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Heterochromatin maintenance following DNA damage in mammalian cells

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ABSTRACT

Maintien de l'hétérochromatine en réponse aux dommages à l'ADN dans des cellules de mammifères

Dans les noyaux cellulaires des organismes eucaryotes, l'organisation de l'ADN avec des protéines histones sous forme de chromatine est une source d'information épigénétique qui dicte l'expression des gènes et l'identité cellulaire. Cependant, la chromatine est déstabilisée lors de toutes les transactions impliquant l'ADN, comme lors de la réparation des dommages à l'ADN. Pendant ma thèse, j'ai abordé la question fondamentale du maintien de l'épigénome au cours de la réparation des lésions de l'ADN dans les cellules mammifères. J'ai concentré mon travail sur le maintien de l'hétérochromatine, qui est fortement condensée, très peu transcrite et caractérisée par des ensembles spécifiques de modifications post-traductionnelles d'histones. Étant données ces particularités de l'hétérochromatine, j'ai émis l'hypothèse que des mécanismes spécifiques pourraient exister pour permettre de restituer la structure et la fonction de l'épigénome à la suite de lésions de l'ADN dans les domaines hétérochromatiques. Pour répondre à cette question, j'ai développé des approches innovantes pour cibler des dommages UVC aux domaines d'hétérochromatine péricentrique dans des cellules vivantes de mammifères et pour suivre en temps réel la réponse à ces dommages. Ainsi, j'ai montré que le maintien des modifications d'histones spécifiques de l'hétérochromatine était découplé de la recompaction de ces domaines et j'ai découvert un rôle critique pour le senseur de dommages UV DDB2 (DNA damage binding protein 2) dans l'orchestration des changements de compaction de l'hétérochromatine pendant la réparation. J'ai également observé que la réparation des dommages causés par les UVC s'effectuait efficacement dans l'hétérochromatine péricentrique et de manière simultanée à l'incorporation de nouvelles histones H3.3 par la chaperone d'histones HIRA (Histone Regulator A). Mes découvertes révèlent également une coopération étroite entre les chaperones d'histones et les enzymes de modification dans le maintien des modifications post-traductionnelles caractéristiques de l'hétérochromatine lors de la réparation des dommages causés par les UV. Dans l'ensemble, cette étude met en lumière des mécanismes fondamentaux impliqués dans le maintien des domaines de chromatine d'ordre supérieur à la suite de lésions dans l'ADN.

Mots clefs: Réparation des dommages à l'ADN, hétérochromatine, dynamique des histones, maintien de l'épigénome

Heterochromatin maintenance following DNA damage in mammalian cells

In eukaryotic cell nuclei, DNA wrapping around histone proteins in the form of chromatin is a source of epigenetic information that specifies gene expression and therefore, cell identity. However, chromatin organization is challenged during all DNA transactions, including DNA damage repair. During my thesis, I addressed the fundamental question of epigenome maintenance following DNA damage and repair in mammalian cells. I focused my work on the maintenance of highly folded heterochromatin domains, which are mostly silent and characterized by specific sets of histone post-translational modifications. Given the particularities of heterochromatin, I hypothesized that specialized mechanisms may exist to allow the restoration of the epigenome structure and function following DNA damage in heterochromatin domains. To tackle this question, I developed an innovative approach for targeting UVC damage to pericentric heterochromatin domains in live mammalian cells and for tracking the response to heterochromatin damage in real time. Thus, I showed that the maintenance of heterochromatin-specific histone modifications was uncoupled from heterochromatin folding and I uncovered a critical role for the UV damage sensor DDB2 (DNA damage binding protein 2) in orchestrating heterochromatin compaction changes during repair. I also observed that UVC damage repair took place efficiently within pericentric heterochromatin concomitantly with *de novo* deposition of H3.3 histones mediated by the histone chaperone HIRA (Histone Regulator A). My findings also unveil a tight cooperation between histone chaperones and modifying enzymes in the maintenance of heterochromatic histone marks upon UV damage. Altogether, this study sheds light on the fundamental mechanisms involved in the maintenance of higher-order chromatin structures following DNA damage.

Keywords: DNA damage repair, heterochromatin, histone dynamics, epigenome maintenance

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Thanks for all your encouragement!

ABBREVIATIONS

(6-4)PP	(6-4)Pyrimidine-Pyrimidone
53BP1	p53-Binding Protein 1
5mC	5methylCytosine
8-oxo-dG	8-oxo-deoxyguanosine
Ac	Acetylation
A-EJ	Alternative End Joining
ALC1	Amplified in Liver Cancer 1
ARR	Access-Repair-Restore
ATAC-see	Assay of Transposase-Accessible Chromatin with visualization
ATM	Ataxia-Telangiectasia Mutated
ATP	Adenosine Tri-Phosphate
ATR	Ataxia-Telangiectasia mutated and Rad3-Related
ATRX	Alpha Thalassemia/Mental Retardation Syndrome X-Linked
BER	Base Excision Repair
CAF-1	Chromatin Assembly Factor 1
CD	Chromo Domain
CENP-A	Centromeric Protein A
CHD3	Chromodomain Helicase DNA binding protein 3
cis-Pt	Cisplatin
COFS	Cerebro-Oculo-Facio-Skeletal syndrome
CPD	Cyclobutane Pyrimidine Dimers
CS	Cockayne Syndrome
CSD	Chromo Shadow Domain
CUL4A, B	Cullin 4A, B
DAPI	4',6-diamidino-2-phenylindole
DAXX	Death Domain-Associated Protein
DDB1, DDB2	DNA Damage-Binding protein 1, 2
DDR	DNA Damage Response
DNA	Deoxyribonucleic Acid
DNMT1, 3a-c	DNA Methyl-Transferase 1, 3a-c
DSB	Double-Strand Break
DUB	DeUbiquitinase
EC	Euchromatin
EU	Ethynyl-Uridine
ERCC	Excision-Repair-Cross-Complementing
EZH2	Enhancer of Zeste 2
FACT	Facilitates Chromatin Transcription
FRAP	Fluorescence Recovery After Photobleaching
GFP	Green Fluorescent Protein
GG-NER	Global Genome NER
H2A.Bbd	H2A Barr body deficient
H2BFWT	Histone H2B type WT
HAT	Histone Acetyl-Transferase
HC	Heterochromatin
HD	Hinge Domain

HDAC	Histone DeAcetylase
HIRA	Histone Regulator A
HJURP	Holliday Junction Recognition Protein
HP1α, β, γ	Heterochromatin Protein 1 α, β, γ
HR	Homologous Recombination
IF	Immunofluorescence
IR	Ionizing Radiation
ISWI	Imitation Switch
KAP1	KRAB-associated protein 1
KDM4A	Lysine DeMethylase 4A
KMT	Lysine Methyl-Transferase
LAD	Lamina-Associated Domain
LINE	Long Interspread Transposable Element
lncRNA	Long non-coding RNA
LTR	Long Terminal Repeats
me1, 2, 3	Mono-, di-, tri-methylation
MMC	Mitomycin
MMR	Mismatch Repair
MNase	Miccrococal Nuclease
NAD	Nucleolus-Associated Domains
ncRNA	Non-coding RNA
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End-Joining
NuRD	Nucleosome Remodeling Deacetylase
P / ph	Phosphorylation
PARP	Poly (ADP-Ribose) Polymerase
PCNA	Proliferating Cell Nuclear Antigen
PRC2	Polycomb Repressive Complex 2
PTM	Post-Translational Modification
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
RNAPII	RNA Polymerase II
RNF2	Ring Finger protein 2
ROS	Reactive Oxygen Species
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
SCAI	Suppressor of Cancer Cell Invasion
SENP7	SUMO1/Sentrin Specific Peptidase 7
SETDB1	SET Domain Bifurcated 1
SINE	Short Interspread Transposable Element
Sir	Silent information regulator
SMC-5/6	Structural Maintenance of Chromosomes 5/6
Suv39H1, 2	Suppressor of Variegation 3-9 Homolog 1, 2
TAD	Topologically Associated Domain
TAU	Triton-Acid-Urea
TC-NER	Transcription-Coupled NER

TDD	Trichothiodystrophy
TET	Ten-Eleven Translocation
TFIIH	Transcription initiation Factor IIH
TRF1, 2	Telomeric Repeat binding Factor 1, 2
TSH2B	Testis-Specific Histone H2B
Ub	Ubiquitin / Ubiquitination
UV	Ultraviolet
UV-DDB	UV-damage DNA-binding protein
$UV^{S}S$	UV-sensitive Syndrome
UVSSA	UV-Stimulated Scaffold Protein A
WB	Western Blot
Xi, Xa	Inactive / Active X chromosome
Xist	X-inactive-specific-transcript
XP (A, B, C, D, E, F, G)	Xeroderma Pigmentosum group A, B, C, D, E, F, G
XR-seq	Excision Repair sequencing

,

OUTLINE

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Reviews:

The response to DNA damage in heterochromatin domains.

Fortuny A., Polo S.E (2018)

Chromosoma, 127: 291–300

Epigenome maintenance in response to DNA damage.

Dabin J.*, Fortuny A.* and Polo S.E. (2016)

Mol Cell, 62: 712-727.

*: equal contribution

Method chapter:

Live imaging of parental histone variant dynamics in UVC-damaged chromatin.

Dabin J.*, Fortuny A.*, Piquet S. and Polo S.E. (2018)

Methods Mol Biol, 1832: 243–253.

*: equal contribution

Preview:

Genome and epigenome maintenance by keeping histone turnover in check.

Fortuny A., Polo S.E. (2017).

Mol Cell, 66: 3-4

FIRST PART

INTRODUCTION

CHAPTER 1

Chromatin and epigenetic information

1.1. Epigenetic information

1.1.1. Origin and definition of epigenetics

How can a single fertilized egg give rise to a complex organism with cells of varied phenotypes? In the late 19th century, this question generated an intense debate among embryologists. On one side, defenders of "preformation" posited that all adult characters were present in the embryo and just needed to unfold during development, while supporters of "epigenesis" advocated for a more complex developmental plan involving multiple chemical reactions among soluble components of the embryo (Felsenfeld, 2014). Despite not being completely accurate, those initial views conveyed the opposing ideas of a static ("preformation") vs. dynamic nature of gene expression ("epigenesis"). It is through the combination of these two concepts that Conrad Waddington later coined the term "epigenetics" to refer to the branch of biology that studied the interactions between genes and their products leading to specific cellular phenotypes (Waddington, 1942) (**Figure 1**).

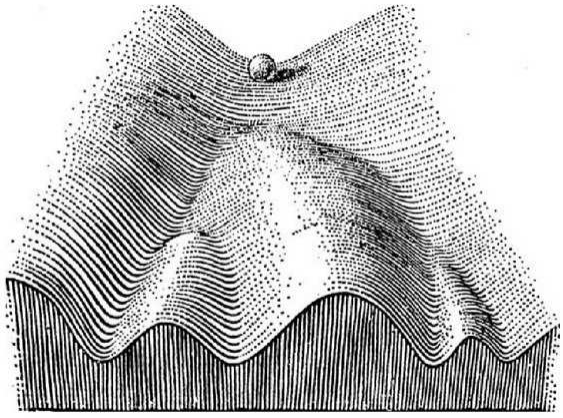

FIGURE 1 – Epigenetic landscape, by C. Waddington. In this illustration, a cell is envisioned as a ball at the top of a slope composed of various hills and valleys, which represent the various developmental pathways a cell might take towards differentiation. Illustration taken from "The Strategy of the Genes", by Conrad Waddington.

Towards the end of the 20th century, the field of epigenetics moved further away from developmental processes and some important additions refined Waddington's original definition. The notion of heritability of epigenetic changes was introduced by Robin Holliday, i.e. the persistence of epigenetically-defined gene expression patterns across cell generations (Holliday, 1994). This led to the current working definition of epigenetics as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo et al., 1996). More recently, it was added that the mechanisms initiating a new epigenetic state should be different from the one required to maintain it (Berger et al., 2009). In other words, epigenetic changes in gene expression are maintained through cell generations in the absence of the original stimulus.

1.1.2. Functional importance of epigenetic information

Epigenetic mechanisms represent an additional layer of information to the one carried by the DNA sequence. By regulating gene expression programs, that is, determining which genes are expressed or are not expressed in a given cell at a given time, the epigenome dictates cell-type identity (reviewed in Allis and Jenuwein, 2016). Recently, the development of single-cell epigenomics has uncovered the heterogeneity of epigenetic marks in cell populations, boosting our understanding of the regulatory diversity of the epigenome (Shema et al., 2019).

While all cells in a eukaryotic organism contain the same genetic material, epigenetic information differs in distinct cell types and developmental stages, and can change in response to environmental factors. Epigenetic information is, therefore, dynamic. Such plasticity is due to the reversibility of epigenetic modifications, which is key for fine-tuning gene expression (reviewed in Soshnev et al., 2016).

Epigenetic changes are of fundamental importance during organism development, and for the response/adaptation of cells and organisms to the environment. Alterations in epigenetic marks and mutations in epigenetic factors are strongly correlated with disease (Zoghbi and Beaudet, 2016) in particular with cancer progression (reviewed in Flavahan et al., 2017; Shen and Laird, 2013). Indeed, epigenetic changes can directly alter transcriptional programs, which can lead to cancer progression by increasing cancer cell plasticity or by silencing tumor suppressor genes. For more information about epigenome maintenance following DNA damage, see **Annex 1**.

From an evolutionary point of view, it is likely that the packaging of genetic information in the form of chromatin (see **Section 1.2**) first served a genome defense role, as nucleosome

assembly hampers transcription. Moreover, nucleosomes enabled a more efficient packaging and condensation of genomes, probably facilitating genome expansion and evolution (Talbert et al., 2019).

1.2. Chromatin and its building block: the nucleosome

From a molecular point of view, epigenetic information is contained in the form of chromatin. Visualized for the first time by Ada et Donald Olins (Olins and Olins, 1974), chromatin was described as a beads-on-a-string structure where DNA wraps around histone proteins, giving rise to the basic unit of chromatin: the nucleosome (Kornberg, 1977; Oudet et al., 1975). In 1997, Luger and colleagues solved the 2.8A crystal structure of this fundamental chromatin building block, providing an atomic depiction of the nucleosome core particle consisting of two copies each of the four core histone proteins H2A, H2B, H3 and H4 around which wrap 1.7 turns of DNA, or about 147 base pairs (Arents et al., 1991; Luger et al., 1997) (**Figure 2**). Each nucleosome is formed through the assembly of a H3-H4 tetramer flanked by two H2A-H2B heterodimers, giving rise to a 11 nm x 5.5 nm cylindrical structure. In addition to the structured histone fold cores, several disordered N- and C-terminal tails protrude from the nucleosome thus constituting preferred targets for recognition and covalent modification by enzymatic machineries (see **Section 1.3.1**).

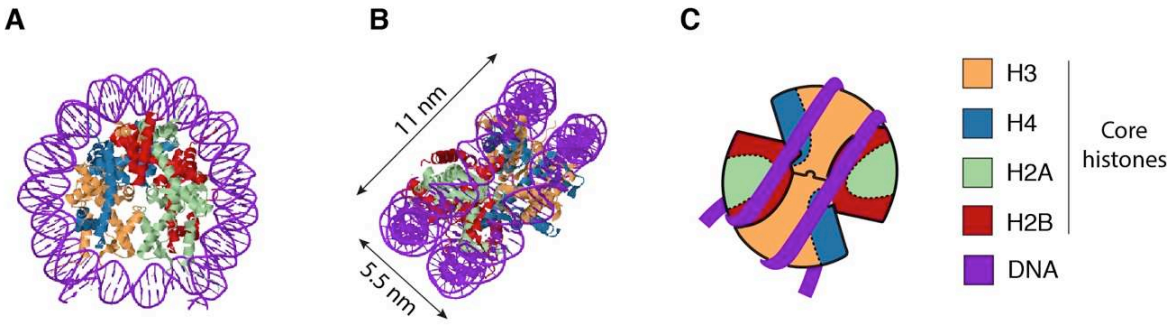

FIGURE 2 – Global structure of the nucleosome. (A, B) Different perspectives (top and side view) of the nucleosome core particle crystal structure at 2.5A resolution (Harp et al., 2000), where 147 base pairs of DNA are wrapped around a histone octamer. PBD ID: 2CV5, visualized with JSmol viewer. (C) Schematic representation of a nucleosome that will be used during this thesis, oriented as in (B). Protruding histone tails are not represented.

Each nucleosome is connected to the adjacent nucleosome through a segment of linker DNA. Nucleosome occupancy and positioning along the chromatin fiber are determined by the DNA sequence and the action of chromatin remodelers and constitute important parameters in transcription regulation (Lai and Pugh, 2017). Moreover, nucleosomes are completed by the addition of linker histone H1, which binds the nucleosome core particle and stabilizes both nucleosome structure and higher-order chromatin architecture (reviewed in Fyodorov et al., 2017, see **Section 1.3.3**).

1.3. Modulations of chromatin structure convey epigenetic information

Owing to its structural properties, the nucleosome serves three main functions. First, it provides the first level of chromatin compaction. Second, by displaying combinatorial arrays of post-translational modifications on histone proteins it can act as a scaffold for chromatin modifying enzymes and readers of modifications regulating chromatin folding (Bannister and Kouzarides, 2011; Bowman and Poirier, 2014). And third, it can self-assemble and give rise to higher-order chromatin structures (Fierz and Poirier, 2019). Therefore, modulations of nucleosome structure can affect chromatin compaction, thus altering gene function and making the nucleosome a key carrier of epigenetic information.

1.3.1. Histone post-translational modifications

Beyond DNA packaging, histones are key vectors of epigenetic information, as they carry multiple post-translational modifications (PTMs, **Figure 3**). We now know that a large number of PTMs exist in different histone tails (Bannister and Kouzarides, 2011) and histone core domains (Tessarz and Kouzarides, 2014), which are deposited, propagated and removed by specific sets of enzymes (**Table 1**).

Histone modifications exert their effects via two main mechanisms. The first involves the modifications directly influencing the overall structure of chromatin, by altering histone-histone and histone-DNA electrostatic interactions (reviewed in Tolsma and Hansen, 2019). A good example for this would be the acetylation of H4K16, which interferes with the H4-H2A interaction, thus disrupting a critical contact between nucleosomes and resulting in a opening of chromatin structure (Allahverdi et al., 2011; Shogren-Knaak et al., 2006). Similarly, ubiquitination of H2BK120 or H2AK15, results in a steric inhibition of H2A-H2B contacts and in chromatin opening (Debelouchina et al., 2017).

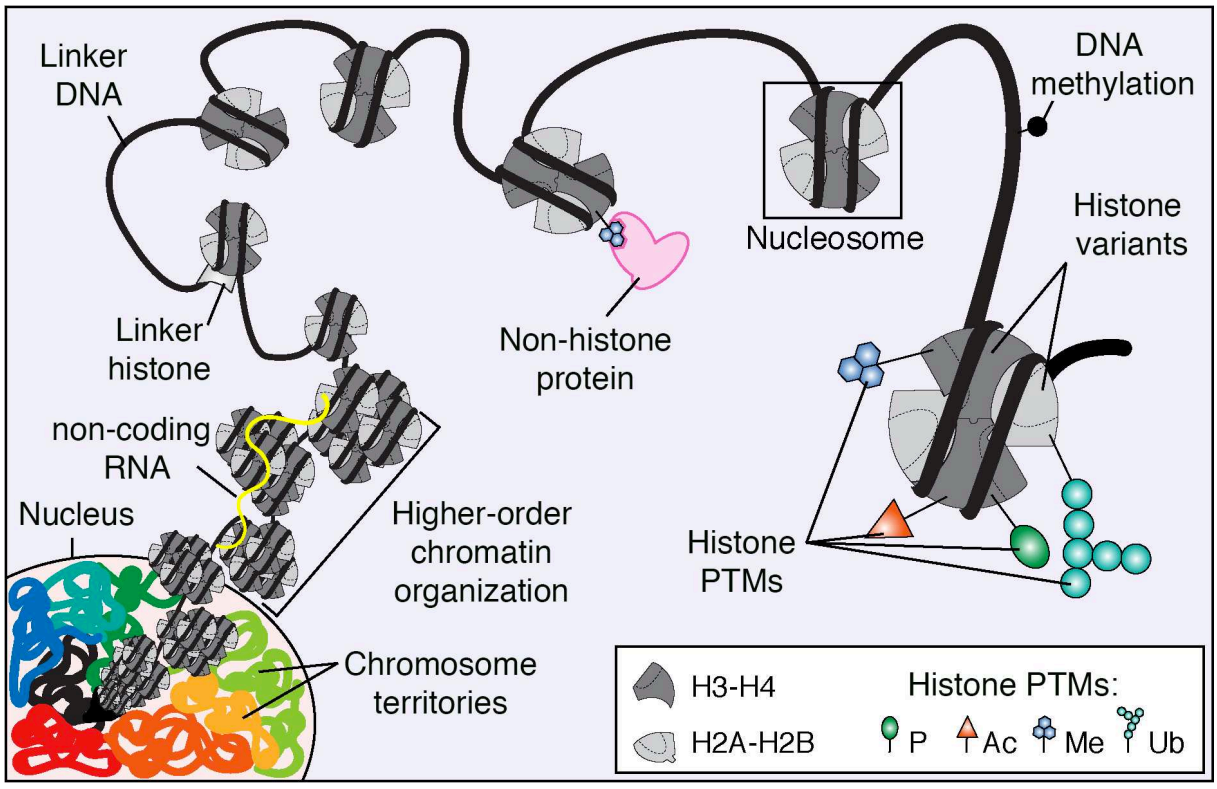

FIGURE 3 - Schematic representation of chromatin organization. Different levels of organization go from chromosome territories in the cell nucleus down to the basic unit of chromatin, the nucleosome. Chromatin consists of DNA wrapped around histone proteins decorated by post-translational modifications (PTMs) including phosphorylation (P), acetylation (Ac), methylation (Me) and ubiquitylation (Ub). Adapted from an original figure by J. Dabin.

The second mechanism involves the modifications regulating the binding of effector molecules. Indeed, histone PTMs are recognized by specific factors known as "readers" (reviewed in Su and Denu, 2016), which in turn can recruit remodeling enzymes or other effectors to promote a biological response. This second mechanism of action is associated with the regulation of gene expression (Su and Denu, 2016) and DNA damage signaling (Dantuma and van Attikum, 2016). Reader proteins have been identified for all major histone modifications, including acetylation, methylation, phosphorylation and ubiquitination (**Table 1**). They recognize and bind histone modifications through specific reader domains, such as bromodomains, chromodomains, tudor domains, BRCT/FHA domains, ubiquitin binding motifs, and others (Patel and Wang, 2013). In particular, histone tail methylation alters chromatin compaction and transcription by promoting or blocking the binding of effectors. For instance, trimethylation of H3K9 recruits heterochromatin protein 1 (HP1) via its chromodomain, which can then self-oligomerize and act as a scaffold for H3K9me3-related methyltransferases and other silencing proteins (KAP1, SETDB1, NuRD) via its chromoshadow domain (see **Chapter 2**).

Main histone modifications	Target residues	Operating enzymes (writers / erasers)	Associated functions (examples)
Acetylation (ac)	Lysine (K)	HAT / HDAC	Chromatin decondensation, transcriptional activation (**H3K9ac**, **H4K16ac**)
Methylation (me1, 2, 3)	Lysine (K) Arginine (R)	KMT / KDM	Transcriptional activation (**H3K4me3**) or silencing (**H3K9me3**, **H4K20me3**, **H3K27me3**)
Phosphorylation (ph)	Serine (S) Threonine (T) Tyrosine (Y)	Kinase / Phosphatase	DNA damage signaling (**H2A.XS139P**) Chromosome condensation during mitosis (**H3S10P**)
Ubiquitination (ub)	Lysine (K)	E3-ubiquitin ligase / DUB	DNA damage signaling (**H2AK13K15ub**) Regulation of transcription (**H2AK118K119ub**)
DNA modifications			
Methylation (5mC)	Cytosine (C)	DNMT1, 3a-c / TET proteins	Transcriptional silencing

TABLE 1 – Main histone post-translational modifications, associated enzymes and biological functions. HAT: Histone Acetyltransferase; HDAC: Histone Deacetylase; KMT: Lysine Methyltransferase; KDM: Lysine Demethylase; DUB: Deubiquitinase; DNMT: DNA Methyltransferase; TET: Ten-eleven Translocation. KMT enzymes modify the appropriate residues to a specific degree (i.e., mono-, di- and/or tri-methyl state). For review: (Rothbart and Strahl, 2014).

In addition to histone modifications, chemical modifications of DNA bases are also important modulators of gene expression. In particular, methylation of cytosines (5mC) in gene promoters is generally associated with transcriptional silencing (reviewed in Schübeler, 2015).

1.3.2. Histone variants

In addition to bearing post-translational modifications, histone proteins exist in different forms named variants, which endow chromatin with special properties thus constituting another layer of epigenetic information (reviewed in Buschbeck and Hake, 2017). Originally discovered by triton-acid-urea (TAU) gel electrophoresis (Franklin and Zwidler, 1977), histone variants are present in all eukaryotes (Talbert et al., 2012). In humans, sequence variants of the core histones H2A, H2B and H3 (**Figure 4**), and of linker histone H1, have been described. Notably, the different histone variants are distinct and unique in their gene and protein sequences, the

processing of their RNA and the timing of their transcription and deposition on DNA during the cell cycle.

Generally, a distinction is made between "canonical" histones, which account for the majority of any given histone species in any cell (i.e. H2A, H2B, H3.1/2, H4, H1), and histone variants. Canonical histones are also referred to as "replication-coupled" histones, as they are massively synthesized during S-phase and deposited during DNA replication. The generation of large and equal amounts of all four nucleosome core histone proteins can be envisioned as a means to provide a constant source of nucleosomes at newly replicated DNA. On the other hand, histone variants are known as "replacement" histones, and their transcription and deposition extends throughout the cell cycle. In addition, the expression of some replacement histones is tissue-specific, with some variants being exclusively or predominantly found in the male germline (Govin et al., 2004; Hoghoughi et al., 2018) (**Figure 4**).

Each of the canonical histones is encoded by multiple genes that group into clusters throughout the genome (Albig and Doenecke, 1997). By contrast, replacement variants are generally encoded by few isolated genes. For instance, while 10 genes encode for the replicative variant H3.1, only 1 and 2 genes encode for the centromere protein A (CENP-A) and H3.3 replacement variants, respectively (Maze et al., 2014).

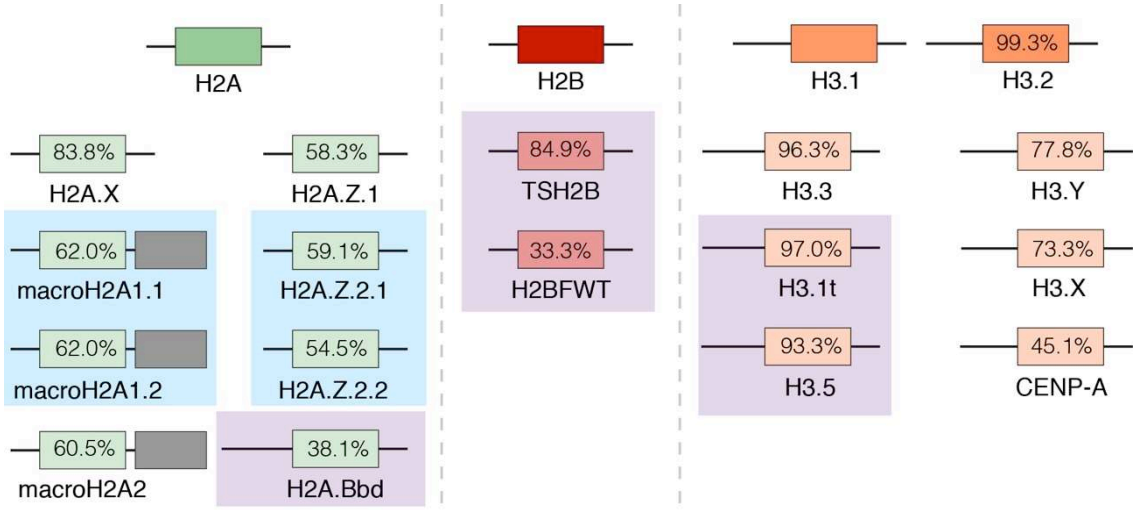

FIGURE 4 – Human core histone variants. Variants of the human core histones H2A (green), H2B (red) and H3 (orange) are shown. No variants of H4 have yet been discovered in humans (Talbert et al., 2012). Rectangles represent core regions, and lines represent flexible histone tails. Testis-specific histone variants are highlighted by light purple boxes, and alternative splicing isoforms by light blue boxes. Percentages indicate total amino acid sequence conservation (% sequence identity) of the variants relative to their replication-coupled counterparts (for H3, two replication-coupled isoforms are present: H3.1 and H3.2). H2A.Bbd: H2A Barr body deficient; TSH2B, testis-specific histone H2B; H2BFWT: histone H2B type WT; CENP-A: histone H3-like centromeric protein A. Adapted from (Buschbeck and Hake, 2017).

Replication-coupled and replacement histones also differ in the structure and processing of their encoding RNAs. Remarkably, replication-coupled histone genes lack introns, whereas several histone variant genes contain introns that are spliced during RNA processing, providing the chance to generate alternative splice isoforms and, in turn, to further increase histone and nucleosome diversity (Bönisch and Hake, 2012) (**Figure 4**).

Histone variants play critical roles in diverse cellular processes including transcription, DNA repair and recombination, chromosome segregation and chromatin remodeling. Hence, mutations on histone variants contribute to disease, as shown for specific forms of cancer (reviewed in Maze et al., 2014). Indeed, histone variants confer unique properties to distinct chromatin regions. Besides directly changing nucleosome structure and stability and thus affecting nucleosome-nucleosome interactions, histone variants can indirectly influence chromatin via the acquisition of PTMs and the recruitment of chromatin modifying enzymes and of histone variant-interacting proteins. Among the last ones, histone chaperones have a crucial role as they guide the assembly of nucleosomes at specific genomic regions (Hammond et al., 2017) (**Figure 5**).

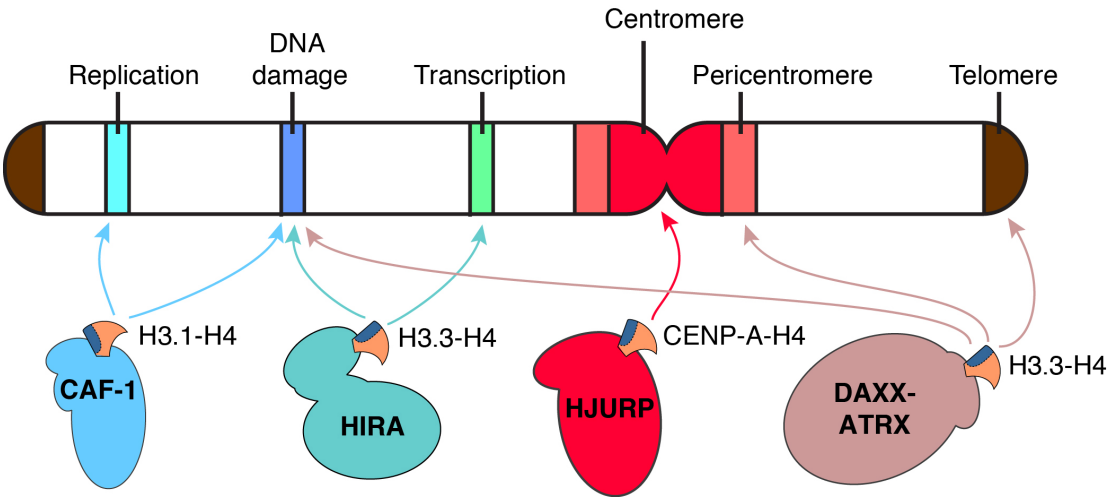

FIGURE 5 – Genomic distribution of histone H3 variants by the corresponding chaperone complexes. While replication-coupled histones are distributed rather equally in chromatin, histone variants show specific and unique distributions. Histones are deposited in distinct chromatin regions by specific histone chaperones. CAF-1: Chromatin Assembly Factor 1; HIRA: Histone Regulator A; HJURP: Holliday Junction Recognition Protein; DAXX: Death Domain-Associated Protein; ATRX: Alpha Thalassemia/Mental Retardation Syndrome X-Linked. CENP-A: histone H3-like centromeric protein A.

In this thesis, I will focus on H3 histones, in particular on the deposition of the replicative variant H3.1 and of the replacement variant H3.3 during DNA repair. The H3.1 variant is deposited by the histone chaperone complex CAF-1 (Chromatin Assembly factor-1) at sites of ongoing DNA synthesis during replication (Tagami et al., 2004) and repair (Li and Tyler, 2016; Polo et al., 2006) in human cells. Among H3.3-specifc histone chaperones, HIRA (Histone Regulator A) deposits H3.3 within transcribed euchromatin in mammalian cells (Goldberg et al., 2010; Ray-Gallet et al., 2011) and in damaged chromatin in human cells (Adam et al., 2013; Li and Tyler, 2016), while DAXX (Death Domain-Associated Protein) with its binding partner ATRX (Alpha Thalassemia/Mental Retardation Syndrome X-Linked) promotes H3.3 enrichment at repeated sequences including subtelomeric and pericentric heterochromatin (Drané et al., 2010; Goldberg et al., 2010; Wong et al., 2010) and in chromatin regions undergoing recombinational repair (Juhasz et al., 2018).

1.3.3. Higher-order folding of chromatin

In the interphase nucleus of higher eukaryotes, the intertwining of DNA with histone proteins forms a 10 nm chromatin fiber. The binding of additional factors such as cations (Visvanathan et al., 2013), linker histones (H1) (Zhou and Bai, 2019), non-histone proteins (i.e. HP1, James and Elgin, 1986) and non-coding RNAs (Yao et al., 2019) further contribute to the higher-order folding of chromatin and to the formation of specialized nuclear domains (Dixon et al., 2016; Sewitz et al., 2017) (**Figure 6**). Notably, the 3D chromatin organization in the cell nucleus greatly influences gene regulation and cell fate decisions (reviewed in Bonev and Cavalli, 2016).

In the last few years, the development of superresolution imaging techniques, together with computational modeling and genome-wide chromosome conformation capture approaches (3C, 4C, 5C and Hi-C) have revealed the principles of higher-order chromatin organization and dynamics (Bonev and Cavalli, 2016; Fierz and Poirier, 2019). Several higher-order organization levels have been proposed to give rise to increasing chromatin compaction (reviewed in Woodcock and Ghosh, 2010), with the highest compaction (10.000-20.000 fold) being achieved in metaphase chromosomes. At the smallest scale of chromatin organization beyond the nucleosome, we find nucleosome-nucleosome interactions. While the original model of 10 nm fibers folding into 30 nm structures within the interphase nucleus does not appear to hold in vivo (Maeshima et al., 2019), it seems increasingly clear that nucleosomes are arranged in heterogeneous groups of varying sizes named "clutches" (Ricci et al., 2015), which are

interspersed with nucleosome-depleted regions. The addition of cohesins to the chromatin fiber determines the formation of loops delineated by CTCF binding sites (Haarhuis et al., 2017; Rao et al., 2014), which in turn help define topologically associated domains (TADs) (Dixon et al., 2016) (**Figure 6**). Chromatin loops are key to bring distinct regulatory elements into spatial proximity, such as enhancers and their target promoters, and may thus have an important role in the regulation of gene expression (Kagey et al., 2010). On the other hand, TADs represent physically and functionally isolated regions of the genome (<1 Mb), and their boundaries correlate with many linear genomic features such as histone modifications, coordinated gene expression, association with the lamina and DNA replication timing. Long-range interactions between TADs are also observed, giving rise to distinct cell-type specific compartments of genes that are in the same transcriptional state, namely compartments A (active) and B (inactive) (Lieberman-Aiden et al., 2009; Wang et al., 2016) (**Figure 6**). Such compartmentalization of the genome allows the distinction between two main chromatin compartments (1-10 Mb): euchromatin (EC) and heterochromatin (HC) (Rowley and Corces, 2018), which are established based on chromatin states through phase separation mechanisms (see **Chapter 2**). Ultimately, chromosomes, divided into active and inactive compartments, occupy discrete territories in the cell nucleus (Cremer and Cremer, 2001) (**Figure 6**).

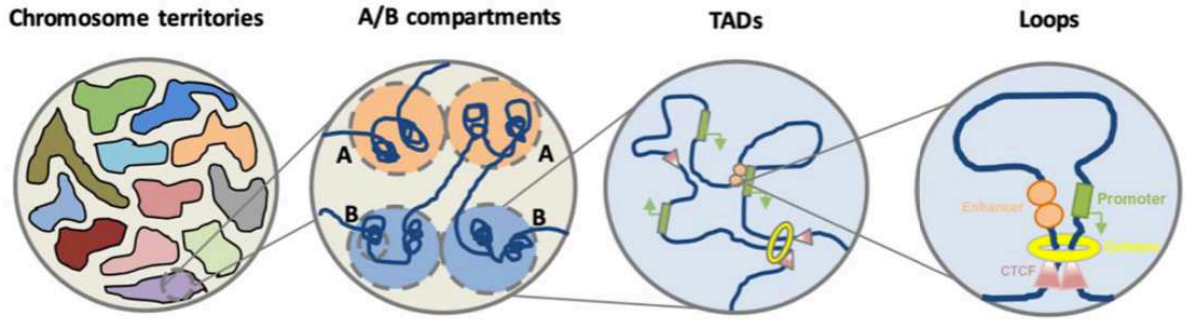

FIGURE 6 – Hierarchical genome organization in the eukaryotic cell nucleus. Individual chromosomes occupy independent chromosome territories (>10Mb) that are divided into active (A) and inactive (B) chromatin compartments (1-10Mb). Each compartment comprises several topologically associating domains (TADs, <1Mb) with CTCF chromatin loops (median length 185kb). TADs: topologically associated domains. Figure from Wright et al., 2019.

CHAPTER 2

Heterochromatin domains

2. 1. Definition of heterochromatin

Back in the 1920s, the development of chromatin staining methods allowed the botanist Emil Heitz to visualize chromosomal regions that did not undergo postmitotic decondensation. He then introduced the term "heterochromatin" to describe the compact and genetically-inert regions of chromosomes, as opposed to less compact, gene-containing "euchromatin" regions (Alberto Ciccia, 2010). Nowadays, heterochromatin domains can be visualized by electron microscopy techniques as electron-dense (dark) regions enriched at the nuclear and nucleolar peripheries (**Figure 7**). Heitz initial observations were followed by multiple studies that contributed to define the main features of heterochromatin. In the 1930s, Hermann Muller established a relationship between heterochromatin and the phenomenon he termed "position-effect variegation", after observing the silencing of Drosophila active genes that were translocated adjacent to heterochromatic regions (Muller and Altenburg, 1930). In the second half of the century, successive studies unveiled the structural and molecular characteristics of heterochromatin, including its repetitive nature, its organization within the nuclear space and its coupling with silencing histone marks and associated proteins (see **Section 2.2.1**).

All the above findings contributed to the idea that heterochromatin was a rigid compartment in which gene-poor, transcriptionally inactive regions of chromatin were densely packed and inaccessible to the cellular machinery. However, this view has been challenged in the recent years. Today, heterochromatin is regarded as a nuclear domain integral to many cellular processes and essential for genome integrity and organismal health (see **Section 2.2.3**). Forming a physically and functionally distinct genomic compartment, heterochromatin is actually a fairly dynamic and plastic domain characterized by its relatively low gene density, enrichment for repetitive DNA sequences, highly compact –but still accessible- chromatin architecture, and late onset of DNA replication (Allshire and Madhani, 2017).

2.2. General principles of heterochromatin structure and function

2.2.1. Molecular features of heterochromatin

Heterochromatin is a major component of the eukaryotic nucleus, composing ~25% to 90% of multicellular eukaryotic genomes (Lander et al., 2001; Vicient and Casacuberta, 2017). It localizes mainly close to the centromeres (Saksouk et al., 2015), the telomeres (Schoeftner and Blasco, 2009), and at genomic regions that interact with specific nuclear structures, such as the nuclear and nucleolar periphery (LADs and NADs, Matheson and Kaufman, 2016; van Steensel and Belmont, 2017). It is also enriched at the nucleolus, where repetitive blocks of ribosomal RNA genes are packaged in heterochromatic structures (Németh and Grummt, 2018) (**Figure 7**).

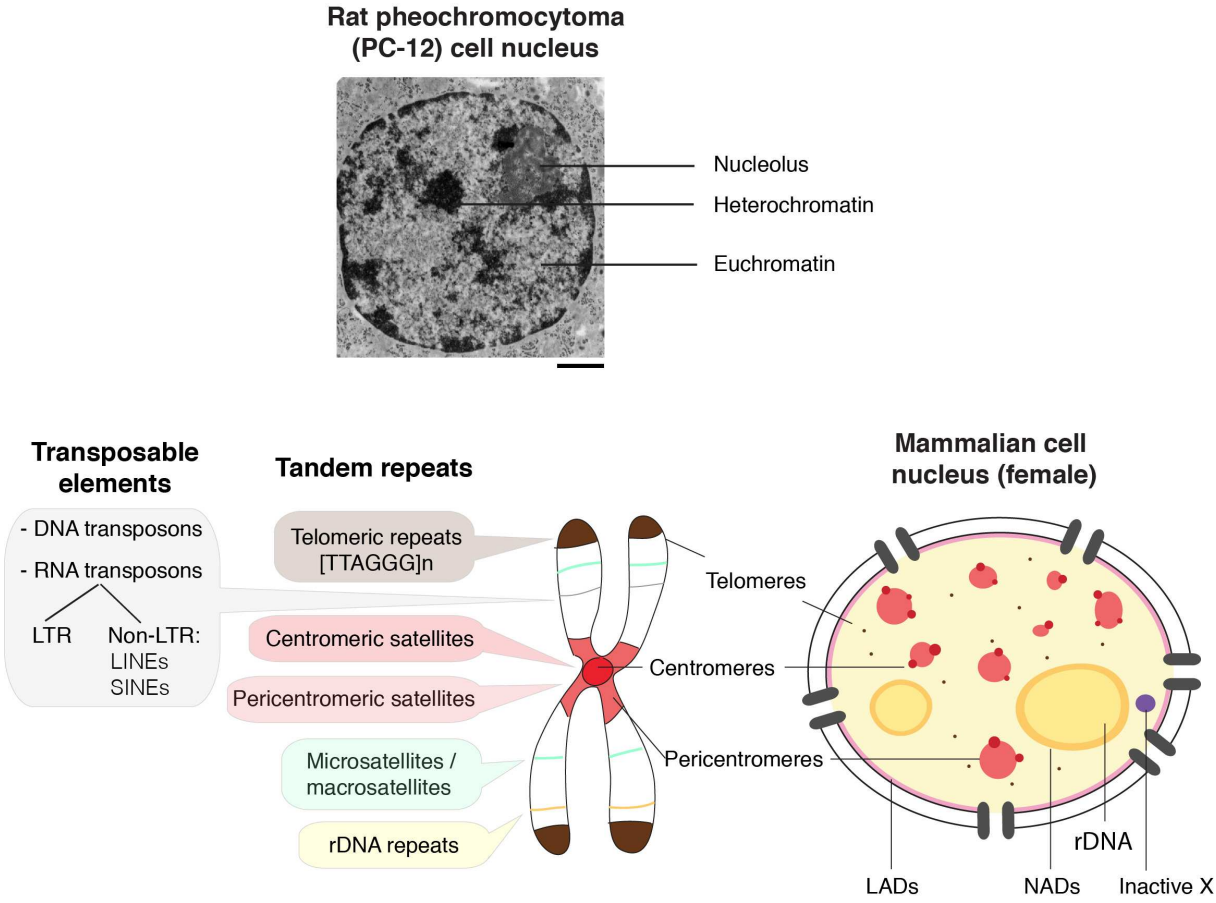

FIGURE 7 – Composition and localization of main heterochromatin domains. Transposable elements and short tandem repeats are the main constituents of heterochromatin domains, which occupy different locations in the cell nucleus. LTR: long terminal repeats; LINEs/SINEs: long/short interspread transposable elements; rDNA: ribosomal DNA; LADs: lamina-associated domains; NAD: nucleolus-associated domains. Brightfield electron microscopy image taken from www.cellnucleus.com. Scale bar, 1µm.

Although distinct heterochromatin regions exist, there are some common elements that identify heterochromatin domains:

Repetitive DNA sequences

Heterochromatin domains are rich in repetitive elements, including short tandem repeats and dispersed transposable elements (**Figure 7**). Such sequences are repeated hundreds or thousands of times. For example, specific mouse 234-bp sequences are repeated thousands of times to give rise to 6 Mb arrays of A/T-dense "major" satellite repeats (Joseph et al., 1989) (see **Section 2.4**).

Notably, repetitive DNA sequences vary significantly among different types of repeats and among species, indicating that the presence of repetitive DNA is sufficient for driving heterochromatin nucleation, independently of sequence (Padeken et al., 2015). Because of its repetitive nature, heterochromatin can hinder basic cellular processes such as replication and repair (see **Section 2.2.3**), and therefore several cellular mechanisms have evolved to compact and silence these regions.

