Post-translational modifications in the DNA damage response

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Doctoral Thesis

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ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
53BP1	p53-binding protein 1
9-1-1	Rad9-Rad1-Hus1
AAD	ATR activating domain
alt-NHEJ	Alternative non-homologous end joining
Arg	Arginine
Asf1	Anti-silencing function protein 1
ATM	Ataxia telangiectasia mutated protein
ATR	Ataxia telangiectasia and Rad3-related protein
BARD1	BRCA1-associated RING domain protein 1
BER	Base excision repair
BFP	Blue fluorescent protein
BLM	Bloom syndrome protein
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BRCT	BRCA1 C-terminal
c-NHEJ	Canonical non-homologous end joining
Cdc25A	Cell division cycle 25 homolog A
Cdc25C	Cell division cycle 25 homolog C
CDK	Cyclin-Dependent Kinase
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CHX	Cycloheximide
CPT	Camptothecin
CtIP	CtBP-interacting protein
DAPI	6-diamino-2-phenylindole
DDR	DNA damage response
D-loop	Displacing loop
DMEM	Dulbecco's modified Eagle medium
DNA2	DNA replication helicase/nuclease 2
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	Double-strand break
DUB	Deubiquitinating enzyme
EDTA	Ethylenediaminetetraacetic acid

Abbreviations

EJ	End joining
ETAA1	Ewing tumor-associated antigen 1
ETP	Etoposide
EV	Empty vector
EXD2	Exonuclease 3'-5' domain containing 2
EXO1	Human exonuclease 1
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HAT	Histone acetyltransferase
HAT1	Histone acetyltransferase 1
HBS	HEPES-buffered saline
HDAC	Histone deacetylase
HR	Homologous recombination
HRP	Horseradish peroxidase
IF	Immunofluorescence
IR	Ionizing radiation
Jmj	Jumonji domain
KAT	Lysine acetyltransferase
kD	Kilodalton
KDAC	Lysine deacetylase
KDM	Lysine demethylase
KDM4A	Lysine-specific demethylase 4A
KMT	Lysine methyltransferase
Lys	Lysine
LIG4	Ligase 4
Luc	Luciferase
L3MBTL1	Lethal(3)malignant brain tumor-like protein 1
MDC1	Mediator of DNA checkpoint protein 1
Mdm2	Murine double minute 2
MAD2L2/Rev7	Mitotic arrest deficient 2-like protein 2
MOI	Multiplicity of infection
MMR	Mismatch repair
Mre11	Meiotic recombination 11
MRN	Mre11-Rad50-Nbs1

Nbs1	Nijmegen breakage syndrome 1
NEM	N-ethylmaleimide
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
Noco	Nocodazole
PALB2	Partner and localizer of BRCA2
PARP	Poly-(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PHD	Plant homeodomain
PHF2	PHD Finger Protein 2
PI	Propidium iodide
PIKK	Phosphatidylinositol 3-kinase-related kinase
Plk1	Polo-like kinase 1
Pol	Polymerase
PPM1D	Protein phosphatase, Mg2+/Mn2+ Dependent 1D
PRMT	Protein arginine methyltransferase
PTIP	Pax transactivation-domain interacting protein
PTM	Post-translational modification
RAP80	Receptor-associated protein 80
Rbap46	Rb-associated protein 46
RFC	Replication factor C
RIF1	Rap1-interacting factor 1 homolog
RNF168	Ring finger protein 168
RNF8	Ring finger protein 8
ROS	Reactive oxygen species
RT	Room temperature
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulphate
SDSA	Synthesis-dependent strand annealing
SSA	Single-stranded annealing
SSB	Single-strand break
ssDNA	Single-stranded DNA
SUV39H1	Suppressor of variegation 3-9 homolog 1
TBS	Tris-buffered saline

Abbreviations

Thr	Threonine
Thym	Thymidine
TIP60	60 kDa Tat-interactive protein
TopBP1	DNA topoisomerase 2-binding protein 1
Tyr	Tyrosine
UBF	Upstream binding factor
Ubls	Ubiquitin-like proteins
USP7	Ubiquitin-specific protease 7
UV	Ultraviolet
WCE	Whole cell extract
Wee1	WEE1-like protein kinase
XLF	XRCC4-like factor
XRCC4	X-ray repair cross-complementing protein 4

INTRODUCTION

1. The DNA Damage Response

The integrity of the human genome is constantly threatened by a variety of endogenous and environmental factors. Cells need to repair the damage suffered in their genetic material to faithfully transmit the information it contains to the next generation. To do so, eukaryotes have evolved an extremely efficient and complex response to DNA lesions, collectively known as the DNA Damage Response (DDR) (Jackson and Bartek, 2009).

Each cell of the human body can suffer up to 10^5 spontaneous DNA lesions every day (Hoeijmakers, 2009). The simplest form of DNA damage is the spontaneous hydrolysis of the N-glycoside bond between the base and the deoxyribose, generating what are known as abasic or AP (apurinic/apyrimidinic) sites (Lindahl, 1993). Another type of spontaneous hydrolysis that takes place in the cell is the deamination of DNA bases carrying exocyclic amino groups (Lindahl, 1993). This process can transform cytosine into uracil, adenine into hypoxanthine and guanine into xanthine (Kow, 2002; Krokan *et al.*, 2002). Additionally, bases can be modified by alkylation, a reaction in which a methyl group is transferred from S-adenosylmethionine (SAM) to guanine or adenine, generating O^6-methylguanine and 3-methyladenine respectively (Lindahl and Barnes, 2000).

DNA damage can also arise from metabolites generated during different cellular processes. Especially toxic for cells are reactive oxygen species (ROS), such as O_2^- or H_2O_2, which can cause several types of damage, mainly base modifications, strand-breaks or DNA-crosslinks (Apel and Hirt, 2004). Furthermore, ROS (as well as some endogenous reactive nitrogen species like nitric oxide) can generate oxidative DNA adducts, being the most prevalent and studied example the formation of 8-oxoguanine (Dexheimer, 2013).

Errors in DNA-processing events can also lead to the formation of aberrant structures in the chromatin. During replication, polymerases can wrongly introduce dNTPs, generating mismatches, insertions and deletions (Kunkel and McCulloch, 2008). Other error prone processes include recombination or DNA repair, which can cause breaks or strand-crosslinks amongst other types of damage (Linn *et al.*, 2001).

Besides all the endogenously generated DNA damage, cells need to face many different environmental DNA-damaging sources, which can be divided into physical and chemical agents. Examples of physical genotoxic agents include ionizing radiation (IR) and ultraviolet (UV) light. IR, originated either from natural (cosmic and γ radiation) or artificial (medical treatments employing X-rays or radiotherapy) sources, mainly causes double-strand breaks (DSBs), but can also generate ROS and lead to base oxidation (Ward, 1988). UV light, on the other hand, generates pyrimidine dimers and 6–4 photoproducts that prevent replication fork movement, and eventually lead to single-strand breaks (SSBs) (Ravanat *et al.*, 2001). Chemical agents can also cause severe damage to DNA. Especially toxic are antitumor agents, which act in diverse ways: alkylating agents, like methyl methanesulfonate (MMS) attach alkyl groups to DNA bases, while topoisomerase inhibitors, like camptothecin (CPT) or etoposide (ETP) generate SSBs or DSBs by trapping topoisomerase–DNA covalent complexes, and crosslinking agents such as mitomycin C introduce covalent links between bases of the same or different DNA molecules (intrastrand and interstrand crosslinks respectively) (Wogan *et al.*, 2004; Irigaray and Belpomme, 2010).

Introduction

The DDR is comprised of different pathways that are used to repair specific types of lesions (Table 1). Mismatches and insertions/deletions in the DNA sequence are repaired by mismatch repair (MMR), in which a single-strand incision is created, processed by nucleases to remove the damaged region and then filled by polymerases and ligases (Jackson and Bartek, 2009). Other repair pathways that involve the removal of the lesion and the flanking region, followed by re-synthesis of the gap are base excision repair (BER) and nucleotide excision repair (NER). In BER, damaged bases (usually by oxidative processes) are recognized and removed by DNA glycosylase enzymes, whereas NER operates on helix-distorting lesions and the oligonucleotide removed is bigger than in the rest of the excision-repair pathways (Laat *et al.*, 1999; David *et al.*, 2010).

Table 1. Overview of the different types of DNA damage, main causing sources and preferred repair pathway

Type of lesion	DNA-damaging agent	Repair Pathway
Abasic or AP (apurinic/apyrimidinic) sites	Spontaneous hydrolysis	Base excision repair (BER)
	ROS	
	Alkylating agents	
Base deamination	Spontaneous deamination	Base excision repair (BER)
	ROS	
	Alkylating agents	
Bulky adducts	UV light	Nucleotide excision Repair (NER)
Pyrimidine dimers	UV light	Nucleotide excision Repair (NER)
Insertions/Deletions	Replication errors	Mismatch repair (MMR)
Interstrand crosslinks	Ionizing radiation	Non-homologous end joining (NHEJ) / Homologous recombination (HR)
	Antitumor agents	
Intrastrand crosslinks	Ionizing radiation	Non-homologous end joining (NHEJ) / Homologous recombination (HR)
	Antitumor agents	
	UV light	
DNA mismatches	Replication errors	Mismatch repair (MMR)
Single-strand breaks	UV light	Base excision repair (BER)
	ROS	
	Antitumor agents	
	Alkylating agents	

Double-strand breaks	Ionizing radiation	Non-homologous end joining (NHEJ) / Homologous recombination (HR)
	ROS	
	Antitumor agents	

While lesions in one of the DNA strands are repaired by excision-repair mechanisms, double-strand breaks (DSBs) are mainly repaired by the non-homologous end joining (NHEJ) and homologous recombination (HR) pathways (see section 5 of introduction).

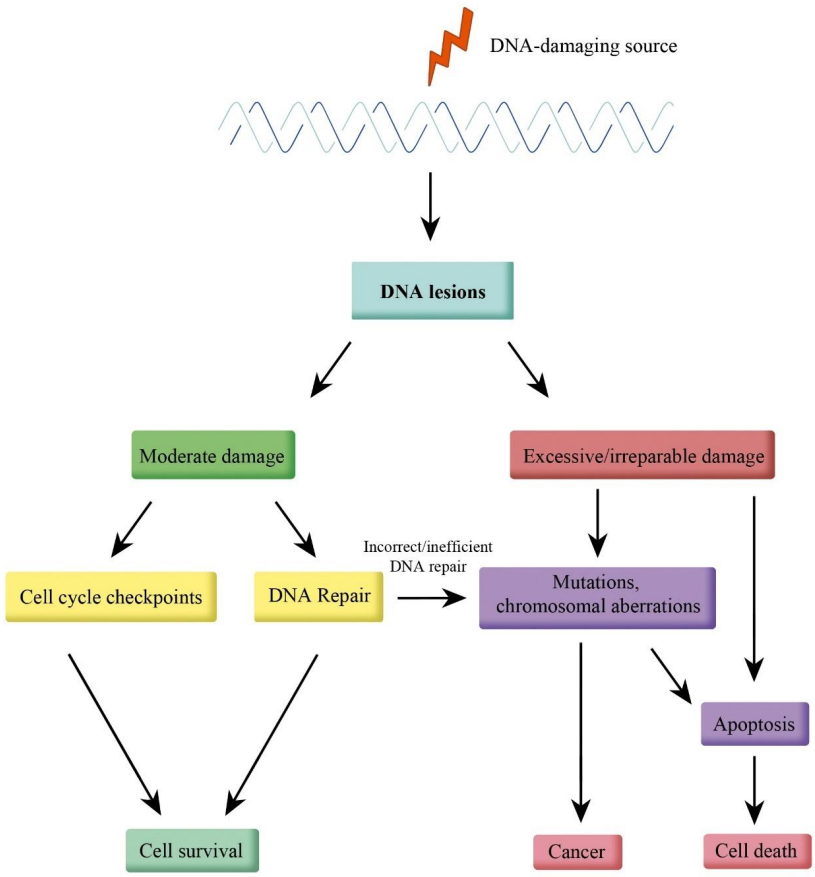

Figure 1. Possible outcomes of DNA damage. The integrity of the human genome is under constant threat of endogenous and exogenous sources of genotoxic stress and subsequently the fate of the cells depends on the extent and severity of the damage suffered. In case of moderate damage, the DDR is activated: cell cycle progression is stopped through checkpoint activation and the different repair pathways repair the DNA lesions, reestablishing genomic stability and leading to cell survival. Upon overwhelming genotoxic stress, cells can activate the apoptotic pathway, so that damaged genetic material is not transmitted to the next generation. However, excessive damage, and sometimes errors in repair processes, can also cause mutations and/or chromosomal aberrations that eventually lead to the onset of cancer.

Upon genotoxic stress, cells stop cell cycle progression through activation of the cell cycle checkpoints (see section 4 of introduction), to prevent the replication and transmission of the damaged DNA to the next generation, and to allow repair by different pathways. Inefficient or inaccurate repair can lead to mutations, cell death and genomic instability, one of the hallmarks of the early stages of cancer (Jackson and Bartek, 2009; Negrini *et al.*, 2010) (fig. 1).

2. Post-translational modifications

After their synthesis in the ribosomes, proteins can be modified by the linkage of different small molecules that are overall known as post-translational modifications (PTMs). PTMs usually occur at amino acid side chains and are mediated by enzymatic activity. These modifications can be critical for protein function, as they might influence their localization, stability, activity and interaction with other molecules. Importantly, PTMs can occur during any step in the "life-cycle" of the protein, and some of them can be reversed or used as a mark for a new modification.

Although, to date, as many as 200 different types of PTMs have been documented (Knorre *et al.*, 2009), in this work we will focus on four of the most common ones: phosphorylation, ubiquitination, acetylation and methylation.

2.1. Histone post-translational modifications

In eukaryotic cells, the genetic material is organized in a complex structure formed by the association of DNA and proteins called chromatin. The fundamental unit of chromatin is the nucleosome, composed by an octamer of the four core histones (H3, H4, H2A and H2B), around which 147 base pairs of DNA are wrapped. Nucleosomes are the most basic form of compaction in the chromatin and are linked between each other through linker histones (H1 in higher eukaryotes).

These histone H1-linked nucleosomes structure can turn coils into a 30 nm diameter helical structure known as the 30 nm fiber or filament, and this filament can itself be folded into more complex structures, that will end up giving rise to the most compact form of chromatin: chromosomes.

Many processes, such as replication, transcription or repair, need DNA in a relaxed state to be functional. Therefore, the genetic material needs to be versatile enough to change to different conformations depending on the needs of the cell. A big part of this versatility is achieved through PTMs in histones. Furthermore, the recruitment of proteins involved in different processes affecting the chromatin also depends on histone modifications.

Histones have a globular structure, except for their N-terminal unstructured "tails". The residues present on these tails are the target of the majority of PTMs suffered by this family of proteins (Kouzarides, 2007). Although eight different types of modifications have been found in histones so far, the focus of this work will be on the above-mentioned: phosphorylation, methylation, acetylation and ubiquitination.

2.2. Phosphorylation

Phosphorylation is the most extended and studied PTM. It involves the addition of a covalently bound phosphate group from ATP to a protein in a reversible process in which the binding is catalyzed by a protein kinase and the removal by a protein phosphatase (Hunter, 2013). In eukaryotes, phosphorylation mostly occurs on three different amino acids: serine (Ser), threonine (Thr) and tyrosine (Tyr), although it has also been reported to happen on lysine (Lys) or histidine (His) residues (Cieśla *et al.*, 2011).

A single protein can be phosphorylated on multiple residues or a single site can be phosphorylated and serve as a priming location for subsequent phosphorylation events. Either way, phosphorylation causes conformational changes, that can affect activity, affinity for other molecules or stability (Loughrey Chen *et al.*, 2002). Moreover, a single protein can be phosphorylated by many kinases, and a single kinase might phosphorylate different proteins, creating a scenario in which one protein can have different activities not only depending on its phosphorylation status, but also on the residue that has been subjected to phosphorylation, and the kinase responsible for it (Singh *et al.*, 2017).

The tails of the four core histones can be phosphorylated, and such phosphorylation occurs on Ser, Thr or Tyr residues (Rossetto *et al.*, 2012). Histone phosphorylation plays a key role in the DDR, the most studied example being phosphorylation of histone H2AX (γ-H2AX), one of the first events to take place after DNA DSBs and the start of a cascade of PTMs that lead to the recruitment of different DNA repair factors (Rogakou *et al.*, 1998) (see section 5.5 of introduction). Histone phosphorylation is also important for other cellular processes, such as transcription regulation, chromatin condensation or mitosis (Rossetto *et al.*, 2012). For instance, phosphorylation of histone H3 on its Ser10 by the Aurora A and B kinases is essential for the chromosomal condensation prior to mitosis (Crosio *et al.*, 2002).

2.3. Methylation

Methylation involves the addition of a methyl group to the side chains of Lys or arginine (Arg) residues. While the ε-amino group of Lys can be mono-, di- or tri-methylated in a SAM-dependent manner, Arg can only be either mono- or di-methylated, using the same methyl group donor as in the case of Lys (Bedford and Richard, 2005; Paik *et al.*, 2007).

Like phosphorylation, Lys methylation is a reversible process, with the addition of the methyl group being catalyzed by Lys-specific methyltransferases (KMTs) and the removal by Lys demethylases (KDMs) (Biggar and Li, 2015). Arg methylation is carried out by Arg methyltransferases (PMRTs), but no enzyme capable of demethylating this amino acid has been identified so far (Yang and Bedford, 2013).

Methylation has been mainly studied in histones, where it serves as an epigenetic mark, regulating transcription or as a mark for the recruitment of proteins involved in different processes (Kouzarides, 2007). For example, dimethylation of histone H4 on Lys20 (H4K20me2) is needed for the recruitment of the NHEJ-promoting factor p53-binding protein 1 (53BP1) to the chromatin after a DNA DSB (Botuyan *et al.*, 2007). However, non-histone proteins methylation has emerged as an interesting field for the last few years, given its implications in a wide variety of cellular functions (Biggar and Li, 2015). For instance, it was recently reported that methylation of the mediator of DNA checkpoint protein 1 (MDC1)

regulates the recruitment of the ataxia telangiectasia mutated (ATM) protein to sites of damage in the early stages of the response to DSBs (Watanabe *et al.*, 2018).

2.3.1. PHF2

PHD Finger Protein 2 (PHF2), also known as KDM7C or JHDM1E, belongs to the Lys demethylase 7 family of the Jumonji C domain superfamily of histone demethylases. It contains both a plant homeodomain (PHD) and a Jumonji C (JmjC) domain in its N-terminal region. The PHD domain recognizes methylated Lys residues and the JmjC domain catalyzes the removal of methyl groups from this amino acid, using Fe^{2+} and 2-oxoglutarate as cofactors (Fortschegger and Shiekhattar, 2011). Functionally, it has been demonstrated that PHF2 forms a complex with the AT-rich interaction domain 5B (ARID5B), a DNA binding protein, and reverses dimethylation of histone H3 at Lys 9 (H3K9me2) after being phosphorylated and subsequently activated by protein kinase A (PKA) (Baba *et al.*, 2011). The dimethylation of Lys 9 of histone H3 is a known mark for transcriptional repression and therefore PHF2 acts as a transcriptional activator by demethylating H3K9me2. In order to perform its role on H3K9me2, PHF2 needs to interact with trimethylated histone 3 in Lys 4 (H3K4me3), a PTM abundant on active promoters (Wen *et al.*, 2010). PHF2 was also shown to demethylate trimethylated histone 4 at Lys 20 (H4K20me3), another transcriptional repression mark (Stender *et al.*, 2013).

However, PHF2 does not always function as a transcriptional activator. For example, it has been observed that PHF2 inhibits rDNA transcription, competing with PHD Finger Protein 8 (PHF8), another member of the same family of histone demethylases, for the binding to H3K4me2/3 on promoters and by recruiting the H3K9me2/3 methyltransferase suppressor of variegation 3-9 homolog 1 (SUV39H1) (Shi *et al.*, 2014).

This histone demethylase has been implicated in a wide variety of processes, such as adipogenesis inflammatory response and cell differentiation (Okuno *et al.*, 2013; Stender *et al.*, 2013; Kim *et al.*, 2014; Lee *et al.*, 2014).

Mutations and alterations in PHF2 levels have been observed in different types of cancers and this protein is known to act as a tumor suppressor with p53 (Sinha *et al.*, 2008; Sun *et al.*, 2013; Lee *et al.*, 2015; C. Lee *et al.*, 2017; J. H. Lee *et al.*, 2017). Furthermore, it was recently reported that PHF2 depletion leads to DNA damage and genomic instability in neural stem cells (Pappa *et al.*, 2019).

2.4. Acetylation

In acetylation, the acetyl group donated by the metabolite acetyl-Coenzyme A is transferred to the ε-amino group of a Lys (Arnesen *et al.*, 2001). The enzymes that catalyze this reaction were first termed as histone acetyltransferases (HATs), since at that point histones were the only known proteins to be acetylated (Allfrey and Mirsky, 1964). Nowadays it is known that non-histone proteins can also be acetylated, and these enzymes have consequently been renamed to Lys acetyltransferases (KATs). In agreement with this, the enzymes responsible for reverting acetylation have been renamed from histone deacetylases (HDACs) to Lys deacetylases (KDACs) (Allis *et al.*, 2007).

Although, like methylation, Lys acetylation has been more studied in histones, where it serves as a transcriptional repression mark, acetylation can also occur in non-histone proteins and regulate many different processes. For example, central DDR protein 53BP1 acetylation

inhibits its recruitment to DSBs, thereby switching repair from NHEJ to HR (Guo *et al.*, 2018), and the acetylation of DNA topoisomerase 2-binding protein 1 (TopBP1) has been shown to be crucial for its role in the G_2/M checkpoint activation (Liu *et al.*, 2016).

2.4.1. HAT1

Histone acetyltransferase 1 (HAT1) was the first histone acetyltransferase described and belongs to the family of type B histone acetyltransferases. These differ from type A histone acetyltransferases in their substrate specificity and cellular localization. While enzymes belonging to the type A family localize in the nucleus and modify only chromatin-bound histones, type B histone acetyltransferases can also be found in the cytoplasm and have the ability to acetylate free, but not nucleosomal histones (Richman *et al.*, 1988; López-Rodas *et al.*, 1991; Brownell and Allis, 1996; Parthun *et al.*, 1996).

HAT1 is a highly conserved protein that has been shown to be essential for different cellular functions in mammalian cells (Nagarajan *et al.*, 2013). HAT1 diacetylates newly synthesized histone H4 on Lys 5 and 12, and displays a minor activity towards Lys 5 of histone H2A (Kleff *et al.*, 1995; Parthun *et al.*, 1996; Tafrova and Tafrov, 2014). Acetylation of histone H4 by this enzyme is highly dependent on the histone chaperone Rb-associated protein 46 (Rbap46), which functions in promoting the interaction between HAT1 and the histone (Parthun *et al.*, 1996). This diacetylation occurs immediately after the synthesis of the histones and is removed once the nucleosome has been assembled (Annunziato and Hansen, 2000). Diacetylated histone H4 associates in the cytoplasm with methylated histone H3, thereby forming dimers that are then transferred to the histone chaperone anti-silencing function protein 1 (Asf1) through the direct association of Asf1 with the HAT1/Rbap46/H3/H4 complex. It was thought that the Asf1/H3/H4 complex then dissociates from HAT1/Rbap46 and is transported into the nucleus with the help of different importins, allowing the incorporation of the H3/H4 dimers into the nascent chromatin (Blackwell *et al.*, 2007; Barman *et al.*, 2008; Alvarez *et al.*, 2011; Campos *et al.*, 2011). Recent evidence, however, suggests that the HAT1/Rbap46 complex localizes to newly replicated DNA with kinetics similar to H4 Lys 5 and 12 acetylation and is evicted from chromatin during the chromatin maturation process (Garcia *et al.*, 2017). In conclusion, HAT1 plays a significant role in chromatin assembly, although the exact role of H4 diacetylation in this process remains to be clarified.

Histone H4 acetylation on Lys 5 and 12, and consequently HAT1, have also been linked to other cellular processes, such as DNA damage repair and centromere dynamics (Nagarajan *et al.*, 2013; Shang *et al.*, 2016).

2.5. Ubiquitination

In ubiquitination, a small molecule of 76 amino acid residues called ubiquitin is linked to the ε-amino group of a Lys residue. Unlike phosphorylation, methylation or acetylation, ubiquitination is not catalyzed by a single enzyme, but rather requires the coordinated action of three enzymes, termed E1, E2 and E3. First, an E1 activates the ubiquitin residue in an energy-dependent reaction. Next, the activated ubiquitin is transferred to the E2 conjugating enzyme. The ubiquitin-bound E2 then binds the E3 ligase, responsible for the recognition of the substrate, to which the ubiquitin is finally transferred, either by the E2 or by the E3, depending on the E2 ligase involved in the reaction (Hochstrasser, 1995; Hershko and Ciechanover, 1998).

Ubiquitination can be reversed by a group of enzymes termed deubiquitinating enzymes (DUBs) or ubiquitin hydrolases (Hershko and Ciechanover, 1998).

Proteins can be mono-, multimono-, di- or polyubiquitinated. After the binding of the first ubiquitin to the substrate, additional ubiquitin residues can be linked through each of the seven Lys residues (Lys6, Lys11, Lys27, Lys229, Lys33, Lys48 and Lys63) present in the ubiquitin molecule and the glycine (Gly) 76 of the already bound ubiquitin residue (Dikic *et al.*, 2009). Furthermore, polyubiquitin chains can be assembled using only one Lys (homotypic polyubiquitination) or through different Lys residues, forming mixed-linkage chains (Komander, 2009). This complexity of ubiquitination makes it a highly versatile system, in which the outcome of the ubiquitinated protein depends on the type of ubiquitination it has been subjected to, and the Lys residues used for the linkage in the case of polyubiquitination. While mono- and diubiquitination lead to functional changes in the protein, such as activation or changes in cellular localization, polyubiquitination can have different effects (Hershko and Ciechanover, 1998). The most studied ubiquitination is the Lys48-linked polyubiquitination, known to mark proteins for degradation by the 26S proteasome (Johnson *et al.*, 1992). Polyubiquitination through Lys29 and Lys11 also target proteins for degradation in this complex (Johnson *et al.*, 1995; Bremm and Komander, 2011). Lys63 polyubiquitin chains, however, have a signaling role, allowing protein re-localization or interaction with other factors, but do not involve proteasomal degradation (Tenno *et al.*, 2004).

2.5.1. USP7

USP7 is a member of the USP (ubiquitin-specific proteases) family of DUBs. It was first reported as an herpesvirus-binding protein that could regulate its lytic replication in cell culture, and was therefore initially termed herpesvirus-associated ubiquitin-specific protease (HAUSP) (Everett *et al.*, 1997).

The most characterized role of this protein is the stabilization of the tumor suppressor p53. It was first observed that USP7 positively regulated p53 levels by preventing its proteasomal degradation through direct deubiquitination in response to DNA damage (Li *et al.*, 2002). However, later reports showed that, in unperturbed cells, USP7 has the opposite effect. Through deubiquitination and consequent stabilization of the E3 ligase murine double minute 2 (Mdm2), USP7 indirectly controls p53 levels, as Mdm2 ubiquitinates p53 and sends it to proteasomal-mediated degradation (Li *et al.*, 2004). Therefore, the effect of USP7 on p53 levels changes depending on the presence or absence of DNA lesions, making it a key regulator of the DDR and a very promising target in cancer therapy (Khusbu and Chen, 2018).

Since its discovery, USP7 has been found to be involved in many cellular processes through its deubiquitinating activity, such as transcriptional regulation, DNA methylation, replication and, important for this work, the ATR-CHK1 pathway of the DDR through deubiquitination of the checkpoint adaptor Claspin (Knaap *et al.*, 2005; Faustrup *et al.*, 2009; Lecona *et al.*, 2016; Yamaguchi *et al.*, 2017).

2.5.2. Ubiquitin-like proteins

Since the discovery of ubiquitin, a new family of small proteins, overall known as ubiquitin-like proteins (Ubls), has been defined (Hochstrasser, 2000). The members of this family have a similar three dimensional structure as the ubiquitin molecule, and are also attached to their

substrate through a C-terminal Lys residue in a three-step process involving an E1, an E2 and an E3 enzyme (Kerscher *et al.*, 2006).

Modification of proteins through Ubl-binding can have different effects, including degradation, activation or re-localization, and these proteins have been linked to different processes in the cell, such as autophagy or transcription (Annemarthe and Ploegh, 2012). Relevant for this work, during the past decades Ubls were found to be an essential part of the DDR, especially SUMO, Nedd8 and ISG15 (Wang *et al.*, 2017). For example, it is known that the E3 SUMO-ligases protein inhibitor of activated STAT 1 and 4 (PIAS1 and PIAS4, respectively) are required for effective DSB signaling and repair (Galanty *et al.*, 2010).

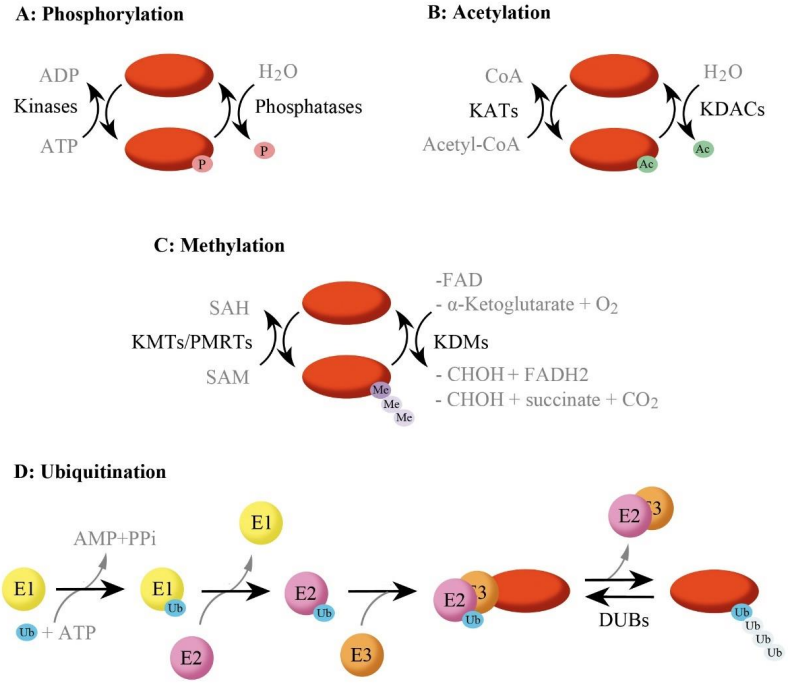

Figure 2. Representation of different PTMs. A) In phosphorylation, kinases transfer a phosphate group from an ATP molecule to the substrate. This phosphate group can be hydrolyzed by phosphatases. B) The transference of an acetyl group from acetyl-CoA to the substrate is termed acetylation. It is catalyzed by KATs and can be reversed by KDACs. C) KMTs and PMRTs can transfer methyl groups from SAM to Lys and Arg, respectively. More methyl groups can be added (up to three on Lys and two in the case of Arg), forming mono-, di- or trimethylated proteins. Lys methylation can be reversed by KDMs, while no enzyme capable of reverting Arg methylation has been reported so far. D) Ubiquitination is a multi-step enzymatic reaction. First, an E1 binds the ubiquitin molecule in an ATP-dependent process. Next, the E1-bound ubiquitin is transferred to an E2-conjugating enzyme. This E2 binds the E3 ligase, responsible for substrate recognition, and the ubiquitin is finally transferred to the substrate, either by the E2 or the E3 depending on the proteins involved. Ubiquitin chains can be further elongated through different Lys residues or hydrolyzed by DUBs.

3. The DDR master kinases: ATM and ATR

The DDR signaling pathways are controlled by two members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family: the protein kinases ataxia telangiectasia and Rad3-related protein, or ATR, and the ataxia telangiectasia mutated protein, or ATM (Linn *et al.*, 2001). Importantly, while ATM is activated by DSBs in a manner that is dependent on the MRN complex, formed by the meiotic recombination 11 (Mre11), Rad50, and Nijmegen breakage syndrome 1 (Nbs1) proteins, the presence of Replication Protein A (RPA)-bound single-strand DNA (ssDNA) stretches triggers ATR activation (Zou and Elledge, 2003; Lee and Paull, 2005; Maréchal and Zou, 2017).

ATM and ATR phosphorylate numerous proteins of the pathway, but the two main downstream targets of ATR and ATM are the effector kinases Checkpoint kinase 1 (CHK1) and Checkpoint kinase 2 (CHK2), respectively (Bartek and Lukas, 2003); these two kinases regulate the activity of key proteins that ultimately coordinate cell cycle progression with DNA repair (Smith *et al.*, 2010).

3.1. The ATR-CHK1 pathway

The presence of ssDNA arising from stalled replication forks, during NER or resected DSBs activates the ATR-CHK1 pathway (MacDougall *et al.*, 2007; Cimprich and Cortez, 2009; Maréchal and Zou, 2017). This ssDNA is rapidly covered by the RPA complex, a heterotrimeric protein with subunits of apparent molecular masses of 70, 32 and 14 kDa, respectively known as RPA70 or RPA1, RPA32 or RPA2, and RPA14 or RPA3 (Wold, 1997). RPA-coated ssDNA allows ATR recruitment through its partner ATR-interacting protein (ATRIP) (Zou and Elledge, 2003). In parallel to this process, the Rad17-Replication factor C (RFC) complex binds damaged chromatin and recruits the Rad9-Rad1-Hus1 (also known as 9-1-1) complex (Melo *et al.*, 2001; Zou *et al.*, 2002). TopBP1 then binds the Rad9 subunit of the 9-1-1 complex, interacts with ATR and subsequently activates ATR through its ATR-activating domain (AAD) thereby allowing the subsequent ATR-mediated phosphorylation events (Kumagai *et al.*, 2006; Delacroix *et al.*, 2007; Lee *et al.*, 2007; Mordes *et al.*, 2008) (fig. 3).

Even though this has been the accepted mechanism for the recruitment of TopBP1 during the last decades, recent data have started to challenge the role that the 9-1-1 complex plays in this process. First, it was observed that disturbing the Rad9-TopBP1 interaction delayed but did not completely abolished the recruitment of TopBP1 to stalled forks (Ohashi *et al.*, 2014). Second, TopBP1 was found to interact with RPA-bound ssDNA independently of Rad9 and, finally, TopBP1 depletion was reported to decrease the recruitment of the 9-1-1 complex to chromatin after treatment with UV or HU in human cells (Gong *et al.*, 2013; Ohashi *et al.*, 2014; Acevedo *et al.*, 2016). These data suggest that TopBP1 binds both Rad9 and RPA-bound ssDNA, and that its recruitment stabilizes the binding of Rad9.

TopBP1 remained as the only known ATR-activating protein until 2016, when various groups reported a new protein able to stimulate ATR kinase activity, termed ETAA1 (Ewing tumor-associated antigen 1). This protein is recruited to ssDNA-bound RPA through direct interaction with the RPA complex and activates ATR in a TopBP1-, Rad17- and 9-1-1 complex-independent manner (Feng *et al.*, 2016; Haahr *et al.*, 2016; Y. Lee *et al.*, 2016; Glick *et al.*, 2017). ETAA1-activated ATR phosphorylates RPA2 on Ser33 and is critical for DNA replication stress response and maintenance of genome integrity, but the data about its role on

CHK1 phosphorylation is somewhat contradictory. Some reports claim that ETAA1 and TopBP1 both contribute to this process, and that depleting either of them only causes a minor effect on CHK1 phosphorylation, while a double knock-down completely abolishes CHK1 phosphorylation (Haahr *et al.*, 2016; Y. Lee *et al.*, 2016). However, ETAA1 knockout cells were also reported to display no defects in CHK1 phosphorylation, indicating that ETAA1-activated ATR only has kinase activity towards RPA, but not towards CHK1 (Glick *et al.*, 2017).

As mentioned before, the primary phosphorylation target of ATR is CHK1, critical for the regulation of the G_2/M checkpoint and DNA repair. CHK1 is activated after phosphorylation by ATR on its residues Ser317 and Ser345 (Liu *et al.*, 2000; Walworth and Bernards, 2016). The mediator protein Claspin is needed for CHK1 phosphorylation by ATR, serving as a "platform" that bridges both proteins (Kumagai *et al.*, 2004). Claspin is phosphorylated in an ATR-dependent manner by a still to be dilucidated kinase, thereby preventing its proteasomal-mediated degradation and facilitating its interaction with CHK1 (Chini and Chen, 2006; Chini *et al.*, 2006) (fig. 3).

The ATR-CHK1 axis has been thoroughly studied and, during the past years, new factors regulating this pathway have been revealed. For example, the Rad9, Hus1, Rad1-interacting nuclear orphan protein 1 **(**RHINO), a novel interactor for the 9-1-1 complex and TopBP1, was found to be important for ATR-mediated phosphorylation of CHK1 (Cotta-ramusino *et al.*, 2011; Lindsey-boltz *et al.*, 2015). The interaction of the Timeless/Tipin complex with RPA was also reported to play a role in this pathway (Yoshizawa-Sugata and Masai, 2007; Kemp *et al.*, 2010).

