UNIVERSITÀ VITA-SALUTE SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA
INTERNAZIONALE IN MEDICINA MOLECOLARE

Curriculum in Cellular and Molecular Biology

The histone demethylase KDM6A is a tumor suppressor controlling the genomic integrity and the DNA damage response

DoS: Prof. Giovanni Tonon
Second Supervisor: Prof. Jan Hoeijmakers

Tesi di DOTTORATO di RICERCA di Gemma Crupi
matr. 015610
Ciclo di dottorato XXXV
SSD BIO/11 - BIOLOGIA MOLECOLARE

Anno Accademico 2021/2022

CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

La sottoscritta / *I* **Gemma Crupi**
matricola / *registration number* 015610
nata a / *born at* Cosenza (CS)
il / *on* 18/11/1994
autrice della tesi di Dottorato di ricerca dal titolo / *author of the PhD thesis entitled*: The histone demethylase KDM6A is a tumor suppressor controlling the genomic integrity and the DNA damage response

NON AUTORIZZA la consultazione della tesi per 12 mesi/ *NOT AUTHORIZES the Consultation of the thesis for 12 months*

a partire dalla data di conseguimento del titolo e precisamente / *from the PhD thesis date, specifically*

Dal / *from* 18/04/2023 Al / *to* 18/04/2024 Poiché / *because*:

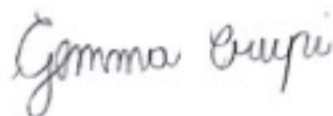
□ l'intera ricerca o parti di essa sono potenzialmente soggette a brevettabilità / *The whole project or part of it might be subject to patentability*;
X ci sono parti di tesi che sono già state sottoposte a un editore o sono in attesa di pubblicazione / *Parts of the thesis have already been submitted to a publisher or are in press*;

□ la tesi è finanziata da enti esterni che vantano dei diritti su di esse e sulla loro pubblicazione / *The thesis project is financed by external bodies that have rights over it and on its publication*.

È fatto divieto di riprodurre, in tutto o in parte, quanto in essa contenuto / *It is not allowed to copy, in whole or in part, the data and the contents of the thesis*.

Data / *Date* 14/02/2023

Firma / *Signature*



DECLARATION

This thesis has been:
- composed by myself and has not been used in any previous application for a degree. Throughout the text I use both 'I' and 'We' interchangeably.
- has been written according to the editing guidelines approved by the University.

Permission to use images and other material covered by copyright has been sought and obtained. For the following images in Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6, and Figure 9 A and B, it was not possible to obtain permission and are therefore included in thesis under the "fair use" exception (Italian legislative Decree no. 68/2003).

All the results presented here were obtained by myself, except for:

1) **RNA sequencing**. For instance:
   RNA sequencing experiments to investigate the differential gene expression between shSCR and shKDM6As (Results, figure 18), were performed in collaboration with Dr. Dejan Lazarevic, Center for Omics Sciences, San Raffaele Scientific Institute, Milan, Italy.

2) **Bioinformatic analysis.** For instance:
   Analysis of transcriptomic data from cBioportal (Results, figure 17) were performed in collaboration with Dr. Jose Garcia Manteiga Center for Omics Sciences, San Raffaele Scientific Institute, Milan, Italy.

All sources of information are acknowledged by means of reference.

ABSTRACT

KDM6A is a histone demethylase frequently altered across several cancer types. By specifically removing the H3K27me2/3 epigenetic repressive marks, KDM6A counteracts Polycomb function promoting target gene activation. Despite its relevance as a cancer gene, KDM6A pathogenic role remains poorly defined.

We revealed that U-2OS cells were KDM6A was depleted displayed an increased genomic instability. Since the aberrations observed are reminiscent of an accumulation of unrepaired breaks, we hence decided to investigate the putative implication of KDM6A in the DNA damage response (DDR). The evaluation of the repair efficacy through reporter-constructs system revealed a specific impairment in the Homologous recombination (HR) repair pathway. Also, KDM6A deficient cells were extremely vulnerable to the PARP inhibitor, Olaparib. The increased susceptibility to the treatment is likely ascribed to the hampered repair upon KDM6A loss. Remarkably, DNA damage signaling, and checkpoint were not affected suggesting that KDM6A action is specifically exerted at the DNA repair level. To gain insight on the molecular mechanisms underlying the role of KDM6A, we exploited The Cancer Genome Atlas (TCGA) RNA-seq dataset. Interestingly, we found that in Bladder Cancer, first for the prevalence of KDM6A alterations, HR repair genes positively correlated with *KDM6A* levels. Despite a full body of literature implicating KDM6A in the transcriptional modulation of developmentally regulated genes, its catalytic activity is dispensable for most of KDM6A tumor suppression functions explored. Our results and data from others point to a pivotal role of KDM6A in the regulation of HR genes transcription.

Moreover, our work expanded the understanding in the regulation of KDM6A levels proposing an unanticipated role of the apical kinase ATM in the modulation of KDM6A in both basal and damage conditions. Collectively, our findings could usher novel avenues to target tumor and to more effectively overcome resistance to drugs.

TABLE OF CONTENTS

ACRONYMS AND ABBREVIATIONS *3*

LIST OF FIGURES *4*

1. INTRODUCTION *5*

1.1 Chromatin architecture 5
1.1.1 Chromatin dynamics and histone modifications 5
1.1.2 The histone modification H3K27me3 7

1.2 KDM6A 8
1.2.1 KDM6A: A Histone H3K27 Demethylase 8
1.2.2 Interactome and Functions of KDM6A-Associated Proteins 11

1.2.3 KDM6A in cancer 12

1.3 DNA damage 16
1.3.1 Genomic instability and sources of damage 16
1.3.2 DNA Damage Response pathway 17
1.3.3 DNA Damage sensing and signaling 18
1.3.4 The DNA repair pathways 20
1.3.5 DDR checkpoint 24
1.3.6 Targeting DNA repair in cancer 26

1.4 Chromatin dynamics in DNA damage 28
1.4.1 Histone dynamics modulate chromatin decompaction and mobility upon damage 28
1.4.2 Histone variants and PTMs promote the repression of transcription at damage sites 29
1.4.3 DNA repair pathway choice is mediated by chromatin alterations at DNA damage sites 30
1.4.4 Chromatin restoration following damage 31
1.4.5 Chromatin shields DNA from damage 32
1.4.6 Polycomb and H3K27me3 in damage 32

2. AIM OF THE WORK *34*

3. RESULTS *35*

3.1 KDM6A loss elicits genomic instability and affects DNA repair 35
3.1.1 KDM6A Knockdown cells are genomically instable 35
3.1.2 DNA repair is impaired in KDM6A deficient cells 39
3.1.3 Altered damage signaling in KDM6A depleted cells 42
3.1.3 KDM6A loss increases sensitivity to the PARP inhibitor Olaparib 44
3.1.4 Cell cycle distribution is only marginally affected by KDM6A loss 47
3.2.1 KDM6A protein increases in response to damage 48

3.3. KDM6A mediates the transcriptional regulation of HR genes 54

3.3.1 The role of KDM6A in the transcriptional regulation in patients 54
3.3.2 Gene expression analysis reveals an enrichment of HR genes in shKDM6A U-2OS 56

4.H3K27me3 levels are dynamically regulated upon IR....................58

4. DISCUSSION 60

5. MATERIALS AND METHODS 67
5.1 Cell Culture 67
5.2 Lentiviral infection 67
5.3 Lentiviral Titer Calculation 68
5.4 Irradiation 68
5.5 Drug treatments 68
5.6 Colony formation assay 68
5.7 Ligand-inducible I-SceI cleavage assay 69
5.8 Immunofluorescence 69
5.9 Western blot 70
5.10 Cell Cycle distribution 70
5.11 Cytogenetics analysis 71
5.12 RNA extraction and real-time quantitative PCR 71
5.13 RNA-seq data analysis 72
5.14 GSEA analysis of patient transcriptome analysis 72
5.15 Statistical analysis .... **Errore. Il segnalibro non è definito.**

6. REFERENCES 74

ACRONYMS AND ABBREVIATIONS

ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
BER	base excision repair
BRCA1/2	breast cancer 1/2
CDK	cyclin-dependent kinase
CtIP	CtBP-interacting protein
DAPI	4', 6-diamidino-2-phenylindole
DDR	DNA damage response
dsDNA	double-stranded DNA
ETO	etoposide
Gy	gray
HDAC	histone deacetylase
HR	homologous recombination
IR	ionizing radiation
KD	knock-down
MLL2/3	mixed leukemia lineage
MRN	Mre11-Rad51-Nbs1 complex
mRNA	messenger RNA
NER	nucleotide excision repair
NHEJ	non-homologous end joining
PARP	poly(ADP-Ribose) polymerase
PFA	paraformaldehyde
PolII	RNA Polymerase II
PTM	post translational modification
ROS	reactive oxygen species
RPA	replication protein A
shRNA	short hairpin RNA
ssDNA	single-stranded DNA
UV	ultraviolet
γH2AX	phosphorylated-histone H2AX on Ser 139

LIST OF FIGURES

Figure 1. Schematic representation of mouse KDM6 histone demethylases. 11
Figure 2. KDM6A functionally interacts with the MLL3/4 complex, CBP/p300 and the BRG1 chromatin remodeler at enhancers. 12
Figure 3. KDM6A is frequently altered in a broad spectrum of cancers 15
Figure 4. DNA damage sensors and signaling proteins action in response to DSBs 20
Figure 5. HR and NHEJ are the two major pathways for the repair of DSBs repair 24
Figure 6. Cyclines and CDKs involved in the activation of cell cycle checkpoints upon damage. 26
Figure 7. KDM6A inactivation elicits genomic instability 36
Figure 8. Micronuclei frequently form in KDM6A depleted cells 38
Figure 9. KDM6A is required for efficient repair 41
Figure 10. Robust checkpoint activation upon KDM6A KD. 43
Figure 11. KDM6A KD increases vulnerability to DNA damaging agents 45
Figure 12. KDM6A silenced cells are insensitive to Etoposide 46
Figure 13. KDM6A only mildly affects cell cycle 48
Figure 14. KDM6A protein increases upon damage 50
Figure 15. KDM6A protein increases upon damage but not its mRNA levels 51
Figure 16. ATM is responsible for KDM6A increase upon IR 53
Figure 17. DNA repair genes transcription is downregulated upon KDM6A expression reduction in bladder cancer patients 55
Figure 18. Homologous recombination genes are downregulated upon KDM6A KD ... 57
Figure 19. H3K27me3 is dynamically modulated upon damage 59

1. INTRODUCTION

1.1 Chromatin architecture

In the nucleus of eukaryotes, DNA is packed into chromatin. Chromatin is a hierarchically organized structure consisting of DNA, histones, and several chromosomal proteins. The basic unit of chromatin, the nucleosome, includes 147 base pairs of DNA wrapped around an octamer of core histone proteins made of two copies of each histone (H2A, H2B, H3, and H4). Repeated nucleosome units are connected by linker DNA of variable length forming motifs referred to as "beads on a string" which are further partitioned into chromatin domains (Hammond *et al*, 2017a; Clapier *et al*, 2017). Long-distance interactions between those domains result in the formation of chromatin compartments (Allshire & Madhani, 2018).
Following their synthesis, histones are bound by specialized chaperones that ensure accurate nucleosome assembly and prevent histone degradation. Mobility within chromatin is conferred by nucleosome remodeling complexes which define histone variants composition and alter nucleosome position by evicting, sliding, and spacing nucleosomes along the DNA sequence.

1.1.1 Chromatin dynamics and histone modifications

To preserve cell identity and permit the appropriate response to stimuli, organisms require an optimal equilibrium between stability and reversibility in gene expression patterns. Chromatin dynamics and transcription are controlled by the combined action of several players, including transcription factors, chromatin remodelers, DNA methylation, and unique histone variations and modifications.
Histones tails feature a variety of post-translational modifications (PTMs) such as methylation, acetylation, ubiquitylation and phosphorylation (Strahl & Allis, 2000). An additional layer of control is provided by the degree of methylation at histones, since lysines on histone tails may undergo mono-, di-, or trimethylation, and arginines mono- or dimethylation. This array of modification patterns confers an extensive functional potential, although not all combinations will be simultaneously present on the same

histone at the same time. The timing of appearance is dynamic and depends on signals within the cell. Chromatin is not an inert structure, but is shaped by environmental conditions via histone PTMs and DNA methylation (Bollati & Baccarelli, 2010).

The deposition of various groups on histones is controlled by the dynamic interplay of complex enzymatic machineries. Epigenetic players are categorized as "writers" responsible for the deposition of PTMs which are subsequently removed by "erasers". There is an additional class of enzymes in charge of the recognition of the modification, known as "readers". Deregulation of this machinery alters chromatin configuration thus disrupting transcriptional programs.

Histone modifications may favor the recruitment of proteins to chromatin (Bannister & Kouzarides, 2011). Thus, based on the modification pattern on a histone, certain proteins can access or are excluded from chromatin. Once recruited, these proteins may tether enzymatic activities which further modify chromatin or affect transcription thereby influencing proliferation, differentiation or cell death.

Proteins utilize distinct domains to recognize and bind histone modifications. Methylation is detected by proteins which include chromo-like domains of the Royal family (chromo, tudor, MBT) and PHD domains, whereas acetylation is bound by bromodomains.

Histone tails protrude from the histone core and interact with neighboring nucleosomes and linker DNA. Mechanistically, these histone PTMs may dictate higher-order chromatin structures by disrupting contacts among different histones in adjacent nucleosomes (Bannister & Kouzarides, 2011). Alternatively, PTMs may alter chromatin configuration by loosening DNA-histone interaction to ensure the execution of certain functions. Due to the abundance of lysine and arginine residues, histones are positively charged. Lysine residues often undergo acetylation which unfold chromatin by neutralizing the electronic charge on histones facilitating the release of the negatively charged DNA.

Histone PTMs coordinate more restricted functions, including the transcription of a specific gene or DNA repair, alternatively may act at the genome-wide level, by affecting DNA replication or chromosome condensation.

With respect to the accessibility for nuclear proteins, modifications distinguish the genome into distinct categories defined as euchromatin where DNA is on an open configuration, or heterochromatin where chromatin is inaccessible. Transcriptionally

silent and active chromatin are depicted by a different pattern of histone tail modifications. Euchromatin is preferentially present on actively transcribed genes as well as regulatory elements like promoters and enhancers (Morrison & Thakur, 2021). Euchromatin is characterized by lysine acetylation and by the trimethylation of H3K4 (H3K4me3) and H3K36 (H3K36me3). A distinguishing trait of the chromatin found in promoter regions proximal to transcriptional start site (TSS) is the H3K4me3. H3K27 acetylation (H3K27ac) coexists with H3K4me3 at promoter region and at the TSS, while gene bodies are endowed with H3K36me3.

In the nuclei, heterochromatin is spatially segregated from euchromatin and is selectively confined in the nuclear periphery and around the nucleolus. Additional classifications of heterochromatin comprise constitutive and facultative heterochromatin. Constitutive heterochromatin is depicted by the H3K9me3, which is imparted in highly repetitive and gene-poor zones like centromere, telomeres and transposons. Conversely, facultative heterochromatin is assembled on developmental genes or on whole chromosomes (X chromosome) and is depicted by H3K27me3 and ubiquitylation of H2AK119.

Genes coding for master transcription factors are often kept in a poised state in embryonic stem cells (ESCs) by presenting both activating and repressive modifications on their regulatory elements (Bernstein *et al*, 2006). These bivalent domains shatter the simplistic notion that activating and repressing PTMs define distinct kinds of chromatin environments. In ESCs, bivalent promoters are typically marked by the trimethylation of both H3K27 and H3K4 (Voigt *et al*, 2012). However, when ESCs differentiate, the repressive H3K27me3 is lost, and target genes are transcribed.

1.1.2 The histone modification H3K27me3

H3K27me3 is the most extensively investigated histone PTM in terms of establishment of facultative heterochromatin and the suppression of developmental related genes. The methylation reaction is catalyzed by the Polycomb Repressive Complex 2 (PRC2) which is a member of the Polycomb-group proteins (PcG proteins). PRC2 consists of four core subunits, EZH1/2, SUZ12, EED, and RBAP46/8 (Kuzmichev *et al*, 2002; Margueron & Reinberg, 2011). The catalytically active component is the SET domain-containing protein EZH2 (or the related EZH1).

PRC1, the other member of the PcG family proteins, recognizes the H3K27 methylation mark and determines H2A ubiquitination. In concert, PRC1 and PRC2 are responsible for the maintenance of transcription silencing through deposition of the H3K27me3.

PRC2 can mono-, di-, and trimethylate H3K27, with each methylation state being functionally distinct. H3K27me3 deposition is centered around the promoter (Zhao *et al*, 2007), intergenic (Cui *et al*, 2009) and subtelomeric regions (Rosenfeld *et al*, 2009) and in long-terminal repeat retrotransposons (Leeb *et al*, 2010). The other two modifications are less studied. While H3K27me1 is distributed through the body of actively transcribed genes in ESCs, H3K27me2 has a more broad distribution (Barski *et al*, 2007; Ferrari *et al*, 2014).

In ESCs, PRC2 and the related H3K27me3 are highly enriched at promoters of developmental genes, including the Hox gene clusters, and at the inactive X chromosome (Plath *et al*, 2003; Bracken *et al*, 2006; Silva *et al*, 2003).

H3K27me3 forms large domains in mouse and human embryonic fibroblast known as broad local enrichments (BLOCs), often visualized over repressed differentiation genes (Pauler *et al*, 2009; Margueron & Reinberg, 2011). Notably, H3K27me3 domains in human ESCs contain developmentally regulated genes as well as neural specific promoters that expand throughout differentiation to suppress genes that are no longer required.

1.2 KDM6A

1.2.1 KDM6A: A Histone H3K27 Demethylase

The existence of histone demethylases remained contentious for several years. There was the widespread perception that histones modifications were stable and inert marks on chromatin, until Yang Shi and colleagues (Shi *et al*, 2004) discovered LSD1/KDM1A. Since then, many other demethylases have been identified. In 2007, a cluster of studies characterized UTX (Ubiquitously transcribed tetratricopeptide repeat, X chromosome) known also as lysine specific demethylase 6A gene (KDM6A) as a JmjC domain-containing demethylase (Agger *et al*, 2007; Hong *et al*, 2007; Lan *et al*, 2007; Lee *et al*, 2007).

The KDM6A protein has tetratricopeptide repeat (TPR) domains at the N terminal domain which facilitate protein-protein interactions (Smith *et al*, 1995) (**Figure 1**). The C terminus possesses a Jumonji C (JmjC) domain that specifically catalyzes histone demethylation thereby promoting gene activation. Target specificity toward the H3K27 mark is assured by a zinc-binding domain which prevents interaction with the near-cognate H3K9 mark (Kim & Song, 2011; Sengoku & Yokoyama, 2011). Interestingly, KDM6A possesses an intrinsically disordered region between the TPRs and the JmjC domain that forms phase-separated liquid condensates (Banani *et al*, 2017; Shi *et al*, 2021).

Although the TPR domains are not strictly essential for the catalysis, the disruption of the N-terminal region results in the reduced demethylation of targets (Lee *et al*, 2007).

KDM6A gene resides on the X chromosome, but escapes X-chromosome inactivation leading to a dosage imbalance between males and females (Greenfield *et al*, 1998). KDM6A has two paralogs in humans, JMJD3 (Jumonji Domain-Containing Protein 3 or KDM6B) and UTY (Ubiquitously Transcribed Tetratricopeptide Repeat Protein, Y-Linked) (Hong *et al*, 2007; Walport *et al*, 2014), all of three proteins containing the JmjC domain (Shpargel *et al*, 2012). As UTY is localized on the Y-chromosome, males possess both KDM6A and UTY, whereas KDM6B is autosomal and situated at 17p13.1. Notably, despite a conserved JmjC domain and a 83% sequence homology with the KDM6A, the demethylase activity of UTY is lower with respect to that of its paralogs (Walport *et al*, 2014; Hong *et al*, 2007; Lan *et al*, 2007; Shpargel *et al*, 2012) (**Figure 1**).

On the other side, the lack of TRP domains in the JMJD3 protein may underlie the alternative reported functions of KDM6A and JMJD3.

Mechanistically, KDM6A counteracts PRC2 by removing the di- and trimethyl repressive marks from H3K27 (Hong *et al*, 2007). Their antagonistic roles are necessary for the stringent epigenetic regulation of transcription during tissue-specific differentiation and development (Agger *et al*, 2007; Ringrose & Paro, 2007; Schuettengruber *et al*, 2007). In particular, KDM6A and PRC2 contribute to animal body patterning by controlling the spatio-temporal expression of the HOX gene clusters by modulating the levels of H3K27me3 at their promoters (Agger *et al*, 2007; Lan *et al*, 2007). As cells differentiate, specific genes loose the H3K27me3 from their promoters, so that the developmental program may start (Ku *et al*, 2008).

Knockout (KO) experiments have shown critical functions for KDM6A across several developmental processes, including cardiac development (Lee *et al*, 2012; Welstead *et al*, 2012), myogenesis (Seenundun *et al*, 2010; Wang *et al*, 2013), hematopoiesis (Thieme *et al*, 2013; Liu *et al*, 2012), and aging (Maures *et al*, 2011; Jin *et al*, 2011). KDM6A loss impairs the development of the caudal trunk in zebrafish (Lan *et al*, 2007) and gonads in *C. elegans* (Agger *et al*, 2007). In mice, KDM6A homozygous mutant females display developmental delay, neural tube closure, and cardiac defects (Welstead *et al*, 2012), whereas heterozygous female mice are viable and fertile. Conversely, hemizygous KDM6A mutant male mice prematurely die, with only a few surviving due to the remaining UTY (Welstead *et al*, 2012; Shpargel *et al*, 2012). Mice deficient for both KDM6A and UTY phenocopy the KDM6A homozygous mutant females, suggesting redundant functions in embryonic development (Shpargel *et al*, 2012). In addition, UTY can partially rescue embryonic lethality in KDM6A KO male mice (Lee *et al*, 2012; Welstead *et al*, 2012; Wang *et al*, 2012; Shpargel *et al*, 2012).

Loss-of-function defects in KDM6A have been reported in individuals with a rare genetic disorder, the Kabuki syndrome (Miyake *et al*, 2013b, 2013a). The Kabuki syndrome, which affects roughly one in every 32,000 live births, is recognized by intellectual impairment, skeletal and facial deformities typified by prominent protruding ears, and postnatal growth retardation (Niikawa *et al*, 1981).

Whole-exome sequencing experiments first identified nonsense and frameshift mutations in the Mixed-Lineage-Leukaemia 2 (MLL2) gene as the etiology of the Kabuki syndrome (Ng *et al*, 2010) in 74% of patients. Nevertheless, in addition to the more prevalent alterations in MLL2, focal deletions and mutations targeting KDM6A were discovered in Kabuki patients (Lederer *et al*, 2012; Miyake *et al*, 2013b).

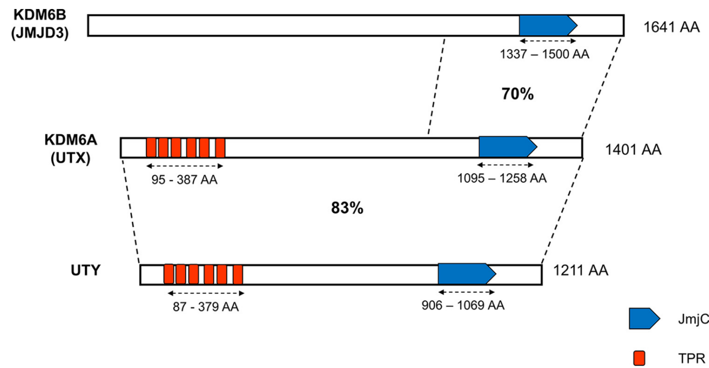


Figure 1. Schematic representation of mouse KDM6 histone demethylases.
The catalytic JmjC and the TRP domains are depicted. KDM6A shares an 83% sequence homology with UTY. All orthologs possess the JmjC domain, while TRPs domains are missing in the KDM6B protein.
(Tran et al, 2020).

1.2.2 Interactome and Functions of KDM6A-Associated Proteins

We previously referred to KDM6A as a histone demethylase implicated in the axis PRC2-KDM6A. Additionally, KDM6A is engaged in a vast network of protein complexes. KDM6A is a component of the MLL 2/3 or COMPASS complex (also called MLL3/MLL4) that promotes the deposition of histone H3 lysine 4 (H3K4) methylation, an epigenetic mark of open and active chromatin (Issaeva *et al*, 2007). It includes 12 proteins, including MLL2, MLL3, ASH2L, RBBP5, WDR5, NCOA6, DPY30, PAXIP and coordinates the transcriptional activation of several biochemical processes through the dynamic interplay between H3K4 methylation and H3K27me2/me3 demethylation (Shilatifard, 2008; Bochyńska *et al*, 2018; Cho *et al*, 2007) (**Figure 2**).
Moreover, KDM6A promotes general chromatin remodeling by functionally interacting with the BRG1-containing SWI/SNF remodeling complex (Miller *et al*, 2010) and H3K27 acetyltransferases p300 or CBP implying a synergistic effort to counteract Polycomb-mediated gene silencing (Tie *et al*, 2012) (**Figure 2**).
KDM6A was also implicated in transcriptional elongation via associations with the SUPT6H-RNA Polymerase II complex (Wang *et al*, 2017; Faralli & Dilworth, 2013). Importantly, KDM6A interacts with distinct transcriptional activators, including pluripotency stem cell factors and the tumor suppressor p53. In addition to the COMPASS

component RBBP5, KDM6A interacts with and affects retinoblastoma (Rb) binding protein transcription, thereby regulating cell cycle stages and cell differentiation (Van der Meulen *et al*, 2014).

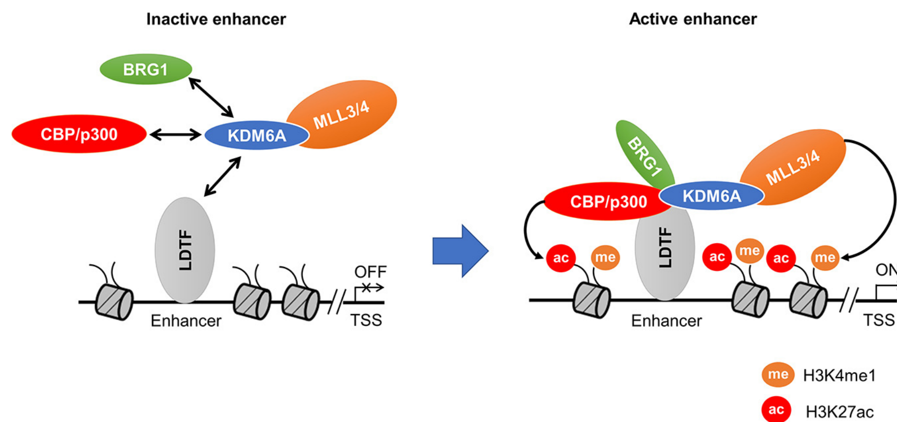


Figure 2. KDM6A functionally interacts with the MLL3/4 complex, CBP/p300 and the BRG1 chromatin remodeler at enhancers.
The synergistic action of histone methylation and acetylation, as well as chromatin remodeling activates gene expression.
(Tran et al, 2020)

1.2.3 KDM6A in cancer

Two years after its discovery, it was found that KDM6A is genetically altered in a broad range of human malignancies, although at very different frequencies (van Haaften *et al*, 2009). KDM6A defects were reported, among others, in human clear cell renal cell carcinoma (ccRCC) (Dalgliesh *et al*, 2010), and T-ALL (Van der Meulen *et al*, 2015), although the highest prevalence is observed in bladder cancer (Gui *et al*, 2011).
The Cancer Genome Atlas (TCGA) (An *et al*, 2016; Rodriguez-Vida *et al*, 2018) reports that the cancer cells of 29.2% of patients affected by urothelial carcinoma possess KDM6A genetic alterations (**Figure 3**). Mutations in the TPR motifs and in the JmjC domain disrupt histone substrate or protein interaction, as well as its enzymatic activity. Nevertheless, a significant number of missense mutations were found throughout the gene, even outside of the two well-defined domains. Conversely, defects in KDM6B and

UTY in cancer are considerably more unusual than those in KDM6A. Of note, due to its location on the Y chromosome, UTY deletions are usually missed in genome-wide studies, and are usually more prevalent in dedicated investigations (Ahn *et al*, 2016; Hurst *et al*, 2017).