Histone post-translational modifications and DNA methylation

Histone tails in heterochromatic regions harbor a specific set of modifications that contribute to heterochromatin compaction and silencing. The most representative histone post-translational modification in mammalian heterochromatin is the di- and trimethylation of H3K9 residues (H3K9me2/3) (**Table 1**). Heterochromatin histones are also typically hypoacetylated as compared to euchromatic ones. Such histone tail modifications affect nucleosome-nucleosome interactions and are recognized by reader enzymes (see **Chapter 1**), which in turn can act as platforms for binding proteins that will compact and silence heterochromatin (**Figure 8**).

Linked to H3K9 methylation, DNA methylation is also a main heterochromatin feature (Du et al., 2015). Methylation of cytosine residues within CpG islands reduces chromatin accessibility and gene expression (reviewed in Jones and Liang, 2009).

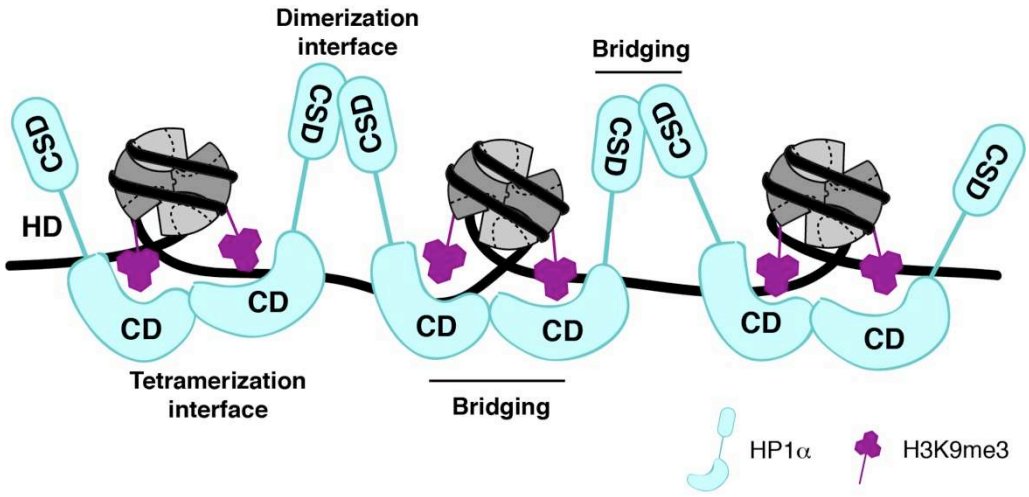

Figure 8 – HP1a binds H3K9me3-enriched heterochromatin. HP1 binds H3K9me3 via its chromo domain (CD) and bridges adjacent nucleosomes through HP1 dimerization via its chromo shadow domain (CSD). The HP1 hinge domain (HD) is also involved in chromatin binding through interactions with H3K9me3 and RNA.

Heterochromatin associated proteins and biophysical properties of heterochromatin

Histone post-translational modifications are recognized and bound by specific factors such as Heterochromatin Protein 1 (HP1, James and Elgin, 1986), which binds trimethylated H3K9 residues (Bannister et al., 2001; Lachner et al., 2001; Watanabe et al., 2018). HP1 is in fact a major structural component of heterochromatin, with its different isoforms – three in mammals- HP1α (also known as CBX5), HP1β (CBX1) and HP1γ (CBX3) showing distinct subnuclear distributions and playing different roles in heterochromatin organization (Bosch-Presegué et al., 2017). In particular, the isoform HP1α is found mainly in pericentric heterochromatin, where it bounds dynamically to chromatin, as revealed by fluorescence recovery after photobleaching (FRAP) (Bryan et al., 2017; Phair et al., 2004), and its homodimerization promotes the crossbridging between neighboring nucleosomes, thus compacting chromatin fibers (Azzaz et al., 2014; Canzio et al., 2011; Verschure et al., 2005) (**Figure 8**).

Repetitive sequences, histone modifications and DNA methylation are not enough to explain the dynamic behavior of heterochromatin. Indeed, despite its compaction, heterochromatin remains a permeable domain allowing fast protein diffusion. In an attempt to explain such apparently contradictory observations, it has recently been suggested that the formation of membrane-less heterochromatin domains could be mediated by phase separation (Boeynaems et al., 2018; Strom et al., 2017). This physicochemical process results from the multivalent interactions between DNA, RNA and proteins, and leads to the formation of phase-separated liquid droplets with a denser liquid phase (similar to oil droplets in water). In light of recent data, HP1α has been proposed to play an important role in chromatin

compartmentalization by its liquid-liquid demixing properties (Larson et al., 2017; Strom et al., 2017).

2.2.2. Establishment and maintenance of heterochromatin

The formation of heterochromatin domains can be distinguished into three steps: initiation, spreading, and maintenance. In fission yeast, non-coding RNAs (ncRNAs) that are processed into small RNAs seem to play an important role in the initial steps of heterochromatin formation (Volpe et al., 2002; Zaratiegui et al., 2011). However, the exact mechanisms initiating heterochromatin formation in mammals have not yet been elucidated. Recent modeling experiments suggest that interactions between heterochromatin regions and with the lamina are key for the organization of heterochromatin domains in the nuclear space (Falk et al., 2019). Following the initial heterochromatin nucleation, a cascade of protein recruitment, including HDAC and HMT, results in a hypo-acetylation of histones and hyper-methylation of H3K9 at the nucleation sites. These initial molecular features are key for the spreading step, as additional HMT and heterochromatin proteins will bind H3K9me3 and recruit multiple chromatin modifiers that will spread the heterochromatin state along large domains. Notably, while specific DNA sequences seem to be required to initiate heterochromatin formation at nucleation sites, the spreading of the heterochromatic state is DNA sequence-independent (Ragunathan et al., 2015).

During DNA replication, parental histones with existing modifications are incorporated into both daughter strands behind the replication fork (Alabert and Groth, 2012). The heterochromatic state is maintained in the daughter cells through a positive feedback mechanism involving enzymes that recognize the pre-existing histone modifications and catalyze the same modifications on newly deposited histones (reader-writer model). In particular, H3K9me-mediated recruitment of H3K9 methyltransferases leads to the stable maintenance of this epigenetic state through generations (Almouzni and Probst, 2011; Hall et al., 2002; Yu et al., 2018).

2.2.3. Functional importance of heterochromatin

Although being highly enriched for repetitive sequences and poorly transcribed, heterochromatin has a major role in cell viability and function. Indeed, numerous studies have related dysfunctional heterochromatin with disease, and in particular with cancer progression

(reviewed in Janssen et al., 2018). Heterochromatin integrity defects often result in changes in transcriptional programs and lead to increased chromosome instability.

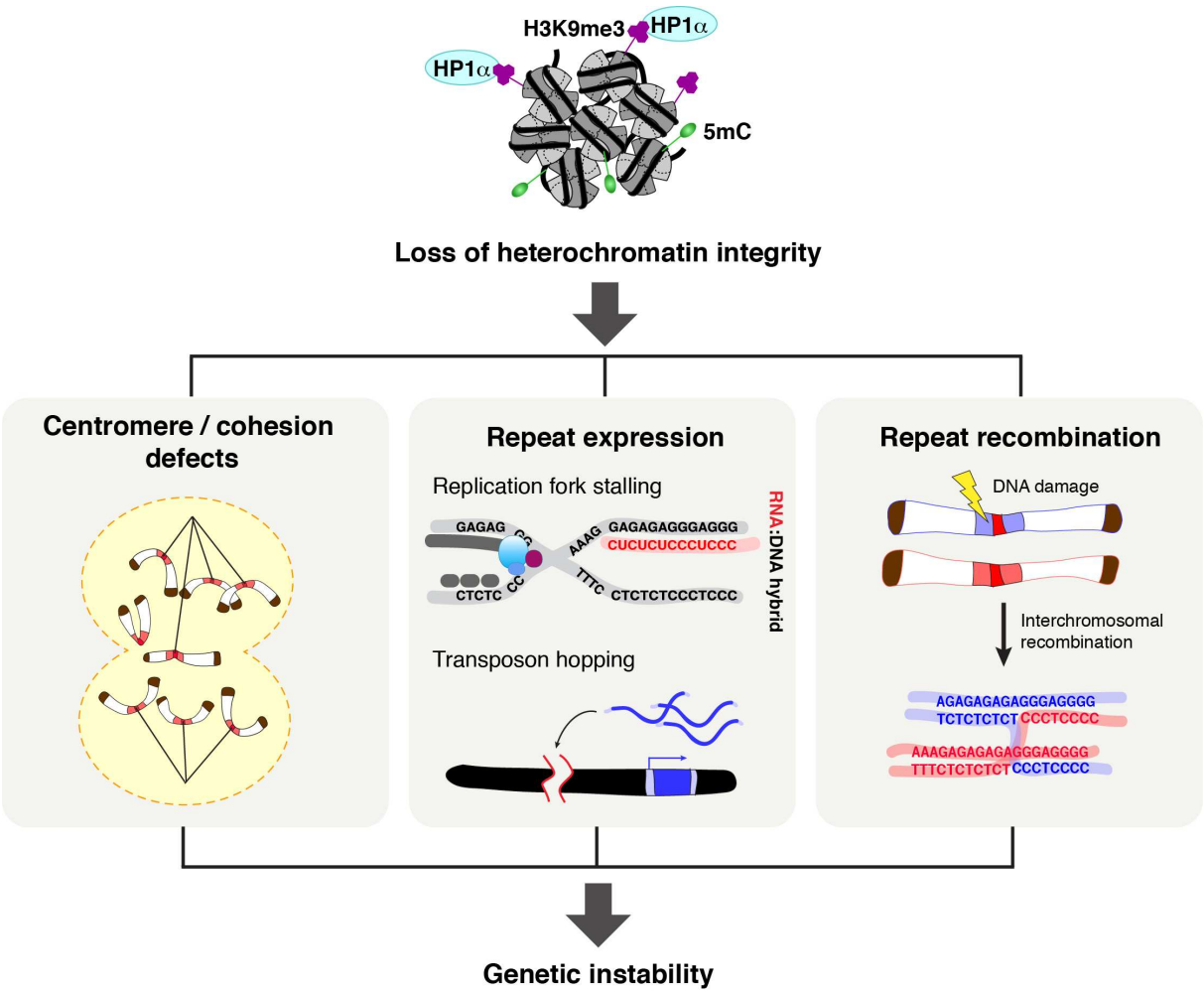

FIGURE 9 – Loss of heterochromatin integrity results in increased genome instability. Alterations of heterochromatin structure can generate a variety of genome stability defects, including chromosome missegregation, increased DNA damage due to replication fork stalling or from transposon hopping, and aberrant recombination between nonhomologous chromosomes. 5mC: 5methylCytosine (adapted from Janssen et al., 2018).

The pivotal role of heterochromatin in maintaining genome stability is achieved through diverse mechanisms (**Figure 9**). One of the most evident contributions of heterochromatin to genome stability is its role for the proper segregation of chromosomes during mitosis. Studies in fission yeast have revealed that pericentromeric heterochromatin indeed contributes to the assembly of the centromere-specific histone variant CENP-A, thus ensuring proper kinetochore-microtubule attachments (Folco et al., 2008). It also strengthens sister-chromatid cohesion by trapping high levels of centromeric cohesin (Bernard et al., 2001). Consequently, loss of

heterochromatin integrity results in chromosome missegregation events, leading to chromosome losses and gains, and to the formation of micronuclei (**Figure 9**).

Heterochromatin is also crucial to silence transcription of repetitive DNA elements. Although repeat RNAs do play a role in heterochromatin formation and function (Johnson et al., 2017; Smurova and De Wulf, 2018), aberrant overexpression of heterochromatin repeats can lead to the formation of DNA:RNA hybrids, causing replication fork stalling and resulting in DNA damage (Aguilera and García-Muse, 2013) (**Figure 9**). In addition, heterochromatin silencing of transposons contributes to restrain the hopping of these mobile elements into other genomic regions, thus preventing the potential disruption of genomic coding regions (Slotkin and Martienssen, 2007) (**Figure 9**).

Finally, heterochromatin prevents homologous recombination between repeats on nonhomologous chromosomes following DNA damage, which could result in chromosomal translocations (**Figure 9**, see **Chapter 4**).

Altogether, heterochromatin integrity greatly contributes to the maintenance of genome stability, and is thus crucial for organism viability. In addition to cancer progression, heterochromatin organization is also important during organism development, and loss of heterochromatic features has been associated both with normal and pathological ageing in several organisms (Brandt et al., 2008; Haithcock et al., 2005; Jones, 2015).

2.3. Types of heterochromatin

Beyond the general features presented above, heterochromatin actually exists in various forms that are structurally and functionally distinct. Regions that remain condensed and transcriptionally silent throughout development and cell division are known as "constitutive heterochromatin" (Saksouk et al., 2015), while "facultative heterochromatin" (Trojer and Reinberg, 2007) corresponds to regions of the genome where gene silencing is dynamically regulated.

2.3.1. Facultative heterochromatin

The most prominent example of facultative heterochromatin is the inactive X chromosome (Xi) in female mammals, a mechanism of dosage compensation of X-linked genes (Gendrel and Heard, 2014). This inactive X chromosome can be visualized with a microscope as a DAPI-dense, shapeless stain, known as the Barr body.

Several general features of gene silencing characterize the Xi, including DNA hypermethylation, histone H3 and H4 hypoacetylation and late replication in S phase. In addition to H3K9me3, facultative heterochromatin is typically enriched for trimethylated H3K27, a silencing mark deposited by the Polycomb repressive complex PRC2 (**Table 1**, Plath et al., 2003; Rougeulle et al., 2004). A central player in the X-inactivation process is a 17 Kb long non-coding RNA (lncRNA) named Xist (X-inactive-specific-transcript), which is expressed exclusively from the Xi and associates in *cis* along the length of the chromosome (Brown et al., 1991; 1992). The Xi is also characterized by a distinct nucleosome composition, with an enrichment in macroH2A1 and macroH2A2 histone variants (Costanzi and Pehrson, 1998; Sun and Bernstein, 2019). Notably, hypermutation of the inactive X chromosome has been found to be a common feature of female cancer genomes occurring across a wide range of tumor types (Chaligné et al., 2015; Jäger et al., 2013).

2.3.2. Constitutive heterochromatin

Unlike facultative heterochromatin, which contains mainly silenced genes, constitutive heterochromatin consists primarily of repetitive elements located at the pericentromeric and telomeric regions of chromosomes (**Figure 7**). The silencing mark H3K9me2/3 represents the defining molecular feature in constitutive heterochromatin (Peters et al., 2001; Saksouk et al., 2015), as it lies upstream of and controls several other heterochromatin marks. On pericentromeres, SUV39H1/2 are the main methyltransferases responsible for H3K9me3, a histone mark recognized by HP1 proteins, which in turn recruit SUV420H and DNMTs, leading to H4K20me3 and DNA methylation, respectively (Fuks et al., 2003; Lehnertz et al., 2003; Schotta et al., 2004). Importantly, H4K20me3 is critical for the timely replication of heterochromatin regions in mammalian genomes (Brustel et al., 2017). Methylation of other histone tail residues, including the less characterized trimethylation of H3K64, complete the molecular signature of heterochromatin and help create the repressive chromatin state (Lange et al., 2013).

2.4. Focus on pericentric heterochromatin

Among constitutive heterochromatin domains, pericentromeric regions are the easiest to observe cytologically as they form clusters in certain cell types, including mouse, plant and Drosophila cells. Indeed, as early as in 1908, the Italian botanist Pasquale Baccarini reported dark stained bodies in the interphase nucleus of plants, which he termed *chromocentri* or chromocenters

(Baccarini, 1908). Such structures result from the clustering of pericentromeric regions from multiple chromosomes in association with HP1α proteins (**Figure 10**). The acrocentric nature of mouse chromosomes facilitates such clustering. In contrast, chromocenters are not observed in human cells, where repetitive sequences are distributed more evenly across the genome and do not cluster to the same degree as in mouse cells.

In mouse cells, chromocenters comprise mainly two types of repetitive DNA: major satellites (6 Mb repeats of 234 bp units) and minor satellites (600 kb repeats of 120 bp units). In situ hybridization on metaphase chromosomes revealed that major satellite sequences are located pericentrically (hence the name "pericentric" heterochromatin), while minor satellite sequences coincide with the centric constriction (centromeric heterochromatin). While the observed clustering into chromocenters is due to the coalescence of major satellites in association with HP1α, minor satellites are located in a surrounding domain as several separate entities (Guenatri et al., 2004) (**Figure 10**). Major satellite transcripts play an important structural role in pericentric heterochromatin (Maison et al., 2002) and were recently shown to bind and stabilize SUV39H1/2 enzymes in constitutive heterochromatin (Johnson et al., 2017; Shirai et al., 2017; Velazquez Camacho et al., 2017).

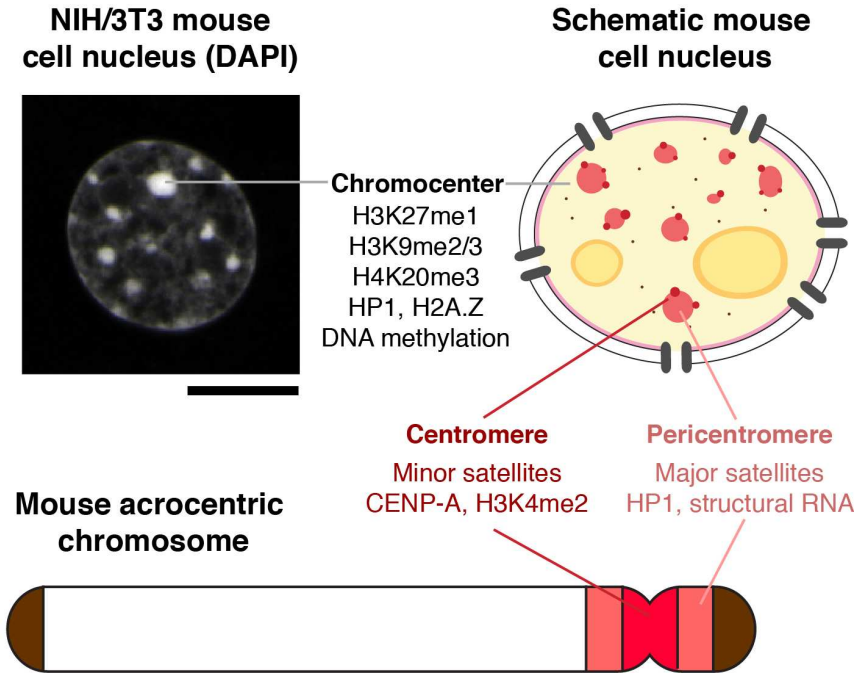

FIGURE 10 – Organization of pericentric heterochromatin into chromocenters in mouse cells. Mouse chromocenters are detectable with DAPI staining, and correspond to the clustering of major satellite regions with minor satellites at the periphery (top). Schematic representation of a typical mouse acrocentric chromosome (bottom). DAPI: 4',6-diamidino-2-phenylindole; HP1: Heterochromatin Protein 1; CENP-A: centromere protein A.

Although the biological significance of chromocenters remains essentially unknown, it has been proposed that such structures might serve to create a local environment where silencing factors concentrate and contribute to the maintenance of the heterochromatin status. In addition, pericentric heterochromatin boundaries have been shown to effectively block heterochromatin spreading to euchromatic regions (Wang et al., 2014). Because of their physical proximity to centromeres, pericentromeres are also essential for the correct segregation of chromosomes (see **Section 2.2.3**). As chromocenters cluster regions of different chromosomes, they might also play a role in genome organization in the cell nucleus (Wijchers et al., 2015).

All the above characteristics make mouse pericentric heterochromatin an attractive model system for studying heterochromatin structure and organization, which will be used in this thesis to study heterochromatin alterations in response to DNA damage.

CHAPTER 3

DNA damage challenges chromatin integrity

3.1. DNA damage and repair

Cells are continuously faced with endogenous and exogenous stress sources that can ultimately lead to DNA damage (reviewed in Chatterjee and Walker, 2017) (**Figure 11**). Endogenous DNA damage arises mainly from DNA engaging in hydrolytic and oxidative reactions with water and reactive oxygen species (ROS), respectively, which are byproducts of cell metabolism. In addition, replication errors by DNA polymerases and programmed DNA breaks during transcription or during somatic and meiotic recombination (Prochazkova and Loizou, 2016; Puc et al., 2017) also damage the DNA sequence. On the other hand, exogenous DNA damage results from the action of environmental physical and chemical agents. Examples of genotoxic agents include ultraviolet (UV) and ionizing radiation (IR), and alkylating and crosslinking agents. Altogether, endogenous damage sources account for an estimated 100.000 lesions per day per human cell, a number that could dramatically increase when considering environmentally-caused lesions (Hoeijmakers, 2009; Lindahl, 1993).

On the 21st anniversary of the historical paper describing the double helical structure of the DNA, Francis Crick stated: "We totally missed the possible role of enzymes in repair, although I later came to realize that DNA is so precious that probably many distinct repair mechanisms would exist. Nowadays, one could hardly discuss mutation without considering repair at the same time" (Crick, 1974). Indeed, to preserve genomic integrity, eukaryotic cells are equipped with sophisticated systems that protect the DNA by signaling and removing or tolerating the damage (**Figure 11**). These mechanisms are collectively referred to as the DNA damage response (DDR, Ciccia and Elledge, 2010; Giglia-Mari et al., 2011).

To ensure the removal of the vast diversity of DNA lesions, dedicated repair pathways have evolved. A common repair mechanism consists in the excision of lesions affecting one DNA strand. There are three types of excision repair: mismatched DNA bases during replication are replaced with correct bases by mismatch repair (MMR, Kunkel and Erie, 2015), while small chemical alterations of DNA bases are repaired by base excision repair (BER, Wallace, 2014) through excision of the damaged base. Nucleotide excision repair (NER, Marteijn et al., 2014; Schärer, 2013), on the other hand, excises several nucleotides at a time to remove bulky lesions

that distort the DNA double helix or inter-strand crosslinks (see **Section 3.2**). The repair of double strand breaks (DSBs) is mainly achieved by homologous recombination (HR) or by non-homologous end-joining (NHEJ) (Ceccaldi et al., 2016; Her and Bunting, 2018).

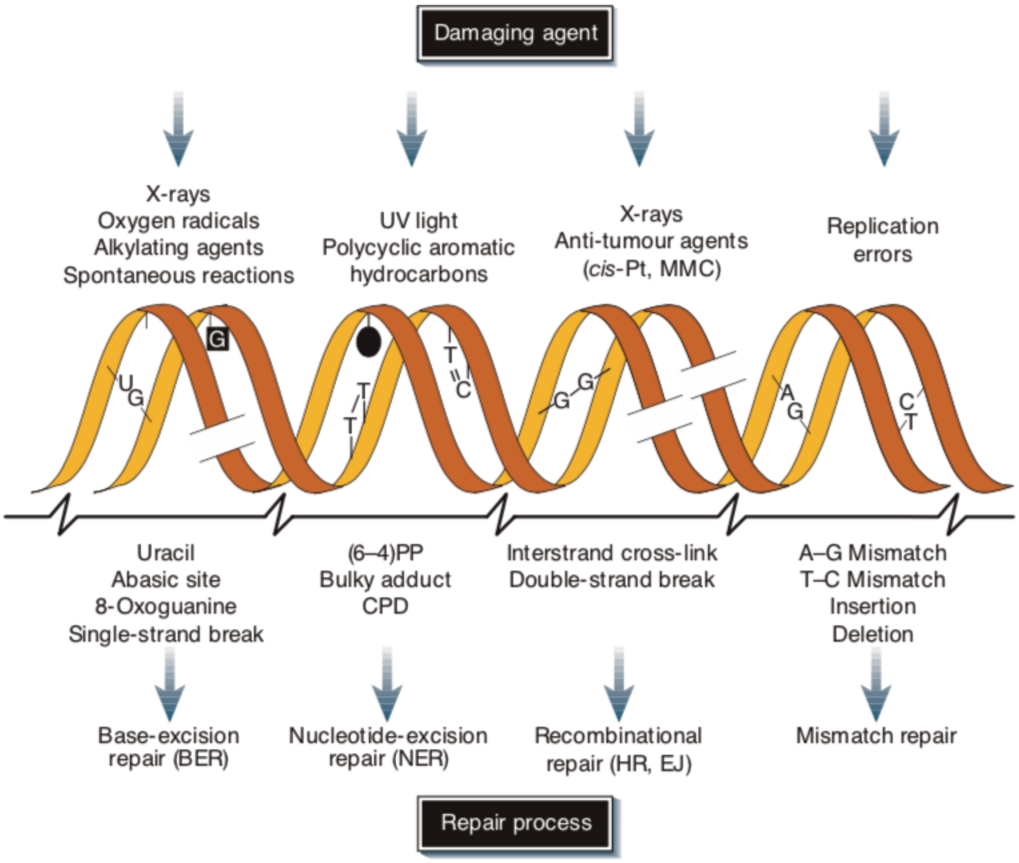

FIGURE 11 – DNA damage and repair pathways. Common exogenous and endogenous DNA damaging agents (top); examples of DNA lesions induced by these agents (middle); and most relevant DNA repair mechanisms responsible for the removal of the lesions (bottom). cis-Pt: cisplatin; MMC: mitomycin C; UV: ultraviolet; (6-4)PP: (6-4)pyrimidine-pyrimidone photoproducts; CPD: cyclobutane pyrimidine dimers; HR: homologous recombination; EJ: end joining. Figure from Hoeijmakers, 2001.

In addition to the factors involved in these repair pathways, additional factors recognize and signal the lesion to downstream effector proteins that will activate cell cycle checkpoints (Shaltiel et al., 2015) to allow the temporary blockage of the cell cycle during repair. Importantly, defects in DNA damage signaling and repair machineries result in pathologies with a broad spectrum of features including immune deficiency, neurodegeneration, premature ageing and susceptibility to develop cancers (Hoeijmakers, 2001; Jackson and Bartek, 2009; Lord and Ashworth, 2012).

3.2. Repair of UV damage by the Nucleotide Excision Repair pathway

3.2.1. UV radiation and UV photoproducts

Different wavelengths of ultraviolet radiation emerge form the Sun, including UVA (315–400nm), UVB (280–315 nm) and UVC (<280 nm), which induce different types of DNA lesions (reviewed in Rastogi et al., 2010) (**Figure 12**). In contrast to UVA and UVB, most of the UVC radiation is absorbed in the Earth's atmosphere by the ozone layer. This, together with the higher yields of DNA photoproducts produced by UVC with respect to UVB, makes UVC radiation a convenient tool to mimic solar UV radiation in a controlled way in the laboratory.

In my thesis, I have focused on the DNA lesions arising from UVC exposure, which comprise covalent linkages between two adjacent pyrimidines. The main photoproducts following UVC irradiation are cyclobutane pyrimidine dimers (CPDs) and (6-4)pyrimidine-pyrimidone photoproducts [(6-4)PPs] in a ratio of 3:1 (Kobayashi et al., 2001) (**Figure 12**). (6-4)PPs, which cause major distortions of the DNA double helix that frequently cause T-to-C mutations during replication, are easily detected by the general NER machinery and removed within 2-4h after UV. CPDs, on the contrary, only mildly destabilize the DNA helix and usually escape recognition, and are therefore repaired more slowly (24-48h) (Adar et al., 2016).

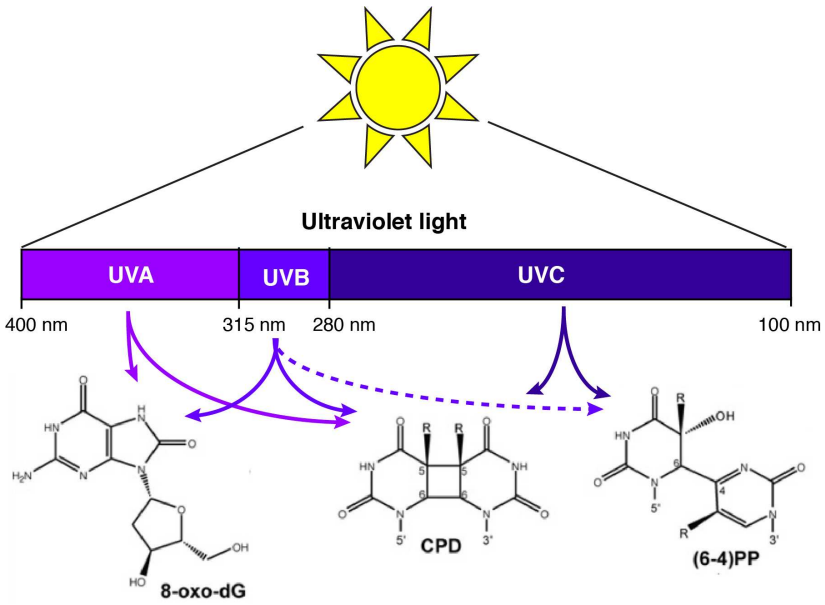

FIGURE 12 – Different UV wavelengths coming from the Sun induce the formation of distinct UV photoproducts. The diagram shows the subdivision of the solar UV spectrum and the main UV photoproducts induced by the different UV wavelengths. UV (A, B, C): ultraviolet light (type A, B, C), 8-oxo-dG: 8-oxo-deoxyguanosine, CPD: cyclobutane pyrimidine dimer; (6-4)PP: (6-4)pyrimidine-pyrimidone photoproduct.

3.2.2. The Nucleotide Excision Repair pathway

The NER pathway has been highly conserved throughout evolution, from bacteria to human (Hoeijmakers, 1993b; 1993a), to repair a wide variety of bulky DNA lesions, including UV photoproducts (**Figure 12**). In all organisms, NER consists of five steps: (1) recognition of the DNA lesion, (2) incision of the damaged strand on both sides of the lesion and at some distance from it, (3) excision of the lesion-containing oligonucleotide, (4) DNA synthesis using the undamaged strand as template and (5) ligation (reviewed in Marteijn et al., 2014; Schärer, 2013).

In addition to NER, other simpler solutions emerged during evolution to reverse UV-induced DNA damage, such as photolyases, which directly recognize UV photoproducts and use the energy of visible light to split pyrimidine dimers into monomers (Weber, 2005). Although photolyases are not conserved in mammals, which have to rely on NER to repair UV lesions, the ability of photolyases to bind UV lesions in a highly sensitive and dose-dependent manner has been recently used to quantify DNA damage load and to determine repair kinetics in real time (Steurer et al., 2019).

NER factors were defined based on complementation groups in rare human syndromes including XP (Xeroderma Pigmentosum), which is characterized by defective UV damage repair (see **Section 3.2.3**). Nevertheless, some NER factors have later been found to be involved in other repair pathways. For instance, DSB repair involves the action of several NER factors, including CSB, XPG and ERCC1-XPF (Ahmad et al., 2008; Al-Minawi et al., 2008; Batenburg et al., 2015; Trego et al., 2016; Yasuhara et al., 2018).

3.2.2.1. Global Genome-NER vs. Transcription-Coupled-NER

In eukaryotic NER, two subpathways exist that differ in their mechanism of initiating damage recognition: transcription-coupled NER (TC-NER) and global genome NER (GG-NER). In the case of TC-NER (Hanawalt and Spivak, 2008), recognition is initiated by the stalling of RNAPolII as it encounters damaged DNA during transcription (Geijer and Marteijn, 2018), which results in the rapid repair of DNA lesions on the actively transcribed strand. On the other hand, GG-NER slowly repairs DNA damage in the rest of the genome, and is thus the predominant repair pathway in heterochromatin domains.

3.2.2.2. Damage recognition in GG-NER

Damage recognition is the rate-limiting step of NER initiation. In addition to UV photoproducts, NER also eliminates numerous bulky chemical adducts, intra-strand crosslinks caused by drugs such as cisplatin and ROS-generated cyclopurines. In this sense, NER is exceptional among the distinct DNA repair pathways in its ability to eliminate the widest spectrum of structurally unrelated DNA lesions. The basis of this versatility is that it circumvents recognition of the lesion itself and instead focuses on common features shared by many different lesions.

During NER, lesion discrimination is executed by the concerted action of two complexes: the UV damage sensor protein Xeroderma Pigmentosum, complementation group C (XPC) (Sugasawa et al., 1998) and the DNA damage-binding protein 1-2 (DDB1-DDB2) heterodimer (UV-DDB complex) (**Figure 13**), which together with Cul4B and Ring1B forms a complex with ubiquitin ligase activity. While XPC easily recognizes the major distortions caused by 6-4PP, it requires previous binding of the UV-DDB complex for effective detection of CPDs (Fitch et al., 2003; Tang et al., 2000; Wakasugi et al., 2001). Indeed, DDB2 allows XPC binding to CPDs by ubiquitylation and potentially by promoting chromatin opening (Luijsterburg et al., 2012; Wang et al., 2006). Importantly, the recognition of UV photoproducts by UV-DDB occurs within a nucleosome context (Matsumoto et al., 2019; Osakabe et al., 2015). After damage recognition, DDB2 is ubiquitylated and subsequently degraded.

3.2.2.3. Molecular mechanisms of NER

Following the detection of the lesion, the subsequent steps of TC-NER and GG-NER converge into a common mechanism (**Figure 13**). Excision of the lesion is mediated by XPG and ERCC1-XPF nucleases, which is followed by filling of the DNA gap and sealing by ligase I. In higher eukaryotic cells, NER excises 24-32-nt DNA fragments containing the lesions with extreme accuracy. The generation of such fragments has been recently exploited for mapping regions undergoing NER in the human genome by excision repair sequencing (XR-seq, Hu et al., 2017).

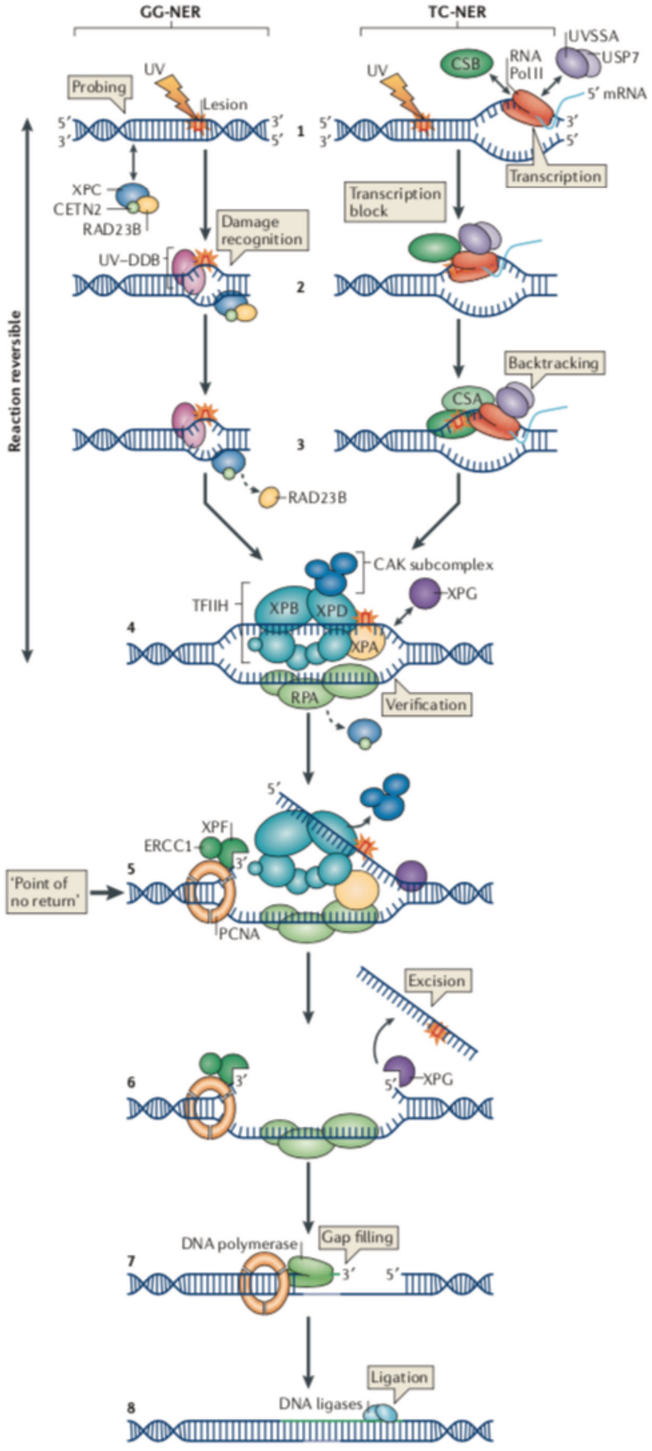

FIGURE 13 – Nucleotide excision repair. *STEPS 1-3.* In the GG-NER subpathway (left), the damage sensor XPC, in complex with RAD23B and CETN2, constantly probes the DNA for helix-distorting lesions, which are recognized with the help of the UV–DDB complex. In the TC-NER subpathway (right), damage is indirectly recognized by the stalling of RNA Pol II at a lesion. The transient interaction of UVSSA, USP7 and CSB with RNA Pol II renders the DNA lesion accessible for repair.

STEPS 4-8: After damage recognition, the helicase activity of TFIIH further opens the double helix around the lesion. The endonuclease XPF–ERCC1 is the recruited to the damaged strand and creates an incision 5' to the lesion. Once this 'point of no return' is reached, XPG cuts the damaged strand 3' to the lesion, which excises the lesion within a 24–32 nucleotide-long strand. PCNA then recruits DNA Polimerases for gap-filling DNA synthesis. The NER reaction is completed through sealing the final nick by DNA ligases. Figure from Marteijn et al., 2014.

3.2.3. Defective NER

The significance of functional NER is illustrated by the severe clinical consequences associated with mutations in NER genes in humans. This includes several pathologies, including xeroderma pigmentosum (XP), and the rare TC-NER-deficiency disorders known as cockayne syndrome (CS), cerebro-oculo-facio-skeletal syndrome (COFS), trichothiodystrophy (TDD) and UV-sensitive syndrome ($UV^{S}S$) (summarized in Vermeulen and Fousteri, 2013). In particular, XP is a high-carcinogenic disease, generally characterized by hypersensitivity to UV light (Tang et al., 2000). XP includes eight genetic complementation groups (A, B, C, D, E, F, G and V), corresponding to eight gene products involved in NER (**Figure 14**). Among them, XPE patients have loss-of-function mutations in the UV damage sensor DDB2 (Chu and Chang, 1988; Hwang et al., 1998) and are characterized by mild photosensitivity, slow development of the disease but high occurrence of skin cancers.

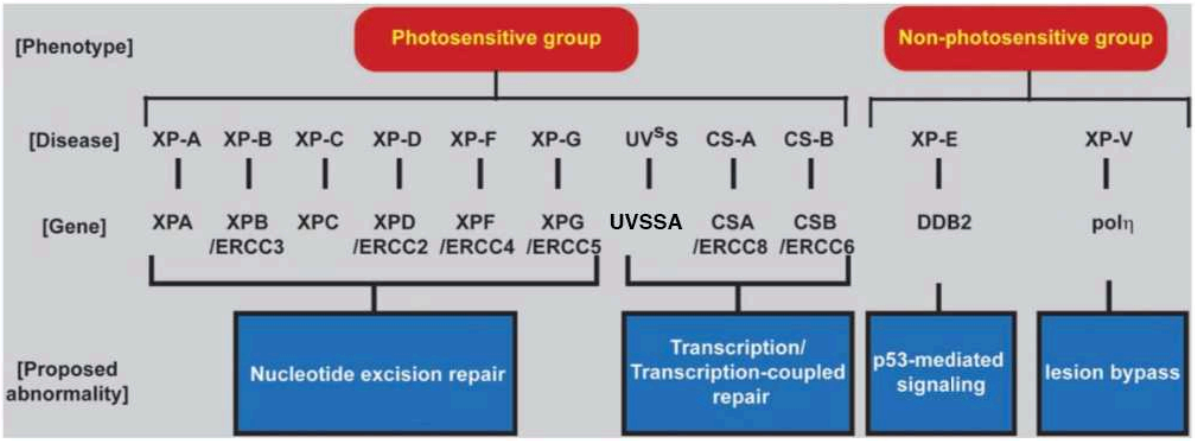

FIGURE 14 – Proposed classification of photosensitive diseases. XP: Xeroderma pigmentosum; ERCC: excision-repair-cross-complementing; $UV^{S}S$: UV-sensitive syndrome; UVSSA: UV-stimulated scaffold srotein A; CS: cockayne syndrome; DDB2: DNA damage-binding protein 2. Adapted from Itoh, 2006.

3.3. Chromatin dynamics following UV damage

3.3.1. Access-Repair-Restore model

The access-repair-restore model was established in 1991 (Smerdon, 1991), based on pioneering studies assessing the accessibility of damaged DNA to nucleases in UVC-irradiated human fibroblasts (Smerdon and Lieberman, 1978). Although originally described in response to NER of UV damage, it can also apply for other types of damage and repair, including DSB

repair. This model states that chromatin dynamics in response to DNA damage follows two main steps: the disorganization/decondensation of chromatin to facilitate the access to the lesion by the repair machinery, followed by a restoration step.

Access

To facilitate the access of the repair machinery to the damaged DNA, chromatin surrounding the lesion suffers several structural rearrangements. These rearrangements were first suggested by Smerdon MJ, who reported a higher sensitivity to micrococal nuclease (MNase) digestion of DNA regions undergoing repair synthesis in human cells as compared to bulk DNA (Smerdon and Lieberman, 1978). Later studies used imaging techniques in mammalian cells to confirm a loss of density for histones H2A, H4 and H1 (Luijsterburg et al., 2012) (see **Section 3.3.2**). Notably, the binding of the NER factor DDB2 has been involved in these rearrangements ((Adam et al., 2013; Luijsterburg et al., 2012) and this study). In addition to factors involved in the repair process, ATP-dependent chromatin remodelers may also be involved, as these are factors recruited to damage sites (reviewed in Lans et al., 2012). For instance, the chromatin-remodeling enzyme Amplified in Liver Cancer 1 (ALC1), which is recruited to DSBs in a Poly (ADP-ribose) polymerase (PARP)-dependent manner, was shown to promote chromatin remodeling during DSB repair following UVA laser irradiation (Ahel et al., 2009). However, the connection between repair factors (like DDB2) and these chromatin modifiers is still missing. Post-translational modifications on chromatin could also be involved in damage-mediated chromatin rearrangements, as observed for acetylation (Smerdon et al., 1982), which can affect nucleosome-nucleosome interactions to promote chromatin opening.

Notably, additional mechanisms may be needed to provide access to lesions arising in compact chromatin regions, including heterochromatin domains (see **Chapter 4**).

Restore

DNA repair is followed by the restoration of chromatin organization. The repair-coupled deposition of newly synthesized histones, reported in response to UV damage (Adam et al., 2013; Dinant et al., 2013; Piquet et al., 2018; Polo et al., 2006) and DNA double-strand breaks (DSBs) (Juhasz et al., 2018; Luijsterburg et al., 2016) likely contributes to the re-establishment of chromatin organization (see **Section 3.3.2**). Chromatin modifiers and remodelers could further contribute to the maintenance of histone modifications and the nucleosome repositioning, although this has not been fully characterized. Whether specific mechanisms exist for the restoration of different chromatin domains, including heterochromatin domains with their

characteristic PTMs and higher-order folding, also remains an open question, and will be the subject of investigation in this study.

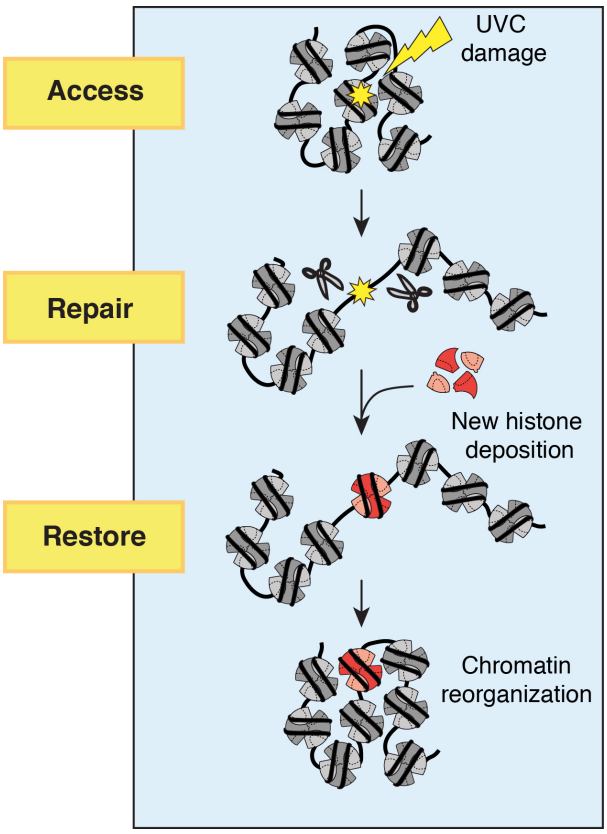

FIGURE 15 – Access-Repair-Restore model describing histone dynamics in response to DNA damage in human cells. In response to UVC damage, chromatin surrounding the lesion is disorganized, which facilitates the access of the repair machinery to the damaged DNA strand. The restoration step involves the deposition of newly synthesized histones and the reorganization of chromatin.