Once activated, CHK1 controls cell cycle arrest and other cellular processes, such as recombination through phosphorylation of Rad51 and breast cancer type 2 susceptibility protein (BRCA2) or DNA damage-dependent inhibition of transcription through phosphorylation of histone H3 (Sørensen *et al.*, 2005; Bahassi *et al.*, 2008; Huang *et al.*, 2008; Shimada *et al.*, 2008).

3.2. The ATM-CHK2 pathway

In unperturbed cells, ATM exists as an inactive dimer. After detection of a DSB, ATM autophosphorylates *in trans* on Ser1981, Ser367 and Ser1893 residues, leading to monomerization and formation of the active form of the protein (Bakkenist and Kastan, 2003; Kozlov *et al.*, 2006; Pellegrini *et al.*, 2006). Active ATM monomers are then recruited to damaged chromatin via interaction with the MRN complex, allowing phosphorylation of numerous substrates (Lee and Paull, 2004, 2005). ATM targets include, among others, H2AX, MDC1, 53BP1, breast cancer type 1 susceptibility protein (BRCA1) and the kinase CHK2 (Chaturvedi *et al.*, 1999).

ATM phosphorylates CHK2 on the N-terminal Thr68 residue and these phosphorylated CHK2 molecules form active dimers due to intramolecular autophosphorylation (Ahn *et al.*, 2000, 2002) (fig. 4). Like CHK1, CHK2 is involved in the regulation of multiple cellular processes, such as cell cycle arrest, apoptosis, and gene transcription (Lukas *et al.*, 2003).

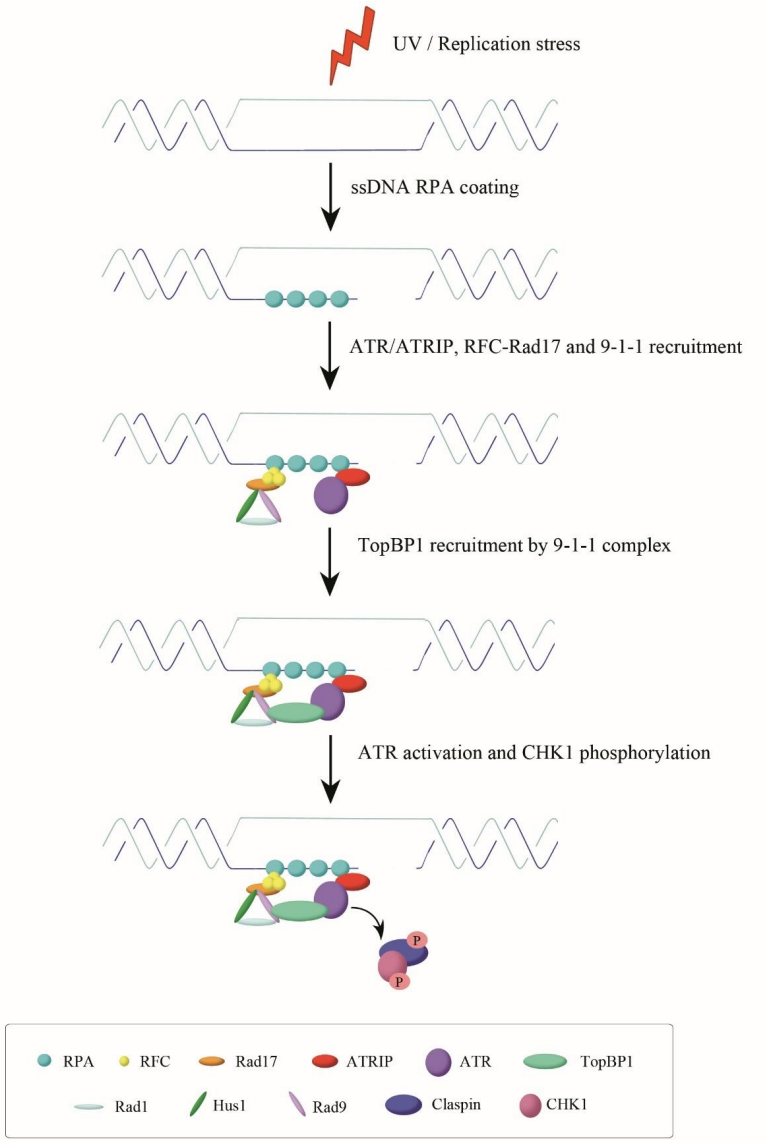

Figure 3. CHK1 activation by ATR. ssDNA is rapidly coated by the RPA complex, which is bound by ATRIP/ATR and the RFC/Rad17 complex. The 9-1-1 complex is then loaded on damaged chromatin through its interaction with the RFC/Rad17 clamp loader. TopBP1 binds the Rad9 subunit of the 9-1-1 complex, allowing interaction with and activation of ATR. Finally, activated ATR phosphorylates CHK1 on residues Ser317 and Ser345 with the help of the mediator protein Claspin, generating an active form of CHK1.

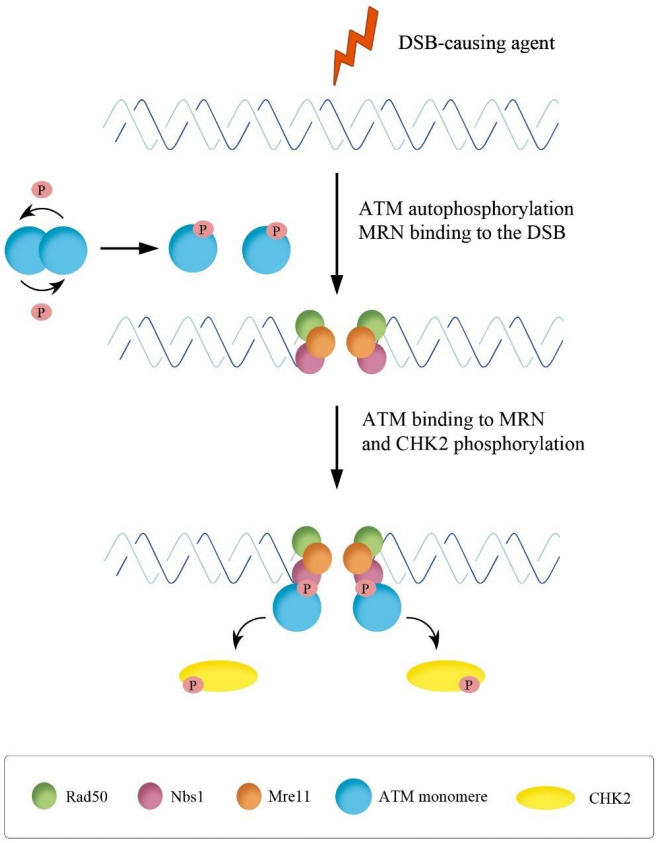

Figure 4. CHK2 activation by ATM. DNA DSBs induce ATM autophosphorylation in several residues of inactive dimers, resulting in the formation of active phosphorylated monomers. In parallel, DSBs ends are bound by the MRN complex, which recruit ATM to damaged chromatin and allows phosphorylation of many targets, including CHK2. ATM-mediated phosphorylation of CHK2 on Thr68 leads to the activation of CHK2.

4. The G_1/S and G_2/M cell cycle checkpoints

As already mentioned in this work, upon DNA damage cells activate cell cycle checkpoints to ensure that lesions are repaired before the DNA is replicated or passed on to the next generation. While the G_1/S checkpoint stops cell cycle progression in G_1, avoiding S phase entry and consequently DNA replication, G_2/M checkpoint activation arrests cells in the G_2 phase of the cell cycle, thereby preventing entry into mitosis (Smith *et al.*, 2010; Warmerdam and Kanaar, 2010; Shaltiel *et al.*, 2015). Cell cycle checkpoints are mainly achieved through prevention of the activity of cyclin-dependent kinases (CDK) by different mechanisms (Kastan and Bartek, 2004; Bartek and Lukas, 2007) (fig. 5).

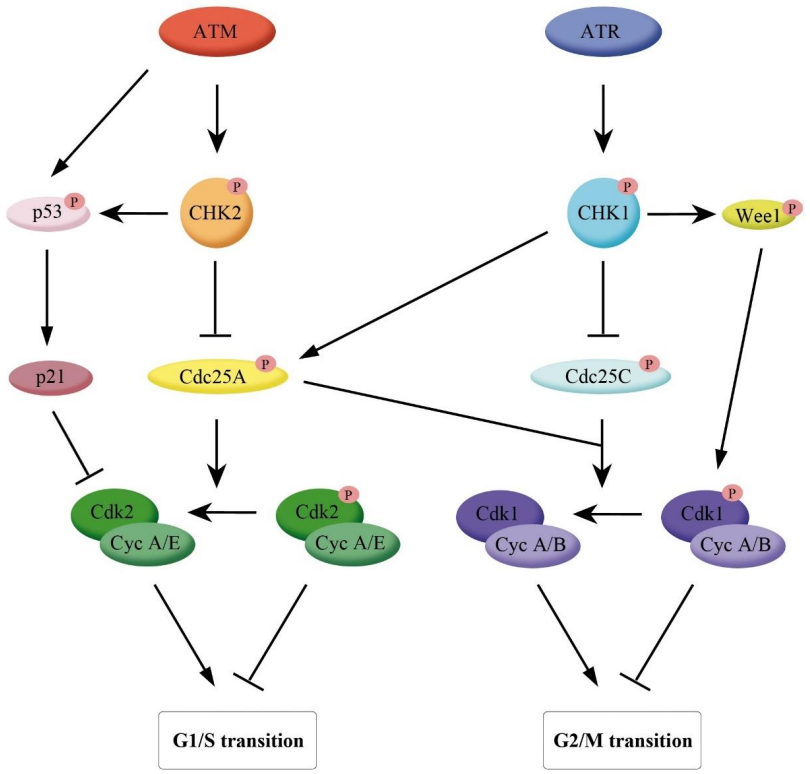

Figure 5. Role of the ATR/CHK1 and ATM/CHK2 pathways in cell cycle checkpoints. ATM-phosphorylated CHK2 and ATR-phosphorylated CHK1 exert their kinase activity on the Cdk2-phosphatase Cdc25A, targeting it for proteasomal degradation. Consequently, the phosphorylated inactive form of Cdk2 accumulates, inhibiting G_1/S transition and arresting cells in G_1. Moreover, ATM and ATM-phosphorylated CHK2 activate p53 via phosphorylation, allowing transcriptional activation of the *P21* gene. p21 inhibits Cdk2 activity, blocking cells in G_1 phase. The G_2/M checkpoint is mainly controlled by the ATR/CHK1 axis: ATR-activated CHK1 phosphorylates Wee1 and Cdc25C, resulting in their activation and inactivation, respectively. Both processes lead to accumulation of phosphorylated (inactive) Cdk1 and the concomitant G_2 arrest. Adapted from (Mimitou PE, 2010).

The G_1 arrest is mediated by the stabilization of the tumor suppressor protein p53 by ATM and CHK2 (Kastan and Bartek, 2004). p53 activates the transcription of the *P21* gene, allowing expression of the CDK inhibitor p21, which silences the G_1/S-promoting Cyclin E/CDK2 kinase and thereby causes a G_1 arrest (Harper *et al.*, 1995). ATM also contributes to the G_1 checkpoint by activating the p38 MAPK family of proteins, which stabilize p21-encoding RNA (Reinhardt *et al.*, 1958; Lafarga *et al.*, 2009). CHK2, and CHK1 in late G_1 phase, promote the G_1 checkpoint arrest by phosphorylating the phosphatase Cell division cycle 25 homolog A (Cdc25A), targeting it for ubiquitination and subsequent proteasomal degradation. This leads to downregulation of Cdc25A protein levels and consequently to accumulation of phosphorylated Cyclin E(A)/CDK2 complexes, unable to promote the G_1/S transition (Donzelli

and Draetta, 2003). Since this response is transcription-independent, it is though that the CHK1/CHK2-Cdc25A checkpoint arrest is implemented rapidly, and sustained until p53 activates the transcription of *P21*, which prolongs the G_1 arrest (Kastan and Bartek, 2004).

The G_2/M checkpoint is mainly governed by the ATR-CHK1 axis. ATR-phosphorylated CHK1 controls cell cycle progression by regulating CDK inhibitory phosphorylation through its targets Cdc25A, Cdc25C and Wee1. The mechanism by which CHK1 inhibits Cdc25A and Cdc25C activities is different: while Cdc25A phosphorylation targets the protein for degradation, CHK1-phosphorylated Cdc25C is sequestered in an inactive form through association with 14-3-3 proteins (Peng *et al.*, 1997; Welcker *et al.*, 2000; Xiao *et al.*, 2003). In contrast, Wee1 is activated by CHK1 phosphorylation (Lee *et al.*, 2001). These three processes lead to the accumulation of phosphorylated (inactive) CDK1 and consequently to a G_2 arrest. Furthermore, p21 is known to participate in the maintenance of the G_2 arrest (Smits *et al.*, 2000).

Although the exact role of ATM and CHK2 in the G_2/M checkpoint still remains controversial, most data suggests that this pathway contributes to the G_2 arrest, but is dispensable for its maintenance (Smith *et al.*, 2010).

4.1. Checkpoint recovery

Once the DNA damage suffered by the cells has been correctly repaired, the checkpoint signaling must be inactivated to allow cell cycle progression to resume, a process known as checkpoint recovery (Chaudhury and Koepp, 2016). The recovery from the G_2 checkpoint arrest, in which this work will focus, is an active process that requires the coordinated activity of different kinases and phosphatases. The main kinase in the G_2 checkpoint recovery is polo-like kinase 1 (Plk1): once the DNA lesions have been repaired, the mediator protein Claspin is phosphorylated by Plk1, allowing the binding of the βTrCP ubiquitin ligase, which leads to proteasomal degradation of Claspin and consequent checkpoint termination (Mailand *et al.*, 2006; Mamely *et al.*, 2006; Peschiaroli *et al.*, 2006). This checkpoint inhibition also requires the activity of the protein phosphatase 1D (PPM1D), which dephosphorylates different DDR-involved proteins, such as p53, γ-H2AX or CHK1 (Lu *et al.*, 2005; Lindqvist *et al.*, 2009; Macůrek *et al.*, 2010). Furthermore, Aurora A (stimulated by its cofactor Bora) phosphorylates Plk1 on Thr210, allowing subsequent phosphorylation and activation of Cdc25C, and consequently CDK1/Cyclin B-promoted cell cycle resumption (Smits and Medema, 2001; van Vugt *et al.*, 2004; Macůrek *et al.*, 2008).

In addition, it is known that restoration of the original structure of the chromatin after repair of the DNA lesions is necessary for checkpoint recovery, but the factors involved in this process still remain largely unknown (Koundrioukoff *et al.*, 2004; Chen and Tyler, 2008).

5. Double-strand break repair

DSBs are the most hazardous lesions DNA can suffer and, as such, their detection and efficient repair are essential for cell survival (Van Gent *et al.*, 2001). There are two types of DSBs: one-ended DSBs arise when an SSB is left unrepaired and is encountered by the replication fork, while two-ended DSBs appear when two SSBs are generated in close proximity in the complementary strands or when both strands of linear dsDNA are broken simultaneously (fig. 6) (Ranjha *et al.*, 2018).

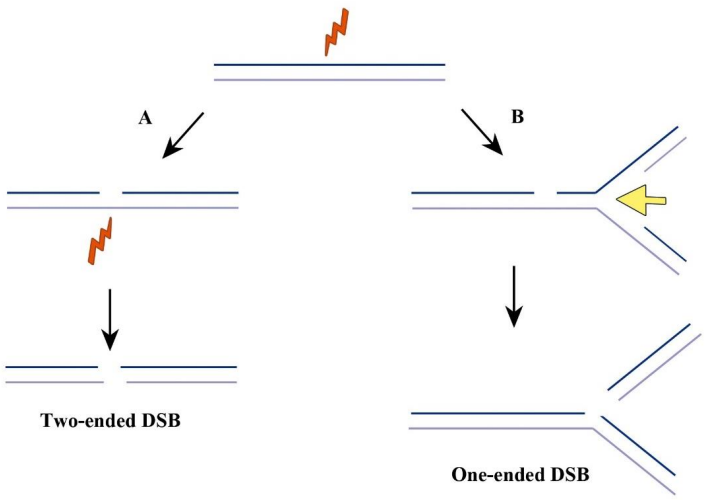

Figure 6. One-ended and two-ended DSBs. When a DNA damaging agent causes a break in one of the DNA strands, a DSB can be generated through two different processes. A new break can be generated in the complementary strand in immediate proximity by an independent event, generating a two-ended DSB (A). This type of DSBs, although less frequently, are also generated by a single event that breaks both strands in an adjacent region (not shown). If DNA replication meets the initial SSB, the replication fork falls apart and a one-ended DSB is generated (B).

There are different exogenous DNA-damaging agents that can lead to the formation of DSBs. IR causes both SSBs and DSBs, either directly or indirectly through the formation of ROS, generating one-ended and two-ended DSBs, respectively (Ward, 1988; Thompson, 2012). Topoisomerase inhibitors are another common source of DSBs. Topoisomerase I relieves the torsional stress associated with DNA replication by inducing SSBs in DNA. The binding of the inhibitor stabilizes the complex formed by the enzyme and the DNA and prevents re-ligation of the break, so that when the replication fork reaches this point, it collapses and a one-ended DSB is generated (Pommier *et al.*, 1998). Topoisomerase II, on the other hand, removes supercoils and tangles from chromosomes by cutting both strands and passing a second DNA duplex through the break. Some topoisomerase II poisons, such as ETP, inhibit the re-ligation of the break, leading to the formation of a two-ended DSB (Caldecott *et al.*, 1990).

DSBs can be repaired by at least four different pathways: the two main pathways are canonical non-homologous end joining (c-NHEJ) and homologous recombination (HR), while Single-Stranded Annealing (SSA) and alternative non-homologous end joining (alt-NHEJ) were considered “back-up” pathways that mainly function when HR or c-NHEJ cannot progress (Iliakis *et al.*, 2004). Nevertheless, it was recently demonstrated that these last two pathways are also functional when NHEJ and HR are operative (Truong *et al.*, 2013).

5.1. Canonical non-homologous end joining

The c-NHEJ repair pathway is mediated by a relatively small number of proteins and consists in the direct re-ligation of both ends of the DNA break. DSBs are rapidly bound by the Ku heterodimer (Ku70 and Ku80) which adopts a preformed ring-shaped structure that completely encircles the DNA duplex (Walker *et al.*, 2001). The Ku-DNA complex then recruits and activates the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), generating what is known as the DNA-PK holoenzyme, which initiates the c-NHEJ pathway (Mahaney *et al.*, 2009). The binding of the DNA-PKcs molecules on opposing DSB ends promotes synapsis or tethering of the two DNA molecules, which results in autophosphorylation of DNA-PKcs in the ABCDE cluster (also known as the Thr2609 cluster), which allows the DNA termini to become accessible (DeFazio *et al.*, 2002; Meek *et al.*, 2008). Like most DNA repair processes, depending on the type and complexity of the DSB break, DNA ends may require modification prior to ligation. DNA termini containing single-stranded overhangs can be made ligatable through either DNA polymerase-mediated fill-in carried out by two members of the X family DNA polymerases, Pol μ and Pol λ or nucleolytic resection, mainly performed by the ARTEMIS and, to less extent, the aprataxin and PNK-like factor (APLF) nucleases in an ATM-dependent manner (Lieber *et al.*, 2008; Mahaney *et al.*, 2009). Excessive end processing is then prevented by DNA-PKcs autophosphorylation on the five-residue PQR cluster (also known as the Ser2056 cluster), which helps protect the DNA ends (Meek *et al.*, 2008). After processing of the DNA, the complex formed by the X-ray repair cross-complementing protein 4 (XRCC4) and ligase 4 (LIG4), stimulated by the XRCC4-like factor (XLF), perform the re-ligation of both DNA ends (Ahnesorg *et al.*, 2006) (fig. 7).

5.2. Alternative non-homologous end joining

The alt-NHEJ pathway (also known as Pol θ-mediated end joining) shares some factors and processes with the HR and c-NHEJ pathways. On one hand, alt-NHEJ involves an initial resection step which requires many (if not all) of the elements that constitute the HR end-resection machinery (Lieber, 2011; Chang *et al.*, 2017). On the other hand, like c-NHEJ, the alt-NHEJ pathway does not need a homologous template, although microhomology that ranges between 2 bp and 20 bp is usually required (Truong *et al.*, 2013; Chang *et al.*, 2017). The type of alt-NHEJ that uses short homologous sequences is termed microhomology-mediated end joining (MM-EJ).

The endonuclease function of MRN, which is stimulated by phosphorylated CtBP-interacting protein (CtIP), seems to initiate alt-NHEJ by processing DNA ends to generate 15–100-nucleotide 3′ overhangs, followed by 3’ to 5’exonucleolytic digestion by MRN (Garcia *et al.*, 2012; Cannavo and Cejka, 2014; Daley *et al.*, 2015). This process is promoted by the Poly-(ADP-ribose) polymerase 1 (PARP1), which also recognizes the DNA lesion (Robert *et al.*, 2009).

It was recently demonstrated that Pol θ has a role in alt-NHEJ (Mateos-Gomez *et al.*, 2016). Pol θ has been shown to stabilize the annealing of two long 3′ ssDNA overhangs, often known as 3′ tails, with as little as 2 bp of homology, extending one 3′ DNA end by using the annealing partner as a template (Wood and Genetics, 2016). This creates a more stable intermediate form of DNA that can be sealed by DNA ligases I or III (Lieber, 2011).

Importantly, Pol θ displaces RPA from ssDNA and also interacts with Rad51, inhibiting Rad51-dependent HR, thereby competing with the HR machinery for resected DNA ends and promoting alt-NHEJ over HR (Ceccaldi *et al.*, 2015; Mateos-Gomez *et al.*, 2016).

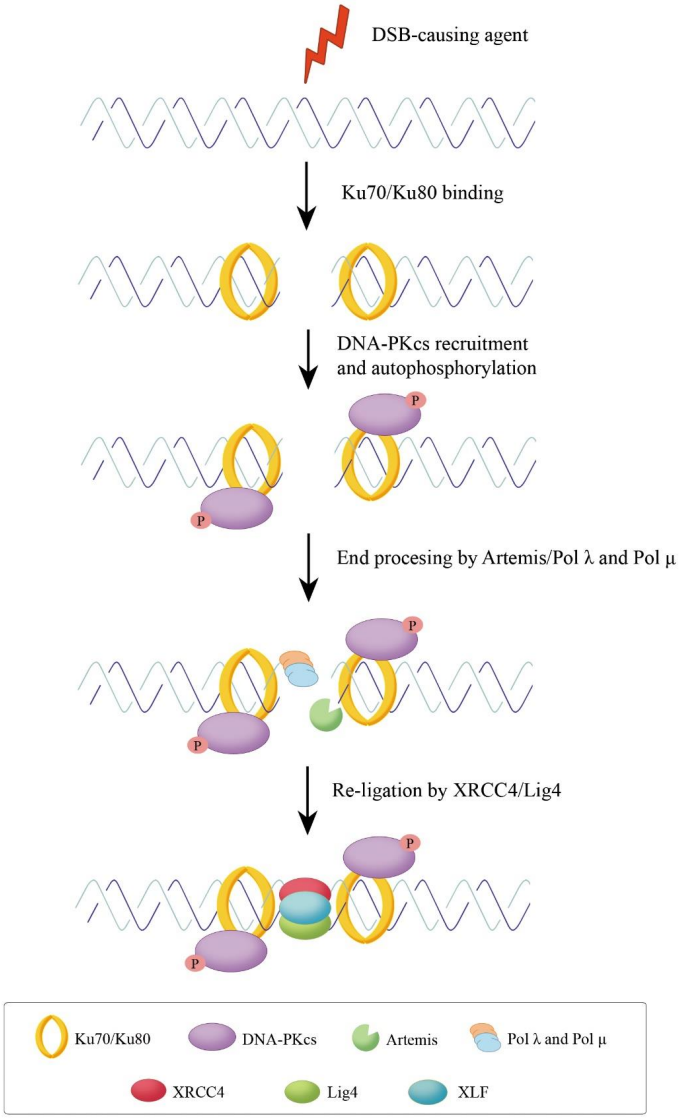

Figure 7. Mechanism of the c-NHEJ pathway. DSBs are rapidly bound by the Ku70/Ku80 dimer, protecting the ends from nuclease activity and serving as a docking point for other proteins in the pathway. The DNA-bound Ku recruits DNA-PKcs which, through autophosphorylation and phosphorylation of other substrates, allows the recruitment of end-processing enzymes, mainly ARTEMIS and the polymerases Pol μ and Pol λ. Once the ends of the DSB are processed, direct re-ligation is performed by the XRCC4/LIG4 complex, in an XLF dependent manner.

5.3. Single-strand annealing

SSA, like HR, requires longer resection of the DSB than the alt-NHEJ pathway (Hartlerode and Scully, 2009). This 5' to 3' resection at both ends is CtIP dependent and exposes single-strand regions with complementary sequences of greater than 25 nucleotides that reside within tandem repeats (Bennardo *et al.*, 2008; Muñoz *et al.*, 2012). Therefore, this model requires the presence of a DSB between homologous repeats. After resection, the flanking repeats are annealed and non-homologous 3′ ssDNA tails are removed, processes that are carried out by Rad52 and the excision repair cross-complementation group 1 (ERCC1) protein, respectively (Bhargava *et al.*, 2017). The final steps in the pathway are gap filling, DNA synthesis and ligation, although the specific polymerases and ligases required for completion of SSA remain poorly understood (Sallmyr and Tomkinson, 2018).

5.4. Homologous recombination

HR starts with the recognition of the DSB by the MRN complex, which promotes the activation of ATM and the preparation of DNA for HR. Rad50 connects the complex to the DNA by interacting with Mre11 and the DSB. Mre11 stabilizes DNA ends, and has endonuclease and exonuclease activities important for resection (Williams *et al.*, 2007). Finally, Nbs1 is the subunit responsible for the interaction and subsequent recruitment of ATM to the DSB (Lee and Paull, 2005; Jazayeri *et al.*, 2008; Kanaar and Wyman, 2008).

After MRN detection, 3' ssDNA is generated by resection of the 5' end of the DSB, directing the repair to either HR, SSA or alt-NHEJ, and inhibiting c-NHEJ (Symington and Gautier, 2011; Kass and Jasin, 2014). This first resection step requires the activity of both CtIP and Mre11, where phosphorylated CtIP acts a cofactor of this member of the MRN complex, stimulating its endonuclease activity (Limbo *et al.*, 2007; Sartori *et al.*, 2007; Anand *et al.*, 2016). These two proteins, together with BRCA1 and the other members of the MRN complex form the BRCA1-C complex (Huen *et al.*, 2009). Importantly, the function of CtIP in resection requires phosphorylation by CDKs (detailed in section 5.6), ubiquitination by BRCA1 and SUMOylation by SUMO E3 ligase chromobox protein homolog 4 (CBX4) (Yu *et al.*, 2006; Soria-Bretones *et al.*, 2017).

It is worth noting that Mre11 only possess 3' to 5' exonuclease activity, while generating 3' overhang requires the opposite (Stracker and Petrini, 2011). In order to achieve this goal, the MRN complex performs an endonucleolytic cleavage of the 5' terminated strand away from the break, followed by a 3' to 5' digestion towards the break, thus generating a 3' overhang (Shibata *et al.*, 2014). It was recently reported that this process is mediated by the exonuclease 3'-5' domain containing 2 (EXD2) protein (Broderick *et al.*, 2016).

The first stretch of ssDNA generated by CtIP and MRN activity is rapidly coated by the RPA complex, that protects ssDNA from nuclease activity and prevents secondary structure formation (De Laat *et al.*, 1998). Long-range resection is then carried out, where the length of ssDNA stretches is extended through 5' to 3' nuclease activity (Nimonkar *et al.*, 2011). The enzymes responsible for this second resection step are exonuclease 1 (Exo1) and DNA replication helicase/nuclease 2 (DNA2), both depending on the helicase activity of the Bloom syndrome protein (BLM) (Genschel *et al.*, 2008; Zhu *et al.*, 2009; Nimonkar *et al.*, 2011) (fig. 8).

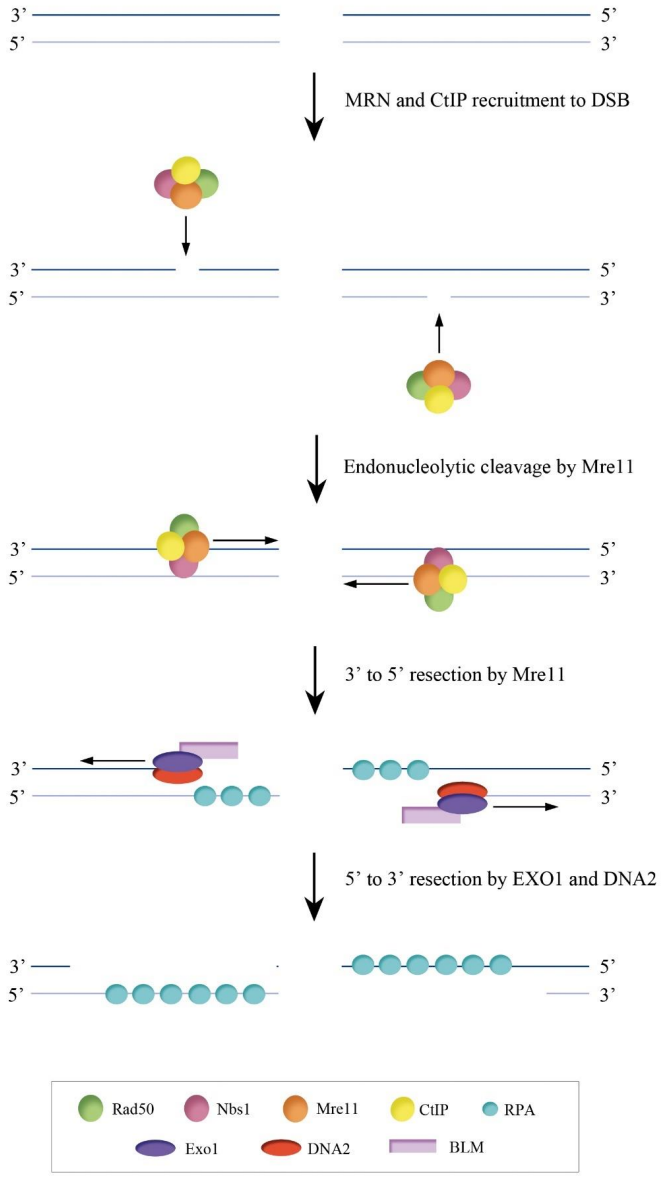

Figure 8. DSB resection in the HR pathway. DSB ends are bound by the MRN complex, which recruits CtIP. CtIP-stimulated Mre11 then cleaves the 5' terminated strand away from the DSB end and performs a 3' to 5' resection, generating a first stretch of ssDNA that is rapidly coated by the RPA complex. Longer stretches of ssDNA are then generated by the 5' to 3' exonuclease activity of Exo1 and DNA2, in collaboration with the BLM helicase, and are also bound by the RPA complex.

ssDNA-bound RPA is known to undergo several PTMs that are important for HR progress and serve as a signal for other DDR pathways (Maréchal and Zou, 2015). Especially relevant for this work is the phosphorylation of the N-terminus of the RPA32 subunit: high levels of ssDNA-bound RPA trigger what is known as RPA32 hyperphosphorylation (phosphorylation at five or more sites), mostly on Ser 4, 8 and 33 (Maréchal and Zou, 2015). During resection, RPA32 Ser33 is progressively phosphorylated by ATR, in a process that requires Nbs1 and TopBP1, but not Rad17 (Morishima *et al.*, 2007; Tahara and Zou, 2013). This event primes RPA32 for phosphorylation on Ser4/Ser8 by DNA-PKcs (Ashley *et al.*, 2014). Phosphorylation of RPA32 is needed for HR progression and, importantly, for checkpoint activation by CHK1 (Ashley *et al.*, 2014).

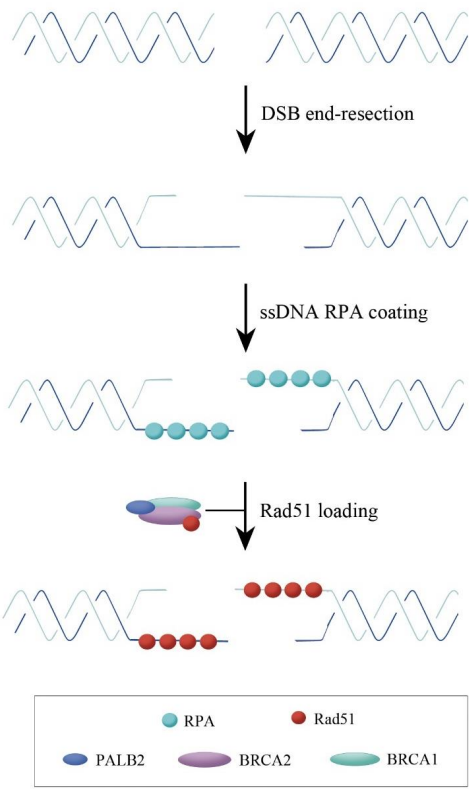

Figure 9. Rad51 nucleofilament formation in the HR pathway. Resection by MRN/CtIP first and EXO2/DNA2 (see fig. 7), generates long stretches of RPA-coated ssDNA. The BRCC complex, formed by PALB2, BRCA2 and BRCA1 then catalyzes the removal of RPA and the replacement by Rad51, generating the nucleofilament that performs the strand invasion, the first step in recombination.

In the next step of HR, Rad51 replaces RPA on the ssDNA, forming the nucleoprotein filament that invades the sister chromatid and allows the recombinational events required for repair of the break (Woese *et al.*, 1998). Rad51, however, has lower affinity for ssDNA than RPA, so several mediator proteins are required to catalyze the removal of the RPA complex and the

subsequent Rad51 loading (Papers *et al.*, 2003). This process is mainly catalyzed by the BRCC complex, formed by BRCA1, BRCA2, partner and localizer of BRCA2 (PALB2) and Rad51 itself. BRCA2 is responsible for the RPA-Rad51 turnover while PALB2 promotes the recruitment of BRCA2 to damaged sites through its interaction with BRCA1 (Sy *et al.*, 2009; Buisson *et al.*, 2010; Etchin *et al.*, 2010; Jensen *et al.*, 2010; Liu *et al.*, 2010; Zhang *et al.*, 2011) (fig. 9). It was recently discovered that the accumulation of the BRCA2-PALB2 complex at DNA lesions is also dependent on the E3 ubiquitin ligase RNF168, which acts redundantly with BRCA1 in the loading of PALB2 (Zong *et al.*, 2019). Once bound to the ssDNA, Rad51 catalyzes strand exchange, a process in which the ssDNA invades homologous duplex DNA forming a displacement loop (D-loop). (West, 2003). The formed D-loop has different fates, but there are two main pathways for solving this structure (San Filippo *et al.*, 2008). In the synthesis-dependent strand annealing (SDSA) pathway, the 3' end of the DSB is extended by DNA repair synthesis and the newly synthesized strand is displaced and annealed with the second resected DSB end (Nassif *et al.*, 1994; Ferguson and Holloman, 1996). Alternatively, the second DSB can be captured in the D-loop to form an intermediate that contains two Holliday junctions, accompanied by gap-filling DNA synthesis and ligation. These Holliday junctions can be solved in different ways, leading to crossover or non-crossover products depending on the endonucleases involved (San Filippo *et al.*, 2008) (fig. 10).

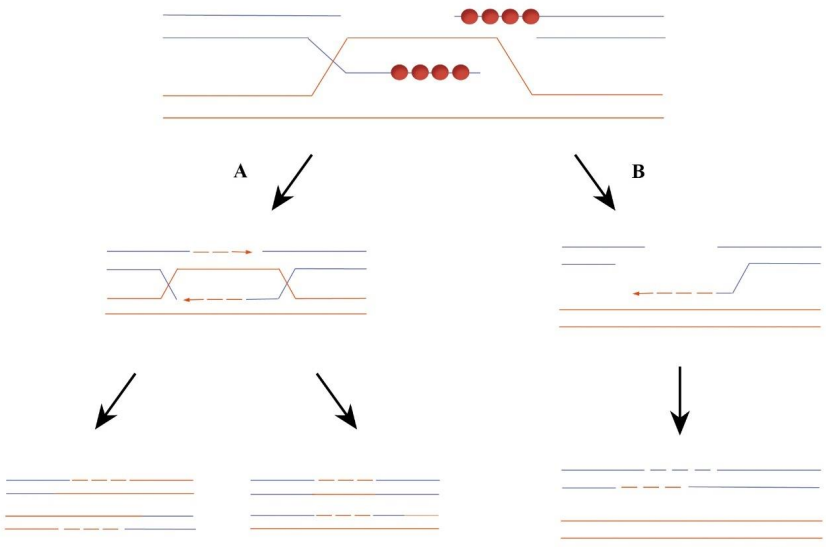

Figure 10. Strand invasion and D-loop resolution in the HR pathway. Rad51 catalyzes the strand exchange between the ssDNA generated from resection with homologous duplex DNA from the sister chromatid, forming a D-loop. This D-loop is resolved by two different mechanisms: A) If the other DSB end is captured, an intermediate containing two structures known as Holliday junctions is formed. These are solved in different ways, that can produce crossover or non-crossover products. B) In SDSA, the 3' end of the DSB is elongated by polymerase activity using the information from the sister chromatid. This newly synthesized DNA is then displaced and used to extend the other resected end of the DSB.