Other studies provided additional evidence of genomic alterations affecting KDM6A in different human solid tumors and hematological malignancies (Bailey *et al*, 2018; Mar *et al*, 2012; Kim & Song, 2011; Robinson *et al*, 2012; Grasso *et al*, 2012).

A large-scale, prospective clinical sequencing initiative (Zehir *et al*, 2017), based on 10,945 tumor samples, identified 386 mutations on KDM6A over all cancer types (i.e. 3.5% on average, with 223 truncating mutations, 144 missense mutations and 10 in-frame InDels). Furthermore, KDM6A was among the 127 most significantly mutated genes in a whole-exome sequencing study from TCGA based on 3,281 tumors derived from 12 tumor types (Kandoth *et al*, 2013). These findings support the notion that KDM6A is a recurring mutational target for several cancer types.

KDM6A overall mutational spectrum is suggestive of a tumor suppressor. However, the functional significance of several missense mutations is uncertain.

While all mutations associated with Kabuki syndrome impair KDM6A function, the landscape in tumors is unclear as some mutations are harmful in certain cancers, suggesting a tumor-suppressive function, whereas in other tumors KDM6A supports oncogenic factors (Schulz *et al*, 2019).

In breast cancer, KDM6A mediates epithelial mesenchymal transition (EMT) modulating the transcription of several factors, such as SNAI and ZEB1/2 (Choi *et al*, 2015). Furthermore, KDM6A was found to support the oncogenic functions of the estrogen receptor α (ERα) (Kim *et al*, 2014; Xie *et al*, 2017).

An additional pro-oncogenic role was demonstrated in cervical cancer, where increased KDM6A expression activates cell cycle through the HPV E7 protein while also mitigating the consequences of replicative stress caused by ectopic proliferation (Soto *et al*, 2017).

The dual role of KDM6A in cancer underscores the context-dependent essence of oncogenes and tumor suppressor genes and possibly implies that H3K27me3 have different activities in distinct cell types.

Notably, both KDM6A and KDM6B, are required for the transcriptional regulation of T-cell development (Manna *et al*, 2015). While KDM6A is frequently genetically

inactivated and functions as a tumor suppressor in human T cell acute lymphoblastic leukemia (T-ALL), KDM6B was ascribed an oncogenic function (Ntziachristos *et al*, 2014). Importantly, KDM6A does not act as a tumor suppressor in all T-ALL subtypes. In the TAL1- driven T-ALL, KDM6A functions as a pro-oncogenic cofactor essential for leukemia maintenance (Benyoucef *et al*, 2016).

Despite the reported implication of the KDM6A catalytic function in tumorigenesis, KDM6A demethylase activity is often dispensable for tumor initiation or progression (Gozdecka *et al*, 2018; Andricovich *et al*, 2018; Shpargel *et al*, 2012). Homozygous KDM6A inactivation may induce squamous-like pancreatic tumors in female mice by affecting the interaction with the COMPASS complex. On the other hand, the simultaneous loss of KDM6A and UTY in the male counterpart was required for the development of this tumor subtype, indicating a restricted demand for H3K27 demethylase activity (Andricovich *et al*, 2018).

Additional insights on KDM6A role in cancer comes from a recently published article, were the ability of KDM6A to phase separate was reported as an underlying mechanism behind the modulation of tumor suppressive gene expression programs (Shi *et al*, 2021). KDM6A was also able to regulate genome-wide histone PTMs and higher-order chromatin interactions in a condensation-dependent manner and to form co-condensates along with its interacting proteins (e.g., MLL4 and p300) through the TRP domains.

As KDM6A function is likely contingent on its interacting partners, KDM6A loss consequences aren’t consistent over different cancer types. Whether KDM6A inhibits or stimulates cancer initiation is intimately connected to its involvement in other complexes. Any KDM6A mutations that impair the interaction between KDM6A, and its partners may impact the function of these complexes, contributing to cancer.

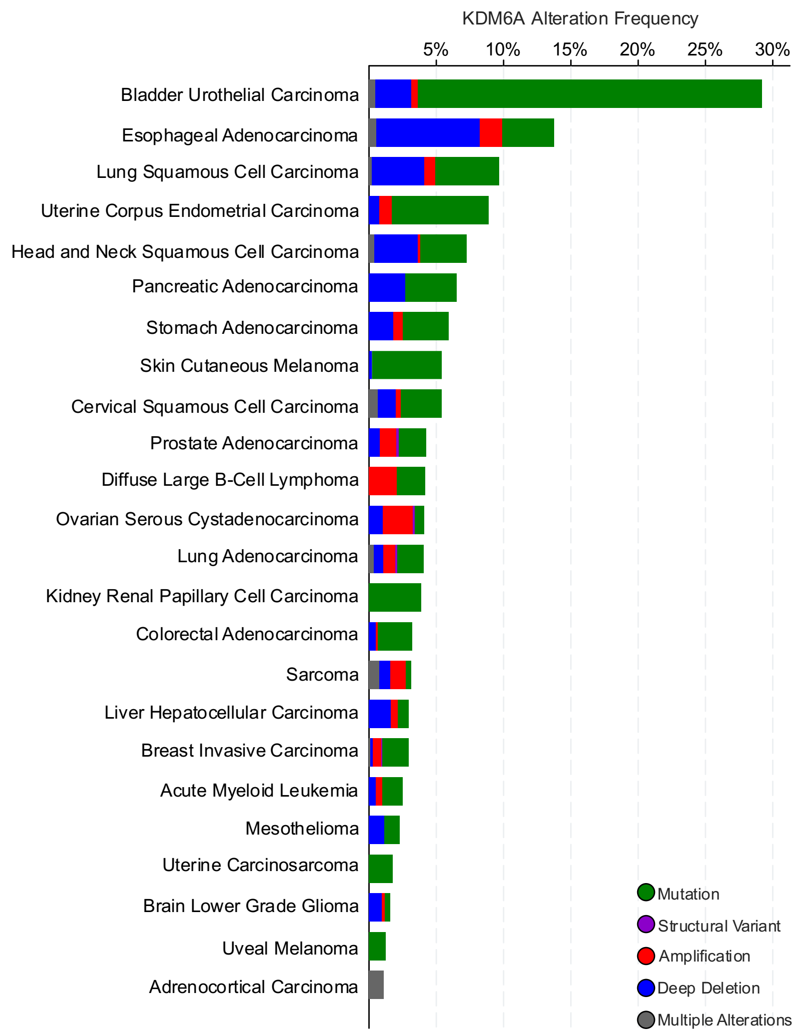


Figure 3. KDM6A is frequently altered in a broad spectrum of cancers
The alteration frequency of KDM6A from the cBioportal is represented. The highest percentage of mutations is found in Bladder Cancer patients (29.2%) (Cerami et al, 2012).

1.3 DNA damage

1.3.1 Genomic instability and sources of damage

Mutations are source of genetic diversity and drivers of natural selection and evolution. On the other hand, the preservation of genome integrity is required for survival and for the faithful transmission of genomic information across generations. DNA is the template for replication and transcription and, if damaged, it cannot be replaced resulting in the accumulation of stochastic DNA lesions, and loss of genetic information (Jackson & Bartek, 2009; Tubbs & Nussenzweig, 2017). As a consequence of mutations, tumor-suppressor genes can be loss or oncogenes improperly activated, ultimately triggering uncontrolled cellular proliferation (Bartek *et al*, 2007).

Cells continuously cope with exogenous sources of stress, such as physical or chemical agents. Exogenous DNA damaging agents include anticancer chemotherapeutic drugs, as alkylating agents (like methyl methane sulfonate), crosslinking agents (mitomycin C and cisplatin among others), and radiomimetic compounds including bleomycin.

Additionally, DNA integrity is threatened by endogenous sources arising during physiologic metabolism (Lindahl, 1993; Bartek *et al*, 2007). Every time a cell divides, $6x10^9$ nucleotides must be faithfully replicated by DNA polymerases. DNA replication itself is not devoid of mistakes and generate mutations at a low but constant rate, potentially leading to DNA breakage, rearrangements and chromosome mis-segregation. During its progression, the elongating replication fork may encounter impediments such as damage sites, proteins, or non-B DNA structure. Each of these circumstances could result to a transient replication pause or, in a worst scenario, to a persistent replication fork stall or collapse (Bartek *et al*, 2004). Cells have evolved a damage tolerance mechanism, the translesion synthesis (TLS), which enables low-fidelity DNA polymerases to efficiently complete DNA replication encompassing the lesion, contributing to the unrelenting accumulation of mutations (Goodman & Woodgate, 2013).

The transcription machinery acts as a natural obstacle for fork advancement, and collisions between the two machineries foster replication stress and genome instability (Sankar *et al*, 2016; Goodman & Woodgate, 2013).

Oncogene activation stimulates the firing of multiple replication forks, altering

replication timing and progression (Halazonetis *et al*, 2008). Analogously, hormonal signaling, and inflammatory responses drive to higher transcriptional levels or replication stress.

DNA breaks can result from by-products of cellular metabolism, such as the reactive oxygen species (ROS) resulted from respiration and lipid peroxidation (De Bont & van Larebeke, 2004). One of the most prevalent lesions produced is the 8-oxoguanine (8-oxoG), which can trigger G>T substitutions if it is not promptly removed prior to DNA synthesis. Moreover, ROS generate inter- and intra- strand crosslinks, and foster DNA–protein crosslinks (Jena, 2012).

It has been predicted that up to 10^5 lesions occur every day in a cell (Lindahl & Barnes, 2000), which can be potentially converted into mutations. The most frequently occurring lesions are single-strand breaks (SSBs) (75%), which are interruptions in a single strand of the DNA double helix arising from oxidative damage, irradiation, base hydrolysis, or during DNA replication. ssDNA can be converted in the more deleterious DNA double-strand breaks (DSBs) when the replicative DNA polymerase encounters an unrepaired SSB and fork collapses (Branzei & Foiani, 2010; Jena, 2012). DSBs interest both DNA strands and therefore lack an intact complementary strand that could be used as a template for DNA repair. Apart from SSB, DSBs can result from IR, radio-mimetic chemicals, ROS or deliberately as intermediates of physiological biological events, including class switch and V(D)J recombination in developing lymphoid cells (Jackson, 2002).

1.3.2 DNA Damage Response pathway

To safeguard genome integrity, cells have evolved intricated mechanisms to rapidly detect DNA lesions, signal their presence to effectors and coordinate their repair, or induce senescence or cell death when the repair capacity is overwhelmed. These signal transduction pathways are collectively known as DNA damage response (DDR). DDR is an exceedingly sensitive and accurate system since it is activated by few DNA lesions, or even just one. To ensure an efficient signaling and subsequent repair, DDR factors do not assemble and disassemble at damage sites as a preformed complex, but in a sequential and coordinated fashion (Polo & Jackson, 2011). DDR proteins accumulation results in discrete foci at the site of damage subjected to a fine spatiotemporal modulation (Bekker-Jensen *et al*, 2006). The assembly of the DDR cascade is achieved through

distinct PTMs, including phosphorylation, ubiquitination, and sumoylation (Bergink & Jentsch, 2009, 2009; Harper & Elledge, 2007).

1.3.3 DNA Damage sensing and signaling

Based on the type and complexity of the lesion, cells engage divergent cellular signaling cascades to properly respond to the genotoxic stress. DDR is primarily mediated by four partially independent sensors proteins, including poly (ADP-ribose) polymerase (PARP) family members, Ku70/Ku80, and MRN factors.

Immediately following a SSB or DSB, PARP1 and 2 are activated and catalyze the polymerization of ADP-ribose moieties (poly-ADP-ribosylation, PARylation) on target proteins to permit the recognition and rapid recruitment of DDR factors to the lesion (Gagné *et al*, 2006; Rouleau *et al*, 2010). PARP target proteins include histone H1 and H2B, and PARP1 itself.

DSBs might also be recognized by the MRE11-RAD50-NBS1 (MRN) complex, that is responsible for ATM induction (Williams *et al*, 2007). ATM is a serine/threonine kinase, member of phosphatidylinositol 3-kinase-like protein kinase (PIKKs) family together with ATR and DNA-PK, which phosphorylates substrates on a sequence including a serine or threonine followed by glutamine, the S/TQ motif (Langerak & Russell, 2011). When recruited to DSBs, ATM is activated through its autophosphorylation at Ser1981 position (Jazayeri *et al*, 2008; Williams *et al*, 2008; Kanaar & Wyman, 2008) (**Figure 4**). ATM activation results in the phosphorylation of the histone variant H2AX at the Ser139 residue (γH2AX) which functions as a docking site for the accumulation of other DDR players. Phosphorylation of downstream factors fuel a positive feedback loop that triggers the spreading of γH2AX regions over 1-2 megabases around the lesion site (Iacovoni *et al*, 2010) and the recruitment of additional ATM molecules, thus sustaining the DDR (Bekker-Jensen *et al*, 2005; Stucki *et al*, 2005). In response to damage, downstream effector proteins are either phosphorylated by ATM/ATR or by their targets, the Checkpoint kinase 1 (CHK1) and 2 (CHK2) (Harper & Elledge, 2007).

In response to a SSB or during replication stress, resulting from nucleotide depletion or replication blockage, the main kinase involved is ATR (Nam & Cortez, 2011; Zhou & Elledge, 2000). When replication is blocked, DNA polymerase releases the replicative helicase (Byun *et al*, 2005) generating tracks of ssDNA that are promptly coated by the

trimeric ssDNA-binding protein RPA. ATR is recruited at the break site in association with its interacting partner, ATRIP which directly binds ssDNA-RPA regions. ATR activation and phosphorylation of the downstream CHK1 necessitate the intervention of two mediator proteins, TopBP1 and Claspin. ATR recruits repair proteins and, once the stall has been resolved, it stabilizes the replication fork to let the replication proceed. The maintenance of the replication machinery at the fork is essential to prevent the generation of unwanted DSBs. If SSBs have not been effectively resolved they inexorably degenerate in DSBs, and both ATR and ATM are recruited.

Similarly to the MRN complex, the Ku heterodimer (Ku70 and Ku80) is a DNA lesion sensor (de Jager *et al*, 2001; Delacroix *et al*, 2007) which displays a toroidal structure that encircles DNA via its central ring domain (Walker *et al*, 2001) (**Figure 4**). Ku rapidly localizes at DSBs where it recruits and activates the apical kinase DNA-PK (Dvir *et al*, 1992; Walker *et al*, 2001).

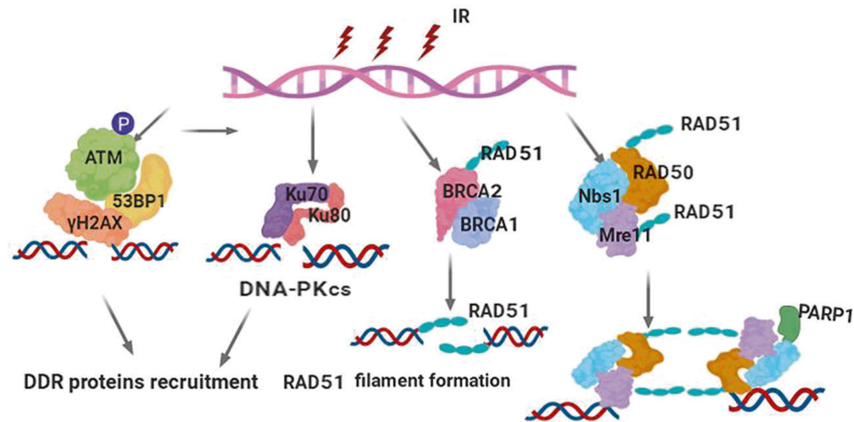


Figure 4. DNA damage sensors and signaling proteins action in response to DSBs
DNA damage sensor such as the apical kinase ATM and ku70/ku80 are rapidly recruited at the DNA damage site to initiate DNA damage signaling cascade.
(Huang & Zhou, 2020)

1.3.4 The DNA repair pathways

DNA repair must occur in a temporal, spatial, and lesion-appropriate manner in order to prevent unnecessary and potentially harmful DNA aberrations.

In the case of SSBs or subtle changes to the DNA sequence, where only one of the two strands of a double helix is severed, three divergent excision repair mechanisms intervene to remove the damaged nucleotide and replace it through the normal DNA replication. The Base Excision Repair (BER) is the primary process for repairing damaged DNA bases throughout the entire cell cycle (Wilson & Bohr, 2007). Nucleotide Excision Repair (NER) removes bulky DNA adducts, such as those caused by UV irradiation and chemicals occurring at the G1 stage. Finally, misincorporations, small insertions or deletions resulting from faulty replication could be excised by a third mechanism, the **M**is**m**atch **R**epair (MMR) pathway (Li, 2008).

Overall, these mechanisms share an overlapping repair process that consists first in the lesion recognition followed by DNA cleavage. Degraded nucleotides are subsequently replaced with newly synthesized DNA.

When SSBs occur, PARP1/2- dependent PARylation promotes the recruitment of the scaffold protein XRCC1, the DNA ligase 3 (LIG3), and accessory repair proteins at the lesion in order to re-ligate the break.

Conversely, DSBs can be repaired by at least four distinct mechanisms: Homologous Recombination (HR), Non-Homologous End Joining (NHEJ), alternative end joining (alt-EJ), and single-strand annealing (SSA). While HR and NHEJ are more faithful processes, the latter may contribute to genome rearrangements and oncogenic transformation.

NHEJ acts throughout the entire cell cycle and despite its high efficiency, is prone to mutations, promoting the potentially inaccurate religation of the two severed DNA ends. Conversely, HR is a conservative pathway that allows the precise repair during S and G2 stages (Karpenshif & Bernstein, 2012).

The first step in NHEJ pathway is the recognition and binding of the Ku70/80 heterodimer to the DSB which recruits DNA- PK and permits the subsequent activation of the catalytic subunit DNA-PKcs. DNA-PKcs, through a cascade of phosphorylation reactions, stabilize DSB ends and prevent undesired end resection events (Meek *et al*, 2008). Once the two ends are juxtaposed, DNA-PKcs autophosphorylation triggers its detachment from the DNA so that end processing enzymes such as the endonuclease ARTEMIS (Meek *et al*, 2008) can access. At this point, relegation of the damaged ends mediated by XRCC4 and Cer-XLF can occur. If DNA ends are complementary and undamaged, they can be directly joined by the DNA ligase 4 (LIG4) (Reynolds *et al*, 2012). In the other case, further end processing by ARTEMIS and APLF nucleases and the PNK kinase/phosphatase is required (Mahaney *et al*, 2009).

Backup pathways are invoked when NHEJ proteins are mutated or loss and the canonical NHEJ is impeded. These pathways often rely on microhomology regions for the joining reaction and implicate factors that mainly act in HR or SSB repair, such as the MRN complex, PARP1, XRCC1, and DNA Ligase 1 or 3.

DSB repair through HR starts with DNA end resection, a multi-step process, in which MRE and CtlP initiate a limited resection, while the exonuclease EXO1 or the helicase BLM mediate more extensive processing of the 5' DNA end to generate fully resected 3' ssDNA ends.

DNA-end resection is a finely regulated process, relegated only to the S and G2 cell cycle phases, when CtIP is phosphorylated, and the sequence homology of a sister chromatid is available to regenerate the broken chromosome.
In this time window, BRCA1 interacts with and ubiquitinates CtIP facilitating its association with the DNA lesion (Huen *et al*, 2010).
Nevertheless, limited DSB resection by CtIP occurs in G1 in a BRCA1-independent way to boost alt-NHEJ (You & Bailis, 2010; Yun & Hiom, 2009).
RPA binds and stabilizes the 3' overhanging ssDNA flanking the break resulting from these resection events and is then displaced by the RAD51 recombinase in a BRCA1 and BRCA2-dependent fashion (West, 2003). The interaction between RAD51 and BRCA2 is confined to S and G2 stages thus preventing the resection of DNA ends outside these phases of the cell cycle (Holliday, 1964; Hartlerode & Scully, 2009). This cell cycle control restricts exchanges between homologous chromosomes preventing loss of heterozygosity events.
ATM and ATR play additional regulatory roles in those subsequent steps of HR by phosphorylating RAD51(Sørensen *et al*, 2005, 1) and BRCA2 (Matsuoka *et al*, 2007).
RAD51 in turn mediates homology search and strand invasion of the 3’ overhang into the homologous undamaged sequence of the sister chromatid displacing a DNA strand and resulting in a so-called D-loop. On the opposite side of the D-loop, an “X” shaped structure, the Holliday Junction, is generated (Holliday, 1964). DNA synthesis beyond the break site, using the sister chromatid as a template, will restore the missing sequence information. Once repair synthesis is complete, the sliding of the Holliday Junction toward the 3’ end allows the release of the invading strand and the newly synthesized 3’ single-stranded end can then anneal to the other side of the break. Final processing to resolve HR intermediates, removing flaps, filling in gaps, and ligating remaining nicks requires Ligase I, and RECQ helicases to yield two intact DNA molecules.
In the context of repetitive DNA sequences which are repaired by SSA, RAD52 promotes annealing of resected 3’ ssDNA, followed by the XPF/ERCC1 removal of DNA flaps and LIG1-mediated DNA ligation (Hartlerode & Scully, 2009; Motycka *et al*, 2004).
The execution of a certain repair mechanism could be determined by the negative control of one pathway by another. For example, the crucial NHEJ component 53BP1 can impede DSB resection by enhancing the stability and mobility of DNA breaks, allowing DNA

ends to find one another for successful ligation (Difilippantonio *et al*, 2008; Bunting *et al*, 2010). Conversely, BRCA1 might suppress 53BP1 action at DNA lesions to favor end resection (Bouwman *et al*, 2010; Bunting *et al*, 2010). Similarly to BRCA1, PARP1 competes with Ku binding to DNA ends to boost HR (Hochegger *et al*, 2006).

Additional regulatory steps that determine which repair pathway is active are the cell-cycle stage at which the DSB is generated and the extent of DNA end processing. In contrast to alt-NHEJ, HR, and SSA (where end resection is restricted for alt-NHEJ but more extensive for HR and SSA), the classical NHEJ is end-resection-independent (Hartlerode & Scully, 2009). After end resection, the canonical NHEJ repair pathway is indeed impeded, and any of the three homology-based mechanisms (SSA, alt-EJ, and HR) can be employed.

The alt-NHEJ and SSA are both error-prone mechanisms that only intervene when the more accurate repair pathways (NHEJ or HR) are disrupted (Groelly *et al*, 2022). The observation that POLQ (an alt-NHEJ polymerase) is increased in HR-deficient tumors supports this notion. Generally, POLQ-dependent DSB repair is prevented until mitosis begins as a final attempt to repair DSBs that would otherwise be carried into mitosis leading to micronuclei and further disastrous outcomes (Llorens-Agost *et al*, 2021; Blackford & Stucki, 2020). These findings imply that changes in the optimal balance of DSB repair pathways might result in genomic instability.

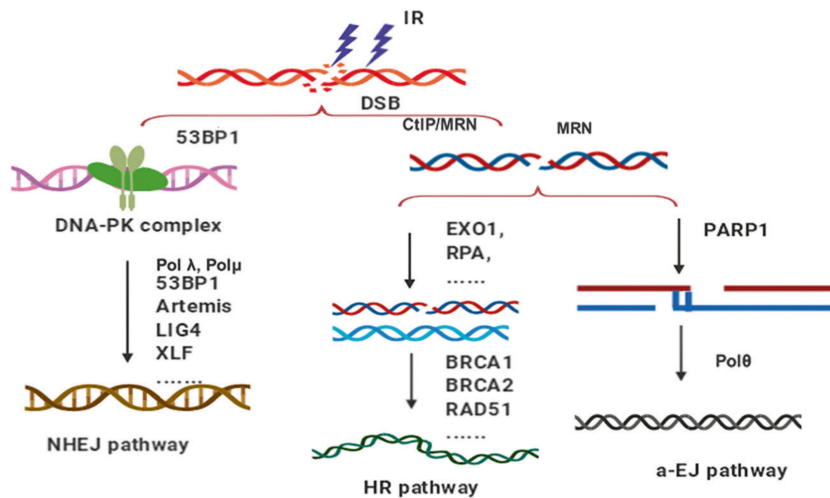


Figure 5. HR and NHEJ are the two major pathways for the repair of DSBs
The major pathways and the key players involved in the repair of DSBs are illustrated.
(Huang & Zhou, 2020)

1.3.5 DDR checkpoint

DDR checkpoints are regulatory mechanisms that act in coordination with cell cycle, transiently slowing or blocking its progression to signal the break. Once repair occurred and the signaling cascade is switched off, checkpoint proteins allow cycle resumption (**Figure 6**). Sophisticated cell cycle checkpoint mechanisms evolved to withstand unforeseen failures in DNA replication. Cell cycle progression is driven by oscillations in the activity of cyclin-dependent kinases (CDKs) which are maintained active through the dephosphorylation catalyzed by CDC25 phosphatases. ATM and ATR, once activated in the presence of genotoxic stress, induce CHK2 and CHK1, which in turn phosphorylate and lead to the proteolytic degradation of CDC25A, thus preventing S-phase entry (Donzelli & Draetta, 2003).

While predominantly controlled through posttranslational modifications, essential decisional processes are established at the transcriptional level allowing for the integration of information over time. The key player in this response is p53, which is phosphorylated and stabilized by ATM and CHK2 when DSBs occur (Zhou & Elledge, 2000). p53, in turn, causes extended cell-cycle arrest in G1, apoptosis, or senescence by

transcriptionally controlling the CDK inhibitor p21 or the proapoptotic factors BAX and PUMA (Riley *et al*, 2008).

Unrepaired damage, which escapes the G1/S checkpoint, switches on the intra-S-phase checkpoint in order to remove DNA lesion prior to the resumption of DNA synthesis. By inhibiting the formation of new replication forks, this intra-S phase checkpoint reduces the likelihood that lesions will be encountered by the replicative DNA polymerases thus preventing fork breakdowns. Furthermore, an intra-S checkpoint may also guarantee the time window necessary for HR occurrence.

ATR additionally exerts a key role in the S–G2 checkpoint which is a surveillance mechanism that prevents the premature entry into mitosis before replication completion (Simoneau & Zou, 2021; Riley *et al*, 2008).