3.3.2. Histone dynamics coupled to UV damage DNA repair

Following the definition of the ARR model, numerous studies addressed the issue of histone dynamics in response to UV damage. The first evidence of this phenomenon came from studies *in vitro*, which identified the histone chaperone CAF-1 as responsible for NER-coupled nucleosome assembly (Gaillard et al., 1996). Later on, the direct interaction of CAF-1 with the polymerase sliding clamp proliferating cell nuclear antigen (PCNA) was discovered, which constituted the molecular basis for coupling histone deposition and repair synthesis (Moggs et al., 2000).

The development of novel techniques allowing the tracking of histone proteins *in vivo* took the study of repair-coupled chromatin dynamics one step further. Using such techniques, the dynamics of newly synthesized histones following local UVC irradiation were analyzed in cultured human cells focusing on specific histone variants. These techniques include analyzing the redistribution of transiently transfected HA-Flag-tagged histones, or fluorescently-tagged histones after photobleaching, or SNAP-tagged histones upon local UVC irradiation of the cells (see **Annex 2** for details). These techniques allowed the observation of new histone deposition in UVC-damaged chromatin in human cells (H3.1, H3.3, H2A, H2A.X) (Adam et al., 2013; Dinant et al., 2013; Piquet et al., 2018; Polo et al., 2006). Remarkably, H3 variants are deposited in damage chromatin in a sequential manner by dedicated histone chaperones (**Figure 16**).

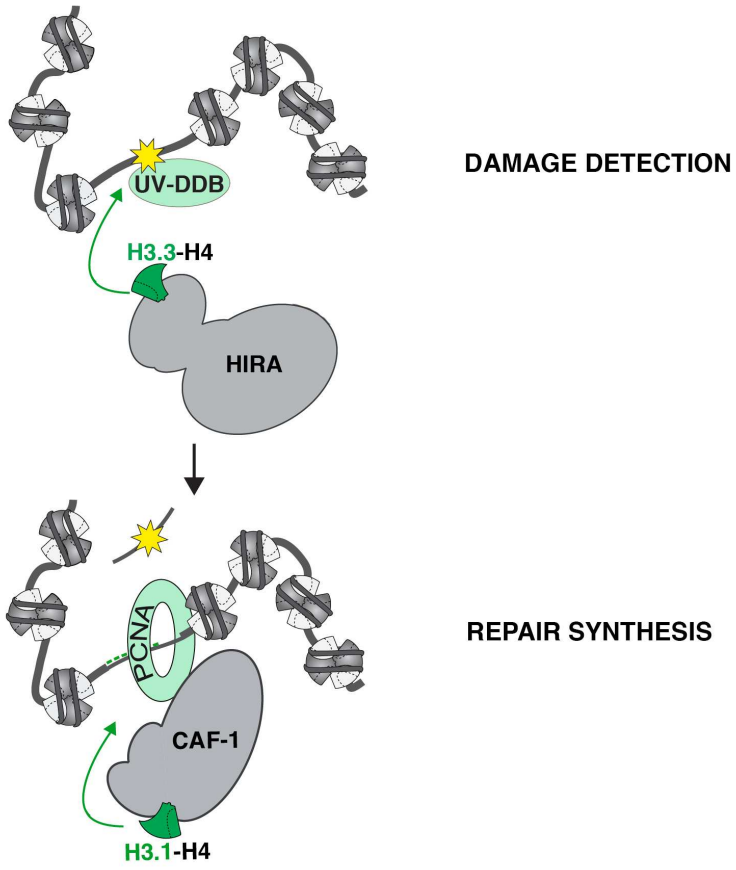

FIGURE 16 – New H3 histone deposition during UV damage repair in human cells. Sequential deposition of new H3 histone variants in UV-damaged chromatin in human cells. H3.3 deposition by the histone chaperone HIRA is coupled to UV-DDB dependent ubiquitylation events at the time of damage detection, and is followed by H3.1 deposition by CAF-1, coupled to repair synthesis. UV-DDB: UV-damage DNA-binding protein; Ub: Ubiquitin; HIRA: Histone Regulator A; PCNA: Proliferating Cell Nuclear Antigen; CAF-1: Chromatin Assembly Factor-1.

The SNAP-tagging approach also revealed different behaviors regarding the dynamics of parental histone variants in damaged chromatin: with a conservative redistribution of H3-H4 (Adam et al., 2016) and an eviction of the histone variant H2A.Z (Piquet et al., 2018).

3.3.2.1 Histone modifications associated with the UV damage response

Phosphorylation of serine 139 in the histone variant H2A.X (Rogakou et al., 1998) is a bona fide DDR histone modification, and a central element for DNA damage signaling (Dantuma and van Attikum, 2016). In response to UV damage, this phosphorylation is dependent on the ataxia-telangiectasia mutated and Rad3-related (ATR) kinase (Hanasoge and Ljungman, 2007). In addition to phosphorylation, the ubiquitylation of histones around the lesion may be involved in chromatin decompaction to facilitate the access of the repair machinery to the lesions. This ubiquitylation could be mediated by two main ubiquitin ligases in the NER pathway, i.e. UV-DDB and RNF2 (Gracheva et al., 2016; Kapetanaki et al., 2006). Another histone post-translational modification that could promote the accessibility of UV-damaged chromatin is acetylation (Ramanathan and Smerdon, 1986). In particular, the K56 residue in H3 histones seems to be deacetylated following UV irradiation in a DDB2-dependent manner (Zhu et al., 2015), and then reacetylated by the action of the histone acetyltransferase p300 in a CAF-1-dependent manner (Battu et al., 2011).

In addition to the modification of parental histones in damaged chromatin, whether newly deposited histones also get modified in a DDR-dependent manner remains unknown.

3.3.3. Modulation of transcription following UV damage

Local inhibition of transcription is a consequence of various types of DNA lesions, as it has been best characterized for RNA polymerase II (RNAPII)-dependent transcription (Chou et al., 2010; Iacovoni et al., 2010; Moné et al., 2001; Pankotai et al., 2012; Shanbhag et al., 2010; Tornaletti, 2009). Such DNA damage-induced transcription arrest is critical to prevent the production of aberrant transcripts and to avoid interference between transcription and repair machineries (Marnef et al., 2017).

UV photoproducts induce strong RNAPII stalling, thus locally affecting transcription of damaged genes, but they also have a more global effect and cause the inhibition of genes distant from the damage (Geijer and Marteijn, 2018). Following excision of the lesion, the displacement

of stalled RNAPII allows the completion of DNA repair and the subsequent restart of transcriptional activity (Gaillard and Aguilera, 2016). However, what triggers and controls transcription restart once repair is complete is still poorly understood. So far, it is known that transcription restart involves histone chaperones such as facilitates chromatin transcription (FACT, Dinant et al., 2013), and HIRA (Adam et al., 2013). While FACT was recently shown to participate to TC-NER by controlling UVSSA recruitment (Wienholz et al., 2019), the mechanisms underlying HIRA-mediated transcription recovery still remain to be characterized. The effects of UV damage and repair on silenced chromatin regions, including heterochromatin, have not yet been characterized.

CHAPTER 4

DNA damage and repair in heterochromatin domains

For this section of the introduction, I include a review that I wrote in 2018 for *Chromosoma* and that extensively covers the topic of DNA damage and repair in heterochromatin domains.

Review:

The response to DNA damage in heterochromatin domains

Fortuny A., Polo S.E.

Chromosoma, 127:291–300, 2018

In this review, we describe the aftermath of genotoxic stress challenges in heterochromatin domains, from DNA damage formation and signaling up to DNA repair. Focusing on distinct heterochromatin domains in various eukaryotic species, we explore the contribution of heterochromatin to the compartmentalization of DNA repair in the cell nucleus and to repair pathway choice. We also present the main heterochromatin alterations associated with the DNA damage response, exploring the mechanisms involved and the biological impact of such alterations. Finally, we discuss the role of the DNA damage response in heterochromatin in the maintenance of genome stability following genotoxic stress.

Since our review was published, interesting studies have come out regarding DNA damage formation and repair in heterochromatin, repair pathway choice and the relocation of DSBs outside heterochromatin domains. In particular, an innovative approach was been recently used to assess the effect of epigenetic silencing in imprinted genes on the mutagenic repair of CRISPR-Cas9 DSBs (Kallimasioti-Pazi et al., 2018). Their results show that heterochromatin organization burdens damage formation and slows down genome editing, but such differences in damage kinetics were not reflected in the outcome of repair.

Another recent study unveiled the role of the Drosophila histone demethylase dKDM4A in controlling timely DSB repair and the relative utilization of HR and NHEJ pathways in pericentromeric heterochromatin (Janssen et al., 2019). These results put forward the importance of histone-modifying activities for proper DSB repair in heterochromatin domains.

Finally, a recent paper from Irene Chiolo's lab sheds new light on the mechanisms controlling the relocation of DSBs outside heterochromatin domains (Caridi et al., 2018). This study follows up and builds on previous work showing that Drosophila DSBs relocate to the nuclear periphery (Ryu et al., 2015). They now identified nuclear actin filaments and myosins as effectors of heterochromatic DSB directed motion both to the nuclear periphery in Drosophila and outside heterochromatic chromocenters during HR in G2 mouse cells.

Chromosoma
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REVIEW

The response to DNA damage in heterochromatin domains

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Abstract

Eukaryotic genomes are organized into chromatin, divided into structurally and functionally distinct euchromatin and heterochromatin compartments. The high level of compaction and the abundance of repeated sequences in heterochromatin pose multiple challenges for the maintenance of genome stability. Cells have evolved sophisticated and highly controlled mechanisms to overcome these constraints. Here, we summarize recent findings on how the heterochromatic state influences DNA damage formation, signaling, and repair. By focusing on distinct heterochromatin domains in different eukaryotic species, we highlight the heterochromatin contribution to the compartmentalization of DNA damage repair in the cell nucleus and to the repair pathway choice. We also describe the diverse chromatin alterations associated with the DNA damage response in heterochromatin domains and present our current understanding of their regulatory mechanisms. Finally, we discuss the biological significance and the evolutionary conservation of these processes.

Keywords Chromatin reorganization · DNA damage repair · Heterochromatin · Nuclear domains

Introduction

In eukaryotic cell nuclei, the genetic information is packaged in the form of chromatin (Kornberg 1977) where DNA wraps around histone proteins to form nucleosomes (Luger et al. 1997) and higher-order structures (Bonev and Cavalli 2016). The different levels of chromatin organization are central to cell function as they constitute key vectors of epigenetic information, which dictates cell identity (Allis and Jenuwein 2016). Among higher-order chromatin structures, heterochromatin domains are critical chromatin compartments with a major influence on chromosome segregation and stability (Allshire and Madhani 2017). Originally defined as chromosomal regions that remain compact throughout the cell cycle (Heitz 1928), heterochromatin domains are generally gene poor, mostly transcriptionally silent, and are characterized by specific sets of histone modifications and associated proteins. Recent advances in super-resolution microscopy have provided a refined three-dimensional picture of chromatin in vivo at a nanoscale resolution, revealing that heterochromatin domains are formed by larger, denser, and less mobile nucleosome clutches compared to euchromatin (Ricci et al. 2015; Nozaki et al. 2017; Ou et al. 2017). Beyond these general features, heterochromatin actually exists in various forms that are structurally and functionally distinct: while constitutive heterochromatin remains condensed and mostly transcriptionally silent throughout development and cell divisions (Saksouk et al. 2015), facultative heterochromatin corresponds to regions of the genome where gene silencing is dynamically regulated (Trojer and Reinberg 2007). A typical example of facultative heterochromatin is the inactive X chromosome in female mammals (Gendrel and Heard 2014), but it also includes genomic regions that interact with specific nuclear structures, such as the lamina-associated domains (LADs) located at the nuclear periphery (van Steensel and Belmont 2017) and nucleolus-associated domains (NADs; Matheson and Kaufman 2016). Constitutive heterochromatin is found at subtelomeric regions (Schoeftner and Blasco 2009) and at pericentromeres (Saksouk et al. 2015), which surround repetitive centromeric DNA (McKinley and Cheeseman 2016). Each of these heterochromatin domains is defined epigenetically by specific histone post-translational modifications, histone variants, and associated proteins (Fig. 1), in addition to DNA methylation, which contributes to transcriptional silencing.

In recent years, a growing number of studies focused on understanding how heterochromatin domains are established during development and then perpetuated through replication

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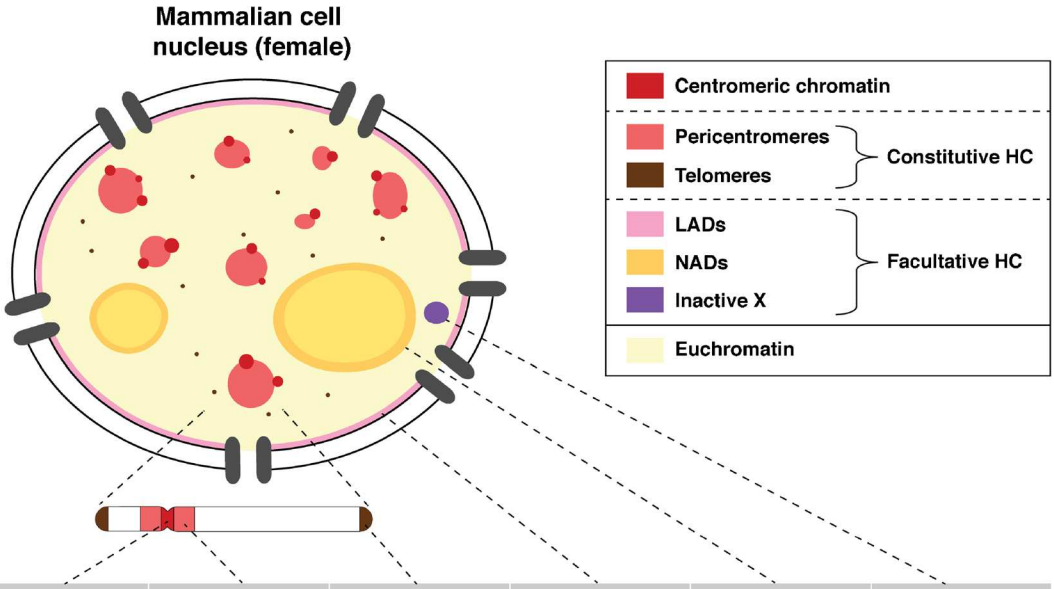

	Centromere	Pericentromere	Telomere	LAD	NAD	Inactive X
Histone variants	CENP-A					MacroH2A
Histone modifications	H3K4me2 H3K36me2	H3K9me3 H3K27me1 H3K56me3 H4K20me3	H3K9me3 H4K20me3	H3K9me2 H3K27me3	H3K9me3 H3K27me3 H4K20me3	H3K27me3
Associated proteins / complexes	CENPs	HP1	Shelterin TRF1/2	Lamins		PRC2

Fig. 1 Main heterochromatin domains and their distinctive features in mammalian cells. Constitutive and facultative heterochromatin domains are depicted and their characteristic histone variants, modifications, and associated proteins are listed. Although it is not heterochromatin per se, we also consider centromeric chromatin, which is rich in repetitive sequences and surrounded by constitutive heterochromatin domains. CENP centromere protein, HC heterochromatin, HP1 heterochromatin protein 1, LAD lamina-associated domain, NAD nucleolus-associated domain, PRC2 polycomb repressive complex 2, TRF1/2 telomeric repeat binding factor 1/2

and cell division. Another major challenge for heterochromatin maintenance is the response to DNA damage, which poses a constant threat to both genome and epigenome stability (Dabin et al. 2016). Furthermore, with the exception of LADs, heterochromatin is highly enriched for repetitive sequences, including tandem satellite sequences and transposable elements (Padeken et al. 2015), which compromises faithful DNA replication and repair, with a risk of aberrant homologous recombination between ectopic repeats leading to chromosome rearrangements and aneuploidy (Peng and Karpen 2008). Silencing of transposable elements through heterochromatinization is also critical for genome stability (Padeken et al. 2015). The issue of genome and epigenome maintenance is thus particularly prominent in heterochromatin.

Here, we review recent advances in our understanding of DNA damage formation, signaling, and repair in heterochromatin domains and describe heterochromatin reorganization associated with the DNA damage response. We focus mainly on the response to DNA double-strand breaks (DSBs) and UV photoproducts in diverse eukaryotic cell systems, including yeast, *Drosophila*, and mammalian cells. We highlight that even though they share common features, not all heterochromatin domains are treated equal following a genotoxic stress challenge.

DNA damage formation in heterochromatin domains

Chromatin organization in the cell nucleus has a significant impact on the DNA damage response, from damage formation to repair. Indeed, chromatin loops were recently identified as a source of topoisomerase 2-mediated DNA breaks in mammalian cells, putting forward chromatin organization as a major driver of genome fragility (Canela et al. 2017). Heterochromatin organization in particular markedly impacts genome stability, as illustrated by higher mutation rates in human cancer cells, both in constitutive (Schuster-Böckler and Lehner 2012) and in facultative heterochromatin (Jäger et al. 2013). Furthermore, mutation patterns strongly associate with nuclear organization, with heterochromatin at the nuclear

periphery, LADs in particular, displaying higher mutation frequencies in various cancer types (Smith et al. 2017). These studies suggest that DNA damage formation and/or repair is influenced by higher-order chromatin organization in the cell nucleus. Over the last few years, several studies have addressed how tridimensional chromatin organization and compaction affect the susceptibility of DNA to damage. In vitro manipulation of chromatin compaction by adjusting magnesium concentration on permeabilized human nuclei and on mitotic chromosomes revealed that the levels of DSBs induced by ionizing radiation in compact chromatin were 5 to 50-fold lower than in decondensed chromatin, implying that chromatin compaction protects genomic DNA from radiation damage (Takata et al. 2013). The question of DSB generation in different chromatin domains was then tackled in vivo both in mouse and in human cells. For this, several genome-wide approaches were developed for mapping DSBs across the genome at single-nucleotide resolution, including BLESS (Crosetto et al. 2013), END-seq (Canela et al. 2016), and DSBCapture (Lensing et al. 2016), which established the higher susceptibility of transcriptionally active euchromatin to endogenous DSB formation. In contrast, breaks induced by aphidicolin were enriched in centromeric and pericentromeric chromatin, most likely reflecting the higher sensitivity of DNA repeats to replication stress. Mechanistic insights into how heterochromatin may hinder endogenous break induction are still lacking. The low levels of transcription in heterochromatin may preserve this chromatin compartment from transcription-induced genome instability (Gaillard and Aguilera 2016). In terms of molecular players, a recent study in *Drosophila* puts forward linker histone H1 as preventing the accumulation of R-loop-induced DNA damage in heterochromatin (Bayona-Feliu et al. 2017). Further work is still needed to fully dissect the mechanisms that control DSB distribution between euchromatin and heterochromatin domains.

While the genome-wide distribution of DSBs is established, contrasting reports continue to emerge regarding the formation of UV-induced DNA lesions in mammalian genomes. Single-nucleotide resolution mapping of cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) by HS-Damage-seq in UV-irradiated human fibroblasts (Hu et al. 2017) showed that the distribution of both types of UV lesions was essentially uniform throughout the genome. In contrast, a concomitant study using a similar genome-wide mapping approach in human fibroblasts showed that lamina-associated heterochromatin at the nuclear periphery was more vulnerable to UV damage than euchromatin (García-Nieto et al. 2017). Furthermore, immunofluorescence-based detection of UV damage revealed that 6-4PP were excluded from pericentromeric heterochromatin in mouse fibroblasts (Han et al. 2016), suggesting that the highly condensed heterochromatin environment may interfere with the formation of some UV lesions. Thus, it is not yet entirely clear whether the UV mutation signature observed in human cancer cells (Smith et al. 2017) results from higher damage formation or from slower repair in heterochromatin. Therefore, the role of nuclear organization and chromatin compaction on DNA damage formation remains an important field of study with broad implications for our understanding of genome stability and mutational landscapes.

Impact of heterochromatin on DNA damage signaling

One of the earliest consequences of DNA damage infliction is the recruitment of DNA damage signaling kinases, which initiates a complex cascade of events leading to cell cycle checkpoint activation. Among the many targets of these kinases, the histone variant H2A.X gets rapidly phosphorylated in large chromatin domains surrounding DSBs, giving rise to γH2A.X foci (Rogakou et al. 1998), which serve as a platform for recruiting downstream checkpoint and repair factors (Smeenk and van Attikum 2013). While this is a general response to DNA damage, several studies in yeast and mammalian cells originally showed that silenced chromatin domains were refractory to H2A.X phosphorylation (Cowell et al. 2007; Kim et al. 2007) and hampered DNA damage checkpoint signaling (Brunton et al. 2011). However, closer examination of the DDR in a time-resolved fashion later showed that H2A.X (H2A.v in *Drosophila*) was phosphorylated within pericentromeric heterochromatin domains in mouse and *Drosophila* cells, while subsequent steps of damage signaling occurred outside heterochromatin domains after a relocation of the breaks to the periphery of the domains (Chiolo et al. 2011; Jakob et al. 2011; Tsouroula et al. 2016; Janssen et al. 2016) or even to the nuclear periphery in *Drosophila* cells (Ryu et al. 2015, 2016). Noteworthy, such relocation specifically affects DSBs repaired by recombination, as discussed in the following sections. In plant cells, the situation is more complex with the existence of a heterochromatin-specific histone variant H2A.W.7, which is phosphorylated in response to damage, while H2A.X phosphorylation takes place primarily in euchromatin (Lorković et al. 2017).

Furthermore, dynamic chromatin compaction appears to play an important regulatory role in DNA damage signaling. Indeed, tethering heterochromatin factors to a LacO array in the absence of DNA damage in human cells induces local chromatin condensation and is sufficient to activate early steps in DNA damage signaling but not downstream effectors (Burgess et al. 2014). While the exact molecular mechanism by which chromatin condensation initiates early damage signaling is unknown, it might involve the repressive histone mark H3K9me3 and its ability to stimulate the acetyltransferase activity of Tip60, which then contributes to the activation of the DNA damage signaling kinase ataxia-telangiectasia

mutated (ATM) (Sun et al. 2005, 2009). However, these assumptions are based on studies performed in euchromatin domains, and further studies are needed to clarify the role of heterochromatin compaction in damage signaling.

Altogether, these studies demonstrate that heterochromatin is permissive for DNA damage signaling and that heterochromatin features including histone marks and chromatin compaction exert a positive role in response to DNA damage by contributing to the checkpoint activation.

Impact of heterochromatin on DNA repair efficiency

In the highly compartmentalized eukaryotic nucleus, both the chromatin state and the nuclear position of DNA lesions have a significant impact on repair pathway choice and repair efficiency (Kalousi and Soutoglou 2016). In this regard, compact heterochromatin domains may be seen as a barrier to repair factor recruitment, underlying higher mutation rates (Fig. 2a). Indeed, it was observed that excision of CPDs, the main UV photoproducts repaired by the nucleotide excision repair (NER) pathway (Marteijn et al. 2014), was significantly slower in H3K9me3-containing chromatin in human cells (Han et al. 2016). Recently, a high-throughput sequencing method, known as XR-seq, was used to analyze oligonucleotide fragments excised during NER in UV-irradiated fibroblasts, further establishing the slower repair associated with heterochromatin regions (Adar et al. 2016). Furthermore, transcription-coupled NER does not operate in poorly transcribed heterochromatin domains. These differences in NER efficiency underly cancer-associated mutagenesis, with an increased mutation density in heterochromatin regions and a reduced mutation rate in euchromatin that is abrogated by loss-of-function of NER factors (Polak et al. 2014; Zheng et al. 2014). Similarly, a lower efficiency of mismatch repair (Jiricny 2013) contributes to higher mutation rates in heterochromatin (Supek and Lehner 2015). Although early steps of DNA break repair proceed efficiently in pericentromeric heterochromatin (Chiolo et al. 2011; Jakob et al. 2011), a slower DSB repair has been observed at chromocenters in mouse cells, where, about 25% of the radiation-induced DSBs are repaired with slow kinetics and they predominantly localize at the vicinity of pericentromeric heterochromatin domains (Goodarzi et al. 2008). In contrast, sequence-specific DSBs induced by the I-SceI endonuclease in *Drosophila* are repaired with similar kinetics in euchromatin and pericentromeric heterochromatin (Janssen et al. 2016). This may reflect differences between species or between DNA ends, radiation-induced breaks requiring more processing than endonuclease-induced breaks. In the future, DSB genome-wide mapping techniques (Crosetto et al. 2013; Canela et al. 2016; Lensing et al. 2016) will be instrumental for analyzing DSB repair efficiency and pathway choice in distinct chromatin compartments.

Impact of heterochromatin on DNA repair pathway choice

In line with the heterogeneity of the eukaryotic nucleus, there are regional differences in DNA repair pathways between euchromatin and heterochromatin compartments. Heterochromatin being mostly transcriptionally silent, global genome NER (GG-NER) is predominant over transcription-coupled NER (TC-NER) in heterochromatin regions with a major role of the GG-NER factor DNA damage binding protein 2 (DDB2) in promoting CPD removal from H3K9me3-containing chromatin (Han et al. 2016). Heterochromatin is also a major determinant in the regulation of DSB repair outcome (Fig. 2b). Repair of genomic DSBs is achieved either by homology-based pathways, i.e., error-free homologous recombination (HR) and mutagenic single strand annealing (SSA), or by non-homologous end joining (NHEJ), with alternative end joining (A-EJ) serving as a backup (Mladenov

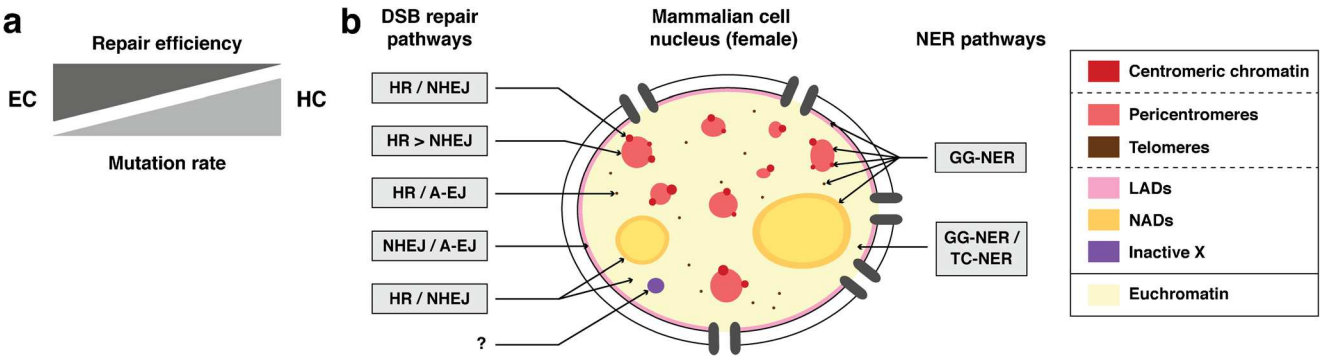

Fig. 2 DNA damage repair in heterochromatin domains. **a** Balance between repair efficiency and mutation rates in euchromatin (EC) and heterochromatin (HC). **b** Compartmentalization of DNA double-strand break (DSB) repair and nucleotide excision repair (NER) pathways in the mammalian cell nucleus. A-EJ alternative end-joining, GG-NER global genome NER, HR homologous recombination, LAD lamina-associated domain, NAD nucleolus-associated domain, NHEJ non-homologous end-joining, TC-NER transcription-coupled NER

et al. 2016). The repetitive nature of heterochromatin increases the risk of illegitimate recombination during repair. Therefore, a tight control of recombination events is critical in these domains. In particular, the silenced chromatin state plays a key role in repressing mitotic recombination at centromeres and telomeres, as revealed in DNA methyltransferase (DNMT)-deficient mouse cells showing increased telomeric and centromeric recombination accompanied by changes in centromere and telomere repeat length (Gonzalo et al. 2006; Jaco et al. 2008). This suggests that the prevention of illicit recombination in these compartments is important to maintain centromere and telomere integrity. Likewise, telomere hyper-recombination and subsequent chromosomal fusions in mouse embryonic stem cells are prevented by the telomere-associated protein Rif1, which mediates heterochromatic silencing by maintaining H3K9me3 levels at subtelomeric regions (Dan et al. 2014). The importance of the silenced chromatin state in controlling recombination has also been observed in *Drosophila* cells, where completion of recombinatorial repair requires a SUMO-dependent relocation of DSBs outside H3K9me2- and HP1a-containing domains (Chiolo et al. 2011; Ryu et al. 2015, 2016). Similarly, in budding yeast, silent information regulators (Sir) inhibit recombinational repair in silenced chromatin domains (Sinha et al. 2009). Interestingly, this inhibition is relieved through the eviction of Sir3p by the SWI/SNF chromatin remodeler (Sinha et al. 2009), suggesting that the constraints on recombinational repair in silenced chromatin can be alleviated by the action of chromatin remodelers. Similar to mitotic recombination, meiotic recombination is also repressed in silenced chromatin, as observed in fission yeast centromeres (Ellermeier et al. 2010). Furthermore, when recombination happens in silenced chromatin, error-free repair pathways are promoted. In budding yeast for instance, subtelomeric Sir3p-repressed chromatin promotes HR by inhibiting excessive DNA-end resection (Batté et al. 2017) and in fission yeast centromeric chromatin, Rad51-dependent HR is favored over SSA (Zafar et al. 2017). Heterochromatic DSBs also rely largely on HR for their repair in G2 mouse cells (Beucher et al. 2009) and in *Drosophila* cultured cells, where pericentromeric heterochromatin appears to be largely repaired through Rad51-dependent HR (Chiolo et al. 2011; Tsouroula et al. 2016). Yet, in fly tissues, which are mostly in G1, NHEJ predominates over HR in pericentromeric heterochromatin (Janssen et al. 2016). Several studies have provided mechanistic insights into how DSB repair could be regulated in heterochromatin based on the involvement of heterochromatin-associated factors in euchromatin repair (Lemaître and Soutoglou 2014). In particular, heterochromatin protein 1 (HP1) has been identified as a main player in the control of DNA-end resection and shown to operate through the recruitment of breast cancer 1 (BRCA1) (Baldeyron et al. 2011; Soria and Almouzni 2013; Lee et al. 2013). In addition to HP1, other heterochromatin-associated factors function with BRCA1 in controlling resection, including the histone H3K9 methyltransferases SET Domain Bifurcated 1 (SETDB1) and Suppressor of Variegation 3-9 Homolog (Suv39H1/2) (Alagoz et al. 2015). Another important player in the repair of heterochromatic DSBs is p53-binding protein 1 (53BP1) (Noon et al. 2010; Kakarougkas et al. 2013). In line with this, Suppressor Of Cancer Cell Invasion (SCAI) has been identified as a 53BP1- and HP1-associated factor that promotes repair of heterochromatic DSBs by facilitating ATM-dependent signaling (Hansen et al. 2016). Together, this intricate network of molecular players is critical for preventing unscheduled repair, thus suppressing mutagenic events in heterochromatin domains.

Heterochromatin domains and compartmentalization of DNA repair

Not all heterochromatin domains have the same impact on repair pathway choice, resulting in a compartmentalization of DNA repair within the eukaryotic nucleus. This has been extensively studied in response to DSBs (Fig. 2b), by tethering DSBs to defined heterochromatin compartments (Lemaître et al. 2014) or by targeted introduction of DSBs into repetitive sequences (Torres-Rosell et al. 2007; van Sluis and McStay 2015; Harding et al. 2015; Tsouroula et al. 2016; Doksani and de Lange 2016). Thus, important differences have emerged regarding how DSBs are processed in distinct silenced chromatin compartments. In mouse cells, both centromeric and pericentromeric DSBs are repaired through HR and NHEJ, but HR is restricted to S/G2 for DSBs arising in pericentromeric heterochromatin while centromeric DSBs recruit the HR factor RAD51 throughout interphase (Tsouroula et al. 2016). Future work will address the molecular bases of these differences by assessing the importance of centromere-specific histone variant and histone modifications in allowing HR of centromeric DSBs in G1 cells. Furthermore, NHEJ repair occurs inside centromeric and pericentromeric chromatin domains in mouse cells as opposed to late steps of HR, which are confined to the periphery of these domains after a relocation of the breaks (Tsouroula et al. 2016). In contrast to what observed at centromeres and pericentromeres, NHEJ does not contribute to repair of telomeric DSBs, which are processed by HR and A-EJ in mouse embryonic fibroblasts (Doksani and de Lange 2016). Striking differences are also found among heterochromatin domains interacting with nuclear structures, with LADs being repaired by error-prone NHEJ and A-EJ (Lemaître et al. 2014), whereas nucleolar DSBs are repaired within NADs by NHEJ and HR (Torres-Rosell et al. 2007; van Sluis and McStay 2015; Harding et al. 2015). The DSB repair pathways that operate in other facultative heterochromatin domains like the inactive X chromosome still remain to be characterized. Future studies will also be needed to fully understand the molecular determinants and the biological relevance of such

compartmentalization of DSB repair in the eukaryotic cell nucleus for genome and epigenome stability.

Heterochromatin reorganization in response to DNA damage

The DNA damage response is accompanied by a marked reorganization of heterochromatin (Fig. 3). In particular, decondensation of damaged heterochromatin has been observed in response to radiation- and nuclease-induced breaks, as reported for pericentromeric heterochromatin in flies (Chiolo et al. 2011) and in mouse embryonic fibroblasts (Jakob et al. 2011; Tsouroula et al. 2016), and for the inactive X chromosome in female human fibroblasts (Müller et al. 2013). This is thus a conserved response between eukaryotic species affecting both constitutive and facultative heterochromatin compartments. Future studies will address whether this is also a general response to various types of DNA lesions besides DSBs. Remarkably, the decompaction of damaged heterochromatin is not accompanied by a detectable loss of heterochromatin-specific histone marks such as H3K9me3 and H4K20me3 at the pericentromere, suggesting that heterochromatin identity may be preserved during this process (Goodarzi et al. 2011; Tsouroula et al. 2016; Natale et al. 2017). Nevertheless, more in-depth studies are needed to fully characterize the local changes in histone marks upon DNA damage in constitutive heterochromatin domains. Whether facultative heterochromatin marks are maintained also remains to be determined. Notably, however, the response to DNA damage in heterochromatin is not always associated with chromatin decondensation as recently reported for uncapped telomeres (Timashev et al. 2017; Vancevska et al. 2017). Indeed, super-resolution imaging revealed that the DNA damage response elicited by removal of shelter in components occurs without substantial telomere decompaction, but is accompanied by telomere clustering. Understanding the molecular mechanisms that trigger heterochromatin decompaction in response to DNA damage may clarify the differences observed between distinct heterochromatin domains.

Among the mechanisms that may drive damaged heterochromatin decompaction, ATM-dependent phosphorylation of the heterochromatin building factor KRAB-domain associated protein 1 (KAP1) was shown to trigger euchromatin relaxation (Ziv et al. 2006) and to facilitate the repair of heterochromatic DSBs at mammalian pericentromeres (Goodarzi et al. 2008). KAP1 phosphorylation indeed results in dissociation of the chromatin remodeler Chromodomain Helicase DNA Binding Protein 3 (CHD3) (Goodarzi et al. 2011), allowing the opposing imitation switch (ISWI) remodeler to promote chromatin relaxation (Klement et al. 2014). In addition to KAP1 phosphorylation, desumoylation of KAP1 by the SUMO1/Sentrin Specific Peptidase 7 (SENP7) also regulates this pathway (Garvin et al. 2013).

Besides chromatin decompaction, another striking feature of the response to DNA damage in heterochromatin domains

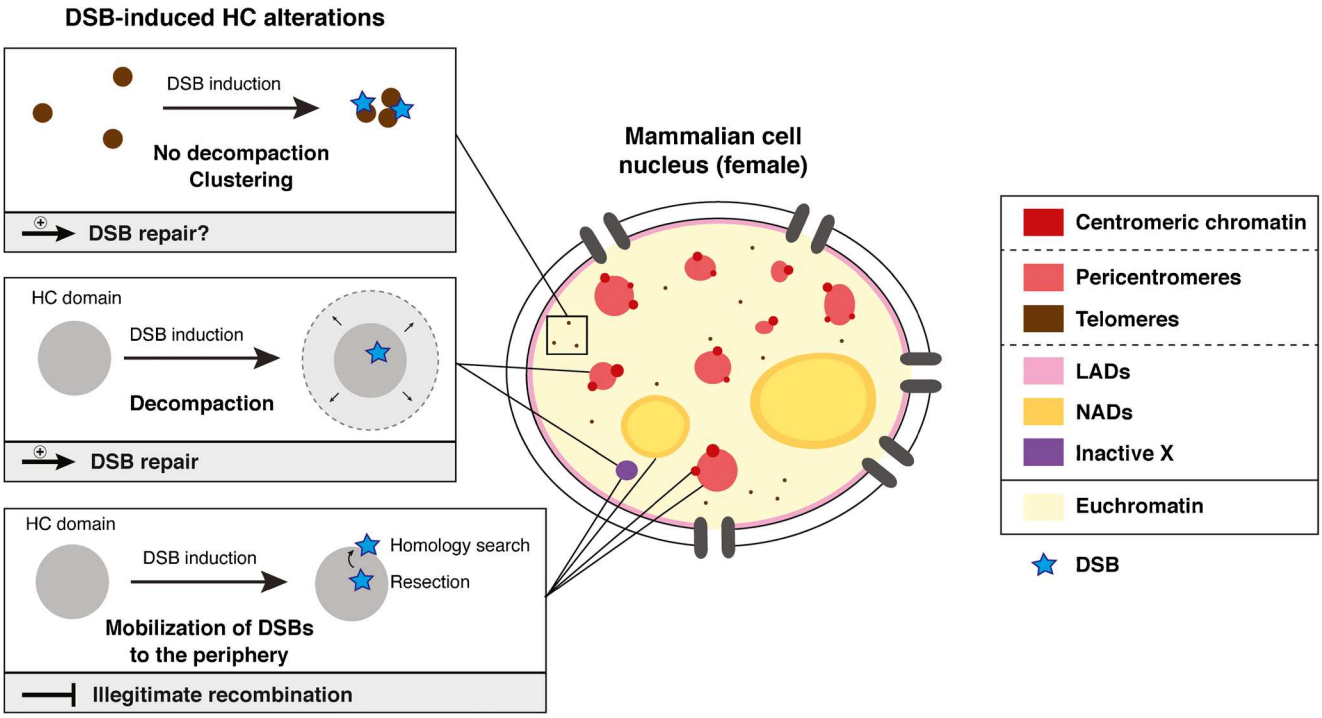

Fig. 3 Heterochromatin reorganization in response to DNA damage. Main alterations of heterochromatin domains in response to DNA double-strand breaks (DSBs, blue stars) and functional relevance. HC heterochromatin, LAD lamina-associated domain, NAD nucleolus-associated domain

is the relocation of DNA lesions (Amaral et al. 2017). Indeed, the decompaction of damaged heterochromatin at pericentromeres (Chiolo et al. 2011; Janssen et al. 2016) and the inactive X (Müller et al. 2013) is accompanied with a relocation of DSBs to the periphery of heterochromatin domains and to the nuclear periphery in *Drosophila*. Notably, a similar relocation of DSBs has been observed at centromeric chromatin (Tsouroula et al. 2016) and nucleoli (Torres-Rosell et al. 2007; van Sluis and McStay 2015; Harding et al. 2015), DSBs being repaired by HR at the periphery of the domains. The mechanisms underlying the relocation of pericentromeric DSBs have been extensively investigated. It has been shown that DSB relocation relies at least in part on the activation of DNA damage checkpoint kinases in *Drosophila* and requires functional DNA end resection both in *Drosophila* and mouse cells (Chiolo et al. 2011; Tsouroula et al. 2016). The molecular details of how resection drives DSB mobility are still elusive. In this respect, it would be important to examine the possible contribution of chromatin remodeling factors, which promote DSB mobility in yeast (Dion and Gasser 2013). Moreover, in light of recent studies involving nuclear actin and myosin in the DNA damage response (Belin et al. 2015; Lottersberger et al. 2015; Kulashreshtha et al. 2016; Aymard et al. 2017), it will be interesting to investigate the role of cytoskeletal and motor proteins in this process. Relocation of DSBs also involves demethylation of the heterochromatin mark H3K56me3 by the Lysine Demethylase 4A (KDM4A) in *Drosophila* cells (Colmenares et al. 2017). Despite the strong similarities between model organisms regarding the mobility of heterochromatic DSBs, there are also mechanistic discrepancies, with pericentromeric DSBs being ultimately relocated to the nuclear periphery in *Drosophila* cells (Ryu et al. 2015, 2016), which so far has not been observed in mouse cells (Tsouroula et al. 2016). In addition, the exclusion of the RAD51 recombinase from heterochromatin domains is dependent on HP1 and Structural Maintenance of Chromosomes (SMC) 5/6 in *Drosophila* (Chiolo et al. 2011) and not in mouse cells (Tsouroula et al. 2016). Functionally, the dynamic relocation of DSBs resulting in their extrusion from heterochromatin domains is thought to be critical for the prevention of illegitimate recombination between heterochromatic repeats through a spatial separation between DNA end resection and homology search (Fig. 3).

Even though heterochromatin is markedly reorganized in response to DNA damage to control and facilitate repair, somehow, surprisingly, chromatin silencing components including HP1 and H3K9me2/3 appear to accumulate at euchromatic damage sites. In particular, the heterochromatin component HP1 is required for DNA repair and is mobilized in response to DNA damage, being recruited to both UV- and laser-induced DNA lesions in a H3K9me3-independent manner in mammalian cells (Luijsterburg et al. 2009; Dinant and Luijsterburg 2009; Baldeyron et al. 2011). HP1 is loaded at DSBs together with the Suv39H1 methyltransferase, which deposits H3K9me3 resulting in local spreading of silencing marks spanning several kilobases around DSBs (Ayrapetov et al. 2014). Interestingly, deposition of silencing epigenetic marks is also favored at sites of replication stress, although the underlying mechanisms are not fully elucidated yet (Nikolov and Taddei 2015). The deposition of silencing marks at euchromatic DSBs was proposed to promote DNA damage signaling (Ayrapetov et al. 2014) and may also contribute to transcriptional silencing in response to DNA damage (Capozzo et al. 2017).

Conclusions and perspectives

DNA lesions arise in all chromatin compartments and among them compact heterochromatin domains pose major constraints to DNA damage repair. In recent years, exciting progress has been made in understanding how heterochromatin regulates DNA damage formation, signaling, and repair, with the characterization of repair pathways operating in distinct heterochromatin domains. Recent studies have also identified important heterochromatin alterations that accompany the DNA damage response. However, mechanistic insights into the reorganization of damaged heterochromatin are still missing, and their functional relevance is not yet completely understood. Most importantly, whether and how the original heterochromatin state is restored after DNA damage repair is still an open question. Future studies will address this important issue and dissect the mechanisms for heterochromatin maintenance following genotoxic stress. This may also shed new light on heterochromatin instability associated with tumorigenesis and on the heterochromatin alterations that arise during cellular aging (Criscione et al. 2016).

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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CHAPTER 5

Aims of the study

5.1. Open questions and objectives

We have seen that DNA damage elicits changes in chromatin organization, thus compromising epigenome integrity. Indeed, the DNA damage response is accompanied by remarkable epigenomic alterations, including substantial changes in chromatin folding, histone dynamics and post-translational modifications. This raises the issue of **epigenome maintenance following DNA damage and repair**, as changes in the epigenetic information contained in damaged chromatin could impact cell function and viability. Previous studies in human cells have shown that cellular mechanisms indeed exist to ensure the restoration of chromatin following DNA damage, including the deposition of newly synthesized histones in damaged chromatin.

Among chromatin regions, highly folded heterochromatin domains are mostly silent and characterized by specific sets of histone marks. Given the particularities of heterochromatin, one could hypothesize that specialized mechanisms exist to allow the restoration of the epigenome structure and function following DNA damage in heterochromatin domains.

In this context, the main objective of my thesis has been to assess **pericentric heterochromatin maintenance following UVC damage in mammalian cells** (Figure 17). I addressed this question by focusing on different aspects:

- Assessing **UV damage repair and repair-coupled histone dynamics** in UVC damaged heterochromatin, in an attempt to test the validity of the current Access-Repair-Restore model in heterochromatin domains.
- Analyzing the **consequences of the repair response on heterochromatin organization and function**, with a special interest in the **higher-order folding of chromatin** and the **maintenance of histone modifications**.