5.5. DSB checkpoint signaling

Concomitant to the above-mentioned processes, the chromatin and DDR proteins at and near the DSB are subjected to multiple PTMs that play a central role in the recruitment of repair complexes. This chain of events starts with the phosphorylation of the histone H2A variant H2AX on C-terminal Ser139 by ATM and other PIKKs. Phospho-H2AX (γ-H2AX) formation occurs within minutes after damage, and extends for up to a megabase from the site of the break, providing a platform for subsequent DDR protein recruitment at DSBs (Rogakou *et al.*, 1998). The γ-H2AX phosphoepitope is recognized by the BRCA1 C-terminal (BRCT) domain of the mediator protein MDC1 (Stucki *et al.*, 2005), which amplifies the signal by creating a positive feedback through interaction with both ATM and γ-H2AX and facilitates the phosphorylation of other H2AX molecules by ATM (Lou *et al.*, 2006).

MDC1 itself is also phosphorylated by ATM, and this process triggers the recruitment of the E3 ubiquitin ligase ring finger protein 8 (RNF8) via phospho-dependent interactions between the RNF8 forkhead-associated (FHA) domain and the ATM-phosphorylated region of MDC1 (Huen *et al.*, 2007; Kolas *et al.*, 2007; Mailand *et al.*, 2007). At first it was thought that RNF8, together with its E2 ubiquitin-conjugating enzyme UBC13, mediated K63-linked polyubiquitination of histones H2A and H2AX surrounding the lesion (Hofmann and Pickart, 1999; Huen *et al.*, 2007; Mailand *et al.*, 2007). This would serve as a dock for the recruitment of another E3 ligase, ring finger protein 168 (RNF168) (Doil *et al.*, 2009; Stewart *et al.*, 2009). However, it was recently demonstrated that RNF8's main target is the linker histone H1 (Thorslund *et al.*, 2015). RNF168 interacts with the H1-linked polyubiquitin chains and amplifies the DNA damage signal by ubiquitinating H2A at Lys13/Lys15 and possibly other proteins to trigger recruitment of DSB repair factors, such as 53BP1 and the BRCA1-Abraxas-receptor-associated protein 80 (RAP80)-MERIT40 (or BRCA1-A) complex (Wang and Elledge, 2007; Doil *et al.*, 2009; Stewart *et al.*, 2009; Fradet-Turcotte *et al.*, 2013; Thorslund *et al.*, 2015) (fig. 11).

5.6. Pathway choice in DSB repair

There are multiple ways through which DSB repair is channeled to the different pathways, in which cell cycle phase is the most critical determinant (Rothkamm *et al.*, 2003). Since HR uses the sister chromatid as a template, it is only functional in S and G_2 phases, while c-NHEJ can be active throughout the cell cycle (Liu *et al.*, 2008). The balance between c-NHEJ and HR in the different cell cycle phases is mainly achieved through the control of resection. As mentioned before, c-NHEJ does not require resection of the DSB, while resection is the triggering process for HR-mediated repair. DSB processing is tightly regulated in a cell cycle dependent manner. In G_1 phase, DSB ends are protected and repair is committed to NHEJ, whereas in S and G_2 phases 5' resection is activated, allowing repair via HR (Symington and Gautier, 2011). In addition to the two major DSB repair pathways, SSA and alt-NHEJ require little resection and homology, functioning even in G_1, where a sister chromatid is absent (Symington and Gautier, 2011).

The antagonistic role of 53BP1 and BRCA1 in the control of resection and thereby the balance between c-NHEJ and HR has been greatly studied over the years. It is widely accepted that 53BP1 promotes c-NHEJ repair by blocking CtIP-dependent resection of DSBs, and that this resection barrier is alleviated by BRCA1 in S/G_2 cells (Bunting *et al.*, 2011) (fig. 12). The resection-inhibiting activity of 53BP1 requires the interaction with rap1-interacting factor 1

homolog (RIF1) through its ATM-phosphorylated N-terminus (Borja-Cacho and Matthews, 2008; Chapman *et al.*, 2013; Daley and Sung, 2013; Escribano-Díaz, CristinaXing *et al.*, 2013). RIF1 promotes the recruitment of the mitotic arrest deficient 2-like protein 2 (MAD2L2) or Rev7, which inhibits 5' DNA end resection by generating blunt ends that are not a suitable target for the nucleases involved in HR (Barazas *et al.*, 2015; Boersma *et al.*, 2015; Sale, 2015). To inhibit HR, REV7 was recently discovered to require other members of the so-called Shieldin complex. This complex, formed by the SHLD1, SHLD2, SHLD3 and REV7 proteins, binds ssDNA through its SHLD2 subunit, blocking access to endonucleases and inhibiting HR-mediated repair (Findlay *et al.*, 2018; Noordermeer *et al.*, 2018). Furthermore, Shieldin interacts with 53BP1-RIF1 and recruits the Ctc1-Stn1-Ten1 (CST) complex, a complex similar to RPA that functions as an accessory factor of polymerase-α, allowing extension of the 3' overhangs generated by the initial resection and switching repair to c-NHEJ (Mirman *et al.*, 2018).

Another 53BP1 partner, Pax transactivation-domain interacting protein (PTIP), is also important in inhibiting DSB resection. PTIP promotes the recruitment of the c-NHEJ nuclease ARTEMIS which, like REV7, trims the DSB ends, preventing resection (Wang *et al.*, 2014). Loss of either RIF1 or PTIP partially alleviates the resection barrier and switches repair to HR (Escribano-Diaz and Durocher, 2013).

While it had always been widely accepted that the inhibiting activity that 53BP1 exerts on HR is exclusively due to its ability to prevent DNA end resection, recent work from Callen *et al*. demonstrated that 53BP1 also inhibits the recruitment of RNF168 to resected DNA in a Shieldin-dependent manner, leading to reduced PALB2 recruitment and thereby inhibiting Rad51 loading and HR-repair completion (Callen *et al.*, 2019).

In the S and G_2 phases of the cell cycle, BRCA1 promotes HR mainly by excluding RIF1 from the sites of damage (Chapman *et al.*, 2012; Escribano-Díaz, CristinaXing *et al.*, 2013). RIF1 release causes 53BP1 to relocate to foci periphery, vacating the central core in a process called foci enlargement and allowing RPA focus formation, a marker for resection (Kakarougkas *et al.*, 2013). BRCA1 promotes protein phosphatase 4 (PP4C)-dependent 53BP1 dephosphorylation, as well as ubiquitin-like-containing PHD and RING finger domains 1 (UHRF1)-mediated Lys63 linked polyubiquitination of RIF1. Both processes disturb the 53BP1-RIF1 interaction, therefore inhibiting c-NHEJ and promoting HR (Zhang *et al.*, 2016; Isono *et al.*, 2017).

It is important to note that BRCA1 not always functions to favor DSB repair by HR. BRCA1 interacts with many proteins and forms different complexes, such as the BRCC and BRCA1-A complexes (Huen *et al.*, 2009). While the BRCC complex has a well-studied role in HR repair, the BRCA1-A complex formation antagonizes this pathway either by restricting resection or by sequestering BRCA1 away from HR sites through RAP80 binding to RNF8/RNF168-ubiquitinated chromatin (Wang and Elledge, 2007; Coleman and Greenberg, 2011; B. Wang *et al.*, 2013; Kakarougkas *et al.*, 2013). Therefore, the balance between different BRCA1-containing complexes plays an important role in DSB repair pathway choice. Furthermore, it has been observed that BRCA1-depleted cells are deficient in both HR and c-NHEJ (Hu *et al.*, 2014; Fouquin *et al.*, 2017), suggesting that this protein might contribute to either pathway depending on the context, although the role that BRCA1 plays in c-NHEJ is still unknown.

The histone marks present in the area surrounding the DSB contribute differently to either 53BP1 or BRCA1 recruitment, and therefore to pathway choice. The accumulation of 53BP1 at damaged sites requires the presence of methylated histone H4 (H4K20me/me2, which is dependent on the RNF8/RNF168-mediated degradation of the histone demethylase KDM4A and is counteracted by the acetylation of the Lys15 of histone H4 (H4K15Ac) by the 60 kDa Tat-interactive protein (TIP60) (Botuyan *et al.*, 2007; Mallette *et al.*, 2012; Tang *et al.*, 2013; Jacquet *et al.*, 2016). Interestingly, the presence of H4K20me2 constitutes a mark of pre-replicative chromatin, signaling that a sister chromatid is absent and that therefore repair cannot be ensued through HR (Simonetta *et al.*, 2018). However, newly synthetized DNA carries unmethylated histone H4, which prevents the recruitment of 53BP1 and promotes the accumulation of BRCA1 through the binding of its partner BRCA1-associated RING domain protein 1 (BARD1) to H4K20me0, thus limiting HR-mediated repair to S and G_2 phases of the cell cycle (Nakamura *et al.*, 2019). Importantly for this work, the methylation status of other histones, such as H3K36me3 or H3K9me2 have also been reported to stimulate HR (Thangue *et al.*, 2013; Wu *et al.*, 2015)

As mentioned, the Ku70-Ku80 dimer binding to the DSB is one of the first events to take place in DSB repair by c-NHEJ, which protects the DNA from exonucleolytic activity (Liang and Jasin, 1996). Given that the Ku dimer has a very high affinity for DNA ends and binds most DSBs, for HR to succeed this resection barrier must be alleviated. This is achieved through different PTMs, that ultimately lead to Ku proteasomal degradation (Feng and Chen, 2012; Ismail *et al.*, 2015; Sites *et al.*, 2015; K. Lee *et al.*, 2016). Furthermore, recent work suggests that the nuclease activity of Mre11, together with CtIP, is also required for the removal of the Ku dimer from DSB ends (Limbo *et al.*, 2007; Anand *et al.*, 2016; Coates *et al.*, 2016).

CtIP phosphorylation is another important factor in the regulation of DNA end resection. For HR, CDK2-mediated phosphorylation of CtIP is needed, which only takes place in S and G_2 phases. Phosphorylation of Thr847 is needed for CtIP localization to sites of damage and for the activation of Mre11 nuclease activity (Huertas and Jackson, 2009), while phosphorylation of Ser327 promotes the CtIP-BRCA1 interaction (Yu and Chen, 2004; Huertas and Jackson, 2009; Yun and Hiom, 2010). Importantly, CtIP was recently demonstrated to be phosphorylated by polo-like kinase 3 (Plk3) on Ser327 in G_1. This phosphorylation leads to DSB resection that is independent on Mre11 endonuclease activity. Consequently, the DNA-PK holoenzyme is not removed from DNA ends and the DSB is repaired through a MM-EJ pathway that uses the same machinery as c-NHEJ (Biehs *et al.*, 2017). Therefore, cell cycle-regulated CtIP phosphorylation inhibits c-NHEJ by promoting resection and targeting repair to MM-EJ and HR in the G_1 and G_2 phases of the cell cycle, respectively.

The DNA structure of the DSB highly influences the pathway used for repair. Importantly, one-ended DSBs lack another DNA end for ligation, so c-NHEJ pathway is inhibited and repair switches to HR (Arnaudeau *et al.*, 2001). In the case of two-ended DSBs that occur in G_2 phase, it is estimated that 70% are repaired through c-NHEJ, while HR is used to repair the remaining 30% (Beucher *et al.*, 2009). Different factors, such as chromatin structure and end-complexity determine this choice (Shibata *et al.*, 2011).

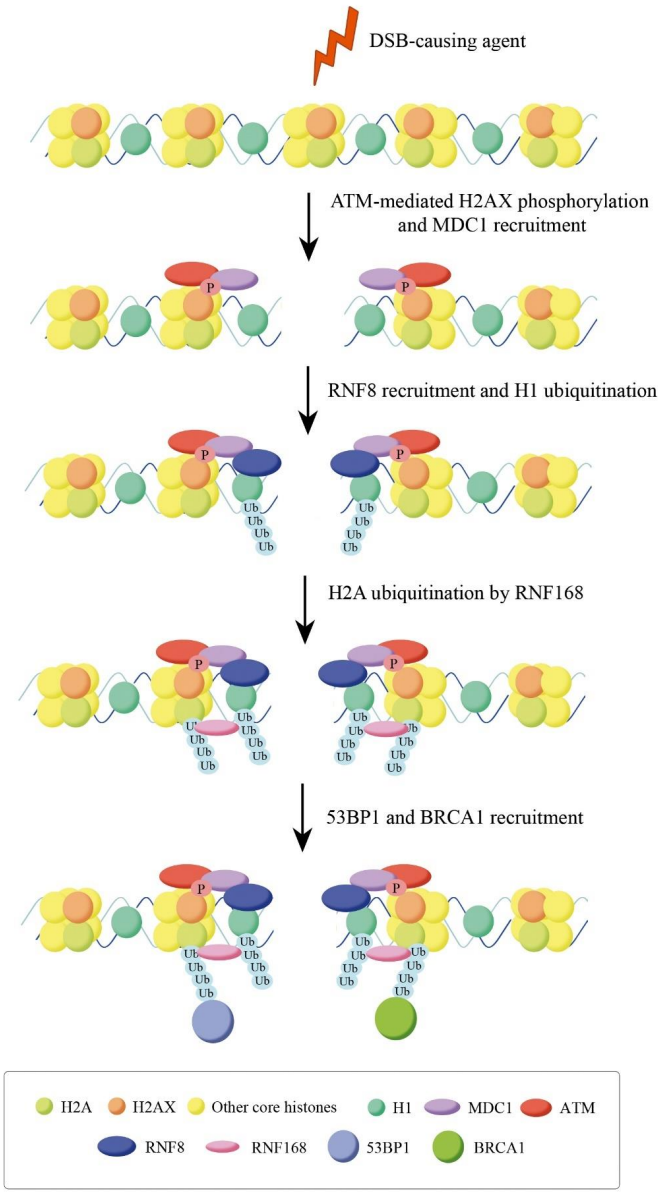

Figure 11. Signaling at DSBs. Shortly after DSB induction, H2AX phosphorylation (γ-H2AX) by ATM takes place. MDC1 is then recruited through interaction with γ-H2AX, and phosphorylated by ATM, triggering RNF8 accumulation. RNF8 binds Lys63-linked polyubiquitin chains to histone H1, which serve as a dock for RNF168 recruitment to damage sites. Finally, RNF168 ubiquitinates histone H2A in multiple Lys, promoting the accumulation of different repair proteins, such as 53BP1 and BRCA1.

Finally, members of the PARP family of proteins were identified to be important players in the pathway choice between HR and c-NHEJ. For example, PARP2 limits 53BP1 accumulation at damaged sites, promoting CtIP-dependent resection and therefore HR repair (Fouquin *et al.*, 2017), while PARP1 was recently identified as a negative regulator of DSB resection (Caron *et al.*, 2019).

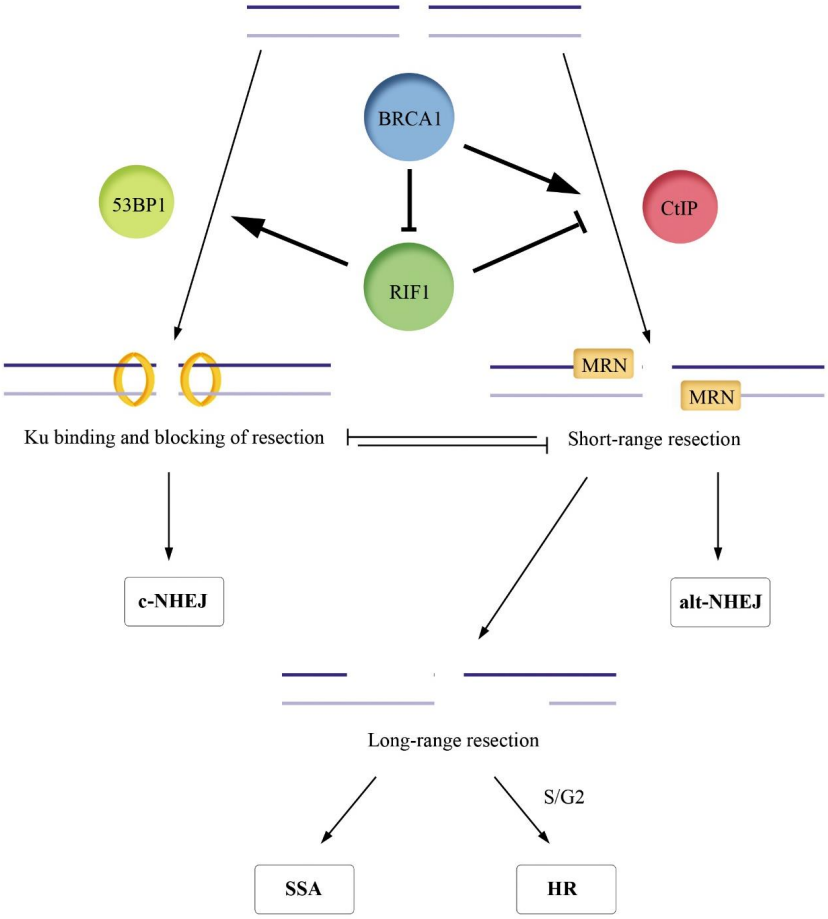

Figure 12. Factors in pathway choice for DSB repair. The competition between BRCA1/CtIP and 53BP1/RIF1 largely determines the DSB repair pathway used. The 53BP1/RIF1 complex blocks resection, promoting Ku binding to the DSB and consequently c-NHEJ-mediated repair. In contrast, BRCA1 relieves the barrier posed by the 53BP1/RIF1 complex, removing the complex from the DSB and allowing CtIP-stimulated resection by the MRN complex. Depending on the length of the resected DNA, different pathways are used: while alt-NHEJ only requires short stretches of ssDNA, whereas both SSA and HR need longer tracts of resected DNA. Furthermore, HR is only viable in S/G_2 phases of the cell cycle, when a sister chromatid is available as a template.

6. Cancer and the DNA damage response

Alterations in chromosome number and/or structure are one of the most prevalent characteristics of cancer cells (Hanahan and Weinberg, 2011). This phenotype, overall known as genomic instability, is usually caused by the combined effect of DNA damage, defects in determined repair pathways and inefficient cell cycle checkpoints (Lord and Ashworth, 2012).

Elevated levels of DNA damage, generated by either exogenous (exposure to carcinogens) or endogenous sources, such as accumulation of stochastic lesions, telomeric shortening or replication stress, and additionally, an active DDR are frequently observed in the early stages of oncogenesis (Tort *et al.*, 2005; Bartek *et al.*, 2007; Luo *et al.*, 2009). While untransformed cells that are not capable of repairing DNA damage normally undergo senescence or apoptosis, thus ensuring that such damage is not passed on to the next generation, tumor cells often carry mutations in DDR genes, such as *P53* or *ATM*, that allow them to bypass these processes and promote cellular proliferation even in the presence of damage (Ciccia and Elledge, 2010). To overcome this problem, new treatments combining chemotherapies that only affect proliferating cells and induction of p53 through either Mdm2 inhibition or low doses of genotoxic agents have been evolved (Smits and Gillespie, 2014). The reasoning behind this treatment is that normal cells stop cell cycle progression due to p53 activation and are unaffected by the chemotherapy, while tumor cells still proliferate, as they carry a mutated form of p53, and therefore die as a consequence of the chemotherapy.

Although the exact DDR defect behind most cancers is not known, there are some examples in which a dysfunction in a determined repair pathway leads to a specific neoplastic phenotype (Spry *et al.*, 2007). For example, mutations in genes encoding proteins involved in the MMR pathway, such as *MLH1*, *MSH2* or *MSH6*, cause hereditary nonpolyposis colorectal cancer (Vasen, 2005). In these cases, the DDR defects present in tumor cells are exploited in cancer treatment though the use of different chemotherapies and radiotherapies Especially well-documented is the association between HR-defects and breast and ovarian cancers. Mutations in the *BRCA1* and *BRCA2* genes are responsible of a high percentage of the familial types of these cancers and, consequently, these tumors are highly sensitive to treatment with PARP inhibitors, like Olaparib or Rucaparib (Farmer *et al.*, 2005; Kaklamanis *et al.*, 2005; Fackenthal and Olopade, 2007). The members of the PARP family are essential for SSB repair, a type of BER and therefore, upon treatment with PARP inhibitors, cells accumulate unrepaired SSBs. A replication fork collapses after reaching an SSB, which generates a one-ended DSB that, in normal cells, are mainly repaired by HR. However, mutations in *BRCA1* or *BRCA2* hinder HR-mediated repair, leading to an increased cell death (fig. 13) (Lord and Ashworth, 2008). Tumors that carry mutations in these two genes usually have low 53BP1 levels, since the partial restoration of the HR-defect by the absence of this protein increases the survivability of cancer cells (Bouwman *et al.*, 2010).

Important for this work, several therapies targeting different PTMs are nowadays successfully used in the treatment of different cancers, such as the proteasome inhibitor Bortezomib in the case of multiple myeloma, or the HDAC inhibitor Vorinostat for certain types of lymphomas (Bross *et al.*, 2004; Mann *et al.*, 2007).

Given the high correlation between DNA damage and cancer, gaining insight into the different DDR pathways is crucial for the development of new treatment against this devastating disease.

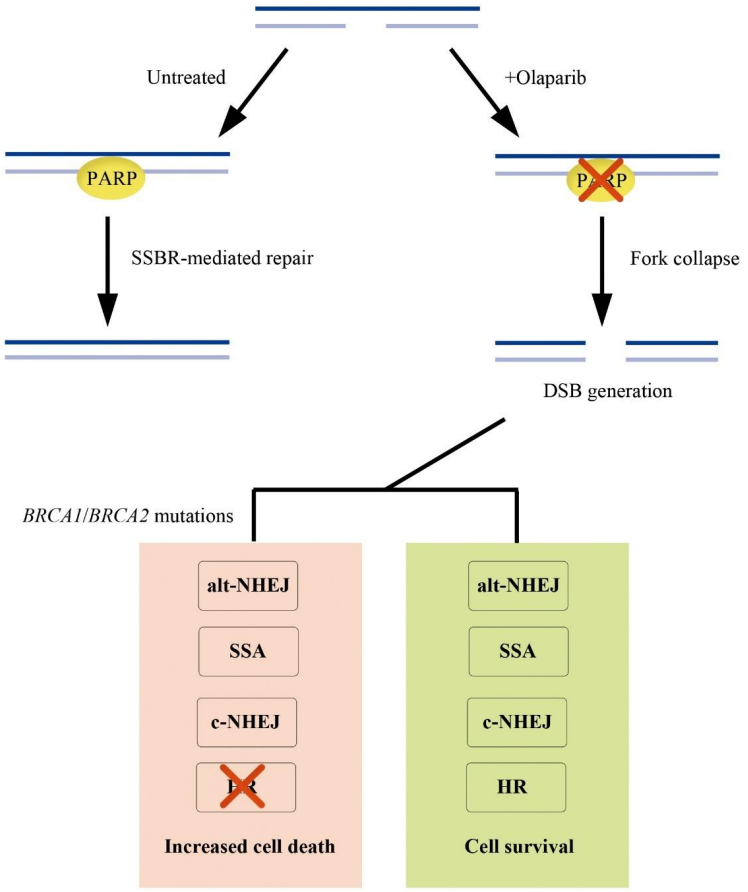

Figure 13. PARP inhibition as cancer therapy. Untreated cells repair SSBs though a PARP-dependent mechanism known as SSB repair. Olaparib and other PARP inhibitors prevent repair though this pathway, and the replication fork eventually stalls at the SSB and collapses, resulting in DSB. Tumors carrying BRCA1 and/or BRCA2 mutations cannot repair these breaks through HR-mediated repair, leading to a decreased survival compared to normal cells.

OBJECTIVES

PTMs play essential roles in gene regulation, cellular function, tissue development and metabolism, in which they can be critical for protein function, by influencing their localization, stability, activity and interaction with other molecules. In particular, the cellular responses to DNA damage involves conserved mechanisms of recruitment, precise control of protein levels and activation of the numerous proteins involved, events for which a multitude of PTMs are required. A correct DDR is essential in maintaining genome integrity and abnormalities in this process can lead to developmental defects, genetic diseases, premature aging and cancer. This is underscored by the observation of signs of replication stress and an activated DDR during the early stages of tumorigenesis. On the other hand, DNA damaging agents are widely used in cancer treatments, due to their toxicity for proliferating cells. Studying the mechanistic details of genome stability, and the role of PTMs in this response, is therefore crucial for the development of therapies against cancer. Moreover, all these modifications are carried out by enzymes and can therefore potentially be inhibited. As a result, regulating these modifications could have a therapeutic impact.

Our general objective is to identify novel enzymes regulating PTMs that are involved in maintaining genome integrity and characterize their molecular action in the DDR. This knowledge is used to understand the process of tumorigeneses and might help to improve cancer treatments.

Specific objectives:

- Study PTMs controlling different levels of the ATR-Chk1 pathway, by regulating CHK1 and TopBP1, crucial proteins in this pathway (chapters I and II)
- Identify novel regulators of the DDR among proteins involved in the structure, maintenance and regulation of chromatin, in different aspects of the response to DNA double-strand breaks by genetic screening (chapters III and IV).

MATERIALS & METHODS

1. Cell culture

The majority of experiments in this study were performed using the following human cell lines:

- U2OS cells, originally known as 2T cells, were obtained from the bone tissue of a patient suffering from osteosarcoma.
- GFP-PHF6 U2OS stable cell line were generated through transfection with a GFP-PHF6 plasmid together with a puromycin resistant cassette. Individual with the integrated plasmid were subsequently selected by puromycin treatment and grown in culture containing this antibiotic (1 mg/ml).
- U2OS 2-6-3 cells expressing inducible FokI-mCherry-LacR (Tang *et al.*, 2013).
- U2OS cells containing a stably integrated copy of either the SA or DR reporter (Gunn and Stark, 2012).
- Human Embryonic Kidney 293T, or HEK293T cells, were obtained from the cell line HEK293, by stable transfection of a mutant version of the SV40 large T antigen.
- HeLa cells were derived from a cervical cancer.
- GC92 human fibroblasts were used to analyze EJ-mediated repair. These cells carry a construct with an I-SceI target that, when repaired through EJ, will express the CD4 receptor (Guirouilh-Barbat *et al.*, 2004; Rass *et al.*, 2009). The expression of this receptor was later detected using an anti-CD4 primary antibody.

The cells were cultured at 37 °C, in a 5% CO_2 atmosphere, using Dulbecco's Modified Eagle's Medium (DMEM, Lonza/Gibco), supplemented with 10% Fetal Bovine Serum (FBS, Gibco-Thermo Fisher Scientific), 1.5 mM L-glutamine (Sigma-Aldrich) and a combination of 150 U/ml penicillin and 150 µg/ml streptomycin (Sigma-Aldrich).

2. Plasmid transfection

Plasmid DNA was transfected into 293T cells at 50-70% confluence using the calcium phosphate transfection method. For 10 cm plates, 10 µg of plasmid DNA were mixed with 450 µl of water and 50 µl of 3.3 mM $CaCl_2$. After gentle vortexing and centrifugation, 500 µl of HBS 2X buffer were added slowly, mixed by pipetting and added to cells. 16 hours after transfection, cells were washed twice with PBS and fresh medium was added. Cells were harvested 24 hours after this last step.

For U2OS cells, growing at 30-60% confluency in 6 cm plates, two different transfection methods were used:

- 3.6 µg of plasmid DNA were mixed with 166 µl of Opti-MEM (Gibco-Thermo Fisher). 12 µl of Fugene (Promega) were added to the solution and added to cells after an incubation of 15 minutes at room temperature (RT). After 4 hours of incubation at 37 °C, the medium was replaced.

- 1 µg of plasmid DNA was diluted in 50 µl of Opti-MEM. In a separate tube, 2 µl of Lipofectamine 2000 (Invitrogen) were diluted in the same way. The content of both tubes was subsequently mixed and, after a 5 minutes incubation period at RT, added to the cells.

Cells were subsequently collected for analysis after 48 hours.

3. Transfection of siRNA oligonucleotides

siRNA oligonucleotides were transfected using Lipofectamine RNAiMax (Invitrogen). 5 µl of oligonucleotide (20 µM) were added to 250 µl of Opti-MEM. In a different tube, 5 µl of Lipofectamine RNAiMax were also added to 250 µl of Opti-MEM. The content from both tubes was mixed and added to cells growing at 30-60% confluency in 6 cm plates containing 2 ml of fresh medium after an incubation of 15 minutes at RT. Depending on the efficiency of the oligo used, this process was repeated 24 hours later. Cells were collected 48 hours after the initial or second transfection.

4. Whole cell extracts

To prepare whole cell extracts (WCE), the cells were washed twice in PBS, lysed in Laemmli buffer 2x and collected into tubes with the help of a cell scraper (Greiner bio-one). Next, extracts were boiled at 95 ºC for 5 minutes and sonicated for 15 seconds at 100% frequency (UP1000H, Hielsher Ultrasonic). For BRCA1 western blot analysis, WCEs were obtained in RIPA buffer and sonicated as indicated above. Protein concentrations were determined using the Bicinchoninic Acid (BCA) protein assay (Novagen). Before loading on SDS-PAGE gel, loading buffer 10x was added.

5. Western blotting

5.1. Electrophoresis

Protein analysis was performed in SDS-polyacrylamide gels, with variable percentage of acrylamide depending on the size of the proteins of interest, ranging from 6% to 12%. The gels included a stacking and a separating layer and were run using a mini-PROTEAN 3 system (BioRad) at 120-160 V in electrophoresis buffer.

5.2. Gel transference and blocking

Proteins were subsequently transferred onto a nitrocellulose membrane (Protran BA, GE Healthcare), using the mini-PROTEANS trans-blot module (BioRad) in transfer buffer for one hour at 295 mA.

After transference was complete, membranes were blocked in 5% skimmed milk (Sveltesse, Nestlé) or 5% BSA (Panreac) depending on the analyzed proteins, in TBS 1x supplemented with 0.1% Tween 20 (Sigma-Aldrich, TBS-T) for one hour in agitation at RT.

5.3. Immunodetection

The membranes were incubated with primary antibodies in the same buffer that was used to block the membrane overnight at 4 ºC. After washing with TBS-T and incubation with horseradish peroxidase (HRP)-coupled secondary antibodies diluted in TBS-T for 1 hour at RT, the membranes were incubated with chemiluminescent substrate solution (Thermo Scientific). Chemiluminescence detection was subsequently carried out using an ImageQuant LAS 4000 mini equipment (GE Healthcare).

6. Immunoprecipitation

Cells were trypsinized and collected by centrifugation at 1,500 rpm for 5 minutes. After washing the pellet with PBS, cells were lysed in 1 ml of EB150 buffer for 20 minutes on ice, and centrifuged at 18,000 rpm for 15 minutes at 4 ºC. The pellet was discarded and 25 µl of the

supernatant were taken and mixed with the same volume of sample buffer 2x (inputs), while the rest of the lysate was incubated with different beads depending on the aim of the study for 2 hours at 4 °C in agitation:

- For immunoprecipitation of Flag-tagged proteins, extracts were incubated with 30 µl of anti-Flag M2 agarose beads (Sigma), previously equilibrated in EB150 buffer.
- For immunoprecipitation of GFP-tagged proteins, 30 µl of protein A Sepharose beads (GE Healthcare) equilibrated in EB150 buffer and 3 µl of GFP antibody were added to lysates.

The resin was collected by centrifugation at 1,500 rpm for 5 minutes and washed three times with 1 ml of ice-cold EB150 buffer. Finally, proteins bound to the beads were eluted by adding 70 µl of sample buffer and heating at 95 °C for 5 minutes.

7. Chromatin fractionation

To study chromatin enrichment of proteins, the method by Méndez and Stillman was used (Mendez and Stillman, 2000). Cells grown on a 10 cm dish were trypsinized and collected by centrifugation at 1,500 rpm for 5 minutes. The pellet obtained was resuspended in 1 ml of PBS, of which 100 µl were spun down and lysed in Laemmli buffer to obtain WCEs. The remaining cells were centrifuged at 1,500 rpm for 5 minutes; the resulting pellet was lysed in 90 µl of buffer A supplemented with Triton X-100 (TX-100) at a final concentration of 0.1%. After incubation on ice for 5 minutes, samples were centrifuged at 1,300 g for 4 minutes at 4 °C. The resulting supernatant, which contained the cytosolic soluble proteins (S_1), was removed and saved in new tubes. The pellets containing the nuclei were washed with 100 µl of buffer A by centrifuging at the same speed and subsequently lysed in 70 µl of buffer B. After incubation on ice for 30 minutes, samples were centrifuged at 1,700 g for 4 minutes at 4 °C. The resulting supernatant, which contained the soluble nuclear proteins (S_2), was taken and added to S_1 to obtain the final soluble fraction (S). The chromatin pellet was washed once in 100 µl of buffer B, cleared by centrifugation at 10,000 g for 1 minute and lysed in 150 µl of Laemmli buffer to obtain the chromatin fraction (P).

8. *In vivo* deubiquitination assay

His-tagged ubiquitin transfected cells were collected by centrifugation at 1,500 rpm for 5 minutes and subsequently lysed and sonicated in 1 ml of buffer A. Next, 50 µl of Ni-NTA agarose beads were added and the samples were incubated at RT in rotation. After two hours, the beads were washed 3 times in buffer A, twice in buffer A 1:4 buffer B and twice in buffer B. Finally, samples were eluted in sample buffer and boiled for 5 minutes at 95 °C.

9. *In vitro* deubiquitination assay

The ubiquitinated protein of interest, purified on Ni-NTA agarose, and the DUB, purified by Flag-immunoprecipitation, were mixed in buffer and incubated for 30 minutes at 37 °C. Sample buffer was added to stop the reaction and samples were analyzed by western blotting.

10. Single Cell Gel Electrophoresis (SCGE) - Comet assay

SCGE was carried out with a kit from Trevigen (CometAssay® ES II), using the following protocol:

Cells transfected with the indicated siRNA oligonucleotides were washed twice with PBS, detached from the plates with 500 µl of Acutase and counted. 200,000 cells were resuspended in 1 ml of DMEM of which 50 µl were transferred to a new tube containing 500 µl of low-melting point 0.5% agarose. After gently pipetting three times, 50 µl of this mixture were placed on microscope slides and left at 4 °C for 30 minutes. Next, the slides were incubated with lysis buffer for 30 minutes at 4 °C, followed by an incubation with an alkaline (300 mM NaOH, 1 mM EDTA pH>13) or TBE (89 mM Tris, 89 mM Boric acid and 2 mM EDTA) buffer, for alkaline and neutral SCGE respectively, for 30 minutes at RT.

Slides were then placed in the electrophoresis slide tray and the electrophoresis was run at 21 V for 30 minutes in alkaline buffer in the case of alkaline SCGE or TBE buffer in the case of neutral SCGE.

The slides were washed twice with H_2O, twice in 70% ethanol and left to dry overnight.

Then, samples were stained with SYBR-Green for 30 minutes, washed twice with H_2O and incubated at 37 °C until dry.

Images were taken using a Leica DM6000B microscope and the tail moment was analyzed with the TriTek CometScore software.

11.I-SceI based DSB repair assays

U2OS cells containing a stably integrated copy of either the SA or DR reporter were used to measure the repair of I-SceI-induced DSBs by SSA (fig. 14 A) and HR (fig. 14 B), respectively (Gunn and Stark, 2012).

60.000 cells per condition were seeded in 6 well plates and transfected with the indicated siRNA oligonucleotides as described earlier.

Then, the cells were infected with lentivirus carrying a copy of BFP-I-SceI, using a multiplicity of infection of 10 (MOI=10). To perform the viral infection, 8 µl of PolyBrene (Sigma) were mixed with the volume of viral solution corresponding to the MOI mentioned above together with 1.5 ml of medium and added to each plate. After 24 hours, cells were washed with PBS and fresh medium was added.

After 24 hours, cells were collected by trypsinization and fixed with 500 µl of 4% paraformaldehyde (PFA) at 4 °C for 1 hour. Then, cells were washed twice with PBS and analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Bioscience). Subsequent analysis was performed using CellQuest Pro Software.

To analyze DNA repair by the c-NHEJ and alt-NHEJ pathways, GC92 (human fibroblasts carrying the end joining substrate shown in figure 14 C) cells were used (Guirouilh-Barbat *et al.*, 2004; Rass *et al.*, 2009).