To avoid that a damaged cell enters in mitosis, cell have developed an additional checkpoint at G2/M transition stage to ensure that the correct genetic material is transferred to daughter cells (Warmerdam & Kanaar, 2010). CHK2 and CHK1 inhibit cycle progression by inactivating CDC25C and stabilizing the kinase Wee1 which deposits inhibitory phosphorylation on CDK1 and CDK2.

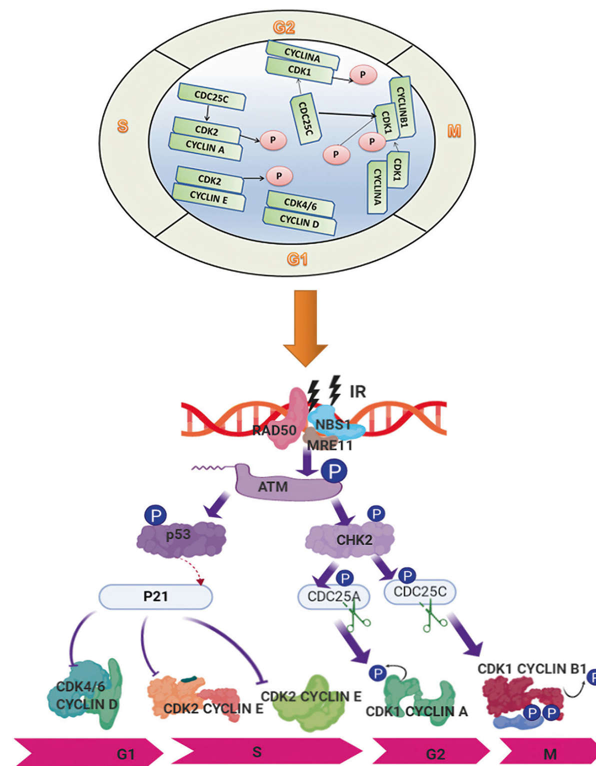


Figure 6. Cyclins and CDKs are involved in the activation of cell cycle checkpoints upon damage.

(Huang & Zhou, 2020)

1.3.6 Targeting DNA repair in cancer

Defects in the DDR are likely to exacerbate the incidence of genomic rearrangements. Normally, functional DDR prevents carcinogenesis by eliciting cellular senescence or death in early tumor cells (Bartek *et al*, 2007). However, mutations in DDR genes like TP53 or ATM may predispose to cancer by encouraging cell death bypass despite the presence of damage (Luo *et al*, 2009). Consistent with this notion, widespread deficiencies in DNA repair genes have been reported in cancer cells and subjects with a genetic susceptibility to cancer (Taylor *et al*, 2019; Pritchard *et al*, 2016; Morganella *et al*, 2016; Nik-Zainal *et al*, 2016).

Inherited DDR factor deficiencies have detrimental outcomes also in a variety of contexts, including immunological impairments, neurodegeneration, and premature aging (Hoeijmakers, 2009, 2001).

Given these premises, it is not surprising that DDR vulnerabilities have been exploited in chemotherapy and radiotherapy regimens. Platinum salts, such as cisplatin, have been proven to be beneficial in ovarian cancer patients because of the defective HR as well as in individuals with ERCC1-negative non-small-cell lung cancers (Friboulet *et al*, 2011). An alternative approach to target cancer cells is the development of drugs that specifically target DDR components in combination with other chemotherapeutic treatments, without affecting cells proficient in DDR. This is because cancer cells experience higher levels of DNA damage than normal cells do, thereby rendering them more vulnerable to attack. Irinotecan and etoposide, two topoisomerase inhibitors, might be regarded as the first generation of these DDR "targeted" drugs (Pommier *et al*, 2010).

Tumors harboring HR gene mutations, such as BRCA1 and BRCA, have been successfully cured with PARP1 inhibitors (Jackson & Bartek, 2009; Farmer *et al*, 2005; Bryant *et al*, 2005).

When PARP-dependent repair is inhibited, SSBs accumulate during replication. It is therefore plausible that the replication fork stalls when it encounters these DNA defects leading to the generation of lethal DSBs. While functioning HR in healthy cells mitigates the impact of PARP inhibition by repairing resultant DSBs, in BRCA1 and BRCA2 deficient cells those lesions are left unrepaired (Lord & Ashworth, 2008). Therefore, inhibition of PARP activity is thought to result in an increased reliance on HR repair.

BRCA1 or BRCA2 germline mutations are uncommon in sporadic breast cancer, although they are prevalent in hereditary breast cancer. However, sporadic tumors, carrying traits in common with tumors lacking BRCA gene (a condition originally known as BRCAness) respond well to PARP inhibitors (Turner *et al*, 2004). BRCAness may arise from somatic mutations in other HR genes or methylation of the BRCA1 gene promoter.

When a combination of gene defects causes cell death despite each individual gene deficiency being compatible with cell survival, this is commonly referred to as "synthetic lethality". In this context, BRCA deficiency is synthetically lethal when PARP is inhibited. The BRCA/PARP model in tumor cells has encouraged the investigation for further DDR synthetic lethalities that might be exploited in the therapy of malignancies.

1.4 Chromatin dynamics in DNA damage

1.4.1 Histone dynamics modulate chromatin decompaction and mobility upon damage

While exploring the DDR in its biological context, it is important to take into account that DNA is wrapped around histone proteins (Kornberg, 1977).
Despite the necessity of maintaining the epigenome stability, all DNA metabolic activities dramatically affect chromatin architecture and its biophysical characteristics. The compaction state of chromatin poses a challenge to transcription, replication and to the detection and repair of DNA damage since the DNA target sequence might be hindered.
DNA repair is extremely challenging for cells since it is mostly unscheduled, and lesions may arise anywhere in the genome and at any time (Hoeijmakers, 2001).
An extensive enzymatic machinery including histone modifiers, chaperones (Hammond *et al*, 2017b), and nucleosome remodelers (Clapier *et al*, 2017) builds a plastic chromatin landscape that rapidly responds to damage by modulating chromatin compaction and transcriptional activity at lesions.
Indeed, DNA damage is accompanied by a dynamic chromatin reshape which relieves nucleosomes constrains thereby increasing chromatin accessibility to the repair machinery (Adam & Polo, 2012; Kruhlak *et al*, 2006; Murga *et al*, 2007; Seeber *et al*, 2018; Lu *et al*, 2019; Chagin *et al*, 2019). Moreover, a facilitated search and invasion of the homologous template sequence, which may be spatially dispersed or confined in another chromatin domain, is required in the case of homology driven DSB repair (Heyer *et al*, 2010).
Despite the widespread view that histones and more generally nucleosomes are stable chromatin structure units, their turnover supports cells in dealing with genotoxic stress. Based on the kind of histone variant and type of damage, histone proteins can be displaced, evicted, or degraded (Adam & Polo, 2012; Luijsterburg *et al*, 2012).
Transient nucleosome disassembly is necessary in yeast and human cells to overcome the barrier posed by nucleosomes to end processing and repair components (Luijsterburg *et al*, 2012; Berkovich *et al*, 2007; Courilleau *et al*, 2012; Clouaire *et al*, 2018; Yang *et al*, 2020; Tsukuda *et al*, 2005; Li & Tyler, 2016; Goldstein *et al*, 2013). This process involves a variety of players, including INO80, p400 nucleosome remodelers (Courilleau *et al*,

2012; Li & Tyler, 2016; van Attikum *et al*, 2007), nucleolin (Goldstein *et al*, 2013), and CHD3/CHD4 (Seeber *et al*, 2013; Jeggo & Downs, 2014).

After exposure to ionizing IR or radiomimetic compounds, a significant degradation of 30%–40% of the core histones was observed (Hauer *et al*, 2017). Limited histone degradation fosters chromatin expansion and decompaction increasing the mobility of the damaged site to encourage homology search (Hauer & Gasser, 2017a, 2017b).

The concept that histones need to be removed and restored after DNA repair has been clarified by the "prime-repair-restore" model. According to this paradigm, a lesion must be rendered accessible ("primed") to let the repair occur. Finally, once the repair is complete, the chromatin environment must be returned to its prelesion state.

In addition to the prominent and well-documented phosphorylation of H2AX, other PTMs impose layers of flexibility on nucleosomes upon damage (Smeenk & van Attikum, 2013; Campos & Reinberg, 2009; Zentner & Henikoff, 2013). Following UVC treatment, the acetyltransferase p300 promotes general chromatin relaxation (Rubbi & Milner, 2003), whilst TIP60 causes nucleosome destabilization at break sites allowing the recruitment of resection-mediating factors (Xu *et al*, 2010; Murr *et al*, 2006). Histone acetylation might also encourage chromatin remodeling by modulating histone proteosome-mediated degradation in response to replication stress (Mandemaker *et al*, 2018), IR and alkylating damage (Qian *et al*, 2013).

By directly acetylating ATM, TIP60 increases its kinase activity (Sun *et al*, 2010) leading to the inactivation of the transcriptional repressor KAP1, which localizes at heterochromatic breaks, and to the subsequently chromatin relaxation (Noon *et al*, 2010). These findings suggest a key role of ATM pathway in the repair of DSBs located in heterochromatin regions (Sun *et al*, 2010).

1.4.2 Histone variants and PTMs promote the repression of transcription at damage sites

The importance of chromatin relaxation for the occurrence of repair has been described; nevertheless, gene silencing at the lesion is required to prevent the creation of erroneous transcripts and harmful collisions between the transcription and repair machinery. Silencing may result from alterations in RNA polymerase progression or from

the modulation of histone PTMs (Geijer & Marteijn, 2018; Caron *et al*, 2019; Machour & Ayoub, 2020).

PRC1 induces local transcriptional suppression through the ubiquitin ligase RNF2-mediated monoubiquitylation on H2AK119 or via the ubiquitin ligase UBR5 ubiquitylation of FACT, hence constraining FACT-mediated transcription elongation (Sanchez *et al*, 2016).

Other players, in addition to the Polycomb-associated proteins, control the ubiquitylation of H2A and the suppression of transcription at damage sites. Polyubiquitination on H2A and H2AX at break sites are catalyzed by RNF8, which lowers transcriptional activity in chromatin that has experienced DNA damaged (Paul & Wang, 2017, 8).

Also KDM5A, by mediating the demethylation on H3K4 around DSBs, enables the recognition of the nucleosome remodeling deacetylase NuRD complex and the local transcription inhibition (Gong *et al*, 2017).

1.4.3 DNA repair pathway choice is mediated by chromatin alterations at DNA damage sites.

While phosphorylation events that occur during cell-cycle (for instance, CtIP phosphorylation by CDKs) allow for the onset of HR during the late S and G2 phases, NHEJ remains the major DSB repair choice throughout interphase (Beucher *et al*, 2009; Karanam *et al*, 2012). The pre-existing chromatin landscape and chromatin changes arising in response to genotoxic insults mediate the DNA repair pathways choice by favoring or preventing the recruitment of specific factors.

An example is the competition between BARD1 (BRCA1 interacting protein) and 53BP1 for the binding of the unmethylated or dimethylated H4K20 to encourage or oppose end resection, respectively (Fradet-Turcotte *et al*, 2013). 53BP1 recognizes and binds H4K20me2 around the break thereby protecting DSB ends from resection (Feng *et al*, 2022; Mirman *et al*, 2022; Ochs *et al*, 2019; Paiano *et al*, 2021) and channeling DSB repair to NHEJ. Conversely, during the S phase the unmethylated histone H4K20 is enriched at nascent chromatin and is recognized by the BRCA1–BARD1 promoting HR. In humans, breaks arising in transcriptionally active genes are preferentially repaired by HR (Aymard *et al*, 2014). Indeed, trimethylation on histone H3 at lysine 4, 36, and 79, as

well as other histone PTMs associated with transcriptional activity, create an environment that is prone to HR repair (Clouaire *et al*, 2018; Pfister *et al*, 2014; Carvalho *et al*, 2014). In heterochromatic regions, the repair of DSBs is channeled by the demethylation of H3K9me3 towards NHEJ (Janssen *et al*, 2019).
Collectively, histone variant and PTMs work together to form a multi-level network that orchestrates the DDR from transcription regulation to repair.

1.4.4 Chromatin restoration following damage

Once DNA repair has occurred, many processes operate to restore chromatin structure surrounding the DSB.
For instance, chromatin restoration following UV damage requires the *de novo* incorporation and deposition of new H3.1 histones by the histone chaperone CAF-1 and HIRA, respectively (Adam *et al*, 2013). The remodeler CHD2 (Luijsterburg *et al*, 2016) and the histone chaperone DAXX in association with ATRX (Juhász *et al*, 2018) were shown to be involved in the deposition of new H3.3 at break sites. In addition, restoration of chromatin architecture entails the re-establishment of linker histone H1 following UVA microirradiation (Strickfaden *et al*, 2016). Although there is an intense attention, it is still unclear whether nucleosomes are fully reconstituted and if their positioning is preserved.
Beyond histone deposition, the re-establishment of preexisting histone modifications and the reversal of those caused by damage is necessary for the completion of repair.
For instance, H2AX dephosphorylation is mediated by a variety of phosphatases (Nazarov *et al*, 2003; Chowdhury *et al*, 2005, 2008; Douglas *et al*, 2010, 6), and several ubiquitin-specific proteases operate enzymatically on H2A/H2AX and H2B (Yu *et al*, 2016; Ting *et al*, 2019; Nicassio *et al*, 2007; Wang *et al*, 2016), facilitating the prompt release of repair components and the licensing of chromatin for transcription resumption. Newly deposited histones differ significantly from their parental histones in terms of modifications (Alabert *et al*, 2015). Despite the considerable effort put into restoring the nucleosomes context, new information might still be introduced in chromatin undergoing repair. One intriguing theory is that new histones may provide a memory of DNA damage infliction by creating scars on chromatin through PTMs. This might assist signaling and repair in chromatin areas more vulnerable to lesions (Ferrand *et al*, 2021).

1.4.5 Chromatin shields DNA from damage

Despite chromatin compaction can affect repair in a variety of ways, as was mentioned above; a growing body of research points to the importance of higher order chromatin structure in protecting the genome. Tightly packed chromatin acts as a barrier by restricting the access to reactive radicals and chemicals (Yoshikawa *et al*, 2008; Falk *et al*, 2008; Valota *et al*, 2003).

On this line, previous in vitro studies reported that DNA compaction suppressed the induction of DSBs generated by γ-rays (Yoshikawa *et al*, 2008; Spotheim-Maurizot *et al*, 1995; Warters *et al*, 1999; Douki *et al*, 2000; Suzuki *et al*, 2009).

The hypothesis that chromatin compaction shield DNA from damage is supported by a recent publication that combined sequencing-based chromatin accessibility data with a radiation-induced DSBs localization assay (BLISS (Yan *et al*, 2017)) in mouse mesothelioma cells (Brambilla *et al*, 2020). The incidence of DSBs had an inverse correlation with respect to nucleosome occupancy (Brambilla *et al*, 2020). This data is consistent with the hypothesis that the likelihood of DSB induction is positively correlated to the accessibility of the DNA surface.

Corroborating these findings, γH2AX foci were commonly observed in euchromatin areas and less recurrently in more compacted heterochromatic portions. Overall, these studies indicate that the degree of chromatin packing determines cell ability to withstand DNA damage.

1.4.6 Polycomb and H3K27me3 in damage

For our knowledge the first study reporting an implication of Polycomb and its related modification H3K27me3 in DNA damage was published in 2008 (O'Hagan *et al*, 2008). Authors induced a defined DSB in an exogenous E-cadherin promoter construct which typically undergoes DNA hypermethylation in epithelial malignancies. In the system employed, EZH2 was recruited at the lesion site along with other proteins linked to the establishment and maintenance of transcriptional silencing such as SIRT1, DNMT1, and DNM3B. The relocalization of these factors was accompanied by the concomitant enrichment of repressing histone PTMs, including hypoacetyl H4K16, H3K9me2/3, and H3K27me3. Authors speculated that these marks prevent the resumption of transcription

before the completion of repair and restrict the damage signals generated initially by chromatin relaxion.

A subsequent chromatin localization screening upon UV- induced damage revealed a PARP-dependent recruitment of PRC2 at the damaged site (Chou *et al*, 2010a). Also in this case, the recruitment of repressive marks at laser microirradiation sites led to the loss of nascent RNA and elongating RNA polymerase from these loci.

It is conceivable that the H3K27me3-mediated transcription repression prevent the interference of the RNA polymerase II with the recruitment of repair factors and the synthesis of truncated mRNAs. H3K27me2/3 deposition was also shown to facilitate NHEJ, since its decrease impeded, through FANCD2 action, the recruitment of 53BP1 upon DSB damage (Zhang *et al*, 2018). Although the EZH2 recruitment to DSBs (Campbell *et al*, 2013; Chou *et al*, 2010b) is well established, contradictory evidence addressing the simultaneous enrichment of H3K27me3 at those loci have been observed (Campbell *et al*, 2013).

Despite a large body of research demonstrating that PRC2-mediated enrichment of H3K27me3 occurs at the lesion site (O'Hagan *et al*, 2008; Abu-Zhayia *et al*, 2018; O'Hagan *et al*, 2011), recent publications revealed that this repressive mark drastically reduces or remains unaltered in response to damage (Li *et al*, 2013; Rath *et al*, 2018; Kakarougkas *et al*, 2014; Campbell *et al*, 2013). Collectively, these observations bring attention to the hypothesis that H3K27me3 levels are dynamically modulated around the break site for an efficient repair.

2. AIM OF THE WORK

Thousands of DNA lesions continually threaten our genome, yet most of them are successfully repaired. When damaged, DNA is the only biologic entity that is fully dependent on the precise repair of already-existing molecules (Dvir *et al*, 1992). Cells have at their disposal a formidable arsenal of proteins to precisely sense and repair DNA lesions to reestablish the continuity and integrity of the genome (Lindahl & Barnes, 2000). However, when this equilibrium is compromised and DNA lesions are left unrepaired, cancer may develop, and organism survival is threatened.

Additional levels of control to the response to damage are conferred by the contribution of chromatin dynamics and specifically of histone PTMs.

PRC2 and the related H3K27me3 mark have been widely implicated in the response to damage, in particular in the transcription suppression at the damage sites. The PRC2 counteractor, KDM6A, is a histone demethylase frequently mutated in cancer. Despite the new advancing, some aspects of its tumor suppressive role have remained unexplored. Based on our preliminary observations, the aim of this dissertation is to investigate the role of KDM6A in the response to damage.

3. RESULTS

3.1 KDM6A loss is associated with genomic instability and DNA repair

3.1.1 KDM6A Knockdown cells are genomically instable

To explore the oncogenic role of KDM6A loss, we selected the osteosarcoma cell line U-2OS and took advantage of shRNA technology in order to downmodulate KDM6A expression. Two independent shRNAs targeting shKDM6A (shKDM6A #1 and shKDM6A #2) were employed, along with a scramble control (shSCR), to avoid off target effects. In order to gain insights on its function, we investigated potential genomic implications of KDM6A depletion by firstly assessing cell nuclear morphology using immunofluorescence assays. Microtubules and actin filaments were stained with alpha-tubulin and phalloidin, respectively, to selectively score cells with proper morphology and an intact cytoplasm.

We found that loss of KDM6A caused a significant accumulation of micronuclei (MN), and a slight increase in nuclear buds (NBUDs) formation, both considered indicators of genomic instability (**Figure** 7).

MN appear as discrete bodies of chromatin in the cytoplasm which eventually arise from mis-segregation events or from acentric chromosomal fragments resulting from unrepaired DSBs (Fenech *et al*, 2011). NBUDs share the same morphology of MN with the exception that they are connected to the main nucleus. NBUDs may result from the process of extrusion of amplified DNA or DNA repair complexes as well as excess chromosomes from aneuploid cells (Fenech *et al*, 2011). NBUDs can also develop when a nucleoplasmic bridge between two nuclei is disrupted, which leads the remaining fragments to shrink back toward the nuclei (Fenech *et al*, 2011).

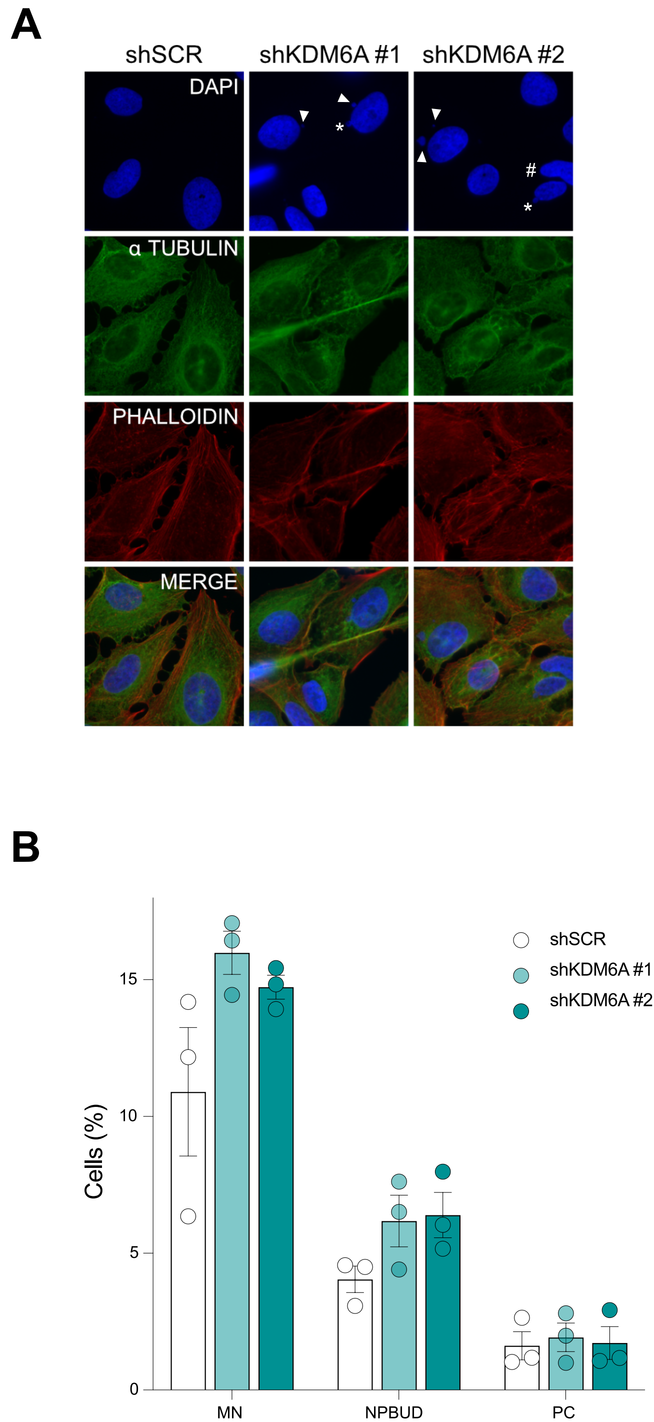


Figure 7. KDM6A inactivation is associated with genomic instability
U-2OS cells were infected with an shSCR or two independent shKDM6As. 72h after selection, cells were fixed and stained for alpha-Tubulin (green), Phalloidin (red), and counterstained with DAPI (blue). (A) Representative pictures of immunofluorescence assays are shown. (B) Quantification of the percentage of plurinucleated cells (PCs) and cell displaying micronuclei (MN) and nuclear buds (NBUDs). White arrow: MN; Asterisk: NBUD; Hashtag: PC. Mean with s.e.m. of n=3 independent experiments (≥ 150 cells scored per line).

These first observations suggested a preferential implication of KDM6A in genome integrity maintenance and, particularly in the response to DSBs, since other common features of a defective mitosis were not significantly different (PCs). Next, we sought to corroborate our results and to determine whether the development of MN was altered following IR-induced damage.

To this end, we employed the cytokinesis-block assay which relies on the Cytochalasin-B agent which disrupts contractile microfilaments by inhibiting actin polymerization thereby preventing the completion of cytokinesis (**Figure 8A**). This assay is the preferred method to measure nuclei anomalies since eventual confounding effects in cell division between experimental conditions are considered. This is important since MN formation is compromised when cytostatic conditions (such as IR) that reduce the number of dividing cells are evaluated. Therefore, performing the assay without Cytochalasin-B may lead to false negative results or to the underestimation of the actual MN frequency. Along with untreated control cells, we irradiated both shSCR and shKDM6As before Cytochalasin-B addition. Once divided binucleated (BN) cells were selectively observed to score aberrations (**Figure 8B**).

After cytokinesis block, KDM6A knock-down (KD) notwithstanding led to an increased presence of micronuclei with respect to the shSCR control, in both basal and damage conditions, suggesting a role for KDM6A loss in eliciting genomic instability (**Figure 8C**).

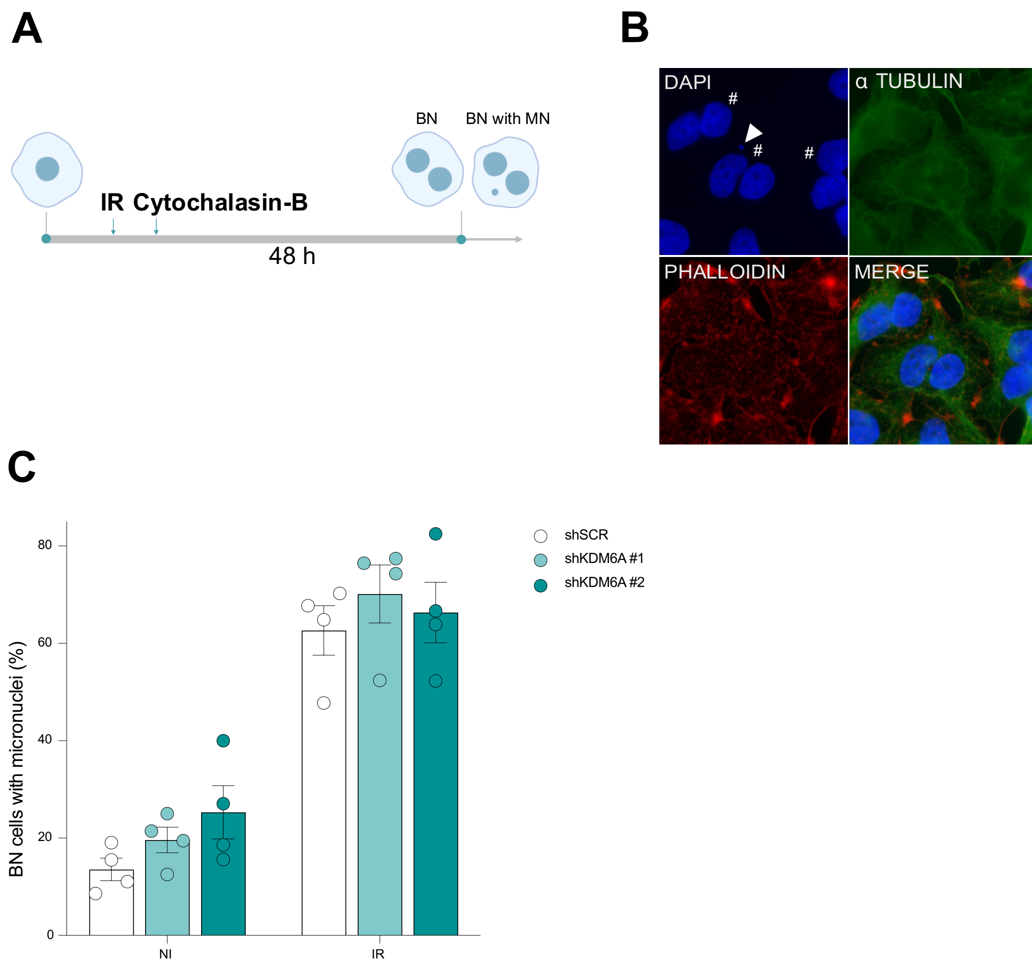


Figure 8. Micronuclei frequently form in KDM6A depleted cells
(A) Schematic representation of Cytochalasin-B mechanism of action. (B) U-2OS cells were infected and Cytochalasin-B (3 ug/ ml) was added to the culturing medium 48h after selection. shSCR and shKDM6Aa cells were subsequently irradiated (4Gy) or left untreated. Cells were returned to the incubator for 48h before fixation. Staining was performed with for α-Tubulin (green) and Phalloidin (red) antibodies, while DNA was stained with DAPI. Representative pictures of immunofluorescence assays are depicted. (C) BN were selectively observed to score MN. White arrow: MN; Plus: BN. Data are represented as mean (+/- s.e.m.) of four independent experiments (≥ 150 cells scored per line).

