d-f) may be due to repositioning of damaged sites to less condensed peripheral heterochromatin regions, as it has been suggested in other models [112, 113]. However, this peculiar distribution of γH2AX foci in replicating/non-replicating interfaces was also observed in early S-phase when the less compact euchromatin replicates (Figure 7, panels a-c). In this respect, BLM-induced DNA lesions could preferentially map at the damage-prone TTR located at the boundaries of early and late replicating compartments.

4. Conclusions

We assayed the influence of eu/heterochromatin replication timing in the distribution of chromatin induced damage using two different approaches: (1) the analysis of UV-C, MMS and etoposide-induced BP in Xp or Xq replicating CHO9 X mitotic chromosome and; (2) the analysis of primary BLM-induced damage (γH2AX foci) in CHO9 early and late replicating interphase nuclei. Our findings support the assumption that induced damage patterns shift according to eu- or heterochromatin replication. The asynchronic replication of eu- or heterochromatin compartments could influence the distribution of primary DNA lesions and CA, prevailing in replicating chromatin regions, irrespective of its eu- or heterochromatic state. Thus, eu/heterochromatin replication timing seems to play an overriding role in the production and localization of chromosome damage in S-phase cells.

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