500,000 cells were seeded in 6 cm dishes and transfected with the indicated siRNA oligonucleotides as described above. 48 hours later, cells were co-transfected with plasmids expressing I-SceI and mCherry, using the Lipofectamine 2000 protocol. After 24 hours, the medium was replaced and the next day cells were collected by trypsinization. Next, incubation with a CD4 antibody was carried out for 20 minutes at RT. Finally, cells were washed in PBS, resuspended in 400 µl of PBS and analyzed by flow cytometry using a LSRII flow cytometer

(BD Bioscience). Subsequent quantification was performed with a FACSDiva software version 5.0.3.

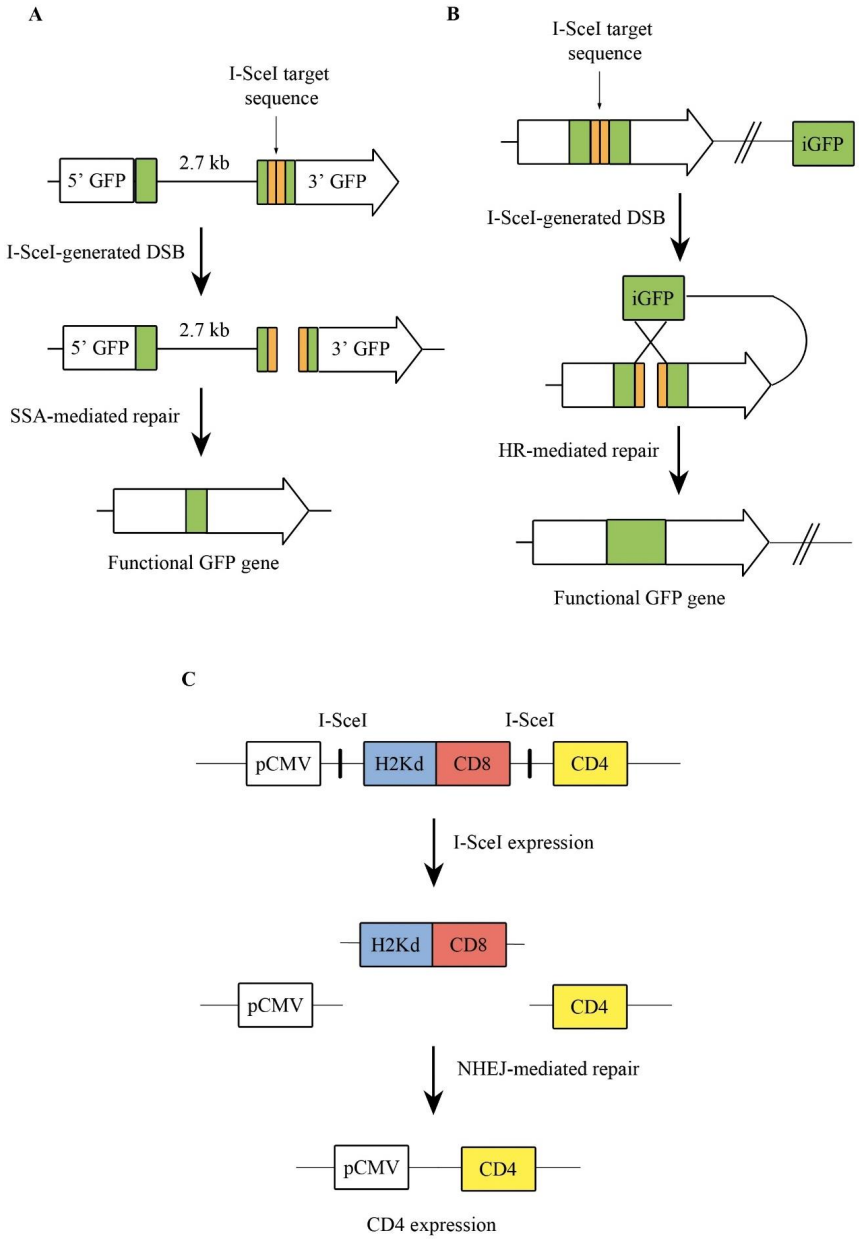

Figure 14. Schematic representation of the systems used to measure DSB repair. A) The SA-GFP reporter system contains a 5' fragment of the GFP gene, separated from the 3' fragment by a 2.7 kb stretch of DNA. The

3' GFP fragment contains an I-SceI target sequence and shares 266 nucleotides of homology with the 5' fragment, so that after induction of the DSB by I-SceI expression, a functional GFP cassette is generated by SSA-mediated repair after annealing of both GFP fragments and DNA-processing events that lead to the loss of the 2.7 kb stretch of DNA. B) The DR-GFP reporter system contains a mutated version of the full length GFP gene, which includes an I-SceI target sequence. After the DSB is created, a downstream 812 pb internal GFP fragment (iGFP) is used by the HR machinery as a homologous sequence donor to restore a functional version of the GFP gene. C) The GC92 cell line contains a stably integrated substrate that includes 3 genes: H2-Kd, CD4 and CD8, of which only H2-Kd is expressed. The H2-Kd/CD8 fragment is flanked by two I-SceI target sites. After expression of the enzyme, this fragment is removed, and the rejoining of the DNA ends by NHEJ allows expression of the CD4 receptor.

11.1. Junction analysis

Junction analysis was performed as described by Taty-Taty *et al.*, 2016. Briefly, GC92 fibroblasts were transfected as indicated above but, instead of being subjected to FACS analysis, their genomic DNA was extracted, followed by PCR and cloning of the amplicons in pGEM T-easy. DNA was transformed and >70 single clones were analyzed by Sanger-sequencing. In-house software was used to analyze the obtained sequences (Schimmel *et al.*, 2017). If the junction was perfectly religated, it was categorized as wild type (WT) and in the case of faulty repair, the junction was categorized into either deletion, insertion or deletion plus insertion (delins). The sequences containing deletions were further categorized into different bins for deletion size and micro homology usage.

12.Lentivirus production

Lentivirus carrying the BFP-I-SceI expression cassette were produced in HEK293T cells. The day before transfection, 6 x 10^6 cells were seeded into 15 cm plates. Then the cells were transfected using the calcium phosphate method. The transfection mix contained 30 µg of BFP-I-SceI plasmid, 20 µg of a plasmid expressing the capsid glycoprotein (VSV-G) and 10 µg of a vector expressing the p89.1 RNA polymerase.

The next day, the cells were washed and the medium was replaced.

After 48 hours, the lentivirus were collected as follows: the medium from the plates was collected, filtered using 0.45 µm sterile filters and subsequently centrifuged at 22,000 rpm for 1.5 hours at 4 °C using an ultracentrifuge. After centrifugation, the supernatant was removed, 500 µl of DMEM were added and the resuspended lentivirus were stored at -80 °C.

13.Immunofluorescence

The cells, growing on 12 mm coverslips, were washed with PBS and then fixed using 4% PFA solution in PBS for 15 minutes at RT. After washing 3 times with PBS, cells were permeabilized with PBS supplemented with 0.2% TX100 for 10 minutes at RT. Then, coverslips were washed with PBS and blocked using PBS + 0.5% FBS for 1 hour at RT. Samples were incubated with the indicated antibodies (diluted in blocking solution) overnight at 4 °C. For some proteins, a pre-extraction protocol was required, through a 5 minutes incubation with ice-cold pre-extraction buffer before the fixation step.

After washing 3 times with PBS with 0.25% FBS, samples were incubated for 1 hour at RT with the corresponding secondary antibodies, diluted in PBS. Finally, coverslips were washed 3 times with PBS, stained with 6-diamino-2-phenylindole (DAPI) and mounted. Images of cells were taken using a Zeiss Cell Observer fluorescent microscope equipped with a 63x NA 1.3

water immersion objective and ZEN imaging software. The number of foci was evaluated in ImageJ.

14.Localization to FokI -induced DSBs

U2OS 2-6-3 cells expressing inducible FokI-mCherry-LacR (Tang *et al.*, 2013) were treated with 300 nM 4-OHT and 1 µM Shield-I for 5 h to induce stabilization and nuclear localization of the expressed product (fig. 15).

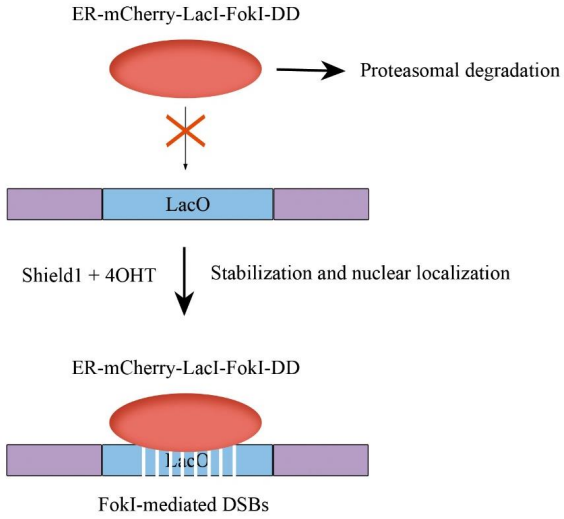

Figure 15. DSB induction in the U2OS 2-6-3 cell line. In normal conditions, the ER-mCherry-LacI-FokI-DD construct expressed is sent for proteasomal degradation due to the presence of the destabilization domain (DD). The addition of Shield1 protects the protein from degradation and 4OHT allows entry of the protein into the nucleus thanks to the estradiol receptor (ER). In the nucleus, the LacI domain interacts with the LacO repeats inserted in the genome and the catalytic domain of FokI creates the DSBs, which can be visualized by fusion to mCherry (adapted from (Rona *et al.*, 2018)).

To detect the recruitment of the protein of interest to the FokI-mediated DSBs, cells were fixed with PFA and immunostained with the indicated antibodies as described above.

15.Multiphoton laser micro-irradiation

Cells grown on 18 mm coverslips were placed in a Chamlide CMB magnetic chamber and the medium was replaced by CO_2-independent Leibovitz's L15 medium (Sigma Aldrich) supplemented with 10% FBS and penicillin-streptomycin. Laser micro-irradiation was carried out on a Leica SP5 confocal microscope equipped with an environmental chamber set to 37 °C. DSB-containing tracks (1.5-2 µm width) were generated with a Mira modelocked titanium-sapphire (Ti:Sapphire) laser (λ = 800 nm, pulse length = 200 fs, repetition rate = 76 MHz, output power = 80 mW) using a UV-transmitting 63x 1.4 NA oil immersion objective (HCX PL APO; Leica). Confocal images were recorded before and after laser irradiation at 5 seconds time intervals over a period of 5 minutes and analyzed using the ImageJ software.

16.Flow cytometry

Cells were collected by trypsinization, washed with PBS and fixed and stored in 70% ethanol at 4 °C. Ethanol was washed off with 10 ml of PBS. The cells were resuspended in 100 µl of PBS with primary antibody and incubated overnight at 4 °C.

The next day, the primary antibody was washed off with 5 ml of PBS, and cells were resuspended in 100 µl of PBS with secondary antibody (conjugated to Alexa 488 or 546) in a 1:300 dilution. Incubation was carried out for one hour at RT, followed by a PBS wash. Finally, the cells were incubated with 500 µl of PBS containing 10 µg/ml propidium iodide (PI, Sigma Aldrich) and 25 µg/ml RNase (Sigma Aldrich). Cells were analyzed using a Macsquant Analyzer and Macsquantify software (Miltenyi).

17.Quantitative-PCR

17.1. RNA extraction

Cells grown in 6 cm plates were washed with PBS and lysed in 500 µl of RiboZol Extraction Reagent (VWR) by passing them several times through the tip of the pipette and incubating them for 10 minutes at RT. Next, 100 µl of chloroform were added, mixed vigorously and incubated at RT for 3 minutes. Samples were then centrifuged at 12,000 g for 15 minutes at 4 °C.

After centrifugation, the upper aqueous phase containing the RNA was removed and transferred to a new sterile tube. The remaining original aqueous phase was re-extracted by adding an equal volume of nuclease free water and centrifuging in the same conditions. The two aqueous phases were then mixed, and the RNA was precipitated by adding 250 µl of isopropanol, incubating for 10 minutes at RT and then centrifuging at 12,000 g for 10 minutes at 4 °C.

The supernatant was removed and the RNA containing pellet was washed twice with 70% ethanol prepared with RNase-free H_2O and centrifuged at 7,500 g for 5 minutes at 4 °C. The resulting pellet was air-dried for 10 minutes and dissolved in 25 µl of RNase-free water.

The RNA concentration was determined by measuring the absorbance at 260 nm, and their purity was assessed by calculating the A260/280 ratio. A Nano-drop 2000 (Thermo-Fisher) system was used to measure the absorbances.

17.2. RT-qPCR

cDNA synthesis and PCR amplification were carried out in the same tube using the qScript One-Step SYBR Green RT-qPCR Kit (Quantabio). For each reaction, 50 ng of RNA were subjected to the following protocol, using a LightCycler480 II (BioRad) system:

Step	Temperature/Length
cDNA synthesis	50 °C, 10 minutes
Taq activation	95 °C, 5 minutes
PCR cycling (40 cycles)	60 °C, 20 seconds 75 °C, 30 seconds (data collection)

18.Clonogenic survival

To determine cellular sensitivity to DNA damaging agents, U2OS or HeLa cells were transfected with the corresponding siRNA oligonucleotides. 48 hours later, 500 cells were seeded in 6 well dishes and treated with the indicated doses of Olaparib (Cayman Chemical) or ionizing radiation. Following 7-10 days in culture, cells were fixed, stained in 0.25% crystal violet, 10% formaldehyde and 50% methanol and colonies were counted. Triplicate cultures were scored for each treatment. Shown is the relative survival as compared to the undamaged control and the error bars present the standard deviation of the mean of three independent experiments.

19.Mutagenesis

For mutagenesis the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) was used.

Three silent mutations (in capitals, gctggaGatCAgGgagcaa) were introduced in the Flag-PHF2 plasmid to make it resistant to siRNA oligonucleotide PHF2#1 (Flag-PHF2*).

Different mutations were introduced in the GFP-PHF6 plasmid to obtain the following constructs: ΔPHD1 mutation (deletion of amino acids 17 to 131) and ΔPHD2 mutation (deletion of amino acids 212 to 329).

20.Knock out by CRISPR/Cas9

Knockout U2OS cell lines were generated using CRISPR/Cas9 genome editing. CRISPR sequences were designed targeting human PHF6 and HAT1 (see targeted exons and sequences in the table below) and cloned into pX458. Cells were transfected with the targeting constructs in addition to a plasmid containing a guide RNA to the zebrafish TIA gene (GGTATGTCGGGAACCTCTCC) and a P2A sequence followed by a puromycin resistance gene, flanked by two TIA target sites. Co-transfection resulted in infrequent integration of the P2A-puromycin cassette at the targeted location, as previously described (Blomen *et al.*, 2015). Successful in frame integration of the gene rendered cells resistant to puromycin. Two days following transfection, the culture medium was supplemented with puromycin (1 mg/ml). Surviving colonies were clonally expanded and screened by western blot for expression of PHF6 and HAT1.

Protein	Exon	Guide RNA sequence
PHF6	2	AATATCTGAAAACCAGAAGG
PHF6	4	CATTGTCCTGGAGCAACAAT
HAT1	2	GGTAGAATATAAGAGTGCAG
HAT1	3	AAGAGTTGATGGGTATACTC

21.Treatments

The treatments in this study were performed as follows, unless stated otherwise:

Agent	Dose	Time
Caffeine (Sigma-Aldrich)	20 µM	1 hour
CHX (Sigma-Aldrich)	35 µg/ml	Variable
CPT (Sigma-Aldrich)	2 µM	1 hour
ETP (Cayman Chemical)	20 µM	1 hour
HU (Chemocare)	10 mM	1 hour
IR	10 Gy	1 hour
MG132 (Calbiochem)	20 µM	3 hours
NEM (Sigma-Aldrich)	2 mM	2 hours
Noco (Sigma-Aldrich)	0.25 µg/µl	3 hours
Olaparib (Cayman Chemical)	Variable	1 hour
P22077 (Cayman Chemical)	30 µM	Variable
Puromycin (Gibco-Thermo Fisher Scientific)	2 µg/ml	Variable
Thym (Sigma-Aldrich)	2.5 mM	24 hours
UV	40 J/m^2	1 hour
VE-821 (Sigma-Aldrich)	10 µM	1 hour

22.Statistical analysis

Statistical analysis was performed with a paired t student test using the PRISM (Graphpad) software (non-significant (ns); P≥0.5; *P<0,5; **P<0.01; ***P<0.001; ****P<0.0001).

APPENDIX

Buffers

Buffer	Composition
Chromatin fractionation A	10 mM Hepes pH 7.8, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na_3VO_4, protease inhibitor cocktail (Calbiochem)
Chromatin fractionation B	3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM Na3VO4, protease inhibitor cocktail
EB150	50 mM Hepes pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM $MgCl_2$, 0.5% NP40, 10% glycerol, protease inhibitor cocktail
Electrophoresis	25 mM Tris, 192 mM Glycine, 0.1% SDS
HBS 2x	280 mM NaCl, 1.5 mM Na_2HPO_4, 12 mM Glucose, 10 mM KCl, 50 mM Hepes pH 7.02
***In vivo* deubiquitination A**	6 M Guanidium-HCl, 0.1 M Na_2HPO_4 pH 8, 10 mM Imidazole
***In vivo* deubiquitination B**	25 mM Tris-HCl, 20 mM Imidazole
***In vitro* deubiquitination**	50 mM Tris-HCl pH 7.5, 4 mM DTT
Laemmli 2x	4% SDS, 20% Glycerol, 120 mM Tris pH 6.8
Loading 10x	0.01% Bromophenol Blue, 5% β-Mercaptoethanol
Pre-extraction	20 mM HEPES pH 8, 20 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40
RIPA	50 mM Tris-HCL pH 8, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, protease inhibitor cocktail
Sample	5% β-Mercaptoethanol, 0.2 % SDS, 2.6% Glycerol, 62.5 mM Tris pH 6.8, 0.01% Bromophenol Blue
Separating gel	0.375 mM Tris pH 8.8, 0.1% SDS, 6-12% acrylamide:bisacrylamide (29:1)
Stacking gel	125 mM Tris pH 6.8, 0.1% SDS, 5% acrylamide:bisacrylamide (29:1)

TBS	25 mM Tris, 10 mM KCl, 270 mM NaCl, pH 7.4
Transfer	25 mM Tris, 192 mM Glycine, 20% Ethanol

Primary antibodies

Antibody	Origin	Source
53BP1 (ab172580)	Rabbit	Abcam
Artemis (NB100-183)	Rabbit	Novus Biologicals
BRCA1	Rabbit	Homemade (Kakarougkas *et al.*, 2013)
BRCA1 (MABC199)	Mouse	Merck-Millipore
CHK1 (G4)	Mouse	Santa Cruz Biotechnology
Claspin	Rabbit	Homemade (Martín *et al.*, 2015)
CtIP (61141)	Mouse	Active Motif
DNA-PKcs (H-163)	Rabbit	Santa Cruz Biotechnology
Flag-M2 (F7425)	Rabbit	Sigma-Aldrich
GAPDH (FL-335)	Rabbit	Santa Cruz Biotechnology
GFP	Rabbit	Homemade (Refolio *et al.*, 2011)
HAT1 (C-20)	Goat	Santa Cruz Biotechnology
Histone H3 (A01502)	Rabbit	GenScript
Immunoglobulin G (Serum)	Rabbit	Jackson Immunoresearch
Ku70 (C-19)	Goat	Santa Cruz Biotechnology
Ku86 (C-20)	Goat	Santa Cruz Biotechnology
Ligase IV (ab193353)	Rabbit	Abcam
Mre11 (ab33125)	Rabbit	Abcam
Nbs1 (ab175800)	Rabbit	Abcam
p53 (DO-1)	Mouse	Santa Cruz Biotechnology
PARP1 (F-2)	Mouse	Santa Cruz Biotechnology
PHF2 (D45A2)	Rabbit	Cell Signaling Technology
PHF2 (NBP1-83080)	Rabbit	Novus Biologicals
PHF2 (PA5-35949)	Rabbit	Invitrogen
PHF6 (NB100-68261)	Rabbit	Novus Biologicals

Phospho-CHK1 Ser317 (AF2054)	Rabbit	R&D Systems
Phospho-CHK1 Ser345 (133D3)	Rabbit	Cell Signaling Technology
Phospho-Histone H2AX Ser139 (γ-H2AX) (A00552)	Rabbit	GenScript
Phospho-Histone H2AX Ser139 (γ-H2AX) (JBW301)	Mouse	Merck-Millipore
Phospho-Histone H3 Ser10 (A00339)	Rabbit	GenScript
Phospho-RPA2 Ser4/8 (A300-245A)	Rabbit	Bethyl Laboratories
Rad50	Rabbit	Homemade
Rad51 (14B4)	Rabbit	Invitrogen
Rif1	Rabbit	Homemade
RPA2 (9H8)	Mouse	Novus Biologicals
TopBP1	Rabbit	Homemade (Danielsen *et al.*, 2009)
Ubiquitinylated proteins, clone FK2 (04-263)	Mouse	Merck-Millipore
USP29	Rabbit	Homemade (Semple *et al.*, 2007)
USP7 (ab190183)	Rabbit	Abcam
XLF (NB100-2258)	Rabbit	Novus Biologicals
XRCC4 (C-4)	Mouse	Santa Cruz Biotechnology
β-Actin	Mouse	GenScript

siRNA oligos

Target	Sequence (5'-3')
CHK1	GCGUGCCGUAGACUGUCCAdTdT
Claspin	GCACAUACAUGAUAAAGAAdTdT
CtIP	UCCACAACAUAAUCCUAAUdTdT
HAT1 #1	GCUACAGACUGGAUAUUAAdTdT
HAT1 #2	GCGUGUUAUUGAACGACUUdTdT

Appendix

Luciferase	UCGAAGUAUUCCGCGUACGdTdT
PHF2 #1	GCUGGAAAUUCGAGAGCAAdTdT
PHF2 #2	GCUAGAGAAGUCGCCUCUAdTdT
PHF2 #3	CCACUUUAAGGACAGCCUUdTdT
PHF6 #1	GCACGAAGCUGAUGUGUU C
PHF6 #2	GCAUGAUAAAGCUCAAAUA
PHF6 #3	AGAUAGGUCUCCACACAGA
USP29	CCCAUCAAGUUUAGAGGAUdTdT
USP7 #1	GGCAACCUUUCAGUUCACUdTdT
USP7 #2	CCCAAAUUAUUCCGCGGCAAAdTdT
USP7 #3	ACCCUUGGACAAUAUUCCUdTdT
XRCC4	AUAUGUUGGUGAACUGAGA

Plasmids

Protein	**Vector**
Claspin	pcDNA3 (HA)
CLOCK	pcDNA4 (Flag-His)
Cul1	pcDNA3 (Flag)
Cul3	pcDNA3 (Flag)
Cul4A	pcDNA3 (Flag)
Cul4B	pcDNA3 (Flag)
Cul5	pcDNA3 (Flag)
DDB1	pcDNA3 (Flag)
EP3000	pCMVbeta
FBXW5	pcDNA3 (Myc)
KAT2A	pCMV (Flag)
KAT2B	pCI (Flag)

KDM1A	pCMV (Flag)
KDM2A	pCAGGS (Flag)
KDM2B	pCAG-IRES (Flag)
KDM3A	pcDNA4 (Myc-His-Flag)
KDM3B	pCMV (HA)
KDM4A	pCMV (HA)
KDM4B	pCMV (HA)
KDM4C	pcDNA3 (Flag-HA)
KDM4D	pcDNA3 (Flag)
KDM5B	pcDNA3 (Myc)
KDM5C	pcDNA3 (Flag)
KDM6A	pCMV (HA)
KDM6B	pCMV (HA)
KDM7	pFASTBAC-HTB (Flag)
KDM8	pLTREX (Flag)
NBS1	pCMV-Tag2B (mCherry)
PHF2 (WT and siRNA resistant)	pcDNA3 (Flag)
PHF6 (WT, ΔPHD1 and ΔPHD2)	pCMV-Tag2B (GFP)
PHF8	JDS74 (Flag)
SKP2	pcDNA3 (Myc)
TAF1	pCS2+(HA)
Ubiquitin	pMT107 (His)
USP7 (WT and CI, C223S)	pCMV-Tag2B (Flag-HA)

Appendix

RT-PCR oligonucleotides

Target	Forward/ Reverse (5'-3')
BRCA1	ACCTTGGAACTGTGAGAACTCT TCTTGATCTCCCACACTGCAATA
CtIP	CAGGAACGAATCTTAGATGCACA GCCTGCTCTTAACCGATCTTCT
GAPDH	GGAGCGAGATCCCTCCAAAAT GGCTGTTGTCATACTTCTCATGG
Mre11	ATGCAGTCAGAGGAAATGATACG CAGGCCGATCACCCATACAAT
PHF2	CTCCCCTACGACGTTACCC CAGTGGTATATGTCGATGTCGG
RPA2	GCACCTTCTCAAGCCGAAAAG CCCCACAATAGTGACCTGTGAAA

RESULTS & DISCUSSION

Chapter I:

USP7 regulates CHK1 levels by deubiquitination

As explained in the introduction of this thesis, CHK1 is a central kinase in the DDR and the critical kinase mediating the G_2 checkpoint arrest. This protein is also important for other vital processes of the cell, such as DNA replication or transcription, which is highlighted by the fact that CHK1 is essential for normal cell proliferation (Takai *et al.*, 2000; Lam *et al.*, 2004; Shechter *et al.*, 2004; Shimada *et al.*, 2008). Correct regulation of CHK1 cellular levels is therefore imperative. It was previously reported that one of the ways in which CHK1 protein regulation occurs is through proteasomal degradation. The Cul1-Cul4A E3 ligases target CHK1 by direct ubiquitination after which it is degraded by the proteasome. This proteasomal degradation is needed for checkpoint termination, but it also regulates CHK1 levels in normal cycling cells (Zhang *et al.*, 2005, 2009). Importantly, CHK1 was found to be stabilized by the ubiquitin-hydrolases USP1 and Ataxin-3, although only the latter was demonstrated to directly deubiquitinate CHK1 (Guervilly *et al.*, 2011; Tu *et al.*, 2017). By the time this work was published, no DUB able to reverse CHK1 polyubiquitination had been reported.

1.1. USP7 depletion reduces CHK1 levels

Previous experiments performed in this laboratory studying the effect of the ubiquitin hydrolase USP7 on the checkpoint mediator protein Claspin showed that USP7 could also affect CHK1 protein stability (Martín *et al.*, 2015). To study this effect in more detail, USP7 was downregulated using three different siRNA oligonucleotides, and the levels of CHK1 were analyzed by western blot. As observed in figure 16 A, transfecting cells with each of the three oligonucleotides targeting USP7 led to a decrease in CHK1 levels.

USP7 was known to regulate Claspin levels, and it had previously been suggested that Claspin levels could affect CHK1 stability (Chini *et al.*, 2006; Faustrup *et al.*, 2009; Lin *et al.*, 2014). The effect of USP7 knockdown on CHK1 could therefore be an indirect effect of lowering Claspin levels. However, Claspin knock down reduced the levels of CHK1 to a lesser extent than USP7 depletion (fig. 16 B), supporting the idea that the observed effect of USP7 downregulation on CHK1 was not an indirect effect through Claspin. Furthermore, depletion of USP29, another DUB known to regulate Claspin levels (Martín *et al.*, 2015), did not affect CHK1 (fig. 16 C). Finally, elevating Claspin protein levels by overexpressing HA-Claspin in USP7-depleted cells did not rescue the decrease in CHK1 levels (fig. 16 D), demonstrating that the observed phenotype after USP7 knockdown was not an indirect effect of the lower Claspin levels in this condition.

To prove that the decrease in CHK1 levels was dependent on the hydrolase activity of USP7, a compound previously reported to inhibit USP7 catalytic activity, P22077 (Altun *et al.*, 2011), was used. As expected, inhibition of USP7 by P22077 reduced CHK1 levels in both U2OS and 239T cells (fig. 16 E).

These data together suggest that USP7 regulates CHK1 protein levels in a Claspin-independent manner.

1.2. USP7 overexpression increases CHK1 levels

Since USP7 downregulation causes a decrease in CHK1 protein levels, it was expected that its overexpression would have the opposite effect. Indeed, transfection of a Flag-tagged version of USP7 into HEK293T cells increased CHK1 levels (fig. 17 A). To study whether this effect of USP7 is due to its catalytic activity, we generated a catalytic inactive (CI) mutant of USP7 by substituting the active site cysteine with arginine (C223S). An *in vitro* activity assay with HA-

Ub-VS as artificial substrate demonstrated that this version of USP7 indeed displayed significant lower catalytic activity compared to the wild type (WT) version (fig. 17 B). As a control, the reaction was carried out in the presence of N-ethylmaleimide (NEM), a DUB inhibitor, which completely inhibited the activity of USP7 WT. Importantly, transfection of the catalytic mutant version of USP7 did not show this effect, supporting the idea that USP7 regulates CHK1 levels through its catalytic activity.

To further study the effect of USP7 on CHK1 stability, protein synthesis was blocked using CHX after overexpression of USP7 and the half-life of CHK1 was measured. As shown in figure 17 C, the half-life of CHK1 increased upon overexpression of USP7 WT, but not the catalytic mutant.

These data demonstrate that USP7 regulates CHK1 levels in a manner that is dependent on the catalytic activity.

1.3. USP7 deubiquitinates CHK1

Since the WT version of USP7 stabilized CHK1, but the CI version, that lacks deubiquitinating activity, did not, we reasoned that such stabilization was achieved through direct deubiquitination, and in such way preventing CHK1 proteasomal degradation. To test this, HEK293T cells were transfected with His-tagged ubiquitin (His-Ub), with or without Flag-USP7. CHK1 polyubiquitination was observed upon transfection with His-Ub and inhibition of the proteasome by incubating the cells with MG132, as observed by CHK1 western blot after His pulldown. However, the levels of CHK1 polyubiquitination were reduced after USP7 overexpression (fig. 18 A), indicating that USP7 regulates CHK1 ubiquitination *in vivo*.

Next, we set out to determine if USP7 directly deubiquitinates CHK1. To do so, an *in vitro* deubiquitination assay was performed with purified proteins. As observed in figure 18 B, USP7 WT, but not the CI version, markedly decreased CHK1 polyubiquitination. Moreover, the addition of the DUB inhibitor NEM also blocked CHK1 deubiquitination by USP7.

Altogether, these results prove that USP7 directly regulates CHK1 deubiquitination in a catalytic activity-dependent manner.

1.4. USP7 is (mono)ubiquitinated

During the course of our experiments, we noticed that upon expression of the USP7 catalytic mutant, an additional band appeared in USP7 western blots (fig. 17 A–C, fig. 18 B). This band was also observed by others and suggested to be (mono-)ubiquitinated USP7 (Faustrup *et al.*, 2009). To demonstrate USP7 ubiquitination, we transfected cells with the WT or the CI versions of USP7, together with His-Ub, to trigger potential USP7 ubiquitination. USP7 was subsequently immunoprecipitated using the Flag-tag, followed by western blotting for USP7 and conjugated ubiquitin (FK2; fig. 19). After immunoprecipitating USP7, the extra band was clearly visible in the USP7 catalytic mutant lane and corresponds to a specific band at the same height and in the same sample of the conjugated ubiquitin western, indicating that USP7 indeed is (mono)ubiquitinated (fig. 19). In addition, the absence of (mono)ubiquitination of the WT version of USP7 strongly suggests that this DUB deubiquitinates itself, possibly in a trans mechanism.

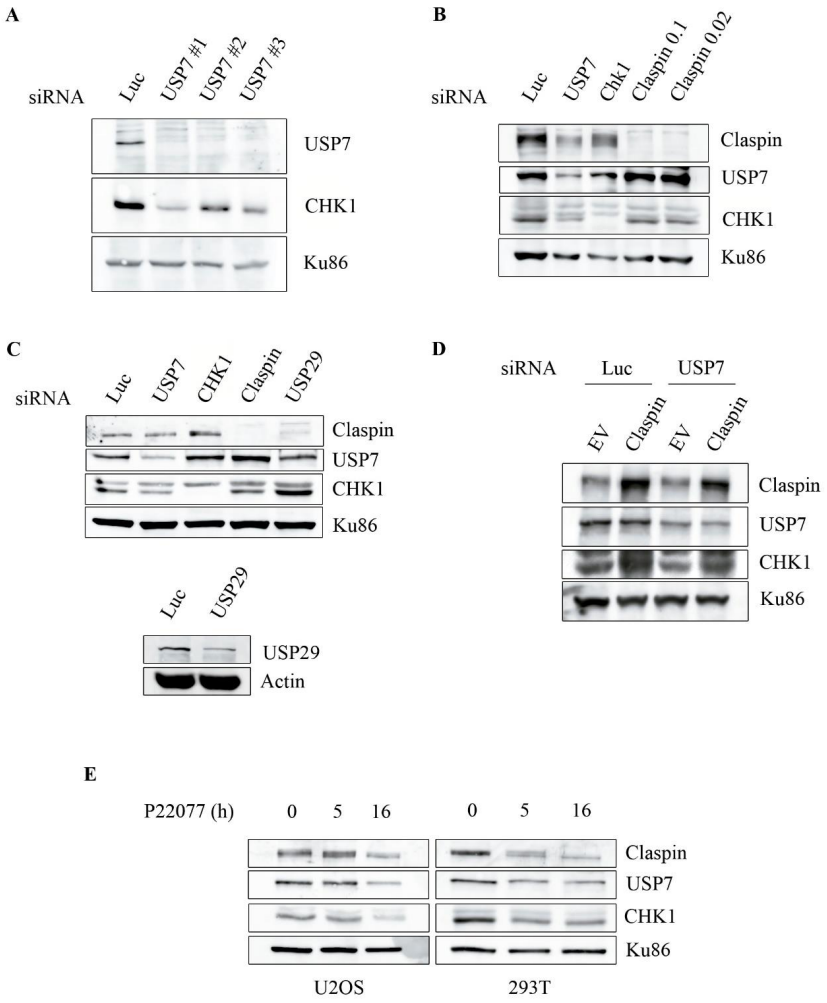

Figure 16. USP7 depletion reduces CHK1 levels in a Claspin-independent manner. A), B) and C) U2OS cells were depleted for luciferase (Luc, control), USP7 (using 3 different oligonucleotides), CHK1, Claspin or USP29 and lysed for analysis by western blot with the indicated antibodies. For Claspin, lower oligo amounts were used (0.1 and 0.02 µM). D) U2OS cells were depleted for Luc or USP7. After 24 h, cells were simultaneously transfected with empty vector (EV) or HA-Claspin and Luc or USP7 siRNA oligos. The next day, cells were lysed and analyzed by western blot using the indicated antibodies. E) 293T and U2OS cells were treated with P22077. At the indicated time points, cells were lysed and analyzed by western blot with the indicated antibodies.

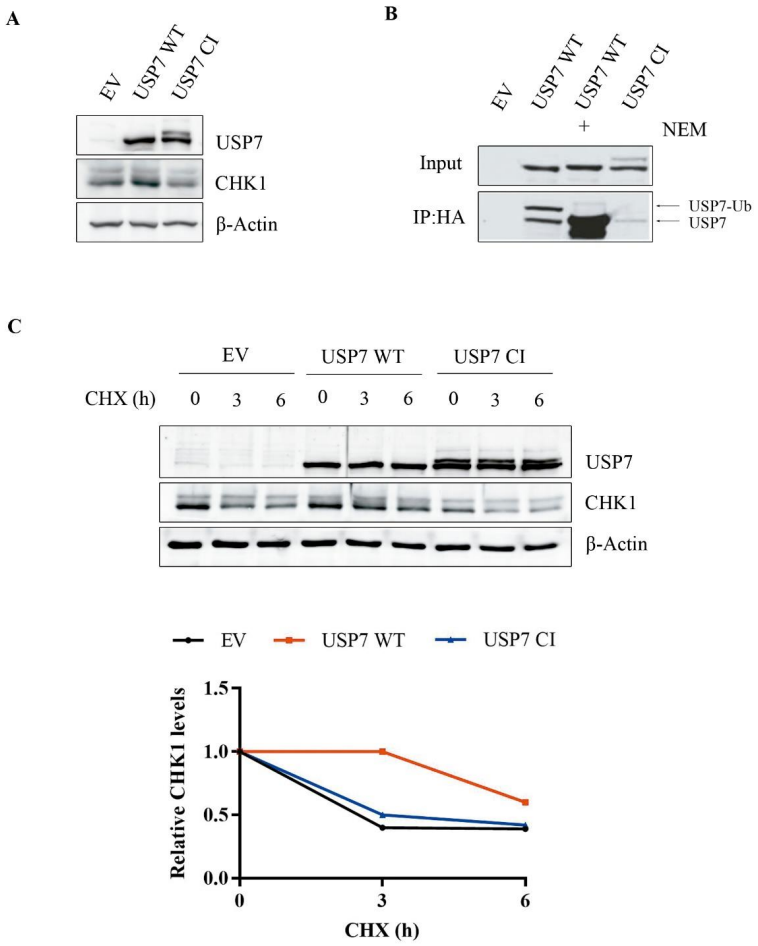

Figure 17. USP7 overexpression elevates CHK1 cellular levels. A) 293T cells were transfected with an EV, Flag-USP7 WT or Flag-USP7 CI (C223S) and analyzed by western blot using the indicated antibodies. B) 293T cells were transfected with EV or the different USP7 expression vectors. After 40 h, cells were lysed, incubated with HA-Ub-VS for 1 h at 37 °C, in presence or absence of N-ethylmaleimide (NEM, 2 mM), followed by an anti-HA immunoprecipitation and western blotting with the indicated antibodies. USP7-Ub is USP7 bound to HA-Ub-VS and therefore represents the active form of the enzyme. C) 293T cells were transfected as in (A) and incubated with CHX for the indicated times. Lysates were then analyzed by western blotting using the indicated antibodies. The lower panel represents the quantification of the CHK1 western. CHK1 was compared to loading control Ku86 and the amount of CHK1 at t = 0 h was put to 1, in each of the conditions (EV, USP7 WT and USP7 CI).