3.1.2 DNA repair is impaired in KDM6A deficient cells

Since it is conceivable that the increased frequency of cells with MN results from the accumulation of DSBs, we assessed whether a defective DNA repair is the underlying cause of the phenotype observed. Two major alternative mechanisms, NHEJ and HR, repair DSBs. We assayed repair efficiency by employing the EJ-DR (EJ-RFP combined with DR-GFP) (Bindra *et al*, 2013), an U-2OS engineered cell line that bears two chromosomally integrated reporter constructs, one that assesses HR, and the other for NHEJ efficiency, thus enabling their simultaneous evaluation. This fluorescence-based technology employs the uncommon cutting endonuclease I-SceI, which is also stably integrated in the cellular system to generate a single site-specific DSB in cells. This I-SceI system is the results of the fusion of the I-SceI gene with the ligand-binding domain of the rat GR. Only when the synthetic ligand triamcinolone (TA) is added, I-SceI translocates from the cytoplasm to the nucleus to induce damage. The I-SceI enzyme additionally presents at the N-terminus a destabilizing domain (dd) which is blocked by the addition of the drug Shield to the culturing medium, thereby increasing I-SceI protein levels. Those additional levels of control enable the fine-tuned regulation of cleavage kinetics. Concerning the DR-GFP construct (**Figure 9A**), the open reading frame (ORF) of the GFP gene has been disrupted by the integration of an I-SceI recognition site, and a truncated GFP gene fragment with the correct ORF sequence is located downstream. Only if the repair of the broken site occurs properly through HR, a functional GFP gene is reconstituted, and a GFP fluorescence signal then can be measured by flow cytometry. In the second construct, the tetracycline (Tet) regulatory circuit has been exploited (**Figure 9B**). Specifically, an I-SceI recognition site is inserted in the TetR gene in addition to a DsRed gene cloned downstream to a TetR binding site (Tet operator). In basal conditions, the DsRed expression is repressed by the binding of the TetR protein to the Tet operator. When ligands are added, the I-SceI endonuclease introduces a DSB and any repair event that disrupts the TetR ORF results in DsRed fluorescence.

EJ-DR cells were infected and after selection ligands were added for 24h to increase I-SceI protein levels and to favor its relocalization in the nucleus. Medium was replaced to let the repair occur and 96h later the efficiency of the two repair pathways was evaluated at the flow cytometer. When compared to the shSCR control, EJ-DRs where KDM6A was knocked down showed a dramatic reduction of HR-repaired GFP-positive population

(**Figure 9C**), as well as the NHEJ-repaired RFP- positive population, albeit to a lesser extent (**Figure 9D**). Overall, these data suggest that KDM6A is required for an efficient repair of damage through HR.

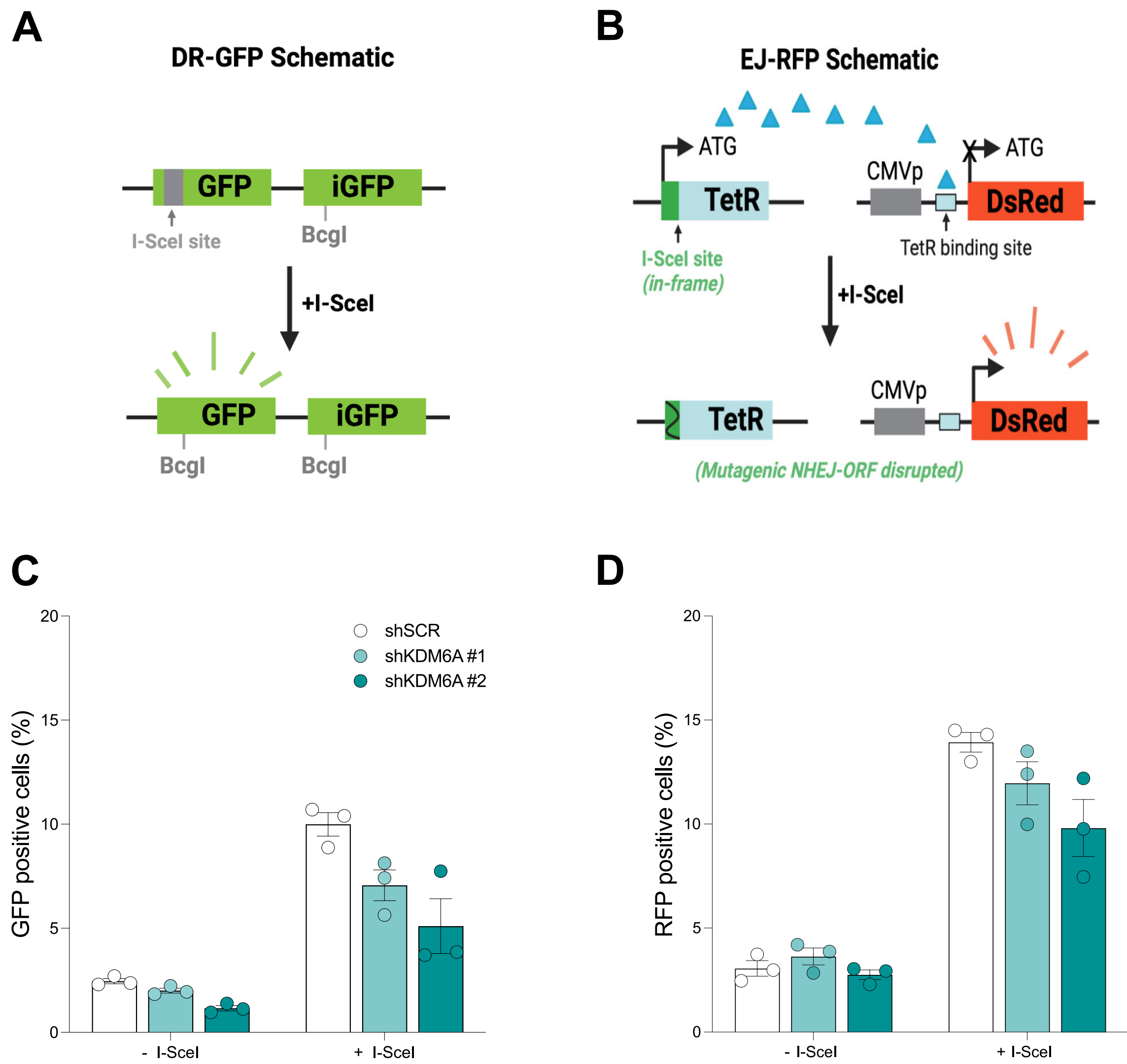


Figure 9. KDM6A is required for efficient DNA repair
(A) Schematic of the DR-GFP assay for the evaluation of HR repair efficiency at an intrachromosomal site. (B) Representation of the EJ-RFP repair system. (C) HR repair efficacy of engineered cells infected with shSCR and shKDM6As is depicted by the percentage of GFP-positive cells. (D) Evaluation of RFP-positive populations representing NHEJ repair accuracy. Error bars show mean +/- s.e.m. from three biological replicates. Depicted schemes are adapted from authors published representations (Brindra et al, 2013).

3.1.3 Altered damage signaling in KDM6A depleted cells

Our results collectively may suggest that the increased genomic instability associated with KDM6A loss, as determined by the formation of MN, may be related to a defective DNA repair. We then reasoned whether an impairment of damage sensing activation was responsible for the phenotype observed. To address this question, we evaluated the recruitment kinetics of the apical kinase involved in the early events of the response to damage, ATM. ATM and ATR are upstream of a cascade of phosphorylation events that result in activation and recruitment of effector proteins to the sites of damage.

Along with assessing the intensity levels of the active phosphorylated form (pATM) signal, the phosphorylation status of its targets was also evaluated. To this end, we used a pS/TQ antibody which detects the levels of proteins containing the ATM/ATR substrate motif, as a documented proxy of pATM activation. When shSCR cells were irradiated, these DDR markers were recruited to DNA lesions and their quantification enabled the estimation of DNA damage signaling and activation dynamics. Interestingly, in KDM6A depleted cells, both pATM and PS/TQ signal intensity increased even in basal conditions. The difference was greater after damage induction, were the DDR activity remained higher when compared to the shSCR (**Figure 10**). Our data demonstrate that KDM6A depletion is associated with an increased activation of pATM and its downstream targets and suggests that its loss does not interfere with the ability of cells to sense and signal DNA damage.

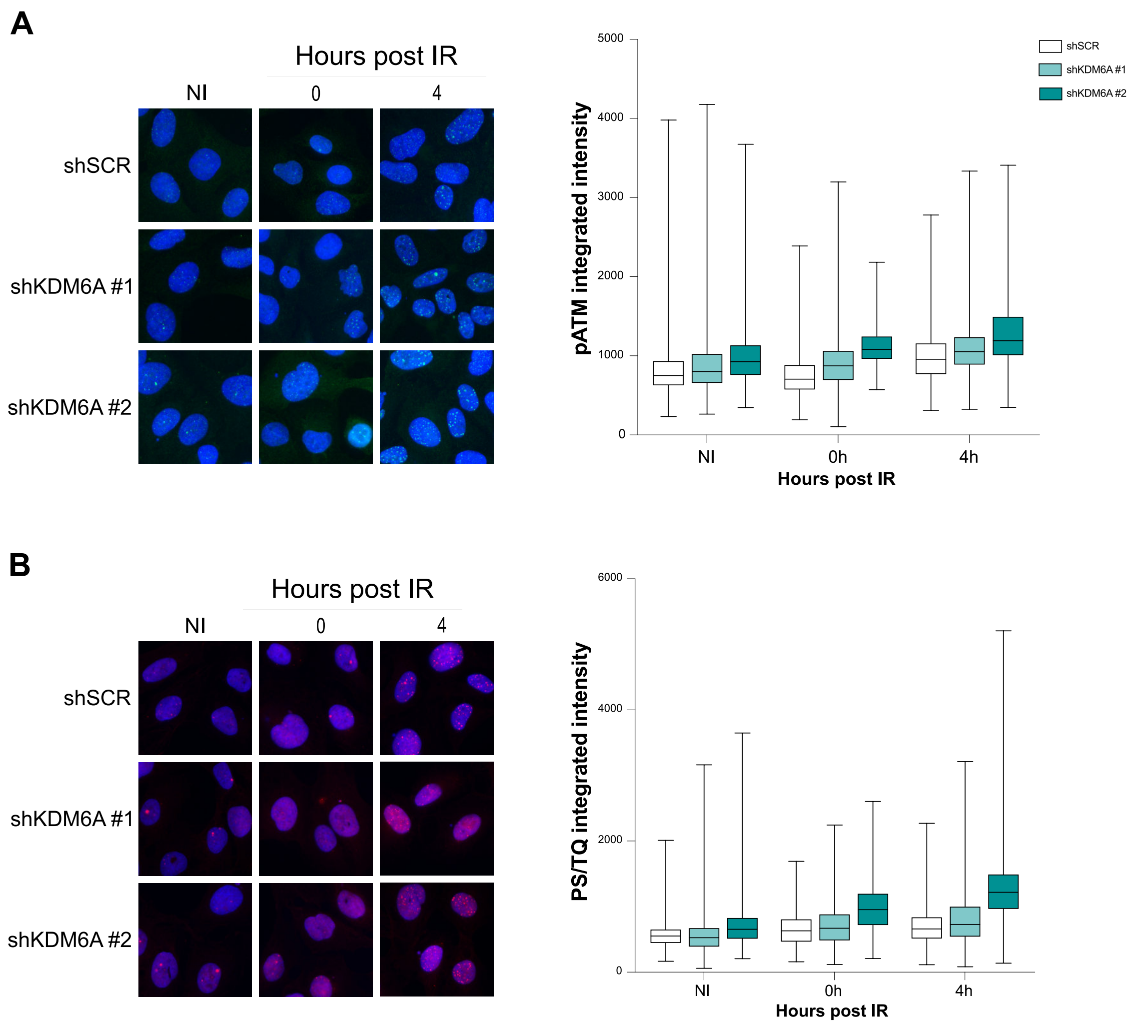


Figure 10. Robust checkpoint activation upon KDM6A KD.
U-2OS cells were infected with shSCR or shKDM6As and 72h after selection, cells were irradiated (4Gy) and fixed at different time points after IR. pATM (A) and PS/TQ (B) antibodies were employed to evaluate DDR activity. The right panel of both figures depicts the quantification of the integrated intensity measured with Cell Profiler. Signal intensity was calculated from two to three biological replicates. At least 150 cells were assessed for each condition.

3.1.3 KDM6A loss increases sensitivity to DNA damaging agents

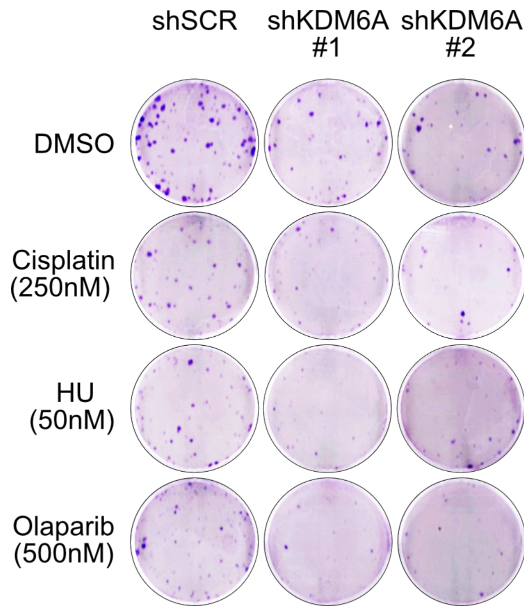
Our data may point toward a disrupted DNA repair process upon KDM6A depletion. Since cells defective in DDR are extremely vulnerable to DNA damaging agents, we asked whether KDM6A silencing may confer enhanced sensitivity upon damage induction. The different sources of damage employed corresponded to a specific insult and kind of lesion generated.

We performed a colony formation assay in control or KDM6As silenced cells treated with the PARP inhibitor Olaparib, the cross-linking agent Cisplatin and Hydroxyhurea (HU) which indirectly leads to replication stress by depleting the pool of dNTPs (**Figure 11A**). We evaluated the colony number, an estimate of the killing effect of the drugs, and the colony dimension, as a measure of the proliferation rate (**Figure 11B**).

ShKDM6As displayed a hindered clonogenic potential (colony number) when compared to the shSCR in untreated condition (DMSO). However, the colony dimension was only marginally affected in the shKDM6A #2 and to a higher extent in the shKDM6A #1. These findings suggest a major impact of KDM6A on clonogenicity rather than proliferation. Interestingly, KDM6A loss decreased cell viability upon all the treatments, and in particular in the case of Olaparib.

Collectively these data may suggest a specific role for KDM6A in the DDR pathway and more specifically in the DNA repair by HR. We additionally performed a colony formation assay by treating cells with the topoisomerase II inhibitor, Etoposide. Surprisingly, when treated, KDM6A depleted cells didn’t show any additional increased sensitivity when compared to the shSCR control (**Figure 12**). The differential vulnerabilities may reside of the distinct mechanism of action of the drugs.

A



B

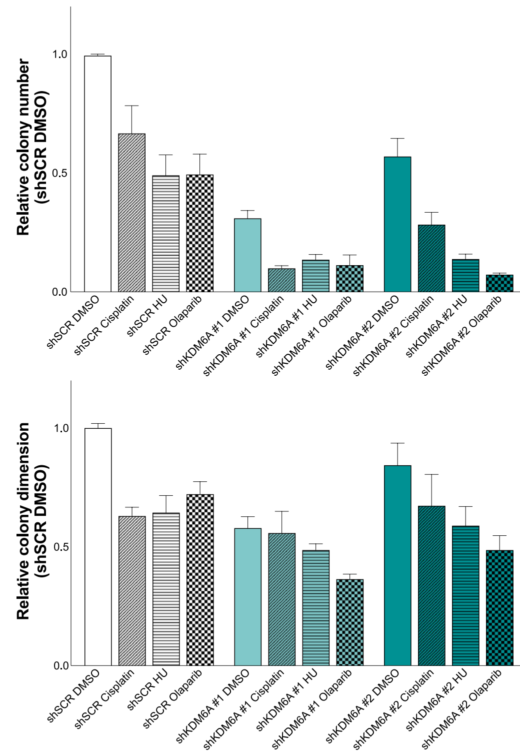


Figure 11. KDM6A KD increases vulnerability to DNA damaging agents

(A) Representative pictures of the clonogenic survival assay of shKDM6A #1 and #2 or shSCR control cells treated with the indicated concentrations of Cisplatin, Hydroxyurea (HU), and Olaparib. (B) Colony number and dimension were assessed from three biological replicates. Error bars show mean +/- s.e.m.

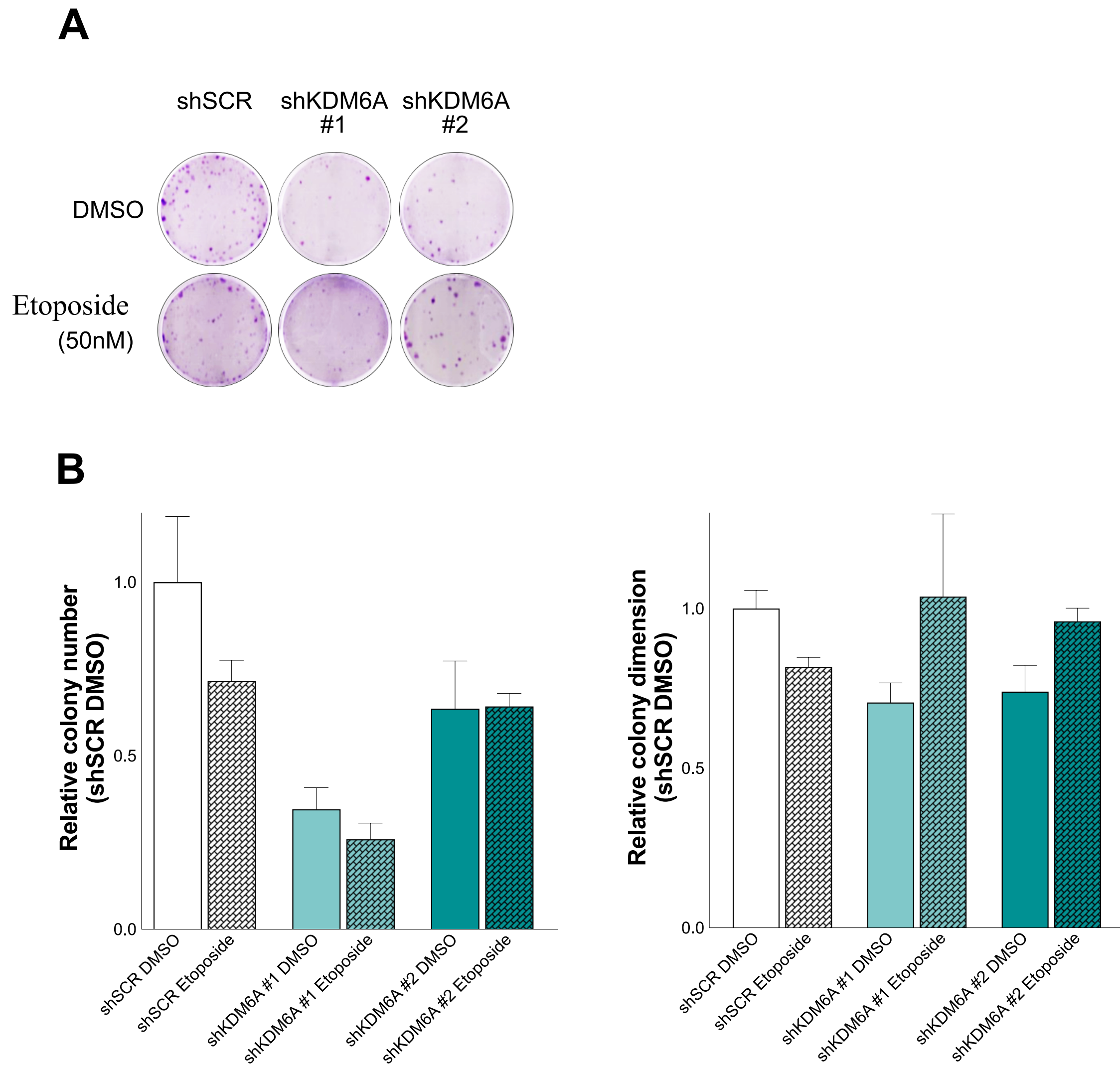


Figure 12. KDM6A silenced cells are insensitive to Etoposide
(A) Representative pictures of the clonogenic survival assay of shKDM6A #1 and #2 or shSCR control cells treated with Etoposide (50nM). (B) Colony number and dimension were assessed from three biological replicates. Error bars show mean +/- s.e.m.

3.1.4 Cell cycle distribution is only marginally affected by KDM6A loss

Colony formation assay showed a remarkable impact of KDM6A depletion on cell survival upon DNA damage induction. The DSB repair mechanism that is engaged following DNA damage is chosen, among other factors, also by the cell cycle phase in which damage occurs. HR is only active during the S and G2 stages, when the sister chromatid is available and functions as a template, while the NHEJ pathway is enlisted throughout the cell cycle. We performed a flow cytometry analysis using propidium iodide as DNA staining agent. The proportion of cell populations in each stage of cell cycle was evaluated with respect to the propidium iodide fluorescence intensity. The cell cycle distribution in KDM6A silenced cells was only marginally affected indicating that the selective reduction of the HR repair pathway that we saw upon KDM6A KD was not driven by the arrest of cells at specific cell cycle stages (**Figure 13**).

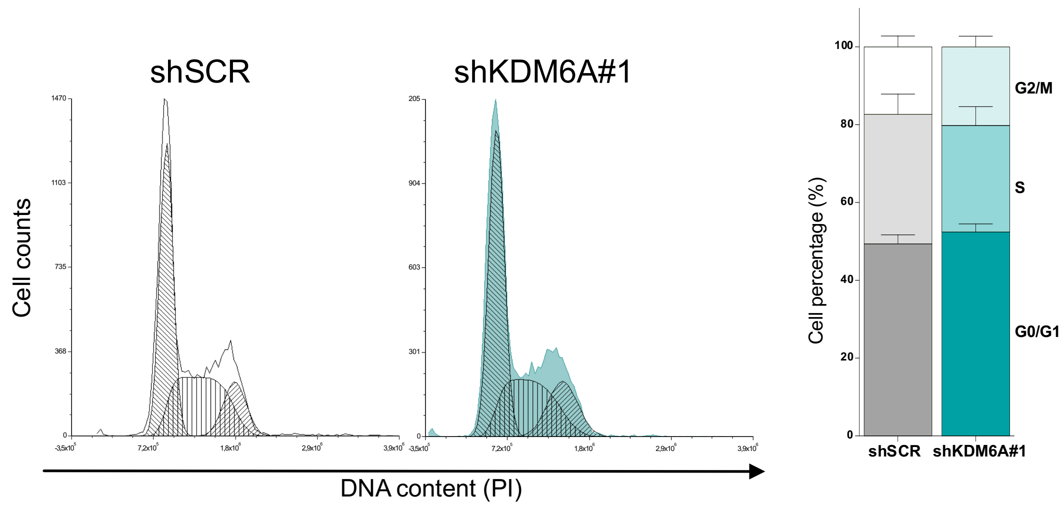


Figure 13. KDM6A loss only mildly affects cell cycle phases

Cell cycle distribution of shSCR and KDM6A #1 silenced cells was obtained by staining cells with propidium iodide (PI). Cell population percentages were assessed through flow cytometry using FCS Express software. Error bars show mean +/- s.e.m. from two biological replicates.

3.2.1 KDM6A protein increases in response to damage

Collectively these data suggest a prominent role for KDM6A in the DDR pathway and more specifically in the DNA repair.

We thus decided to evaluate whether KDM6A is directly engaged as a result of DSBs formation. To this end, we induced DNA damage in U-2OS cells using three different sources. Specifically, Doxorubicin (**Figure 14A**) is an effective chemotherapeutic drug which elicits DNA damage through the intercalation between DNA base pairs and the inhibition of the Topoisomerase II. Bleomycin is a glycopeptide antibiotic that leads to the accumulation of reactive oxygen species resulting in DNA breaks (**Figure 14B**). Finally, gamma IR produces several DNA lesions including DSBs, by directly affecting the DNA structure or, indirectly as a result of the formation of water radicals (**Figure 14C**).

All the treatments led to the rapid phosphorylation/activation of H2AX which in turn functions as a platform for the recruitment of many other DDR proteins. As expected, also pATM levels rose, ultimately leading to the activation of the p53 protein which acts downstream in the response to damage.

Remarkably, all these three different sources of damage led to a time- or dose-dependent increase in KDM6A protein levels, within few hours upon treatment. The kinetics of KDM6A induction paralleled those of the other proteins that promptly respond to damage.