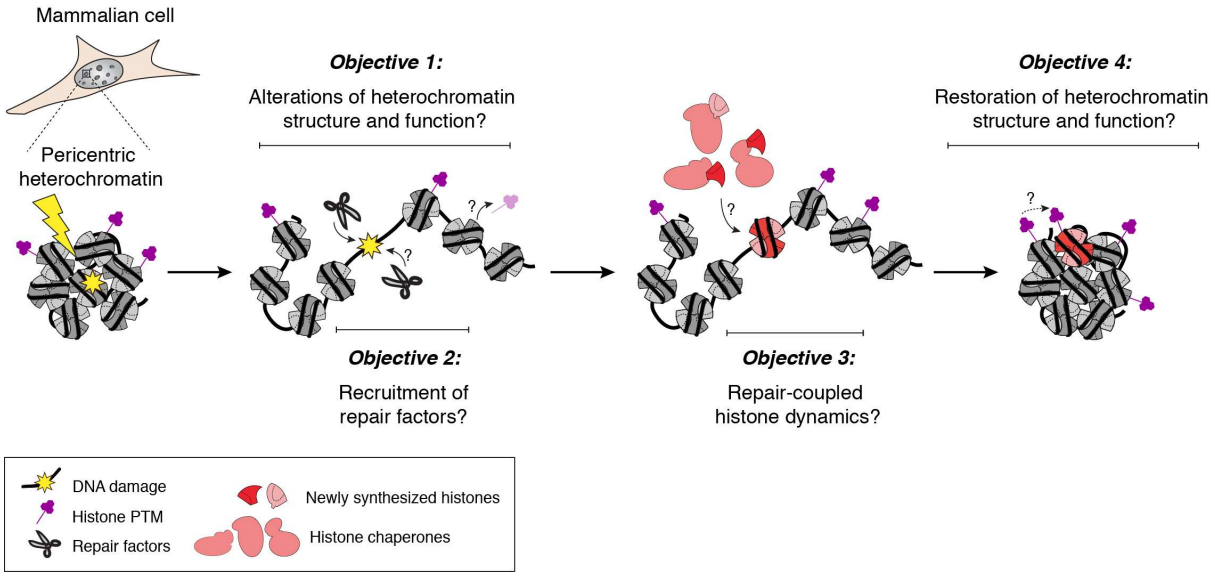

Figure 17. Model for heterochromatin maintenance upon DNA damage: open questions and thesis objectives. Pericentric heterochromatin domains are highly compact and display specific histone marks. Open questions relate to how damage is repaired in heterochromatin domains and with what consequences on heterochromatin structure and function.

5.2. Methodology

In order to study UV damage repair and its consequences on pericentric heterochromatin structure and function, we needed a cellular model where pericentric heterochromatin could be easily distinguished and where DNA repair events and histone deposition into chromatin could be tracked. We thus selected NIH/3T3 mouse embryonic fibroblasts, characterized by a clustering of pericentric heterochromatin domains into chromocenters, which are easily detectable with nuclear staining dyes (Probst and Almouzni, 2011) (**Figure 18**). The low endogenous levels of the UV damage sensor DNA Damage-Binding Protein 2 (DDB2) in mouse fibroblasts were overcomed by the ectopic expression of GFP-tagged human DDB2 (GFP-hDDB2). This, combined with the expression of SNAP-tagged H3.3 histones, resulted in a unique cellular model allowing simultaneous tracking of UVC damage repair and associated histone dynamics in pericentromeric heterochromatin domains (**Figure 18**). Besides, human MCF7 cells were used to confirm our main findings. For more details about our methods for tracking histone proteins following local UVC damage, please refer to **Annex 2**.

To assess UV damage repair in heterochromatin domains, I generated UVC damage in the above-described cellular model. Irradiation of cells with a 254 nm lamp induced pyrimidine dimers distributed over the entire cell nuclei (global damage), while the irradiation through

polycarbonate micropore filters generated DNA damage patches in random nuclear domains (local damage) (Katsumi et al., 2001; Moné et al., 2001) (**Figure 19**). To target the damage specifically to pericentric heterochromatin domains, I used a 266 nm UVC laser coupled to a confocal microscope. This system, which is available at the Curie Institute's imaging platform in Paris, allowed me to damage pericentric heterochromatin domains in live cells and to study the consequences of damage in real time (Dinant et al., 2007) (**Figure 19**).

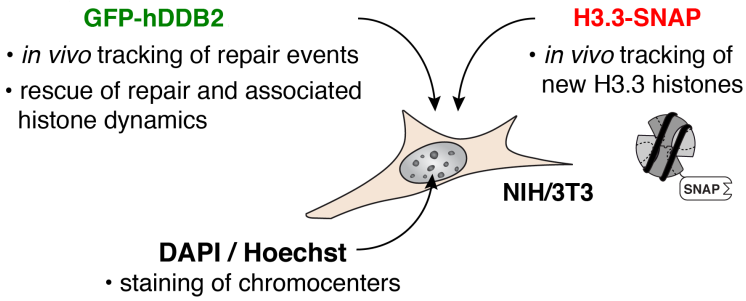

Figure 18 - Cellular model for studying pericentric heterochromatin maintenance in response to UV damage. NIH/3T3 mouse embryonic fibroblasts were engineered to stably express GFP-hDDB2 and H3.3-SNAP, allowing the simultaneous tracking of UVC damage repair and associated histone dynamics in pericentromeric heterochromatin domains.

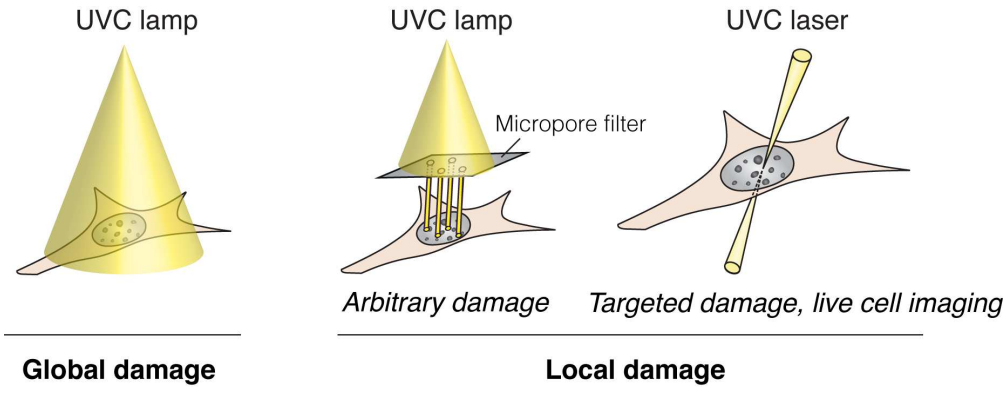

Figure 19 - Technical approaches for generating UVC damage in mammalian cells. Irradiation of cultured mammalian cells with a UVC lamp generates global damage or, when combined with micropore filters, random local damage. Alternatively, pericentric heterochromatin domains can be specifically targeted with a UVC laser coupled to a confocal microscope and followed in real time.

SECOND PART

RESULTS & DISCUSSION

*The main results obtained in this study are presented in the form of a scientific paper currently in preparation (see **Chapter 9**). Additional data is presented in **Chapter 10,** page 146.*

Manuscript in preparation:

Histone modifiers and chaperones cooperate to maintain heterochromatin integrity following DNA damage.

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CHAPTER 6

Consequences of UV damage on pericentric heterochromatin organization and function

6.1. Heterochromatin decompaction following UVC laser irradiation

As a first step towards dissecting the mechanisms for heterochromatin maintenance in response to DNA damage, I examined the consequences of UV damage on pericentric heterochromatin organization in live cells. For this, NIH/3T3 GFP-hDDB2 mouse fibroblasts were stained with Hoechst, which allowed the visualization of DNA in live cell nuclei, including the clustering of pericentric heterochromatin domains into chromocenters. Individual chromocenters were then specifically targeted with a UVC laser and followed by live imaging, which revealed a pronounced and **rapid decompaction of pericentric heterochromatin within minutes after damage** (Figure 1). By measuring the area of damaged chromocenters at different times post-UVC, I quantified the observed decompaction, which reached a maximum of 6-fold 3o minutes after damage. Importantly, the decompaction of damaged heterochromatin was followed by a **recompaction taking several hours.** This recompaction phase started around 2 hours after damage and lasted about 10 hours, during which damaged chromocenters progressively returned to their original size (Figure 1).

6.2. Maintenance of heterochromatin-specific features upon UV damage

To determine if the decompaction of pericentric heterochromatin was accompanied by a loss of silencing histone marks, which could alter heterochromatin transcriptional status, I fixed UV-irradiated cells at the time of maximum heterochromatin decompaction and stained for H3K9me3 and H4K20me3. These analyses revealed that both marks were not lost, but rather

enriched, in damaged heterochromatin, indicating that **UV-induced heterochromatin decompaction was not associated with a loss of silencing histone marks** (Figures 1 and S2).

I then sought to determine if decompaction of damaged heterochromatin triggered the aberrant transcription of pericentromeric repeats. When staining nascent transcripts with Ethynyl-Uridine (EU), I observed a UV-induced reduction of transcription in pericentric heterochromatin domains. Similar results were obtained when performing qRT-PCR of pericentromeric major satellite transcripts following UV damage, indicating that **UV-mediated heterochromatin decompaction is not accompanied by aberrant transcription of heterochromatic repeats** (Figure 1).

6.3. Discussion: is heterochromatin identity preserved upon damage-induced decompaction?

In this first part of our study, we have shown that the UV damage response is accompanied by a marked decondensation of pericentric heterochromatin in mouse fibroblasts (Figure 1). Our results are in line with a number of studies demonstrating the dynamic nature of heterochromatin in response to environmental stress (reviewed in (Wang et al., 2016). Indeed, decompaction of pericentromeric heterochromatin has been reported in response to radiation- and nuclease-induced breaks both in flies (Chiolo et al., 2011) and in mouse embryonic fibroblasts (Jakob et al., 2011; Tsouroula et al., 2016), as well as following heat stress in plants (Pecinka et al., 2010). Besides being a response to environmental stress, reorganization of constitutive heterochromatin also takes place during somatic cell reprogramming to a pluripotent state (Fussner et al., 2011).

While heat shock activates transcription of pericentric heterochromatin repeats, as shown in human cells (Jolly et al., 2004; Rizzi et al., 2004), we provide evidence that pericentromeric heterochromatin remains silenced upon UV-mediated decompaction (Figure 1). This might be explained by the maintenance of silencing histone marks H3K9me3 and H4K20me3 at UV-damaged pericentromeres (Figures 1 and S2). These observations, which are in line with previous studies (Goodarzi et al., 2011; Natale et al., 2017; Tsouroula et al., 2016), suggest that heterochromatin identity may be preserved during damage-mediated decompaction.

In addition to heterochromatin histone marks, another potential player in preserving heterochromatin identity following damage is the H3K9me3-binding factor Heterochromatin Protein 1 alpha (HP1α). We and others have observed that HP1α is recruited to various types of DNA damage, including UVC lesions (Figure 18, panel A - Chapter 10, page 146), in a

H3K9me3-independent manner (Luijsterburg et al., 2009). Owing to its liquid-liquid demixing properties, which are critical for heterochromatin formation, we could hypothesize that HP1α may have a role in the maintenance of heterochromatin organization upon damage, for example by boosting heterochromatin recompaction at later time points post-UVC (**Figure 1**). A more detailed study of HP1α recruitment kinetics and HP1 loss-of-function assays will help elucidate the role of HP1α in the maintenance of heterochromatin integrity following UV damage.

CHAPTER 7

UV damage repair in mouse pericentric heterochromatin

7.1. The UV damage sensor DDB2 regulates heterochromatin compaction

Having observed a transient but marked decondensation of pericentric heterochromatin upon UVC irradiation, I decided to investigate the molecular mechanisms triggering such decompaction. A promising candidate was the early repair factor DDB2, a UV damage sensor acting in the GG-NER pathway that had previously been related to histone redistribution and chromatin relaxation (Adam et al., 2016; Luijsterburg et al., 2012). Supporting this hypothesis, decompaction of UV-damaged heterochromatin was only observed in mouse cells that ectopically express DDB2 and not in the parental cell line (**Figure 2**). Furthermore, Cas9-mediated tethering of GFP-hDDB2 to mouse pericentric heterochromatin in the absence of DNA damage resulted in enlarged and less spherical chromocenters, indicative of a **DDB2-mediated decompaction of pericentric heterochromatin domains** (**Figures 2** and **S3**). Subsequent release of DDB2 from pericentric heterochromatin resulted in chromocenters retrieving their original shape and size, revealing that **DDB2 release allows pericentric heterochromatin recompaction** (**Figures 2** and **S3**). These results imply that the early repair factor DDB2, which is recruited to UVC-damaged heterochromatin, regulates heterochromatin compaction.

7.2. Recruitment of repair factors to heterochromatin domains

I next wondered if the DDB2-mediated decompaction of damaged heterochromatin could provide access to downstream repair factors. I thus examined the recruitment of repair proteins acting downstream of DDB2 in the NER pathway, and observed the effective recruitment of the intermediate factor XPB (Xeroderma Pigmentosum type B), and of the late factor PCNA

(Proliferating Cell Nuclear Antigen) to damaged heterochromatin (**Figure 3**). Importantly, PCNA accumulated within heterochromatin domains during DNA damage repair, while it remained confined to the periphery of these domains during replication (**Figures 3** and **S2**). These observations revealed that, unlike replicative synthesis, repair synthesis takes place inside heterochromatin domains. Altogether, our results indicate that **pericentric heterochromatin is fully permissive for nucleotide excision repair, from early to late repair steps**.

7.3. Discussion: DDB2-mediated heterochromatin decompaction – how and why?

Chromatin movement during DNA repair can be seen as arising from the lesion itself or, alternatively, as being indirectly influenced by repair-associated proteins. Our results go in line with this second view, as the repair factor DDB2 proved to be necessary for heterochromatin decompaction following UVC damage (**Figure 2**). However, the exact mechanism by which DDB2 promotes chromatin decondensation remains elusive. Although it has been shown that the DDB2-binding partners DDB1 and Cul4A/B are not involved in chromatin decompaction (Luijsterburg et al., 2012), it is not clear if DDB2 promotes decompaction by itself or by interacting with other factors such as chromatin remodelers. Given that DDB2 does not have known chromatin remodeling activity or motifs, it is probable that it induces chromatin decompaction indirectly, by promoting the recruitment or, alternatively, the release, of chromatin remodelers from damage sites. In support of this hypothesis, DDB2 kinetics of recruitment to and release from damaged heterochromatin were faster than changes in heterochromatin compaction, indicative of an indirect effect of DDB2 (**Figure 18**, panel B – Chapter 10, page 146). Nevertheless, further studies are needed to fully understand the mechanisms for DDB2-mediated chromatin decompaction. To measure any direct effect of DDB2 on chromatin folding, biophysical approaches could be envisioned using recombinant DDB2 and in vitro reconstituted chromatin templates (Fierz et al., 2012). Proteomic analyses of DDB2-associated factors or of factors released from chromatin after UV damage may help identify the molecular players mediating DDB2 effect on chromatin decompaction.

Decompaction of damaged heterochromatin has been observed in response to various types of DNA lesions, including DSBs and UV photoproducts. In the case of DSBs, such decompaction facilitates the movement of damaged DNA fibers to the periphery of heterochromatin domains, where recombinational repair is completed (Chiolo et al., 2011; Jakob et al., 2011; Janssen et al., 2016; Tsouroula et al., 2016). This spatial segregation of repair events is

seen as a way to avoid ectopic recombination between heterochromatic repeats during homologous repair (HR). NER, in contrast, does not involve recombination. The NER machinery can access pericentric heterochromatin and repair is completed within the domain (**Figure 3**). Then, why is there decompaction of UV-damaged heterochromatin? One could hypothesize that DDB2-mediated heterochromatin decompaction facilitates the access of later repair factors. Even though heterochromatin is readily accessible even to large macromolecules/proteins (Bancaud et al., 2009), efficient recognition of UV lesions might be hindered in compact chromatin domains and would be facilitated by DDB2-mediated chromatin decompaction. In this case, there would not be a spatial regulation of NER - all NER factors being able to access heterochromatin- but a temporal regulation due to the necessary decompaction step. This would explain the slower kinetics of UV damage repair observed in mammalian heterochromatin domains, with DDB2 promoting repair of heterochromatic UV lesions (Han et al., 2016)). To test if DDB2-mediated decompaction made heterochromatin more accessible, I assessed DNA accessibility in UVC-damaged cells by ATAC-see (**Figure 18**, panel C - Chapter 10, page 146), an image-based method, which reveals chromatin regions accessible to Tn5 transposase (Chen et al., 2016). However, Tn5 accessibility appeared to be hindered not only by chromatin compaction (lower ATAC-see signal in heterochromatin) but also in decompacted heterochromatin after damage, possibly due to the abundant repair factor binding to damaged sites. Thus, the hypothesis that DDB2-mediated heterochromatin decompaction enhances accessibility still awaits experimental validation.

CHAPTER 8

Histone dynamics in UVC-damaged heterochromatin

8.1. Deposition of newly synthesized H3 histones in damaged heterochromatin

Having established that pericentric heterochromatin domains are permissive for NER factor recruitment, I decided to investigate whether repair-coupled deposition of newly synthesized histones was taking place in heterochromatin. For this, I focused on the H3 histone variants H3.1 and H3.3, which are deposited de novo in UV-damaged chromatin in human cells **(Adam et al., 2013; Polo et al., 2006)**. By examining the recruitment of H3 variant-specific chaperones, I observed an **accumulation of the H3.1 chaperone CAF-1** (Chromatin Assembly Factor 1)**,** together with the two main **H3.3-specific histone chaperones HIRA** (Histone Regulator A) **and DAXX** (Death Domain Associated Protein) **in UVC-damaged heterochromatin** (**Figures 4** and **S2**). By tracking newly synthesized histones, I also revealed new H3.3 deposition in damaged heterochromatin (**Figure 4**) and through loss-of-function experiments, I identified **HIRA as the main driver of new H3.3 histone deposition in UVC-damaged heterochromatin domains** (**Figure 4**). The functional relevance of DAXX recruitment to UV-damaged heterochromatin remains to be determined, although we cannot formally exclude that this chaperone plays a minor role in new H3.3 deposition in damaged heterochromatin.

8.2. Maintenance of histone modifications on damaged heterochromatin

Newly synthesized H3 histones do not carry the same post-translational modifications as nucleosomal H3 and are largely devoid of trimethylation marks **(Alabert and Groth, 2012;**

Loyola et al., 2006). Thus, I wondered whether and how the newly deposited H3 histones would acquire heterochromatin-specific modifications, including H3K9me3, which seemed enriched at UV-damaged heterochromatin (**Figures 1** and **S2**).

To tackle this issue, I examined the recruitment of several known H3K9 methyltransferases to UVC-damaged heterochromatin, and detected a **strong accumulation of SETDB1** (SET Domain Bifurcated 1) (**Figure 5**). Importantly, SETDB1 recruitment was dependent both on new H3 histone deposition and on the presence of SUV39 enzymes, which maintain parental H3K9me3 (**Figure 5**). Based on these findings, we hypothesize that SETDB1 may trimethylate newly deposited H3 histones in UV-damaged heterochromatin by copying the K9me3 mark from neighboring SUV39-modified parental histones. Altogether, our study reveals that histone modifiers and chaperones cooperate to maintain pericentric heterochromatin integrity following UV damage.

8.3. Discussion: heterochromatin maintenance following UV damage - molecular bases and functional relevance

The specific recruitment of DAXX-ATRX to UVC-damaged heterochromatin suggested the involvement of this complex in heterochromatin maintenance following UV damage. Although we ruled out a major role for DAXX-ATRX in new H3.3 deposition in damaged heterochromatin (**Figure 4**), there are a number of potential alternative functions that could be investigated. Even if DAXX-ATRX are not involved in the deposition of new H3.3 histones, they could still facilitate parental H3.3 recovery during the repair process. They could also regulate the dynamics of other histone variants. Indeed, DAXX is a promiscuous chaperone responsible for ectopic localization of overexpressed CENP-A in cancer cells (Lacoste et al., 2014) while ATRX regulates macroH2A1 dynamics (Kim et al., 2019; Ratnakumar et al., 2012). Another possibility would be that DAXX-ATRX regulate heterochromatin recompaction at late time points post-UV, according to recent data revealing that ATRX-DAXX-mediated deposition of H3.3 is key for chromocenter clustering during myogenic differentiation (Park et al., 2018). Finally, it has been proposed that this complex could regulate repair synthesis as observed during HR (Juhasz et al., 2018), but our preliminary data do not support such a function during NER (data not shown).

Similar to DAXX-ATRX, the histone methyltransferase SETDB1 is recruited specifically to UV-damaged heterochromatin. Although the mechanisms of recruitment of SETDB1 are not

clear, they may involve known SETDB1-binding partners such as the histone methyltransferase SUV39 (Fritsch et al., 2010) and the histone chaperone CAF-1 (Loyola et al., 2009) (**Figure 5**). Interestingly, SETDB1 has a triple Tudor domain that specifically binds to doubly modified histone H3 containing K14 acetylation (H3K14ac) and K9 methylation (H3K9me1/2/3) (Jurkowska et al., 2017). Knowing that newly synthesized H3 histones are enriched in K14ac (Alabert et al., 2015), and are not optimal substrates for trimethylation by SUV39 (Rea et al., 2000), we hypothesize that SETDB1 could bind, via its Tudor domains, the new H3 histones deposited in damaged heterochromatin, and then trimethylate these histones. To test this hypothesis, we could first assess the importance of SETDB1 Tudor domains for SETDB1 recruitment to UV-damaged heterochromatin. Next, we could examine by chromatin immunoprecipitation of H3K9me3 on major satellites if SETDB1 contributes to H3K9 trimethylation in pericentric heterochromatin following UV damage. It is also possible to exploit the SNAP-tag technology to pull down specifically new H3 and assess the effect of SETDB1 knock-down on trimethylation of these new histones post-UV. These approaches would also reveal if trimethylation happens exclusively on new H3 histones or also on parental histones within nucleosomes that would be exposed upon chromatin decompaction.

Finally, one may wonder what is the biological relevance of new histone deposition and methylation in UVC-damaged chromocenters. It is known that pericentric heterochromatin integrity is crucial for chromosome segregation. It would thus be important to test if defective new histone deposition or methylation upon UV damage in heterochromatin affects chromosome segregation, mitotic progression or cell survival.

CHAPTER 9

Scientific article (manuscript in preparation)

The main results obtained in this study are presented in the form of a scientific paper currently in preparation. Additional data is presented in ***Chapter 10*** *(page 146).*

Manuscript in preparation:

Histone modifiers and chaperones cooperate to maintain heterochromatin integrity following DNA damage.

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Histone chaperones and modifiers cooperate to maintain heterochromatin integrity following DNA damage

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SUMMARY

Epigenome integrity, which is central to cell function and identity, is challenged during the response to DNA damage. How epigenetic features, including histone post-translational modifications and higher-order chromatin folding, are maintained during the repair response is unknown. Here, we address this issue by focusing on heterochromatin domains. We establish a unique cellular model in mouse fibroblasts for targeting UV damage to pericentric heterochromatin and for tracking the heterochromatin response to UV damage in real time. Thus, we reveal that the maintenance of heterochromatin-specific histone modifications is uncoupled from heterochromatin folding and we uncover a critical role for the UV damage sensor DDB2 in orchestrating heterochromatin compaction changes during repair. We also unveil a tight cooperation between histone chaperones and modifiers in the maintenance of heterochromatic histone marks upon UV damage. Altogether, this study sheds light on the molecular mechanisms safeguarding higher-order chromatin integrity following DNA damage.

INTRODUCTION

Eukaryotic cell identity and function are governed by the epigenetic information stored in the form of chromatin inside the cell nucleus, where DNA wraps around histone proteins (Allis and Jenuwein, 2016). This information encompasses multiple layers of regulation, from histone modifications (Bannister and Kouzarides, 2011) and histone variants (Buschbeck and Hake, 2017), up to higher-order folding of the chromatin fiber into nuclear domains (Yu and Ren, 2017) which, in concert, control gene expression. The critical importance of an accurate maintenance of the epigenome is emphasized by the profound and sometimes dramatic influence of epigenetic alterations on cell fate transitions during development, reprogramming and disease (Brookes and Shi, 2014; Xu and Xie, 2018).

Nevertheless, epigenome maintenance is challenged during all DNA transactions, including the response to endogenous and exogenous DNA damage (Hoeijmakers, 2009; Tubbs and Nussenzweig, 2017), which impairs both genome and epigenome integrity (Dabin et al., 2016; Hauer and Gasser, 2017). Chromatin rearrangements arising during the repair response include histone and chromatin mobility (Dabin et al., 2016; Dion and Gasser, 2013), changes in histone post-translational modifications (Dantuma and van Attikum, 2016), and alterations in chromatin compaction (Khurana et al., 2014; Kruhlak et al., 2006; Luijsterburg et al., 2012; Sellou et al., 2016; Smeenk, 2013; Strickfaden et al., 2015). These rearrangements are accompanied by transient changes in chromatin transcriptional activity (Capozzo et al., 2017; Geijer and Marteijn, 2018; Marnef et al., 2017).

The destabilization of chromatin organization upon genotoxic stress is followed by a restoration of chromatin structure (Polo and Almouzni, 2015; Smerdon, 1991). However, our knowledge of this fundamental process is still largely incomplete. Whether the epigenome is faithfully restored following DNA damage and repair, and by which mechanisms, are still

open questions. The repair-coupled deposition of newly synthesized histones, reported in response to UV damage (Adam et al., 2013; Dinant et al., 2013; Piquet et al., 2018; Polo et al., 2006) and DNA double-strand breaks (DSBs) (Juhasz et al., 2018; Luijsterburg et al., 2016) likely contributes to re-establishing chromatin organization. However, little is known about the maintenance of histone modifications and the restoration of higher-order chromatin folding during the repair response. Whether histone modifications and chromatin folding are maintained in a concerted manner also remains elusive.

To study the maintenance of these epigenomic features upon DNA damage, we chose to focus on heterochromatin domains, which are highly folded and characterized by specific patterns of histone post-translational modifications (Allshire and Madhani, 2017; Janssen et al., 2018). For instance, pericentric heterochromatin domains (Saksouk et al., 2015) carry a specific chromatin signature including trimethylation on H3 lysine 9 (H3K9me3) and on H4 lysine 20 (H4K20me3) (Martens et al., 2005), which contribute to epigenetic silencing of major satellite repeats. H3K9me3 heterochromatin also plays a pivotal role in defining cell identity by silencing lineage-specific genes during development (Becker et al., 2016). Highly concentrated at pericentromeric and subtelomeric regions, heterochromatin is crucial for chromosome segregation and integrity (Janssen et al., 2018). However, due to its high compaction status and to the abundance of repeated sequences prone to ectopic recombination, heterochromatin represents a challenging environment for the DNA damage response (DDR). Heterochromatic regions indeed pose a barrier to DNA damage signaling (Lemaître et al., 2014) and repair, as described for nucleotide excision repair (Adar et al., 2016; Han et al., 2016; Zheng et al., 2014), DSB repair (Kallimasioti-Pazi et al., 2018; Lemaître and Soutoglou, 2014) and mismatch repair (Supek and Lehner, 2015) in mammalian cells. In line with this, higher mutation rates are found in heterochromatin in human cancer

genomes (Schuster-Böckler and Lehner, 2012; Zheng et al., 2014) and alterations of heterochromatin features are associated with aging and cancer (Janssen et al., 2018).

In the last few years, DNA damage repair in heterochromatin has been extensively studied, mostly in response to DNA breaks (Amaral et al., 2017). In drosophila and mouse cells, DSBs elicit a decompaction of pericentric heterochromatin and relocate to the periphery of heterochromatin domains for the completion of recombinational repair, wich is thought to prevent illegitimate recombination between pericentromeric repeats (Chiolo et al., 2011; Jakob et al., 2011; Janssen et al., 2016; Tsouroula et al., 2016). However, beyond the restoration of genome integrity, the mechanisms underlying the maintenance of heterochromatic features during the repair response remain uncharacterized (reviewed in (Fortuny and Polo, 2018)).

Here, we explore these mechanisms by inflicting UV damage in pericentric heterochromatin domains in mammalian cells. We reveal that the maintenance of heterochromatin-specific histone marks is uncoupled from heterochromatin folding and we uncover a critical role for the UV damage sensor DDB2 in orchestrating heterochromatin compaction changes during the repair response. Our findings also unveil a tight cooperation between histone chaperones and histone modifiers in the maintenance of heterochromatic histone marks following UV damage.

RESULTS

Maintenance of heterochromatin integrity in response to UV damage

In order to study heterochromatin maintenance in response to DNA damage, we first established an appropriate cellular model where heterochromatin domains could be easily distinguished and where DNA repair events and histone deposition into chromatin could be

tracked. For this purpose, we selected NIH/3T3 mouse embryonic fibroblasts, characterized by a clustering of pericentric heterochromatin domains into chromocenters (Probst and Almouzni, 2011) (Fig. 1a), and we focused on the cell response to UVC damage (Fig. S1a). Noteworthy, mouse fibroblasts express the UV damage sensor DNA Damage-Binding Protein 2 (DDB2) at very low levels, which impairs both UVC damage repair (Tang et al., 2000) and repair-coupled histone dynamics (Adam et al., 2013; 2016). To overcome these defects, NIH/3T3 stable cell lines were engineered to ectopically express GFP-tagged human DDB2 (GFP-hDDB2). These cells also stably express SNAP-tagged H3.3, which allows specific tracking of newly synthesized H3.3 histones (Adam et al., 2015) (Fig. S1a, see Fig. S1b-d for a complete characterization of the cell lines). The ectopic expression of DDB2 and H3.3 did not affect pericentric heterochromatin organization as judged by immunostaining for H3K9me3 and Heterochromatin protein 1 α (HP1α) (Fig. 1a). We verified that GFP-hDDB2 expression rescued UVC damage repair and associated histone dynamics in mouse cells, by analyzing the recruitment of the nucleotide excision repair (NER) factor Xeroderma Pigmentosum complementation group B (XPB) and the deposition of newly synthesized H3.3 histones at sites of UVC damage (Fig. S1e).

Using the mammalian cellular model described above, we assessed the importance of heterochromatin integrity for the cellular response to UV damage. We impaired heterochromatin integrity by knocking-down the histone methyltransferases SUV39H1 and 2 (Suppressor Of Variegation 3-9 Homolog1/2), which are the main drivers of H3K9me3 in pericentric heterochromatin (Peters et al., 2001) (Fig. 1a), and tested the ability of SUV39H1/2-depleted cells to survive UVC damage. We observed that SUV39H1/2 knockdown sensitized cells to UVC (Fig. 1a), thus underlining the functional importance of constitutive heterochromatin integrity for cell viability following UV damage.

To determine whether heterochromatin integrity was preserved following a genotoxic stress

challenge, we developed an innovative approach for targeting UVC damage to pericentric heterochromatin domains in live cells and for tracking the response to heterochromatin damage in real time. We employed the live cell DNA stain Hoechst to visualize chromocenters in mouse cells and then inflicted UVC damage specifically to chromocenters of interest by using a UVC laser coupled to a confocal microscope (Fig. 1b). Using this approach, we observed a pronounced and rapid decompaction of damaged heterochromatin within minutes after UVC laser damage, reaching a maximum (up to 6-fold) 30 min to 1 h after irradiation (Fig. 1b and Movie S1). Importantly, damaged heterochromatin decompaction was followed by a slower recompaction phase taking several hours, which restored heterochromatin compaction close to original state (Fig. 1b and Movie S2). Furthermore, immunostaining for H3K9me3 in cells fixed 1 h after UVC laser damage revealed that damaged heterochromatin decompaction was not associated with a reduction of this heterochromatin-specific histone mark, which instead appeared slightly increased on damaged chromocenters (Fig. 1c). Similar results were obtained when staining for H4K20me3 (Fig. S2). In line with these findings, damaged heterochromatin decompaction was not accompanied by a burst of aberrant transcription. The staining of nascent transcripts with Ethynyl-Uridine (EU) indeed revealed that transcription was even further reduced in UV-damaged heterochromatin (Fig. 1d). This UV-induced transcriptional arrest in heterochromatin was confirmed by RT-qPCR of pericentric major satellite transcripts (Fig. 1d). From these observations, we conclude that UVC damage challenges heterochromatin integrity and that maintenance mechanisms operate to restore heterochromatin compaction and to reinforce the heterochromatin-specific histone marks and heterochromatin silencing following UV damage.

The UV damage sensor DDB2 regulates heterochromatin compaction

To characterize the mechanisms underlying heterochromatin maintenance following UVC

damage, we first sought to identify the molecular trigger for damaged heterochromatin decompaction. For this, we examined the potential contribution of the UV damage sensor DDB2, whose binding to chromatin was shown to promote histone redistribution and chromatin relaxation in human cells (Adam et al., 2016; Luijsterburg et al., 2012). Noteworthy, we observed decompaction of UV-damaged heterochromatin domains only in the engineered cell line expressing hDDB2 and not in the parental mouse cell line (DDB2-deficient) (Fig. 2a), supporting the idea that DDB2 was required for heterochromatin decompaction following UVC damage.

To directly test whether DDB2 could drive heterochromatin decompaction, we tethered GFP-hDDB2 to mouse pericentric heterochromatin in the absence of DNA damage by co-expressing catalytically dead Cas9 (dCas9) fused to a GFP nanobody and a guide RNA targeting major satellite repeats (Anton and Bultmann, 2017; Tsouroula et al., 2016) (Fig. 2b and Fig. S3a-b). DDB2 tethering led to substantial changes in the shape and size of pericentric heterochromatin domains, which were enlarged and less spherical compared to control cells, indicative of a decompaction of pericentric heterochromatin domains. This effect was specific to DDB2 tethering as it was not observed upon targeting of another early NER factor, Xeroderma Pigmentosum complementation group C (XPC), to chromocenters (Fig. 2c and Fig. S3c). Noteworthy, when we induced the release of tethered DDB2 from major satellite repeats with the anti-Cas9 bacteriophage protein AcrIIA4, thus mimicking the release of DDB2 from damaged chromatin that occurs during repair progression, the typical size and shape of chromocenters were restored, showing that DDB2 release allows pericentric heterochromatin recompaction (Fig. 2d and Fig. S3d). These findings establish that the UV damage sensor DDB2 is both necessary and sufficient for driving changes in heterochromatin compaction following UVC damage.

UV damage repair within heterochromatin domains

DDB2-mediated decompaction of damaged heterochromatin could provide access to downstream repair factors. We thus examined the recruitment to UVC-damaged heterochromatin of repair proteins acting downstream of DDB2 in the NER pathway, namely the intermediate repair factor XPB, which contributes to opening the damaged DNA double-helix, and the late repair factor Proliferating Cell Nuclear Antigen (PCNA), involved in repair synthesis after damage excision (Fig. 3a). Similar to what observed for GFP-hDDB2, we detected the accumulation of endogenous XPB and PCNA in damaged heterochromatin upon cell exposure to local UVC irradiation (Fig. 3b). Importantly, we noticed that PCNA accumulated within heterochromatin domains during DNA damage repair, as observed both in our mouse cell line model and in human MCF7 cells (Fig 3c and Fig. S4), which contrasts with PCNA peripheral localization during heterochromatin replication (Quivy et al., 2004). This indicates that, unlike replicative synthesis, UV damage repair synthesis takes place inside heterochromatin domains. Altogether, these results establish that pericentromeric heterochromatin is fully permissive for NER factor recruitment up to late repair steps.

Repair-coupled deposition of new H3 histones in heterochromatin domains

UV damage repair elicits the deposition of newly synthesized histones in human cells (Adam et al., 2013; Dinant et al., 2013; Piquet et al., 2018; Polo et al., 2006), including the H3 histone variants H3.1 and H3.3. To investigate whether such repair-coupled histone deposition was taking place in damaged heterochromatin, we examined the recruitment of H3 variant-specific chaperones, starting with the H3.1 histone chaperone Chromatin Assembly Factor-1 (CAF-1), which is known to interact with PCNA during repair (Moggs et al., 2000) and to deposit new H3.1 histones at UVC damage sites (Polo et al., 2006). Similar to PCNA (Fig. 3b-c), we observed that CAF-1 accumulated in damaged heterochromatin upon local

UVC irradiation both in mouse NIH/3T3 GFP-hDDB2 cells and in human MCF7 cells (Fig. 4a and Fig. S4). Regarding H3.3 histone chaperones, both HIRA (Histone Regulator A) and DAXX (Death Domain Associated Protein) can drive H3.3 deposition (reviewed in (Sitbon et al., 2017)). HIRA deposits H3.3 within transcribed euchromatin in mammalian cells (Goldberg et al., 2010; Ray-Gallet et al., 2011) and in UVC-damaged chromatin in human cells (Adam et al., 2013), while DAXX promotes H3.3 enrichment at repeated sequences including subtelomeric and pericentric heterochromatin (Drané et al., 2010; Goldberg et al., 2010; Wong et al., 2010). We thus examined whether one or both of these chaperones were recruited to UVC-damaged heterochromatin. We observed that while HIRA accumulated in a comparable manner in damaged euchromatin and heterochromatin domains (Fig. 4b), DAXX was specifically recruited to damaged heterochromatin (Fig. 4c). Thus, although originally considered to operate in distinct chromatin domains (Elsaesser and Allis, 2010), HIRA and DAXX chaperones co-exist within damaged heterochromatin, which we confirmed in human MCF7 cells (Fig. S4). In light of these findings, we explored the possibility of a co-recruitment of these two chaperones to damaged heterochromatin. However, siRNA-mediated depletion of HIRA did not impair DAXX recruitment and reciprocally, showing that both H3.3 histone chaperones are independently recruited to UVC-damaged heterochromatin (Fig. S5a).

DAXX recruitment being heterochromatin-specific, we further investigated the underlying mechanisms. We noticed a co-enrichment on damaged heterochromatin of the DAXX-binding partner and heterochromatin-associated protein ATRX (Alpha Thalassemia/Mental Retardation Syndrome X-Linked) (Fig. S5b). ATRX knock-down revealed that ATRX was driving DAXX recruitment to damaged heterochromatin (Fig. S5c). Noteworthy, we observed DAXX accumulation in damaged heterochromatin both in and outside S-phase (Fig. S5d), ruling out the possibility that DAXX recruitment could be coupled to heterochromatin

replication, owing to enhanced chromatin accessibility during this process. Analogous to what observed for HIRA recruitment to UV sites in human cells (Adam et al., 2013), DAXX accumulation in UVC-damaged heterochromatin domains was dependent on the UV damage sensor DDB2 (Fig. S5e).

In line with the recruitment of H3.3-specific histone chaperones, we observed an accumulation of newly synthesized H3.3-SNAP histones within damaged heterochromatin domains, comparable to neighboring euchromatin regions (Fig. 4d). Loss-of-function experiments revealed that only HIRA depletion markedly inhibited new H3.3 deposition in UV-damaged chromocenters (Fig. 4e). Although we cannot exclude a minor contribution of DAXX, HIRA thus appears to be the main driver for new H3.3 deposition in damaged heterochromatin. Collectively, these findings demonstrate that UVC damage drives the recruitment of H3 histone chaperones and new H3 deposition in heterochromatin domains.

Maintenance of histone modifications in damaged heterochromatin

Newly synthesized H3 histones do not carry the same post-translational modifications as nucleosomal H3 and are largely devoid of trimethylation marks (Alabert and Groth, 2012; Loyola et al., 2006). Thus, we wondered whether and how the newly synthesized H3 histones deposited in damaged heterochromatin would acquire heterochromatin-specific modifications, including H3K9me3, which we observed was maintained after UVC damage (Fig. 1c). Interestingly, when we examined the recruitment of H3K9 methyltrasnferases, we found that SETDB1 (SET Domain Bifurcated 1) was specifically recruited to damaged pericentric heterochromatin (Fig. 5a) while SUV39H1 displayed only a slight enrichment on UVC-damaged compared to undamaged chromocenters (Fig. S6a). The H3K27 methyltransferase EZH2 (Enhancer Of Zeste Homolog 2) in contrast did not show any significant accumulation in damaged chromocenters (Fig. S6b).

Given the specific recruitment of SETDB1 to damaged heterochromatin, we further investigated its potential role in methylating newly deposited H3 histones. SETDB1 was dispensable for new H3.3 deposition (Fig. S6c). However, abrogation of new H3 histone deposition, achieved by simultaneous depletion of H3.3 and of the H3.1-chaperone CAF-1, impaired SETDB1 recruitment to UVC-damaged heterochromatin (Fig. 5b). Single depletion of H3.3 or CAF-1 had no or very little effect (Fig. S6d). These results indicate that deposition of newly synthesized H3 histones drives SETDB1 recruitment to damaged heterochromatin domains. Interestingly, erasing parental H3K9me3 by knocking down SUV39 also prevented SETDB1 recruitment to damaged heterochromatin (Fig. 5c). Together, these findings suggest that SETDB1 may trimethylate newly deposited H3 histones in UV-damaged heterochromatin by copying the K9me3 mark from neighboring SUV39-modified parental histones.

DISCUSSION

By assessing the consequences of UVC damage on mammalian pericentric heterochromatin domains, we provide important novel insights into the mechanisms for heterochromatin maintenance following DNA damage. We describe damage-mediated alterations in heterochromatin compaction with the retention of silencing histone marks, which may facilitate repair in compact regions of the genome while preserving heterochromatin identity. We also unveil a repair-coupled deposition of newly synthesized histones in damaged heterochromatin, and propose that histone chaperones and chromatin modifiers cooperate to maintain heterochromatin integrity following DNA damage (Fig. 6).

Regulation of heterochromatin compaction following UV damage

Chromatin rearrangements coupled to the early stages of the DNA damage response are considered to be critical for efficient DNA repair. This is particularly relevant in compact

heterochromatin domains. Indeed, decompaction of pericentromeric heterochromatin has been reported in response to radiation- and nuclease-induced breaks both in flies (Chiolo et al., 2011) and in mouse embryonic fibroblasts (Jakob et al., 2011; Tsouroula et al., 2016), as well as following heat stress in plants (Pecinka et al., 2010). Here, we provide evidence for pericentric heterochromatin decompaction following UVC damage in mouse fibroblasts, together with the maintenance of silencing histone marks in decondensed heterochromatin. We propose that by retaining their histone marks, these chromatin domains also maintain their identity, which could be crucial for the re-establishment of the original chromatin state once DNA repair is complete. Our observation that UV damaged chromatin decompacts while retaining heterochromatic histone marks is in line with previous studies in response to DSBs (Natale et al., 2017; Tsouroula et al., 2016), and highlights an uncoupling between chromatin structural and molecular determinants during DNA damage repair. Reciprocally, heterochromatic histone marks can be erased without any significant effect on heterochromatin decompaction, as observed upon SUV39 knockdown.

In addition to heterochromatin histone marks, Heterochromatin Protein 1 alpha (HP1 α), which is recruited to UVC lesions (Luijsterburg et al., 2009), could contribute to preserve heterochromatin identity following DNA damage. Owing to its liquid-liquid demixing properties (Larson et al., 2017; Strom et al., 2017), which are critical for heterochromatin formation, HP1α could stimulate heterochromatin recompaction at later time points post-UVC.

Mechanistically, the regulation of damaged heterochromatin compaction differs in response to distinct types of DNA damage. In response to UV lesions, we have identified the UV damage sensor DDB2 as a master regulator of heterochromatin compaction. Although it has been shown that the DDB2-binding partners DDB1 and Cul4A/B are not involved in controlling

chromatin decompaction (Luijsterburg et al., 2012), it is not clear if DDB2 promotes decompaction by itself or by interacting with other factors such as chromatin remodelers. Recent structural data indicate that the DDB2 complex can expose UV lesions occluded in nucleosomal DNA by promoting DNA shifting (Matsumoto et al., 2019). However, such local activity at the nucleosome level is unlikely to sustain larger scale chromatin decompaction. Given that DDB2 does not harbor known chromatin remodeling activity or motifs, we hypothesize that it induces chromatin decompaction indirectly, by promoting the recruitment or, alternatively, the release of chromatin remodelers from damage sites. Further studies will be needed to fully dissect the molecular bases of DDB2-mediated chromatin decompaction.

It will also be of major interest to assess the impact of damage-mediated decompaction on the three-dimensional chromatin organization in the nuclear space (Rowley and Corces, 2018). Indeed, it is not known whether decompaction entails only local chromatin movement with the loss or enlargement of chromatin loops within topologically associated domains, or more profound and global alterations of chromatin topology.

Functionally, whether heterochromatin decompaction facilitates the access of repair factors to damaged DNA is not entirely clear. Here, we have shown that the NER machinery can access UV lesions in pericentric heterochromatin and that repair can be completed within these domains. This contrasts with the relocalization of repair foci to the periphery of heterochromatin domains for late steps of DSB recombination (Jakob et al., 2011; Tsouroula et al., 2016), as also observed for replication foci (Quivy et al., 2004), thus showing that not all pathways that involve DNA synthesis are excluded from the core heterochromatin domain. Our results are consistent with several studies showing that, even if volume exclusion and moderate diffusive hindrance occur in heterochromatin domains (Bancaud et al., 2009), heterochromatin is accessible to large proteins (Verschure et al., 2003), including non-homologous end joining (NHEJ), single-strand annealing (SSA) and early homologous

recombination (HR) factors (Tsouroula et al., 2016). Considering that NER, unlike HR of DSBs, does not pose a risk for ectopic recombination between heterochromatic repeats, there would be no need for a relocalization of the NER machinery to the heterochromatin periphery and thus no spatial segregation of UV damage repair events. Instead, there is a temporal regulation of NER in heterochromatin, with slower kinetics of UV damage repair (Han et al., 2016), likely due to the necessary decompaction to promote access to lesions buried in heterochromatin.