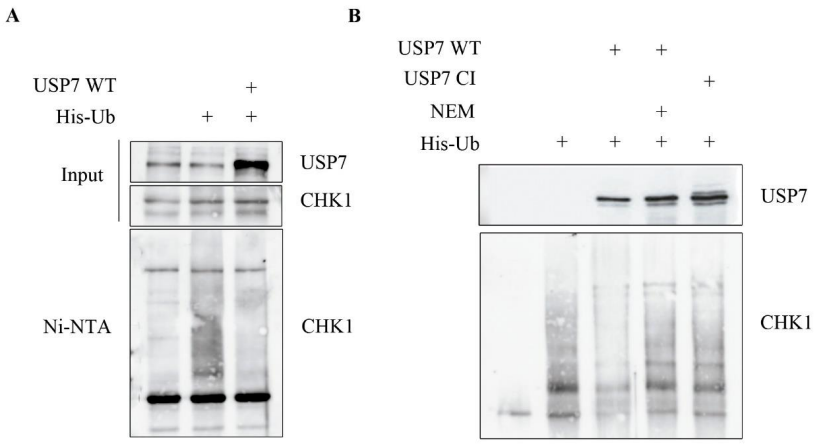

Figure 18. USP7 deubiquitinates CHK1. A) 293T cells were transfected with EV or Flag-USP7 WT and His-tagged ubiquitin (His-Ub) and treated with MG-132. After lysis, a pulldown using Ni-NTA beads was performed and resolved by western blot using the indicated antibodies. B) 293T cells were transfected with EV or His-Ub and ubiquitinated CHK1-bound beads were obtained as in (A). After extensive washing, beads were mixed with purified Flag-USP7 WT or CI in the absence or presence of NEM. Subsequently, samples were resolved by western blot using the indicated antibodies.

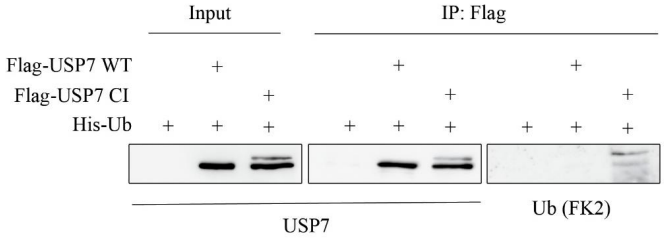

Figure 19. USP7 is monoubiquitinated. 293T cells were transfected with EV, Flag-USP7 WT or Flag-USP7 CI (C223S), together with His-Ub. After 40 h, an anti-Flag immunoprecipitation was performed and immunoprecipitates were resolved by western blot.

DISCUSSION

In this study, USP7 was identified as a novel ubiquitin hydrolase for CHK1. USP7 depletion or inhibition decreases CHK1 protein levels, whereas USP7 overexpression has the opposite effect. USP7 was previously reported to regulate checkpoint adaptor protein Claspin, and it was also suggested that maintaining Claspin levels could be important for CHK1 stability (Chini and Chen, 2006; Chini *et al.*, 2006; Faustrup *et al.*, 2009; Lin *et al.*, 2014). Our results, however, clearly point to a Claspin-independent role for USP7 in CHK1 stability. Claspin depletion only caused a minor effect on CHK1 levels compared to USP7, and Claspin overexpression could not rescue the decrease on CHK1 levels observed after USP7 depletion. Furthermore, depletion

of USP29, a DUB known to regulate Claspin levels (Martín *et al.*, 2015), had no effect on CHK1.

This study also proves that the regulation of CHK1 levels by USP7 is performed by direct deubiquitination, as purified Flag-USP7 WT, but not a CI version, was able to reverse the polyubiquitination of CHK1.

Apart from its role in the ATR-CHK1 pathway through Claspin and CHK1 deubiquitination, USP7 was also described to regulate other processes involved in the maintenance of genome integrity. An example is the extensively analyzed role of USP7 in apoptosis and the G_1/S checkpoint through regulation of p53 levels (Li *et al.*, 2002, 2004). This ubiquitin hydrolase has also been linked to different DNA damage repair pathways. Loss of USP7 destabilizes Rad18 and compromises UV-induced PCNA mono-ubiquitylation and Pol η recruitment to stalled replication forks, thus inhibiting translesion synthesis (TLS) (Zlatanou *et al.*, 2016). USP7 also controls DSB repair through both HR and NHEJ by stabilizing the histone demethylase PHF8 and thereby allowing the recruitment of key factors BLM and Ku70 (Wang *et al.*, 2016). Its relevance in multiple DDR pathways, added to the fact that USP7 levels were found to be altered in several types of cancer, such as breast, cervical or leukemia, point to a role for this DUB in regulating tumorigenesis (Hernández-Pérez *et al.*, 2017; Shan *et al.*, 2018; Su *et al.*, 2018). How CHK1 regulation by USP7 contributes to this process is something that will need to be clarified in the future, but it seems likely that regulation of USP7 activity plays a role in the proteasome-mediated CHK1 degradation that occurs at late timepoints after damage (Zhang *et al.*, 2005).

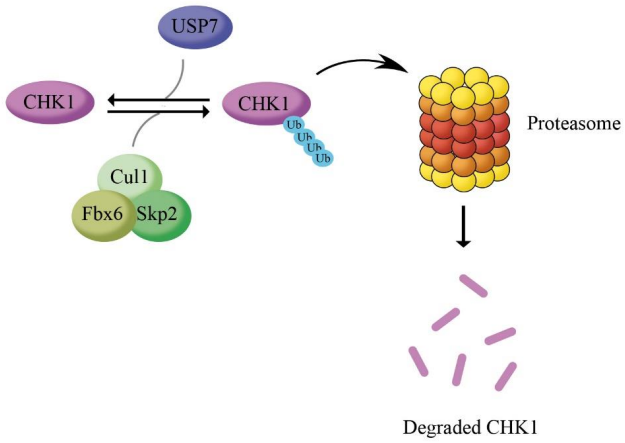

Figure 19. USP7 prevents CHK1-proteasomal degradation by direct deubiquitination. CHK1 protein levels are regulated by ubiquitination: the Skp1-Cul1-Fbx6 complex polyubiquitinates CHK1 and marks it for degradation by the proteasome, while USP7 hydrolyzes these polyubiquitin chains, thereby stabilizing protein levels. The balance between these processes controls CHK1 cellular levels.

This work also shows that USP7 is (mono)ubiquitinated and can possibly auto-deubiquitinate. Other DUBs have been reported to regulate their own ubiquitination, with different outcomes. USP1 auto-deubiquitination inactivates the protein, allowing accumulation of mono-

ubiquitinated PCNA, while USP29 auto-deubiquitinase activity leads to stabilization of the protein (Huang *et al.*, 2006; Mei *et al.*, 2011). The mechanism and the relevance of the here described USP7 auto-deubiquitination needs further investigation, but the fact that the pharmacological inhibition of USP7 leads to decreased levels of this DUB (fig. 16 E) suggests that USP7 controls its own stability through auto-deubiquitination.

USP7 has been proposed as a new target for cancer therapies, and different inhibitors are under development (Tavana *et al.*, 2018; Zhou *et al.*, 2018; Fu *et al.*, 2019). The fact that this DUB controls CHK1 cellular levels by direct deubiquitination is a new factor to consider while designing future USP7-targeted therapies.

Chapter II:

HAT1 plays a role in the G_2/M checkpoint

Given the importance of TopBP1 in the ATR-CHK1 pathway, we were interested in identifying new proteins involved in the regulation of this scaffold protein. In a screening for TopBP1-interacting proteins by mass spectrometry performed by our collaborator Dr. Marcus Smolka (Cornell University, Ithaca, New York, United States), lysine acetyltransferase HAT1 was identified as a possible interaction partner of TopBP1.

Interestingly, in 2014 Liu et al. demonstrated that, in order to interact with Rad9 and to activate ATR, TopBP1 needs to be acetylated in a DNA damage-dependent manner (Liu *et al.*, 2016). Since HAT1 had already been shown to acetylate non-histone proteins (Sadler *et al.*, 2015), we decided to investigate if TopBP1 could be regulated by HAT1 through direct acetylation.

2.1. HAT1 interacts with TopBP1

To confirm the interaction of TopBP1 with HAT1, as detected by mass spectrometry, different immunoprecipitation experiments were performed. First, a GFP-tagged version of TopBP1 was expressed in 293T cells, and exogenous TopBP1 was immunoprecipitated using a GFP antibody. As observed in figure 20 A, HAT1 was detected in these GFP-TopBP1 immunoprecipitations, demonstrating that endogenous HAT1 binds GFP-TopBP1. In addition, HAT1 could also be pulled down after immunoprecipitating endogenous TopBP1 from untransfected cells (fig. 20 B).

These results confirm the data obtained by mass spectrometry and identify HAT1 as a novel interactor of TopBP1 *in vivo*.

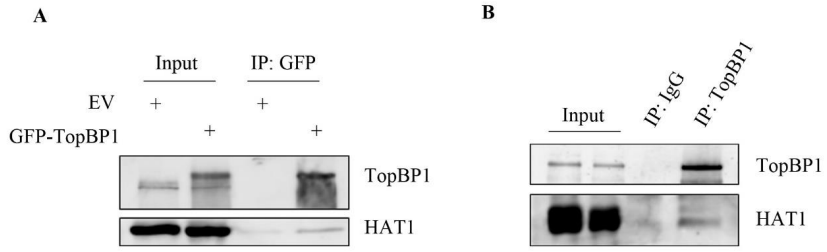

Figure 20. TopBP1 interacts with HAT1 *in vivo*. A) 293T cells were transfected with an EV or with GFP-TopBP1. GFP immunoprecipitates were analyzed by western blot using TopBP1 and HAT1 antibodies. B) 293T cells were lysed and subjected to TopBP1 immunoprecipitation and analyzed as in (A).

2.2. HAT1 facilitates DNA damage-induced recruitment of TopBP1 to the chromatin

As explained in section 3.1 of the introduction, in response to DNA damage TopBP1 is recruited to the chromatin, where it triggers ATR activation. To determine if HAT1 is required for this recruitment, HAT1 was depleted from U2OS cells by siRNA and a chromatin fractionation was carried out after irradiating the cells with UV light. As expected, an increase in the amount of TopBP1 on the chromatin was observed in response to UV light (fig. 21). Downregulating HAT1 protein levels caused a reduction in the amount of chromatin-bound TopBP1 in both damaged and undamaged situations (fig. 21), indicating that HAT1 controls the chromatin binding of TopBP1. Interestingly, a slight increase in the amount of HAT1 in the chromatin fraction after DNA damage as compared to undamaged cells was also observed, suggesting that HAT1 might be recruited to DNA lesions.

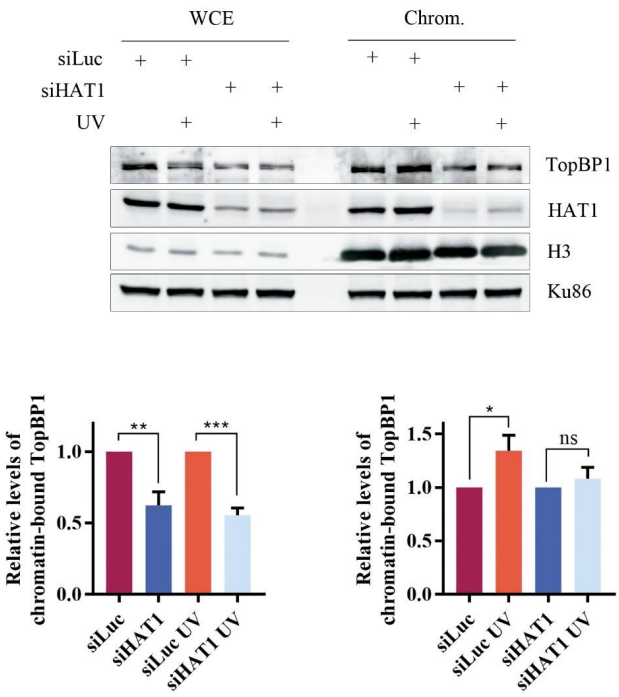

Figure 21. HAT1 is required for the efficient localization of TopBP1 to chromatin (I). U2OS cells were depleted for Luc or HAT1 by siRNA, treated with UV light and subjected to chromatin fractionation (WCE: whole cell extracts and P: chromatin). Samples were then analyzed by western blot using the indicated antibodies. For quantification, chromatin-bound TopBP1 levels were compared to control (Ku86) and the difference between control and HAT1 downregulated cells (left panel) or between untreated and UV-treated cells (right panel) were plotted. Data from three independent experiments was collected and subjected to statistical analysis.

The accumulation of numerous DDR proteins to sites of DNA damage can be visualized by immunofluorescence through the formation of so-called foci. Indeed, TopBP1 forms focal accumulations ("foci") upon treatment with different DNA damaging agents (Mäkiniemi *et al.*, 2001). TopBP1 focus formation upon treatment with UV light was analyzed after knockdown of HAT1 by siRNA in U2OS cells. As expected from the chromatin fractionation experiments, decreasing HAT1 protein levels caused a reduction in the amount of TopBP1 foci-positive cells (fig. 22 A). In contrast, focus formation of RPA2 and Rad9, two proteins that function upstream of TopBP1, was not affected by HAT1 downregulation (fig. 22 A and B). Together, these data support the idea that HAT1 might be involved in the recruitment of TopBP1 to the chromatin in response to DNA damage and during the unperturbed cell cycle.

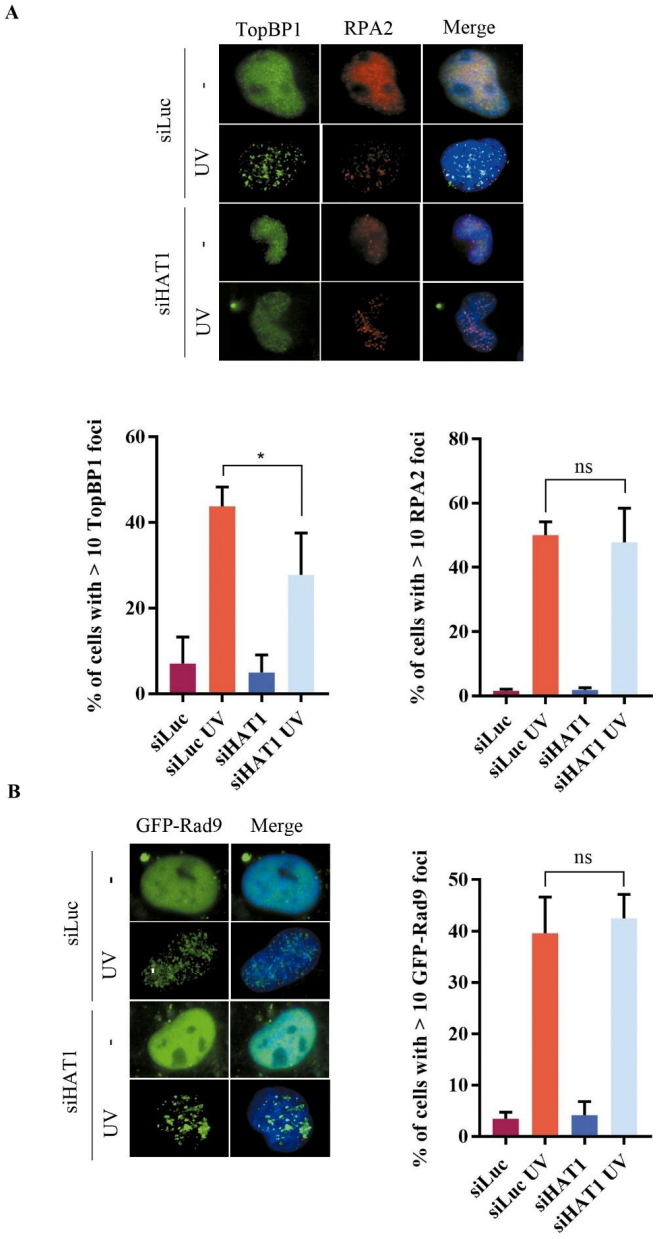

Figure 22. HAT1 is required for the efficient recruitment of TopBP1 to chromatin (II). A) U2OS cells were depleted for Luc or HAT1 and UV-induced focus formation of TopBP1 and RPA2 was analyzed by immunofluorescence. For each experiment, 100 cells were counted. Shown are the mean ± SD from three independent experiments. B) U2OS cells stably expressing GFP-Rad9 were transfected as in (A) and UV-induced GFP-Rad9 focus formation was analyzed by fluorescence. Mean ± SD from three independent experiments (100 cells per experiment) are shown.

2.3. HAT1 promotes the G_2/M checkpoint through regulation of CHK1 phosphorylation

The DNA damage-induced recruitment of TopBP1 triggers the activation of ATR via its AAD and the subsequent phosphorylation of effector kinase CHK1 on Ser 317 and 345. Phosphorylated CHK1 then exerts its kinase activity on different downstream proteins, thereby arresting the cells in G_2. Since chromatin-bound levels of TopBP1 are reduced after HAT1 knockdown, the next step was to study if this affected the activation of ATR and CHK1 in response to DNA damage. Indeed, UV-induced phosphorylation of CHK1 on Ser 317 was reduced after HAT1 downregulation as compared to control (fig. 23 A).

Next, the effect of HAT1 on the IR-induced G_2/M checkpoint was assessed by analyzing mitotic cells by phospho-histone H3 staining and flow cytometry. Whereas IR treatment of control cells inhibited the progression into mitosis, HAT1 knockdown resulted in relatively more mitotic cells and therefore partially prevented the G_2 arrest (fig. 23 B).

Overall, these data demonstrate that HAT1 contributes to an efficient functioning of the ATR-CHK1 pathway, by promoting TopBP1 recruitment to the chromatin, ATR-mediated CHK1 phosphorylation and the subsequent G_2 checkpoint arrest.

2.4. HAT1 knockout cells generated by CRISPR/Cas9 show the same phenotype as siHAT1 transfected U2OS

Although highly reproducible, the effects of HAT1 knockdown on TopBP1 recruitment to sites of DNA lesions, CHK1 phosphorylation and the G_2 checkpoint arrest were relatively small. We reasoned that this could be due to the low efficiency of the HAT1 siRNA oligo used to downregulate the protein. A different siRNA oligonucleotide resulted in similar levels of downregulation (fig. 24 A), which suggests that the HAT1 protein is highly stable and therefore difficult to efficiently downregulate. To solve this problem, CRISPR-Cas9 technology was used to produce stable U2OS knockout cells for HAT1. Two clones targeting HAT1 exon 2 (clone #1) and exon 3 (clone #2) were obtained (fig. 24 B). These cells were then subjected to treatment with UV and phosphorylation of CHK1 was analyzed by western blot. As expected, HAT1 knockout led to a reduced UV-induced CHK1 phosphorylation, as seen after HAT1 depletion by siRNA (fig. 23 A). Strikingly, however, the effect of the complete HAT1 knockout is similar to that of downregulating HAT1 by siRNA and resulted only in a minor decrease on CHK1 phosphorylation upon UV as compared to the control cells (fig. 25 A).

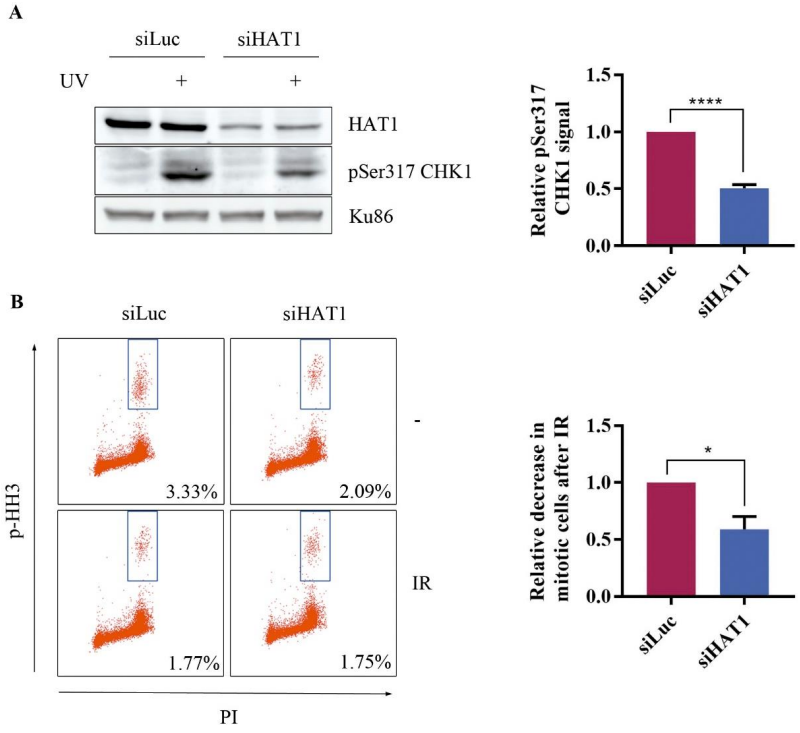

Figure 23. HAT1 is required for efficient CHK1 phosphorylation and the G_2 checkpoint arrest. A) U2OS cells were downregulated for Luc or HAT1 by siRNA and treated with UV light before lysis. Analysis by western blot using the indicated antibodies was performed (left panel). In the quantification (right panel) the UV-induced CHK1 phosphorylation in control transfected cells is put to 1. Mean ± SD from three independent experiments was plotted. B) U2OS cells were transfected as in (A) and treated with IR (2 Gy) and nocodazole for 3 hours. After fixation, cells were analyzed by flow cytometry for PI and pHH3. In the quantification, the decrease in mitotic (pHH3-positive) cells after IR treatment was calculated, and the result obtained for control cells was put to 1. Mean ± SD from three independent experiments was plotted.

Assessing the G_2 checkpoint in HAT1 knockout cells had the same result. Although HAT1 knockout resulted in a small defect in the G_2 checkpoint arrest as compared to wild type cells (fig. 25 B), the effect was not bigger than the one obtained with HAT1 depletion by siRNA (fig. 23 B).

Together, these results clearly demonstrate that the small but reproducible effects on both UV-induced CHK1 phosphorylation and the IR-induced G_2 checkpoint arrest observed after HAT1 partial depletion are not due to the low downregulation efficiency, and suggest that besides HAT1, additional regulatory mechanisms for TopBP1 exist and/or that there is a redundancy of acetyltransferases.

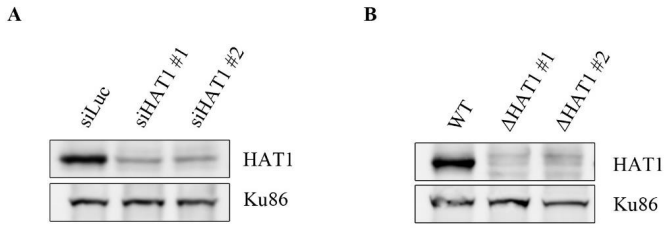

Figure 24. Comparison between HAT depletion by siRNA and CRISPR/Cas9-mediated HAT1 knockout. A) U2OS cells were depleted for Luc or HAT1 using two different siRNA oligos. Lysates were subsequently analyzed by western blot using the indicated antibodies. B) Western blot analysis of U2OS WT and two HAT1 knockout clones obtained using CRISPR-Cas9 technology.

DISCUSSION

TopBP1 regulates several processes due to its ability to interact with many different proteins (Garcia *et al.*, 2005; Rappas *et al.*, 2011). The importance of this protein is highlighted by the fact that its disruption is lethal in most multicellular organisms, including *Drosophila* and mouse (Yamamoto *et al.*, 2000; Jeon *et al.*, 2011). Human TopBP1 is essential for replication initiation, DNA repair and, most relevant for this work, the activation of ATR in response to DNA damage (Papers *et al.*, 2001; Hashimoto and Takisawa, 2003; Morishima *et al.*, 2007; Liu *et al.*, 2017). During the latter process, TopBP1 is recruited to damaged chromatin through interaction with RPA and directs the 9-1-1 complex to these sites (Yan and Michael, 2008; Acevedo *et al.*, 2016). In the suggested model, the interaction of TopBP1 with the 9-1-1 complex stabilizes TopBP1 binding to RPA-coated ssDNA and allows ATR activation, via the AAD of TopBP1 (Kumagai *et al.*, 2006; Mordes *et al.*, 2008; Yan and Michael, 2008). Activated ATR then phosphorylates CHK1, leading to the consequent G_2 checkpoint arrest (Liu *et al.*, 2000).

For a long time, the mechanism by which TopBP1 switches from its DNA replication function to its role in checkpoint initiation remained elusive. In 2016, Liu et al. described that the TopBP1 acetylation status, regulated by the deacetylase SIRT1, controls the differential functions of TopBP1. Acetylation of TopBP1 causes a conformational change that suppresses its association with replication-involved proteins and allows interaction with Rad9, promoting ATR activation and subsequent checkpoint arrest (Liu *et al.*, 2016).

Our experiments point to a novel role for the Lys acetyltransferase HAT1 in the DDR, promoting TopBP1 recruitment to sites of damage and therefore CHK1 phosphorylation by ATR and subsequent checkpoint arrest. HAT1 interacts with TopBP1 in the absence of DNA lesions, as also suggested by the interaction studies in undamaged cells by mass spectrometry data obtained by Dr. Marcus Smolka. However, like TopBP1, HAT1 accumulates on the chromatin after DNA damage. A possible interpretation for these results is that both proteins form a complex, maybe together with another yet to be elucidated factor(s), and that the formation of such complex is needed for the localization of TopBP1 onto the chromatin.

HAT1 knockdown decreases TopBP1 chromatin-bound levels and focus formation in response to UV light, without affecting RPA2 and Rad9 recruitment to damaged sites.

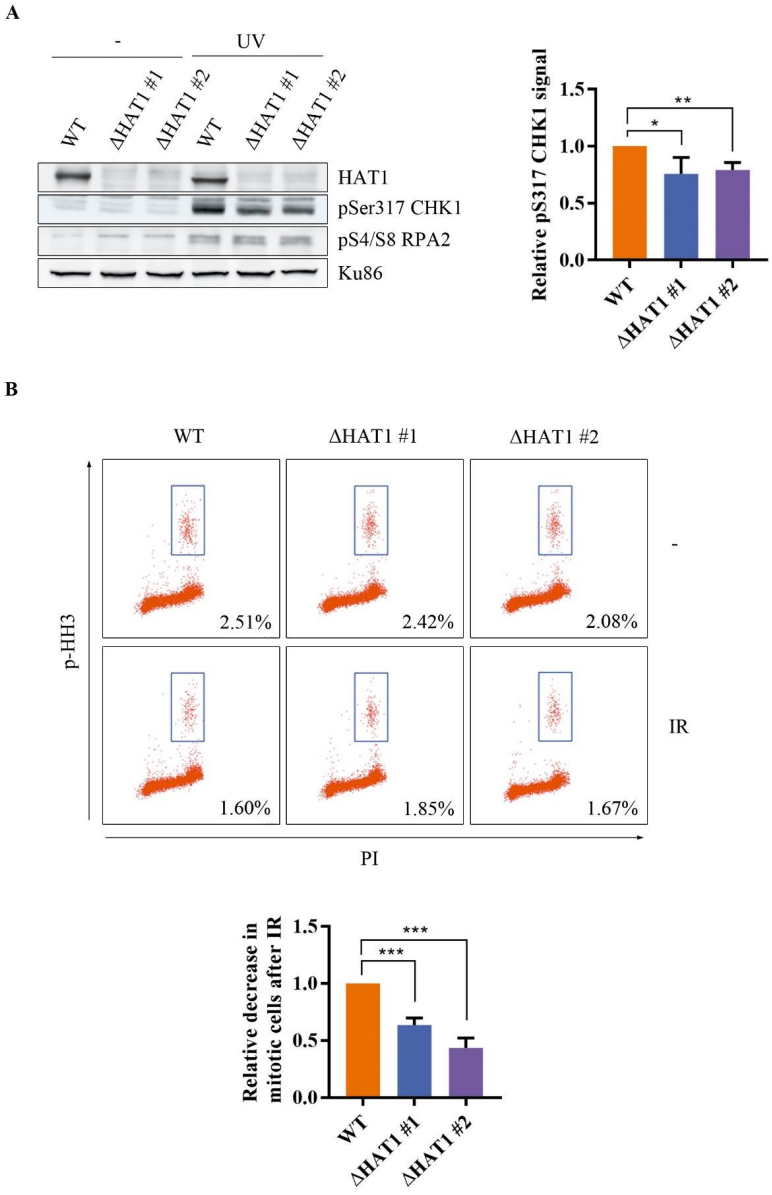

Figure 25. HAT1 knockout results in a similar phenotype as HAT1 knockdowns. A) U2OS WT and HAT1 knockout cells were treated with UV light and, after lysis, analyzed by western blot using the indicated antibodies (left panel). In the quantification (right panel) the UV-induced CHK1 phosphorylation in control (WT) cells is put to 1. Mean ± SD from three independent experiments was plotted. B) U2OS WT and HAT1 knockout cells were treated with IR (2 Gy) and nocodazole for 3 hours. After fixation, cells were analyzed by FACS for PI and pHH3. In the quantification, the decrease in mitotic (pHH3-positive) cells after IR treatment was calculated, and the result obtained for control cells was put to 1. Mean ± SD from three independent experiments were plotted.

This, in addition to the observed interaction between both proteins, supports a direct role for HAT1 in the regulation of TopBP1 function. Accordingly, diminished damage induced CHK1 phosphorylation and G_2 checkpoint arrest were observed after HAT1 knock down. Further experiments are required to clarify how HAT1 contributes to TopBP1 function in the ATR-CHK1 pathway. As recent data demonstrated that TopBP1 acetylation is essential for its role in the DDR (Liu *et al.*, 2016), HAT1 might regulate TopBP1 function via direct acetylation. Mass spectrometry data from our collaborator Dr. Marcus Smolka identified three TopBP1 acetylated residues in HU-treated cells: Lys687, Lys858 and Lys1265/Lys1266. Amongst these residues, Lys687 was especially interesting as it is situated in the BRCT5 domain, responsible for MDC1 binding and TopBP1 focus formation (Wang *et al.*, 2011). Additionally, and according to our collaborator Dr. Miguel Fernandes' structural simulation studies, acetylation of this residue could cause a conformational change in the protein, that could for example lead to a change of function. Therefore, we generated versions of TopBP1 in which these Lys were mutated to compare TopBP1 acetylation in WT and mutated versions of the protein. In addition, the influence of HAT1 on TopBP1 acetylation would be tested upon depletion of HAT1 by siRNA. However, neither of the acetylated-Lys antibodies, commercial or homemade, used in our acetylation experiments could effectively detect Lys acetylation and therefore TopBP1 acetylation by HAT1 could not be demonstrated and analyzed (data not shown). In addition, we were unfortunately also not able to analyze damage-induced focus formation of these TopBP1 mutants, due to technical problems. Interestingly however, Carafa et al. recently demonstrated that HAT1 forms a complex with SIRT1 (Carafa *et al.*, 2018), the only enzyme known to deacetylate TopBP1, which supports the hypothesis that SIRT1-HAT1 complex is important in the regulation of TopBP1 acetylation status.

Apart from direct acetylation, HAT1 could regulate TopBP1 in other ways. It was shown that histone acetylation is critical in regulating the recruitment of other DDR factors to sites of damage. For example, acetylation of histone H4 on Lys16 by TIP60 inhibits the recruitment of the c-NHEJ promoting protein 53BP1, switching repair of DSBs to the HR pathway (Tang *et al.*, 2013). Since HAT1 acetylates histone H4 Lys5 and Lys12, it would be worth studying if H4 acetylation plays a role in the recruitment of TopBP1 to sites of DNA damage. Another possibility is that the acetylation events performed by HAT1 could cause conformational changes in chromatin that eventually would allow TopBP1 recruitment. Such conformational changes caused by different PTMs have been reported to be critical in different DDR processes (Dantuma and Attikum, 2016). For example, acetylation of the H4 tail by the NuA4-Tip60 complex promotes a transition from the repressive state to the open structures in the chromatin after DSB induction that allows the recruitment of several DSB repair factors (Dhar *et al.*, 2017). However, the fact that TopBP1 and HAT1 interact suggests a more direct role for HAT1 in TopBP1 function.

The influence of HAT on TopBP1 role was small but reproducible, even in conditions of HAT1 knockout. Remarkably, several studies demonstrated that the absence of this acetyltransferase does not affect the efficiency of different processes in which it is known to function. For example, combining HAT1 deletion with mutations in the Lys residues of the histones that are known to be acetylated by HAT1 resulted in defects in telomeric silencing and DNA damage sensitivity, while HAT1 knockout alone had no effect on these processes (Kelly *et al.*, 2000; Qin and Parthun, 2002). If the effect of HAT1 on TopBP1 is indeed due to its acetylating activity, functional redundancy with p300, an acetyltransferase known to acetylate TopBP1

(Liu *et al.*, 2016), or another acetyltransferase, could explain why the observed effects after HAT1 knock down and knockout are so small.

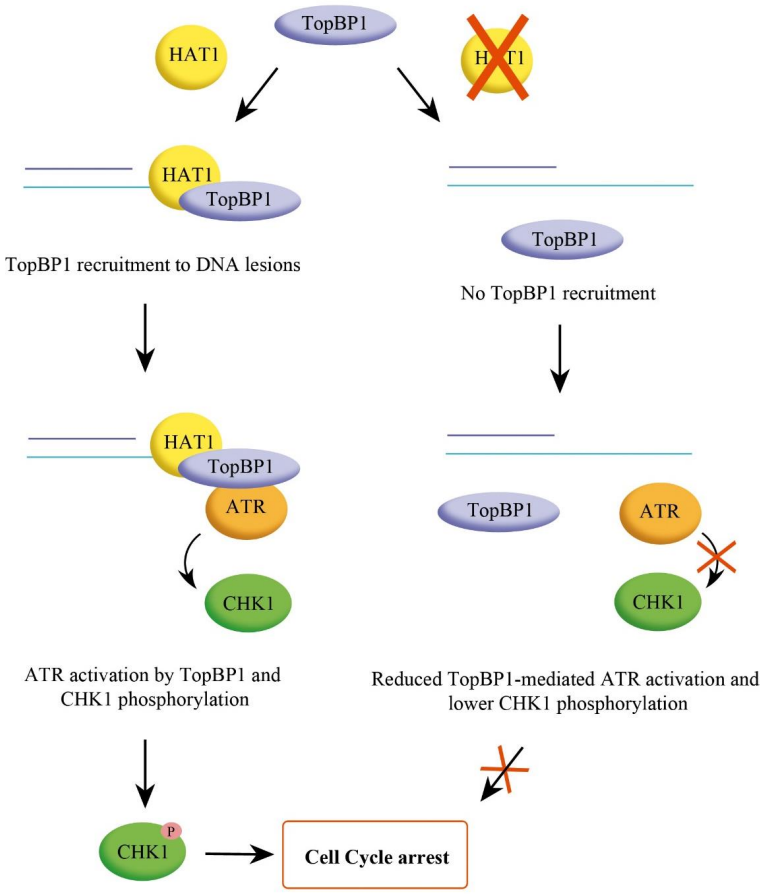

Figure 26. HAT1 in the ATR-CHK1 pathway. By contributing to the accumulation of TopBP1 on the chromatin, HAT1 controls TopBP1 interaction with ATR, subsequent CHK1 phosphorylation and the G_2 checkpoint arrest. In the absence of HAT1, the recruitment of TopBP1 to the chromatin is reduced, decreasing the amount of activated ATR and therefore of phosphorylated CHK1, resulting in an inefficient cell cycle arrest.

In conclusion, this study shows that HAT1 controls the DNA damage G_2 checkpoint through controlling TopBP1 localization to the chromatin and subsequent ATR activation, but further work is needed to clarify how HAT1 exactly contributes to this process.