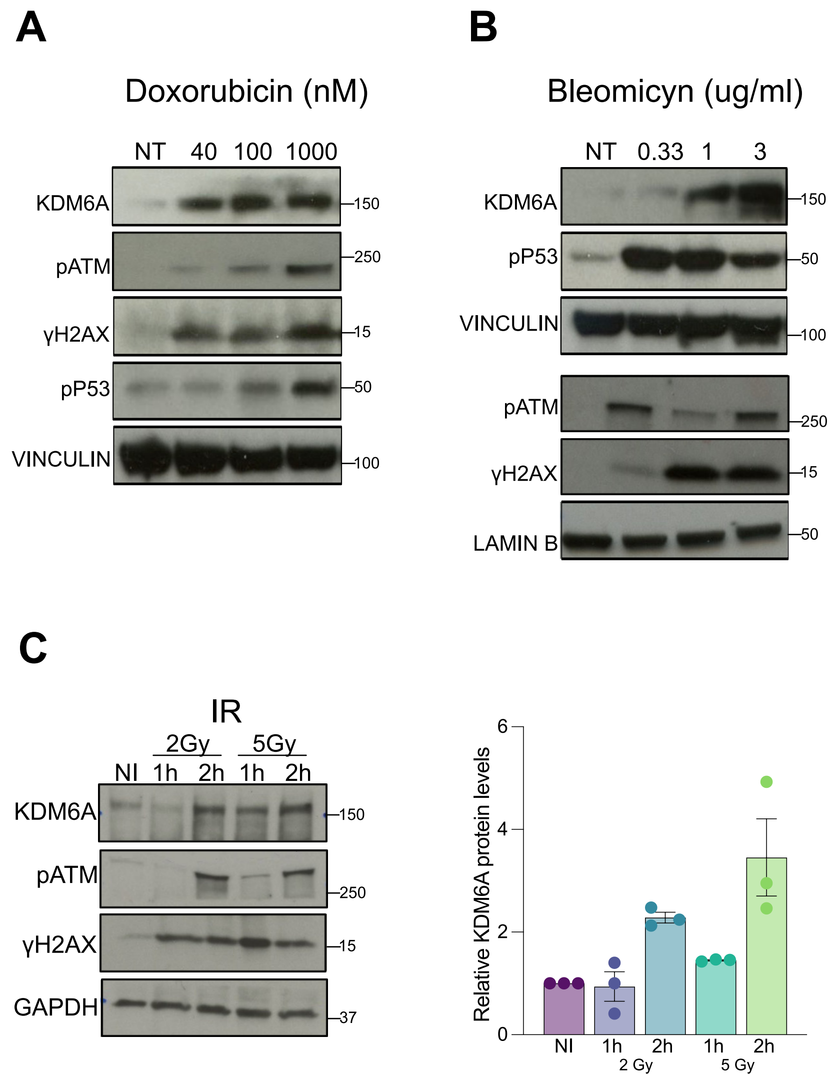


Figure 14. KDM6A protein increases upon damage
U-2OS cells were subjected to DNA damaging agents and cell pellet collected for γH2AX, pP53, pATM, and KDM6A protein content assessment. (A) and (B) U-2OS cells were treated with increasing concentrations of drugs for 30 minutes before samples collection (n=1). (C) Representative Western Blot from three biological replicates of untreated or irradiated U-2OS cells (2Gy and 5Gy) collected 1 and 2h after treatment. The bar plot on the right depicts the quantification of the blot on the left (mean +/- s.e.m.). KDM6A were normalized to the GAPDH protein levels and to the untreated condition (NI).

To define whether the increase in KDM6A protein levels were due to an increased transcription, we collected samples for RNA extraction derived from the same cells and conditions. Interestingly, KDM6A transcription was almost unaffected following IR (**Figure 15B**). All together, these results suggest that KDM6A protein increase upon damage induction is not to be ascribed to an increase in mRNA levels, possibly implying mechanisms of PTMs.

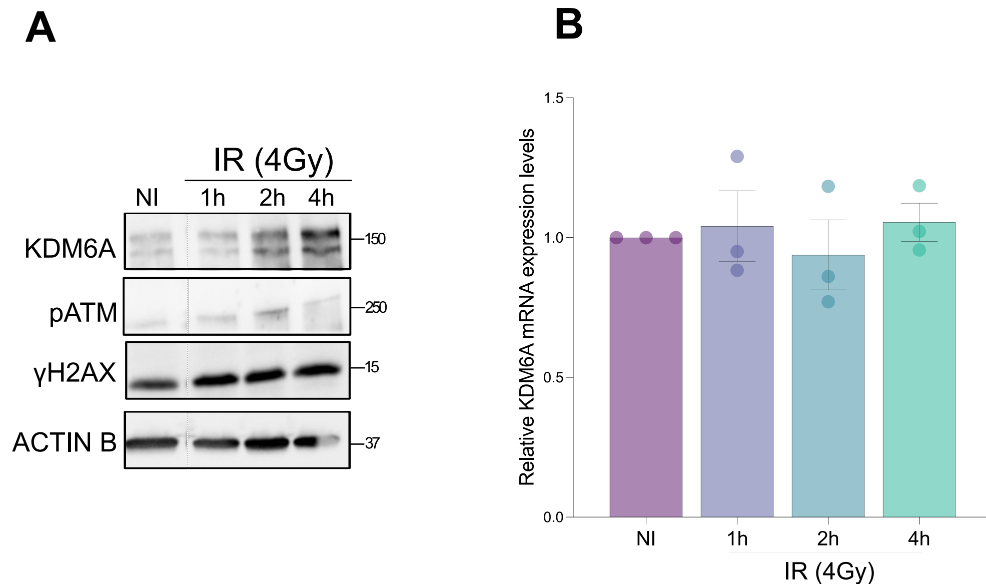


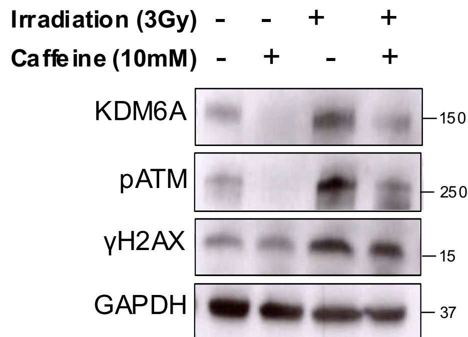
Figure 15. KDM6A protein increases upon damage but not its mRNA levels
U-2OS cells were irradiated, collected at different timepoints after IR, and protein and RNA were extracted from each sample for Western Blot and q-RTPCR analyses. (A) KDM6A protein levels were evaluated along with other DDR proteins, γH2AX and pATM (n=1). (B) KDM6A mRNA levels normalized to ACTIN and to the untreated sample (NI) were plotted. Error bars show mean +/- s.e.m. from three biological replicates.

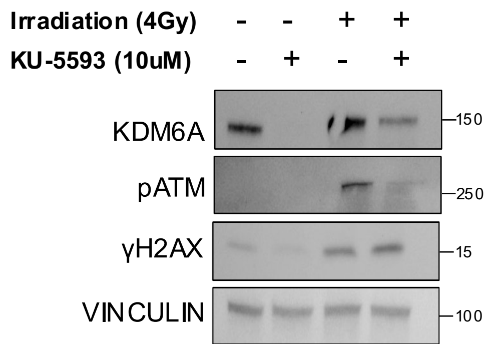
3.2.2 ATM is responsible for KDM6A protein increase upon damage

We next explored the mechanism by which KDM6A levels are increased upon damage. Given the similar kinetic of activation (**Figure 14**), we wondered whether the enhanced KDM6A protein levels were dependent on the activity of the PIKK kinase ATM which is responsible for the phosphorylation and stabilization of many other DDR components (Blackford & Jackson, 2017). We first performed an in-silico analysis of kinase specific phosphorylation sites on KDM6A aminoacidic sequence leveraging a public available software (Netphos-3.1b Server) (Blom *et al*, 2004). Four serine residues (Ser459, Ser464, Ser476, and Ser552) were predicted as highly probable sites of ATM phosphorylation.

To assess a possible implication of ATM in the protein increase of KDM6A upon damage, we pretreated cells with Caffeine, a broad ATM/ATR inhibitor (Sarkaria *et al*, 1999). We exposed cells to a concentration of 10mM of Caffeine for 2 h before IR. Cells were returned to the incubator to let the response to damage occur, and 2h later samples were collected. As anticipated, Caffeine affected the ATM autophosphorylation and the phosphorylation of downstream targets as depicted by the reduced levels of both pATM and γH2AX. Caffeine prevented KDM6A protein increase upon damage, but interestingly its basal level was also reduced (**Figure 16A**).

Next, to assess the individual contribution of the ATM kinase in KDM6A regulation, we employed the ATM specific inhibitor KU-55933 (Hickson *et al*, 2004). Interestingly, we found that treatment with ATM inhibitors reduced KDM6A protein levels (**Figure 16B**). These findings imply a participation of the apical kinase ATM in determining KDM6A protein levels in both physiological and damage conditions.





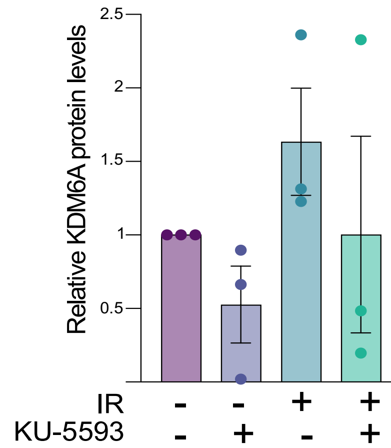


Figure 16. ATM is responsible for KDM6A increase upon IR
(A) and (B) U-2OS cells were pre-treated with Caffeine (10mM) (n=1) or KU (10uM) for two hours before IR. Cells were collected two hours later for Western Blot assay. (B) Representative blot from three biological replicates is depicted. Bands were quantified in the right panel, were KDM6A protein levels were normalized to VINCULIN and to the vehicle control (no IR and no KU-5593). Bar plot represents mean +/- s.e.m. Blots were assayed for KDM6A antibody along with other key components of the DDR.

3.3. KDM6A mediates the transcriptional regulation of HR genes

3.3.1 The role of KDM6A in the transcriptional regulation in patients

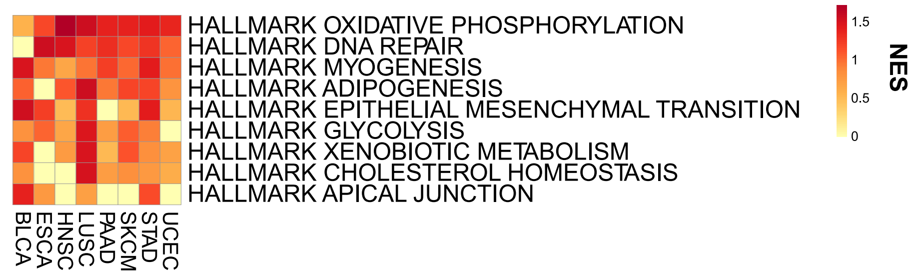
To gain additional insights on the molecular mechanism underlying KDM6A role in carcinogenesis, we performed a comprehensive analysis employing available RNA-seq expression datasets of The Cancer Genome Atlas (TCGA) Pan Cancer Atlas (Cerami *et al*, 2012). We leveraged the cBioportal web site to rank tumor types based on KDM6A defects frequency and selected the top eight tumors. We were seeking to identify genes whose expression varies with respect to the modulation of KDM6A expression. The Gene Set Enrichment Analysis (GSEA) (Subramanian *et al*, 2005) revealed Oxidative phosphorylation and DNA repair as top enriched pathways in patients presenting low levels of KDM6A (**Figure 17A**). A more detailed focus on the DNA repair pathway pointed out an inversed correlation between the expression of KDM6A and that of key genes involved in the transcriptional-coupled repair, a sub pathway of NER. POL2J, ERCC1, POLR2I, and POLR2F were among the most enriched across all the evaluated tumor types except for the Bladder Cancer Dataset.

The Bladder Urothelial Carcinoma represents the tumor with the highest frequency of somatic mutations in the KDM6A gene (29.2 % of cases, TCGA Pan Cancer Atlas).

GSEA revealed a significant down representation of genes involved in the HR repair pathway in this cancer type, in the subset of patients with low expression of KDM6A (**Figure 17C**). This finding may surmise a direct implication of KDM6A transcriptional activity in the regulation of key genes involved in the HR. Since KDM6A acts by removing the H3K27me3 from gene regulatory elements, it is conceivable that an increased deposition of this repressive epigenetic mark might lead to a downregulation of target genes expression in the subset of tumors where KDM6A is inactivated.

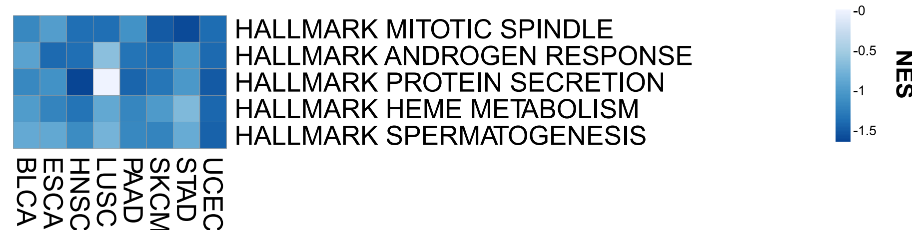
A

Enriched Pathways in KDM6A low patients



B

Enriched Pathways in KDM6A low patients



C

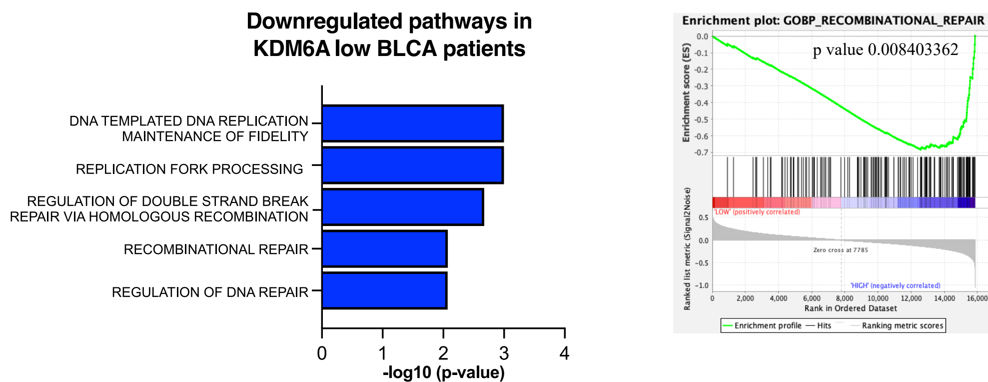


Figure 17. DNA repair genes transcription is downregulated upon KDM6A expression reduction in Bladder Cancer patients

*(A-B) Heatmaps represent the most enriched pathways in KDM6A low patients coming from the first eight tumors ranked by KDM6A alteration frequency from cBioportal Database (**Figure 3**). (C) Downregulated pathways from GSEA of Bladder Cancer (BLCA) patients' transcriptomes comparing low vs high KDM6A expression individuals. DDR pathways coming from Gene Ontology Biological Process (GOBP) were selected among all the pathways with a canonical P value < 0.01. On the right panel the Enrichment plot of the "Recombinational Repair" pathway is depicted.*

BLCA: Bladder Urothelial Carcinoma; ESCA: Esophageal carcinoma; HNSC: Head and Neck Squamous Cell Carcinoma; LUSC: Lung Squamous Cell Carcinoma; PAAD: Pancreatic Adenocarcinoma; SKCM: Cutaneous Melanoma; STAD: Stomach Adenocarcinoma; UCEC: Endometrial Carcinoma.

3.3.2 Gene expression analysis reveals an enrichment of HR genes in shKDM6A U-2OS

Data obtained from TCGA might suggest a transcriptional rewiring upon KDM6A loss in Bladder Cancer which results in the downmodulation of HR genes.
We therefore sought to deepen our understanding on the putative role of KDM6A by evaluating the transcriptional differences emerging when comparing U-2OS shKDM6A to the shSCR control.
U-2OS cells were infected with either shSCR or shKDM6A #1 lentivirus. After selection, RNA was extracted from both cell lines and retrotranscribed to perform RNA-seq. The output of the analysis was a list of differentially expressed genes (DEGs), that were sorted by P-value ($p<0.01$). Then, we analyzed changes in expression by ranking the gene list by Log_2FC.
We were interested in DNA repair related genes, thus we downloaded *GOBP DOUBLE STRAND BREAK REPAIR and GOBP HOMOLOGOUS RECOMBINATION* gene signatures from the GSEA portal (Subramanian *et al*, 2005).
Therefore, we assessed the expression of these genes in our RNA-seq data, and we found that most of the genes required for DSBs repair (**Figure 18A**) and, in particular in the HR pathway (**Figure 18B**) were downregulated upon KDM6A loss.
Although the downmodulation of genes specifically implicated in the HR emerged from our analysis might suggest a direct role of KDM6A in gene transcription regulation, further experiments are required to validate this hypothesis.

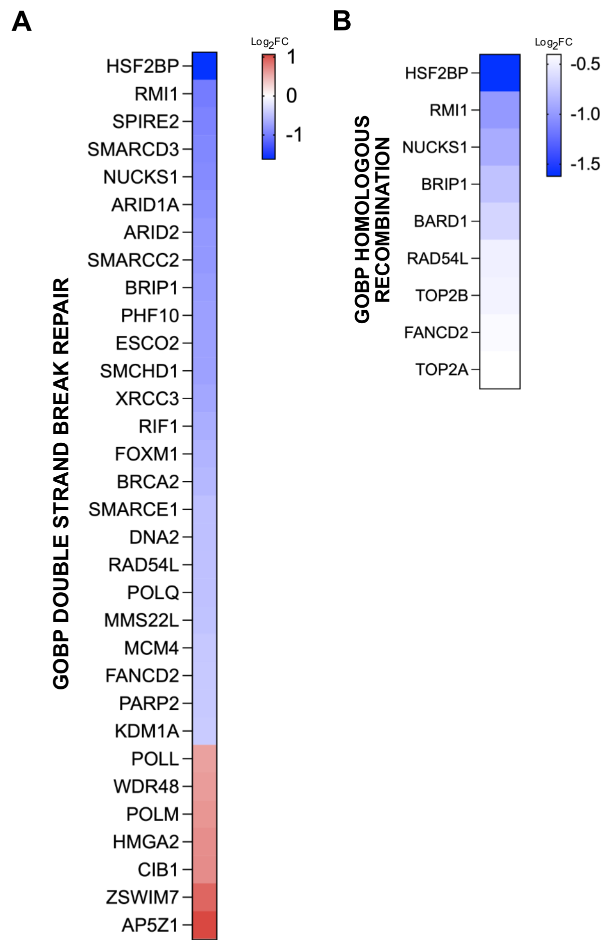


Figure 18. Homologous recombination genes are downregulated upon KDM6A KD
(A-B) Heatmaps represent the differential expression levels of DDR genes derived from GOBP_DOUBLE_STRAND_BREAK_REPAIR and GOBP_HOMOLOGOUS_RECOMBINATION in U-2 OS RNA-seq comparing shKDM6A #1 versus the shSCR control. DEGs with a p-value < 0.01 are shown.

4.H3K27me3 levels are dynamically regulated upon IR

A large body of evidence in literature has shown a direct recruitment of PRC2 at damage sites where transcription suppression is necessary to prevent deleterious consequences. Despite this mechanism is well established, a correspondent increase in the H3K27me3 levels is not always detected (Campbell *et al*, 2013); conversely its decrease has also been reported (Rath *et al*, 2018; Boila *et al*, 2023). We have previously shown a dramatic increase of KDM6A upon IR and we thus asked whether it could be accompanied by a concomitant decrease in H3K27me3 levels. We irradiated U-2OS cells and observed, through immunofluorescence assays, the H3K27me3 signal intensity at different time points following IR. Cells immediately after IR were put on ice to cool down in order to prevent any form of repair and fixed to evaluate H3K27me3 induction (0h). Along with the untreated condition, cells were fixed at 4h after IR when the DDR is occurring. Cells were stained with γH2AX antibody as a control of the correct damage induction and occurrence of repair (**Figure 19**).

H3K27me3 levels rose immediately after damage induction, but decreased almost to the basal levels at 4 hours after IR (**Figure 19**). The kinetic of H3K27me3 levels may suggest a KDM6A-dependent removal during DNA repair, since KDM6A levels are higher in this time-window. Experiments of CUT&TAG are ongoing, to assess genome-wide, and in concomitance with DNA damage, the engagement of KDM6A and the changes of histone modifications. Additionally, we are exploring with GET-seq (Tedesco *et al*, 2022), the technology recently developed in the lab, whether there are differences in chromatin compaction upon KDM6A knock-down, on eu- and heterochromatin regions.

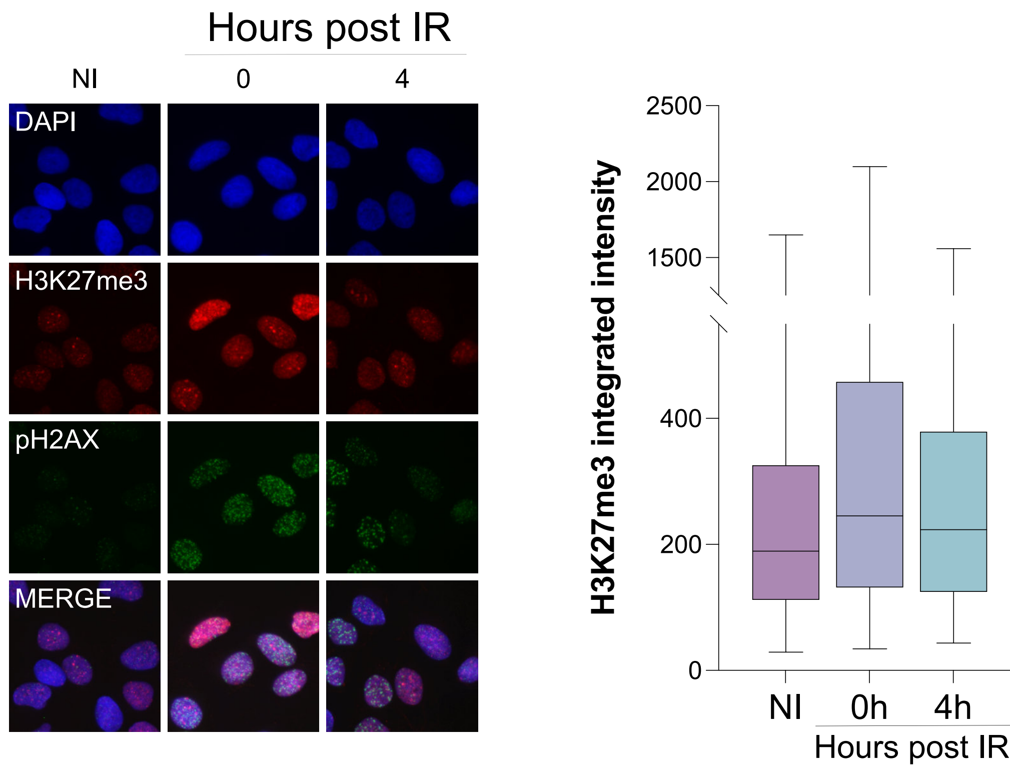


Figure 19. H3K27me3 is dynamically modulated upon damage
Cells were irradiated (4Gy) and fixed at different time points after IR. H3K27me3 and γH2AX antibodies were employed. Box plot on the right depicts the quantification of the integrated intensity assayed through Cell Profiler. Signal intensity was calculated from three biological replicates. At least 150 cells were assessed for each condition.

4. DISCUSSION

In the present study we unraveled a key role of KDM6A in the response to DNA damage. KDM6A loss prevented an efficient repair and led to an increased vulnerability to Olaparib treatment possibly suggesting a direct implication of KDM6A in the specific pathway of HR. Despite the KDM6A gene is frequently altered across different cancer contexts, its implication in tumorigenesis remains unclear. Analysis of the transcriptome of Bladder Cancer patients and from our own RNA-seq in U-2OS suggest a putative transcriptional- dependent role of KDM6A in the regulation of HR repair genes. Our preliminary data may implicate that upon damage induction, KDM6A rapidly increases in order to activate gene transcription. Conversely, in cells deficient for KDM6A, HR repair genes expression is prevented and unrepaired DSBs accumulate leading to the formation of micronuclei.

Our study started from the finding of an increased basal level of genome instability when KDM6A is loss. Indeed, KDM6A depletion elicited a significant increase in the percentage of cells with micronuclei. The formation of these chromatin bodies within the cytoplasm usually stems from ongoing DNA damage. Unrepaired DSBs deriving from previous cell cycles give rise to chromosomal fragments (clastogenic processes) which cause micronuclei formation (Xu *et al*, 2011; Shimizu, 2011). Despite all our experiments point toward this direction, we could not rule out the possibility of an aneugenic process, in which micronuclei arise from lagging chromosomes caused by mitotic errors. While micronuclei resulting from aneugenic events include the whole chromosome, those emerging from faulty repair are acentric and lack the centromere (Fenech *et al*, 2011; Norppa & Falck, 2003; Bakhoum *et al*, 2014). Labeling of centromere proteins such as CREST may help to trace the origin of these defects. Eventual alterations in mitosis which implicate KDM6A in the faithful cell division have not been evaluated in this thesis project but are object of our current investigation.

We next sough to test whether the failure in the maintenance of genome integrity was caused by intrinsic defects in the repair of DSBs. To this end, we took advantage of the U2-OS- EJDRs, an engineered cell line which enables the simultaneous evaluation of the HR and NHEJ efficacy. KDM6A loss markedly impaired HR in both shKDM6As. We did see also an effect of KDM6A knock-down in NHEJ, that we did not explore.

While repair proficient cells can withstand DNA damage since have at their disposal a potent arsenal of proteins to effectively resolve the break, cells deficient in DDR components are particularly susceptible. Since we observed a hindered efficiency of the HR pathway, we interrogated whether KDM6A depletion may impact the sensitivity to damage. To this end, we exposed U-2OS cells to various drugs, including the FDA-approved PARP inhibitor Olaparib, the cross-linking agent Cisplatin, the Topoisomerase II poison Etoposide, and Hydroxyurea. KDM6A depletion profoundly decreased cell viability upon treatment with respect to the control cells, in particular in the case of Olaparib. This phenotype may be reminiscent of a condition known as "BRCAness", were the inhibition of PARP activity results in an increased reliance on HR repair. Upon Olaparib treatment, PARP activity is inhibited and SSBs accumulate during replication. When the replication fork encounters unrepaired SSBs, its progression is hampered and DSBs are generated. Therefore, effective HR repair is required to prevent even more deleterious consequences.

Other drugs did show a less prominent effect as well, suggesting that KDM6A may impact also on other DNA repair pathways, as hinted by the results using the NHEJ U-2OS-EJDRs. In the case of Cisplatin and HU, an additive effect of KDM6A loss was observed on cell viability. Interestingly, while Etoposide decreased of a 30% the viability of shSCR cells, it had no additional effect on the shKDM6A cells. Etoposide is a Topoisomerase II poison that specifically hampers its ligase activity (Singh *et al*, 2022). As a result of the inhibition, the Topoisomerase II enzyme which normally unwinds the torsional structure of the DNA, may cleave, but not relegate DNA ends thereby leading to an increase of DNA breaks. The resistance to Etoposide could not be attributed to differences in the rates of replication between genotypes since shKDM6A cells exhibited increased sensitivity to HU, which also activates the DDR in replicating cells. Moreover, proliferation assays revealed only a modest not significant decrease in cell proliferation. Our findings may suggest a possible implication of KDM6A in the same pathway of the Topoisomerase II, but additional experiments are required to explore this possibility.

We then asked whether the deficient repair was due to an impairment in sensing the damage occurred. For this purpose, cells were irradiated and the activation of pATM and the phosphorylation of its downstream targets were evaluated. Surprisingly, the signaling pathway was not switched off; on the contrary, it was hyperactivated. We envision that

this sustained DDR activation may mirror the attempt of KDM6A depleted cells to mitigate the overload of damaging insults to which they are exposed.

Together, our data provide the compelling evidence that KDM6A directly regulates DSBs repair by affecting the HR process. In support to this notion, we observed an increase in KDM6A protein levels within few hours upon damage induction. Since its increase was concomitant with the induction of key proteins involved in the early events of the DDR, such as pATM and γH2AX, we speculate a direct implication of KDM6A in the response to damage.