Histone deposition in UV-damaged heterochromatin: role of histone chaperones

By assessing the recruitment of H3 variant-specific histone chaperones to UVC damaged heterochromatin, we have identified the histone chaperone HIRA as the main driver of new H3.3 deposition at UVC-damaged heterochromatin. While we cannot formally exclude that the DAXX-ATRX complex has a minor contribution to this process, we can envision alternative roles for this complex, such as stimulating parental H3.3 recovery during the repair response. Known as a promiscuous histone chaperone, the DAXX-ATRX complex could also regulate the dynamics of other histone variants, including CENP-A (Lacoste et al., 2014) and macroH2A.1 (Kim et al., 2019; Ratnakumar et al., 2012). Another potential role would be the control of heterochromatin recompaction at late time points post-UV, in light of recent data revealing that DAXX-ATRX-mediated deposition of H3.3 is key for chromocenter clustering during myogenic differentiation (Park et al., 2018). Finally, it has been proposed that this histone chaperone complex could regulate repair synthesis as observed during HR (Juhasz et al., 2018), but our preliminary data do not support such a function during NER.

Maintenance of silencing histone marks in UV-damaged heterochromatin

We have found that the histone methyltransferase SETDB1 is specifically recruited to UVC-damaged heterochromatin. Although the underlying mechanisms are still unclear, they may involve SETDB1 tandem Tudor domains, reported to bind specifically to dually modified

histone H3 containing both K14 acetylation (H3K14ac) and K9 methylation (H3K9me1/2/3) (Jurkowska et al., 2017). Given that newly synthesized H3 histones are enriched in K14ac (Alabert et al., 2015), and are not optimal substrates for trimethylation by SUV39 (Rea et al., 2000), we hypothesize that SETDB1 could bind, via its Tudor domains, the new H3 histones deposited in damaged heterochromatin, and then trimethylate these histones, thus mirroring SUV39-dependent trimethylation on parental H3. In support of this hypothesis, SETDB1 has already been involved in DNA damage-induced H3K9me3 leading to sex chromosome inactivation in meiosis (Hirota et al., 2018). Future studies will determine whether SETDB1 indeed promotes trimethylation of newly deposited H3 histones in UV-damaged heterochromatin.

Collectively, our work sheds new light on the processes safeguarding pericentric heterochromatin integrity following DNA damage. It would be of interest to determine if similar or distinct mechanisms operate in other heterochromatin domains characterized by different patterns of epigenetic marks, such as telomeric chromatin and facultative heterochromatin. Beyond the DNA damage response, our findings may also provide a framework for understanding heterochromatin maintenance during other disruptive events in both normal and pathological conditions, like DNA replication, cell differentiation, aging and disease.

METHODS

Cell culture

U2OS (ATCC HTB-96, human osteosarcoma, female), MCF7 (ATCC HTB-22, human breast adenocarcinoma, female), and NIH/3T3 cells (ATCC CRL-1658, mouse embryonic fibroblast, male) were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (EUROBIO) and antibiotics

(100 U/ml penicillin and 100 μg/ml streptomycin, Invitrogen) and the appropriate selection antibiotics (Euromedex, Supplementary Table 1). For seeding NIH/3T3 cells on coverslips, coverslips were first coated with 20 μg/ml Collagen Type I (MERCK Millipore) and 2 μg/ml Fibronectin (Sigma-Aldrich) to increase cell adhesion.

Stable cell lines	Selection antibiotics
NIH/3T3 GFP-DDB2	200 μg/ml Hygromycin
NIH/3T3 GFP-DDB2 H3.3-SNAP	200 μg/ml Hygromycin + 500 μg/ml G418
NIH/3T3 H3.3-SNAP	100 μg/ml G418
U2OS H3.3-SNAP	100 μg/ml G418

Supplementary Table 1: Stable cell lines.
Ectopically expressed proteins are of human origin and are more than 80% similar to mouse proteins.
Antibiotics: Hygromycin, G418 (Euromedex)

siRNA and plasmid transfections

siRNA purchased from Eurofins MWG Operon (Supplementary Table 2) were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) following manufacturer's instructions. The final concentration of siRNA in the culture medium was 50-80 nM. Cells were harvested 48-72 hr after transfection.

Designation	Target species	Target sequence	Working conditions
siATRX	Mouse	5'GTACAGAAATCTCGCTCAA3'	50 nM – 72 hours
siCAF-1 p150	Mouse	5'AAGGAGAAGGCGGAGAAGCAG3'	30 nM – 48 hours
siDAXX	Mouse	5'TGACCTTACAAACACTGAA3'	50 nM – 72 hours
siH3.3	Mouse	1:1 combination of siH3.3A:5'CTACAAAAGCCGCTCGCAA3' and siH3.3B: 5'GCCAAGAGAGTCACCATCA3'	50 nM – 48 to 72 hours
siHIRA	Mouse	5' GGAAGGTTGTGATCTGGAA 3'	50 nM – 72 hours
siLUC (Luciferase)	Firefly	5'CGTACGCGGAATACTTCGA3'	50 nM – 48 to 72 hours
siSETDB1	Mouse	5'GCGCAGAGTTAACCGCAAA3'	50 nM – 72 hours
siSUV39H1/2	Mouse	5'ACCTCTTTGACCTGGACTA3'	50 nM – 72 hours
siXPG	Mouse	5'TGATGATAACGATGAGAAA3'	50 nM – 72 hours

Supplementary Table 2: siRNA sequences.

Cells were transfected with plasmid DNA (Supplementary Table 3) using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. For stable cell line establishment (Supplementary Table 1), plasmid DNA was transfected into cells at 1 μg/ml final, 48 hr

before antibiotic selection of clones. For transient transfections, each plasmid was at 0.5 μg/ml final and cells were fixed 48 hr post transfection. For DDB2 tethering to major satellites, plasmids encoding GFP-tagged proteins, GBP-dCas9-mRFP and major satellite gRNA were co-transfected into NIH/3T3 cells 48 hr before cell analysis . For DDB2 detachment from major satellites, NIH/3T3 GFP-DDB2 cells were transfected with GBP-dCas9-mRFP and major satellite gRNA plasmids and 24 hr later with anti-Cas9 plasmid. Cells were fixed 24 hr after the second transfection.

Plasmid	Construct details	Reference/Provider
anti-Cas9 (pJH376-AcrIIA4)	Bacteriophage AcrIIA4 sequence inserted into pcDNA3.1(+)	gift from Joseph Bondy-Denomy (Addgene plasmid # 86842)(Rauch et al., 2017)
GBP-dCas9-mRFP	GFP-binding nanobody (GBP) and mRFP coding sequences cloned into pCAG-dCas9	Gift from Sebastian Butlmann(Anton and Bultmann, 2017)
GFP	pEGFP-C1	Clontech #6084-1
GFP-DDB2	Human *DDB2* coding sequence (Montpellier Genomic Collections) subcloned into GFP-XPC plasmid replacing XPC	(Adam et al., 2016)
GFP-XPC	cDNA encoding GFP-human XPC cloned into pIREShyg vector (Clontech)	Gift from Ryotaro Nishi(Nishi et al., 2009)
H3.3-SNAP	Human *H3F3B* coding sequence cloned into pSNAPm (New England Biolabs)	(Dunleavy et al., 2011)
HIRA-YFP	Human HIRA sequence from (Hall et al., 2001) edited by Genscript and subcloned into pEYFP-N1 (Clontech)	(Adam et al., 2013)
MajSat gRNA	Major satellite guide RNA sequence cloned into pEX-A-U6-gRNA	Gift from Sebastian Butlmann(Anton et al., 2014)

Supplementary Table 3: Plasmids.

UVC irradiation

Cells grown on glass coverslips (12 mm diameter, thickness No.1.5, Thorlabs) were irradiated with UVC (254 nm) using a low-pressure mercury lamp. Conditions were set using a VLX-3W dosimeter (Vilbert-Lourmat). For global UVC irradiation, cells in Phosphate Buffer Saline (PBS) were exposed to UVC doses ranging from 4 to 12 J/m^2 for survival assays and to 10 J/m^2 in other experiments. For local UVC irradiation (Katsumi et al., 2001)·(Moné et al.,

2001), cells were covered with a polycarbonate filter (5 μm pore size, Millipore) and irradiated with 150 or 300 J/m^2 UVC. Irradiated cells were allowed to recover in culture medium for the indicated times before fixation.

For UVC laser micro-irradiation(Dinant et al., 2007), cells were grown on quartz coverslips (25 mm diameter, thickness No.1, SPI supplies) and nuclei were stained by adding Hoechst 33258 (10 μg/mL final, Sigma-Aldrich) to the culture medium 30 min before UVC irradiation. Quartz coverslips were transferred to a Chamlide magnetic chamber on a custom stage insert (Live Cell Instrument) and cells were irradiated for 50 ms using a 2 mW pulsed diode-pumped solid-state laser emitting at 266 nm (RappOptoElectronics, Hamburg GmbH) directly connected to a Zeiss LSM 700 confocal microscope adapted for UVC transmission with all-quartz optics. The laser was attenuated using a neutral density filter OD1 and focused through a 40x/0.6 Ultrafluar glycerol objective.

Cell extracts and western blot

Total extracts were obtained by scraping cells on plates or resuspending cell pellets in Laemmli buffer (50 mM Tris-HCl pH 6.8, 1.6% Sodium Dodecyl Sulfate (SDS), 8% glycerol, 4% β-mercaptoethanol, 0.0025% bromophenol blue) followed by 5 min denaturation at 95°C. For western blot analysis, extracts were run on 4%–20% Mini-PROTEAN TGX gels (Bio-Rad) in running buffer (200 mM glycine, 25 mM Tris, 0.1% SDS) and transferred onto nitrocellulose membranes (Amersham Protran) with a Trans-Blot SD semidry transfer cell (Bio-Rad). Total proteins were revealed by Pierce® Reversible Stain (Thermo Scientific). Proteins of interest were probed using the appropriate primary and Horse Radish Peroxidase (HRP)-conjugated secondary antibodies (Supplementary Table 4), detected using SuperSignal West Pico or Femto chemiluminescence substrates (Pierce) on hyperfilms MP (Amersham).

Flow cytometry

For cell cycle analysis, cells were fixed in ice-cold 70% ethanol before DNA staining with 50 μg/ml propidium iodide (Sigma-Aldrich) in PBS containing 0.05% Tween and 0.5 mg/ml RNase A (USB/Affymetrix). DNA content was analyzed by flow cytometry using a BD FACScalibur flow cytometer (BD Biosciences) and FlowJo software (TreeStar).

SNAP-tag labeling of newly synthesized histones

For specific labeling of newly synthesized histones(Adam et al., 2015; Bodor et al., 2012), cells were grown on glass coverslips and pre-existing SNAP-tagged histones were first quenched by incubating cells with 10 μM of the non-fluorescent substrate SNAP-cell Block (New England Biolabs) for 30 min followed by a 30 min-wash in fresh medium and a 2hr-chase. The new SNAP-tagged histones synthesized during the chase were fluorescently labeled with 2 μM of the red-fluorescent reagent SNAP-cell TMR star (New England Biolabs) during a 15 min-pulse step followed by 30 min wash in fresh medium. Cells were subsequently permeabilized with Triton X-100, fixed and processed for immunostaining. Cells were irradiated with a UVC lamp before the pulse step.

EdU-labeling of replicating cells and repair sites

To visualize replication foci, 10 μM Ethynyl-deoxyUridine (EdU) was incorporated into cells on glass coverslips during 15 min at 37°C and revealed using the Click-It EdU Alexa Fluor 647 Imaging kit (Invitrogen) according to manufacturer's instructions. To localize the sites of UV damage repair, cells were incubated with 10 μM EdU for 1h30 after local UVC irradiation and EdU was revealed using the Click-It EdU Alexa Fluor 488 Imaging kit (Invitrogen).

Nascent RNA labeling

Cells on glass coverslips were incubated in medium supplemented with 0.5 mM Ethynyl-Uridine (EU) for 45 min at 37°C, and EU incorporation was revealed with Click-iT RNA Alexa Fluor 594 Imaging kit (Invitrogen) according to manufacturer's instructions. Coverslips were mounted in Vectashield medium with DAPI (Vector laboratories). EU fluorescence intensity in heterochromatin was measured using ImageJ software. Heterochromatin segmentation was based on DAPI staining.

Immunofluorescence

Cells grown on coverslips were either fixed directly with 2% paraformaldehyde (Electron Microscopy Sciences) for 20 min and permeabilized for 5 min with 0.5% Triton X-100 in PBS or cells were pre-extracted before fixation with 0.5% Triton X-100 in CSK buffer (Cytoskeletal buffer: 10 mM PIPES pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$) for 5 min at room temperature to remove soluble proteins. For PCNA staining, cells were fixed with 100% ice-cold methanol for 15 min. For the detection of UVC photoproducts (CPD), DNA was denatured with 2N HCl for 10 min at 37°C (Euromedex antibody, Supplementary Table 4) or with 0.5 M NaOH for 5 min at room temperature (Kamiya antibody, Supplementary Table 4). Since this denaturation quenches GFP fluorescence, when CPD detection was combined with the visualization of GFP-DDB2, immunofluorescence was performed in two steps starting with GFP immunodetection using a rat anti-GFP antibody (Supplementary Table 4) followed by fixation, denaturation and CPD immunodetection. Samples were blocked for 10 min in 5% BSA (Bovine Serum Albumin, Sigma-Aldrich) in PBT (PBS 0.5% Tween-20), followed by 45 min incubation with primary antibodies and 30 min incubation with secondary antibodies coupled to AlexaFluor dyes (Supplementary Table 4) diluted in blocking buffer. Coverslips were mounted in Vectashield medium with DAPI

(Vector laboratories).

Type	Antibody target	Species	Supplier	Dilution	Application
Primary	**ATRX**	Mouse	Millipore (MABE897)	1:100	IF
				1:500	WB
	CPD	Mouse	Kamiya Biomedical Company (MC-062, clone KTM53)	1:1000	IF
		Mouse	Cosmo Bio (CAC-NM-DND-001, clone TDM2)	1:1000	IF
	DAXX	Rabbit	Santa Cruz Biotechnology (sc-7152)	1:250	IF
			Sigma-Aldrich (HPA008736)	1:100	IF
			Ozyme (4533)	1:100	WB
	DDB2	Mouse	Abcam (ab51017)	1:200	WB
	EZH2	Mouse	BD-Biosciences (612666)	1:100	IF
	GFP	Rat	Santa Cruz Biotechnology (sc-101536)	1:50	IF
		Mouse	Roche Applied Science (11814460001)	1:1000	WB
	γH2A.X	Mouse	MERCK Millipore (05-636, clone JBW301)	1:1000	IF
	H3.3	Rabbit	MERCK Millipore (09-838)	1:1000	WB
	H3K4me3	Rabbit	MERCK Millipore (07-473)	1:5000	IF
	H3K9me3	Rabbit	Active Motif (39765)	1:500	IF
				1:1000	WB
	H4K20me3	Rabbit	Abcam (ab9053)	1:500	IF

	HIRA	Mouse	Active Motif (39557)	1:100	IF
				1:200	WB
	HP1α	Mouse	Millipore (MAB3584)	1:500	IF
	CAF-1 p60	Mouse	Active Motif (39996)	1:500	IF
	CAF-1 p150	Goat	Santa Cruz biotechnology (sc-10206)	1:50	IF
				1:200	WB
	PCNA	Rabbit	Santa Cruz Biotechnology (sc-7907)	1:50	IF
		Mouse	Dako (M0879)	1:1000	IF
	SETDB1	Mouse	Thermo scientific (MA515722)	1:200	IF
		Rabbit	Santa Cruz Biotechnology (sc-66884)	1:200	IF
		Mouse	Abcam (ab107225)	1:1000	WB
	SNAP	Rabbit	Pierce Antibodies (CAB4255)	1:500	IF
				1:1000	WB
	SUV39H1	Rabbit	Cell signaling technology (8729)	1:25	IF
				1:1000	WB
	Tubulin	Mouse	Sigma-Aldrich (T9026)	1:10000	WB
	XPA	Mouse	BD Biosciences (556453)	1:500	IF
	XPB	Rabbit	Santa Cruz Biotechnology (sc-293)	1:400	IF
	XPG	Rabbit	Bethyl Laboratories (A301-484A)	1:500	WB
Secondary	**Goat HRP**	Donkey	Santa Cruz Biotechnology (sc-2020)	1:10000	WB

	Mouse HRP	Goat	Jackson Immunoresearch Laboratories (115-035-068)	1:10000	WB
	Rabbit HRP	Donkey	Jackson Immunoresearch Laboratories (711-035-152)	1:10000	WB
	Goat Alexa Fluor 594	Donkey	Invitrogen (A11058)	1:1000	IF
	Mouse Alexa Fluor 488	Goat	Invitrogen (A11029)	1:1000	IF
	Mouse Alexa Fluor 568	Goat	Invitrogen (A11031)	1:1000	IF
	Mouse Alexa Fluor 594	Goat	Invitrogen (A11032)	1:1000	IF
	Mouse Alexa Fluor 647	Goat	Invitrogen (A21236)	1:1000	IF
	Rabbit Alexa Fluor 488	Goat	Invitrogen (A11034)	1:1000	IF
	Rabbit Alexa Fluor 568	Goat	Invitrogen (A11036)	1:1000	IF
	Rabbit Alexa Fluor 594	Goat	Invitrogen (A11037)	1:1000	IF
	Rat Alexa Fluor 488	Goat	Invitrogen (A11006)	1:1000	IF

Supplementary Table 4: Antibodies
IF: Immunofluorescence; WB: Western-Blot

Image acquisition and analysis

Fluorescence imaging was performed with a Leica DMI6000 epifluorescence microscope using Plan-Apochromat 63x/1.4 oil objective. Images were captured using a CCD camera (Photometrics) and Metamorph software. Images were assembled with Adobe Photoshop. For confocal imaging, samples were observed on a Zeiss LSM710 confocal microscope using a Plan-Apochromat 63x/1.4 oil objective. Live cell imaging coupled to UVC laser micro-irradiation was performed using a 40x/0.6 Ultrafluar Glycerol objective on a Zeiss LSM700 confocal microscope. Images were captured using Zen software, and analysed with ImageJ

(U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/). Nuclei and heterochromatin domains were segmented on 2D confocal images based on DAPI or Hoechst staining, and UVC-damaged regions based on GFP-DDB2 fluorescence using custom-made ImageJ macros. The volume, sphericity and GFP intensity of heterochromatin domains as well as H3K9me3 intensity in damaged heterochromatin were analyzed on 3D images reconstructed from z-stacks using Imaris (Bitplane, http://www.bitplane.com/imaris).

Colony forming assays

Cells were replated 48h after siRNA transfection and exposed to global UVC irradiation (4, 8 and 12 J/m^2) the following day. Colonies were stained 12 days later with 0.5% crystal violet/20% ethanol and counted. Results were normalized to plating efficiencies.

RT- Quantitative PCR

Total RNA was extracted from cells with TRIzol™ Reagent following manufacturer's instructions (Invitrogen) and precipitated in isopropanol. RNA samples were subject to DNA digestion with Turbo DNA-free (Invitrogen) before reverse transcription with Superscript III RT using random primers (200 ng/reaction, Invitrogen). Quantitative PCR reactions were carried out with the indicated primer pairs (Eurofins MWG Operon, 500 nM final concentration, Supplementary Table 5) and Power SYBR® Green PCR Master Mix (Applied Biosystems) and read in MicroAmp® Fast Optical 96-well plates (Applied Biosystems) using a ABI 7500 Fast detection system (Applied Biosystems). Results were normalized to the amount of the GAPDH housekeeping gene product.

Designation	**Sequence**
Major satellite_F	$^{5'}$ GACGACTTGAAAAATGACGAAATC $^{3'}$
Major satellite _R	$^{5'}$ CATATTCCAGGTCCTTCAGTGTGC $^{3'}$
GAPDH_F	$^{5'}$ TGCACCACCAACTGCTTAGC $^{3'}$

GAPDH_R	5' GGCATGGACTGTGGTCATGAG 3'

Supplementary Table 5: qRT-PCR primers
F: forward; R: reverse.

Statistical analyses

Percentages of positively stained cells were obtained by scoring at least 150 cells in each experiment. Statistical tests were performed using GraphPad Prism. P-values for mean comparisons between two groups were calculated with a Student's t-test with Welch's correction when necessary. Multiple comparisons were performed by one-way ANOVA with Bonferroni post-test. Comparisons of clonogenic survival were based on non-linear regression with a polynomial quadratic model. ns: non-significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

SUPPLEMENTARY INFORMATION

Supplementary information includes 6 figures (see below) and 2 movies, which are located in a Google Drive folder and can be accessed by the following link:

https://drive.google.com/open?id=1i0QLZJ3MfecCbrdv8uWcwtCRSy8ZltI1

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AUTHOR CONTRIBUTIONS

A.F., A.C., and S.E.P. designed and performed experiments, analyzed the data and wrote the manuscript. O.C. provided technical assistance and established mouse stable cell lines. O.L. and O.R. implemented the UVC laser technology and helped with image analyses. S.E.P. supervised the project

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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Figure 1

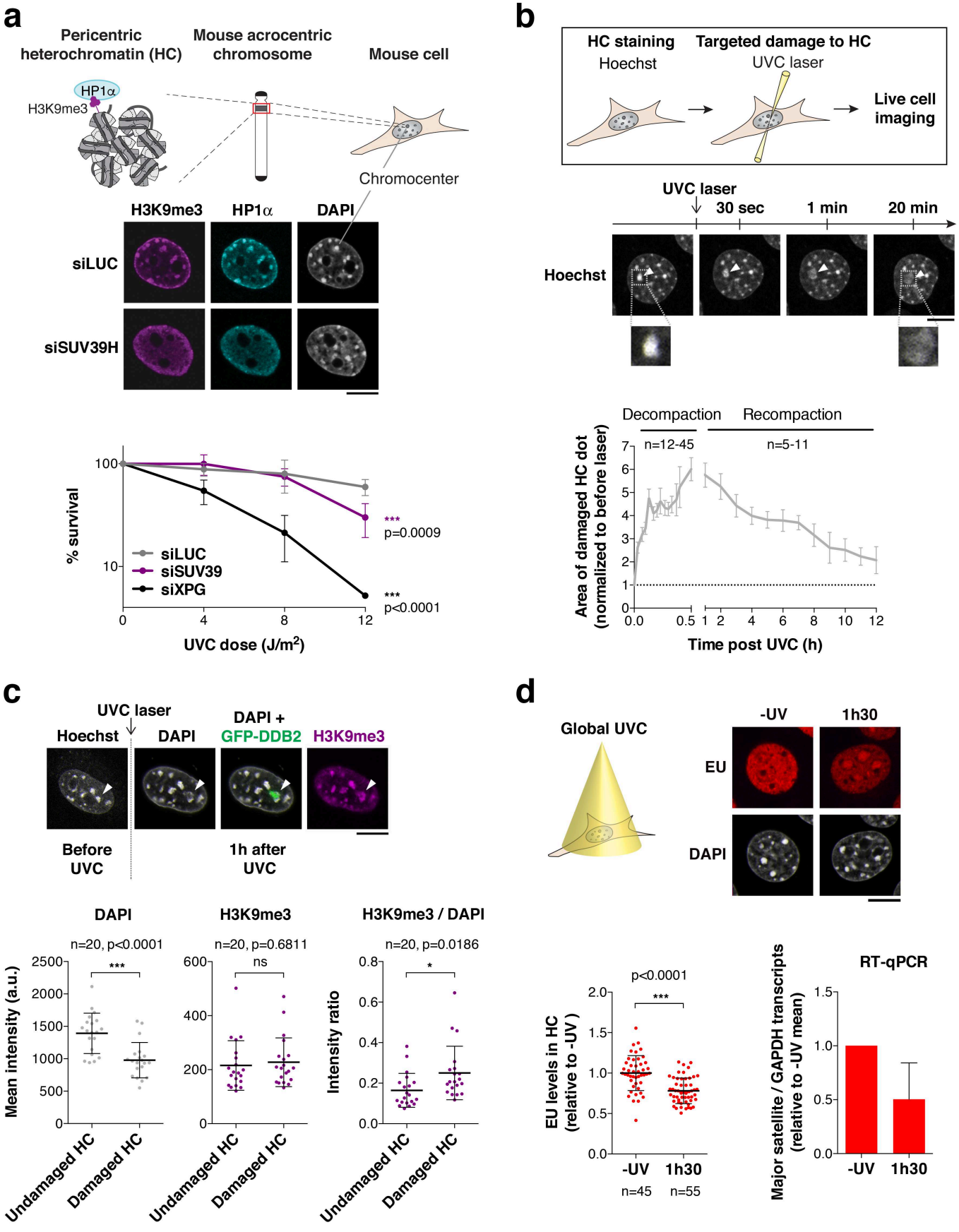
a
Pericentric heterochromatin (HC)
Mouse acrocentric chromosome
Mouse cell
HP1α
H3K9me3
Chromocenter
H3K9me3
HP1α
DAPI
siLUC
siSUV39H
% survival
100
10
siLUC
siSUV39
siXPG
***
p=0.0009
***
p<0.0001
0
4
8
12
UVC dose (J/m2)
b
HC staining
Hoechst
Targeted damage to HC
UVC laser
Live cell imaging
UVC laser
30 sec
1 min
20 min
Hoechst
Decompaction
Recompaction
n=12-45
n=5-11
Area of damaged HC dot (normalized to before laser)
Time post UVC (h)
c
UVC laser
Hoechst
DAPI
DAPI + GFP-DDB2
H3K9me3
Before UVC
1h after UVC
DAPI
H3K9me3
H3K9me3 / DAPI
n=20, p<0.0001
n=20, p=0.6811
n=20, p=0.0186
***
ns
*
Mean intensity (a.u.)
Intensity ratio
Undamaged HC
Damaged HC
d
Global UVC
-UV
1h30
EU
DAPI
p<0.0001
***
EU levels in HC (relative to -UV)
-UV
1h30
n=45
n=55
RT-qPCR
Major satellite / GAPDH transcripts (relative to -UV mean)

Figure 2

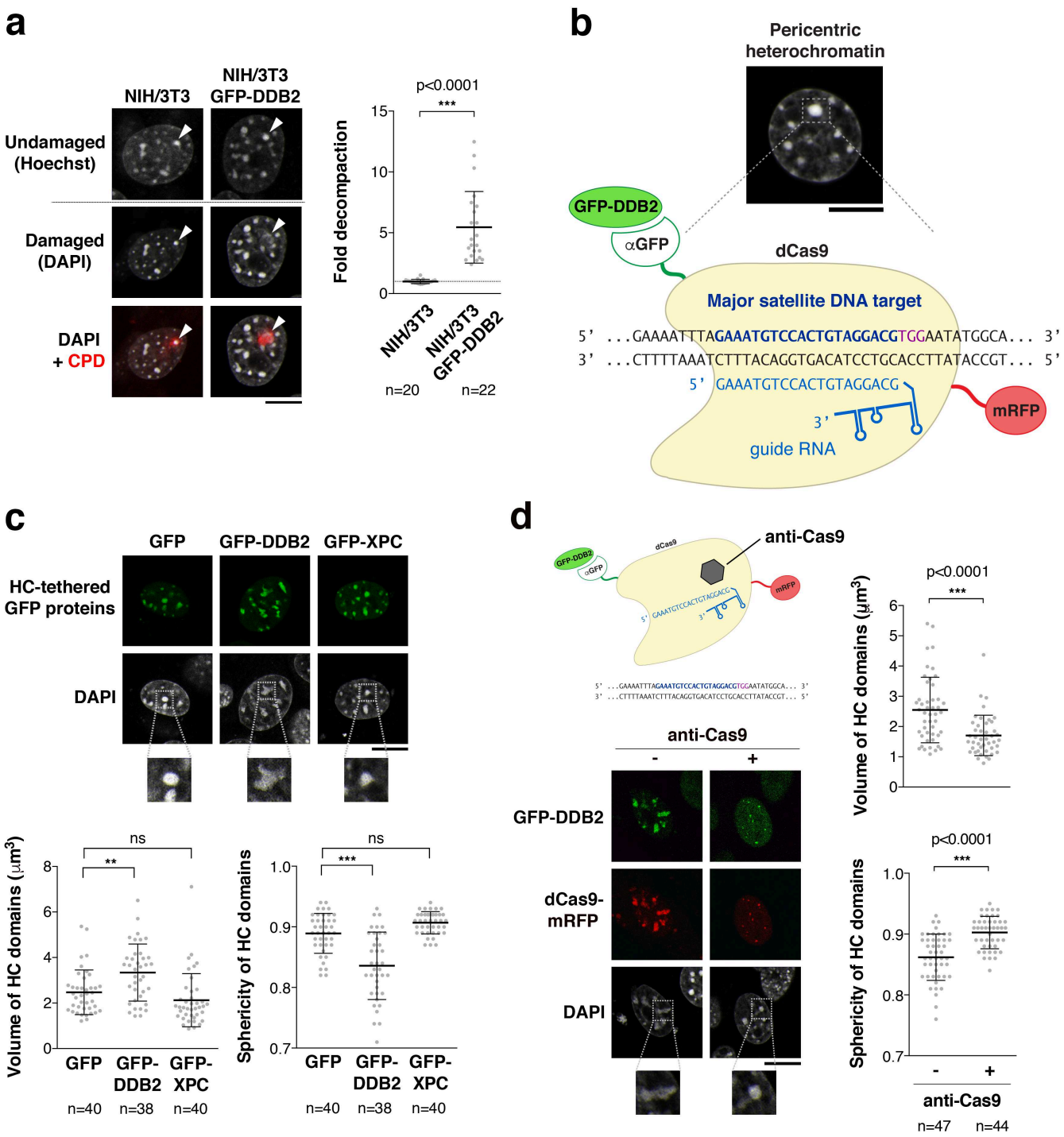
a
NIH/3T3
NIH/3T3 GFP-DDB2
Undamaged (Hoechst)
Damaged (DAPI)
DAPI + CPD
p<0.0001
***
Fold decompaction
15
10
5
0
NIH/3T3
NIH/3T3 GFP-DDB2
n=20
n=22
b
Pericentric heterochromatin
GFP-DDB2
αGFP
dCas9
Major satellite DNA target
5' ...GAAAATTTAGAAATGTCCACTGTAGGACGTGGAATATGGCA... 3'
3' ...CTTTTAAATCTTTACAGGTGACATCCTGCACCTTATACCGT... 5'
5' GAAATGTCCACTGTAGGACG
3'
guide RNA
mRFP
c
GFP
GFP-DDB2
GFP-XPC
HC-tethered GFP proteins
DAPI
Volume of HC domains (μm3)
ns
**
8
6
4
2
0
GFP
GFP-DDB2
GFP-XPC
n=40
n=38
n=40
Sphericity of HC domains
ns
***
1.0
0.9
0.8
0.7
GFP
GFP-DDB2
GFP-XPC
n=40
n=38
n=40
d
anti-Cas9
GFP-DDB2
dCas9-mRFP
DAPI
anti-Cas9
-
+
Volume of HC domains (μm3)
p<0.0001
***
6
5
4
3
2
1
0
Sphericity of HC domains
p<0.0001
***
1.0
0.9
0.8
0.7
-
+
anti-Cas9
n=47
n=44

Figure 3

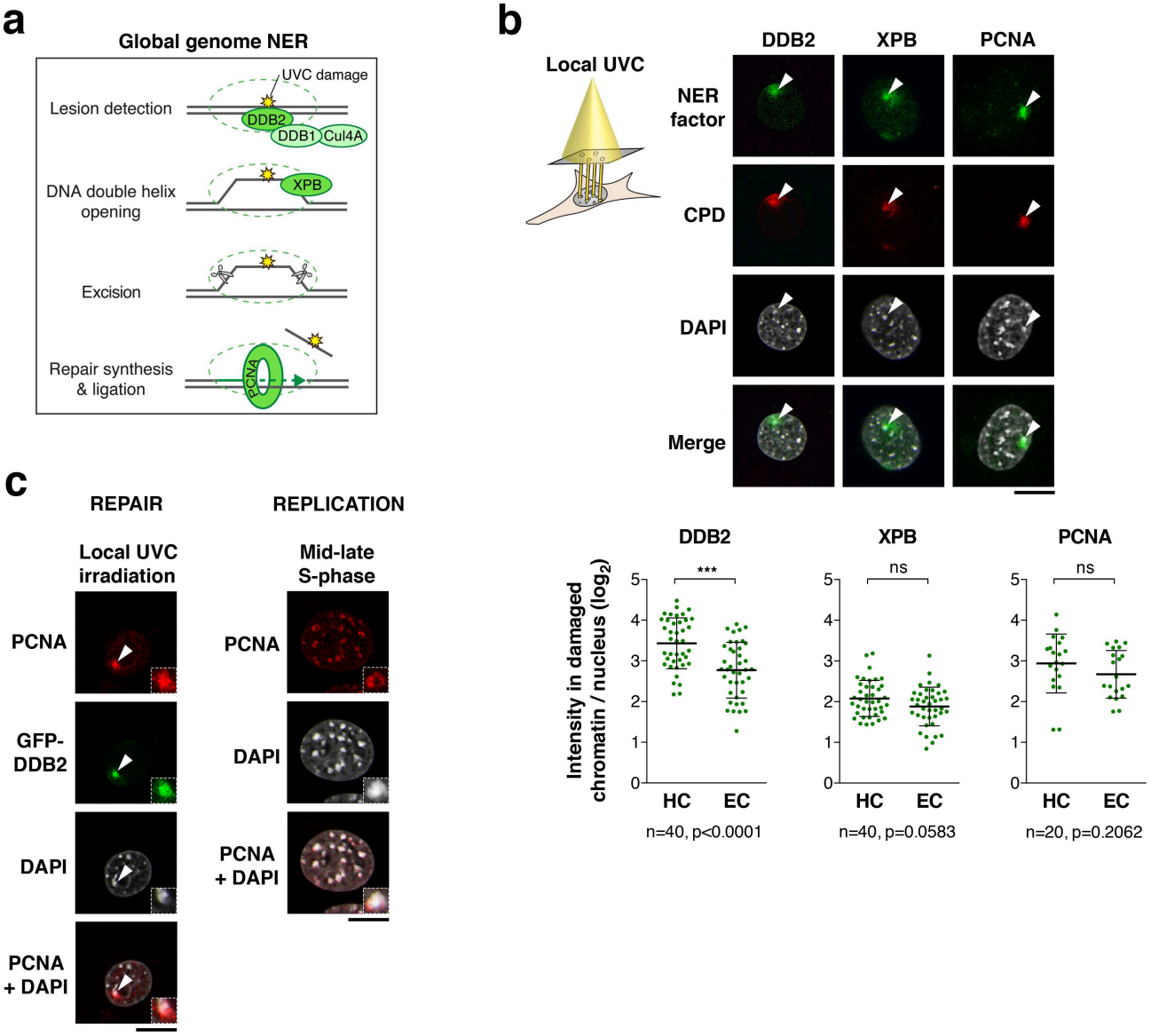
a
Global genome NER
UVC damage
Lesion detection
DDB2
DDB1
Cul4A
DNA double helix opening
XPB
Excision
Repair synthesis & ligation
PCNA
b
Local UVC
DDB2
XPB
PCNA
NER factor
CPD
DAPI
Merge
Intensity in damaged chromatin / nucleus (log2)
***
ns
ns
HC
EC
n=40, p<0.0001
n=40, p=0.0583
n=20, p=0.2062
c
REPAIR
REPLICATION
Local UVC irradiation
Mid-late S-phase
PCNA
GFP-DDB2
DAPI
PCNA + DAPI

Figure 4

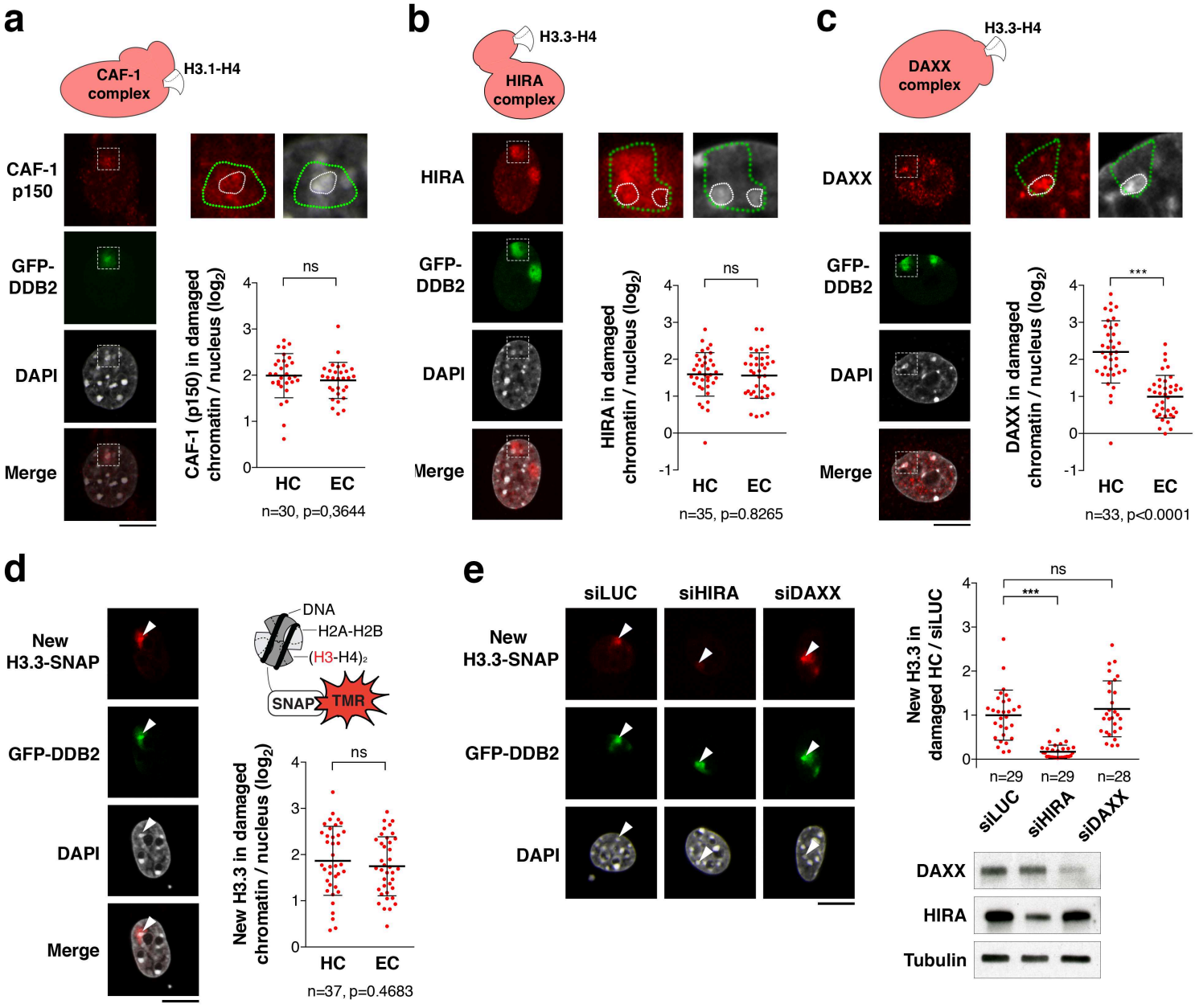
a
CAF-1 complex
H3.1-H4
CAF-1 p150
GFP-DDB2
DAPI
Merge
CAF-1 (p150) in damaged chromatin / nucleus (log2)
ns
HC
EC
n=30, p=0,3644
b
H3.3-H4
HIRA complex
HIRA
GFP-DDB2
DAPI
Merge
HIRA in damaged chromatin / nucleus (log2)
ns
HC
EC
n=35, p=0.8265
c
H3.3-H4
DAXX complex
DAXX
GFP-DDB2
DAPI
Merge
DAXX in damaged chromatin / nucleus (log2)
***
HC
EC
n=33, p<0.0001
d
New H3.3-SNAP
GFP-DDB2
DAPI
Merge
DNA
H2A-H2B
(H3-H4)2
SNAP
TMR
New H3.3 in damaged chromatin / nucleus (log2)
ns
HC
EC
n=37, p=0.4683
e
siLUC
siHIRA
siDAXX
New H3.3-SNAP
GFP-DDB2
DAPI
New H3.3 in damaged HC / siLUC
ns
***
n=29
n=29
n=28
siLUC
siHIRA
siDAXX
DAXX
HIRA
Tubulin

Figure 5

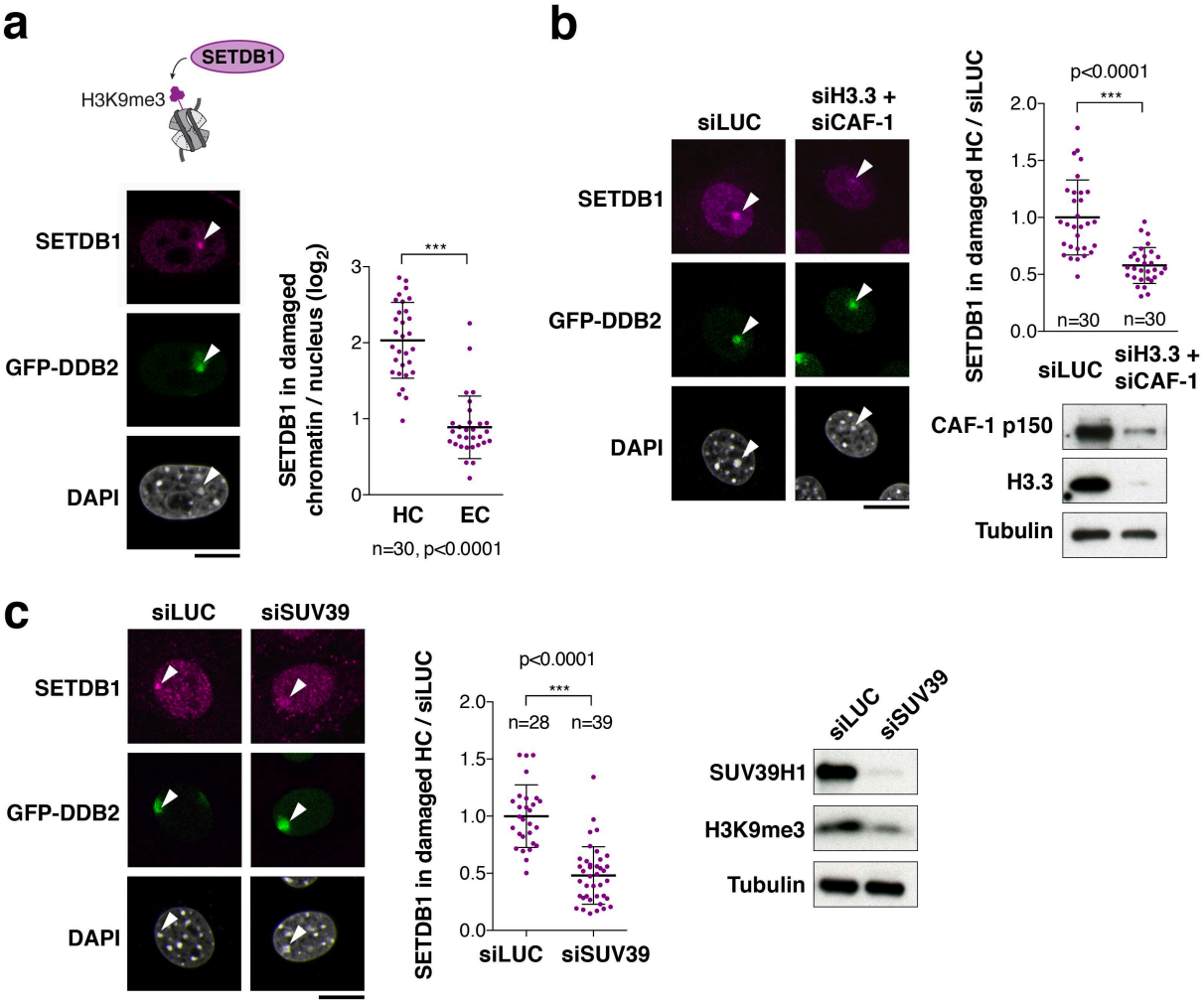
a
SETDB1
H3K9me3
SETDB1
GFP-DDB2
DAPI
SETDB1 in damaged chromatin / nucleus (log2)
***
HC
EC
n=30, p<0.0001
b
siLUC
siH3.3 + siCAF-1
SETDB1
GFP-DDB2
DAPI
p<0.0001
***
SETDB1 in damaged HC / siLUC
n=30
n=30
siLUC
siH3.3 + siCAF-1
CAF-1 p150
H3.3
Tubulin
c
siLUC
siSUV39
SETDB1
GFP-DDB2
DAPI
p<0.0001
***
SETDB1 in damaged HC / siLUC
n=28
n=39
siLUC
siSUV39
siLUC
siSUV39
SUV39H1
H3K9me3
Tubulin