Chapter III:

PHF2 regulates HR-mediated repair by controlling BRCA1 and CtIP levels

DSBs are the most toxic of DNA lesions that cells can suffer and, as such, need to be correctly detected and repaired to guarantee cell survival. As explained in the introduction of this work, DSBs are mainly repaired through two different pathways: c-NHEJ and HR. In c-NHEJ, both ends of the break are directly re-ligated in a process that involves a relatively small amount of proteins and requires little to no-resection of the DSB (Chang *et al.*, 2017). HR, on the other hand, is a more complex pathway that requires long stretches of resected DNA and uses the information contained in the sister chromatid to repair the DSB. Consequently, HR is only active during the S and G_2 phases of the cell cycle (Jasin and Rothstein, 2013).

The competition between BRCA1 and 53BP1 is the main factor that determines the pathway used to repair DSBs in S and G_2 phases. It is widely accepted that 53BP1, together with its partner RIF1, blocks CtIP-dependent resection of DNA ends, whereas BRCA1 alleviates this resection barrier, switching repair from c-NHEJ to HR (Bunting *et al.*, 2011). Therefore, regulation of these two proteins is key in a correct response to DSBs.

Histone Lys methylation plays an important role in DSB repair through different processes. This PTM marks the chromatin for the recruitment of different DDR proteins. For instance, 53BP1 recruitment requires (di)methylation of histone H4 on Lys20 (H4K20me/me2) (Botuyan *et al.*, 2007). This process relies on the RNF8/RNF168-mediated degradation of the histone demethylase KDM4A (Mallette *et al.*, 2012). Importantly, post-replicative DNA has reduced levels of methylated histone H4, inhibiting 53BP1 accumulation, thereby promoting the recruitment of BRCA1 through the binding of its partner BARD1 to histone H4K20me0 and switching repair from c-NHEJ to HR (Nakamura *et al.*, 2019). The retention of the BARD1-BRCA1 complex at damaged sites is also dependent on the presence of dimethylated Lys9 of histone H3 (H3K9me2) (Wu *et al.*, 2015). In addition, histone demethylases and methyltransferases control the expression of many DDR-involved genes through the regulation of transcription repression marks. For example, the histone methyl transferase KMT2C localizes to the promoters of the *ATM, ATR, BRCA1*, and *BRCA2* genes and enhances their expression by adding the transcriptional activation mark H3K4me3, increasing HR-mediated repair by promoting the expression of these factors (Rampias *et al.*, 2019).

3.1. Identification of novel chromatin modifiers involved in the DDR

PTM of histone and associated proteins are critical for a correct DDR. Although several chromatin modifications were reported to play a role in the early responses of the DDR, such as the recruitment of 53BP1 to sites of DNA lesions (Schultz *et al.*, 2000; Anderson *et al.*, 2001), we reasoned that more chromatin modulating enzymes were to be discovered in this response.

In order to identify new chromatin modifying proteins that are involved in the DDR, two screenings were performed in parallel, both using the same small library with expression vectors of different chromatin modifying proteins, mostly demethylases and acetyltransferases, as well as other related enzymes. The first approach consisted on transfecting 293T cells with these plasmids, treating them with IR and subsequently analyzing phosphorylation of histone H2AX on Ser139 (γ-H2AX) used as a marker of DNA damage. In the second approach, U2OS cells were transfected with individual plasmids of the same library, after which 53BP1 focus formation was assessed by immunofluorescence in the absence and presence of DNA damage (IR). The results for all analyzed proteins are summarized in Table 2.

Protein	γ-H2AX signal (Untreated/IR)	53BP1 focus formation	Activity
CLOCK	Normal	Normal	Lys acetyltransferase
CUL1	Normal	Normal	Part of ubiquitin ligase complex
CUL3	Normal	Normal	Part of ubiquitin ligase complex
CUL4A	Normal	Normal	Part of ubiquitin ligase complex
CUL4B	Increased	Decreased	Part of ubiquitin ligase complex
CUL5	Normal	Normal	Part of ubiquitin ligase complex
DDB1	Normal	Normal	Part of ubiquitin ligase complex
EP3000	Normal	Normal	Lys acetyltransferase
FBXW5	Normal	Normal	Part of ubiquitin ligase complex
KAT2A	Normal	Normal	Lys acetyltransferase
KAT2B	Increased	Normal	Lys acetyltransferase
KDM1A	Normal	Normal	Lys demethylase
KDM2A	Normal	Normal	Lys demethylase
KDM2B	Normal	Normal	Lys demethylase
KDM3A	Normal	Normal	Lys demethylase
KDM3B	Normal	Normal	Lys demethylase
KDM4A	Normal	Decreased	Lys demethylase
KDM4B	Normal/Decreased	Increased	Lys demethylase
KDM4C	Normal	Normal	Lys demethylase
KDM4D	Increased/Increased	Normal	Lys demethylase
KDM5B	Normal	Normal	Lys demethylase
KDM5C	Increased/Increased	Normal	Lys demethylase
KDM6A	Normal	Increased	Lys demethylase

KDM6B	Normal	Normal	Lys demethylase
KDM7	Increased/Normal	Normal	Lys demethylase
KDM8	Normal	Normal	Lys demethylase
PHF2	Increased/Normal	Decreased	Lys demethylase
PHF8	Increased/Normal	Normal	Lys demethylase
SKP2	Normal	Normal	Part of ubiquitin ligase complex
TAF1	Normal	Normal	Lys acetyltransferase

Table 2. Observed effects after overexpression of different chromatin modifying proteins and other related enzymes on H2AX phosphorylation and 53BP1 focus formation in 293T and U2OS cells.

The fact that different proteins already known to be involved in the DDR were detected in these screenings supported the validity of the results. For example, in our screening, overexpression of the histone demethylase KDM4B leads to reduced γ-H2AX levels, as previously observed by others (Young *et al.*, 2013; Castellini *et al.*, 2017; Deng *et al.*, 2018). Another histone demethylase, KDM4A, is known to control the recruitment of 53BP1 to DNA lesions through demethylation of H4K20me2, needed for the recruitment of this factor (Mallette *et al.*, 2012). Accordingly, our screening shows that overexpression of KDM4A inhibits 53BP1 focus formation.

Amongst all candidate proteins, the histone demethylase PHF2, also known as KDM7C or JHDM1E, was selected for further analysis. PHF2 is known to function as a transcriptional activator through demethylation of the repressive histone marks H3K9me2 and, to less extent, H3K9me3 (Baba *et al.*, 2011; Stender *et al.*, 2013). In this way, PHF2 has been linked to different processes, including adipogenesis inflammatory response and cell differentiation (Okuno *et al.*, 2013; Stender *et al.*, 2013; Kim *et al.*, 2014; Lee *et al.*, 2014) and, interestingly, was found to be mutated in different cancers (Sinha *et al.*, 2008; Sun *et al.*, 2013; Lee *et al.*, 2015; C. Lee *et al.*, 2017; J. H. Lee *et al.*, 2017) (see introduction).

We were encouraged to study the role of PHF2 in the DDR for two main reasons: this histone demethylase had not been linked to DNA repair before this project started and PHF2 overexpression gave a clear and reproducible phenotype in both readouts. First, its overexpression on 293T cells caused increased γ-H2AX levels in the absence of damage, but not after treatment with IR (fig. 27 A), which suggests that altering the levels of this protein causes DNA lesions. Second, cells with higher levels of PHF2 showed decreased 53BP1 focus formation after treatment with IR (fig. 27 B), suggesting that cells might have problems detecting the damage and/or repairing DSBs.

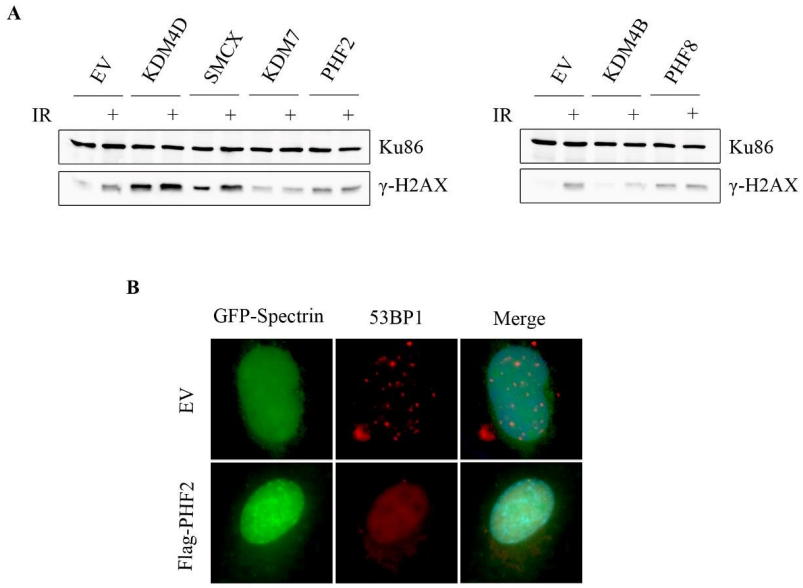

Figure 27. PHF2 as a candidate from two screenings searching for chromatin modifiers involved in the DDR. A) 239T cells were transfected with plasmids expressing different chromatin-modifying proteins, treated with IR (10 Gy, 1 h) and subsequently lysed and resolved by western blot using the indicated antibodies. B) U2OS cells were transfected with Flag-PHF2, fixed and analyzed by immunofluorescence with a 53BP1 antibody (red) after 1 h of IR (10 Gy). Co-transfection with GFP-Spectrin (green) allowed identification of transfected cells.

3.2. PHF2 depletion causes DNA damage and reduces cell viability

Following the results from our screening, we used different approaches to test if altering PHF2 levels indeed causes DNA damage. We started with analyzing the effect of depletion of PHF2 by siRNA. An SCGE, or comet assay, was carried out to directly detect the presence of DNA lesions in individual cells. As observed in figure 28, downregulation of PHF2 led to a pronounced increase of the tail moment in the absence of exogenous damage, indicating that cells present a higher number of DNA breaks. In accordance with this result, cells with lower PHF2 levels showed increased H2AX phosphorylation and γ-H2AX focus formation (fig. 29 A and B respectively).

Next, we performed colony formation experiments to test if PHF2 affects cell viability. As observed in figure 29 C, PHF2 depletion negatively affected cell growth in both U2OS and HeLa cells, demonstrating that downregulation of this protein reduces cell viability, possibly, although probably not exclusively, through affecting genomic stability.

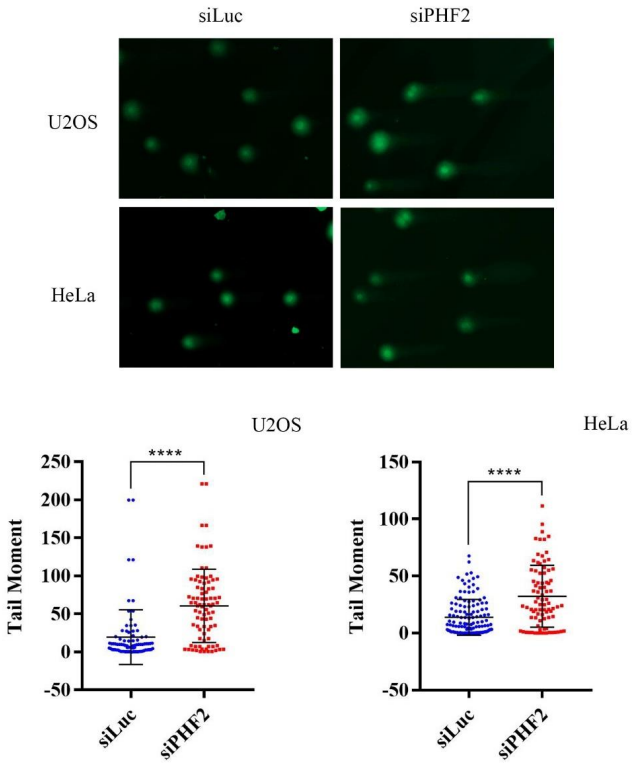

Figure 28. PHF2 downregulation causes DNA damage. U2OS and HeLa cells were depleted for Luc or PHF2 and subjected to alkaline SCGE. Three independent experiments were carried out and 50 cells were scored in each experiment. Average tail moment of the cells was measured, and statistical analysis was performed.

These data confirm that reducing PHF2 levels triggers DNA damage and, together with the overexpression data, suggests that maintaining stable levels of this protein is necessary for a correct maintenance of genomic stability.

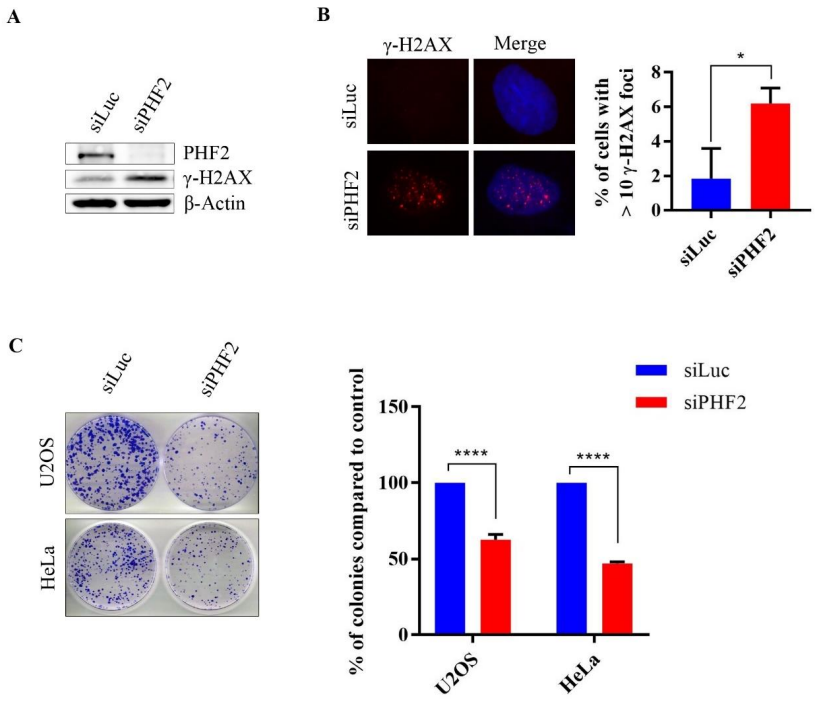

Figure 29. Depletion of PHF2 affects genome stability. A) and B) U2OS cells were depleted for Luc or PHF2, lysed and analyzed by western blot using the indicated antibodies, or fixed and analyzed by immunofluorescence using a γ-H2AX antibody, respectively. For B), three independent experiments were carried out and 100 cells were scored in each experiment. Mean ± SD was calculated and subjected to statistical analysis. C) U2OS and HeLa cells were transfected as in (A) and (B) and an equal number of cells were seeded and left to grow for 10 days. Colonies were then stained and counted. The number of colonies in the control condition (siLuc) was set to 100%. Mean ± SD from three independent experiments was calculated and subjected to statistical analysis

3.3. PHF2 downregulation inhibits DSB repair by HR

The increased tail moment observed in PHF2-depleted cells suggests that these cells are suffering more DNA breaks, or that lesions occurring in physiological conditions are not repaired as efficiently as in control cells. Importantly, alkaline SCGE, which was used here, detects both SSBs and DSBs, so this increased tail moment could be caused by both types of lesions. To test if the repair of DNA breaks was indeed impaired in cells downregulated for PHF2, cells were treated with different DNA-damaging agents and analyzed for CHK1 phosphorylation on Ser345. This phosphorylation is mediated by ATR after detection of RPA-coated ssDNA, which is generated during SSBs or after resection of a DSB, necessary for DSB repair by HR (Zou and Elledge, 2003; Lee and Paull, 2005; Maréchal and Zou, 2017) (see 3.1 of introduction). Upon knockdown of PHF2, the levels of phosphorylated CHK1 were the same as in control-depleted cells after treatment with UV and HU, but after treatment with IR, CPT and ETP, CHK1 phosphorylation was decreased compared to control cells (fig. 30 A). Given that these last three DNA-damaging agents induce DSBs much more efficiently than UV and HU (Mehta and Haber, 2014), this result suggested that PHF2 depletion decreases the presence

of ssDNA, possibly through the inhibition of the resection of DSBs. To test this hypothesis, we analyzed RPA2 phosphorylation on Ser4/Ser8 and RPA2 hyperphosphorylation, two markers commonly used for resection (Maréchal and Zou, 2015), upon DNA damage. As observed in figure 30 B, PHF2 depletion negatively affected RPA phosphorylation after treatment with CPT and ETP, indicating that the resection of DSBs was indeed compromised by PHF2 depletion.

Since DNA end resection usually leads to DSB repair by HR, it is expected that its inhibition would be accompanied by a defect in this repair pathway. Therefore, HR-mediated repair was assessed using the I-SceI-based DR-GFP system in cells depleted for PHF2. Downregulation of CtIP, critical for the resection of DSBs (Sartori *et al.*, 2007), was used as a positive control. Interestingly, depletion of both PHF2 and CtIP led to an inhibition of HR-mediated repair, as compared to control-depleted cells (fig. 30 C). Furthermore, repair by SSA, another resection-dependent repair pathway, was also decreased after PHF2 downregulation (fig. 30 D). Finally, since HR defects, such as BRCA1 depletion, are known to sensitize cells to PARP inhibition (Lord and Ashworth, 2008), we analyzed the survival of PHF2-depleted cells after treatment with the PARP1/2 inhibitor Olaparib. In accordance with the previous results, PHF2 knockdown decreased clonogenic survival after treatment with Olaparib as compared to control, albeit to a lesser extent as the positive control of BRCA1 depletion (fig. 30 E). These data confirm that PHF2 promotes the resection of DSBs and thereby homology-mediated DSB repair.

Because HR is mostly active in S and G_2 phases due to the availability of a sister chromatid as repair template (Liu *et al.*, 2008), the effect of PHF2 on this repair pathway could be caused by the indirect consequence of a change in cell cycle distribution upon PHF2 knockdown. To discard this possibility, PHF2 was depleted from U2OS cells, followed by PI staining and analysis by flow cytometry. Indeed, PHF2 depletion did not affect the cell cycle as compared to control cells (fig. 31), indicating that the HR defect observed in PHF2-depleted cells was not caused by alterations in cell cycle distribution.

3.4. PHF2 recruitment to sites of damage could not be observed

To determine if PHF2 accumulates at sites of damage, a common behavior of many proteins of the DDR, two different approaches were used. First, cells were co-transfected with a Flag-tagged version of PHF2 and mCherry-Nbs1, used as a positive control, subjected to laser-induced damage and stained with antibodies against Flag and γ-H2AX, the latter to identify the track that was damaged. During the analyzed time period, no recruitment of PHF2 to laser-induced tracks could be detected, while mCherry-Nbs1 efficiently localized to damaged sites (fig. 32 A).

Next, U2OS 2-6-3 cells expressing inducible FokI-mCherry-LacR were transfected with Flag-PHF2 and treated with 4-OH and Shield-I to induce FokI activity and consequent DSB generation. In accordance with the previous experiment, no Flag-tagged PHF2 was detected at FokI-induced DSBs (fig. 32 B).

In conclusion, PHF2 recruitment to DSBs could not be detected with the available techniques. Although we formally cannot exclude PHF2 accumulation at DNA damage sites, these results suggest that PHF2 does not localize to sites of DNA lesions and that PHF2 plays a more global role in the DDR.

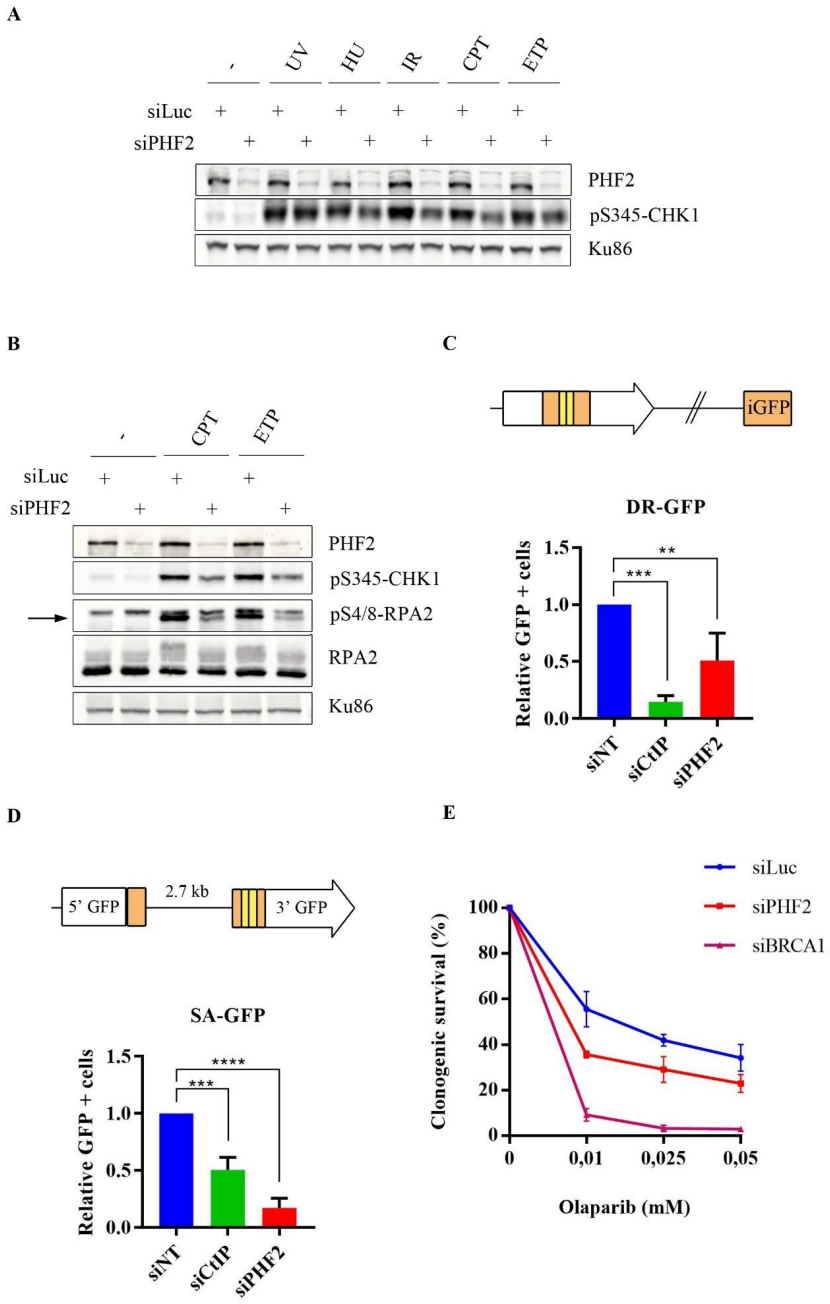

Figure 30. PHF2 depletion impairs resection and HR-mediated repair of DSBs. A) and B) U2OS cells were transfected with siRNA oligos against Luc or PHF2 and treated with either UV, HU, IR, CPT or ETP for 1 hour. Cell lysates were analyzed by western blot using the indicated antibodies. Arrow indicates pS4/8-RPA. C) U2OS

cells stably expressing a single copy of the DR-GFP construct were depleted for CtIP, PHF2 or non-targeting (NT, control) and GFP-positive cells were analyzed by FACS. For quantification, the percentage of GFP-positive cells in control was put to 1. The mean ± SD from three independent experiments was plotted and statistical analysis was performed. D) Same as in (C) but using U2OS cells carrying the SSA reporter construct. E) HeLa cells were depleted for Luc, PHF2 or BRCA1 and an equal number of cells was seeded and incubated for clonogenic survival assays in presence or absence of different concentrations of Olaparib. For each condition, the number of colonies present in the absence of treatment was set to 100%. Mean ± SEM from 4 different independent experiments are shown.

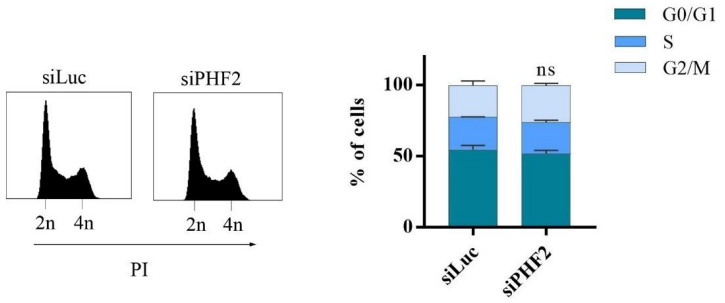

Figure 31. PHF2 depletion does not affect cell cycle distribution. U2OS cells were transfected with siRNA oligos against Luc or PHF2 and analyzed for PI by flow cytometry. Mean ± SD from three independent experiments was plotted and statistical analysis was performed.

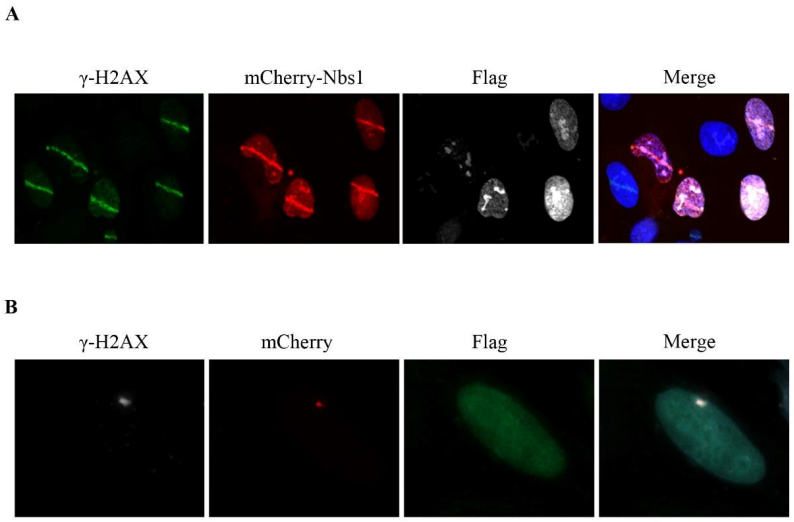

Figure 32. No PHF2 accumulation at DNA damage sites could be observed A) U2OS cells were transfected with Flag-PHF2 and mCherry-Nbs1 and DNA damage was induced through multiphoton laser irradiation. Accumulation of the indicated proteins at laser stripes was analyzed through immunofluorescence using the indicated antibodies. B) U2OS 2-6-3 cells were transfected as in (A) and treated with 4-OHT and Shield1 for 4 hours, to induce FokI expression and nuclear localization. Recruitment of different factors to FokI-induced DSB was then analyzed by immunofluorescence.

3.5. PHF2 regulates BRCA1 and CtIP levels

Since PHF2 acts as a transcriptional activator, mainly by removing the H3K9me2 histone mark (Baba *et al.*, 2011), we reasoned that the effect observed on homology-directed repair of DSBs after depletion of PHF2 could be caused by changes in the expression level of different HR-involved factors. To test this, analysis by western blot were carried out. As observed in figure 33 A, downregulation of PHF2 negatively affected CtIP and BRCA1 levels, but the expression of Rad51, RPA2, 53BP1, Nbs1 and Mre11 remained unchanged. Downregulation of PHF2 performed using two additional oligos also caused a reduction in CtIP and BRCA1 levels, ruling out a possible off-target effect of the siRNA oligo that was initially used. In accordance with these results, overexpression of PHF2 increases BRCA1 and CtIP protein levels (fig. 33 B), and, in addition, partially rescues the defect observed after PHF2 depletion (fig. 33 C and D).

As it was likely that PHF2 controls the levels of BRCA1 and CtIP through transcriptional regulation, we measured the expression of the *BRCA1* and *CtIP* genes by RT-PCR. The genes encoding Mre11 and RPA2, two proteins for which no changes were observed in the western blot analysis, were analyzed as controls. Indeed, PHF2 depletion decreased BRCA1 and CtIP mRNA levels, but not Mre11 or RPA2 (fig. 33 E).

Together, these data indicate that PHF2 acts as a transcriptional activator of the *BRCA1* and *CtIP* genes, controlling their mRNA levels, and consequently regulating BRCA1 and CtIP protein levels in the cell.

3.6. Loss of PHF2 impairs the recruitment of HR factors to DNA damaged sites

As already mentioned in this work, BRCA1 and CtIP are essential for the initiation of HR-mediated repair of DSBs: BRCA1 alleviates the resection barrier posed by 53BP1 during the S and G_2 phases of the cell cycle, switching repair to HR, while CtIP stimulates Mre11 endonuclease activity and thereby DNA end resection (Limbo *et al.*, 2007; Sartori *et al.*, 2007; Bunting *et al.*, 2011; Anand *et al.*, 2016). To test if the observed decreased levels of BRCA1 and CtIP also results in diminished recruitment of BRCA1 and CtIP to damaged sites and consequently prevents them from performing their HR-promoting activity, we analyzed the accumulation of these proteins to DNA lesions after PHF2 depletion. First, BRCA1 focus formation was assessed using a RAP80 KO cell line, to avoid BRCA1-A complex formation and subsequent HR suppression (Wang and Elledge, 2007; B. Wang *et al.*, 2013; Kakarougkas *et al.*, 2013). As observed in fig. 34 A, PHF2 downregulation inhibited IR-induced BRCA1 focus formation. Next, we monitored the accumulation of GFP-CtIP onto laser-induced damage after PHF2 depletion, using mCherry-Nbs1 as a control. PHF2 depletion compromised CtIP but not Nbs1 recruitment to damaged sites (fig. 34 B). In conclusion, PHF2 depletion inhibits the accumulation of BRCA1 and CtIP at DNA DSBs.

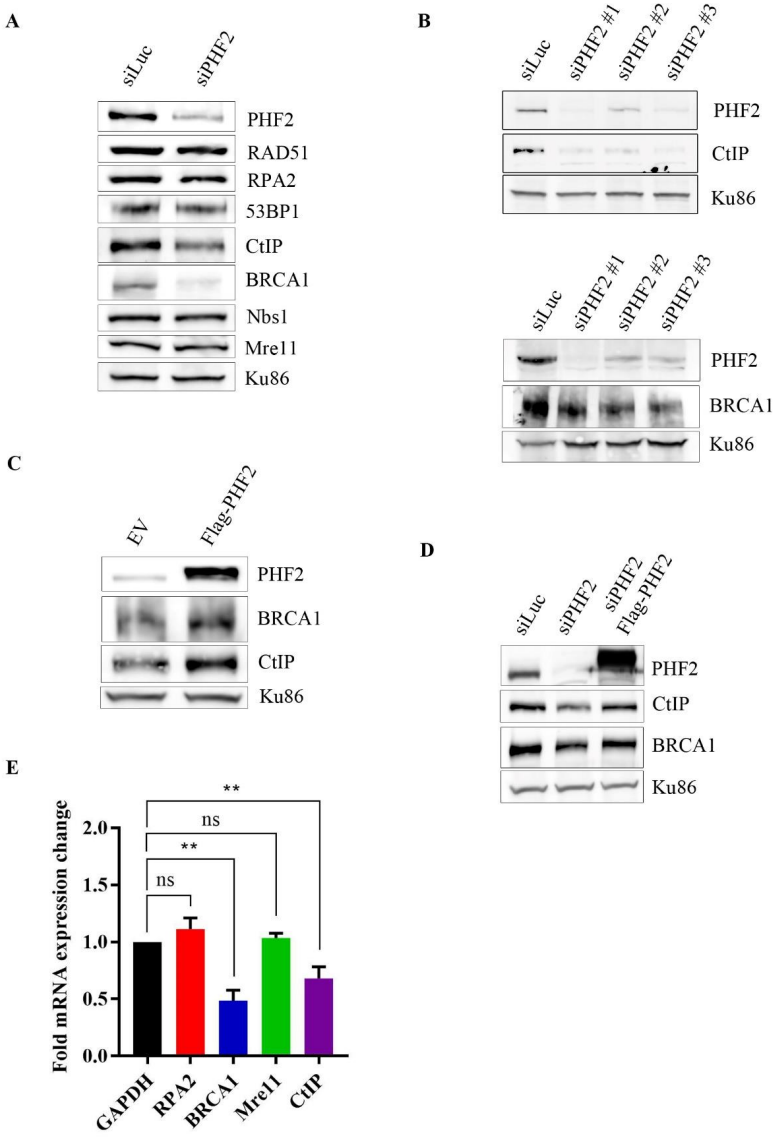

Figure 33. PHF2 controls BRCA1 and CtIP levels. A) U2OS cells were depleted for Luc or PHF2, lysed and analyzed by western blot for the indicated proteins. B) U2OS cells were transfected as in (A) using three different siRNA oligos targeting PHF2, lysed and analyzed by western blot using the indicated antibodies. C) U2OS cells were transfected with an EV or Flag-PHF2, lysed and analyzed by western blot for the indicated proteins. D) U2OS cells were depleted for Luc or PHF2 and, 24 hours later, transfected with EV or Flag-PHF2. Extracts were analyzed by western blot. E) U2OS cells were depleted for Luc or PHF2, after which RNA was purified and analyzed by RT-PCR. Mean ± SD from three independent experiments was calculated and statistical analysis was performed. For each experiment, mRNA levels of control cells were put to 1.

Decreased CtIP recruitment to DNA lesions should affect DSB resection, and thereby diminish the presence of ssDNA. Since the RPA complex binds these structures, RPA accumulation should also be inhibited by PHF2 downregulation. As expected, damage-induced RPA2 focus formation was compromised after PHF2 depletion (fig. 35 A). Consequently, PHF2 depletion also inhibited Rad51 recruitment to DNA lesions, as observed by immunofluorescence (fig. 35 B) and chromatin fractionation (fig. 35 C). Importantly, expressing an siRNA-resistant form of PHF2 reverted the decreased RPA2 and Rad51 focus formation caused by PHF2 depletion (fig. 36 A and B).

Together, these results demonstrate that PHF2 regulates the recruitment of the HR-involved factors CtIP, BRCA1, RPA2 and Rad51 to DSBs.

3.7. PHF2 depletion promotes the retention of 53BP1 at damage sites

HR-mediated repair requires the dephosphorylation of 53BP1 and the consequent release from its partner RIF1. This process is mediated by BRCA1 and causes 53BP1 to vacate DNA damaged sites, allowing the resection machinery to generate the ssDNA needed for the subsequent steps of the HR pathway (Isono *et al.*, 2017). Since loss of PHF2 diminished BRCA1 levels, we reasoned that this event should be accompanied by a change in 53BP1 focus formation dynamics. PHF2 was therefore downregulated after which 53BP1 focus formation was analyzed at different timepoints after irradiation, using CtIP downregulation as a positive control. One hour after IR, most cells were positive for 53BP1 foci, both in control and PHF2 or CtIP depleted cells, as expected. However, although 53BP1 focus formation in control cells diminished al later time points, in cells depleted for PHF2 or CtIP, the percentage of cells with 53BP1 foci remained high (fig. 37 A), indicating that 53BP1 was not released correctly from damaged sites. This was further confirmed by quantifying the number of foci per cell 7 hours after IR (fig. 37 B).

Next, we tested the effect of PHF2 overexpression on 53BP1 focus formation. As suggested by the results of our screening (fig. 27 B), Flag-PHF2 transfection reduced 53BP1 retention at DNA damaged sites (fig. 37 C) and partially rescued the increase caused by PHF2 depletion (fig. 37 D).

In conclusion, changes in PHF2 levels alter 53BP1 focus formation dynamics, further supporting a role for this protein in DSB repair pathway choice.

DISCUSSION

In this study we have uncovered a new role for the histone demethylase PHF2 in DSB repair. In a screening searching for enzymes that modulate chromatin involved in the DDR, we found that overexpression of PHF2 inhibited IR-induced 53BP1 focus formation, while increasing H2AX phosphorylation in undamaged conditions, suggesting that altering the levels of this enzyme leads to an increase in the presence of unrepaired DNA breaks. Further analysis showed that loss of PHF2 impaired the accumulation of key HR factors BRCA1 and CtIP to DNA lesions and thereby inhibited resection, as observed by RPA2 (hyper)phosphorylation and focus formation. Consequently, Rad51 loading was inhibited and HR could not ensue, leading to retention of 53BP1 foci and increased sensitivity to PARP inhibition. Possibly due to the defect in HR repair observed in PHF2-depleted cells, these cells also showed increased DNA lesions in conditions without exogenous damage, as observed by SCGE and H2AX phosphorylation analysis.