KDM6A is a histone demethylase which preferentially removes di- and trimethylation from lysine 27 on histone H3. Its role has been widely characterized in the context of development. Notably, KDM6A demethylase-independent functions have been frequently implicated to its tumor suppressor function.

We thus decided to evaluate whether the defects observed were ascribed to its catalytic activity. To this end, we performed a GSEA analysis on RNA-seq available data from the TCGA cBioportal (Cerami *et al*, 2012). Patients were ranked based on KDM6A expression levels and pathways that were significantly enriched in a least one of the eight tumors were evaluated. The comprehensive analysis of patients' transcriptome revealed an enrichment of transcription-coupled repair (NER) in individual with low levels of KDM6A expression. Of note, Bladder Cancer patients with low levels of KDM6A showed a corresponding decreased expression of genes associated to DNA repair, and specifically in the HR pathway.

This explorative analysis led us to address whether the decreased transcription of genes related to DNA repair is the cause of the defective repair observed in our system. We performed an RNA-seq on cells infected either with shSCR or shKDM6A. We exploited known DNA repair signatures from the GSEA portal and evaluated the expression levels of genes previously related to repair. The analysis revealed a significant downmodulation of genes intimately associated to the HR mechanism. Ongoing CUT&TAG experiments targeting KDM6A protein will eventually validate our finding and confirm whether KDM6A directly lies on the regulatory elements of genes involved in the HR.

A recently published paper indicated KDM6A and KDM6B as crucial proteins for DNA repair in Acute myeloid leukemia (AML) (Boila *et al*, 2023). They propose a mechanism similar to ours in which KDM6A protein is recruited upon damage to the

transcriptional start site (TSS) and promoter-proximal elements of key HR genes to activate their transcription upon damage. Indeed, when KDM6 activities are loss through genetical or pharmacological inhibition (GSK J4), the subsequent increase in H3K27me3 levels at those regulatory elements prevents gene transcription and repair is hampered. Moreover, the SWI/SNF remodeler complex concurs with KDM6A to modulate repair gene transcription. Interestingly, they also reported an increased sensitivity to Olaparib in KDM6 deficient cells. While in this study both KDM6 proteins globally affect DDR transcription impacting signaling and repair, we show a role of KDM6A restricted to the repair since pATM and its targets were fully recruited to the damage sites.

Moreover, authors proposed an increase of KDM6A transcriptional levels upon damage supporting a key role of KDM6A in directly mediating DNA repair. However, the protein increase in our U-2OS cells cannot be attributed to the increased mRNA levels, suggesting a process beyond transcription to account for the rise. Two mechanisms might explain this phenotype: DNA damage could either prevent KDM6A protein degradation as a result of enhanced protein stability, or increased translation may account for the higher protein levels observed in treated cells. This discrepancy may derive from the different degree of damage induction, since, also in the reported experimental conditions, KDM6A transcription increased with a lesser extent when a lower dose of IR was employed. We propose an unanticipated role of ATM in regulating KDM6A levels. Indeed, KDM6A protein increase was prevented when the broad ATM/ATR inhibitor Caffeine or the specific ATM inhibitors KU-5593 were employed. ATM is an apical kinase necessary for the recruitment of DDR proteins. A preliminary in-silico analysis of KDM6A aminoacidic sequence predicted four different serine residues as putative ATM recognition and phosphorylation sites.
ATM has been implicated in the recruitment at the lesion site of many histone modifiers (Udayakumar *et al*, 2015; Cao *et al*, 2016). Interestingly, ATM phosphorylates the KDM6A-counteracting protein EZH2 on Ser734 leading to a reduced protein stability.

A previous study suggested a role of KDM6 proteins in regulating the transcription of DSBs repair genes in diffuse intrinsic pontine glioma cells bearing H3K27M mutation (K27M DIPG). They propose GSK-J4 as a radiosensitizer to prevent damage repair and enhance radiotherapy effects. Additionally, they argue that GSK-J4 prevents HR repair, which takes place in late S and G2 phases, by maintaining a large proportion of the S

phase cell fraction. NHEJ repair efficiency was not affected by GSK-J4 treatment. In 2016 Hofstetter et al. (Hofstetter *et al*, 2016) reported an increased DNA damage state in differentiating but not in undifferentiated embryonic stem cells (ESCs) when KDM6 proteins were depleted or upon GSK-J4 treatment. Transcriptome analyses revealed as differentially expressed genes involved in developmental, cell death and cell proliferation processes. Interestingly, DNA repair genes did not emerge as deregulated upon KDM6A KD. Notably, KDM6A deletion did not reduce the efficiency of HR repair. The authors speculated that KDM6A could be relevant for the switch from HR in undifferentiated cells to NHEJ in differentiated ESCs. This assumption is supported by the co-presence in the MLL2 complex of PTIP, which is known to promote the 53BP1-mediated inhibition of HR (Cloos et al., 2008; Callen et al., 2013). Another evidence implying a role of KDM6A in the transcriptional regulation of the NHEJ factor Ku80 upon damage was reported in Drosophila (Zhang et al., 2013).

We and others have provided the evidence of a direct role of KDM6A in the transcriptional regulation of HR genes and a differential sensitivity to Olaparib treatment. While tumors harboring HR gene mutations have been successfully cured with PARP inhibitors (Jackson & Bartek, 2009; Farmer *et al*, 2005; Bryant *et al*, 2005), repair proficient tumors are insensitive. Thus, we surmise that tumors harboring KDM6A defects may display a BRCAness phenotype which renders them less refractory to treatment.

Our findings extend the knowledge in this field by implicating for the first time the role of ATM in the regulation of KDM6A protein levels. Moreover, we speculated that the increased DNA signaling activation may be pharmacologically exploited to selectively kill KDM6A depleted cells which rely more on the pATM pathway. In addition, we revealed an unprecedented connection between KDM6A loss in Bladder Cancer and transcriptional repression of HR genes which could have an important clinical relevance. Our own data and those from literature suggest the necessity of KDM6 proteins demethylates activity for a proper DNA repair, although its transcriptional activity was shown to be dispensable upon damage in mESCs (Hofstetter *et al*, 2016).

This finding may rise the possibility of alternative or parallel actions of KDM6A upon damage. In particular, the relevance of chromatin dynamics in the response to damage and how its architecture is affected upon KDM6A loss are currently under our

consideration. The complexity of chromatin organization presents a series of challenges to different DNA-based function, such as transcription, replication, and repair. DSBs repair proceeds through a cascade of events that take place within the relatively restricted environment. Nucleosome packing and chromatin structure surrounding the lesion may impair the recruitment of DDR factors and the faithful repair of the damaged DNA. When a DSB occurs, cells preferentially engage HR or NHEJ repair mechanisms to precisely restore DNA integrity. Nevertheless, additional layers of regulation contribute to modulate the DDR. Histones modifiers are considered as key players in the control of chromatin architecture in response to damage, more locally at the damage site or at the genome wide level. Histone methylation has been linked to the DDR by facilitating or limiting, based on the modified residue, the recognition and recruitment of key damage proteins to the lesion site (Huyen *et al*, 2004; Botuyan *et al*, 2006; Ayrapetov *et al*, 2014) or by preventing aberrant gene transcription. Despite the substantial literature published, a direct implication of H3K27me3 in the DDR is contentious. The H3K27me3 writer EZH2 has been involved in different aspects of the response to damage and its recruitment to damage sites (Campbell *et al*, 2013; Chou *et al*, 2010b; Johnson *et al*, 2019) is well established. Conversely, contradictory evidence addressing the simultaneous enrichment of H3K27me3 at lesion loci have been observed (Campbell *et al*, 2013; Johnson *et al*, 2019; Rath *et al*, 2018; Chou *et al*, 2010b). The type of damage induced and the timing of experiments, underlying the alternative roles that H3K27me3 may have in the different stages of the response to damage, may account for the contrasting outcomes observed. Although contentious, it has been shown that PRC2 is rapidly recruited to damage sites to deposit the H3K27me3 mark in order to halt gene transcription. We propose that KDM6A, by removing the epigenetic repressive mark, promotes the formation of an open chromatin that allows the repair machinery to better access and repair the spatially confined region surrounding the lesion. This speculation is supported by our preliminary experiments which revealed an initial increase in H3K27me3 levels immediately after damage induction, followed by a decrease at 4h following IR. A decrease of H3K27me3 deposition upon damage concomitant to KDM6A increase was also observed in AML (Boila *et al*, 2023).

Therefore, we surmise that KDM6A may act as a key factor to coordinate chromatin accessibility to enable DNA repair while also orchestrating transcriptional responses.

We foresee that the elucidation of the connection between a UTX lesion in cancer and ultimately genomic instability, could introduce a novel perspective, providing not only crucial insights on the relationship between chromatin modifications and genome stability, but bearing also important translational implications.

5. MATERIALS AND METHODS

5.1 Cell Culture

U-2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Euroclone) supplemented with 1% antibiotics (Pen/Strep, Euroclone) and 10% fetal bovine serum (FBS, Gibco). For U-2OS-EJDR cells (EJ-RFP and DR-GFP) 10% tetracycline-free FBS (Euroclone) was used to prevent the unintended DsRed gene expression from the EJ-RFP reporter construct due to residual tetracycline in FBS preparations. All cell lines were grown at 37°C in a 5% CO_2 incubator.

5.2 Lentiviral infection

In order to knockdown KDM6A expression, two independent pLKO lentiviral vectors bearing short harpin RNAs targeting the human KDM6A were used. Non-targeting, scrambled shRNA (shSCR) was used as a control. The harpin sequences are provided below:

Scrambled shRNA CAACAAGATGAAGAGCACCAA

KDM6A shRNA #1 ATAGTTGAACTGTGTTCATGC

KDM&A shRNA #2 TGCACTTGCAGCACGAATTAA

We mixed the vector of interest with vectors encoding for the packaging and envelope to enable viral particles production. The solution containing plasmids was added to HEK293T cells that were transfected according to the CaCl2 protocol. After 16 hours, medium was renewed. The day after medium was pelleted at 1,200 rpm and filtered on a 0.22μm filter. Aliquots were stored at – 80° C. The day prior to infection, 180.000 cells were plated in a 6-well plate. The day of infection, the proper amount of virus (MOI=5) was added for 24h. Virus containing medium was replaced and cells were selected with 1 μg/mL puromycin (Invitrogen) or 2 μg/ml in the case of U-2OS- EJDRs for 3 days.

5.3 Lentiviral Titer Calculation

To determine viral titers, 60.000 U-2OS cells were infected with different amounts of virus in a 12-well plate. 24h after infection, each transduction dilution was split in two wells and puromycin was added to one of the two well for each viral dilution. Three days after, cells were counted to determine the percentage of transduced cells comparing the two wells puromycin/non-puromycin for each dilution:

(Puromycin selected / non-selected cells) x 100 = % infected cells

MOI calculation was derived based on the percentage of transduced cells. The concentration of Transducing Units (TU) was determined using the following formula:

TU/ml = (number of cells at day of transduction) × [MOI / (μl of viral suspension used for transduction)]

5.4 Irradiation

IR deriving from a ^{137}Cs source (Biobeam GM device) was employed to generate acute damage and for kinetic experiments. The energy intensity, measured in Grays (Gy), is defined as one joule of radiation energy per kilogram of matter.

5.5 Drug treatments

To study the differential sensitivity to damage a plethora of DNA damaging agents were used: Olaparib (AZD2281, KU005943), Cisplatin (Sigma Aldrich, C2210000), Hydroxyurea (Sigma Aldrich, H8627), and Etoposide (DBA, S1225). To evaluate the implication of ATM in the dynamic modulation of KDM6A upon damage Caffeine (Sigma Aldrich, C0750) and the specific inhibitor KU-5593 (DBA, S1092) were used. To evaluate KDM6A induction upon damage Doxorubicin (DBA, S1208) and Bleomycin (TRC, B595800) were used.

5.6 Colony formation assay

To study the sensitivity of KDM6A silenced cells to DNA damage, U-2OS cells were plated in 6 well dishes and infected with shRNA against KDM6A, as described above. 72 hours following antibiotic selection, 600 cells were plated in triplicate in 6-wells and

treated the following day with Cisplatin (250nM), Olaparib (500nM), Hydroxyurea (50nM), and Etoposide (50nM). Medium was renewed after 6 days, and cells were returned to the incubator. At 14 days from plating, dishes were fixed and stained for colony scoring in Crystal Violet solution (0.5% Crystal violet in 20% methanol). The average number and area of visible colonies for each condition were calculated with Fiji Software (Schindelin *et al*, 2012).

5.7 Ligand-inducible I-SceI cleavage assay

U-2OS EJDR stable cell line was infected with control (shSCR) or shKDM6A #1 and #2. The inducible cleavage by the I-SceI endonuclease was achieved by adding to the culture medium the Schield1(Diatech, 63189) and triamcinolone (TA, Sigm-Aldrich, T6501-250MG) ligands at concentrations of 1µM and 100nM, respectively. 24h after, medium was replaced following 3 washes with pre-warmed PBS and cells were further incubated in DMEM. After 96h cells were collected and the frequency of GFP^{+} and RFP^{+} cells was determined at the flow cytometer CytoFLEX S. Data analysis was performed with FCS Express 6.0 software (De Novo Software).

5.8 Immunofluorescence

Immunofluorescence assay was used to evaluate the relocalization of DDR proteins upon damage and to evaluate nuclear aberrations. Cells were cultured on a 13 mm and fixed for 15 minutes in 4% paraformaldehyde (PFA, SIC). Permeabilization was performed with PBS-T (PBS, 0.3% Triton X-100 (Sigma Aldrich)) while blocking with 3% bovine serum albumin (BSA, Sigma Aldrich) dissolved in PBS (30 minutes). For the staining, coverslips were incubated with primary antibodies diluted in the blocking solution for 1 hour at room temperature. Cells were then washed with PBS and incubated with the secondary antibodies, rhodamine Phalloidin (1:400, Invitrogen, #R415) and DAPI (1:1000, 5 mg/mL, Molecular Probes, #D3571) diluted in blocking solution for 1 hour at RT, in a dark humidified chamber. Finally, Coverslips were mounted on microscope slides using ProLong Gold Antifade Reagent (Invitrogen) and images were acquired at the Axio Imager 2 (ZEISS). Quantification of DDR proteins signal intensity was performed by counting at least 150 cells per condition and analysis were made using

Cell Profiler software (Carpenter *et al*, 2006) and processed with Affinity Designer software (https://affinity.serif.com).
The following primary antibodies were used: anti-ATM pS1981 (Rockland 200-301- 400, 1:200); anti-pS/TQ (Cell Signalling 2851, 1:200); alpha tubulin (MERK, T5168, 1:1000); anti-H3K27me3 (Millipore 07-449, 1:100), anti-pH2AX (Euroclone, 9718s, 1:200). As secondary antibodies: Alexa Fluor 488 Donkey Anti-Mouse IgG, Alexa Fluor 568 Goat Anti-Rabbit IgG (1:1000).

5.9 Western blot

Cells were collected and lysed in laemmli buffer (50% glycerol, 1M Tris-HCl pH6.8, 10% SDS). Lysates were then sonicated using the ultrasonic homogenizers BANDELIN SONOPULS (amplitude 12%, 15', for the required times). Proteins were electrophoretically separated with precasted 4-20% polyacrylamide gels (BioRad) and transferred onto nitrocellulose membrane (Amersham Hybond ECL, GE Healthcare) through a BioRad Trans-Blot system. After blocking with 5% dry milk (BioRad) in PBS-Tween (PBS, 0,1% Tween-20) at RT for 1 hour, membranes were incubated overnight at 4°C with primary antibodies. After washes with PBS-Tween, membranes were incubated with secondary HRP-antibody. Images were acquired at the Chemidoc (BioRad) and processed with the Image Lab Software (BioRad).
The following primary antibodies were used: KDM6A (Euroclone 33410s, 1:1000), γH2AX (Abcam ab2893; 1:1000), pP53 (Euroclone 9284S, 1:1000), pATM (Abcam ab81293, 1:1000), ACTIN (Sigma-Aldrich A3854, 1:10000), GAPDH (Genetex GTX627408, 1:10000), VINCULIN (MERK V9131, 1:10000). As secondary antibodies: ECL Anti-mouse IgG, peroxidase- linked species-specific F(ab')2 fragment (from sheep), GE Healthcare; ECL Rabbit IgG, HRP-linked whole Ab (from donkey), GE Healthcare.

5.10 Cell Cycle distribution

For cell cycle analysis by flow cytometry, U-2OS cells were infected and 48 hours after selection were fixed in pure ethanol for 30 minutes. Cells were then washed with a washing buffer (PBS, 1% BSA), resuspended in a solution containing 50μg/mL propidium iodide (PI, Merck) and 200μg/mL RNase A (Euroclone), and incubated

overnight at 4°C. At least 20000 events per sample were acquired and cell cycle was evaluated by analyzing the acquired events with FCS Express 6.0 software (De Novo Software).

5.11 Cytogenetics analysis

The day prior to IR, infected cells were seeded onto coverslips. In either treated (IR) or untreated cells, Cytochalasin-B (Sigma Aldrich, C2743-200UL) was added at a concentration of 3µg/mL. Cells were returned to the incubator to allow cell division. 48h post treatment, cells were fixed and stained. Alpha-tubulin and rhodamin phalloidin antibodies were used to specifically visualize microtubules and actin filaments, respectively. Only binucleated cells with intact nucleus and cytoplasm were scored. Micronuclei (MN) were recognized as distinct nuclear bodies within the cytoplasm with a diameter not lager than 1/3 of that of the main nucleus. MN refractile, overlapping or connected with the main nucleus were excluded. The frequency of binucleated cells containing MN was measured to evaluate the effect of KDM6A inactivation and IR. In the case of nuclear buds, they appear similar to MN but are linked to the main nucleus through a bridge.

5.12 RNA extraction and real-time quantitative PCR

Total RNA was extracted from cells with QIAquick PCR purification kit (Qiagen, 28106), according to the manufacturer protocol. RNA was quantified with NanoDropTM 8000 (ThermoFisher). The same RNA amount for each sample was retrotranscribed with ImProm-IITM Reverse Transcription System (Promega, #A3800). Quantitative Real Time PCR was performed using SYBR® Green Master Mix (Applied Biosystems, #4364346) on the ViiA7 Real Time PCR System (Applied Biosystems). Expression levels of target genes were normalized to an endogenous control (housekeeping gene): the beta actin. The list of primers is provided below:

BETA ACTIN FWD: GAAGTCCCTTGCCATCCTAAAAG

BETA ACTIN REV: ATGCTATCACCTCCCCTGTGTG

KDM6A ACTIN FWD: TACAGGCTCAGTTGTGTAACCT

KDM6A ACTIN REV: CTGCGGGAATTGGTAGGCTC

5.13 RNA-seq data analysis

U-2OS cells were infected with either shSCR or shKDM6A #1 lentivirus. 48h after selection cells were processed for RNA extraction (see also **RNA extraction and real-time quantitative PCR).** Library preparation and sequencing were performed by Center for Omics Sciences (COSR) at San Raffaele Scientific Institute. RNA counts from RNASeq were analyzed in biological triplicates (3x shSCR and 3x shKDM6A #1). Transcriptomic profiles were obtained by RNA-Seq using mRNA TrueSeq mRNA Illumina Unstranded 2x100 PE in HiSeq2500 (Illumina, Inc) to obtain 30 M clusters of single end 100bp reads. Adapters and low-quality sequences were trimmed using Trimmomatic (Bolger *et al*, 2014). Reads were aligned to the human genome (hg38) using STAR (Dobin *et al*, 2013) (version: STAR_2.5.3a) and gene-level counts were obtained by featureCounts using the gene annotation from GeneCode (version 31). The differentially expressed genes (DEGs) between shKDM6A #1 and shSCR cells were obtained using linear models through the use of the R/Bioconductor package limma (Ritchie *et al*, 2015). Relative expression was analyzed comparing shSCR to the shKDM6A #1 condition. Log_2FC and p-value were investigated: samples were filtered for p-value < 0.01 and ranked by their Log_2FC value. We downloaded DNA repair related signatures (*GOBP DOUBLE STRAND BREAK REPAIR and GOBP HOMOLOGOUS RECOMBINATION*) from the GSEA portal (Subramanian *et al*, 2005). We checked the Log_2FC of genes belonging to these pathways in our RNA-seq data and we plotted them as heatmaps.

5.14 GSEA analysis of patient transcriptome analysis

Gene expression data from TCGA datasets was downloaded from the cBioportal data hub (https://cbioportal-datahub.s3.amazonaws.com) for the selected datasets from the pan-cancer atlas of the TCGA (Cerami *et al*, 2012), in the form of mRNA RNA-Seq RSEM analyzed reads. For each dataset, genes with zero counts were removed. Samples were analyzed for female patients only to run GSEA analysis followed by pathway analysis enrichment. The limma-voom approach was run to normalize RSEM values and KDM6A higher (upper quartile) or lower (lower quartile) expressors were selected. For

GSEA analysis a complete table of voom normalized gene expression for all higher and lower expressors was input to the GSEA algorithm using both Hallmarks (hall.v2022.1.Hs.symbols.gmt) and GOBP (c5.go.bp.v.2022.1.Hs.symbols.gmt) to obtain pathways enriched. Heatmaps of Normalized Enrichment score were constructed both for Upregulated (in lower expressors) or Downregulated (in higher expressors) Hallmarks and GOBP that showed a canonical p-value of at least 0.05 in at least one dataset. An averaged NES was calculated and the top10 pathways were selected for both Hallmarks and GOBP. Heatmaps of Pathways enriched were constructed using Fisher Odds Ratio. For BLCA data, we selected pathways that were significantly downregulated in KDM6A low patients, and we plotted them as a heatmap. Significance threshold was set as nominal p-value < 0.01.

6. REFERENCES

Abu-Zhayia ER, Awwad SW, Ben-Oz BM, Khoury-Haddad H & Ayoub N (2018) CDYL1 fosters double-strand break-induced transcription silencing and promotes homology-directed repair. *Journal of Molecular Cell Biology* 10: 341–357

Adam S & Polo SE (2012) Chromatin Dynamics during Nucleotide Excision Repair: Histones on the Move. *International Journal of Molecular Sciences* 13: 11895–11911

Adam S, Polo SE & Almouzni G (2013) Transcription Recovery after DNA Damage Requires Chromatin Priming by the H3.3 Histone Chaperone HIRA. *Cell* 155: 94–106

Agger K, Cloos PAC, Christensen J, Pasini D, Rose S, Rappsilber J, Issaeva I, Canaani E, Salcini AE & Helin K (2007) UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature* 449: 731–734

Ahn J, Kim KH, Park S, Ahn Y-H, Kim HY, Yoon H, Lee JH, Bang D & Lee DH (2016) Target sequencing and CRISPR/Cas editing reveal simultaneous loss of UTX and UTY in urothelial bladder cancer. *Oncotarget* 7: 63252–63260

Alabert C, Barth TK, Reverón-Gómez N, Sidoli S, Schmidt A, Jensen ON, Imhof A & Groth A (2015) Two distinct modes for propagation of histone PTMs across the cell cycle. *Genes Dev* 29: 585–590

Allshire RC & Madhani HD (2018) Ten principles of heterochromatin formation and function. *Nat Rev Mol Cell Biol* 19: 229–244

An W, Dt S, Mc F, Sj L, De R, N H, Js C, Jd F, N R, Le M, *et al* (2016) Smoking status, usual adult occupation, and risk of recurrent urothelial bladder carcinoma: data from The Cancer Genome Atlas (TCGA) Project. *Cancer causes & control : CCC* 27

Andricovich J, Perkail S, Kai Y, Casasanta N, Peng W & Tzatsos A (2018) Loss of KDM6A Activates Super-Enhancers to Induce Gender-Specific Squamous-like Pancreatic Cancer and Confers Sensitivity to BET Inhibitors. *Cancer Cell* 33: 512-526.e8

van Attikum H, Fritsch O & Gasser SM (2007) Distinct roles for SWR1 and INO80 chromatin remodeling complexes at chromosomal double-strand breaks. *The EMBO Journal* 26: 4113–4125

Aymard F, Bugler B, Schmidt CK, Guillou E, Caron P, Briois S, Iacovoni JS, Daburon V, Miller KM, Jackson SP, *et al* (2014) Transcriptionally active chromatin recruits homologous recombination at DNA double-strand breaks. *Nat Struct Mol Biol* 21: 366–374

Ayrapetov MK, Gursoy-Yuzugullu O, Xu C, Xu Y & Price BD (2014) DNA double-strand breaks promote methylation of histone H3 on lysine 9 and transient formation of repressive chromatin. *Proc Natl Acad Sci U S A* 111: 9169–9174

Bailey MH, Tokheim C, Porta-Pardo E, Sengupta S, Bertrand D, Weerasinghe A, Colaprico A, Wendl MC, Kim J, Reardon B, *et al* (2018) Comprehensive Characterization of Cancer Driver Genes and Mutations. *Cell* 173: 371-385.e18

Bakhoum SF, Silkworth WT, Nardi IK, Nicholson JM, Compton DA & Cimini D (2014) The mitotic origin of chromosomal instability. *Current Biology* 24: R148–R149

Banani SF, Lee HO, Hyman AA & Rosen MK (2017) Biomolecular condensates: organizers of cellular biochemistry. *Nat Rev Mol Cell Biol* 18: 285–298

Banaszynski LA, Wen D, Dewell S, Whitcomb SJ, Lin M, Diaz N, Elsässer SJ, Chapgier A, Goldberg AD, Canaani E, *et al* (2013) Hira-dependent histone H3.3 deposition facilitates PRC2 recruitment at developmental loci in ES cells. *Cell* 155: 107–120

Bannister AJ & Kouzarides T (2011) Regulation of chromatin by histone modifications. *Cell Res* 21: 381–395

Barski A, Cuddapah S, Cui K, Roh T-Y, Schones DE, Wang Z, Wei G, Chepelev I & Zhao K (2007) High-resolution profiling of histone methylations in the human genome. *Cell* 129: 823–837

Bartek J, Bartkova J & Lukas J (2007) DNA damage signalling guards against activated oncogenes and tumour progression. *Oncogene* 26: 7773–7779

Bartek J, Lukas C & Lukas J (2004) Checking on DNA damage in S phase. *Nat Rev Mol Cell Biol* 5: 792–804

Bekker-Jensen S, Lukas C, Kitagawa R, Melander F, Kastan MB, Bartek J & Lukas J (2006) Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. *J Cell Biol* 173: 195–206

Bekker-Jensen S, Lukas C, Melander F, Bartek J & Lukas J (2005) Dynamic assembly and sustained retention of 53BP1 at the sites of DNA damage are controlled by Mdc1/NFBD1. *J Cell Biol* 170: 201–211

Benyoucef A, Palii CG, Wang C, Porter CJ, Chu A, Dai F, Tremblay V, Rakopoulos P, Singh K, Huang S, *et al* (2016) UTX inhibition as selective epigenetic therapy against TAL1-driven T-cell acute lymphoblastic leukemia. *Genes Dev* 30: 508–521

Bergink S & Jentsch S (2009) Principles of ubiquitin and SUMO modifications in DNA repair. *Nature* 458: 461–467

Berkovich E, Monnat RJ & Kastan MB (2007) Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair. *Nat Cell Biol* 9: 683–690

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, *et al* (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 125: 315–326