Figure 6

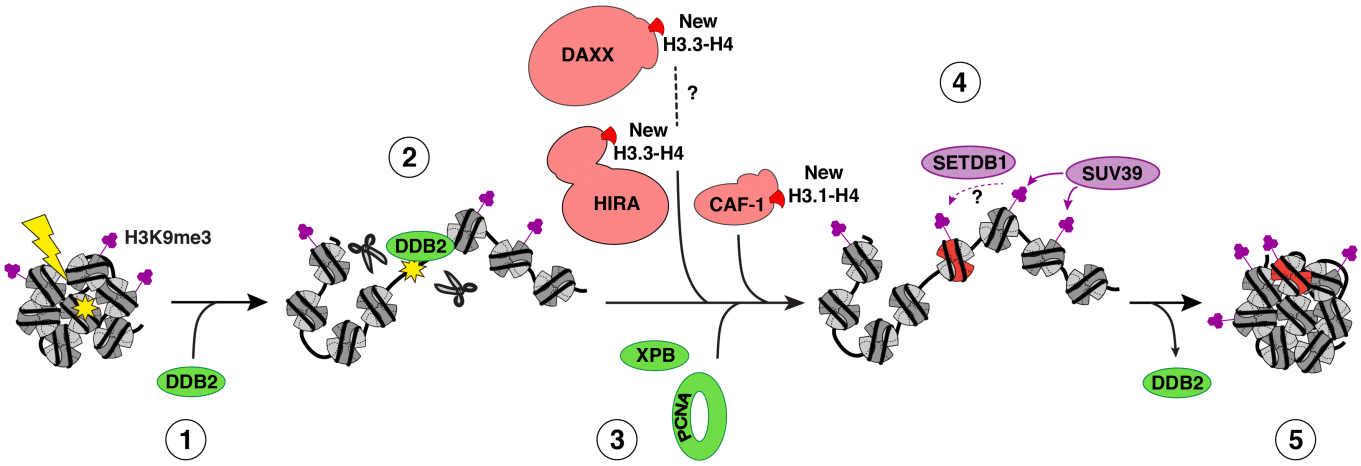
DAXX
New
H3.3-H4
?
New
H3.3-H4
HIRA
CAF-1
New
H3.1-H4
SETDB1
SUV39
?
4
2
H3K9me3
DDB2
DDB2
XPB
PCNA
DDB2
1
3
5

Figure S1

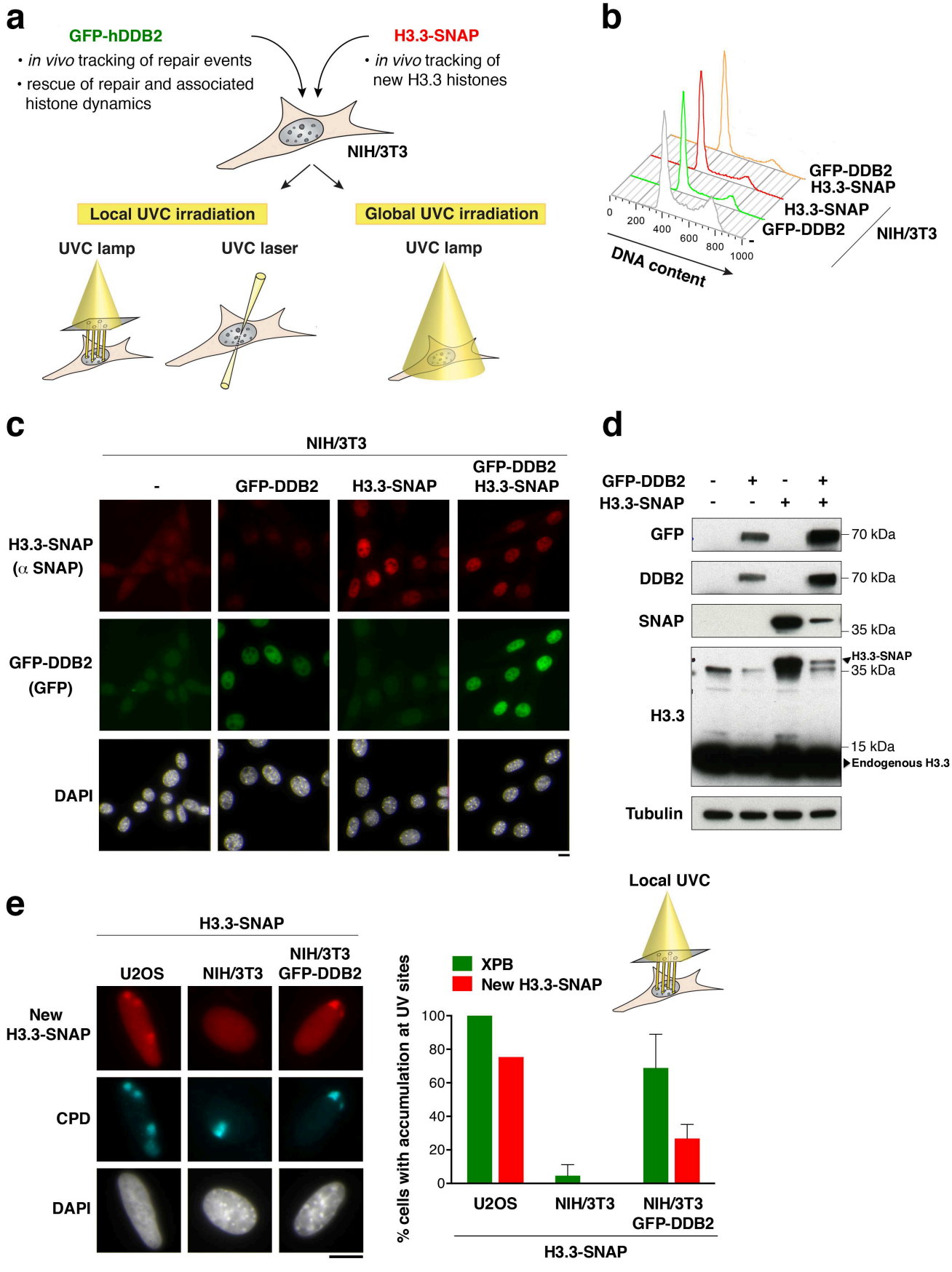
a
GFP-hDDB2
• in vivo tracking of repair events
• rescue of repair and associated histone dynamics
H3.3-SNAP
• in vivo tracking of new H3.3 histones
NIH/3T3
Local UVC irradiation
Global UVC irradiation
UVC lamp
UVC laser
UVC lamp
b
GFP-DDB2
H3.3-SNAP
H3.3-SNAP
GFP-DDB2
NIH/3T3
0
200
400
600
800
1000
DNA content
c
NIH/3T3
-
GFP-DDB2
H3.3-SNAP
GFP-DDB2
H3.3-SNAP
H3.3-SNAP
(α SNAP)
GFP-DDB2
(GFP)
DAPI
d
GFP-DDB2 - + - +
H3.3-SNAP - - + +
GFP
DDB2
SNAP
H3.3
Tubulin
70 kDa
70 kDa
35 kDa
H3.3-SNAP
35 kDa
15 kDa
Endogenous H3.3
e
H3.3-SNAP
U2OS
NIH/3T3
NIH/3T3
GFP-DDB2
New
H3.3-SNAP
CPD
DAPI
Local UVC
XPB
New H3.3-SNAP
% cells with accumulation at UV sites
100
80
60
40
20
0
U2OS
NIH/3T3
NIH/3T3
GFP-DDB2
H3.3-SNAP

Figure S2

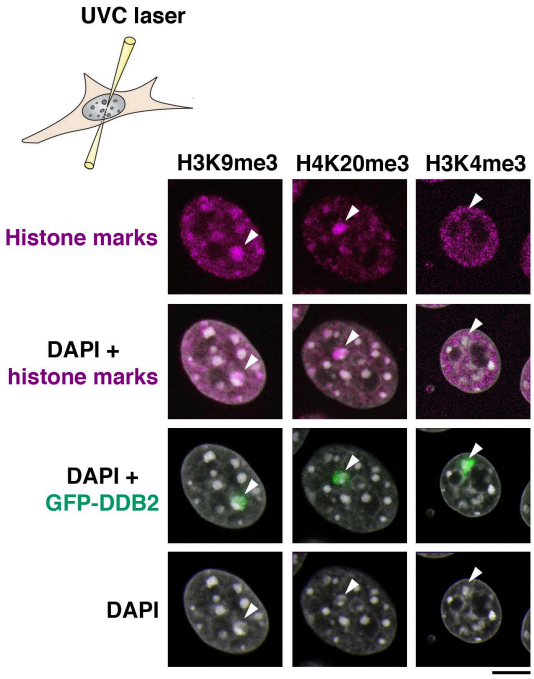
UVC laser
H3K9me3
H4K20me3
H3K4me3
Histone marks
DAPI +
histone marks
DAPI +
GFP-DDB2
DAPI

Figure S3

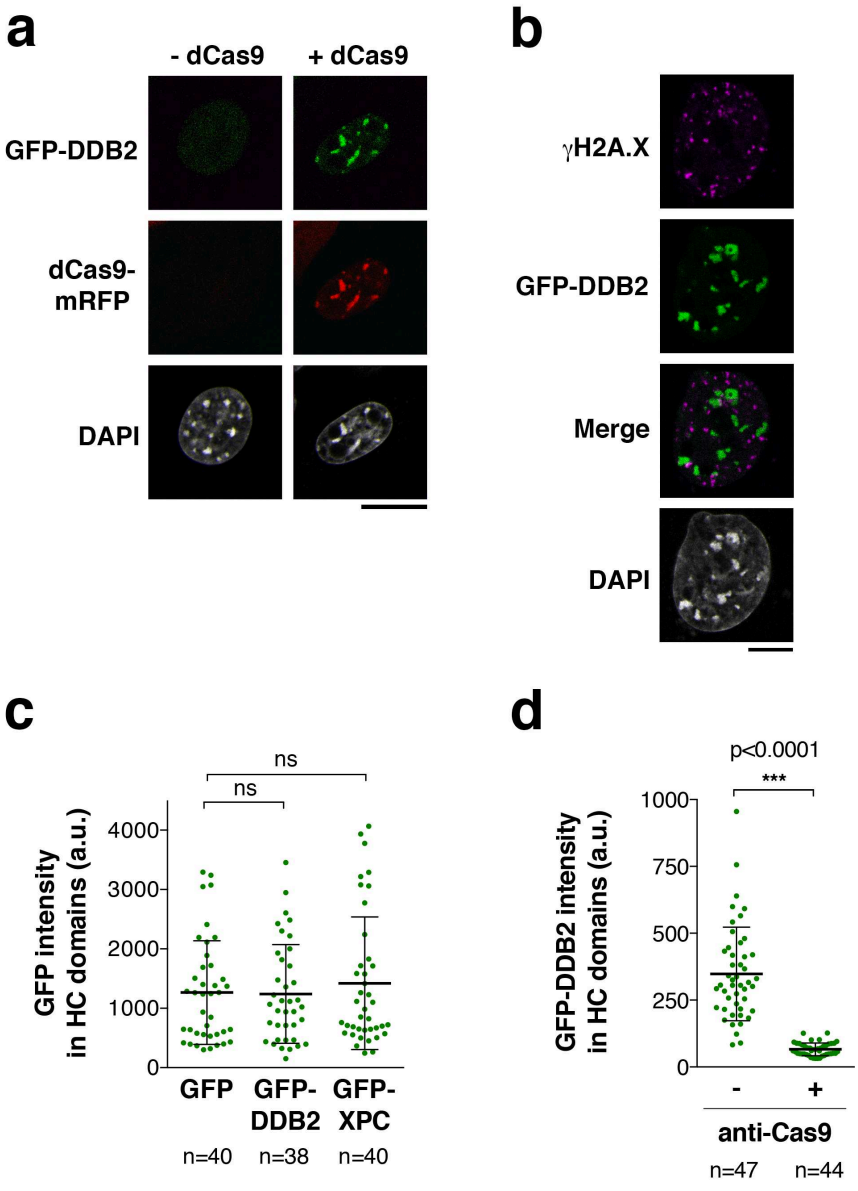
a
- dCas9
+ dCas9
GFP-DDB2
dCas9-mRFP
DAPI
b
γH2A.X
GFP-DDB2
Merge
DAPI
c
GFP intensity in HC domains (a.u.)
ns
ns
4000
3000
2000
1000
0
GFP
GFP-DDB2
GFP-XPC
n=40
n=38
n=40
d
GFP-DDB2 intensity in HC domains (a.u.)
p<0.0001
***
1000
750
500
250
0
-
+
anti-Cas9
n=47
n=44

Figure S4

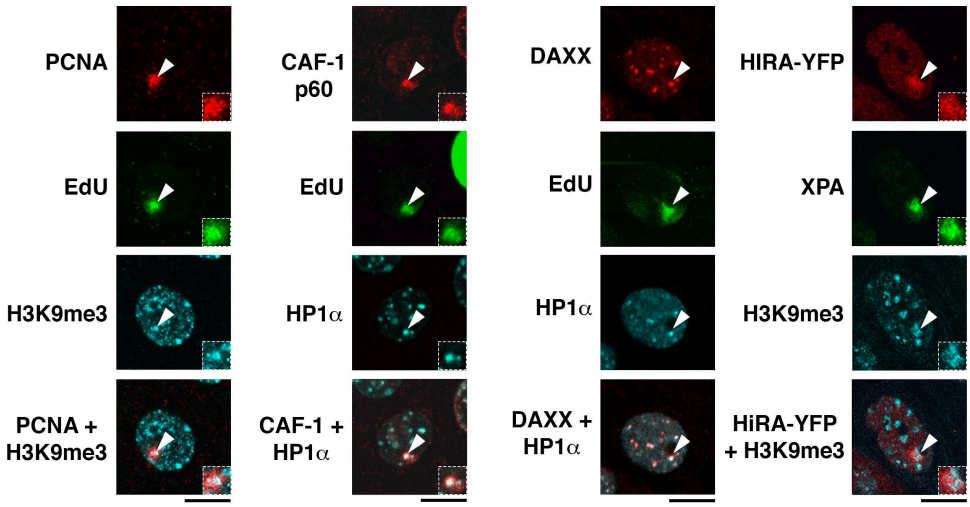
PCNA
CAF-1 p60
DAXX
HIRA-YFP
EdU
EdU
EdU
XPA
H3K9me3
HP1α
HP1α
H3K9me3
PCNA + H3K9me3
CAF-1 + HP1α
DAXX + HP1α
HiRA-YFP + H3K9me3

Figure S5

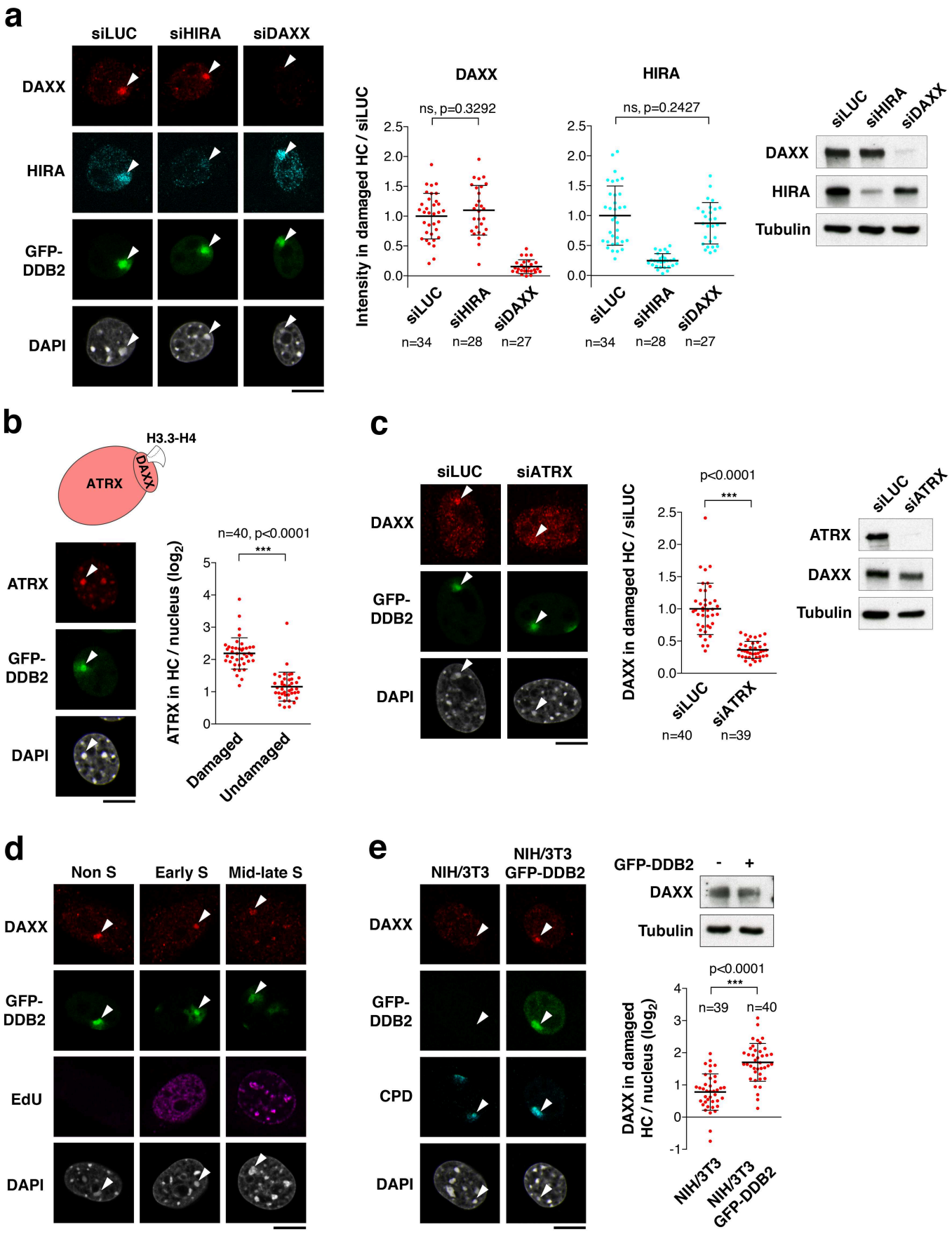
a
siLUC
siHIRA
siDAXX
DAXX
HIRA
GFP-DDB2
DAPI
DAXX
HIRA
Intensity in damaged HC / siLUC
ns, p=0.3292
ns, p=0.2427
n=34
n=28
n=27
siLUC
siHIRA
siDAXX
Tubulin
b
H3.3-H4
ATRX
DAXX
ATRX in HC / nucleus (log2)
n=40, p<0.0001
***
Damaged
Undamaged
c
siLUC
siATRX
DAXX in damaged HC / siLUC
p<0.0001
n=40
n=39
d
Non S
Early S
Mid-late S
EdU
e
NIH/3T3
NIH/3T3 GFP-DDB2
GFP-DDB2
CPD
DAXX in damaged HC / nucleus (log2)

Figure S6

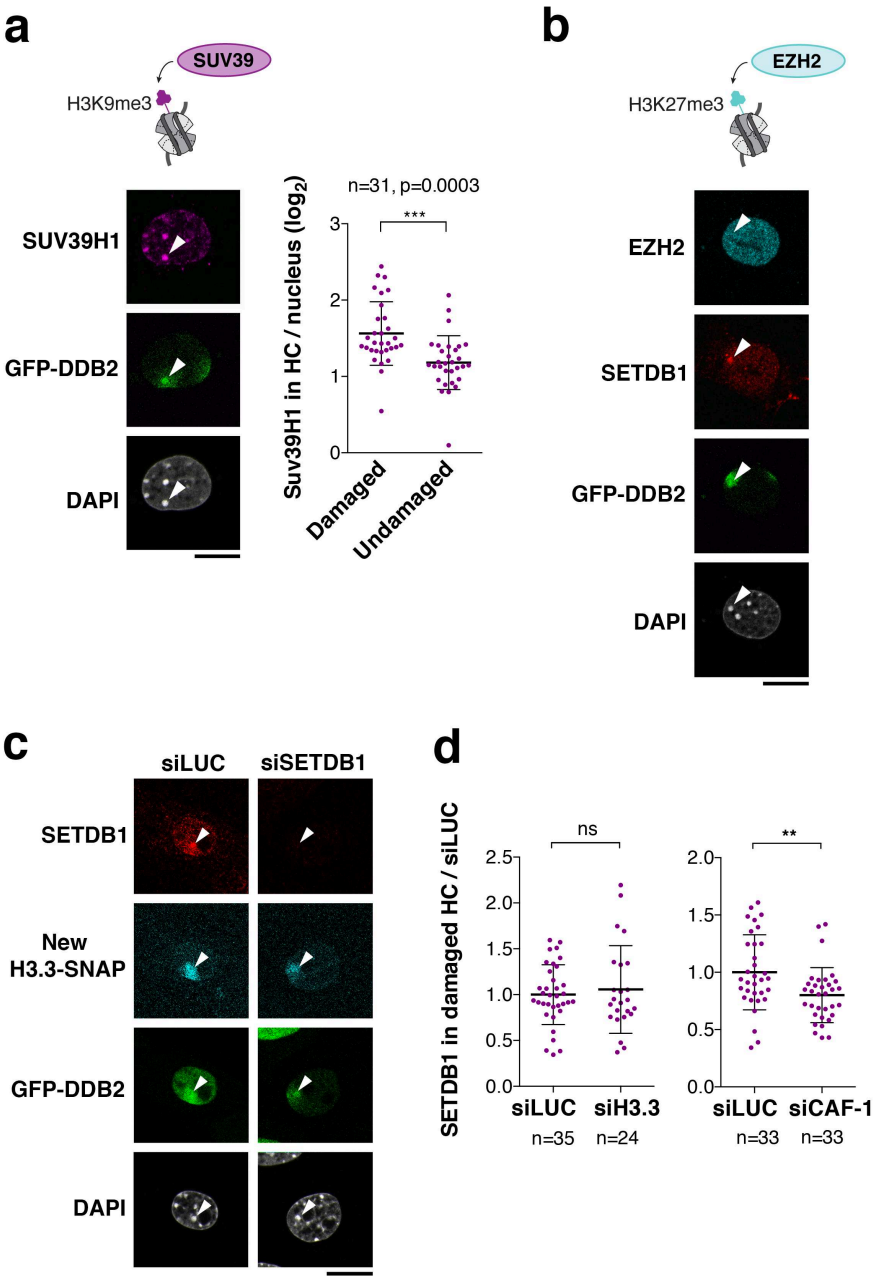
a
SUV39
H3K9me3
SUV39H1
GFP-DDB2
DAPI
n=31, p=0.0003
***
Suv39H1 in HC / nucleus (log2)
3
2
1
0
Damaged
Undamaged
b
EZH2
H3K27me3
EZH2
SETDB1
GFP-DDB2
DAPI
c
siLUC
siSETDB1
SETDB1
New
H3.3-SNAP
GFP-DDB2
DAPI
d
SETDB1 in damaged HC / siLUC
ns
**
2.5
2.0
1.5
1.0
0.5
0.0
siLUC
siH3.3
n=35
n=24
siLUC
siCAF-1
n=33
n=33

FIGURE LEGENDS

Figure 1. Maintenance of heterochromatin integrity in response to UV damage

(**a**) Schematic representation of pericentric heterochromatin domains in mouse cells and delocalization of heterochromatin marks (H3K9me3, HP1α) upon knock-down of SUV39 methyltransferases in NIH/3T3 GFP-DDB2 cells. Clonogenic survival of the same cell line treated with the indicated siRNAs (siLUC, negative control; siXPG, positive control) and exposed to global UVC irradiation. (**b**) Technical approach for targeting UVC damage to pericentric heterochromatin domains (HC) in live murine cells. Heterochromatin compaction changes upon UVC laser micro-irradiation are analyzed by live imaging in NIH/3T3 GFP-DDB2 cells stained with Hoechst. White arrowheads point to UVC-damaged heterochromatin domains. (**c**) H3K9me3 levels in damaged heterochromatin (white arrowheads) analyzed by immunofluorescence in NIH/3T3 GFP-DDB2 cells 1h after UVC laser micro-irradiation. Scatter plots represent DAPI and H3K9me3 levels measured on reconstructed 3D images in damaged heterochromatin domains compared to undamaged heterochromatin in the same nucleus. (d) Heterochromatin transcription analyzed 1h30 after global UVC damage in NIH/3T3 GFP-DDB2 cells by EU staining (left) and by RT-qPCR for major satellite transcripts (right). Error bars, s.d. (s.e.m. for (b) panel only) from n cells scored in at least four independent experiments, or in two experiments for EU staining. a.u., arbitrary units. All microscopy images are confocal sections. Scale bars, 10 μm. Zoomed in views of heterochromatin domains (x2.6).

Figure 2. The UV damage sensor DDB2 regulates heterochromatin compaction

(**a**) Decompaction of damaged pericentric heterochromatin domains (white arrowheads) circa one hour after UVC laser micro-irradiation analyzed by live imaging in the indicated cell

lines. CPD staining in fixed cells highlights the damaged chromocenter. The scatter plots represent the area of the damaged chromocenters normalized to the same chromocenters before UVC laser. (**b**) Procedure for targeting GFP-tagged DDB2 to major satellites sequences in pericentric heterochromatin. (**c, d**) Confocal sections showing the aspect of pericentric heterochromatin domains upon tethering of the indicated GFP-tagged proteins in NIH/3T3 (c) or NIH/3T3 GFP-DDB2 cells (d). Heterochromatin tethering is relieved by expressing an anti-Cas9 peptide (d). The scatter plots show changes in volume and sphericity of heterochromatin domains quantified on reconstructed 3D images. Error bars, s.d. from n cells scored in at least three independent experiments. Scale bars, 10 μm. Zoomed in views of heterochromatin domains (x2.6).

Figure 3. Proficient NER pathway within heterochromatin domains

(**a**) Scheme of the Global Genome Nucleotide Excision Repair factors studied. (**b**) Recruitment to UVC damage (CPD) of early (DDB2), intermediate (XPB) and late (PCNA) repair factors, analyzed by immunofluorescence 30 min after local UVC irradiation through micropore filters in NIH/3T3 GFP-DDB2 cells. Cells with damaged heterochromatin domains (white arrowheads) were selected for the analysis. PCNA accumulation to damaged heterochromatin was analyzed outside S-phase. XPB and PCNA were not stained in green because the cells express GFP-DDB2, but are presented in green for simplicity. Scatter plots represent log2 fold enrichments of repair proteins in damaged heterochromatin (HC) and damaged euchromatin (EC) compared to the whole nucleus. Error bars, s.d. from n cells scored in at least two independent experiments. (**c**) Accumulation of PCNA within heterochromatin domains upon local UVC irradiation and confined to the periphery of replicating heterochromatin in mid-late S-phase. Insets show zoomed in views of heterochromatin domains (x2.3). All microscopy images are confocal sections. Scale bars, 10

μm.

Figure 4. Histone H3 deposition in UVC-damaged heterochromatin

(**a**, **b**, **c**) Recruitment of the H3.1 histone chaperone CAF-1 (p150 subunit) (a), and of the H3.3 histone chaperones HIRA (b) and DAXX (c) to UVC-damaged regions, analyzed by immunofluorescence in NIH/3T3 GFP-DDB2 cells 1h30 after local UVC irradiation through micropore filters. Zoomed in views (x2.6) show damaged regions (delineated by green dotted lines) containing heterochromatin domains (delineated by white dotted lines) (**d**, **e**) Accumulation of newly synthesized H3.3 histones in UVC-damaged heterochromatin regions (white arrowheads) analyzed in NIH/3T3 GFP-DDB2 H3.3-SNAP cells 45 min after local UVC irradiation through micropore filters. H3.3 chaperones are knocked down by siRNA (siLUC, control) (e). siRNA efficiencies are controlled by western blot (Tubulin, loading control). Scatter plots represent log2 fold enrichments of histone chaperones or new H3.3-SNAP histones in damaged heterochromatin (HC) and damaged euchromatin (EC) compared to the whole nucleus (a-d) or normalized to the corresponding siLUC experiment (e). Error bars, s.d. from n cells scored in at least three independent experiments. All microscopy images are confocal sections. Scale bars, 10 μm.

Figure 5: SETDB1 recruitment to UVC-damaged heterochromatin

(**a**) Recruitment of SETDB1 to damaged heterochromatin (white arrowheads) analyzed by immunofluorescence 1h30 after local UVC irradiation through micropore filters in NIH/3T3 GFP-DDB2 cells. (**b**, **c**) SETDB1 recruitment to damaged heterochromatin in cells treated with the indicated siRNAs (siLUC, control). siRNA efficiencies are controlled by western blot (Tubulin, loading control). Scatter plots represent log2 fold enrichments of repair proteins in damaged heterochromatin (HC) and damaged euchromatin (EC) compared to the whole

nucleus (a) or normalized to the corresponding siLUC experiment (b, c). Error bars, s.d. from n cells scored in three independent experiments. All microscopy images are confocal sections. Scale bars, 10 μm.

Figure 6: Model for heterochromatin maintenance following UVC damage

Recognition of UVC damage by the sensor protein DDB2 (1) triggers decompaction of damaged pericentric heterochromatin (2), thus facilitating access of downstream repair factors and histone chaperones (3) to the core of the domain. Histone chaperones promote the incorporation of newly synthesized H3 histones (in red), which subsequently acquire heterochromatin-specific modifications through the action of trimethylating enzymes (4). DDB2 release during repair progression allows heterochromatin recompaction (5).

Figure S1. Mammalian cellular model for studying heterochromatin maintenance in response to UV damage

(**a**) Engineered NIH/3T3 stable cell lines permit to track DNA repair events and to follow H3.3 histone deposition into chromatin in cells exposed to global or local UVC irradiation. (**b**) Cell cycle distribution analyzed by flow cytometry in the NIH/3T3 stable cell lines. (**c**) H3.3-SNAP and GFP-DDB2 expression analyzed by fluorescence microscopy in the indicated cell lines. (**d**) Total cell extracts of NIH/3T3 stable cell lines (same as in c) analyzed by western blot with the indicated antibodies. The top band detected by the H3.3 antibody corresponds to H3.3-SNAP. Tubulin is used as a loading control. (**e**) New H3.3 histone deposition (red) at UVC damage sites (CPD) analyzed by immunofluorescence in the indicated cell lines 45 min after local UVC irradiation through micropore filters. Histograms represent the fraction of cells showing new H3.3 histone accumulation at UV damage sites (red bars). Recruitment of the repair factor XPB to UV damage sites was analyzed by

immunofluorescence 30 min after local UVC irradiation in the same cell lines and plotted on the same histogram (green bars). Error bars, s.d. from two independent experiments scoring 150 cells in each experiment. Scale bars, 10 μm.

Figure S2. Histone marks in damaged pericentric heterochromatin domains

H3K9me3, H4K20me3 and H3K4me3 in damaged heterochromatin (white arrowheads) analyzed by immunofluorescence in NIH/3T3 GFP-DDB2 cells 1h after UVC laser micro-irradiation. H3K9me3 and H4K20me3 are heterochromatin-specific modifications associated with transcriptional silencing while H3K4me3 is a transcriptionally active histone mark used as negative control. Scale bars, 10 μm.

Figure S3. GFP-DDB2 tethering to pericentric heterochromatin domains

(**a**) Confocal sections showing the tethering of GFP-DDB2 to pericentric heterochromatin domains of NIH/3T3 GFP-DDB2 cells in the presence of catalytically dead Cas9 (dCas9). (**b)** Confocal sections showing no overlap between the DNA damage marker γH2A.X and GFP-DDB2 tethered to pericentric heterochromatin**. (c**, **d**) GFP intensity levels in heterochromatin domains quantified on reconstructed 3D images corresponding to Fig. 2c (c) and Fig. 2d (d). Error bars, s.d. from n cells scored in at least three independent experiments. Scale bars, 10 μm.

Figure S4. Validation in human MCF7 cells

Recruitment of the repair factor PCNA and of the histone chaperones CAF-1 (p60 subunit), DAXX and HIRA to UVC damaged heterochromatin domains (white arrowheads) analyzed 30 min (HIRA) or 1h30 (PCNA, CAF-1, DAXX) after local UVC irradiation through micropore filters in MCF7 cells. PCNA, CAF-1 and DAXX are detected by

immunofluorescence and HIRA upon transfection of HIRA-YFP. Constitutive heterochromatin is revealed by H3K9me3 or HP1α immunostaining. Sites of UVC damage repair are marked by Ethynyl-deoxyUridine (EdU, repair synthesis) or by immunodetection of the repair factor XPA. Insets show zoomed in views of heterochromatin domains (x1.8). All microscopy images are confocal sections. Scale bars, 10 μm.

Figure S5. DAXX accumulation in UVC-damaged heterochromatin

(**a**) Recruitment of DAXX and HIRA chaperones to damaged heterochromatin (white arrowheads) analyzed by immunofluorescence 1h30 after local UVC irradiation through micropore filters in NIH/3T3 GFP-DDB2 cells treated with the indicated siRNAs (siLUC, control). siRNA efficiencies are controlled by western blot (Tubulin, loading control). (**b**) Recruitment of ATRX to damaged heterochromatin (white arrowheads) analyzed by immunofluorescence 1h30 after local UVC irradiation in NIH/3T3 GFP-DDB2 cells. (**c**) DAXX recruitment to damaged heterochromatin upon ATRX knock-down (siLUC, control) 1h30 after local UVC irradiation in NIH/3T3 GFP-DDB2 cells. (**d**) Recruitment of DAXX to damaged heterochromatin (white arrowheads) 1h30 after local UVC irradiation in NIH/3T3 GFP-DDB2 cells. Cell cycle stages were defined based on staining of replication foci with Ethynyl-deoxyUridine (EdU). (**e**) Recruitment of DAXX to damaged heterochromatin analyzed in the indicated cell lines 1h30 after local UVC irradiation. DAXX total levels are shown on the western blot (Tubulin, loading control). The scatter plots show DAXX, ATRX and HIRA levels in damaged heterochromatin normalized to the corresponding siLUC experiment (a, c) or log2 fold enrichments compared to the whole nucleus (b, e). Error bars, s.d. from n cells scored in three independent experiments. All microscopy images are confocal sections. Scale bars, 10 μm.

Figure S6. Recruitment of histone methyltransferases to UVC-damaged heterochromatin

(**a**, **b**) Recruitment of the histone methyltransferases SUV39H1 (a) and EZH2 (b) to damaged heterochromatin (white arrowheads) analyzed by immunofluorescence 1h30 after local UVC irradiation through micropore filters in NIH/3T3 GFP-DDB2 cells. Scatter plots represent log2 fold enrichments compared to the whole nucleus. Error bars, s.d. from n cells scored in at least three independent experiments. All microscopy images are confocal sections. Scale bars, 10 μm. (**c**) Accumulation of newly synthesized H3.3 histones in UVC-damaged heterochromatin regions (white arrowheads) upon SETDB1 knockdown (siLUC, control) analyzed in NIH/3T3 GFP-DDB2 H3.3-SNAP cells 1h30 after local UVC irradiation through micropore filters. (**d**) Scatter plots representing log2 fold enrichments of SETDB1 in damaged heterochromatin normalized to the corresponding siLUC experiment upon knock down of H3.3 or CAF-1 (p150 subunit)

Supplementary information also includes 2 movies, which are located in a Google Drive folder and can be accessed by the following link:

https://drive.google.com/open?id=1i0QLZJ3MfecCbrdv8uWcwtCRSy8ZltI1

Movie S1: Pericentric heterochromatin decompaction following UVC laser irradiation (22 min kinetics). Related to Fig. 1b.

Heterochromatin decompaction visualized by Hoechst staining during the first 22 min following local damage with the UVC laser in a NIH/3T3 GFP-hDDB2 mouse fibroblast nucleus. 12 images were captured at 2 min intervals and are displayed at 2 frames/sec. The

resulting motion picture is shown with a superimposed white arrowhead pointing to the laser irradiation site.

Movie S2: Pericentric heterochromatin decompaction and recompaction following UVC laser irradiation (12 h kinetics). Related to Fig. 1b.

Heterochromatin decompaction and recompaction are visualized by Hoechst staining during the first 12 h following local damage with the UVC laser in a NIH/3T3 GFP-hDDB2 mouse fibroblast nucleus. 24 images were captured at the following time points: before UVC, 8 min, 30 min, 1h45, and every 30 min till 12 h, and are displayed at 2 frames/sec. The resulting motion picture is shown with a superimposed white arrowhead pointing to the laser irradiation site.

CHAPTER 10

Additional data

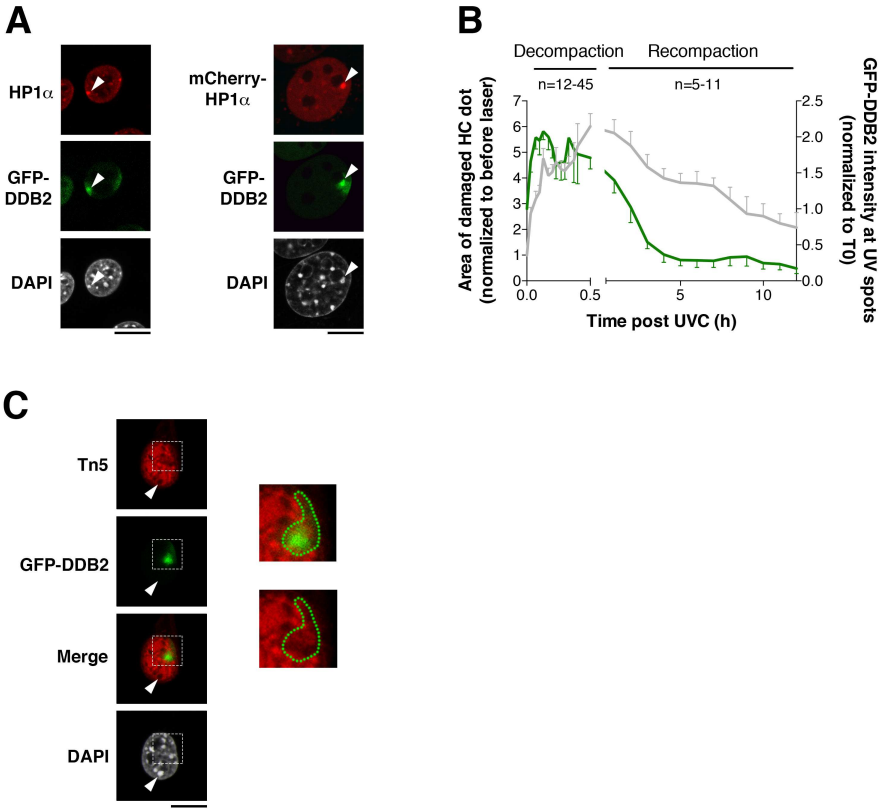

Figure 18 - Additional data. (**A**) Recruitment of HP1a to damaged heterochromatin (white arrowheads) analyzed by immunofluorescence and by transfection of mCherry-HP1a 1h30 after local UVC irradiation through micropore filters in 3T3 GFP-DDB2 cells. (**B**) Heterochromatin compaction changes and DDB2 recruitment kinetics upon UVC laser micro-irradiation are analyzed by live imaging in NIH/3T3 GFP-DDB2 cells stained with Hoechst. (**C**) ATAC-see in locally irradiated 3T3 GFP-DDB2 cells. The hyperactive transposase Tn5 incorporates fluorescent oligonucleotides in accessible chromatin regions. White arrowheads indicate pericentric heterochromatin domains; zoomed in views (x2.6) show damaged regions (delineated by green dotted lines). Error bars, s.e.m. from n cells scored in more than 5 independent experiments. All microscopy images are confocal sections. Scale bars, 10 µm.

THIRD PART

CONCLUSIONS & PERSPECTIVES

As stated in **Chapter 5**, the main objective of this project was to assess heterochromatin maintenance following DNA damage. By studying the response to UVC damage in pericentric heterochromatin domains in mammalian cells, I uncovered both shared (HIRA, CAF-1) and dedicated mechanisms (DAXX, SETDB1) for heterochromatin maintenance with respect to euchromatin domains. My experiments also revealed a critical role for the UV damage sensor DDB2 in orchestrating heterochromatin compaction changes during NER, as well as the uncoupling between heterochromatin folding and the maintenance of heterochromatin-specific histone marks. Finally, my findings support the idea of a tight cooperation between histone chaperones and modifying enzymes for the maintenance of heterochromatin-specific histone marks upon UV damage.

In the following chapters, I discuss the general implications of these findings.

CHAPTER 11

The access-repair-restore model in higher order chromatin domains

The access-repair-restore (ARR) model provides a molecular framework for chromatin dynamics in response to DNA damage. It describes the chromatin rearrangements that prime DNA for repair, together with the events allowing the restoration of chromatin architecture and function following repair completion. However, the current ARR model does not take into account the higher-order organization of chromatin in the cell nucleus, which could pose additional constraints to the accessibility of the repair machinery to the lesions, and where dedicated mechanisms might be needed to restore its distinctive epigenomic features.

11.1. Heterochromatin repair, cancer and evolution

Comparative studies of DNA repair in distinct chromatin domains in mammalian cells have uncovered the slower repair kinetics in heterochromatin with respect to euchromatin regions (Adar et al., 2016; Goodarzi et al., 2008; Han et al., 2016). The lower efficiency of DNA repair in heterochromatin is reflected in higher mutation rates in human cancers in heterochromatic regions (Schuster-Böckler and Lehner, 2012; Zheng et al., 2014). Noteworthy, besides contributing to tumorigenesis, high mutation rates in heterochromatin appear to be a driver of evolution. Indeed, the sequence composition of heterochromatic regions from even closely related species is often distinct (Makova and Hardison, 2015). Elucidating the connections between higher-order chromatin organization and mutation rates will thus provide important information about both fundamental evolutionary processes in the genome and about processes in diseases such as cancer.

In this study, we have seen that the UV damage sensor DDB2 promotes chromatin decompaction, which may facilitate the access of downstream NER factors to the core of UV-damaged heterochromatin domains. In humans, the absence of functional DDB2 results in the rare genetic condition Xeroderma Pigmentosum, complementation group E (XPE), characterized by a high predisposition to UV-induced skin cancers without any major UV photosensitivity (Chu and Chang, 1988; Tang et al., 2000). Based on this peculiar phenotype and on the fact that DDB2 is dispensable for NER in vitro, several studies have dismissed the role of DDB2 in DNA

repair, while highlighting its role in p53-mediated apoptosis (Itoh et al., 2003; Kulaksiz et al., 2005), thus suggesting that DDB2 deficiency leads to cancer not by defective NER repair, but by decreased apoptosis and the subsequent accumulation of mutations. Collectively, these data seem to indicate that DDB2 performs multiple functions in the cell, with DDB2 mutations leading to UV-induced skin cancers potentially by defects both in heterochromatic DNA repair and in several other pathways that control cellular responses to DNA damage, including p53-mediated cell cycle regulation and apoptosis. Understanding the exact role(s) of DDB2 in response to UV damage will open new avenues for the prevention of cancers in XPE patients.

Interestingly, in several cell lines and primary tissues from rodents, including the NIH/3T3 mouse embryonic fibroblasts used in this study, loss of the p53 responsive element in the DDB2 gene results in DDB2-deficient cells (Tan and Chu, 2002). In addition, the promoter of DDB2 in rodent cells is silenced by promoter methylation (Hwang et al., 1998). However, rodents are mostly nocturnal animals with fur, which may shield them from UV exposure and thus protect them from UV-induced carcinogenesis. These particularities of rodent cells should be taken into account when considering their validity as models for studying the XPE syndrome, DDB2 function and UV damage repair in general.

11.2. Restoration of heterochromatin following UV damage: histone dynamics

Deposition of newly synthesized histones at UV damage sites in human cells has been so far observed for different H3 (H3.3, H3.1) and H2A histone variants (H2A, H2A.X) (Adam et al., 2013; Dinant et al., 2013; Piquet et al., 2018; Polo et al., 2006). However, owing to the distinct histone variant composition of different chromatin regions, one could envision a differential deposition of newly synthesized histones, for example, in heterochromatin domains. Likewise, distinct histone chaperones could be involved in new histone deposition in the different domains, and their interactions with specific chromatin modifiers could further contribute to restore the particular identity of each chromatin region.

In our study, we focused on the dynamics of H3 histone variants and their chaperones in UV-damaged pericentric heterochromatin domains. The observed recruitment of the histone chaperone DAXX specifically to heterochromatin domains indeed pointed to heterochromatin-specific mechanisms governing post-UV histone dynamics in these domains. However, DAXX recruitment and function following DNA damage need to be further characterized. We also observed new H3.3 histone deposition by the histone chaperone HIRA in constitutive

heterochromatin. H3.1 dynamics could not be examined due to technical issues, but based on CAF-1 recruitment it is reasonable to assume that new H3.1 histones also get deposited in UV-damaged heterochromatin. Whether the newly synthesized histones are deposited in a nucleosomal form remains to be characterized. To complete this characterization, it will be of interest to examine the de novo deposition of other histone variants in damaged heterochromatin, including the H2A variants that have previously been found at UV damage sites (Dinant et al., 2013; Piquet et al., 2018). In light of the contribution of linker histones to chromatin compaction, it would also be important to investigate histone H1 dynamics in UV-damaged heterochromatin, which could potentially contribute to the observed heterochromatin decompaction/recompaction.