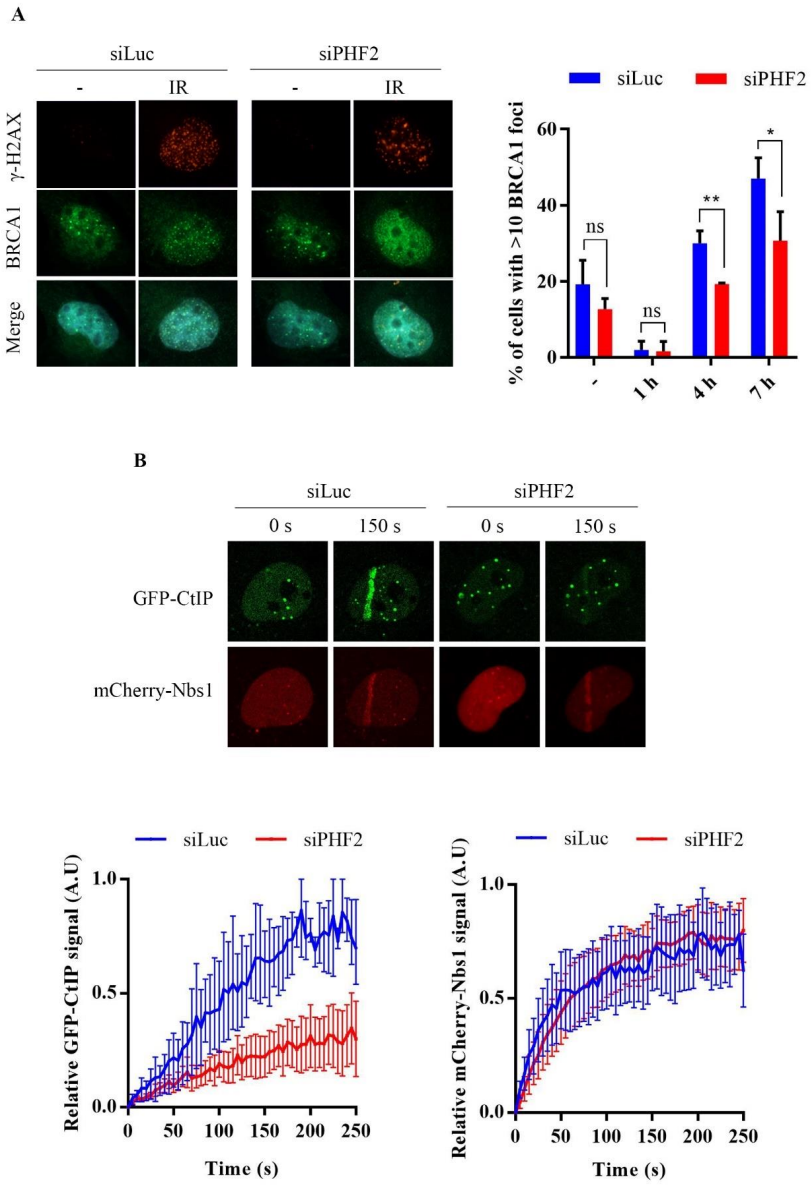

Figure 34. PHF2 promotes the recruitment of CtIP and BRCA1 to damage sites. A) U2OS RAP80 knockout cells were depleted for Luc or PHF2, treated with IR (10 Gy) and analyzed by immunofluorescence using the indicated antibodies at the indicated timepoints. For each experiment, 100 cells were analyzed. Mean ± SD from three independent experiments was plotted and statistical analysis was performed. B) U2OS cells were depleted as in (A) and transfected with GFP-CtIP and mCherry-Nbs1. The recruitment of CtIP and Nbs1 to laser-induced

damage was assessed by fluorescent microscopy. For the quantification, the maximum detected fluorescence was put to 1. Three independent experiments were carried out, 50 cells were analyzed in each of them and mean ± SD from was plotted.

This defect in HR-mediated repair was caused, at least partially, by the observed decrease in BRCA1 and CtIP protein levels after PHF2 downregulation. Importantly, such decrease could be induced through depletion of PHF2 by three different siRNA oligos. Moreover, overexpression of PHF2 had the opposite effect and it could partially rescue the decrease observed after PHF2 depletion, demonstrating that the reduction of BRCA1 and CtIP protein levels was not due to off-target effects. Finally, the fact that BRCA1 and CtIP mRNA levels were reduced after PHF2 depletion strongly suggests that PHF2 regulates BRCA1 and CtIP transcription in a direct manner.

Further studies are needed to clarify if PHF2 inhibits HR-mediated repair through additional processes, other than *BRCA1* and *CtIP* transcriptional regulation. PHF2 depletion reduced BRCA1 recruitment to damage sites in a RAP80 KO background, but did not seem to affect this protein's role in replication, since cell cycle distribution and BRCA1 focus formation in undamaged cells were unaffected. It is possible that the decreased BRCA1 levels observed after PHF2 depletion was not high enough to affect its role in replication, but sufficient to prevent accumulation of this protein at damage sites, but an active role for this protein in the recruitment of BRCA1 to DSBs cannot be discarded. PHF2 depletion also hindered the accumulation of CtIP to DNA lesions, induced by laser microirradiation. It is known that BRCA1 ubiquitinates CtIP and this ubiquitination promotes CtIP recruitment to DNA lesions (Yu *et al.*, 2006). Given that PHF2-depleted cells displayed reduced BRCA1 levels, lower CtIP ubiquitination could account for this effect, although we cannot exclude the possibility of PHF2 regulating CtIP at different levels, as in the case of the recently reported splicing factor SF3B, which controls both CtIP mRNA levels and recruitment of CtIP to chromatin, but in independent manners (Prados-Carvajal *et al.*, 2018). However, since PHF2 does not seem to localize to DNA lesions, and no interaction with CtIP or BRCA1 could be detected (data not shown), it seems unlikely that this histone demethylase functions directly at damaged sites.

Future work will clarify the mechanism through which PHF2 inhibits the transcription of the *BRCA1* and *CtIP* genes. PHF2 frequently acts a transcriptional activator by erasing the dimethylated Lys9 of histone H3 (H3K9me2) and, although to less extent, the trimethylated Lys20 of histone H4 (H4K20me) repression marks (Baba *et al.*, 2011; Stender *et al.*, 2013). The transcriptional effect of PHF2 on *BRCA1* and *CtIP* is likely achieved through demethylation of either or both the above-mentioned repression marks. A chromatin immunoprecipitation (ChIP) analysis of the H3K9me2 and H4K20me marks at the promoters of these two genes after PHF2 depletion could confirm this hypothesis. Moreover, it has been reported that PHF2 can inhibit transcription independently of its demethylase activity. Instead, PHF2 represses transcription by competition with PHF8 for binding to the promoter and by recruiting SUV39H1, the H3K9me2/3 methyltransferase (Shi *et al.*, 2014). This form of transcriptional repression could be an alternative mechanism of regulating *BRCA1* and *CtIP*.

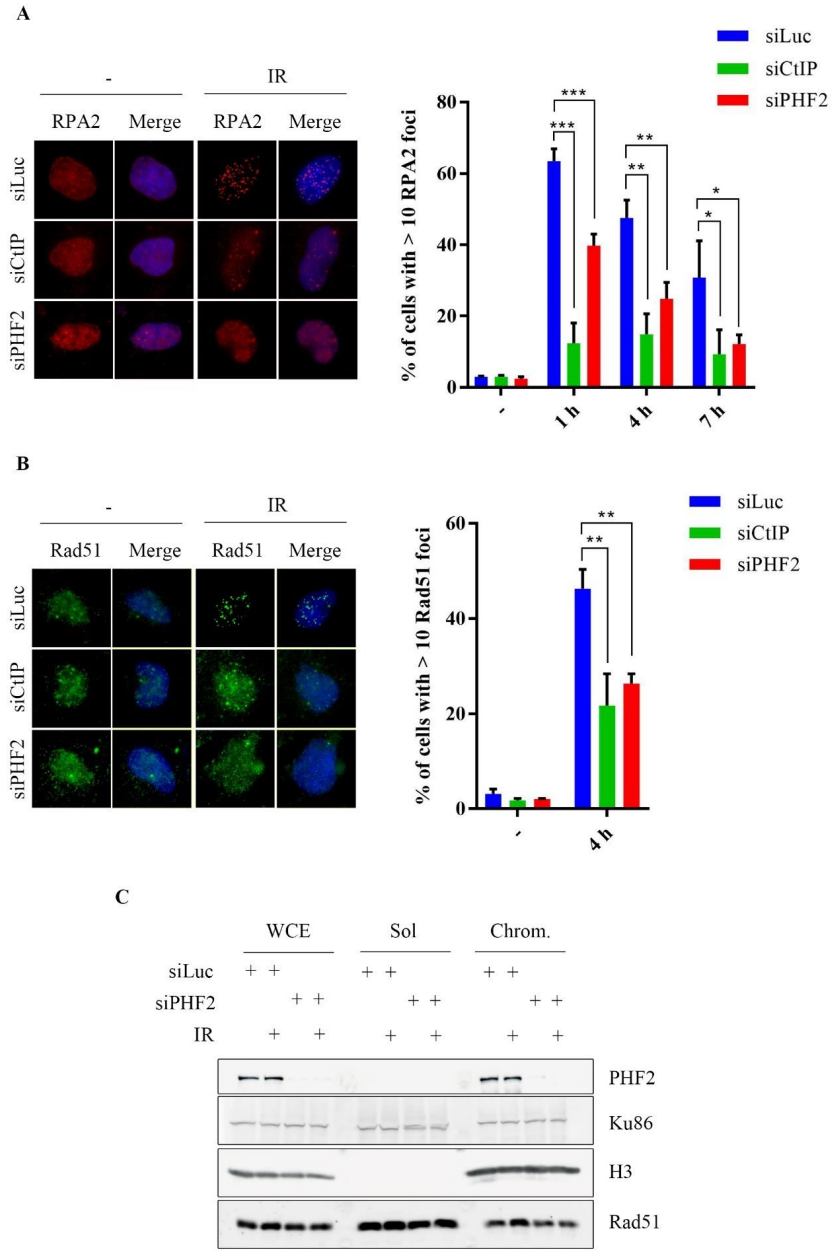

Figure 35. PHF2 depletion impairs RPA2 and Rad51 accumulation at DNA damaged sites. A) and B) U2OS were depleted for Luc or PHF2. treated with IR (10 Gy) and analyzed by immunofluorescence using the indicated antibodies after the indicated timepoints. For each experiment, 100 cells were analyzed. Mean ± SD from three

independent experiments was plotted and statistical analysis was performed. C) U2OS cells were transfected as in (A) and (B), subjected to chromatin fractionation and analyzed by western blot using the indicated antibodies.

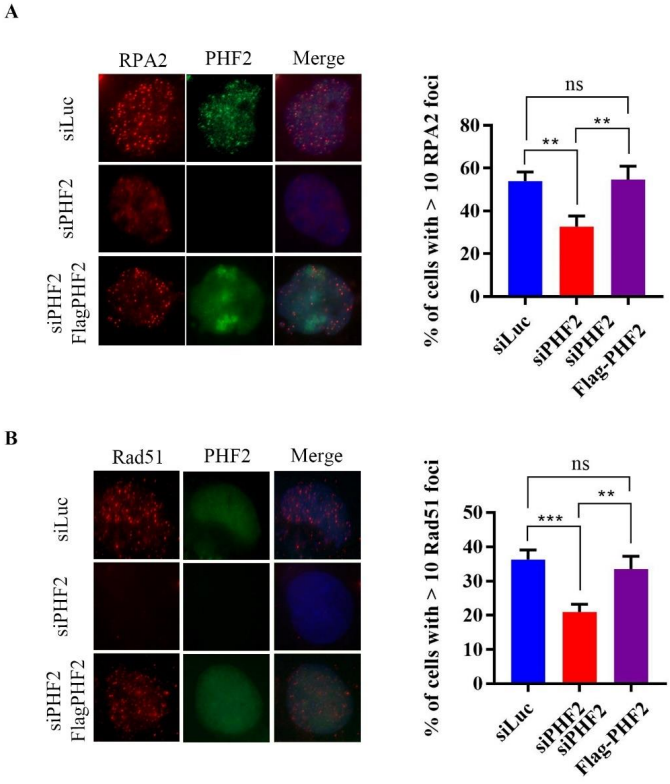

Figure 36. Restoration of PHF2 expression rescues RPA2 and Rad51 focus formation. A) U2OS cells were depleted for Luc or PHF2. 24 hours later, transfection with siRNA-resistant Flag-PHF2 was carried out. Cells were then treated with IR (10 Gy) and, after 4 hours, analyzed by immunofluorescence using PHF2 and RPA2 antibodies. B) Cells were transfected as in A), treated with IR (10 Gy) and, after 1 hour, analyzed by immunofluorescence using PHF2 and Rad51 antibodies. For both A) and B), 50 transfected cells in each experiment were analyzed. Mean ± SD from three independent experiments was plotted and statistical analysis was performed.

Besides transcriptional regulation, PHF2 could affect HR efficiency in additional ways. One possibility is that the lack of PHF2 prevents the demethylation of Lys residues needed for the recruitment of certain DSB repair factors. Furthermore, PHF2 could compete for the binding to methylated histones with c-NHEJ-promoting factors that, in the absence of this histone demethylase, would accumulate at a higher rate, thereby inhibiting HR-mediated repair. This kind of allosteric competition has been reported to regulate the recruitment of 53BP1. For example, both 53BP1 and lethal(3)malignant brain tumor-like protein 1 (L3MBTL1) compete for the binding to histone H4K20me2. Upon DNA damage, L3MBTL1 is ubiquitinated in a RNF8/RNF168-dependent manner and consequently removed by p97, increasing the binding of 53BP1 to this methylated residue and thereby promoting c-NHEJ-mediated repair (Acs *et*

al., 2011). Finally, it is also worth mentioning that PHF2 was reported to demethylate non-histone proteins and that the methylation of some DDR proteins, such as MDC1, is critical for their function (Kim *et al.*, 2014; Watanabe *et al.*, 2018).

Overexpression of PHF2 was observed in esophageal and renal cell carcinomas, whereas the *PHF2* gene is deleted or hypermethylated in its promoter region in breast cancer and mutated in colon and gastric cancers (Sinha *et al.*, 2008; Sun *et al.*, 2013; C. Lee *et al.*, 2017; J. H. Lee *et al.*, 2017). Altogether, these observations indicate that PHF2 plays a role in the onset and/or development of cancer. The new function of PHF2 as a new regulator of the homology-directed DSB repair found here suggests that this histone demethylase might have an important role in controlling genomic stability and could be important in the understanding and treatment of these cancers. Our results showing that PHF2-defective cells display increased sensitivity to PARP inhibition could be important for the treatment of breast and ovarian cancers without mutations in BRCA1/2 or other known HR proteins. Finally, it is worth mentioning that the use of histone demethylase inhibitors in cancer treatment is under current investigation and has a promising future (Højfeldt *et al.*, 2013; Morera *et al.*, 2016).

In conclusion, we propose that the histone demethylase PHF2 controls the resection of DSBs and thereby HR-mediated repair through transcriptional regulation of the *BRCA1* and *CtIP* genes. Such regulation could be achieved through demethylation of the histone residues that act as transcriptional repression marks present in the promoters of these genes (fig. 38).

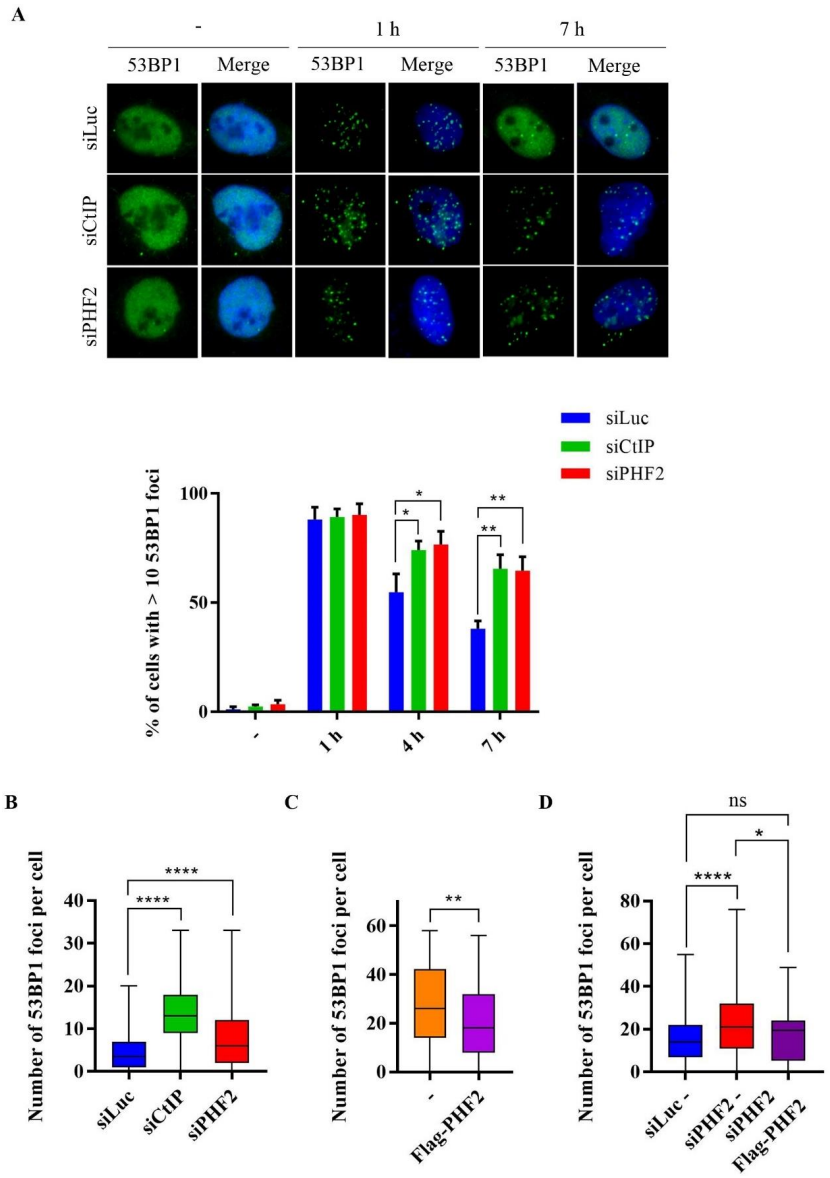

Figure 37. PHF2 knock down alters 53BP1 focus formation dynamics. A) U2OS cells were depleted for Luc, CtIP or PHF2, treated with IR (3 Gy) and analyzed by immunofluorescence using a 53BP1 antibody at different timepoints. B) U2OS cells were transfected as in (A), treated with IR (3 Gy) and, after 7 h, fixed and analyzed by immunofluorescence for 53BP1. C) U2OS cells were transfected with an EV (-) or Flag-PHF2, treated with IR (3 Gy) and, after 7 hours, analyzed by immunofluorescence using Flag and 53BP1 antibodies. D) U2OS cells were depleted for Luc or PHF2. 24 hours later, transfection with either an EV or an siRNA-resistant Flag-PHF2 was carried out. Cells were then treated with IR (3 Gy) and, after 7 hours, analyzed by immunofluorescence as in (C).

For each experiment, 100 cells, in the case of A and B, or 50 transfected cells (in C and D) were analyzed. Mean ± SD from three independent experiments was plotted and statistical analysis was performed.

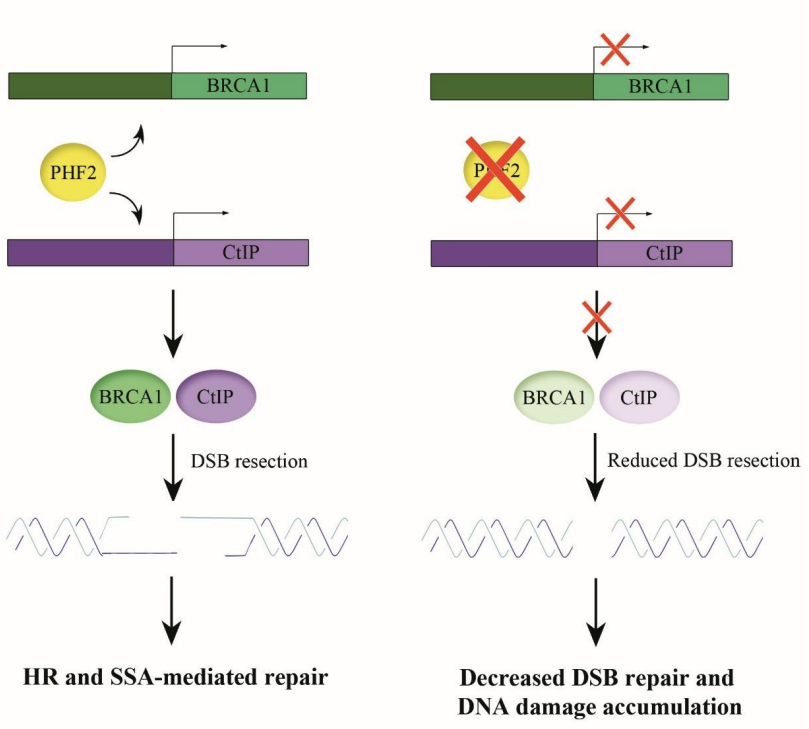

Figure 38. PHF2 regulates HR-mediated repair by controlling the expression of *BRCA1* and *CtIP*. In unperturbed conditions, PHF2 binds the *BRCA1* and *CtIP* promoters, stimulating transcription of these two genes, possibly by removing silencing histone methylation marks. Consequently, the presence of adequate levels of BRCA1 and CtIP allow DNA end resection and homology-directed repair. In the absence of PHF2, BRCA1 and CtIP levels decrease, preventing resection and therefore efficient DSB repair, leading to the accumulation of DNA damage.

Chapter IV:

A novel role for PHF6 in the DDR

Recovery from the G_2 DNA damage checkpoint is a controlled process which involves active shut down of ATR-mediated signaling and activation of CDK1 to promote entry into mitosis (see introduction). However, in addition to reparation of the DNA lesion, the chromatin structure needs to be restored before cells can continue the cell cycle. To identify chromatin-associated regulators involved in the recovery from the G_2 DNA damage checkpoint, an RNA interference screen was performed in human U2OS cells by our collaborator Dr. Daniël O. Warmerdam. An siRNA-based library targeting 529 genes related to the structure, maintenance or modification of chromatin (Baas *et al.*, 2014), was used to screen for the efficiency of cell cycle committed cells to recover after treatment with a non-lethal dose of ionizing radiation (IR). Cells, synchronized in G_2, were irradiated and mitotic entry was determined using immunofluorescence analysis of phosphorylated histone H3. The plant homeodomain finger protein 6 (PHF6), amongst others, showed a reduced checkpoint recovery. PHF6 is a member of the NuRD chromatin remodeling complex that was found to be mutated in the Börjeson-Forssman-Lehmann (BFL) syndrome, a rare genetic disease that causes intellectual disability, obesity, and growth defects (Lower *et al.*, 2002). Mutations in PHF6 have also been linked to leukemia (Mullighan and Holmfeldt, 2010; Van Vlierberghe *et al.*, 2014) and PHF6 was identified as a potential sensitizing factor for drug treatment in resistant glioblastoma (Hiddingh *et al.*, 2014). Together, these data point to a role for this protein in tumorigenesis. At a cellular level, PHF6 has been linked to rDNA transcription repression (J. Wang *et al.*, 2013; Todd *et al.*, 2016), but little is known about how this protein might function in other cellular processes. Here we studied the mechanism by which PHF6 regulates DNA damage checkpoint recovery.

4.1. PHF6 depletion hinders recovery from IR-induced G_2 arrest

To confirm that PHF6 was involved in the recovery from the G_2 checkpoint arrest and rule out possible off-target effects of the siRNA oligonucleotides used in the initial screening, CRISPR/Cas9 technology was used to stably knock out PHF6 in U2OS cells. As observed in figure 39 A, several clones lacking PHF6 were successfully obtained. Two of these knockout clones were then used to analyze recovery from IR-induced G_2 arrest following the same protocol used in the screening: cells were synchronized in G_1/S by thymidine and subsequently released for 7 hours for cells to be in the G_2 phase of the cell cycle. Next, cells were either treated with IR (2 Gy) or left untreated and nocodazole was added one hour later to arrest cells in mitosis. After 16 hours, the cells were collected and analyzed by flow cytometry for phospho-histone H3 (pHH3), a marker of mitosis (fig. 39 B). As observed in figure 39 C, relative recovery was decreased in clones #1 and #2 compared to the control, although clone #1 showed a stronger phenotype. This confirmed the results obtained in the initial screening, which identified PHF6 as an important factor for the recovery after IR-induced G_2 arrest.

Next, we tested if knockout of PHF6 had any effects on cell cycle distribution, since this could explain the differences observed in the G_2 checkpoint recovery assays. However, the absence of PHF6 did not affect the cell cycle distribution (fig. 40 A). As explained in section 3 of the introduction, the G_2 DNA damage checkpoint is mainly governed by the ATR/CHK1 pathway of the DDR. Upon the induction of DNA damage, ATR is activated and phosphorylates CHK1 on Ser317 and Ser345 (Liu *et al.*, 2000; Walworth and Bernards, 2016). CHK1 then phosphorylates different targets, including Cdc25, thereby preventing CDK1 activation and progression into mitosis (Peng *et al.*, 1997; McGowan *et al.*, 1998; Lee *et al.*, 2001; Donzelli and Draetta, 2003; Xiao *et al.*, 2003). As the observed decrease in the recovery from the G_2 arrest in PHF6 knockout cells could be due to a more efficient checkpoint arrest, we analyzed

CHK1 phosphorylation in these cells at different times after DNA damage induction. Importantly, IR-induced CHK1 phosphorylation was not altered in the absence of PHF6 after IR treatment (fig. 40 B), demonstrating that knockout of PHF6 does not affect the ATR-CHK1. Accordingly, IR treatment caused the same decrease in mitotic cells in PHF6 knockout cells as compared to WT (fig. 41 C), showing that the G_2 arrest is functioning correctly in these conditions.

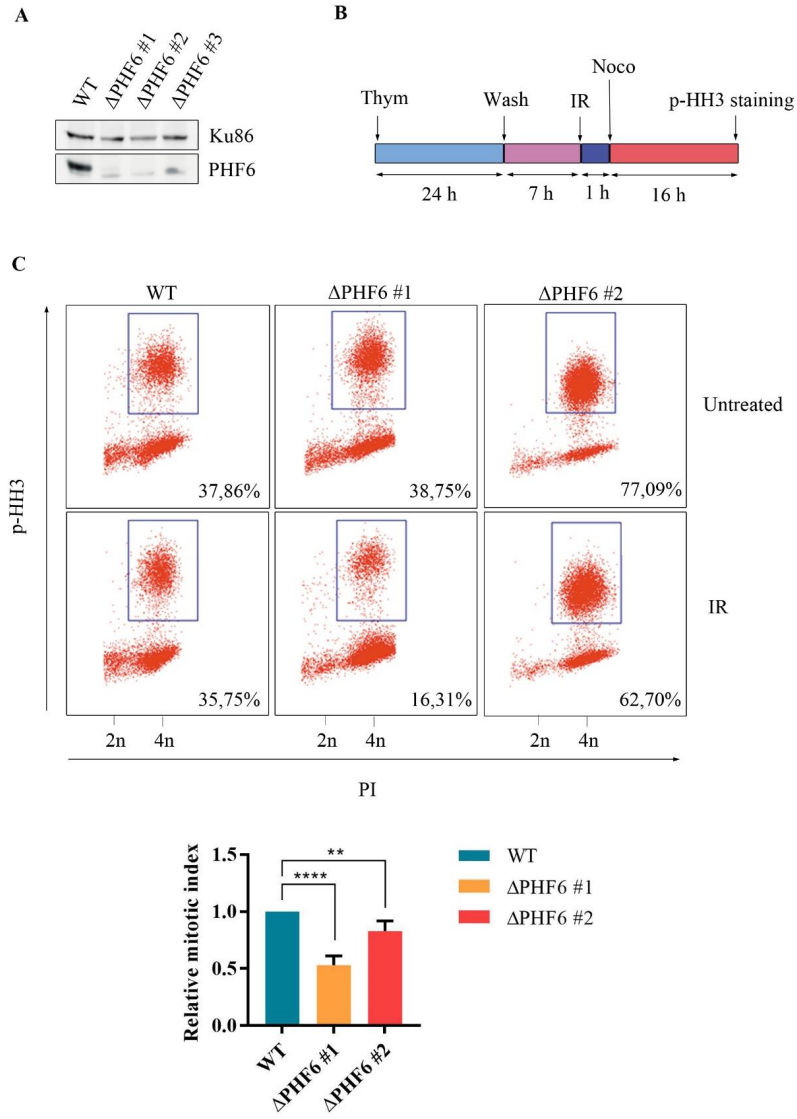

Figure 39. PHF6 knockout compromises recovery from the G_2 checkpoint arrest. A) Western blot showing CRISPR/Cas9-mediated PHF6 knockout in U2OS cells. B) Representation of the assay used to analyze recovery from G_2 checkpoint arrest. C) FACS analysis of mitotic cells in WT and PHF6 knockout U2OS cells, using the

assay shown in B) (upper panel). For each condition, the reduction in mitotic cells from untreated to IR-treated cells was calculated, and the reduction observed in WT cells was put to 1. Mean ± SD of three experiments was calculated and statistical analysis was performed (lower panel).

To confirm that the absence of PHF6 was responsible for the defect in the G_2 checkpoint recovery observed and rule out possible off-target effects derived from the CRISPR/Cas9-mediated knockout procedure, a GFP-tagged version of PHF6 was re-introduced into knockout cells (fig. 41 A) and their ability to recover from the IR-induced G_2 checkpoint arrest was assessed as explained before. Indeed, expression of exogenous PHF6 partially restored the ability of these cells to recover from DNA damage-induced cell cycle arrest (fig. 41 B).

Altogether, these data demonstrate that PHF6 knockout restrains efficient recovery from the IR-induced G_2 arrest without affecting the cell cycle or the ATR-CHK1 pathway.

4.2. PHF6 is involved in DSB repair

Since G_2 arrested cells are thought only to progress to mitosis once the damage has been repaired, we wondered if PHF6 could be involved in DNA repair, instead of playing an active role in recovery. To test if the lack of PHF6 resulted in more DNA lesions, neutral SCGE (comet assay) analysis was used to determine the extent of DNA damage in WT and PHF6 knockout cells in unperturbed conditions and 1 hour after treatment with IR. The absence of PHF6 led to an increased tail moment in both untreated and treated conditions, indicating that these cells accumulate more DNA lesions (specifically DSBs, as these are detected in the neutral assay) than the WT cells (fig. 42). Next, we confirmed these data by analyzing γ-H2AX focus formation at different timepoints after IR. As observed in figure 43 A, the absence of PHF6 caused an increase in γ-H2AX foci, suggesting that PHF6 knockout cells had problems repairing the IR-induced DNA breaks. Accordingly, these cells were more sensitive to IR in a clonogenic survival analysis (fig. 43 B).

The increased sensitivity to IR and the presence of more DNA lesions suggested a decreased DSB repair efficiency upon PHF6 knock out. Interestingly, GFP-PHF6 rapidly accumulated at laser-induced damage (fig. 44), which suggests that PHF6 plays an active role at the sites of DNA breaks.

Next, to directly test if DSB repair was affected by the absence of PHF6, we used the DR-GFP and the GC92 cell lines. Both cell lines express constructs containing an I-SceI target site that allows DSB induction through transfection with the I-SceI enzyme. In the DR-GFP system, repair of the construct through HR leads to the expression of a GFP gene, while, in the case of the GC92 system, repair through EJ generates a functional CD4 receptor (Guirouilh-Barbat *et al.*, 2004; Rass *et al.*, 2009; Gunn and Stark, 2012). As observed in figure 45 A, depletion of PHF6 by three different oligos inhibited EJ-mediated repair to the same extent as Ku80, a main component of the c-NHEJ pathway (Featherstone and Jackson, 1999). In contrast, PHF6 downregulation with oligonucleotides #1 and #2 led to increased HR-mediated repair, something that is usually observed in cells defective for EJ (fig. 45 B). However, depleting PHF6 by oligo #3 did not result in decreased HR-efficiency, despite having a similar downregulation efficiency (fig. 45 C). We figured that this discrepancy could be due to an effect of oligo #3 on cell cycle distribution. We therefore analyzed the cell cycle after PHF6 downregulation with the different siRNA oligos and observed a small decrease in S phase cells after depleting PHF6 with oligo #3 as compared to control cells and cells transfected with oligos

#1 and #2 (fig. 45 D). However, we do not think this small effect on the cell cycle could explain the defective HR after depleting PHF6 using this oligonucleotide.

Together, these results suggest that, in the absence of PHF6, cells accumulate damage in the form of DSBs, due to a decreased efficiency in the repair of this type of lesion by EJs.

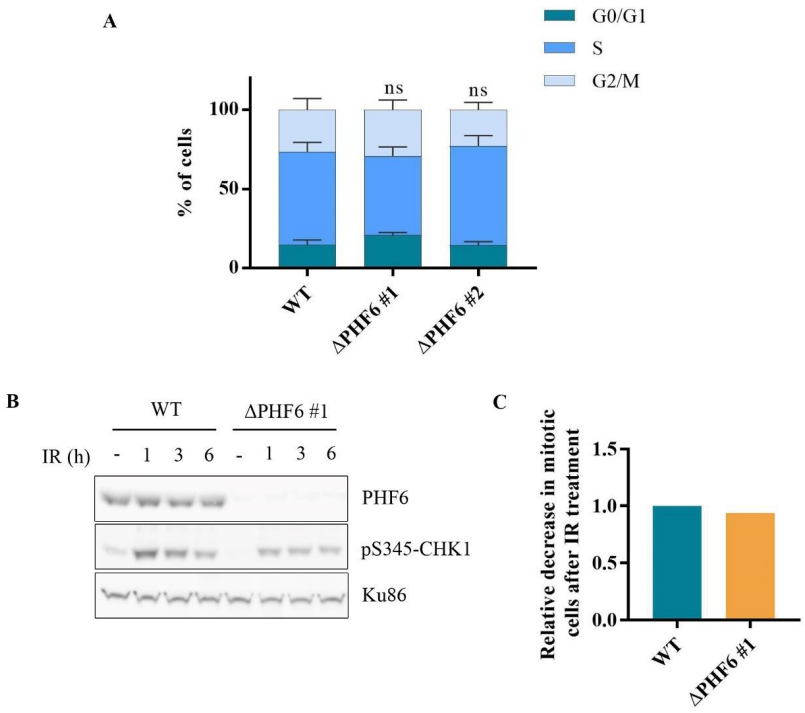

Figure 40. Cell cycle distribution and checkpoint activation are not affected by PHF6 knockout. A) U2OS WT and PHF6 knockout cells were analyzed for PI by flow cytometry. Mean ± SD from three independent experiments was plotted and statistical analysis was performed. B) U2OS WT and PHF6 knockout cells were treated with IR and, after different timepoints, analyzed by western blot using the indicated antibodies. C) U2OS WT and PHF6 knockout cells were treated with IR (2 Gy) or left untreated. After 1 hour, cells were fixed and analyzed by FACS for PI and pHH3. In the quantification, the decrease in mitotic (pHH3 positive) cells after IR treatment was calculated, and the result obtained for the WT control was put to 1.

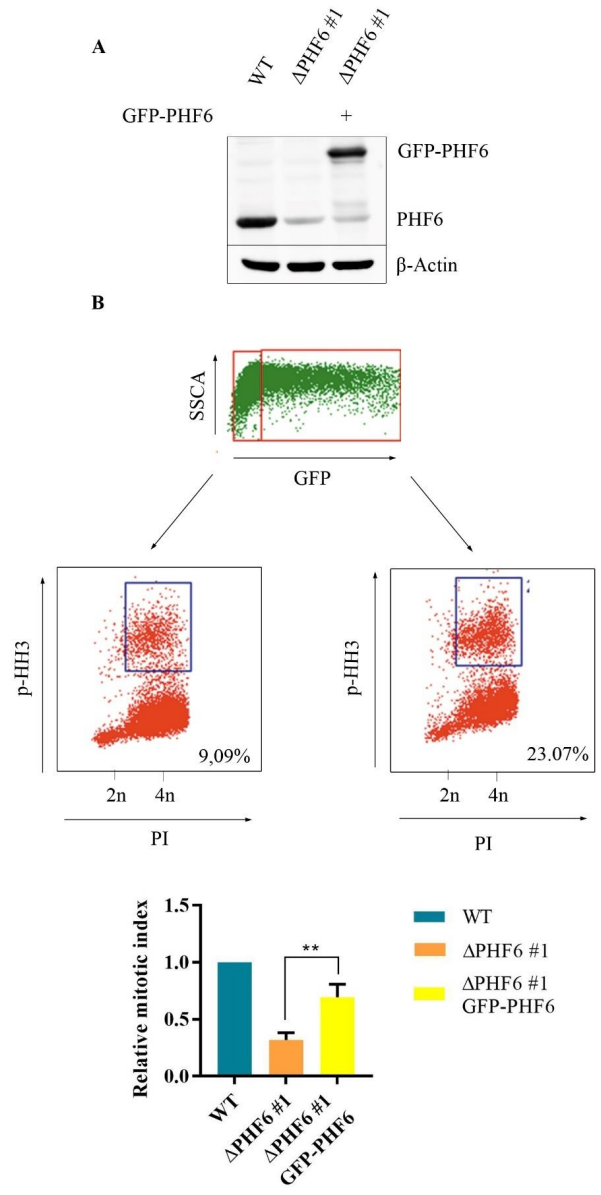

Figure 41. Expression of exogenous PHF6 in PHF6 knockout cells partially rescues the G_2 checkpoint recovery defect. A) U2OS WT cells were transfected with EV and U2OS PHF6 knock out cells with EV or GFP-PHF6 and analyzed by western blot. B) FACS analysis of mitotic cells in U2OS WT and PHF6 knockout cells transfected as in (A). For each condition, the reduction on mitotic cells from untreated to IR-treated cells was calculated, and the reduction observed in WT cells was put to 1. Mean ± SD of three experiments was calculated and statistical analysis was performed.