Beucher A, Birraux J, Tchouandong L, Barton O, Shibata A, Conrad S, Goodarzi AA, Krempler A, Jeggo PA & Löbrich M (2009) ATM and Artemis promote homologous recombination of radiation-induced DNA double-strand breaks in G2. *EMBO J* 28: 3413–3427

Bindra RS, Goglia AG, Jasin M & Powell SN (2013) Development of an assay to measure mutagenic non-homologous end-joining repair activity in mammalian cells. *Nucleic Acids Res* 41: e115

Blackford AN & Jackson SP (2017) ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. *Molecular Cell* 66: 801–817

Blackford AN & Stucki M (2020) How Cells Respond to DNA Breaks in Mitosis. *Trends in Biochemical Sciences* 45: 321–331

Blom N, Sicheritz-Pontén T, Gupta R, Gammeltoft S & Brunak S (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* 4: 1633–1649

Bochyńska A, Lüscher-Firzlaff J & Lüscher B (2018) Modes of Interaction of KMT2 Histone H3 Lysine 4 Methyltransferase/COMPASS Complexes with Chromatin. *Cells* 7: 17

Boila LD, Ghosh S, Bandyopadhyay SK, Jin L, Murison A, Zeng AGX, Shaikh W, Bhowmik S, Muddineni SSNA, Biswas M, *et al* (2023) KDM6 demethylases integrate DNA repair gene regulation and loss of KDM6A sensitizes human acute myeloid leukemia to PARP and BCL2 inhibition. *Leukemia*: 1–14

Bolger AM, Lohse M & Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114–2120

Bollati V & Baccarelli A (2010) Environmental epigenetics. *Heredity* 105: 105–112

Botuyan MV, Lee J, Ward IM, Kim J-E, Thompson JR, Chen J & Mer G (2006) Structural basis for the methylation state-specific recognition of histone H4-K20 by 53BP1 and Crb2 in DNA repair. *Cell* 127: 1361–1373

Bouwman P, Aly A, Escandell JM, Pieterse M, Bartkova J, van der Gulden H, Hiddingh S, Thanasoula M, Kulkarni A, Yang Q, *et al* (2010) 53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers. *Nat Struct Mol Biol* 17: 688–695

Bracken AP, Dietrich N, Pasini D, Hansen KH & Helin K (2006) Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. *Genes Dev* 20: 1123–1136

Brambilla F, Garcia-Manteiga JM, Monteleone E, Hoelzen L, Zocchi A, Agresti A & Bianchi ME (2020) Nucleosomes effectively shield DNA from radiation damage in living cells. *Nucleic Acids Research* 48: 8993–9006

Branzei D & Foiani M (2010) Maintaining genome stability at the replication fork. *Nat Rev Mol Cell Biol* 11: 208–219

Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, Kyle S, Meuth M, Curtin NJ & Helleday T (2005) Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 434: 913–917

Bunting SF, Callén E, Wong N, Chen H-T, Polato F, Gunn A, Bothmer A, Feldhahn N, Fernandez-Capetillo O, Cao L, *et al* (2010) 53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks. *Cell* 141: 243–254

Byun TS, Pacek M, Yee M, Walter JC & Cimprich KA (2005) Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. *Genes Dev* 19: 1040–1052

Campbell S, Ismail IH, Young LC, Poirier GG & Hendzel MJ (2013) Polycomb repressive complex 2 contributes to DNA double-strand break repair. *Cell Cycle* 12: 2675–2683

Campos EI & Reinberg D (2009) Histones: annotating chromatin. *Annu Rev Genet* 43: 559–599

Cao L-L, Wei F, Du Y, Song B, Wang D, Shen C, Lu X, Cao Z, Yang Q, Gao Y, *et al* (2016) ATM-mediated KDM2A phosphorylation is required for the DNA damage repair. *Oncogene* 35: 301–313

Caretti G, Di Padova M, Micales B, Lyons GE & Sartorelli V (2004) The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. *Genes Dev* 18: 2627–2638

Caron P, van der Linden J & van Attikum H (2019) Bon voyage: A transcriptional journey around DNA breaks. *DNA Repair* 82: 102686

Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, *et al* (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biology* 7: R100

Carvalho S, Vítor AC, Sridhara SC, Filipa BM, Ana CR, Desterro JMP, Ferreira J & de Almeida SF (2014) SETD2 is required for DNA double-strand break repair and activation of the p53-mediated checkpoint. *eLife* 2014

Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, *et al* (2012) The cBio Cancer Genomics Portal: An Open

Platform for Exploring Multidimensional Cancer Genomics Data. *Cancer Discovery* 2: 401–404

Chagin VO, Reinhart B, Becker A, Mortusewicz O, Jost KL, Rapp A, Leonhardt H & Cardoso MC (2019) Processive DNA synthesis is associated with localized decompaction of constitutive heterochromatin at the sites of DNA replication and repair. *Nucleus* 10: 231–253

Cho Y-W, Hong T, Hong S, Guo H, Yu H, Kim D, Guszczynski T, Dressler GR, Copeland TD, Kalkum M, *et al* (2007) PTIP Associates with MLL3- and MLL4-containing Histone H3 Lysine 4 Methyltransferase Complex. *J Biol Chem* 282: 20395–20406

Choi H-J, Park J-H, Park M, Won H-Y, Joo H-S, Lee CH, Lee J-Y & Kong G (2015) UTX inhibits EMT-induced breast CSC properties by epigenetic repression of EMT genes in cooperation with LSD1 and HDAC1. *EMBO Rep* 16: 1288–1298

Chou DM, Adamson B, Dephoure NE, Tan X, Nottke AC, Hurov KE, Gygi SP, Colaiácovo MP & Elledge SJ (2010a) A chromatin localization screen reveals poly (ADP ribose)-regulated recruitment of the repressive polycomb and NuRD complexes to sites of DNA damage. *Proceedings of the National Academy of Sciences* 107: 18475–18480

Chou DM, Adamson B, Dephoure NE, Tan X, Nottke AC, Hurov KE, Gygi SP, Colaiácovo MP & Elledge SJ (2010b) A chromatin localization screen reveals poly (ADP ribose)-regulated recruitment of the repressive polycomb and NuRD complexes to sites of DNA damage. *Proceedings of the National Academy of Sciences* 107: 18475–18480

Chowdhury D, Keogh M-C, Ishii H, Peterson CL, Buratowski S & Lieberman J (2005) γ-H2AX Dephosphorylation by Protein Phosphatase 2A Facilitates DNA Double-Strand Break Repair. *Molecular Cell* 20: 801–809

Chowdhury D, Xu X, Zhong X, Ahmed F, Zhong J, Liao J, Dykxhoorn DM, Weinstock DM, Pfeifer GP & Lieberman J (2008) A PP4-Phosphatase Complex Dephosphorylates γ-H2AX Generated during DNA Replication. *Molecular Cell* 31: 33–46

Clapier CR, Iwasa J, Cairns BR & Peterson CL (2017) Mechanisms of action and regulation of ATP-dependent chromatin-remodelling complexes. *Nat Rev Mol Cell Biol* 18: 407–422

Clouaire T, Rocher V, Lashgari A, Arnould C, Aguirrebengoa M, Biernacka A, Skrzypczak M, Aymard F, Fongang B, Dojer N, *et al* (2018) Comprehensive Mapping of Histone Modifications at DNA Double-Strand Breaks Deciphers Repair Pathway Chromatin Signatures. *Molecular Cell* 72: 250-262.e6

Courilleau C, Chailleux C, Jauneau A, Grimal F, Briois S, Boutet-Robinet E, Boudsocq F, Trouche D & Canitrot Y (2012) The chromatin remodeler p400 ATPase

facilitates Rad51-mediated repair of DNA double-strand breaks. *Journal of Cell Biology* 199: 1067–1081

Cui K, Zang C, Roh T-Y, Schones DE, Childs RW, Peng W & Zhao K (2009) Chromatin Signatures in Multipotent Human Hematopoietic Stem Cells Indicate the Fate of Bivalent Genes during Differentiation. *Cell Stem Cell* 4: 80–93

Dalgliesh GL, Furge K, Greenman C, Chen L, Bignell G, Butler A, Davies H, Edkins S, Hardy C, Latimer C, *et al* (2010) Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. *Nature* 463: 360–363

De Bont R & van Larebeke N (2004) Endogenous DNA damage in humans: a review of quantitative data. *Mutagenesis* 19: 169–185

Delacroix S, Wagner JM, Kobayashi M, Yamamoto K & Karnitz LM (2007) The Rad9–Hus1–Rad1 (9–1–1) clamp activates checkpoint signaling via TopBP1. *Genes Dev* 21: 1472–1477

Difilippantonio S, Gapud E, Wong N, Huang C-Y, Mahowald G, Chen HT, Kruhlak MJ, Callen E, Livak F, Nussenzweig MC, *et al* (2008) 53BP1 facilitates long-range DNA end-joining during V(D)J recombination. *Nature* 456: 529–533

Dimitrova N, Chen Y-CM, Spector DL & de Lange T (2008) 53BP1 promotes non-homologous end joining of telomeres by increasing chromatin mobility. *Nature* 456: 524–528

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M & Gingeras TR (2013) STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29: 15–21

Donzelli M & Draetta GF (2003) Regulating mammalian checkpoints through Cdc25 inactivation. *EMBO Rep* 4: 671–677

Douglas P, Zhong J, Ye R, Moorhead GBG, Xu X & Lees-Miller SP (2010) Protein phosphatase 6 interacts with the DNA-dependent protein kinase catalytic subunit and dephosphorylates γ-H2AX. *Molecular and Cellular Biology* 30: 1368–1381

Douki T, Bretonniere Y & Cadet J (2000) Protection against radiation-induced degradation of DNA bases by polyamines. *Radiation Research* 153: 29–35

Dvir A, Peterson SR, Knuth MW, Lu H & Dynan WS (1992) Ku autoantigen is the regulatory component of a template-associated protein kinase that phosphorylates RNA polymerase II. *Proc Natl Acad Sci U S A* 89: 11920–11924

Falk M, Lukášová E & Kozubek S (2008) Chromatin structure influences the sensitivity of DNA to γ-radiation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1783: 2398–2414

Fang J, Feng Q, Ketel CS, Wang H, Cao R, Xia L, Erdjument-Bromage H, Tempst P, Simon JA & Zhang Y (2002) Purification and functional characterization of

SET8, a nucleosomal histone H4-lysine 20-specific methyltransferase. *Curr Biol* 12: 1086–1099

Faralli H & Dilworth FJ (2013) Chaperoning RNA Polymerase II through repressive chromatin. *The EMBO Journal* 32: 1067–1068

Farmer H, McCabe N, Lord CJ, Tutt ANJ, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, *et al* (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434: 917–921

Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, Norppa H, Eastmond DA, Tucker JD & Thomas P (2011) Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis* 26: 125–132

Feng S, Ma S, Li K, Gao S, Ning S, Shang J, Guo R, Chen Y, Blumenfeld B, Simon I, *et al* (2022) RIF1-ASF1-mediated high-order chromatin structure safeguards genome integrity. *Nat Commun* 13: 957

Ferrand J, Plessier A & Polo SE (2021) Control of the chromatin response to DNA damage: Histone proteins pull the strings. *Semin Cell Dev Biol* 113: 75–87

Ferrari KJ, Scelfo A, Jammula S, Cuomo A, Barozzi I, Stützer A, Fischle W, Bonaldi T & Pasini D (2014) Polycomb-dependent H3K27me1 and H3K27me2 regulate active transcription and enhancer fidelity. *Mol Cell* 53: 49–62

Fradet-Turcotte A, Canny MD, Escribano-Díaz C, Orthwein A, Leung CCY, Huang H, Landry M-C, Kitevski-LeBlanc J, Noordermeer SM, Sicheri F, *et al* (2013) 53BP1 is a reader of the DNA-damage-induced H2A Lys 15 ubiquitin mark. *Nature* 499: 50–54

Friboulet L, Barrios-Gonzales D, Commo F, Olaussen KA, Vagner S, Adam J, Goubar A, Dorvault N, Lazar V, Job B, *et al* (2011) Molecular Characteristics of ERCC1-Negative versus ERCC1-Positive Tumors in Resected NSCLC. *Clinical Cancer Research* 17: 5562–5572

Gagné J-P, Hendzel MJ, Droit A & Poirier GG (2006) The expanding role of poly(ADP-ribose) metabolism: current challenges and new perspectives. *Curr Opin Cell Biol* 18: 145–151

Geijer ME & Marteijn JA (2018) What happens at the lesion does not stay at the lesion: Transcription-coupled nucleotide excision repair and the effects of DNA damage on transcription in cis and trans. *DNA Repair* 71: 56–68

Gillet LCJ & Schärer OD (2006) Molecular mechanisms of mammalian global genome nucleotide excision repair. *Chem Rev* 106: 253–276

Goldstein M, Derheimer FA, Tait-Mulder J & Kastan MB (2013) Nucleolin mediates nucleosome disruption critical for DNA double-strand break repair. *Proceedings of the National Academy of Sciences* 110: 16874–16879

Gong F, Clouaire T, Aguirrebengoa M, Legube G & Miller KM (2017) Histone demethylase KDM5A regulates the ZMYND8–NuRD chromatin remodeler to promote DNA repair. *Journal of Cell Biology* 216: 1959–1974

Goodman MF & Woodgate R (2013) Translesion DNA polymerases. *Cold Spring Harb Perspect Biol* 5: a010363

Gozdecka M, Meduri E, Mazan M, Tzelepis K, Dudek M, Knights AJ, Pardo M, Yu L, Choudhary JS, Metzakopian E, *et al* (2018) UTX-mediated enhancer and chromatin remodeling suppresses myeloid leukemogenesis through noncatalytic inverse regulation of ETS and GATA programs. *Nat Genet* 50: 883–894

Grasso CS, Wu Y-M, Robinson DR, Cao X, Dhanasekaran SM, Khan AP, Quist MJ, Jing X, Lonigro RJ, Brenner JC, *et al* (2012) The mutational landscape of lethal castration-resistant prostate cancer. *Nature* 487: 239–243

Greenfield A, Carrel L, Pennisi D, Philippe C, Quaderi N, Siggers P, Steiner K, Tam PP, Monaco AP, Willard HF, *et al* (1998) The UTX gene escapes X inactivation in mice and humans. *Hum Mol Genet* 7: 737–742

Groelly FJ, Fawkes M, Dagg RA, Blackford AN & Tarsounas M (2022) Targeting DNA damage response pathways in cancer. *Nat Rev Cancer*: 1–17

Gui Y, Guo G, Huang Y, Hu X, Tang A, Gao S, Wu R, Chen C, Li X, Zhou L, *et al* (2011) Frequent mutations of chromatin remodeling genes in transitional cell carcinoma of the bladder. *Nat Genet* 43: 875–878

van Haaften G, Dalgliesh GL, Davies H, Chen L, Bignell G, Greenman C, Edkins S, Hardy C, O'Meara S, Teague J, *et al* (2009) Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. *Nat Genet* 41: 521–523

Halazonetis TD, Gorgoulis VG & Bartek J (2008) An oncogene-induced DNA damage model for cancer development. *Science* 319: 1352–1355

Hammond CM, Strømme CB, Huang H, Patel DJ & Groth A (2017a) Histone chaperone networks shaping chromatin function. *Nat Rev Mol Cell Biol* 18: 141–158

Hammond CM, Strømme CB, Huang H, Patel DJ & Groth A (2017b) Histone chaperone networks shaping chromatin function. *Nat Rev Mol Cell Biol* 18: 141–158

Harper JW & Elledge SJ (2007) The DNA damage response: ten years after. *Mol Cell* 28: 739–745

Hartlerode AJ & Scully R (2009) Mechanisms of double-strand break repair in somatic mammalian cells. *Biochem J* 423: 157–168

Hauer MH & Gasser SM (2017a) Chromatin and nucleosome dynamics in DNA damage and repair. *Genes Dev* 31: 2204–2221

Hauer MH & Gasser SM (2017b) Chromatin and nucleosome dynamics in DNA damage and repair. *Genes Dev* 31: 2204–2221

Hauer MH, Seeber A, Singh V, Thierry R, Sack R, Amitai A, Kryzhanovska M, Eglinger J, Holcman D, Owen-Hughes T, *et al* (2017) Histone degradation in response to DNA damage enhances chromatin dynamics and recombination rates. *Nat Struct Mol Biol* 24: 99–107

Hawkins RD, Hon GC, Lee LK, Ngo Q, Lister R, Pelizzola M, Edsall LE, Kuan S, Luu Y, Klugman S, *et al* (2010) Distinct Epigenomic Landscapes of Pluripotent and Lineage-Committed Human Cells. *Cell Stem Cell* 6: 479–491

Heyer W-D, Ehmsen KT & Liu J (2010) Regulation of homologous recombination in eukaryotes. *Annu Rev Genet* 44: 113–139

Hickson I, Zhao Y, Richardson CJ, Green SJ, Martin NMB, Orr AI, Reaper PM, Jackson SP, Curtin NJ & Smith GCM (2004) Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res* 64: 9152–9159

Hochegger H, Dejsuphong D, Fukushima T, Morrison C, Sonoda E, Schreiber V, Zhao GY, Saberi A, Masutani M, Adachi N, *et al* (2006) Parp-1 protects homologous recombination from interference by Ku and Ligase IV in vertebrate cells. *EMBO J* 25: 1305–1314

Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. *Nature* 411: 366–374

Hoeijmakers JHJ (2009) DNA Damage, Aging, and Cancer. *N Engl J Med* 361: 1475–1485

Hofstetter C, Kampka JM, Huppertz S, Weber H, Schlosser A, Müller AM & Becker M (2016) Inhibition of KDM6 activity during murine ESC differentiation induces DNA damage. *J Cell Sci* 129: 788–803

Holliday R (1964) A mechanism for gene conversion in fungi. *Genetics Research* 5: 282–304

Hong S, Cho Y-W, Yu L-R, Yu H, Veenstra TD & Ge K (2007) Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. *Proc Natl Acad Sci U S A* 104: 18439–18444

Huang R-X & Zhou P-K (2020) DNA damage response signaling pathways and targets for radiotherapy sensitization in cancer. *Signal Transduct Target Ther* 5: 60

Huen MSY, Sy SMH & Chen J (2010) BRCA1 and its toolbox for the maintenance of genome integrity. *Nat Rev Mol Cell Biol* 11: 138–148

Hurst CD, Alder O, Platt FM, Droop A, Stead LF, Burns JE, Burghel GJ, Jain S, Klimczak LJ, Lindsay H, *et al* (2017) Genomic Subtypes of Non-invasive Bladder Cancer

with Distinct Metabolic Profile and Female Gender Bias in KDM6A Mutation Frequency. *Cancer Cell* 32: 701-715.e7

Huyen Y, Zgheib O, Ditullio RA, Gorgoulis VG, Zacharatos P, Petty TJ, Sheston EA, Mellert HS, Stavridi ES & Halazonetis TD (2004) Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. *Nature* 432: 406–411

Iacovoni JS, Caron P, Lassadi I, Nicolas E, Massip L, Trouche D & Legube G (2010) High-resolution profiling of gammaH2AX around DNA double strand breaks in the mammalian genome. *EMBO J* 29: 1446–1457

Issaeva I, Zonis Y, Rozovskaia T, Orlovsky K, Croce CM, Nakamura T, Mazo A, Eisenbach L & Canaani E (2007) Knockdown of ALR (MLL2) reveals ALR target genes and leads to alterations in cell adhesion and growth. *Mol Cell Biol* 27: 1889–1903

Jackson SP (2002) Sensing and repairing DNA double-strand breaks. *Carcinogenesis* 23: 687–696

Jackson SP & Bartek J (2009) The DNA-damage response in human biology and disease. *Nature* 461: 1071–1078

de Jager M, van Noort J, van Gent DC, Dekker C, Kanaar R & Wyman C (2001) Human Rad50/Mre11 is a flexible complex that can tether DNA ends. *Mol Cell* 8: 1129–1135

Janssen A, Colmenares SU, Lee T & Karpen GH (2019) Timely double-strand break repair and pathway choice in pericentromeric heterochromatin depend on the histone demethylase dKDM4A. *Genes Dev* 33: 103–115

Jazayeri A, Balestrini A, Garner E, Haber JE & Costanzo V (2008) Mre11–Rad50–Nbs1-dependent processing of DNA breaks generates oligonucleotides that stimulate ATM activity. *EMBO J* 27: 1953–1962

Jeggo PA & Downs JA (2014) Roles of chromatin remodellers in DNA double strand break repair. *Exp Cell Res* 329: 69–77

Jena NR (2012) DNA damage by reactive species: Mechanisms, mutation and repair. *J Biosci* 37: 503–517

Jin C, Li J, Green CD, Yu X, Tang X, Han D, Xian B, Wang D, Huang X, Cao X, *et al* (2011) Histone demethylase UTX-1 regulates C. elegans life span by targeting the insulin/IGF-1 signaling pathway. *Cell Metab* 14: 161–172

Johnson DP, Spitz-Becker GS, Chakraborti K & Bhaskara S (2019) Assessment of epigenetic mechanisms and DNA double-strand break repair using laser micro-irradiation technique developed for hematological cells. *EBioMedicine* 43: 138–149

Juhász S, Elbakry A, Mathes A & Löbrich M (2018) ATRX Promotes DNA Repair Synthesis and Sister Chromatid Exchange during Homologous Recombination. *Molecular Cell* 71: 11-24.e7

Kakarougkas A, Ismail A, Chambers AL, Riballo E, Herbert AD, Künzel J, Löbrich M, Jeggo PA & Downs JA (2014) Requirement for PBAF in Transcriptional Repression and Repair at DNA Breaks in Actively Transcribed Regions of Chromatin. *Molecular Cell* 55: 723–732

Kanaar R & Wyman C (2008) DNA Repair by the MRN Complex: Break It to Make It. *Cell* 135: 14–16

Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, Xie M, Zhang Q, McMichael JF, Wyczalkowski MA, *et al* (2013) Mutational landscape and significance across 12 major cancer types. *Nature* 502: 333–339

Karanam K, Kafri R, Loewer A & Lahav G (2012) Quantitative Live Cell Imaging Reveals a Gradual Shift between DNA Repair Mechanisms and a Maximal Use of HR in Mid S Phase. *Molecular Cell* 47: 320–329

Karpenshif Y & Bernstein K (2012) From yeast to mammals: recent advances in genetic control of homologous recombination. *DNA Repair (Amst)* 11: 781–788

Kim E & Song J-J (2011) Diverse ways to be specific: a novel Zn-binding domain confers substrate specificity to UTX/KDM6A histone H3 Lys 27 demethylase. *Genes Dev* 25: 2223–2226

Kim J-H, Sharma A, Dhar SS, Lee S-H, Gu B, Chan C-H, Lin H-K & Lee MG (2014) UTX and MLL4 coordinately regulate transcriptional programs for cell proliferation and invasiveness in breast cancer cells. *Cancer Res* 74: 1705–1717

Kleine H & Lüscher B (2009) Learning how to read ADP-ribosylation. *Cell* 139: 17–19

Kornberg RD (1977) Structure of Chromatin. *Annual Review of Biochemistry* 46: 931–954

Kruhlak MJ, Celeste A, Dellaire G, Fernandez-Capetillo O, Müller WG, McNally JG, Bazett-Jones DP & Nussenzweig A (2006) Changes in chromatin structure and mobility in living cells at sites of DNA double-strand breaks. *Journal of Cell Biology* 172: 823–834

Ku M, Koche RP, Rheinbay E, Mendenhall EM, Endoh M, Mikkelsen TS, Presser A, Nusbaum C, Xie X, Chi AS, *et al* (2008) Genomewide analysis of PRC1 and PRC2 occupancy identifies two classes of bivalent domains. *PLoS Genet* 4: e1000242

Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P & Reinberg D (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev* 16: 2893–2905

Lan F, Bayliss PE, Rinn JL, Whetstine JR, Wang JK, Chen S, Iwase S, Alpatov R, Issaeva I, Canaani E, *et al* (2007) A histone H3 lysine 27 demethylase regulates animal posterior development. *Nature* 449: 689–694

Langerak P & Russell P (2011) Regulatory networks integrating cell cycle control with DNA damage checkpoints and double-strand break repair. *Philos Trans R Soc Lond B Biol Sci* 366: 3562–3571

Lederer D, Grisart B, Digilio MC, Benoit V, Crespin M, Ghariani SC, Maystadt I, Dallapiccola B & Verellen-Dumoulin C (2012) Deletion of KDM6A, a Histone Demethylase Interacting with MLL2, in Three Patients with Kabuki Syndrome. *Am J Hum Genet* 90: 119–124

Lee MG, Villa R, Trojer P, Norman J, Yan K-P, Reinberg D, Di Croce L & Shiekhattar R (2007) Demethylation of H3K27 Regulates Polycomb Recruitment and H2A Ubiquitination. *Science* 318: 447–450

Lee S, Lee JW & Lee S-K (2012) UTX, a histone H3-lysine 27 demethylase, acts as a critical switch to activate the cardiac developmental program. *Dev Cell* 22: 25–37

Leeb M, Pasini D, Novatchkova M, Jaritz M, Helin K & Wutz A (2010) Polycomb complexes act redundantly to repress genomic repeats and genes. *Genes Dev* 24: 265–276

Li G-M (2008) Mechanisms and functions of DNA mismatch repair. *Cell Res* 18: 85–98

Li J, Hart RP, Mallimo EM, Swerdel MR, Kusnecov AW & Herrup K (2013) EZH2-mediated H3K27 trimethylation mediates neurodegeneration in ataxia-telangiectasia. *Nat Neurosci* 16: 1745–1753

Li X & Tyler JK (2016) Nucleosome disassembly during human non-homologous end joining followed by concerted HIRA- and CAF-1-dependent reassembly. *eLife* 5

Lindahl T (1993) Instability and decay of the primary structure of DNA. *Nature* 362: 709–715

Lindahl T & Barnes DE (2000) Repair of endogenous DNA damage. *Cold Spring Harb Symp Quant Biol* 65: 127–133

Liu J, Mercher T, Scholl C, Brumme K, Gilliland DG & Zhu N (2012) A functional role for the histone demethylase UTX in normal and malignant hematopoietic cells. *Exp Hematol* 40: 487-498.e3

Llorens-Agost M, Ensminger M, Le HP, Gawai A, Liu J, Cruz-García A, Bhetawal S, Wood RD, Heyer W-D & Löbrich M (2021) POLθ-mediated end joining is restricted by RAD52 and BRCA2 until the onset of mitosis. *Nat Cell Biol* 23: 1095–1104

Lord CJ & Ashworth A (2008) Targeted therapy for cancer using PARP inhibitors. *Current Opinion in Pharmacology* 8: 363–369

Lu H, Saha J, Beckmann PJ, Hendrickson EA & Davis AJ (2019) DNA-PKcs promotes chromatin decondensation to facilitate initiation of the DNA damage response. *Nucleic Acids Research* 47: 9467–9479

Luijsterburg MS, de Krijger I, Wiegant WW, Shah RG, Smeenk G, de Groot AJL, Pines A, Vertegaal ACO, Jacobs JJL, Shah GM, *et al* (2016) PARP1 Links CHD2-Mediated Chromatin Expansion and H3.3 Deposition to DNA Repair by Non-homologous End-Joining. *Molecular Cell* 61: 547–562