Another critical point that should be addressed is the relative contribution of new and parental histones in repaired heterochromatin. Unpublished work in our laboratory shows that new H3.3 histones account for approx. 10-15% of total H3.3 at UV damage sites in human cells, but these proportions could be different in densely packed heterochromatin domains. This information could be of major importance to understand the maintenance of histone PTMs following damage, as newly synthesized histones come with a particular and distinctive pattern of histone modifications. To address the above issues, the establishment and further use of additional cell lines stably expressing SNAP-tagged histone variants will be instrumental to allow the specific tracking of newly synthesized vs. parental histones and to study their dynamics in UV-damaged heterochromatin domains.

11.3. Restoration of heterochromatin following UV damage: uncoupling between heterochromatin features

Histone post-translational modifications are key epigenetic features controlling chromatin compaction and transcription. However, in our experiments we observed that UVC damage-mediated heterochromatin decompaction did not correlate with a loss of repressive histone marks, which implies that changes in higher-order chromatin organization may be governed by additional players other than histone PTMs and associated factors. These players might include factors involved in the DDR, such as repair factors or their interactors. Indeed, we have unveiled an important role for the early repair factor DDB2 in heterochromatin decompaction, and we have determined that DDB2 release is necessary to license chromatin recompaction. Additional factors coming into play might include the heterochromatin-specific chaperone DAXX or the heterochromatin factor HP1.

Interestingly, such uncoupling between histone PTMs and chromatin compaction changes had been previously observed in heterochromatin decompaction following DSB induction (Natale et al., 2017; Tsouroula et al., 2016) and in the formation of senescence-associated heterochromatic foci (SAHF, Chandra et al., 2012; Narita et al., 2003) in human fibroblasts. Indeed, SAHF formation results from the spatial rearrangement of pre-existing heterochromatin without changes in the linear epigenomic profiles of H3K9me3 and H3K27me3 repressive marks. Reciprocally, erasing H3K9me3 from pericentromeric heterochromatin domains by knocking down SUV39 enzymes does not trigger significant heterochromatin decompaction, further illustrating the uncoupling between histone PTMs and compaction in heterochromatin.

Regarding DNA methylation, a key epigenetic feature highly enriched in heterochromatin domains (Schübeler, 2015), little is known about its maintenance following DNA damage. However, owing to the reported evidence highlighting a crosstalk between DNA methylation and H3 methylation on several residues, including K9 (Jeltsch et al., 2018), a similar behavior to H3K9me3 could be expected for DNA methylation during heterochromatin repair. In addition, the fact that DDB2 has proved to be important for DNA methylation in plants (Schalk et al., 2016) establishes a potential link between UV damage repair and the maintenance of DNA methylation. Further studies will be needed to clarify the mechanistic connections among the different heterochromatin features during the restoration of heterochromatin organization and function following disruptive events such as DNA damage repair or DNA replication.

CHAPTER 12

Maintenance of the three-dimensional genome organization

Chromatin is organized within the 3D nuclear space, and the maintenance of such an organization is crucial for cell viability. Here, I discuss the potential consequences that disruptive events including DNA damage repair and DNA replication may have on the three-dimensional organization of different heterochromatin domains.

12.1. Effects of DNA damage and repair on higher-order heterochromatin organization

Damage-mediated decompaction of heterochromatin has been observed mainly using imaging approaches. It would be useful to complement these analyses with molecular approaches such as chromosome conformation capture techniques to fully understand the impact of such decompaction on the 3D organization of heterochromatin. Indeed, it is not known to which extent chromatin organization is altered upon damage-mediated decompaction: while decompaction could involve only local chromatin movements with the loss or enlargement of chromatin loops within topologically associated domains, it could also require more profound and global alterations of chromatin topology. In support of an interplay between the DDR and the 3D genome organization, cohesins have been found to accumulate at DSBs both in yeast and in human cells (Litwin et al., 2018). Chromatin immunoprecipitation studies further revealed that cohesins delineate γH2AX domains following DSB induction in human cells, thus controlling γH2AX spreading (Caron et al., 2012), and more recently, cohesins were found to inhibit transcription near DSBs (Meisenberg et al., 2019). However, the possible contribution of cohesins to (hetero)chromatin reorganization after UV damage still needs to be investigated. Interestingly, the heterochromatin factor HP1α has been found to interact with CTCF at TAD borders (Bosch-Presegué et al., 2017). Thus, it is tempting to speculate that HP1α recruitment to damaged heterochromatin could contribute to maintain/recover heterochromatin higher-order organization by impacting 3D genome folding.

12.2. Maintenance of distinct heterochromatin domains following DNA damage

While chromatin decondensation has been reported in response DNA damage in pericentric heterochromatin domains (Chiolo et al., 2011; Jakob et al., 2011; Tsouroula et al., 2016) and in the inactive X chromosome (Xi) in female cells (Müller et al., 2013), the DNA damage response in higher-order chromatin domains is not always accompanied by chromatin opening. In telomeres, for example, DNA damage drives telomere clustering instead (Timashev et al., 2017; Vancevska et al., 2017). Different heterochromatin domains bear distinct epigenetic marks and associated proteins, with pericentromeres being highly enriched in H3K9me3, H4K20me3 and HP1 proteins, while shelterin proteins associate with telomeric repeats and H3K27me3 characterizes the Xi. These differences may entail specific mechanisms to recover heterochromatin organization and function following DNA damage repair. Supporting this idea, DSB repair is achieved by different repair pathways in centromeric vs. pericentromeric heterochromatin regions (Tsouroula et al., 2016). Considering this, it would be interesting to determine if, similar to what observed in damaged pericentromeric heterochromatin domains, facultative heterochromatin marks are maintained following decompaction of the inactive X chromosome (Müller et al., 2013). Moreover, the study of heterochromatin maintenance in this particular heterochromatin context would allow for an interesting comparison between the response to damage in the heterochromatic inactive (Xi) and the euchromatic active (Xa) chromosomes, which are genetically very similar but differ in epigenetic features. The SAHFs (Zhang et al., 2005) in senescent cells are another interesting heterochromatin context to study the response to DNA damage owing to their peculiar 3D organization in the cell nucleus.

12.3. Heterochromatin maintenance during other disruptive events

Besides the DNA damage response, several other cellular events transiently disrupt heterochromatin organization. Among them, DNA replication prior to mitotic cell division is of particular interest, as it involves chromatin rearrangements similar to those observed during DNA damage. Indeed, new histones are deposited onto replicating DNA (reviewed in Alabert and Groth, 2012), which resembles the repair-coupled histone deposition of new histones after

damage. Therefore, it is reasonable to assume that analogous mechanisms could be involved in higher-order chromatin dynamics during repair and replication.

Interestingly, the histone chaperone CAF-1, which deposits newly synthesized H3.1 histones in damaged chromatin (Polo et al., 2006) and also accumulates at UV-damaged heterochromatin domains (this study), is critical for the duplication of pericentric heterochromatin through its interaction with HP1 in mouse cells (Quivy et al., 2004). Moreover, during replication, the HP1-CAF-1 complex associates with the histone methylatransferase SETDB1, which contributes to the recovery of heterochromatin hypermethylation by imposing H3K9me1 to histones that will later be trimethylated in mouse pericentric regions (Loyola et al., 2009). Given that CAF-1, HP1 and SETDB1 are recruited to UV-damaged heterochromatin, it is probable that they play a similar role in the maintenance of chromatin integrity following DNA damage. Supporting this hypothesis, SETDB1 has already been involved in DNA damage-induced H3K9me3 leading to sex chromosome inactivation in meiosis (Hirota et al., 2018).

Even though they share many common mechanisms, there are also important differences between heterochromatin maintenance during replication and repair. Notably, the deposition of new H3.3 histones detected at repair sites is normally not observed at replication foci (Ray-Gallet et al., 2011), and while PCNA can access the core of heterochromatin domains during repair (this study), it remains confined to the periphery of the domains during replication synthesis (Quivy et al., 2004).

Altogether, it will be interesting to see how many of these heterochromatin re-establishment mechanisms are shared with the de novo heterochromatin formation during development, which is a research area of intense investigation (Jansz and Torres-Padilla, 2019). Future studies will hopefully reveal how much we can learn from studying heterochromatin alterations post damage to answer fundamental questions regarding the importance of chromatin higher-order organization and its maintenance during various disruptive events.

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FORTH PART

ANNEXES

ANNEX 1:

Epigenome maintenance in response to DNA damage

Epigenome maintenance in response to DNA damage

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*: equal contribution

In this review, we describe the alterations of the chromatin landscape during DNA damage repair. We present the molecular players involved in the coordinated maintenance of genome and epigenome integrity, and explore the impact of chromatin organization into nuclear domains on the DNA damage response. Finally, we discuss the transient or definitive nature of chromatin alterations upon DNA damage, as well as their potential impact on the faithful maintenance of the epigenome.

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Epigenome Maintenance in Response to DNA Damage

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Organism viability relies on the stable maintenance of specific chromatin landscapes, established during development, that shape cell functions and identities by driving distinct gene expression programs. Yet epigenome maintenance is challenged during transcription, replication, and repair of DNA damage, all of which elicit dynamic changes in chromatin organization. Here, we review recent advances that have shed light on the specialized mechanisms contributing to the restoration of epigenome structure and function after DNA damage in the mammalian cell nucleus. By drawing a parallel with epigenome maintenance during replication, we explore emerging concepts and highlight open issues in this rapidly growing field. In particular, we present our current knowledge of molecular players that support the coordinated maintenance of genome and epigenome integrity in response to DNA damage, and we highlight how nuclear organization impacts genome stability. Finally, we discuss possible functional implications of epigenome plasticity in response to genotoxic stress.

Introduction: Functional Importance of Epigenome Integrity

The diversity of cell types in multicellular organisms is established during development through fine-tuned cell differentiation processes consisting of the activation and repression of specific transcription programs (Semrau and van Oudenaarden, 2015). Differential gene expression thus contributes to defining specialized functions and identities in cells sharing the same genome. It relies on epigenetic modifications acquired during development and stably inherited through cell divisions.

The epigenome, which supports such heritable changes in gene expression, consists of genome packaging into chromatin in the cell nucleus, where DNA is wrapped around histone proteins to form nucleosomes (Kornberg, 1977; Luger et al., 1997). DNA methylation (Schübeler, 2015), histone post-translational modifications (Bannister and Kouzarides, 2011), and the existence of histone variants that differ in their protein sequences (Maze et al., 2014) confer variability at the nucleosome level. Variations in nucleosome positioning, non-histone chromatin components, and higher-order chromatin folding further contribute to chromatin plasticity (Li and Reinberg, 2011). Chromatin can thus be viewed as a multifaceted signal integration platform that responds to intracellular and environmental cues by dynamic changes in epigenetic marks (Badeaux and Shi, 2013).

While epigenome plasticity is a key feature underpinning cell differentiation, epigenome maintenance is critical for the robustness of phenotypic traits, which is of fundamental importance not only during embryonic development but also for adult tissue homeostasis. Accordingly, failure to maintain epigenome integrity can have deleterious consequences for the organism, resulting in aging and disease (Zane et al., 2014).

However, DNA metabolic activities, such as transcription, replication, and repair, trigger dramatic chromatin alterations and thus pose a major challenge to epigenome maintenance. Among them, DNA damage repair is unique in the sense that it is generally non-programmed, since DNA damage can occurr anywhere in the genome at any time, and also versatile, owing to the variety of DNA lesions (Ciccia and Elledge, 2010; Hoeijmakers, 2009; Jackson and Bartek, 2009). Here, we focus on chromatin dynamics during the repair response in the mammalian cell nucleus. We review recent progress in delineating by which mechanisms and to what extent epigenome integrity is affected and restored after DNA damage, underlying cell fate maintenance.

DNA Damage Challenges Epigenome Integrity

DNA damage poses a serious threat to cell viability by compromising both genome and epigenome integrity. Indeed, the DNA damage response (DDR) is accompanied by significant alterations of chromatin structure, affecting intrinsic chromatin components and epigenetic marks, with major implications for epigenome functions.

Chromatin Rearrangements in Response to DNA Damage

A large number of studies based on biochemical and imaging approaches in several eukaryotic cell systems highlight the marked rearrangements of damaged chromatin (Figure 1A). In particular, chromatin decondensation, accompanied by a loss of density of core and linker histones, has been reported in human cells exposed to local UVC irradiation (Luijsterburg et al., 2012) or to laser microirradiation-induced DNA breaks (Kruhlak et al., 2006; Smeenk et al., 2013; Strickfaden et al., 2016), in a poly(ADP)ribose polymerase (PARP)-dependent manner. Moreover, there is accumulating evidence for nucleosome loss from the damaged region, increase in core histone extractability, and even histone eviction from damaged chromatin, with partial or complete nucleosome disruption (reviewed in Adam et al., 2015a; Bakkenist and Kastan, 2015; Rodriguez et al., 2015). Epigenome perturbations after DNA damage culminate with the degradation of chromatin components, as described for acetylated histones in yeast and mouse cells exposed to alkylating damage or ionizing radiation (IR) (Qian et al., 2013).

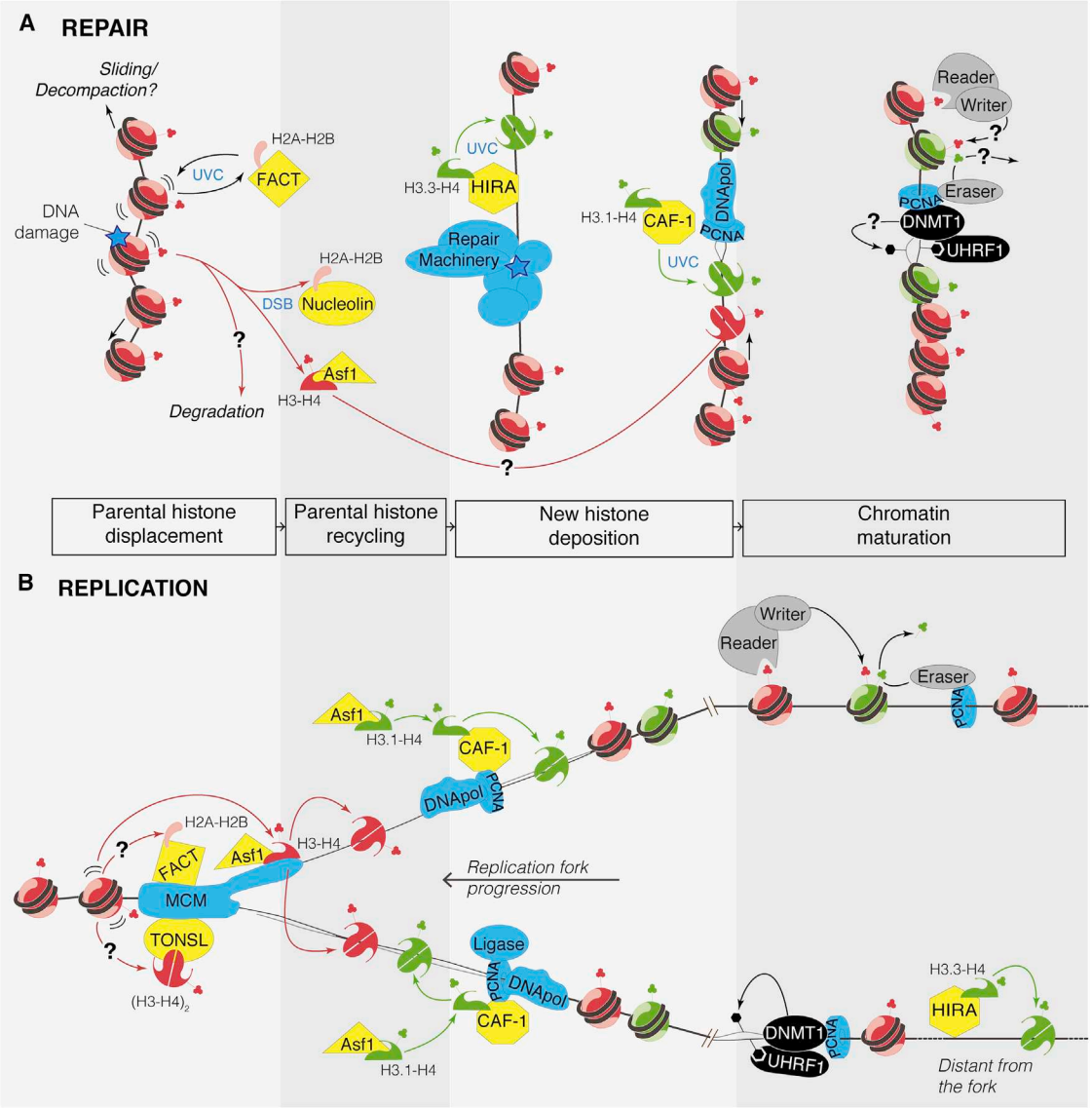

Figure 1. Epigenome Maintenance in Mammalian Cells: Parallel between Replication and Repair

DNA repair (A) and replication (B) involve similar chromatin dynamics with shared histone chaperones (yellow) promoting the mobilization of parental histones (red) and the deposition of newly synthesized histones (green). For simplicity, shared chromatin remodeling complexes are not represented. Both at the replication fork and at UV damage repair sites, new H3.1 deposition by CAF-1 is coupled to DNA synthesis while new H3.3 histones are deposited by HIRA independently of DNA synthesis. The histone chaperone FACT also promotes histone dynamics in response to UVC damage, while ASF1 and Nucleolin are involved in the response to DSBs. Subsequent chromatin maturation involves erasure of naive histone marks (green), transmission of parental histone marks (red) to newly synthesized histones, and maintenance of DNA methylation (black). Although such processes have been described at the replication fork, related mechanisms at repair sites are still to be characterized. Repair and replication factors are represented in blue.

Likely candidates for promoting histone mobility in response to DNA damage are chromatin remodeling factors, which use the energy of ATP hydrolysis to slide, exchange, and/or evict histone proteins from chromatin (Bartholomew, 2014). Indeed, all major families of remodelers are recruited to damaged chromatin and contribute to DNA damage repair (reviewed in Aydin et al., 2014b; Lans et al., 2012; Seeber et al., 2013). Furthermore, remodelers can have a direct effect on chromatin compaction and histone mobilization at sites of DNA damage, as recently shown for the human remodeling factor chromodomain helicase DNA-binding protein 2 (CHD2) in response to DNA double-strand breaks (DSBs) (Luijsterburg et al., 2016). In parallel, and independently of ATP hydrolysis, histone chaperones (Gurard-Levin et al., 2014) contribute to histone dynamics in damaged chromatin. This is exemplified at DSBs induced by the site-specific endonuclease I-PpoI in human cells, where the Nucleolin and anti-silencing function 1 (ASF1) chaperones promote nucleosome disassembly by evicting outer and inner core histones, respectively (Goldstein et al., 2013). It is important to stress that while the alteration of chromatin organization is a general

response to DNA damage, different types of DNA lesions likely involve distinct chromatin rearrangements and underlying mechanisms.

Non-histone chromatin components also undergo dynamic changes upon DDR activation in mammalian cells, with the accumulation of silencing factors like heterochromatin protein 1 (HP1), polycomb group proteins, histone deacetylases, histone methyltransferases, and DNA methyltransferases (DNMTs) at sites of DNA damage, accompanied by chromatin modifications including DNA methylation and repressive histone marks (Alagoz et al., 2015; Dinant and Luijsterburg, 2009; Gong and Miller, 2013; Gursoy-Yuzugullu et al., 2016; O'Hagan, 2014; Soria et al., 2012; Vissers et al., 2012).

More generally, a plethora of damage-induced post-translational modifications, affecting histone proteins and chromatin-associated components, have been identified (reviewed in Dantuma and van Attikum, 2016). Among them, phosphorylation, acetylation, ubiquitylation, and PARylation are the best studied, a prominent example being the evolutionarily conserved phosphorylation of a carboxy-terminal serine in the H2AX variant, so-called γH2AX, which plays a pivotal role in response to DSBs (reviewed in Scully and Xie, 2013). Proteomic screens have been instrumental for identifying the multiple targets of post-translational modifications occurring in response to UV, IR, oxidative, and alkylation damage in human cells (Elia et al., 2015; Jungmichel et al., 2013; Matsuoka et al., 2007; Povlsen et al., 2012). However, much remains to be learned about the specific, and possibly combinatorial, roles of chromatin modifications after a genotoxic stress challenge, especially when considering that several of these modifications are not necessarily unique to the DDR.

Spatiotemporal Regulation of Chromatin Alterations

Chromatin alterations in response to DNA damage undergo a tight spatiotemporal regulation that only begins to be unraveled. Indeed, several recent studies in human cells have shown that transient compaction follows chromatin relaxation at sites of DNA breaks (Ayrapetov et al., 2014; Burgess et al., 2014; Khurana et al., 2014; Strickfaden et al., 2016), which could be driven by the antagonistic activities of chromatin remodeling complexes, as proposed for CHD3 and imitation switch (ISWI) in response to heterochromatic DSBs (Klement et al., 2014). The temporal dynamics of chromatin changes following DNA damage further emphasize the fluid nature of chromatin organization during repair. Rearrangements of damaged chromatin are also regulated during the cell cycle: a complete disruption of nucleosomes has been observed around DSBs in asynchronous human cells, with a loss of all core histones, while nucleosomes are only partially disrupted in G1-arrested cells (Goldstein et al., 2013). This disparity likely reflects the predominance of distinct DSB repair pathways in different cell-cycle stages, non-homologous end joining (NHEJ) being the preferred repair pathway in G1, while homologous recombination (HR) predominates in S/G2 (Ceccaldi et al., 2016).

In addition, chromatin dynamics in response to DNA damage are spatially regulated. Indeed, it has been shown in human cells that chromatin is more accessible at laser damage sites and more compact in adjacent regions (Hinde et al., 2014). This observation emphasizes that changes in chromatin organization are not restricted to the damaged area. Genome-wide rearrangements of the epigenetic landscape also take place during the DDR, such as global reprogramming of H3K27ac upon UV irradiation in mammalian cells, which underlies expression changes of critical genes during the stress response (Schick et al., 2015). Similarly, UV irradiation triggers genome-wide hyperacetylation on H3 and H4 in budding yeast (Yu et al., 2005). Further highlighting that the response to DNA damage is integrated in the nuclear space, an increased mobility of chromatin surrounding DSBs has been reported in yeast (Dion and Gasser, 2013; Miné-Hattab and Rothstein, 2013), for heterochromatic breaks in *Drosophila* (Ryu et al., 2015), and for damaged or uncapped telomeres in mammalian cells (Chen et al., 2013; Cho et al., 2014; Dimitrova et al., 2008) in a microtubule-dependent manner (Lottersberger et al., 2015).

Functional Relevance of Chromatin Alterations

The above-described changes in chromatin organization after DNA damage have important functional consequences on DNA damage signaling and repair. As proposed several decades ago in the access-repair-restore (ARR) model (Polo and Almouzni, 2015; Smerdon, 1991), transient disruption of chromatin organization is thought to facilitate access of the repair machinery to damaged DNA. Recent work in human cells supports this hypothesis by showing that CHD2 remodeling activity promotes chromatin expansion at DSBs and the recruitment of NHEJ factors (Luijsterburg et al., 2016). Furthermore, damage-induced histone modifications, including γH2AX, H2AK15 ubiquitination, and H4K20 methylation, are critical for DNA damage signaling and repair progression as they provide a structural basis for the coordinated recruitment of DDR effectors through the binding of histone readers (Figure 2; reviewed in Dantuma and van Attikum, 2016; Smeenk and van Attikum, 2013). Notably, such modifications are not restricted to core histones, as linker histone ubiquitylation was recently identified as a key player in DNA damage signaling in response to IR in human cells (Thorslund et al., 2015). The prominent role of histone modifications in this process highlights the importance of the balance between histone-modifying enzymes—writers and erasers—in fine-tuning the DDR. Histone dynamics also contribute to regulating repair pathway choice and repair efficiency, as reported for H2A.Z, whose dynamic exchange at DSBs in human cells appears to control DNA end resection, DSB repair by HR, and NHEJ (Alatwi and Downs, 2015; Gursoy-Yuzugullu et al., 2015; Xu et al., 2012). Similarly, the dynamics of silencing factors like HP1 and the co-repressor KRAB-associated protein 1 (KAP1) are of major importance as their sustained retention on chromatin impairs DSB repair by HR (Kalousi et al., 2015).

While one can appreciate the functional relevance of chromatin opening and dynamics in the DDR, it was not straightforward to understand the role of chromatin compaction that precedes its relaxation and was also observed at the periphery of damaged areas. Recent studies have demonstrated that transient chromatin condensation is actually necessary and sufficient for stimulating DNA damage signaling in human cells (Burgess et al., 2014). It is also conceivable that local chromatin compaction can facilitate the ligation of broken DNA ends by keeping them in close proximity. Finally, the recruitment of silencing factors and the establishment of repressive histone

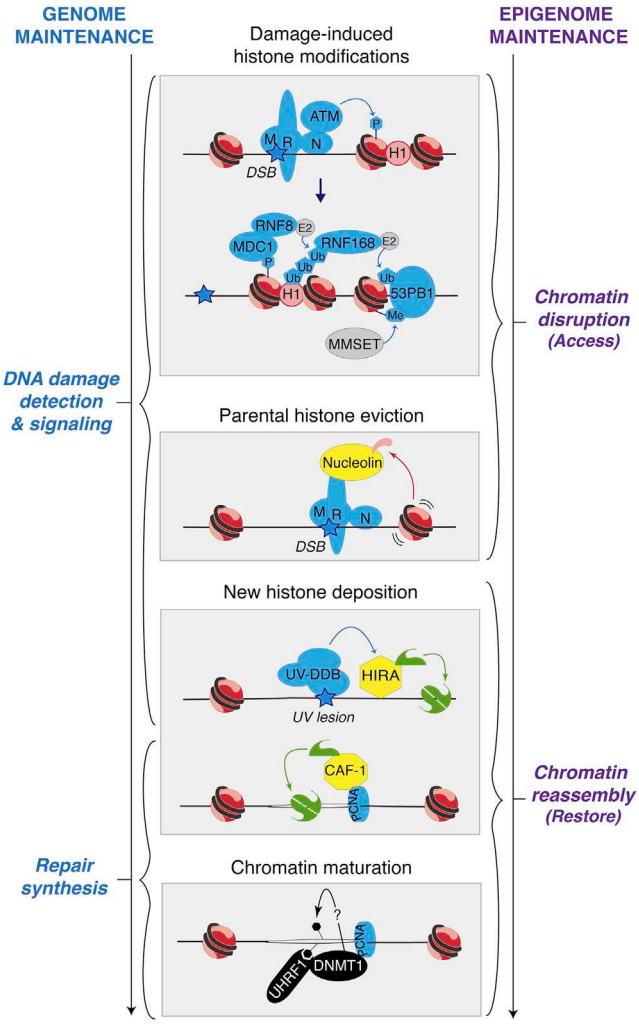

Figure 2. Coordination between Genome and Epigenome Maintenance in Response to DNA Damage

The coordinated maintenance of genome integrity and epigenome stability along the repair process in mammalian cells is supported by direct molecular interactions of DDR factors (blue) with histone modifications (P, phosphorylation; Ub, ubiquitination; Me, methylation), histone chaperones (yellow), and the DNA methylation machinery (black). DNA damage signaling factors are as follows: ATM, ataxia telangiectasia mutated; MDC1, mediator of DNA damage checkpoint 1; RNF, RING finger protein; MMSET, multiple myeloma SET domain; 53BP1, TP53-binding protein 1.

marks on damaged chromatin can participate in silencing transcription at sites of DNA damage, thus avoiding deleterious interference between transcription and repair machineries (Chou et al., 2010; Gong et al., 2015; Kakarougkas et al., 2014; Ui et al., 2015).

Restoration of Epigenome Integrity after DNA Damage

New Histone Deposition

In order to preserve cell identity and function, epigenome integrity must be restored after DNA damage repair. A salient feature of this restoration step is the deposition of newly synthesized histones into damaged chromatin, as first characterized in response to local UVC damage in human cells (Figure 1A). Transient transfection of cells with epitope-tagged histones indeed revealed the de novo incorporation of the H3.1 histone variant and of the outer core histone H2A at sites of local UVC irradiation (Dinant et al., 2013; Polo et al., 2006). Combination of local UVC damage with specific tracking of newly synthesized histones using the SNAP-tag technology (Adam et al., 2015b) then established that the histone variant H3.3, but not the centromeric H3 variant centromeric protein A (CENPA), was also deposited de novo in damaged chromatin (Adam et al., 2013). Notably, both high-frequency point mutations and transcription repression of genes encoding H3.3 have been uncovered in several human cancers, particularly in brain tumors (Gallo et al., 2015; Kallappagoudar et al., 2015). It will be interesting to investigate whether these alterations impact epigenome restoration after DNA damage, thus contributing to tumorigenesis. More generally, how cancer-associated chromatin alterations (Zane et al., 2014) may affect DNA repair and the resetting of epigenetic marks during repair deserves to be explored.

Among the molecular players in repair-coupled chromatin assembly, histone chaperones play pivotal roles in the restoration of chromatin integrity after DNA damage by promoting new histone deposition. In particular, the histone chaperone chromatin assembly factor-1 (CAF-1) escorts H3.1 in human cells (Tagami et al., 2004) and stimulates its de novo incorporation into UVC-damaged chromatin in a manner coupled to repair synthesis (Polo et al., 2006). In vitro experiments, revealing the direct interaction and functional synergy between the human chaperones CAF-1 and ASF1 during UVC damage repair, raise the possibility that ASF1 could also be involved in new histone deposition upon cell exposure to damaging agents, potentially as a histone donor for CAF-1 (Mello et al., 2002). Furthermore, the recent identification of CAF-1 as a chaperone for the H3.2 histone variant (Latreille et al., 2014) suggests an even wider role for CAF-1 in depositing newly synthesized H3 variants during UV-damaged chromatin restoration. Contrary to CAF-1-mediated H3.1 deposition that is coupled to late repair steps, newly synthesized H3.3 histones are deposited early after damage by the H3.3-specific chaperone histone regulator A (HIRA), which is recruited to UVC-damaged chromatin in response to Cullin4-dependent ubiquitylation events occurring upon damage detection (Adam et al., 2013). Intriguingly, HIRA is also required for the deposition of new H3.1, thus revealing a possible crosstalk between H3 variant deposition pathways at repair sites. Finally, the histone chaperone facilitates chromatin transcription (FACT) presumably stimulates new H2A deposition in damaged chromatin because it is responsible for the accelerated turnover of H2A at sites of UVC irradiation (Dinant et al., 2013). Notably, FACT-mediated H2A exchange and HIRA-dependent H3.3 incorporation at damaged sites are shared mechanisms between UVC damage and DSB responses in human cells (Heo et al., 2008; Yang et al., 2013). Furthermore, the budding yeast orthologs of CAF-1 and HIRA likely contribute to chromatin restoration also at meiotic DSBs (Brachet et al., 2015).

Moreover, there is now compelling evidence for an important role of ATP-dependent chromatin remodelers at damage sites (reviewed in Lans et al., 2012; Smeenk and van Attikum, 2013). Current evidence suggests that chromatin remodelers function mostly in providing access to chromatin and regulating repair.

These remodelers may also assist histone chaperones and/or directly participate in new histone deposition coupled to repair, as observed during transcription. For example, p400, CHD1, and CHD2 deposit H3.3 in transcriptionally active chromatin regions in mammalian cells (Harada et al., 2012; Pradhan et al., 2016; Siggens et al., 2015). Interestingly, the CHD2 remodeler was recently shown to promote new H3.3 accumulation at sites of laser microirradiation-induced damage in human cells (Luijsterburg et al., 2016). One can also hypothesize that chromatin remodelers may help restore nucleosome positioning/spacing after damage.

Beyond the restoration of chromatin structure by new histone deposition, histone chaperones also contribute to the re-establishment of a transcriptional program after UVC damage, as shown for HIRA (Adam et al., 2013) and FACT in human cells (Dinant et al., 2013). Besides histone chaperones, the histone-modifying enzyme DOT1-like H3K79 methyltransferase (DOT1L) is also required for transcription recovery after UVC damage in mouse cells (Oksenych et al., 2013). However, the molecular bases for how histone dynamics mediated by these chromatin-associated factors contribute to transcription restart are still under investigation. Interestingly, the significant delay observed between HIRA release from damaged chromatin and transcription restart opens up the possibility that new H3.3 histones deposited by HIRA in damaged chromatin could act as a bookmark, licensing chromatin for transcription restart once repair is complete (Adam et al., 2013).

The deposition of new histones at damaged sites also raises the fundamental question of the contribution of parental histones to chromatin restoration after repair. Since parental histones are present in chromatin before damage and carry the original epigenetic information, it will be important to investigate whether they are replaced by newly synthesized histones during chromatin repair and to what extent they participate in the maintenance of epigenome integrity after damage.

DNA and Histone Modifications

While deciphering the contribution of histone dynamics to restoring chromatin integrity is in progress, how the parental histone and DNA modifications are maintained in response to damage is still uncharacterized. The recruitment of the maintenance DNA (cytosine-5-)-methyltransferase 1 (DNMT1) to repair sites upon UVA laser microirradiation (Mortusewicz et al., 2005), oxidative damage (O'Hagan et al., 2011), and site-specific DSBs in human cells (O'Hagan et al., 2008) suggests that, like during DNA replication, DNMT1 may contribute to methylating newly synthesized DNA at repair sites. Another key epigenetic regulator of DNA methylation, ubiquitin-like with PHD and ring finger domains 1 (UHRF1), which maintains cytosine methylation and facilitates DNMT1 accumulation, is recruited to DNA interstrand crosslinks and DSBs in human cells (Liang et al., 2015; Tian et al., 2015; Zhang et al., 2016). Although no evidence for UHRF1 involvement in DNA methylation maintenance at repair sites has been provided so far, UHRF1 interacts with repair factors and regulates lesion processing.

Even less is known about the maintenance of histone modifications at sites of DNA damage repair. Since histone post-translational modifications (PTMs) are key epigenetic marks in the control of gene expression and are challenged by DNA damage, it is important that DNA damage-induced changes in histone PTMs are erased and original PTMs are re-established. However, whether and by which mechanisms this actually occurs is still an open question. Moreover, the fact that newly synthesized histones deposited into chromatin during repair come with their own set of modifications (Loyola et al., 2006) further complicates the maintenance of the original histone PTM landscape. Future work will determine if parental histone marks are transferred to newly deposited histones after repair.

Altogether, this illustrates the lack of mechanistic insights into how DNA and histone modifications are preserved after DNA damage, a question that has been approached in the context of DNA replication.

Lessons from Chromatin Replication

During the duplication of the genetic material in S phase of the cell cycle, chromatin undergoes dramatic alterations (reviewed in Alabert and Groth, 2012; Annunziato, 2015; Ramachandran and Henikoff, 2015) that bear striking similarity with chromatin rearrangements that occur during DNA damage repair (Figure 1).

Parallel between Epigenome Maintenance during Replication and Repair

Similar to the transient chromatin rearrangements observed at sites of DNA damage, DNA replication leads first to the disruption of pre-existing nucleosomes ahead of the replication fork. Parental histones then symmetrically segregate between the leading and lagging DNA strands, and contribute to nucleosome reassembly downstream of the replication fork together with newly synthesized histones. New histones are deposited onto replicating DNA by a replication-coupled nucleosome assembly process (reviewed in Alabert and Groth, 2012; Ramachandran and Henikoff, 2015), which resembles the repair synthesis-coupled deposition of new histones after damage. Notably, the coupling between chromatin assembly and DNA replication also applies to lagging strand synthesis as new histone deposition is required for the termination of Okazaki fragments in budding yeast (Smith and Whitehouse, 2012). However, while the replication process results into a semi-conservative mode of inheritance at the genome and epigenome levels with a 2-fold dilution of parental histones (Alabert et al., 2015), the relative contributions of parental and new histones to the composition of repaired chromatin are still to be determined. Furthermore, in contrast to the massive synthesis of canonical replicative histone variants that occurs in S phase and provides the necessary supply for new histone deposition at the replication fork (Marzluff et al., 2008), there is no evidence for an induction of histone synthesis in response to DNA damage. Canonical histone synthesis is even inhibited due to DNA damage checkpoint activation, as described in human cells exposed to IR (Su et al., 2004). Despite these discrepancies, epigenome maintenance during replication and that during repair have much in common. Studying chromatin duplication can thus give interesting insights into how chromatin integrity is preserved in response to DNA damage.

Shared Molecular Players: Focus on Histone Chaperones

Chromatin dynamics during replication and repair share several molecular players (Figure 1), in particular histone chaperones

and chromatin remodelers. Indeed, as is the case at repair sites, the histone chaperone CAF-1 deposits new H3.1 at replication foci in human cells (Ray-Gallet et al., 2011; Smith and Stillman, 1991). Furthermore, the direct interaction of CAF-1 with the polymerase sliding clamp proliferating-cell nuclear antigen (PCNA) ensures proper coordination between new histone deposition and DNA synthesis both at replication (Shibahara and Stillman, 1999) and repair sites (Moggs et al., 2000). Similarly, the interaction of the histone chaperone ASF1 with the replicative helicase subunit MCM2 (minichromosome maintenance 2) through an H3-H4 histone bridge likely coordinates replication fork progression with parental histone transfer in human cells (Groth et al., 2007; Huang et al., 2015; Richet et al., 2015; Wang et al., 2015). Interestingly, human ASF1 participates in parental H3-H4 histone eviction at I-PpoI cut sites (Goldstein et al., 2013). It is thus tempting to speculate that this chaperone could play a central role in parental histone recycling in response to DNA damage, as proposed at the replication fork. In addition, human ASF1 functions as a histone buffer upon replication fork stalling, chaperoning H3-H4 histones within a multi-chaperone complex also containing the histone chaperone nuclear autoantigenic sperm protein (NASP) (Groth et al., 2005). NASP protects soluble H3-H4 from degradation (Cook et al., 2011), hence making it a good candidate for chaperoning evicted histones in response to DNA damage. Importantly, the replicative helicase minichromosome maintenance (MCM) associates with several histone chaperones in addition to ASF1. The human histone chaperone tonsoku-like-MMS22-like (TONSL- MMS22L) bound to MCM5 is thought to have a role in replication-coupled histone eviction (Campos et al., 2015). Since TONSL localizes to UV damage sites in human cells (Hill et al., 2014) and promotes HR of replication-associated DSBs (Duro et al., 2010; O'Connell et al., 2010; O'Donnell et al., 2010), it is tempting to speculate that this chaperone could stimulate histone dynamics in damaged chromatin. The histone chaperone FACT also forms a complex with MCM and histones in yeast and human cells (Foltman et al., 2013; Tan et al., 2006). Considering that FACT disrupts nucleosome structure by competing with DNA for binding H2A-H2B (Hondele et al., 2013; Kemble et al., 2015), this chaperone could initiate nucleosome disassembly at the replication fork. Notably, even though the literature does not report a direct role for FACT in nucleosome disassembly at repair sites, FACT promotes chromatin exchange of the damage-responsive histone variant H2AX (Heo et al., 2008) and H2A turnover at sites of UVC irradiation in human cells (Dinant et al., 2013).

Inheritance of Histone Marks

Beyond histone dynamics promoted by histone chaperones, the inheritance of histone marks at the replication fork is another critical aspect of epigenome maintenance that has stimulated intense research. Differential labeling of old and new histones, combined with immunodetection or quantitative proteomics of histone PTMs, has been instrumental for monitoring epigenome inheritance at the replication fork (reviewed in Annunziato, 2015; Huang et al., 2013). A recent study in human cells thus demonstrates that parental histones are recycled with their PTMs at replication forks and that all parental PTMs are restored within one cell cycle (Alabert et al., 2015). This is achieved by the modification of new histones with parental marks, except for H3K9 and K27 trimethylation marks, which require continuous modification of both parental and new histones. It contrasts with the situation in *Drosophila* embryos, where parental histone methylation marks are erased during replication and re-established through the stable association of histone methyltransferases with replicating chromatin (Petruk et al., 2012). While these studies shed light on how histone PTMs are propagated during replication, further work is needed to characterize the inheritance of histone marks after DNA damage repair. Isolation of nascent chromatin at repair sites, employing similar experimental strategies as those developed for replication forks (Alabert et al., 2014), or pulling down specifically UVC-damaged chromatin (Zavala et al., 2014) could be useful tools to explore this issue. Likewise, the induction of transient site-specific DSBs, combined with histone mark profiling (Aymard et al., 2014; Massip et al., 2010), appears to be a powerful technique to follow changes in histone modifications during DNA damage repair. To approach this issue, it could also be interesting to focus on chromatin domains characterized by a specific set of histone marks like heterochromatin (Saksouk et al., 2015).

Duplication of Higher-Order Chromatin Structures

How heterochromatin affects DNA damage repair has been the focus of intense research in recent years (detailed below). Reciprocally, it will be important to investigate how higher-order chromatin structures are altered during the DDR and re-established after repair. It is reasonable to assume that similar mechanisms could support higher-order chromatin dynamics during repair and replication. In this respect, the interaction of the histone chaperone CAF-1 with the heterochromatin protein HP1 is critical for the duplication of pericentric heterochromatin domains in mouse cells (Quivy et al., 2004, 2008). The HP1-CAF-1 tandem also associates with the histone methyltransferase SET domain, bifurcated 1 (SETDB1), which imposes H3K9me1 for subsequent trimethylation in mouse pericentric regions (Loyola et al., 2009), and with methyl-CpG binding domain protein 1 (MBD1), which couples replication of methylated DNA with maintenance of the H3K9me3 heterochromatic mark in human cells (Sarraf and Stancheva, 2004). Given that HP1, SETDB1, and CAF-1 are involved in chromatin dynamics in response to DNA damage, it will be worthwhile investigating how they contribute to maintaining heterochromatin integrity.

Future work will determine if the mechanisms and molecular players highlighted in the above studies focused on DNA replication also underlie epigenome maintenance during DNA damage repair.

Coordinated Maintenance of Genome and Epigenome Integrity in Response to DNA Damage

Safeguarding both genetic and epigenetic information after damage entails orchestrating chromatin dynamics with the DDR to ensure timely and efficient repair. Recent studies indeed put forward genome and epigenome maintenance as an integrated process, supported by direct molecular interactions of DDR factors with chromatin modifiers and histone modifications (Figure 2).

In particular, histone modifications resulting from the activity of DNA damage checkpoint proteins are integral components of the DNA damage signaling cascade (reviewed in Smeenk

and van Attikum, 2013). Another critical step, early on in the DDR, is the coordination between DNA damage detection and chromatin dynamics, which is thought to facilitate access to downstream repair factors. This is exemplified with the histone chaperone Nucleolin, which is rapidly recruited to DSBs via its interaction with the RAD50 subunit of the DSB recognition complex MRE11-RAD50-NBS1 (MRN), thereby mediating nucleosome disruption and the recruitment of downstream repair factors at I-PpoI cut sites in mammalian cells (Goldstein et al., 2013). Nucleosome destabilization at DSBs is also promoted by the ATPase p400 and the acetyltransferase Tip60 (Xu et al., 2012; 2010). Notably, Tip60 is targeted to DSBs by the MRN complex (Sun et al., 2009), and p400 recruitment requires the checkpoint protein mediator of DNA damage checkpoint 1 (MDC1) (Xu et al., 2010), thus providing additional molecular bases for coordinating damage detection and signaling with histone dynamics.

Similarly, during the UVC damage response, new histone deposition by HIRA is linked to damage detection by the human UV-damaged DNA-binding complex (UV-DDB) (Adam et al., 2013). Later on during repair synthesis, the polymerase sliding clamp PCNA acts as a molecular anchor for the CAF-1 histone chaperone (Moggs et al., 2000; Polo et al., 2006). PCNA is also thought to coordinate completion of mismatch repair with CAF-1-mediated nucleosome assembly (Schöpf et al., 2012). Contrary to CAF-1 and HIRA, the recruitment of the histone chaperone FACT to damaged chromatin has not been directly connected to DNA damage repair, and the mechanisms underlying FACT recruitment to UVC damage sites and IR-induced foci remain to be elucidated (Dinant et al., 2013; Oliveira et al., 2014). Like histone chaperones, chromatin remodelers also have direct connections to damage signaling and repair machineries, as exemplified by the human SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 5 (SMARCA5), which interacts with the damage signaling factor ring finger protein 168 (RNF168) in a PARP-dependent manner, stimulating RNF168 accumulation at DSBs and downstream signaling events (Smeenk et al., 2013). The SMARCA5-associated subunit ATP-utilizing chromatin assembly and remodeling factor 1 (ACF1) directly interacts with the NHEJ factor Ku70 and promotes Ku accumulation at DSBs (Lan et al., 2010). SMARCA5 also associates, directly or indirectly, with the early repair factor Cockayne syndrome B protein (CSB) and stimulates CSB recruitment to UVC-damaged chromatin (Aydin et al., 2014a).