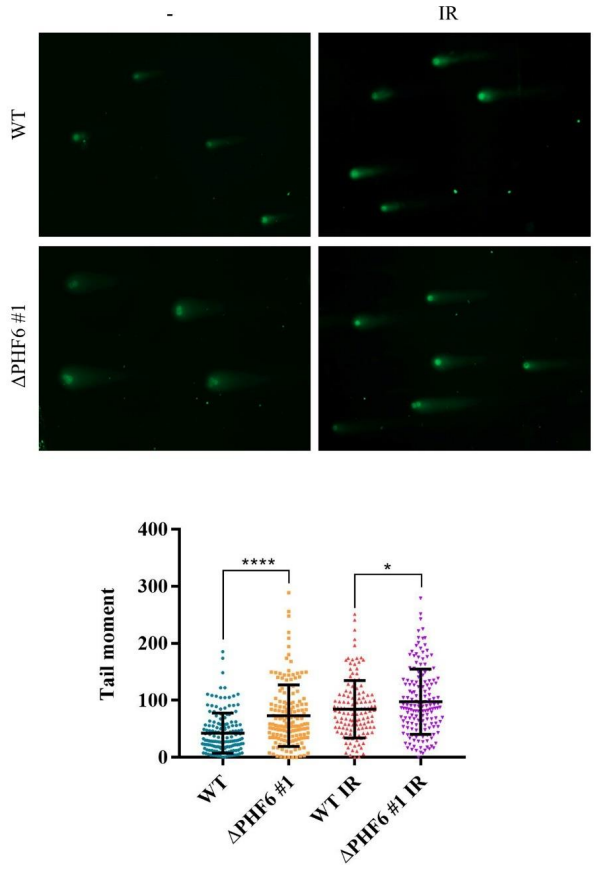

Figure 42. Loss of PHF6 sensitizes cells to IR (I). A) U2OS WT and PHF6 knockout cells were treated with IR (3 Gy). After 1 h, cells were subjected to neutral SCGE (top panel). Three independent experiments were carried out and 50 cells were scored in each of them. Mean ± SD of the tail moment was measured, and statistical analysis was performed (bottom panel).

4.3. PHF6 regulates c-NHEJ-mediated repair

As PHF6 was described as a transcriptional regulator (J. Wang *et al.*, 2013) a possible way to control EJ-mediated repair of DSBs was by regulating (a) critical factor(s) in this repair pathway. We therefore examined the levels of a broad range of proteins directly or indirectly involved in DSB repair by western blot. As observed in figure 46, knockout or knockdown of PHF6 did not cause any changes in the levels of the analyzed proteins.

The GC92 system measures all EJ-mediated repair pathways, which include c-NHEJ and alt-NHEJ (Rass *et al.*, 2009). We set on to investigate which of these pathways was affected in the absence of PHF6. Crucial in the DSB repair pathway choice is 53BP1. By inhibiting DNA end resection, 53BP1 promotes DNA repair by c-NHEJ, which does not require resection (Bunting *et al.*, 2011). Interestingly, irradiating PHF6 knockout cells resulted in less 53BP1 foci per cell

and reduced 53BP1 focus intensity as compared to the WT cells, specially at shorter timepoints after damage, suggesting a role for PHF6 in the c-NHEJ pathway of the DNA repair (fig. 47 A). Furthermore, we performed clonogenic survival assays upon IR in WT and PHF6 knockout cells after depletion of XRCC4, a core protein in c-NHEJ (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997). Knockout of PHF6 or depletion of XRCC4 led to increased sensitivity to IR (fig. 47 B). Importantly however, XRCC4 depletion in PHF6 knockout cells did not lead to a further increase in IR sensitivity, indicating that these two proteins participate in the same pathway and suggesting that PHF6 controls classical, rather than alternative NHEJ (fig. 47 B). To demonstrate that this is the case, the repaired break sites in the GC92 system were analyzed through PCR amplification and subsequent sequencing of the repair junctions, as previously described (Taty-Taty *et al.*, 2016). Like Ku80 depletion (used as a positive control), PHF6 downregulation resulted in a decrease in direct re-ligation events, concomitantly with a rise in the number and the length of deletions (fig. 48 A and B), and an increased usage of microhomology sequences (fig. 48 C). These results indicate that repair of DSB switches from c-NHEJ to alt-NHEJ in the absence of PHF6 and thereby demonstrate that PHF6 controls c-NHEJ.

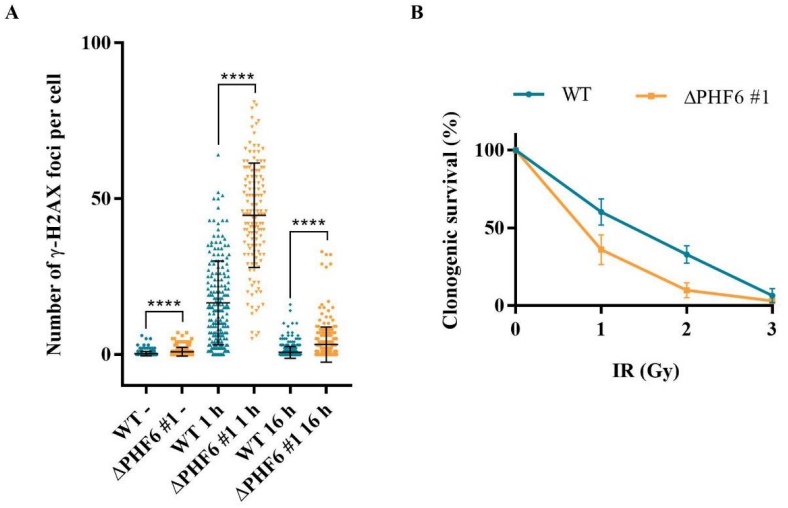

Figure 43. Loss of PHF6 sensitizes cells to IR (II). A) U2OS WT and PHF6 knockout cells were treated with IR (3 Gy). At the indicated timepoints, cells were analyzed by immunofluorescence for γ-H2AX. Each experiment was performed three times, the mean ± SD was calculated, and statistical analysis was performed. B) U2OS WT and PHF6 knockout cells were irradiated with the indicated doses of IR and left in culture for 7-10 days, after which the colonies were stained. Colonies from three independent experiments were counted, the number of colonies present in the untreated plates was set to 100% and the mean ± SEM was obtained.

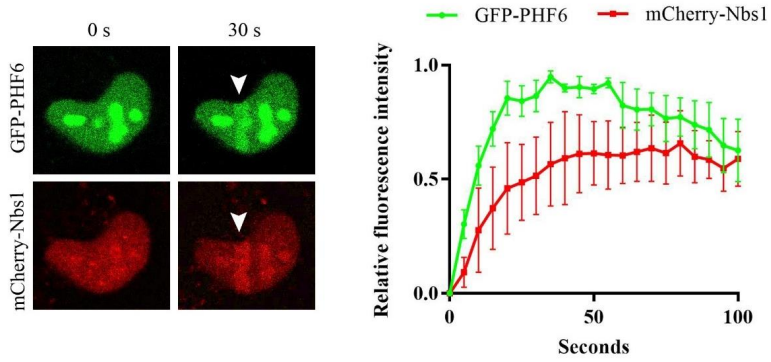

Figure 44. PHF6 is recruited to laser-induced DNA damage. U2OS cells stably expressing GFP-PHF6 were transfected with mCherry-Nbs1. The recruitment of PHF6 and NBS1 to laser-induced damage was analyzed by fluorescent microscopy and live-cell imaging. For quantification, the maximum detected fluorescence was put to 1. Three independent experiments were carried out, 50 cells were analyzed in each of them and mean ± SD was plotted.

4.4. PHF6 role on DSB repair is PARP dependent

Since PHF6 had been previously identified as a putative target of ATM/ATR in a proteomic screening (Matsuoka *et al.*, 2007), we hypothesized that the activity of these DDR kinases could control the role of PHF6 in the DDR. To test this, GFP-PHF6 recruitment to laser stripes was analyzed upon treatment with ATM and ATR inhibitors. Inhibition of these two kinases had no effect on PHF6 accumulation at damage sites (fig. 49 A), indicating that this process is independent of ATR and ATM activity.

Recent data identified PARP1 as an important factor for the recruitment of chromatin modifying proteins and consequently c-NHEJ core factors, during the first steps of DNA repair by c-NHEJ (Luijsterburg *et al.*, 2016). Since PHF6, that controls c-NHEJ, is rapidly recruited to DNA lesions (fig. 44), we tested if PARP1 activity is necessary for the recruitment of PHF6 to DNA damage sites by measuring GFP-PHF6 accumulation to laser stripes in the presence of the PARP1/2 inhibitor Olaparib. Interestingly, PARP inhibition drastically reduced PHF6 recruitment to laser-induced damage (fig. 49 B), suggesting that the role of PHF6 in DSB repair is PARP-dependent.

4.5. The PHD1 and PHD2 domains of PHF6 are important for its role in the DDR

PHF6 has two zinc-finger-like domains termed PHD domains (PHD1 and PHD2) known to be involved in chromatin regulation (Aasland *et al.*, 1995). The PHD1 domain was suggested to interact with upstream binding factor (UBF), a ribosomal DNA transcription factor (Zanto *et al.*, 2011; J. Wang *et al.*, 2013; Todd *et al.*, 2016). The PHD2 domain on the other hand, was reported to bind double-stranded DNA in vitro, and similar domains have been found in chromatin-associated proteins (Liu *et al.*, 2014). To study the involvement of these domains in the role of PHF6 in DNA repair and therefore in the recovery from the G_2 checkpoint, deletion mutants of this protein that lack either of the two PHD domains were generated (fig 50 A). The PHF6 knockout cells were subsequently complemented with either WT PHF6 or each of the

ΔPHD mutants, after which IR-induced 53BP1 and γ-H2AX focus formation were analyzed. Interestingly, while complementation with the WT version of PHF6 rescued the defect in 53BP1 and the increase in γ-H2AX focus formation, both ΔPHD mutants did not, showing a similar phenotype as the PHF6 knockout cells (fig. 50 B and C). In accordance to this, PHF6 knockout cells expressing the ΔPHD mutants failed to recover from an ETP-induced G_2 checkpoint arrest, while expressing a WT version of PHF6 resulted in increased recovery (fig. 50 C).

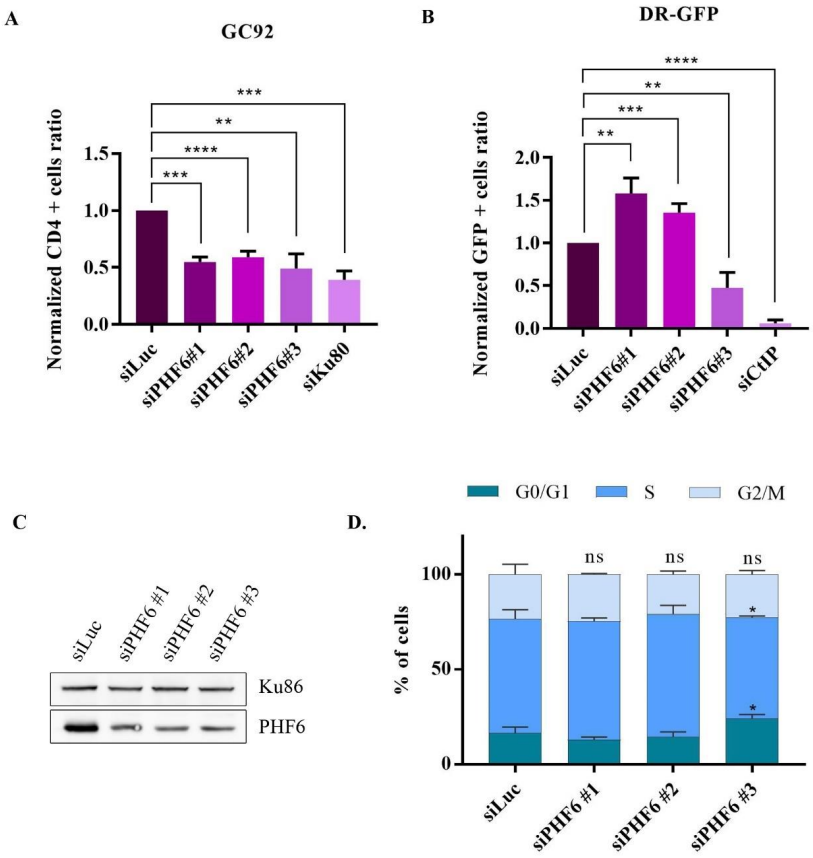

Figure 45. PHF6 regulates end joining-mediated repair of DSBs. A) GC92 human fibroblasts were depleted for Luc, Ku80 (positive control) or PHF6 and CD4-positive cells were analyzed by flow cytometry. B) U2OS cells stably expressing a single copy of the DR-GFP construct were depleted for Luc, CtIP (positive control) or PHF6 and GFP-positive cells were analyzed by FACS. In both A) and B), the percentage of GFP or CD4-positive cells in control was put to 1. The mean ± SD from three independent experiments was plotted and statistical analysis was performed. C) Western blot showing the downregulation efficiency obtained with the three siRNA oligonucleotides used in (A) and (B). D) U2OS cells transfected as in (C) were analyzed for PI by flow cytometry. Mean ± SD from three independent experiments was plotted and statistical analysis was performed.

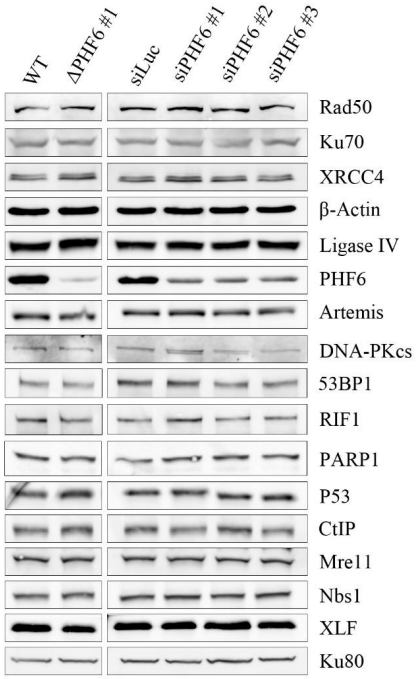

Figure 46. PHF6 does not affect the levels of proteins in DSB repair. U2OS WT, PHF6 knockout cells and U2OS WT cells depleted for Luc or PHF6 using the indicated siRNA oligos were analyzed by western blot using the indicated antibodies.

Together, these data shows that both PHD domains of PHF6 are needed for the role of this protein in the DDR.

DISCUSSION

Upon DNA damage, cells activate cell cycle checkpoints that stop or slow down cell cycle progression to allow time to repair the lesions before the DNA is replicated and transmitted to the new generation of cells (Warmerdam and Kanaar, 2010). Specifically, the G_2/M checkpoint arrests cells in the G_2 phase of the cell cycle, preventing entrance into mitosis of cells with damaged DNA (Smith *et al.*, 2010). This arrest is mainly mediated by the ATR/CHK1 axis: phosphorylation of its targets Cdc25, Cdc25C and Wee1 inactivates these enzymes and thereby prevents CDK activation and subsequent transition to mitosis (Peng *et al.*, 1997; Welcker *et al.*, 2000; Lee *et al.*, 2001; Xiao *et al.*, 2003).

Once the DNA damage is repaired, cell cycle progression is resumed in a process known as checkpoint recovery (Van Vugt and Medema, 2004). Recovery from the G_2 checkpoint arrest is a complex process orchestrated by different kinases and phosphatases (amongst other proteins) that requires chromatin remodeling to reestablish the original structure that was present before the DNA repair events (Lu *et al.*, 2005; Chen and Tyler, 2008; Macůrek *et al.*, 2008, 2010; Lindqvist *et al.*, 2009; Soria *et al.*, 2012). For example, dephosphorylation of the critical DDR factors p53, CHK1 and γ-H2AX by the phosphatase PPM1D and the degradation

of Claspin through the activity of the ubiquitin ligase βTrCP are needed to stop DNA damage signaling and subsequent checkpoint switch off (Lu *et al.*, 2005; Mailand *et al.*, 2006; Mamely *et al.*, 2006; Peschiaroli *et al.*, 2006; Lindqvist *et al.*, 2009; Macůrek *et al.*, 2010). However, the factors that contribute to checkpoint recovery by chromatin remodeling remain largely unknown.

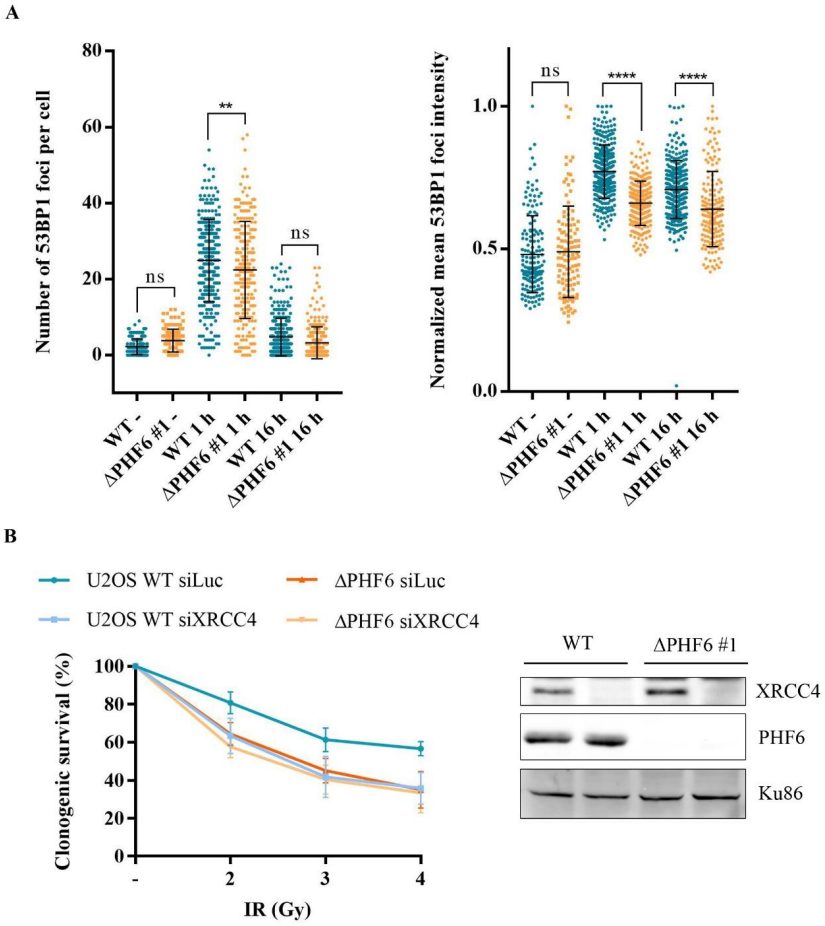

Figure 47. PHF6 promotes DSB repair by c-NHEJ (I). A) U2OS WT and PHF6 knockout cells were treated with IR (3 Gy) and analyzed by immunofluorescence using a 53BP1 antibody after the indicated timepoints. Quantified was the number of 53BP1 foci per cell (left panel) and the focus intensity (right panel). B) U2OS WT and PHF6 knockout cells were depleted for Luc and/or XRCC4 and an equal number of cells were seeded, treated with the indicated doses of IR and left to grow for 7-10 days. For each condition, the number of colonies present without IR treatment was set to 100%. Mean ± SEM from three independent experiments are shown.

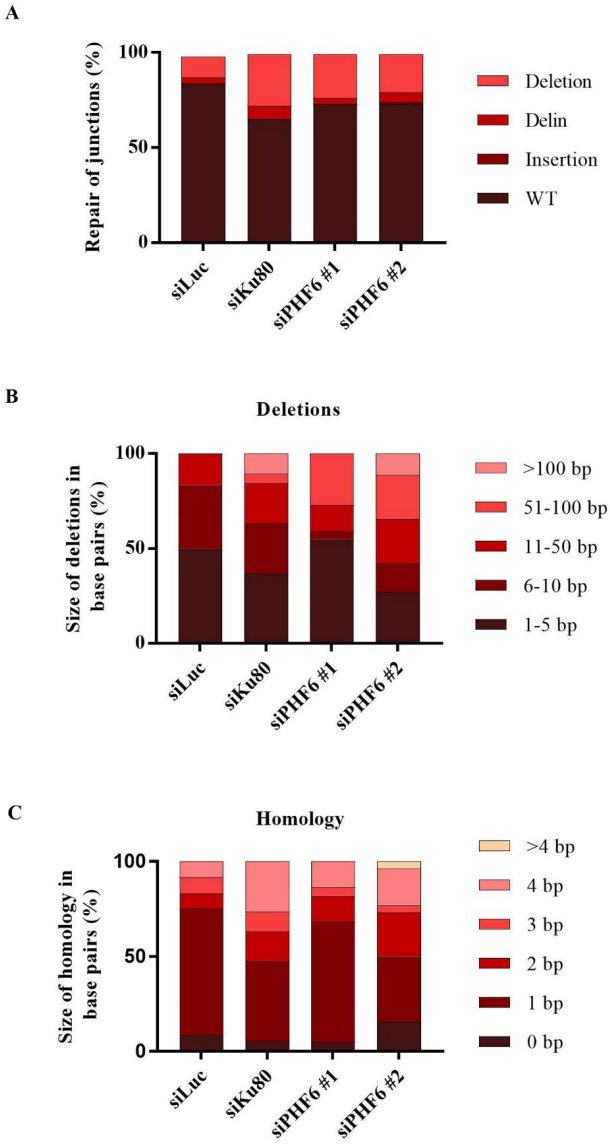

Figure 48: PHF6 promotes DSB repair by c-NHEJ (II). GC92 human fibroblasts were depleted for Luc, Ku80 (positive control) or PHF6 and genomic DNA was extracted. Junction analysis was performed as described in the materials & methods. In A) the percentage of events that restore the WT sequence, and the events that involve deletions, insertions or delins (insertions plus deletions) are represented, while B) and C) show the length of the deletions and the extent of microhomology, respectively.

The work here presented follows a large siRNA screening performed by Dr. Daniël O. Warmerdam to identify new chromatin remodeling factors involved in the recovery after IR-induced G_2 checkpoint arrest, in which it was observed that PHF6 depletion led to decreased checkpoint recovery efficiency.

However, certain discrepancies were observed during the original study, using different siRNA oligos to deplete PHF6, possibly due to the existence of at least three transcript variants of PHF6 (Voss *et al.*, 2007; Van Vlierberghe *et al.*, 2014). To overcome this obstacle and to validate the results obtained in the screening, CRISPR/Cas9 technology was used to generate PHF6 knockout cells. We selected two clones for further analysis and determined that these cells also show a defect in the DNA damage checkpoint recovery. Importantly, such phenotype is not caused by alterations in cell cycle distribution or CHK1 phosphorylation and can be partially rescued by restoring PHF6 expression through plasmid transfection.

Further analysis showed that PHF6 knockout cells accumulate more DNA damage than WT cells after IR treatment, leading to reduced cell survival. These results led us to investigate if PHF6 could be involved in the repair of the DNA lesions caused by IR. PHF6 efficiently localizes to laser-induced damage in an ATM- and ATR-independent, but a PARP-dependent manner, and its depletion inhibits EJ-mediated repair. Interestingly, PARP1 has recently been shown to promote the recruitment of the chromatin remodeling factor CHD2, and such recruitment is critical for the subsequent accumulation of the core factor XRCC4 and for the correct functioning of the c-NHEJ pathway (Luijsterburg *et al.*, 2016). PHF6, like CHD2, is recruited to DNA lesions in a PARP-dependent manner, although further work is needed to clarify if such recruitment allows any type of chromatin modification needed for c-NHEJ.

The predominant pathways to repair DNA DSBs are c-NHEJ or HR, but cells also rely on two other minor pathways: alt-NHEJ and SSA. Importantly, the GC92 system used in this study detects all EJ events, including c-NHEJ and alt-NHEJ (Rass *et al.*, 2009). Since the activity of PARP1 is important for both pathways (Robert *et al.*, 2009; Luijsterburg *et al.*, 2016), PHF6 could be participating in any of them. However, the fact that 53BP1 focus formation after IR is prevented in the absence of PHF6 pointed to a role for this protein in the c-NHEJ pathway. Indeed, junction analysis demonstrated that PHF6 depletion led to an increased number and length of depletions, a phenotype that indicates that repair is being pushed towards alt-NHEJ under these conditions.

PHF6 does not have enzymatic activity, but its PHD domains have been reported to be important for some of its described functions: the PHD1 domain was reported to interact and negatively regulate UBF, a critical factor in promoting ribosomal DNA transcription, while PHD2 was reported to bind double-stranded DNA *in vitro* (Zanto *et al.*, 2011; J. Wang *et al.*, 2013; Liu *et al.*, 2014; Todd *et al.*, 2016). Importantly, similar domains have been found in chromatin-associated proteins, some of which have also been implicated in the DDR (Liu *et al.*, 2014). For example, the plant homeodomain finger protein 11 (PHF11) is known to regulate resection of DSBs through the stimulation of Exo1 activity. This process requires the binding of PHF11 to RPA-coated ssDNA through the PHF11 PHD domain (Gong *et al.*, 2017). Taking this into account, we generated two mutant versions of PHF6 lacking each of the PHD domains and performed different complementation experiments to examine if the PHD domains are required for the function of PHF6 in DSB repair. First, both mutants were unable to rescue the decrease in 53BP1 focus formation observed in the knockout cells as compared to the WT,

suggesting that both domains are important for the role of PHF6 in the c-NHEJ pathway. Next, while complementation with a WT version of PHF6 decreased the number of γ-H2AX foci to levels similar to the ones observed in WT cells, the ΔPHD1 and ΔPHD2 mutants failed to do so, indicating that more damage was left unrepaired in these conditions. Finally, and in accordance with our previous results, complementation with both PHD mutants could not rescue the recovery defect observed in PHF6 knockout cells. These data indicate that the PHD1 and PHD2 domains are needed for PHF6 to function in c-NHEJ, and that upon mutation or depletion of these domains, cells fail to efficiently repair DNA lesions, leading to a prolonged G_2 checkpoint arrest.

Given the recent reports linking PHF6 mutations to leukemia and glioblastoma (Mullighan and Holmfeldt, 2010; Hiddingh *et al.*, 2014; Van Vlierberghe *et al.*, 2014), it will be interesting to study if and how such mutations affect the role of PHF6 in the DDR. In addition, it is important to note that (co)treatment with PARP1/2 inhibitor is expected to be unbeneficial for the therapy of tumors harboring PHF6 loss-of-function mutations.

In conclusion, we have identified PHF6 as a new member of the DDR: PHF6 is recruited to DNA damage sites in a PARP-dependent manner, where it regulates c-NHEJ-mediated repair of DSBs. In the absence of this protein, correct DNA repair fails to ensue, cells accumulate damage and do not recover from the G_2 checkpoint arrest (fig. 51). Although the exact mechanism through which PHF6 contributes to c-NHEJ is something that needs to be addressed in future studies, its PHD domains, and therefore the ability of PHF6 to bind and/or modulate the chromatin, are important for this function.

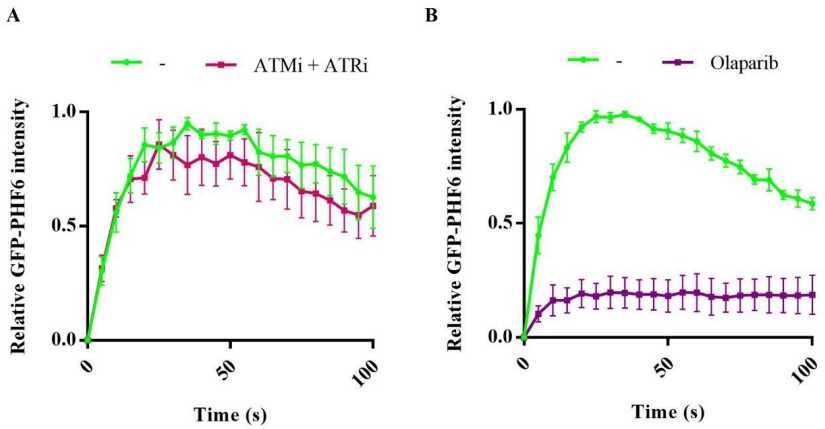

Figure 49. The recruitment of PHF6 to DNA damage sites is ATM/ATR-independent but dependent on PARP. GFP-PHF6 stable cells were pre-treated with ATM and ATR inhibitors (caffeine and VE-821) (A) or with a PARP inhibitor (Olaparib, 10 μM) for 1 h (B). The recruitment GFP-PHF6 to laser-induced damage was assessed by fluorescent microscopy and live-cell imaging. For quantification, the maximum detected fluorescence was put to 1. Three independent experiments were carried out, 50 cells were analyzed in each of them and the mean ± SD from was plotted.

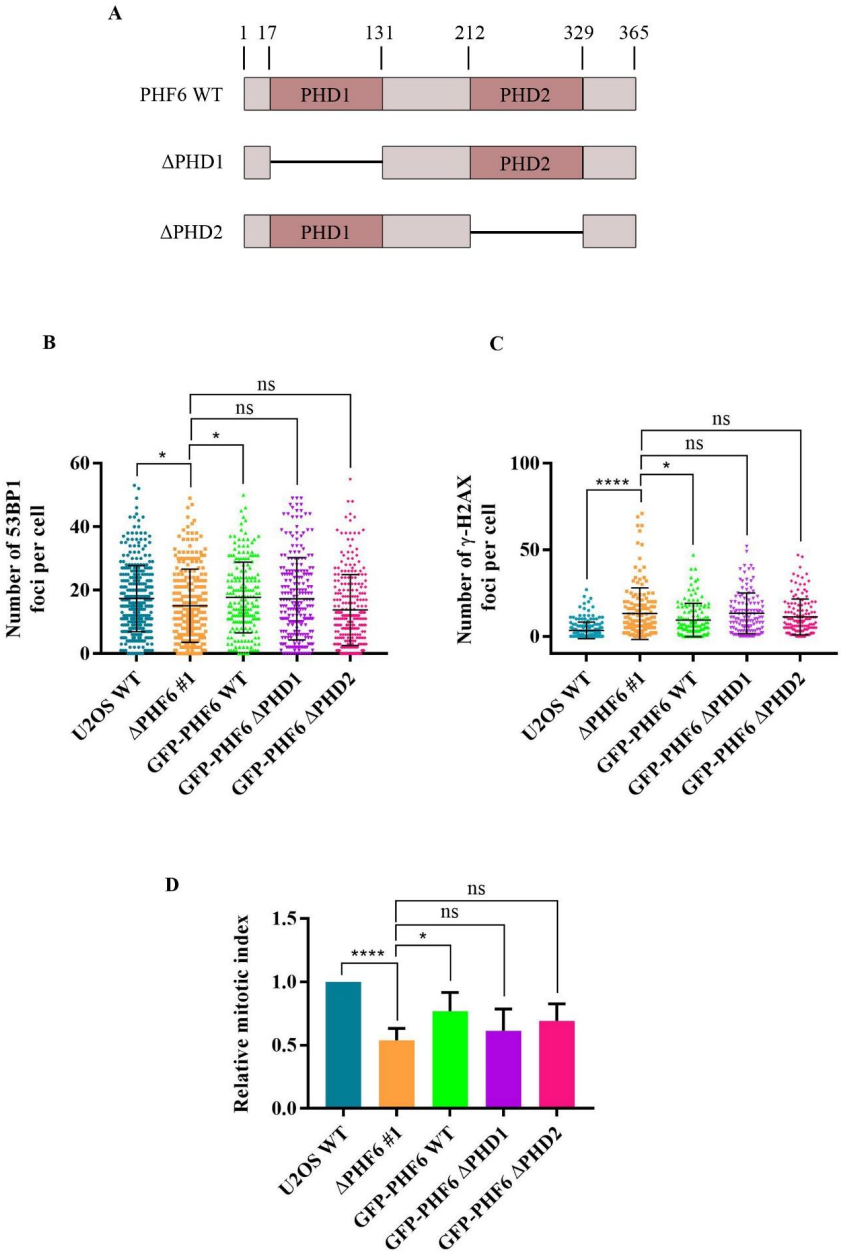

Figure 50. The PHD domains of PHF6 are required for its role in the DDR. A) Schematic representation of PHF6 WT and the ΔPHD1 and ΔPHD2 mutants. B) and C) PHF6 knockout cells were transfected with the indicated constructs and U2OS WT cells were transfected with an EV and analyzed by immunofluorescence 1 hour after 1 IR for 53BP1 (B) or 24 hours after IR for γ-H2AX (C). D) Cells were transfected as in (B) and (C) and their ability to recover from an ETP-induced (3 μM) G_2 checkpoint arrest was analyzed by flow cytometry.

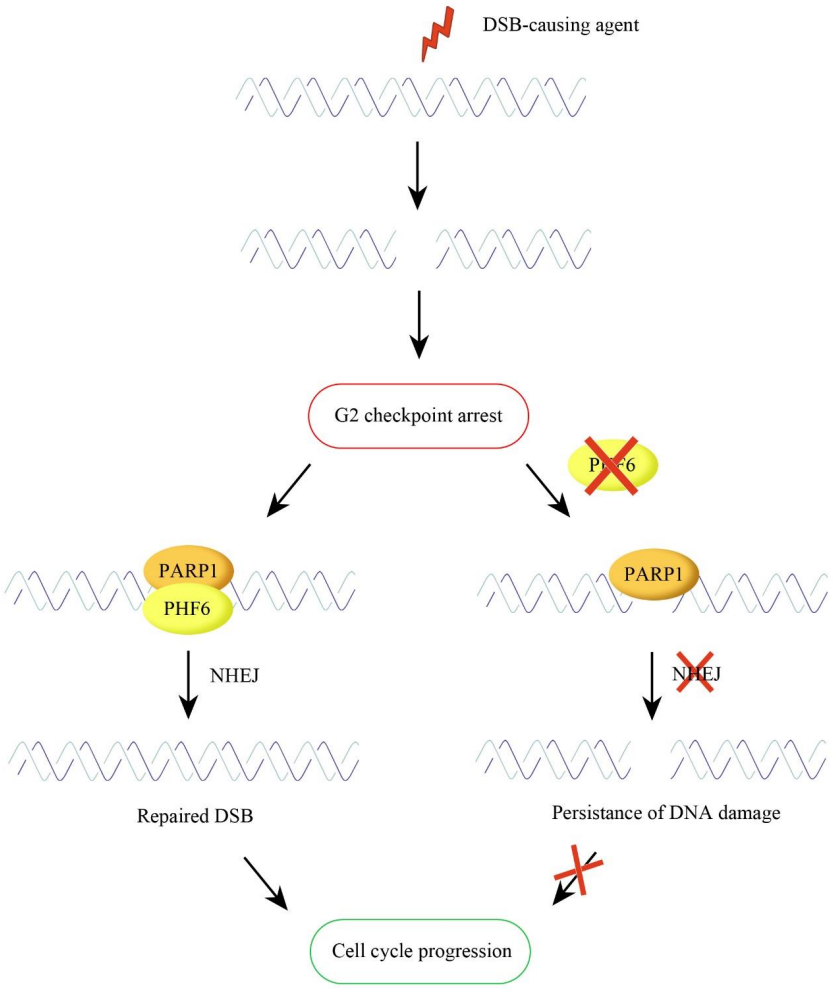

Figure 51: PHF6 promotes the recovery from the G_2 checkpoint arrest. DNA DSB induction triggers checkpoint arrest and activation of the different DNA repair pathways, including c-NHEJ. PARP1 is rapidly recruited to DSBs and allows PHF6 accumulation, leading to proper re-ligation of the ends and subsequent cell cycle progression. In the absence of PHF6, DNA lesions are not repaired as efficiently and in consequence cells accumulate damage and do not recover from the G_2 arrest.

CONCLUSIONS

1. Ubiquitin hydrolase USP7 and histone acetyltransferase HAT1 are novel regulators of the ATR-CHK1 pathway of the DNA damage response.

2. USP7 directly deubiquitinates CHK1, thereby controlling CHK1 protein levels by preventing its proteasomal degradation.

3. USP7 is able to auto-deubiquitinate.

4. HAT1 controls the G_2/M checkpoint by regulating the localization to the chromatin of TopBP1, a critical activator of ATR.

5. Genetic screenings identified PHF2 and PHF6 as new regulators of the DNA damage response.

6. Alterations in the levels of PHF2 lead to genomic instability.

7. PHF2 regulates the resection of double-strand breaks (DSBs), and thereby homology-directed DSB repair, by controlling the transcription of BRCA1 and CtIP.

8. PHF6 is required for efficient recovery from G_2 DNA damage checkpoint arrest.

9. PHF6 controls DSB repair by classical Non-Homologous End Joining (c-NHEJ) in a PHD domain-dependent manner.

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