Luijsterburg MS, Lindh M, Acs K, Vrouwe MG, Pines A, van Attikum H, Mullenders LH & Dantuma NP (2012) DDB2 promotes chromatin decondensation at UV-induced DNA damage. *Journal of Cell Biology* 197: 267–281

Luo J, Solimini NL & Elledge SJ (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* 136: 823–837

Machour FE & Ayoub N (2020) Transcriptional Regulation at DSBs: Mechanisms and Consequences. *Trends in Genetics* 36: 981–997

Mahaney BL, Meek K & Lees-Miller SP (2009) Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. *Biochem J* 417: 639–650

Mandemaker IK, Geijer ME, Kik I, Bezstarosti K, Rijkers E, Raams A, Janssens RC, Lans H, Hoeijmakers JH, Demmers JA, *et al* (2018) DNA damage-induced replication stress results in PA200-proteasome-mediated degradation of acetylated histones. *EMBO reports* 19: e45566

Manna S, Kim JK, Baugé C, Cam M, Zhao Y, Shetty J, Vacchio MS, Castro E, Tran B, Tessarollo L, *et al* (2015) Histone H3 Lysine 27 demethylases Jmjd3 and Utx are required for T-cell differentiation. *Nat Commun* 6: 8152

Mar BG, Bullinger L, Basu E, Schlis K, Silverman LB, Döhner K & Armstrong SA (2012) Sequencing histone-modifying enzymes identifies UTX mutations in acute lymphoblastic leukemia. *Leukemia* 26: 1881–1883

Margueron R & Reinberg D (2011) The Polycomb complex PRC2 and its mark in life. *Nature* 469: 343–349

Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, *et al* (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* 316: 1160–1166

Maures TJ, Greer EL, Hauswirth AG & Brunet A (2011) The H3K27 demethylase UTX-1 regulates C. elegans lifespan in a germline-independent, insulin-dependent manner. *Aging Cell* 10: 980–990

Meek K, Dang V & Lees-Miller SP (2008) DNA-PK: the means to justify the ends? *Adv Immunol* 99: 33–58

Miller SA, Mohn SE & Weinmann AS (2010) Jmjd3 and UTX play a demethylase-independent role in chromatin remodeling to regulate T-box family member-dependent gene expression. *Mol Cell* 40: 594–605

Mirman Z, Sasi NK, King A, Chapman JR & de Lange T (2022) 53BP1–shieldin-dependent DSB processing in BRCA1-deficient cells requires CST–Polα–primase fill-in synthesis. *Nat Cell Biol* 24: 51–61

Miyake N, Koshimizu E, Okamoto N, Mizuno S, Ogata T, Nagai T, Kosho T, Ohashi H, Kato M, Sasaki G, *et al* (2013a) MLL2 and KDM6A mutations in patients with Kabuki syndrome. *Am J Med Genet A* 161A: 2234–2243

Miyake N, Mizuno S, Okamoto N, Ohashi H, Shiina M, Ogata K, Tsurusaki Y, Nakashima M, Saitsu H, Niikawa N, *et al* (2013b) KDM6A point mutations cause Kabuki syndrome. *Hum Mutat* 34: 108–110

Morganella S, Alexandrov LB, Glodzik D, Zou X, Davies H, Staaf J, Sieuwerts AM, Brinkman AB, Martin S, Ramakrishna M, *et al* (2016) The topography of mutational processes in breast cancer genomes. *Nat Commun* 7: 11383

Morrison O & Thakur J (2021) Molecular Complexes at Euchromatin, Heterochromatin and Centromeric Chromatin. *International Journal of Molecular Sciences* 22: 6922

Motycka TA, Bessho T, Post SM, Sung P & Tomkinson AE (2004) Physical and functional interaction between the XPF/ERCC1 endonuclease and hRad52. *J Biol Chem* 279: 13634–13639

Murga M, Jaco I, Fan Y, Soria R, Martinez-Pastor B, Cuadrado M, Yang S-M, Blasco MA, Skoultchi AI & Fernandez-Capetillo O (2007) Global chromatin compaction limits the strength of the DNA damage response. *Journal of Cell Biology* 178: 1101–1108

Murr R, Loizou JI, Yang Y-G, Cuenin C, Li H, Wang Z-Q & Herceg Z (2006) Histone acetylation by Trrap–Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks. *Nat Cell Biol* 8: 91–99

Nam EA & Cortez D (2011) ATR signalling: more than meeting at the fork. *Biochem J* 436: 527–536

de Napoles M, Mermoud JE, Wakao R, Tang YA, Endoh M, Appanah R, Nesterova TB, Silva J, Otte AP, Vidal M, *et al* (2004) Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. *Dev Cell* 7: 663–676

Nazarov IB, Smirnova AN, Krutilina RI, Svetlova MP, Solovjeva LV, Nikiforov AA, Oei S-L, Zalenskaya IA, Yau PM, Bradbury EM, *et al* (2003) Dephosphorylation of histone γ-H2AX during repair of DNA double-strand breaks in mammalian cells and its inhibition by calyculin A. *Radiation Research* 160: 309–317

Ng SB, Bigham AW, Buckingham KJ, Hannibal MC, McMillin MJ, Gildersleeve HI, Beck AE, Tabor HK, Cooper GM, Mefford HC, *et al* (2010) Exome sequencing identifies MLL2 mutations as a cause of Kabuki syndrome. *Nat Genet* 42: 790–793

Nicassio F, Corrado N, Vissers JHA, Areces LB, Bergink S, Marteijn JA, Geverts B, Houtsmuller AB, Vermeulen W, Di Fiore PP, *et al* (2007) Human USP3 Is a Chromatin Modifier Required for S Phase Progression and Genome Stability. *Current Biology* 17: 1972–1977

Niikawa N, Matsuura N, Fukushima Y, Ohsawa T & Kajii T (1981) Kabuki make-up syndrome: a syndrome of mental retardation, unusual facies, large and protruding ears, and postnatal growth deficiency. *J Pediatr* 99: 565–569

Nik-Zainal S, Davies H, Staaf J, Ramakrishna M, Glodzik D, Zou X, Martincorena I, Alexandrov LB, Martin S, Wedge DC, *et al* (2016) Landscape of somatic mutations in 560 breast cancer whole-genome sequences. *Nature* 534: 47–54

Noon AT, Shibata A, Rief N, Löbrich M, Stewart GS, Jeggo PA & Goodarzi AA (2010) 53BP1-dependent robust localized KAP-1 phosphorylation is essential for heterochromatic DNA double-strand break repair. *Nat Cell Biol* 12: 177–184

Norppa H & Falck GC-M (2003) What do human micronuclei contain? *Mutagenesis* 18: 221–233

Ntziachristos P, Tsirigos A, Welstead GG, Trimarchi T, Bakogianni S, Xu L, Loizou E, Holmfeldt L, Strikoudis A, King B, *et al* (2014) Contrasting roles of histone 3 lysine 27 demethylases in acute lymphoblastic leukaemia. *Nature* 514: 513–517

Ochs F, Karemore G, Miron E, Brown J, Sedlackova H, Rask M-B, Lampe M, Buckle V, Schermelleh L, Lukas J, *et al* (2019) Stabilization of chromatin topology safeguards genome integrity. *Nature* 574: 571–574

O'Hagan HM, Mohammad HP & Baylin SB (2008) Double Strand Breaks Can Initiate Gene Silencing and SIRT1-Dependent Onset of DNA Methylation in an Exogenous Promoter CpG Island. *PLOS Genetics* 4: e1000155

O'Hagan HM, Wang W, Sen S, DeStefano Shields C, Lee SS, Zhang YW, Clements EG, Cai Y, Van Neste L, Easwaran H, *et al* (2011) Oxidative Damage Targets Complexes Containing DNA Methyltransferases, SIRT1, and Polycomb Members to Promoter CpG Islands. *Cancer Cell* 20: 606–619

Paiano J, Zolnerowich N, Wu W, Pavani R, Wang C, Li H, Zheng L, Shen B, Sleckman BP, Chen B-R, *et al* (2021) Role of 53BP1 in end protection and DNA synthesis at DNA breaks. *Genes Dev* 35: 1356–1367

Pan G, Tian S, Nie J, Yang C, Ruotti V, Wei H, Jonsdottir GA, Stewart R & Thomson JA (2007) Whole-Genome Analysis of Histone H3 Lysine 4 and Lysine 27 Methylation in Human Embryonic Stem Cells. *Cell Stem Cell* 1: 299–312

Paul A & Wang B (2017) RNF8- and Ube2S-Dependent Ubiquitin Lysine 11-Linkage Modification in Response to DNA Damage. *Molecular Cell* 66: 458-472.e5

Pauler FM, Sloane MA, Huang R, Regha K, Koerner MV, Tamir I, Sommer A, Aszodi A, Jenuwein T & Barlow DP (2009) H3K27me3 forms BLOCs over silent genes and intergenic regions and specifies a histone banding pattern on a mouse autosomal chromosome. *Genome Res* 19: 221–233

Pfister SX, Ahrabi S, Zalmas L-P, Sarkar S, Aymard F, Bachrati CZ, Helleday T, Legube G, La Thangue NB, Porter ACG, *et al* (2014) SETD2-Dependent Histone H3K36 Trimethylation Is Required for Homologous Recombination Repair and Genome Stability. *Cell Reports* 7: 2006–2018

Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, de la Cruz CC, Otte AP, Panning B & Zhang Y (2003) Role of histone H3 lysine 27 methylation in X inactivation. *Science* 300: 131–135

Polo SE & Jackson SP (2011) Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. *Genes Dev* 25: 409–433

Pommier Y, Leo E, Zhang H & Marchand C (2010) DNA Topoisomerases and Their Poisoning by Anticancer and Antibacterial Drugs. *Chemistry & Biology* 17: 421–433

Pritchard CC, Mateo J, Walsh MF, De Sarkar N, Abida W, Beltran H, Garofalo A, Gulati R, Carreira S, Eeles R, *et al* (2016) Inherited DNA-Repair Gene Mutations in Men with Metastatic Prostate Cancer. *N Engl J Med* 375: 443–453

Qian M-X, Pang Y, Liu CH, Haratake K, Du B-Y, Ji D-Y, Wang G-F, Zhu Q-Q, Song W, Yu Y, *et al* (2013) Acetylation-Mediated Proteasomal Degradation of Core Histones during DNA Repair and Spermatogenesis. *Cell* 153: 1012–1024

Rath BH, Waung I, Camphausen K & Tofilon PJ (2018) Inhibition of the histone H3K27 demethylase UTX enhances tumor cell radiosensitivity. *Mol Cancer Ther* 17: 1070–1078

Reynolds P, Anderson JA, Harper JV, Hill MA, Botchway SW, Parker AW & O'Neill P (2012) The dynamics of Ku70/80 and DNA-PKcs at DSBs induced by ionizing radiation is dependent on the complexity of damage. *Nucleic Acids Research* 40: 10821–10831

Riley T, Sontag E, Chen P & Levine A (2008) Transcriptional control of human p53-regulated genes. *Nat Rev Mol Cell Biol* 9: 402–412

Ringrose L & Paro R (2007) Polycomb/Trithorax response elements and epigenetic memory of cell identity. *Development* 134: 223–232

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W & Smyth GK (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43: e47

Robinson G, Parker M, Kranenburg TA, Lu C, Chen X, Ding L, Phoenix TN, Hedlund E, Wei L, Zhu X, *et al* (2012) Novel mutations target distinct subgroups of medulloblastoma. *Nature* 488: 43–48

Rodriguez-Vida A, Lerner SP & Bellmunt J (2018) The Cancer Genome Atlas Project in Bladder Cancer. *Cancer Treat Res* 175: 259–271

Rosenfeld JA, Wang Z, Schones DE, Zhao K, DeSalle R & Zhang MQ (2009) Determination of enriched histone modifications in non-genic portions of the human genome. *BMC Genomics* 10: 143

Rouleau M, Patel A, Hendzel MJ, Kaufmann SH & Poirier GG (2010) PARP inhibition: PARP1 and beyond. *Nat Rev Cancer* 10: 293–301

Rubbi CP & Milner J (2003) p53 is a chromatin accessibility factor for nucleotide excision repair of DNA damage. *EMBO Journal* 22: 975–986

Sanchez A, De Vivo A, Uprety N, Kim J, Stevens SM & Kee Y (2016) BMI1–UBR5 axis regulates transcriptional repression at damaged chromatin. *Proc Natl Acad Sci USA* 113: 11243–11248

Sankar TS, Wastuwidyaningtyas BD, Dong Y, Lewis SA & Wang JD (2016) The nature of mutations induced by replication–transcription collisions. *Nature* 535: 178–181

Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM & Abraham RT (1999) Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res* 59: 4375–4382

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, *et al* (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9: 676–682

Schuettengruber B, Chourrout D, Vervoort M, Leblanc B & Cavalli G (2007) Genome Regulation by Polycomb and Trithorax Proteins. *Cell* 128: 735–745

Schulz WA, Lang A, Koch J & Greife A (2019) The histone demethylase UTX/KDM6A in cancer: Progress and puzzles. *International Journal of Cancer* 145: 614–620

Schwartz YB & Pirrotta V (2007) Polycomb silencing mechanisms and the management of genomic programmes. *Nat Rev Genet* 8: 9–22

Seeber A, Hauer M & Gasser SM (2013) Nucleosome remodelers in double-strand break repair. *Curr Opin Genet Dev* 23: 174–184

Seeber A, Hauer MH & Gasser SM (2018) Chromosome Dynamics in Response to DNA Damage. *Annual Review of Genetics* 52: 295–319

Seenundun S, Rampalli S, Liu Q-C, Aziz A, Palii C, Hong S, Blais A, Brand M, Ge K & Dilworth FJ (2010) UTX mediates demethylation of H3K27me3 at muscle-specific genes during myogenesis. *EMBO J* 29: 1401–1411

Sengoku T & Yokoyama S (2011) Structural basis for histone H3 Lys 27 demethylation by UTX/KDM6A. *Genes Dev* 25: 2266–2277

Shi B, Li W, Song Y, Wang Z, Ju R, Ulman A, Hu J, Palomba F, Zhao Y, Le JP, *et al* (2021) UTX condensation underlies its tumor-suppressive activity. *Nature* 597: 726–731

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA & Shi Y (2004) Histone Demethylation Mediated by the Nuclear Amine Oxidase Homolog LSD1. *Cell* 119: 941–953

Shilatifard A (2008) Molecular implementation and physiological roles for histone H3 lysine 4 (H3K4) methylation. *Curr Opin Cell Biol* 20: 341–348

Shimizu N (2011) Molecular mechanisms of the origin of micronuclei from extrachromosomal elements. *Mutagenesis* 26: 119–123

Shpargel KB, Sengoku T, Yokoyama S & Magnuson T (2012) UTX and UTY demonstrate histone demethylase-independent function in mouse embryonic development. *PLoS Genet* 8: e1002964

Silva J, Mak W, Zvetkova I, Appanah R, Nesterova TB, Webster Z, Peters AHFM, Jenuwein T, Otte AP & Brockdorff N (2003) Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. *Dev Cell* 4: 481–495

Simon JA & Kingston RE (2013) Occupying chromatin: Polycomb mechanisms for getting to genomic targets, stopping transcriptional traffic, and staying put. *Mol Cell* 49: 808–824

Simoneau A & Zou L (2021) An extending ATR–CHK1 circuitry: the replication stress response and beyond. *Current Opinion in Genetics & Development* 71: 92–98

Singh V, Johansson P, Ekedahl E, Lin Y-L, Hammarsten O & Westerlund F (2022) Quantification of single-strand DNA lesions caused by the topoisomerase II poison etoposide using single DNA molecule imaging. *Biochemical and Biophysical Research Communications* 594: 57–62

Smeenk G & van Attikum H (2013) The chromatin response to DNA breaks: leaving a mark on genome integrity. *Annu Rev Biochem* 82: 55–80

Smith RL, Redd MJ & Johnson AD (1995) The tetratricopeptide repeats of Ssn6 interact with the homeo domain of alpha 2. *Genes Dev* 9: 2903–2910

Sørensen CS, Hansen LT, Dziegielewski J, Syljuåsen RG, Lundin C, Bartek J & Helleday T (2005) The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. *Nat Cell Biol* 7: 195–201

Soto DR, Barton C, Munger K & McLaughlin-Drubin ME (2017) KDM6A addiction of cervical carcinoma cell lines is triggered by E7 and mediated by p21CIP1 suppression of replication stress. *PLOS Pathogens* 13: e1006661

Spotheim-Maurizot M, Ruiz S, Sabattier R & Charlier M (1995) Radioprotection of DNA by Polyamines. *International Journal of Radiation Biology* 68: 571–577

Strahl BD & Allis CD (2000) The language of covalent histone modifications. *Nature* 403: 41–45

Strickfaden H, McDonald D, Kruhlak MJ, Haince J-F, Th'ng JPH, Rouleau M, Ishibashi T, Corry GN, Ausio J, Underhill DA, *et al* (2016) Poly(ADP-ribosyl)ation-dependent Transient Chromatin Decondensation and Histone Displacement following Laser Microirradiation*. *Journal of Biological Chemistry* 291: 1789–1802

Stucki M, Clapperton JA, Mohammad D, Yaffe MB, Smerdon SJ & Jackson SP (2005) MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. *Cell* 123: 1213–1226

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, *et al* (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences* 102: 15545–15550

Sun Y, Jiang X & Price BD (2010) Tip60: Connecting chromatin to DNA damage signaling. *Cell Cycle* 9: 930–936

Suzuki M, Crozatier C, Yoshikawa K, Mori T & Yoshikawa Y (2009) Protamine-induced DNA compaction but not aggregation shows effective radioprotection against double-strand breaks. *Chemical Physics Letters* 480: 113–117

Taylor AMR, Rothblum-Oviatt C, Ellis NA, Hickson ID, Meyer S, Crawford TO, Smogorzewska A, Pietrucha B, Weemaes C & Stewart GS (2019) Chromosome instability syndromes. *Nat Rev Dis Primers* 5: 1–20

Tedesco M, Giannese F, Lazarević D, Giansanti V, Rosano D, Monzani S, Catalano I, Grassi E, Zanella ER, Botrugno OA, *et al* (2022) Chromatin Velocity reveals epigenetic dynamics by single-cell profiling of heterochromatin and euchromatin. *Nat Biotechnol* 40: 235–244

Thieme S, Gyárfás T, Richter C, Özhan G, Fu J, Alexopoulou D, Muders MH, Michalk I, Jakob C, Dahl A, *et al* (2013) The histone demethylase UTX regulates stem cell migration and hematopoiesis. *Blood* 121: 2462–2473

Tie F, Banerjee R, Conrad PA, Scacheri PC & Harte PJ (2012) Histone demethylase UTX and chromatin remodeler BRM bind directly to CBP and modulate acetylation of histone H3 lysine 27. *Mol Cell Biol* 32: 2323–2334

Ting X, Xia L, Yang J, He L, Si W, Shang Y & Sun L (2019) USP11 acts as a histone deubiquitinase functioning in chromatin reorganization during DNA repair. *Nucleic Acids Research* 47: 9721–9740

Tran N, Broun A & Ge K (2020) Lysine Demethylase KDM6A in Differentiation, Development, and Cancer. *Mol Cell Biol* 40: e00341-20

Tsukuda T, Fleming AB, Nickoloff JA & Osley MA (2005) Chromatin remodelling at a DNA double-strand break site in Saccharomyces cerevisiae. *Nature* 438: 379–383

Tubbs A & Nussenzweig A (2017) Endogenous DNA Damage as a Source of Genomic Instability in Cancer. *Cell* 168: 644–656

Turner N, Tutt A & Ashworth A (2004) Hallmarks of 'BRCAness' in sporadic cancers. *Nat Rev Cancer* 4: 814–819

Udayakumar D, Horikoshi N, Mishra L, Hunt C & Pandita TK (2015) Detecting ATM-Dependent Chromatin Modification in DNA Damage Response. *Methods Mol Biol* 1288: 317–336

Valota A, Ballarini F, Friedland W, Jacob P, Ottolenghi A & Paretzke HG (2003) Modelling study on the protective role of OH radical scavengers and DNA higher-order structures in induction of single- and double-strand break by gamma-radiation. *International Journal of Radiation Biology* 79: 643–653

Van der Meulen J, Sanghvi V, Mavrakis K, Durinck K, Fang F, Matthijssens F, Rondou P, Rosen M, Pieters T, Vandenberghe P, *et al* (2015) The H3K27me3 demethylase UTX is a gender-specific tumor suppressor in T-cell acute lymphoblastic leukemia. *Blood* 125: 13–21

Van der Meulen J, Speleman F & Van Vlierberghe P (2014) The H3K27me3 demethylase UTX in normal development and disease. *Epigenetics* 9: 658–668

Voigt P, LeRoy G, Drury WJ, Zee BM, Son J, Beck DB, Young NL, Garcia BA & Reinberg D (2012) Asymmetrically Modified Nucleosomes. *Cell* 151: 181–193

Walker JR, Corpina RA & Goldberg J (2001) Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature* 412: 607–614

Walport LJ, Hopkinson RJ, Vollmar M, Madden SK, Gileadi C, Oppermann U, Schofield CJ & Johansson C (2014) Human UTY(KDM6C) Is a Male-specific Nϵ-Methyl Lysyl Demethylase. *J Biol Chem* 289: 18302–18313

Wang AH, Zare H, Mousavi K, Wang C, Moravec CE, Sirotkin HI, Ge K, Gutierrez-Cruz G & Sartorelli V (2013) The histone chaperone Spt6 coordinates histone H3K27 demethylation and myogenesis. *EMBO J* 32: 1075–1086

Wang C, Lee J-E, Cho Y-W, Xiao Y, Jin Q, Liu C & Ge K (2012) UTX regulates mesoderm differentiation of embryonic stem cells independent of H3K27 demethylase activity. *Proc Natl Acad Sci U S A* 109: 15324–15329

Wang S-P, Tang Z, Chen C-W, Shimada M, Koche RP, Wang L-H, Nakadai T, Chramiec A, Krivtsov AV, Armstrong SA, *et al* (2017) A UTX-MLL4-p300 Transcriptional Regulatory Network Coordinately Shapes Active Enhancer Landscapes for Eliciting Transcription. *Mol Cell* 67: 308-321.e6

Wang Z, Zhang H, Liu J, Cheruiyot A, Lee J-H, Ordog T, Lou Z, You Z & Zhang Z (2016) USP51 deubiquitylates H2AK13,15ub and regulates DNA damage response. *Genes Dev* 30: 946–959

Warmerdam DO & Kanaar R (2010) Dealing with DNA damage: Relationships between checkpoint and repair pathways. *Mutation Research/Reviews in Mutation Research* 704: 2–11

Warters RL, Newton GL, Olive PL & Fahey RC (1999) Radioprotection of Human Cell Nuclear DNA by Polyamines: Radiosensitivity of Chromatin Is Influenced by Tightly Bound Spermine. *Radiation Research* 151: 354–362

Welstead GG, Creyghton MP, Bilodeau S, Cheng AW, Markoulaki S, Young RA & Jaenisch R (2012) X-linked H3K27me3 demethylase Utx is required for embryonic development in a sex-specific manner. *Proc Natl Acad Sci U S A* 109: 13004–13009

West SC (2003) Molecular views of recombination proteins and their control. *Nat Rev Mol Cell Biol* 4: 435–445

Williams RS, Moncalian G, Williams JS, Yamada Y, Limbo O, Shin DS, Groocock LM, Cahill D, Hitomi C, Guenther G, *et al* (2008) Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. *Cell* 135: 97–109

Williams RS, Williams JS & Tainer JA (2007) Mre11-Rad50-Nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template. *Biochem Cell Biol* 85: 509–520

Wilson DM & Bohr VA (2007) The mechanics of base excision repair, and its relationship to aging and disease. *DNA Repair* 6: 544–559

Wip1 phosphatase is associated with chromatin and dephosphorylates γH2AX to promote checkpoint inhibition | Oncogene

Xie G, Liu X, Zhang Y, Li W, Liu S, Chen Z, Xu B, Yang J, He L, Zhang Z, *et al* (2017) UTX promotes hormonally responsive breast carcinogenesis through feed-forward transcription regulation with estrogen receptor. *Oncogene* 36: 5497–5511

Xu B, Sun Z, Liu Z, Guo H, Liu Q, Jiang H, Zou Y, Gong Y, Tischfield JA & Shao C (2011) Replication Stress Induces Micronuclei Comprising of Aggregated DNA Double-Strand Breaks. *PLOS ONE* 6: e18618

Xu Y, Sun Y, Jiang X, Ayrapetov MK, Moskwa P, Yang S, Weinstock DM & Price BD (2010) The p400 ATPase regulates nucleosome stability and chromatin ubiquitination during DNA repair. *Journal of Cell Biology* 191: 31–43

Yan WX, Mirzazadeh R, Garnerone S, Scott D, Schneider MW, Kallas T, Custodio J, Wernersson E, Li Y, Gao L, *et al* (2017) BLISS is a versatile and quantitative method for genome-wide profiling of DNA double-strand breaks. *Nature Communications* 8

Yang G, Chen Y, Wu J, Chen S-H, Liu X, Singh AK & Yu X (2020) Poly(ADP-ribosyl)ation mediates early phase histone eviction at DNA lesions. *Nucleic Acids Research* 48: 3001–3013

Yoshikawa Y, Mori T, Magome N, Hibino K & Yoshikawa K (2008) DNA compaction plays a key role in radioprotection against double-strand breaks as revealed by single-molecule observation. *Chemical Physics Letters* 456: 80–83

You Z & Bailis JM (2010) DNA damage and decisions: CtIP coordinates DNA repair and cell cycle checkpoints. *Trends Cell Biol* 20: 402–409

Yu M, Liu K, Mao Z, Luo J, Gu W & Zhao W (2016) USP11 Is a Negative Regulator to γH2AX Ubiquitylation by RNF8/RNF168*. *Journal of Biological Chemistry* 291: 959–967

Yun MH & Hiom K (2009) CtIP-BRCA1 modulates the choice of DNA double-strand break repair pathway throughout the cell cycle. *Nature* 459: 460–463

Zehir A, Benayed R, Shah RH, Syed A, Middha S, Kim HR, Srinivasan P, Gao J, Chakravarty D, Devlin SM, *et al* (2017) Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. *Nat Med* 23: 703–713

Zentner GE & Henikoff S (2013) Regulation of nucleosome dynamics by histone modifications. *Nat Struct Mol Biol* 20: 259–266

Zhang Y, Chang J-F, Sun J, Chen L, Yang X-M, Tang H-Y, Jing Y-Y, Kang X, He Z-M, Wu J-Y, *et al* (2018) Histone H3K27 methylation modulates the dynamics of FANCD2 on chromatin to facilitate NHEJ and genome stability. *Journal of Cell Science* 131: jcs215525

Zhao XD, Han X, Chew JL, Liu J, Chiu KP, Choo A, Orlov YL, Sung W-K, Shahab A, Kuznetsov VA, *et al* (2007) Whole-Genome Mapping of Histone H3 Lys4 and 27 Trimethylations Reveals Distinct Genomic Compartments in Human Embryonic Stem Cells. *Cell Stem Cell* 1: 286–298

Zhou B-BS & Elledge SJ (2000) The DNA damage response: putting checkpoints in perspective. *Nature* 408: 433–439