Similar to damage-induced histone dynamics and chromatin remodeling, the maintenance of DNA methylation is likely to be directly coupled to DNA damage signaling and repair. Indeed, recent studies in human cells report that the maintenance DNA methyltransferase DNMT1 is recruited to DSBs via PCNA and the checkpoint factors Chk1 and 9-1-1 (Ha et al., 2011), and via mismatch repair proteins to sites of oxidative damage (Ding et al., 2015).

Interestingly, the coupling between chromatin dynamics and the DDR also provides a means to coordinate damaged chromatin disruption and reassembly, as both are connected to repair. To validate this hypothesis, it will be useful to examine the possible crosstalks between parental and newly synthesized histone dynamics at damage sites.

Furthermore, the coupling between genome and epigenome maintenance after damage actually goes both ways, with chromatin restoration having a feedback effect on DNA damage repair and genome stability in some instances. Although new H3.1 and H3.3 deposition in UVC-damaged chromatin are dispensable for UVC damage repair in human cells (Adam et al., 2013; Polo et al., 2006), H3.3 is important for maintaining genome stability in mouse embryonic fibroblasts (Jang et al., 2015) and for replication fork progression in response to UV damage in chicken cells (Frey et al., 2014). Furthermore, HIRA-mediated histone replacement contributes to protecting DNA from damage during mouse oogenesis (Nashun et al., 2015), arguing that histone dynamics can also be crucial for the maintenance of genome integrity.

In addition to the direct coupling between chromatin dynamics and the DDR, which takes place during DNA damage repair, epigenome maintenance may also rely on chromatin maturation events after completion of DNA repair, similar to those that occur after DNA replication (Figure 1). It will be of major interest to further investigate the stepwise restoration of chromatin after damage to determine when and by which mechanisms the original epigenetic information is fully re-established (in terms of histone modification, histone variant and DNA methylation patterns, and nucleosome spacing). In particular, how repair completion and termination of checkpoint signaling are coordinated with the erasure of DNA damage-induced histone modifications remains to be elucidated. Ultimately, understanding the coordination between DNA damage repair and the re-establishment of higher-order chromatin structures and nuclear domains would complete our view of epigenome maintenance in response to DNA damage.

Impact of Nuclear Organization on Genome Maintenance in Response to DNA Damage

One of the most fundamental properties of the eukaryotic cell nucleus is the non-random organization of the genome within the tridimensional space of the nucleus, where chromosomes occupy discrete territories and which determines long-range chromatin interactions (Dekker and Misteli, 2015; Sexton and Cavalli, 2015). Nuclear organization plays a critical role not only in the regulation of gene expression, but also in the maintenance of genome stability (Misteli and Soutoglou, 2009).

Role of Chromosome Territories in the Biogenesis of Chromosomal Translocations

The spatial proximity of genomic regions located in neighboring chromosome territories is a major determinant in the frequency of chromosomal translocations (Figure 3A). Indeed, genomic regions whose nuclear territories are proximal or overlapping recombine more efficiently than spatially distant sequences both in yeast and mammals (Agmon et al., 2013; Roukos et al., 2013; Soutoglou et al., 2007). In mammalian cells, this can be explained by the relative positional stability of broken chromosomes (Soutoglou et al., 2007), although the mobility of broken ends, observed mostly in other systems, can contribute to homology search (Dion and Gasser, 2013). The idea that spatial proximity can guide illegitimate joining of DSBs genome-wide is supported by several studies focusing on the genesis of recurrent translocations that drive tumor development in mouse

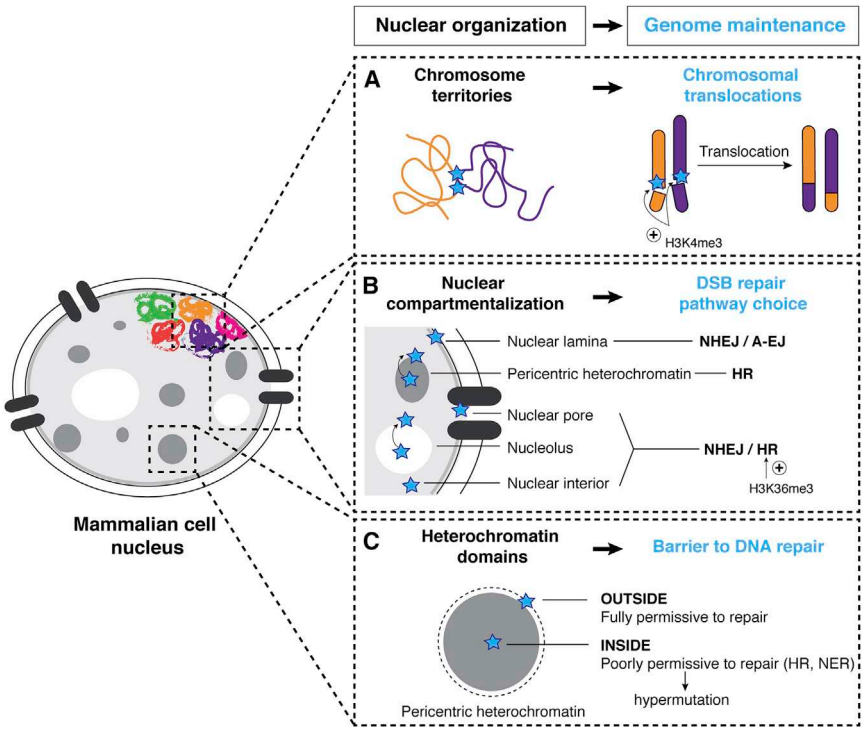

Figure 3. Impact of Nuclear Organization on Genome Maintenance in Response to DNA Damage in Mammalian Cells

(A) The spatial proximity of chromosome territories in the mammalian cell nucleus determines partner selection in chromosome translocations. Chromosome breakpoints (blue stars) are characterized by an enrichment in the transcription-associated histone mark H3K4me3, which facilitates DSB formation.

(B) Nuclear position of DSBs (blue stars) dictates repair pathway choice. NHEJ, non-homologous end joining; A-EJ, alternative end joining; HR, homologous recombination. DSBs located in actively transcribed genes are targeted to HR repair via the transcription elongation-associated histone mark H3K36me3.

(C) Highly compact heterochromatin domains pose a barrier to repair of DNA damage (blue star). HR, homologous recombination; NER, nucleotide excision repair.

lymphoid cells (Chiarle et al., 2011; Hakim et al., 2012; Klein et al., 2011; Rocha et al., 2012; Zhang et al., 2012). It is also in lymphoid cells that the molecular features defining recurrent chromosome breakpoints were originally unraveled: chromatin loops and H3K4me3 focus and stimulate the V(D)J endonuclease, and also potentially the class-switch recombinase, leading to the programmed recombination of antigen receptor genes (Hu et al., 2015; Shimazaki et al., 2009; Stanlie et al., 2010). Chromosomal translocations arise from defects during these programmed genome rearrangements. A recent study in human hematopoietic cells further strengthens the prominent role of the local chromatin environment and of histone modifications—H3K4 methylation in particular—in predisposing chromosome regions to breakage and translocations (Burman et al., 2015). Interestingly, H3K4me3 also specifies meiotic recombination hotspots in yeast and mammals by promoting the recruitment of the meiotic nuclease complex (reviewed in Baudat et al., 2013). Altogether, these studies put forward chromosome positioning and epigenetic marks as key determinants in shaping chromosomal recombination and translocation patterns.

Nuclear Compartmentalization Governs DSB Repair Pathway Choice

In addition to chromosome territories, the eukaryotic cell nucleus contains a variety of functionally distinct compartments and chromatin domains that can direct DNA repair, particularly DSB repair pathway choice (Figure 3B). Targeting DSBs to distinct nuclear compartments in human cells was instrumental for demonstrating the effect of the subnuclear localization of the damage on favoring different repair mechanisms (Lemaître et al., 2014). While DSBs located in the nuclear interior and at nuclear pores can be repaired both by HR and NHEJ, the presence of compact heterochromatin associated with the lamina at the inner nuclear membrane delays DNA damage signaling and impairs HR (Lemaître et al., 2014). Furthermore, DSBs within lamina-associated domains do not migrate to HR-permissive compartments but are repaired in situ by NHEJ or alternative end joining (A-EJ). By contrast, heterochromatic breaks in *Drosophila* move to the nuclear periphery to complete HR (Ryu et al., 2015). Interestingly, DSB repair compartmentalization at the nuclear periphery is conserved in yeast: persistent breaks migrate to the nuclear periphery, where their anchoring with nuclear pores or with the nuclear envelope determines different repair outcomes, error-prone repair or error-free HR, respectively (Horigome et al., 2014). Furthermore, in a given chromatin domain, pre-existing histone modifications also contribute to dictating DSB repair pathway choice, as demonstrated for H3K36me3, which, in mammalian cells, enhances repair by HR, while the dimethylation of the same residue stimulates NHEJ (reviewed in Clouaire and Legube, 2015). Notably, H3K36me3 also positively influences mismatch repair in human cells (Li et al., 2013). Thus, DNA damage repair is tightly integrated within nuclear architecture as it operates in response to a specific nuclear localization in a defined chromatin environment.

Heterochromatin Domains: Barriers to Repair

Among chromatin domains in the eukaryotic cell nucleus, highly condensed and poorly transcribed heterochromatin (Saksouk et al., 2015) has a major impact on genome stability. Indeed, heterochromatin high compaction levels present a barrier to DNA damage repair (Figure 3C), suggesting that cells need to use specific mechanisms for accessing damage within heterochromatin. Slower repair kinetics have been observed for IR-induced damage associated with pericentromeric heterochromatin in mouse cells (Goodarzi et al., 2008) and for UVC damage in H3K9me3-enriched chromatin in human fibroblasts (Han et al., 2016). Other studies, both in yeast and mammalian systems, found that heterochromatin damaged by IR, radiomimetic drugs, or endonuclease cut was devoid of γH2AX (Cowell et al., 2007;

Kim et al., 2007), thus suggesting that heterochromatin domains are refractory to DNA damage signaling and repair. Indeed, the DDR is impaired in human perinuclear heterochromatin, as described above (Lemaître et al., 2014). In line with this, higher point mutation rates have been observed in heterochromatin in human cancers (Schuster-Böckler and Lehner, 2012). Interestingly, heterochromatin constraints on the DDR are relieved by replication (Cowell et al., 2007) and also by transcription, as shown in the context of nucleotide excision repair (NER) of UV damage (Zheng et al., 2014). Strikingly, even though compact heterochromatin appears refractory to the DDR, it has been shown that dynamic compaction of chromatin is required for the activation of DNA damage signaling (Burgess et al., 2014). In fact, early DDR steps efficiently operate in heterochromatin and only late steps are inhibited, as observed in *Drosophila* cells exposed to IR (Chiolo et al., 2011). Late steps of DNA damage signaling and repair occur at the periphery of heterochromatin domains, after heterochromatin expansion and extrusion of repair foci. The dynamic relocalization of repair centers to the periphery of heterochromatin domains, observed both in *Drosophila* and mammalian cells (Chiolo et al., 2011; Jakob et al., 2011), is thought to play a critical role in preventing ectopic recombination between heterochromatic repeats. Importantly, there are substantial differences in heterochromatin organization between model organisms, which may underlie mechanistic discrepancies in the repair of heterochromatic damage. As observed for late repair steps, DNA replication is also confined to the periphery of pericentric heterochromatin domains in mouse cells (Quivy et al., 2004), suggesting a common mechanism for repair and replication of these heterochromatin regions. Similarly, in nucleoli, DSBs are relocalized from the nucleolar interior to anchoring points at the periphery, where ribosomal DNA becomes accessible to repair factors, as reported both in yeast and mammalian cells (Harding et al., 2015; Torres-Rosell et al., 2007; van Sluis and McStay, 2015) (Figure 3B). These data illustrate how different chromatin contexts, characterized by the presence of repeated sequences, impose similar constraints limiting unscheduled DNA repair.

While nuclear organization, through chromosome territories, chromatin domains, and nuclear compartments, has a major impact on genome stability, how it affects epigenome maintenance after damage is still elusive. Targeting DNA damage to specific chromatin domains and nuclear territories, via site-specific endonucleases or laser microirradiation techniques, will be critical for addressing this question. Further studies will also be needed to assess whether nuclear organization, and in particular the presence of compact heterochromatin domains, poses boundaries to chromatin restoration after damage.

Epigenome Integrity versus Plasticity after DNA Damage

As described in the previous sections, we have increasing knowledge of the epigenetic alterations that take place in damaged chromatin and of the molecular players involved in these chromatin rearrangements. However, whether the epigenome is faithfully restored after DNA damage repair is still a matter of debate. A repaired state strictly identical to the original one would ensure the maintenance of epigenome integrity; alternatively, the epigenetic landscape could be reshaped in response to DNA damage, owing to the inherent plasticity of epigenetic information, which is reversible in nature. A damage scar with altered epigenetic marks could then persist on chromatin. As such, chromatin repair can be seen as a window of opportunity for modulating epigenetic information and shaping gene expression in response to genotoxic stress (Figure 4).

Damage Scar on Chromatin

Changes in DNA methylation, histone variants, histone PTMs, and even histone density at damage sites can all contribute to leaving a mark of DNA damage repair on chromatin. Indeed, the base excision repair machinery is involved in erasing DNA methylation in several eukaryotic systems (Wu and Zhang, 2014), while DSB repair by HR stably alters the DNA methylation pattern and gene expression at recombination sites in HeLa cells (Morano et al., 2014). In addition to DNA methylation, modification of histone patterns can also participate in marking sites of DNA damage. The de novo histone deposition taking place at UV damage sites in human cells (Adam et al., 2013; Dinant et al., 2013; Polo et al., 2006) raised the question of whether it changes the histone variant and PTM landscape. Since new histone marks differ from the parental ones (Loyola et al., 2006), it is likely that new histone deposition at least transiently affects histone PTMs at damage sites. Importantly, PTM changes associated with genome instability can have a profound and long-lasting impact on transcription profiles, as shown in chicken cells, where replication defects generate epigenetic instability by uncoupling DNA synthesis from parental histone recycling. The failure to recycle pre-existing histone marks results in alterations in gene expression (Papadopoulou et al., 2015; Sarkies et al., 2010, 2012). Finally, the local accumulation of histones observed after repair at laser microirradiation sites in human U2OS cells (Strickfaden et al., 2016) suggests that changes in histone density could also be part of the damage scar on chromatin.

In contrast to the different types of DNA lesions that form in response to various sources of genotoxic stress, such chromatin scar could constitute a common epigenetic signature of DNA damage. Future challenges will include determining whether the observed epigenetic changes are transient or long term, if they occur in a pathological context—contributing to aging or tumorigenesis—or in a programmed manner, and how relevant they are to the cellular response to DNA damage.

Functional Relevance of Epigenome Plasticity after DNA Damage

Regarding the significance of epigenome plasticity after DNA damage, several hypotheses could be raised based on recent studies. On one hand, the persistence of a damage scar on chromatin could potentially serve as a "damage memory" mark, facilitating the response to a second genotoxic stress insult, analogous to immune memory. Note that the persistence of the damage scar may depend on the repair pathway engaged, the transcriptional activity of the damaged region, and/or the cell type affected. On the other hand, recent work in *Drosophila* highlights the importance of new histone deposition for the maintenance of stem cell identity. Indeed, during asymmetric division of *Drosophila* male germline stem cells, chromatin containing newly synthesized histones is segregated to differentiating cells, while parental histones are retained in the stem cells (Tran et al.,

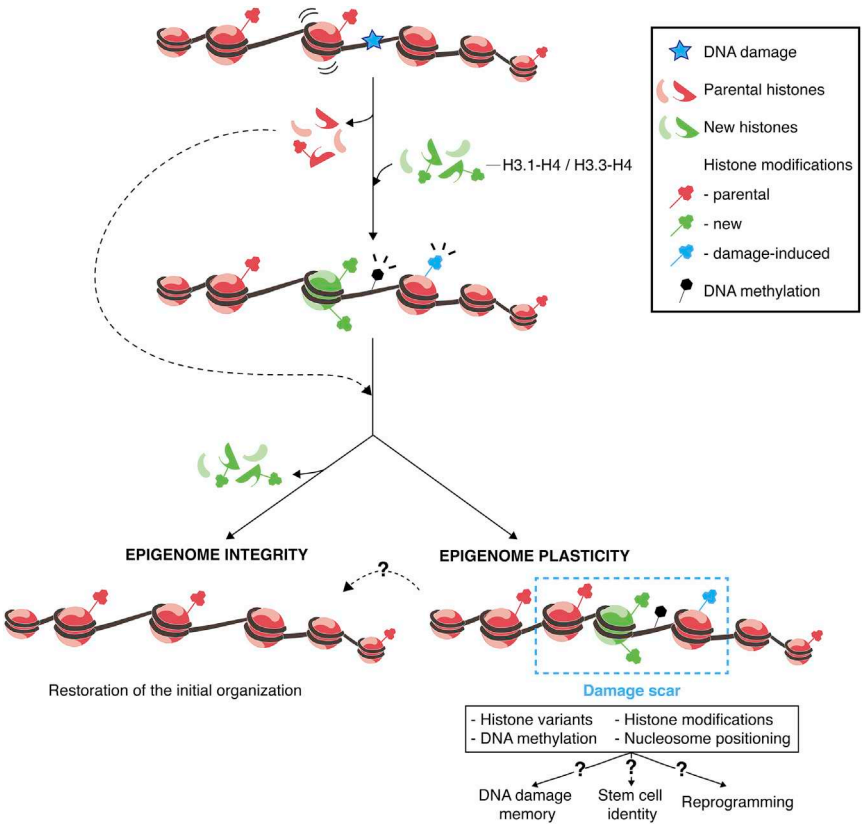

Figure 4. Epigenome Integrity versus Plasticity in Response to DNA Damage in Mammalian Cells

DNA damage (blue star) elicits substantial chromatin rearrangements, with a loss of parental information (red) due to the mobilization of pre-existing histones, and the incorporation of new information with histone variant exchange and deposition of newly synthesized histones (green), DNA damage-responsive PTMs (blue), and DNA methylation (black). For simplicity, factors escorting and mobilizing histones and modifying enzymes for histones and DNA are not represented. Future challenges in the field will be to determine whether or not the pre-existing chromatin landscape is ultimately faithfully restored after genotoxic stress, thus allowing the maintenance of epigenome integrity. Alternatively, the persistence of a damage scar on chromatin (dotted line box) could contribute to damage memory, maintenance of stem cell identity, or reprogramming.

2012). This relies on differential phosphorylation of parental and new H3 (Xie et al., 2015). One could hypothesize that a similar mechanism may operate upon DNA damage in mammalian cells. New histone deposition coupled to repair would ensure that only undamaged information is kept in stem cells, thus preserving stem cell molecular properties. Another possible function of a persistent damage scar on chromatin could relate to cell reprogramming. Indeed, efficient reprogramming relies on chromatin alterations (Apostolou and Hochedlinger, 2013) and also requires DNA damage repair activities in mammalian cells, such as the HR pathway (González et al., 2013) and the NER complex containing xeroderma pigmentosum, complementation group C (XPC) (Fong et al., 2011). In line with this, histone variants and chaperones involved in chromatin dynamics in response to DNA damage also play a major role in development, reprogramming, and regulating cell pluripotency (Filipescu et al., 2013; Skene and Henikoff, 2013). In particular, the histone chaperone HIRA and the H3.3 histone variant have been implicated both in cell reprogramming and transcription plasticity in *Xenopus*, mouse, and human systems (Banaszynski et al., 2013; Jullien et al., 2012; Lin et al., 2014; Maze et al., 2015; Ng and Gurdon, 2008; Wen et al., 2014). The histone chaperone ASF1 is also required for the maintenance of pluripotency and human cell reprogramming (Gonzalez-Muñoz et al., 2014). In contrast, the histone chaperone CAF-1 has been characterized as a negative regulator of reprogramming in mouse cells (Cheloufi et al., 2015; Ishiuchi et al., 2015) and was shown to promote cell differentiation during neurodevelopment in the nematode (Nakano et al., 2011). Future work will determine if a persistent damage scar on chromatin indeed contributes to local or global reprogramming of transcription in response to genotoxic stress, with possible consequences on cell identity.

Conclusions and Future Challenges

In recent years, it has become increasingly clear that safeguarding genome function entails reshaping chromatin. The coordinated maintenance of genome and epigenome integrity in response to DNA damage is supported by direct molecular interactions between DDR factors and chromatin modifiers. Recent studies also emphasize a critical interplay between genome maintenance pathways and the topological organization of the genome in the cell nucleus. Studying the maintenance of chromatin integrity after DNA damage thus appears as a multifaceted research field at the crossroads of epigenetics, genome stability, and nuclear organization.

Nevertheless, much remains to be learned about how the epigenetic landscape is altered and in which form it is ultimately re-established during the course of DNA damage repair. Furthermore, while the impact of nuclear organization on genome maintenance is clearly established, it will be important to understand how DNA damage repair affects chromatin nuclear domains and genome topology. Combining genotoxic stress with Hi-C techniques for genome-wide analysis of chromatin interactions, now applicable at the single-cell level (Nagano et al., 2013; Risca and Greenleaf, 2015), could be extremely valuable to address this issue. In addition, the crosstalks between damaged chromatin dynamics and transcriptional activity (Adam and Polo, 2014), but also with post-transcriptional events such as splicing regulation (Tresini et al., 2015), and the importance of non-coding RNAs in the DDR (d'Adda di Fagagna, 2014) are emerging topics that will surely shape future research in the field. Another remarkable feature that has become increasingly recognized is the strong influence of cell metabolism on both epigenetic marks and the DDR (Janke et al., 2015; Shimizu et al., 2014), which could provide mechanistic insights into how damaged chromatin

plasticity is regulated. In fine, in-depth understanding of the cellular mechanisms underlying epigenome maintenance in response to genotoxic stress could open up new strategies for modulating damaged chromatin dynamics and driving phenotypic changes with therapeutic benefit.

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ANNEX 2:

Real-time tracking of histones in UVC-damaged chromatin

Live imaging of parental histone variant dynamics in UVC-damaged chromatin.

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Methods Mol Biol, 1832:243–253, 2018

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This method paper describes a step-by-step protocol to visualize the dynamics of parental histones, which characterize the chromatin state before damage, in cultured human cells. Staining of specific parental histone variants using either the SNAP technology or photoactivation is combined with local micro-irradiation of the cells with a UVC laser. This allows the tracking in real-time of parental histones in damaged chromatin regions.

Chapter 13

Live Imaging of Parental Histone Variant Dynamics in UVC-Damaged Chromatin

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Abstract

In eukaryotic cell nuclei, all DNA transactions, including DNA damage repair, take place on a chromatin substrate, the integrity of which is central to gene expression programs and cell identity. However, substantial chromatin rearrangements accompany the repair response, culminating in the deposition of new histones. How the original epigenetic information conveyed by chromatin may be preserved in this context is a burning question. Elucidating the fate of parental histones, which characterize the pre-damage chromatin state, is a key step forward in deciphering the mechanisms that safeguard epigenome stability. Here, we present an in vivo approach for tracking parental histone H3 variant dynamics in real time after UVC laser-induced damage in human cells.

Key words Live-cell imaging, Parental histone variants, Photoactivation, SNAP-tag technology, UVC laser damage

1 Introduction

In eukaryotic cells, the DNA associates with histone proteins in the form of chromatin, the basic unit of which is the nucleosome [1]. Chromatin organization is modulated at the nucleosomal level through the existence of histone variants [2] and their posttranslational modifications [3], and via the association of non-histone chromatin components, but also at the level of higher-order folding of the chromatin fiber into nuclear domains [4]. Altogether, these structural variations convey epigenetic information, which drives genome functions and cell identity [5]. Maintaining epigenome integrity is thus of critical importance. However, epigenome stability is challenged during all DNA transactions that take place on the chromatin substrate, including DNA damage repair

Juliette Dabin and Anna Fortuny equally contributed to this work.

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[6]. Substantial chromatin rearrangements indeed accompany the repair response, as originally described in the Access-Repair-Restore model [7, 8]. This model postulates that damaged chromatin is first transiently disorganized to allow access to the repair factors, followed by restoration of chromatin structure and function. Our understanding of the molecular events underpinning such chromatin rearrangements has greatly improved in recent years, owing to the emergence of innovative methods that allow a more detailed examination of chromatin dynamics following DNA damage.

In particular, the development of novel imaging techniques, combining local induction of DNA damage and specific tagging of histone proteins, has been instrumental for visualizing a loss of histone density accompanied by chromatin decondensation at sites of UVC irradiation [9] and UVA laser micro-irradiation [10–12] in human cells. These approaches also proved invaluable for detecting the deposition of newly synthesized H2A and H3 histone variants at sites of DNA damage [13–17]. While new histones likely contribute to restoring chromatin structure following DNA damage, they also bring in new information [18], which raises the question of how the epigenetic landscape may be preserved. To address this issue, one needs to focus on parental histones, which characterize the pre-damage chromatin state and carry the original epigenetic information.

Here, we describe two complementary methods for real-time tracking of parental histone variants in response to local UVC irradiation in human cells [19]. The specific labeling of parental histones relies on histone protein tagging with a SNAP-tag [20], or a PhotoActivatable Green Fluorescent Protein (PA-GFP) [21]. We combine these technologies with cell micro-irradiation using a UVC laser implemented on a confocal microscope. Thus, we can follow parental histone redistribution and recovery during repair progression in UVC-damaged chromatin [19].

2 Materials

2.1 Cell Culture

1. Human U2OS cell lines stably expressing H3.3-SNAP or H3.3-PA-GFP (*see* **Note 1**). The *H3F3B* sequence encoding human H3.3 is cloned into plasmids encoding the SNAP-tag (New Englands Biolabs) or PA-GFP [22]. Stable cell lines are established by transfection followed by antibiotic selection of clones [19].
2. Dulbecco's Modified Eagle Medium with high glucose, GlutaMAX, sodium pyruvate, and phenol red (DMEM high glucose GlutaMAX™ Supplement, Gibco) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml

streptomycin, 100 µg/ml G418 disulfate salt solution and 200 µg/ml hygromycin B. Store at 4 °C.

3. Dulbecco's Phosphate-Buffered Saline without calcium, magnesium, and phenol red (DPBS 1×).
4. Sterile round quartz coverslips 25 mm, thickness No. 1 (Neyco) (*see* **Note 2**).
5. Six-well cell culture plates (TPP).

2.2 SNAP Reagent

1. Fluorescent SNAP substrate (200 µM stock solution): dissolve 30 nmol SNAP-Cell® TMR-STAR (New England Biolabs) into 150 µl sterile dimethyl sulfoxide (DMSO). Store 30 µl aliquots at −20 °C (*see* **Note 3**).

2.3 Microscopy Equipment (see Note 4)

1. Zeiss fully motorized Axiovert microscope with a LSM700 confocal module (Carl Zeiss) adapted for UVC transmission with all-quartz optics, a temperature-controlled chamber and Zen software (Carl Zeiss).
2. UVC laser: 2 mW pulsed (7.8 kHz) diode-pumped solid-state laser emitting at 266 nm (Rapp OptoElectronics, Hamburg GmbH), directly coupled to the microscope stand. A neutral density filter OD1 (10% T) can be added to the light path. The UVC laser is fixed but the position of the damage spot can be precisely controlled by moving the motorized stage of the microscope via a custom macro on Zen software (*see* **Notes 5** and **6**).
3. Photoactivation laser: 405 nm laser diode, 5 mW power.
4. Imaging lasers: 405 nm, 5 mW power; 488 nm, 10 mW power; 555 nm, 10 mW power.
5. Objectives: Quartz 40x/0.6 Ultrafluar glycerol objective (Carl Zeiss) and LD LCI Plan-Apochromat 25x/0.8 multi-immersion objective for oil, water or glycerol immersion (Carl Zeiss).
6. Glycerol for immersion.

2.4 Live-Cell Imaging

1. Chamlide CMB 35 mm dish type 1-well magnetic chamber for round coverslip (Live Cell Instrument).
2. Thermoregulated microscope chamber with CO_2 entry (*see* **Note 7**).

2.5 Image Analysis and Processing

1. ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).
2. Microsoft® Excel® or equivalent spreadsheet software.
3. Adobe® Photoshop® or equivalent image processing software.

3 Methods

Here, we describe a protocol for monitoring the in vivo dynamics of parental histone H3.3 variants at sites of DNA damage in human cells. This method combines either SNAP-tag-based labeling [20] or photoactivation [21] of parental histones (Fig. 1) with local UVC irradiation of cells, followed by live-cell imaging. First, parental SNAP-tagged H3.3 histones are labeled with a fluorescent SNAP substrate (**step 1**). Alternatively, PA-GFP-tagged H3.3 histones are photoactivated with a 405 nm laser diode (**step 2**). Next, cells are locally irradiated with a UVC laser (266 nm) coupled to a confocal microscope to induce damages in subnuclear regions of interest (**step 3**). Live-cell imaging following UVC irradiation allows visualization of parental histone dynamics during the repair response (**step 4**). Quantification of the fluorescence associated with parental histones (**step 5**) reveals their dynamics in damaged chromatin (Fig. 2, *see* also **Note 8**). The whole protocol can be completed in 4 to 6 days (*see* **Note 9**).

3.1 SNAP Labeling of Parental Histones (see Note 10)

1. Grow U2OS cells stably expressing H3.3-SNAP and GFP-XPC on quartz coverslips in 6-well plates with 2.5 ml culture medium per well so that they reach 70–80% confluency 3 days later. Incubate in a humidified 37 °C incubator with 5% CO_2 overnight.
2. The next day, dilute the fluorescent SNAP substrate in fresh culture medium to a final concentration of 2 μM (*see* **Note 11**). Pulse-label all SNAP-tagged histones by incubating cells with 1 ml of this solution per well for 20 min at 37 °C, 5% CO_2.
3. Wash out the excess of fluorescent substrate by rinsing cells twice with 3 ml PBS (*see* **Note 12**), then incubate cells with 2.5 ml culture medium per well for 30 min.
4. Chase time: rinse cells twice with 3 ml PBS and incubate cells in 2.5 ml culture medium for 48 h at 37 °C, 5% CO_2 (*see* **Notes 13** and **14**).

3.2 Photoactivation of Parental Histones (see Note 10)

1. Grow U2OS cells stably expressing H3.3-PA-GFP and RFP-XPC on quartz coverslips in 6-well plates with 2.5 ml culture medium per well so that they reach 70–80% confluency 3 days later. Incubate overnight in a humidified 37 °C incubator with 5% CO_2.
2. The next day, transfer each coverslip to a Chamlide magnetic chamber and place the chamber on the confocal microscope stage with controlled temperature and CO_2 conditions (37 °C, 5% CO_2).

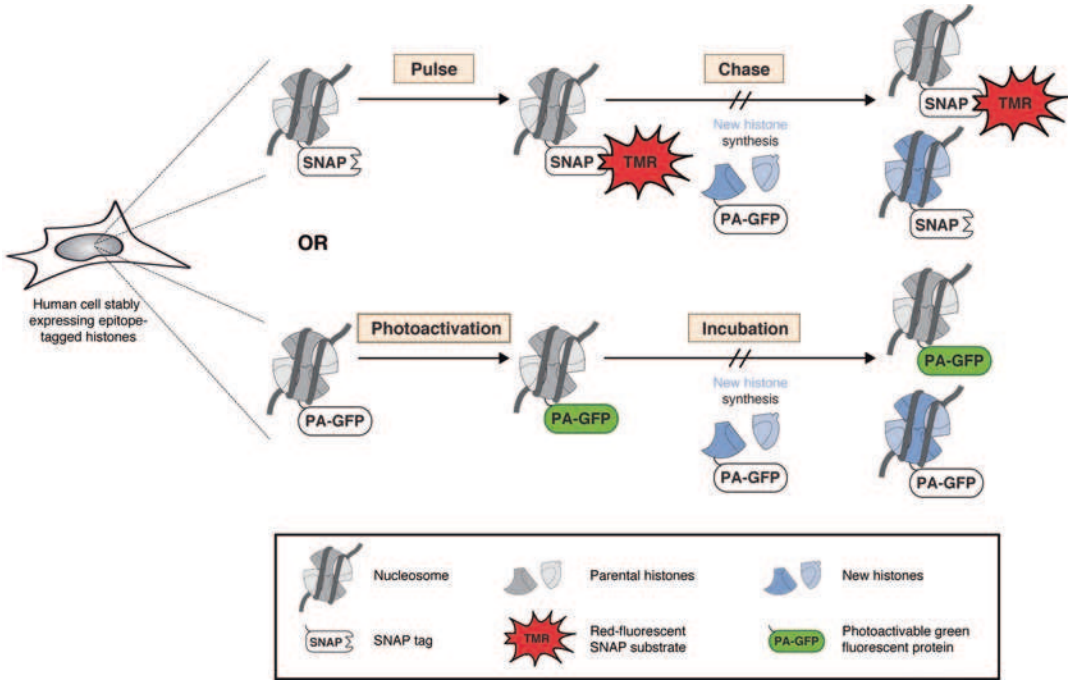

Fig. 1 Scheme of the assay for labeling parental histones. Two days prior to UVC irradiation, U2OS cells stably expressing SNAP- or PA-GFP-tagged histones are pulsed with a red fluorescent SNAP reagent (TMR, top) or photo-activated with a 405 nm laser (bottom), respectively. The subsequent chase/incubation time ensures that the pulse-labeled or photo-activated histones are properly incorporated into chromatin

3. Using the 40× glycerol immersion objective (*see* **Note 15**), photoactivate parental histones by bleaching a whole field of cells with the 405 nm laser diode using the following settings: maximum power, 5 iterations, 6.30μs/pixel scan speed. Cell nuclei can be visualized in transmitted light or via the fluorescently labeled repair factor. Repeat until 100 cell nuclei have been photoactivated (*see* **Note 16**).
4. Clean carefully the glycerol on the bottom of the coverslip before transferring it back to the cell culture plate and incubate for 48 h at 37 °C, 5% CO_2 (*see* **Note 13**).

3.3 Local UVC Irradiation of Cells

1. Two days after parental histone labeling or photoactivation, transfer each coverslip to a Chamlide magnetic chamber (*see* **Notes 17** and **18**) and place the chamber on the confocal microscope stage with controlled temperature and CO_2 conditions (37 °C, 5% CO_2).
2. Localize the fields of interest containing fluorescent nuclei (i.e., fluorescently labeled parental histones) and acquire an image of each field before UVC irradiation using the 25× multi-immersion objective with glycerol (*see* **Notes 15** and **19–21**). Use the appropriate imaging lasers depending on the

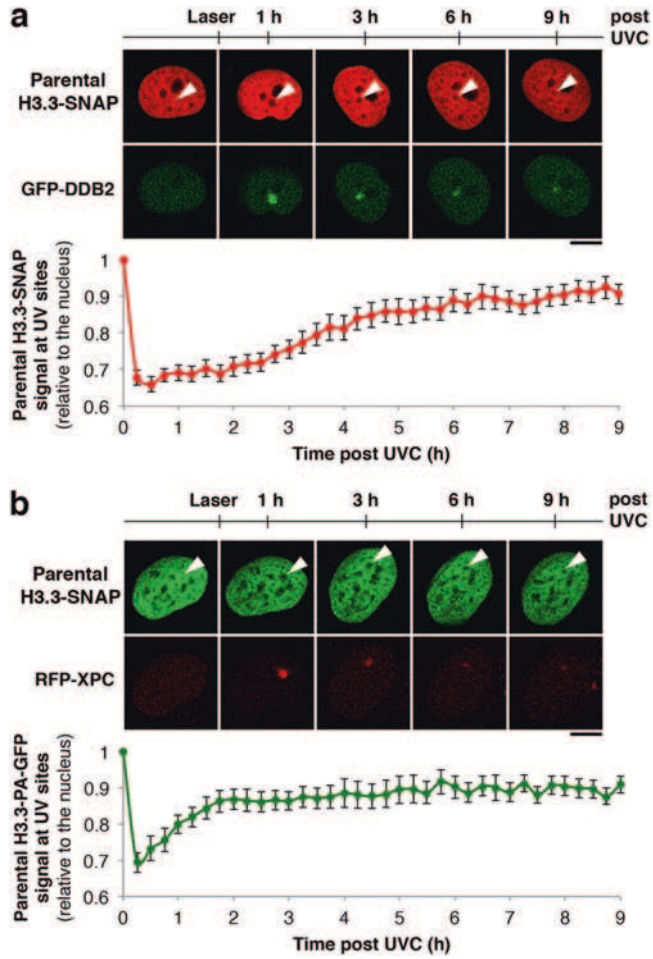

Fig. 2 Parental H3.3 redistribution and recovery at UVC damage sites. (**a**) Dynamics of parental histones H3.3 (red) at the indicated time points after UVC laser micro-irradiation in U2OS cells stably expressing H3.3-SNAP and GFP-DDB2. (**b**) Dynamics of parental histones H3.3 (green) at the indicated time points after UVC laser micro-irradiation in U2OS cells stably expressing H3.3-PA-GFP and RFP-XPC. White arrowheads point to the sites of laser impact. The graphs show quantification of red (**a**) and green (**b**) fluorescence in irradiated areas. Error bars represent SEM from n cells scored in two independent experiments. Scale bars: 10 μm. Adapted from ref. 19

fluorescence of parental histones (488 nm laser for H3.3-PA-GFP, 555 nm laser for H3.3-SNAP-tag-TMR). Save the position of each field of cells before moving to the next one to retrieve the cells more easily.

3. For UVC irradiation, switch to the 40× quartz objective with glycerol (*see* **Note 15**) and insert the neutral density filter (*see* **Note 22**).

4. Using the UV macro, mark the positions to be targeted by the UVC laser (266 nm) in as many cell nuclei as possible in a given field (one damage spot per nucleus, *see* also **Note 23**). Each cell nucleus is irradiated for 50 ms at maximum laser power (*see* **Notes 24–26**).

3.4 Live-Imaging of Parental Histones

1. Immediately following UVC irradiation, switch back to the 25× multi-immersion objective with glycerol to acquire images of the irradiated cells.
2. Capture one image every 15 min for several hours to overnight (*see* **Notes 19**, **21**, and **27**). Use the appropriate imaging lasers depending on the fluorescence of parental histones, repair factors, and DNA if needed. Retrieve each field of cells using the positions saved previously. Cells should be kept at 37 °C, 5% CO_2 throughout the acquisition.
3. Once the acquisition is over, the quartz coverslip can be fixed with 2% paraformaldehyde if immunostaining is necessary or it can be recycled (*see* **Note 2**).
4. Clean the Chamlide magnetic chamber with distilled water and then 70% ethanol for reuse.

3.5 Image Analysis and Processing (see Note 28)

1. Open images with the ImageJ software.
2. Using the wand tool, select the UVC-damage region in an irradiated nucleus based on the fluorescence of the repair factor (XPC or DDB2) 15 min after irradiation. The selected area is then manually copied on each other image of the same cell nucleus before and after irradiation (*see* **Note 29**).
3. Measure the fluorescence signal (mean gray value) associated with parental histones within this region at each time point before and after UVC irradiation.
4. Also measure parental histone fluorescence intensity in the whole cell nucleus at each time point before and after UVC irradiation. The position of the nucleus is determined using the wand tool, based on the fluorescence of parental histones at each time point. Always measure background fluorescence in the same field.
5. Repeat **steps 2** to **4** for all damaged cells (*see* **Note 29**).
6. Export the results to an Excel spreadsheet.
7. Subtract background fluorescence from all measurements.
8. Divide the histone fluorescence intensity in the UVC-damaged area by the intensity measured in the corresponding nucleus. Normalize the results relative to before irradiation.
9. Selected images are mounted using Adobe Photoshop.

4 Notes

1. This protocol is optimized for U2OS cells stably expressing epitope-tagged H3.3, but it can be adapted to other cell types, stably or transiently expressing SNAP- or PA-GFP-tagged histones (H3 variants or other histone variants). Stable monoclonal cell lines are preferred to ensure minimal cell-to-cell variability in transgene expression. The co-expression of a fluorescently tagged repair factor, such as GFP- or RFP (Red Fluorescent Protein)-tagged XPC (Xeroderma Pigmentosum, complementation group C), is recommended to be able to visualize the damage site and assess repair progression. Other repair factors can also be used for the same purpose like DNA Damage Binding protein 2 (DDB2), as shown in Fig. 2.
2. Quartz coverslips are used to allow the transmission of UVC light through the coverslip to the sample. Cells normally do not detach from the coverslip but if this happens, it can be avoided by coating the coverslips with poly-L-lysine or collagen-fibronectin before seeding the cells. Quartz coverslips can be recycled after an experiment by performing the following washes (10 min each at room temperature): one wash in 1% SDS (Sodium Dodecyl Sulfate, MP Biomedicals), three washes in distilled water, two washes in 100% ethanol.
3. Centrifuge the fluorescent SNAP substrate before pipetting to avoid aggregates. The fluorescent SNAP substrate must be protected from light and is sensitive to repeated freeze-thaw cycles. It is thus advisable to prepare aliquots of the stock solution.
4. Any equivalent confocal microscope can be used as long as optics are changed to quartz and that it can be coupled to a 266 nm laser.
5. The UVC laser setup that we use for our experiments is available at the Cell and Tissue Imaging Facility of the Institut Curie (Paris, France). To date, the only other confocal microscope equipped with a 266 nm UVC laser and all-quartz optics is a Zeiss Axiovert 200 M with LSM 510 confocal module (Erasmus MC, Rotterdam, the Netherlands) [23].
6. Local UVC irradiation can also be performed on the bench with a UVC lamp (254 nm) through micropore filters [24].
7. Instead of CO_2, HEPES buffer can be added to the culture medium (25 mM final concentration) to maintain the pH.
8. For a deeper understanding of the mechanisms underlying histone dynamics at UV sites, this protocol can be combined with siRNA-mediated depletion of histone chaperones and repair factors or with inhibition of enzymatic activities involved in the UV damage response [19].

9. The total duration of the experiment may vary depending on the duration of live cell imaging (a few hours to overnight) and on the final number of cells to be analyzed.
10. Subheadings 3.1 and 3.2 are to be executed alternatively rather than sequentially.
11. In cells that do not express a GFP-tagged repair protein, the red-fluorescent substrate SNAP-Cell® TMR-star can be replaced by the green fluorescent SNAP-Cell® Oregon-Green® at a final concentration of 4 μM from a 1 mM stock solution in DMSO.
12. The washing procedures are critical to minimize background signal. The specificity of SNAP labeling can be controlled on the parent U2OS cell line, which does not express SNAP-tagged proteins and thus should not be stained.
13. The 48 h chase/incubation time allows incorporation of labeled parental histones into chromatin and can be adapted depending on the turnover of histone proteins.
14. Parental histone labeling can be combined with specific labeling of newly synthesized histones using distinct fluorescent SNAP substrates for new and parental histones (red-fluorescent SNAP-Cell® TMR-star and green-fluorescent SNAP-Cell® Oregon-Green®) [17, 19].
15. The same immersion medium, i.e., glycerol, should be used for photo-bleaching, imaging, and UVC irradiation.
16. Photoactivation efficiency is monitored by imaging with the 488 nm laser. The indicated settings should lead to maximal photoactivation of PA-GFP-tagged histones, but they may need to be adjusted depending on the objective and laser power available.
17. Following SNAP-tag labeling, it is preferable to rinse the cells in PBS before transferring the coverslip to the chamber to remove remaining aggregates of fluorescent SNAP substrate from the culture medium.
18. If DNA visualization is wanted during live-cell imaging, incubate cells before UVC irradiation with Hoechst 33258 nuclear staining dye at a final concentration of 10 μg/ml for 30 min.
19. Acquisitions can be performed in 2D or in 3D (z-stacks). You can save your images in any file format supported by the ImageJ software.
20. The 25× objective is preferred for image acquisitions due to a larger numerical aperture.
21. You can use the autofocus mode for image acquisitions (focusing on the fluorescent parental histones).
22. For safety reasons, it is important to close the microscope chamber during the UVC irradiation procedure and the lamp

shutter should also be closed to avoid transmission of the UVC light through the eyepieces.

23. As there is no scanning mode for the UVC laser, only damage spots can be obtained. It is not possible to draw lines.
24. The damage spot is 2 μm in diameter corresponding to ca. 2% of the nuclear volume. The UVC dose delivered at the site of laser micro-irradiation is estimated at 600 J/m^2 and does not cause major cytotoxicity: the mortality rate during an overnight imaging experiment is only around 10%, the damaged cells do repair and can go through mitosis.
25. Other laser wavelengths can be used for generating other types of DNA damage including DNA breaks [23].
26. We did not observe bleaching of TMR star, Oregon green, Hoechst, GFP, or RFP fluorescence by the UVC laser in the conditions used for local UVC irradiation.
27. To avoid phototoxicity during long-term imaging such as overnight experiments, it is recommended to adjust the acquisition settings in order to minimize the laser power and the number of total captured images (4 images/h maximum). Images can be acquired more frequently in case of shorter kinetics.
28. Image analysis can be performed on 2D acquisitions or on projections of 3D acquisitions.
29. The quantification procedure cannot be easily automated due to cell movement during long-term imaging.

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